ELSEVIER

Journal of Chromatography A, 880 (2000) 189–202

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Review

C_{30} Stationary phases for the analysis of food by liquid chromatography ☆

Lane C. Sander*, Katherine E. Sharpless, Matthias Pursch[1]

Analytical Chemistry Division, National Institute of Standards and Technology (NIST), Gaithersburg, MD 20899-8392, USA

Abstract

The introduction of a polymeric C_{30} liquid chromatographic column by Sander et al. [Anal. Chem., 66 (1994) 1667] designed for the separation of carotenoid isomers, has led to the development of improved analytical methods for these compounds. Subsequent commercial availability of polymerically bonded C_{30} columns has facilitated these advances, and applications to a wide variety of separation problems with biological samples have been described. This report provides a comprehensive review of applications of polymeric C_{30} columns, utilized in the determination of carotenoids, retinoids, and other nutrients and related compounds in complex, natural-matrix samples. © 2000 Published by Elsevier Science B.V.

Keywords: Stationary phase, LC; Food analysis; Reviews; Carotenoids; Retinoids; Tocopherols; Vitamins; Carotenes

Contents

1. Introduction

The liquid chromatographic (LC) separation of certain classes of isomers can present a significant challenge to the analyst. In environmental analyses, polycyclic aromatic hydrocarbons (PAHs) represent one such separation challenge. Early in the develop-

☆Contribution of the National Institute of Standards and Technology. Not subject to copyright.

*Corresponding author.

[1]Current address: Dow Deutschland Inc., Analytical Sciences, 77836 Reinmünster, Germany.

0021-9673/00/$ – see front matter
PII: S0021-9673(00)00121-7

ment of LC methods for PAHs it was recognized that C_{18} columns from various manufacturers differed considerably in their ability to resolve isomers [2–6]. Further studies revealed that the differences were related to the way in which the C_{18} stationary phases were synthesized [7]. Stationary phases prepared under anhydrous conditions and/or prepared with monofunctional (e.g., monochloro or monoalkoxy) silanes are designated 'monomeric', whereas phases prepared with trifunctional silanes in the presence of water are termed 'polymeric'. In general, better separations of complex PAH isomer mixtures have been demonstrated with polymeric C_{18} columns than with the more common monomeric C_{18} columns [8]. A variety of nutrients also exist in isomeric forms in foodstuffs and biological sources. Examples of such isomer classes include carotenoids, retinoids, and tocopherols and related compounds. Carotenoids are naturally occurring pigments that are found in a wide variety of plant and animal sources including most fruits and vegetables, certain animal tissues, and biological fluids. Carotenoids and retinoids are essential nutrients in the human diet. They are required for vision, act as antioxidants and as free radical scavengers, and may function in the prevention of disease. Due to their constrained polyene structure, carotenoids can exist in *cis* and *trans* isomeric forms (see Fig. 1). These isomers differ in biological activity, and separation of individual species is required for the assessment of potential health benefits. As with PAHs, subtle differences in molecular structure provide a basis for separation by LC that is dependent on stationary phase properties. Although polymeric C_{18} columns offer improved separation of these compounds compared to monomeric C_{18} columns [9], even better separations have been demonstrated with polymeric C_{30} columns [1].

The use of C_{30} stationary phases in LC was first reported in 1987 for the separation of PAHs [10] and oligonucleotides [11], and later for alkylbenzenes [12], aldehyde and ketone derivatives [13], and fullerenes [14]. Pesek et al. described a novel approach to the synthesis of alkyl stationary phases (including C_{30} phases) based on hydrosilylation of a silica hydride surface with α-olefins [15,16]. The phases were somewhat lower in bonding density, and linkages to the silica surface were different from those resulting from reaction with chlorosilanes. Bell et al. synthesized C_{34} stationary phases for comparison with C_{30} phases [17]. Differences in selectivity were relatively minor for geometric carotenoid isomers, but separations were slightly improved for the C_{34} column. In general it has been observed that separations of isomeric compounds were often improved with increasing alkyl chain length of the stationary phase [10]. The ability of a column to discriminate among isomers (or other closely related compounds) on the basis of molecular shape is referred to as shape selectivity, and a recent review of this topic has been presented [18].

The application of C_{30} stationary phases to the separation of carotenoid isomers was first reported by Sander et al. [1]. Efforts were directed toward the development of a stationary phase that would permit improved separations of complex mixtures containing polar and nonpolar carotenoid isomers. Research with other classes of shape-constrained solutes indicated that selectivity is strongly influenced by certain stationary phase properties, including bonding density [19], stationary phase chemistry (i.e., monomeric vs. polymeric surface modification) [7], and alkyl chain length [10]. The separation of polar carotenoids is also influenced by silanol activity [20]; however, nonpolar carotenoids are relatively insensitive to this property. Each of these parameters was studied individually in an effort to optimize selectivity for carotenoid isomers. Initial separations of carotenoids with monomeric and polymeric C_{18} phases indicated that better separations of geometric isomers were usually possible with polymeric stationary phases. Similarly, better separations of isomers resulted for densely bonded phases (e.g., greater than 4 $\mu mol/m^2$) compared to phases with lower surface coverages. Because carotene molecules are relatively large compared with the thickness of C_{18} stationary phases (≈30 Å vs. ≈21 Å, respectively), it was thought that more extensive interactions might occur with thicker stationary phases. C_{30} stationary phases were prepared to test this hypothesis, and the resulting separations of carotenoid isomers were improved over separations with C_{18} phases.

After consideration of the effects of these parameters, a stationary phase was prepared with properties tailored for the separation of polar and nonpolar carotenoid isomers [1]. The stationary phase was

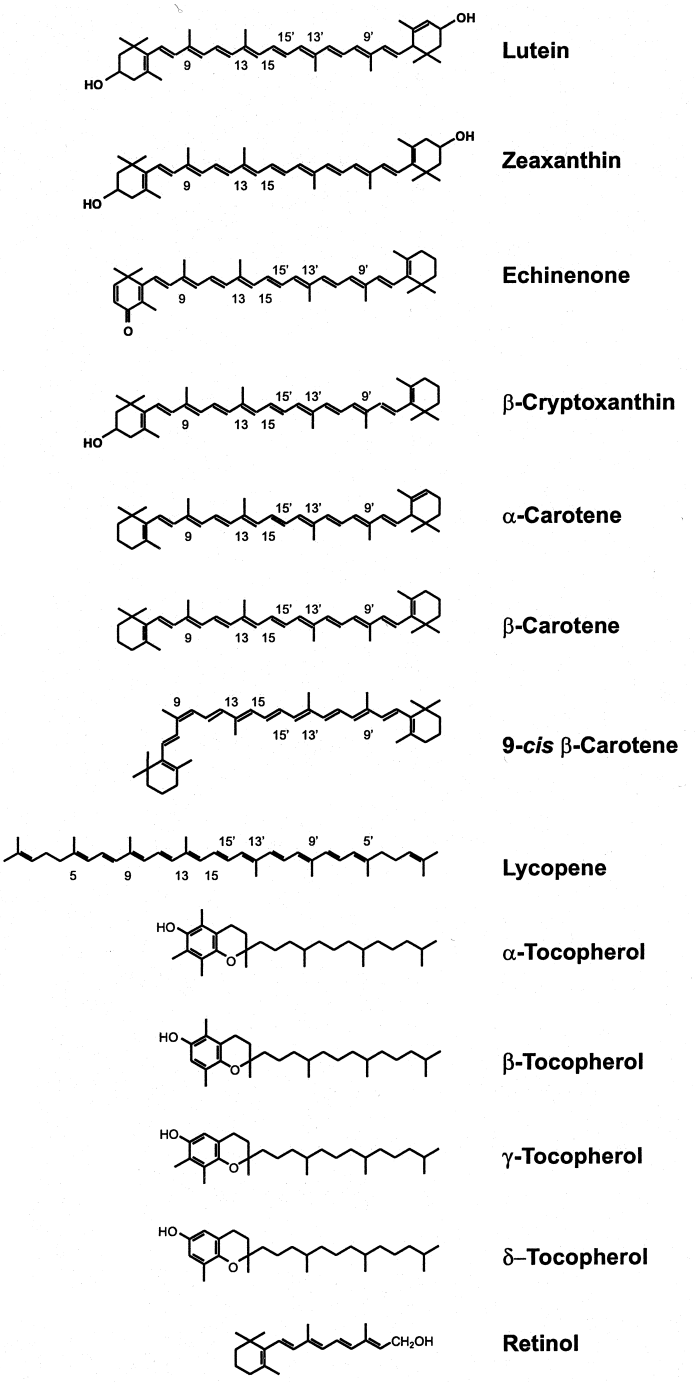

Fig. 1. Structures of selected carotenoids, retenoids and tocopherols.

based on C_{30} polymeric modification of a moderate pore size (≈200 Å), moderate surface area (≈200 m^2/g) silica, without subsequent silanol deactivation. Choice of silica is important to the overall properties of the column. To achieve sufficient retention of polar carotenoids, a stationary phase with relatively high carbon loading is required (≈18% mass fraction). Carbon loading is proportional to the surface area of the silica substrate, and is inversely proportional to the pore diameter. However, polymeric stationary phases are more shape selective when prepared on wide-pore silica substrates (>150 Å) [21]. Thus, the choice of a 200 Å, 200 m^2/g silica represents a compromise between absolute retention and selectivity that contributes to the desired chromatographic retention behavior. Currently the authors are aware of four manufacturers who offer columns with C_{30} surface modification: Waters/YMC (Milford, MA, USA), Micra Scientific (Northbrook, IL, USA), Bischoff (Leonberg, Germany), and Nomura Chemicals (Seto, Japan).

Considerable research effort has been directed toward an understanding of the physical and chromatographic properties of stationary phases. The study of C_{30} phases has benefitted from recent advances in ^{1}H, ^{13}C, and ^{29}Si solid state nuclear magnetic resonance spectroscopy (NMR), and a review on the topic has been published [22]. Albert et al. have reported several studies of C_{30} phase investigations by solid-state NMR, and a few conclusions can be summarized [23–27]. Alkyl chain mobility and order (i.e., *gauche* and *trans* conformations) are interrelated, and are temperature dependent. At reduced temperature, alkyl chains are more rigid and ordered than at elevated temperature. It is hypothesized that these changes are the primary cause of selectivity changes that are also observed with temperature. Temperature-dependent changes in alkyl chain conformation and selectivity have also been reported for C_{18} phases, as indicated by infrared spectroscopy and chromatographic studies [28,29].

2. Applications in liquid chromatography

2.1. Carotenoids — fundamental studies

The use of C_{30} stationary phases for the separation of xanthophylls and hydrocarbon carotenoids was first demonstrated for mixtures of standards, and later for food extracts and other complex-matrix samples. A comparison of the separation of 14 polar and nonpolar carotenoid standards is shown in Fig. 2, for monomeric and polymeric C_{18} columns, as well

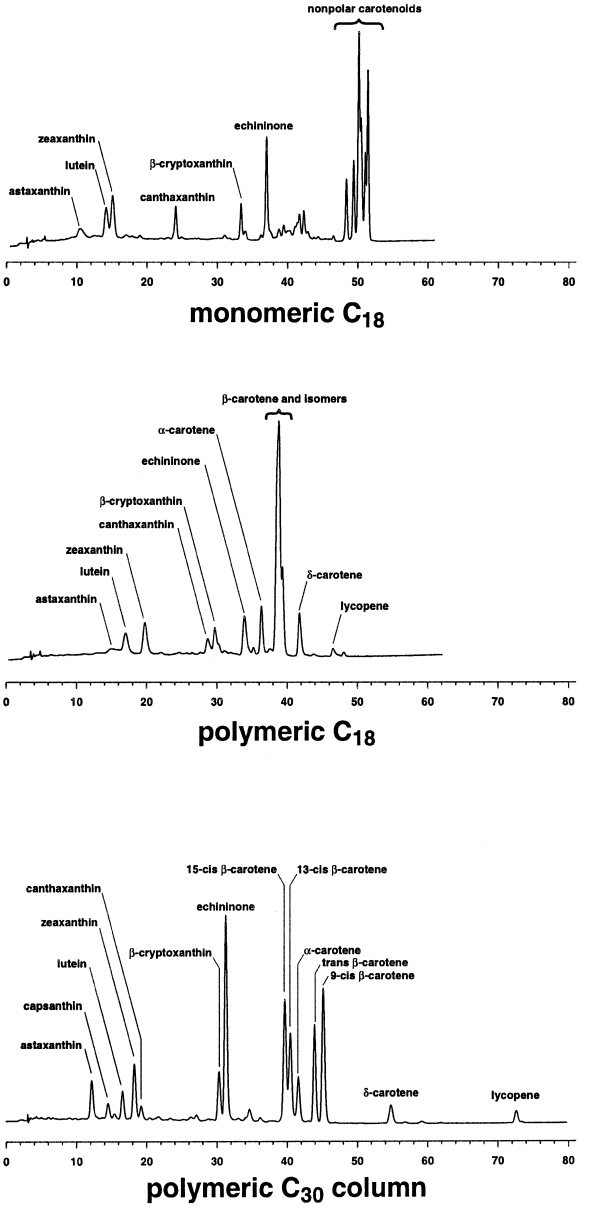

Fig. 2. Separation of carotenoid standards on (a) monomeric C_{18} column; (b) polymeric C_{18} column; and (c) polymeric C_{30} column. Time scale in min (from Ref. [1]; used with permission).

as for a polymeric C_{30} column [1]. With the monomeric C_{18} column, nonpolar carotenoid isomers were poorly resolved, and lutein and zeaxanthin were not separated. Better separation of the hydrocarbon carotenoids was possible with the polymeric C_{18} column; however, *cis*/*trans* isomers of β-carotene were unresolved. Using the same mobile phase gradient, near baseline resolution of the components was achieved with the polymeric C_{30} column. Interestingly, the retention behavior of lycopene varies dramatically with stationary phase properties. With monomeric C_{18} columns, lycopene usually elutes before α- and β-carotene, whereas with polymeric C_{18} and C_{30} columns, lycopene is strongly retained and elutes after these carotenoids.

Selectivity is strongly dependent on the choice of the mobile phase modifier, and this can be useful in the development of a separation method. For example, column selectivity toward *cis* and *trans* isomers of β-carotene differs with the use of methanol or acetonitrile in the mobile phase [9]. The separation of polar carotenoids is also influenced by the presence of water, whereas nonpolar carotenoids are relatively insensitive to this parameter. With the exception of β-cryptoxanthin and echinenone, nearly complete separations of the standards shown in Fig. 2 were possible with a nonaqueous methanol–methyl *tert*.-butyl ether (MTBE) gradient. MTBE was utilized as a modifier to facilitate elution of lycopene, which is strongly retained in a methanol environment. Acetone has also been used in combination with methanol to reduce absolute retention of lycopene with C_{30} columns.

Emenhiser and co-workers utilized a polymeric C_{30} column to separate mixtures of geometric isomers created by iodine-catalyzed photoisomerization [30,31]. The resulting mixtures typically contained three to five components identified as *cis* and *trans* isomers, and a number of other components at lower levels identified as di-*cis* isomers. Isomerized mixtures of the following compounds were studied: lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, and lycopene. Peak identifications were tentatively assigned based on UV–vis absorbance spectra collected with a diode array detector. In the case of α-carotene isomers, unambiguous identification of peaks was made by off-line NMR measurements [31]. The elution order of the predominant α-carotene isomers was reported: 13-*cis*-, 13′-*cis*-, *trans*-, 9-*cis*-, 9′-*cis*-α-carotene. Higher-resolution separations of similar isomerized mixtures were later demonstrated by Sander et al. using a polymeric C_{30} capillary column operated in the electrochromatography mode [32] (see Fig. 3). Strohschein et al. employed coupled LC–NMR to provide on-line peak identification of β-carotene isomers [33]. Their results confirmed the earlier assignments of Emenhiser, and provided identification of two di-*cis* isomers (13, 15-di-*cis*- and 9, 13-di-*cis*-β-carotene). This on-line LC–NMR approach reduces the potential for compound oxidation and isomerization prior to identification. In each case, better separations of the isomer mixtures were achieved with polymeric C_{30} columns compared with C_{18} columns. These examples demonstrate the potential offered by C_{30} columns for improved measurements of complex carotenoid mixtures.

Changes in C_{30} column selectivity with temperature have been investigated by a variety of chromatographic, spectroscopic, and computational approaches [24], and the influence of temperature on the separation of carotenoid isomers has been studied in some detail [24,34–36]. Unusual changes in retention have been reported for *cis* isomers over certain temperature intervals. For example, the retention of 13-*cis*- and 15-*cis*-, α- and β-carotene actually increases with increasing temperature above ≈35°C. This behavior was thought to result from increased solute–stationary phase interactions at elevated temperature. Van 't Hoff plots for these isomers were nonlinear, in contrast to linear plots observed for *trans* carotenoids. Zhu et al. studied the influence of both temperature and mobile phase composition on selectivity, for a variety of compounds [36]. A polymeric C_{30} column was included in this work, with various carotenes as probe solutes. In contrast to other compounds studied with monomeric C_{18} columns, temperature was found to influence selectivity to a greater extent than changes in mobile phase composition. Pursch et al. investigated the influence of temperature on C_{30} stationary phase conformation by ^{13}C cross polarization magic angle spinning NMR [35]. The *trans*/*gauche* ratio of conformations within the stationary phase was observed to increase steadily with decreasing temperature, thus indicating chain straightening at low temperature. Because of the sensitivity of carotenoid retention to changes in temperature, it is recom-

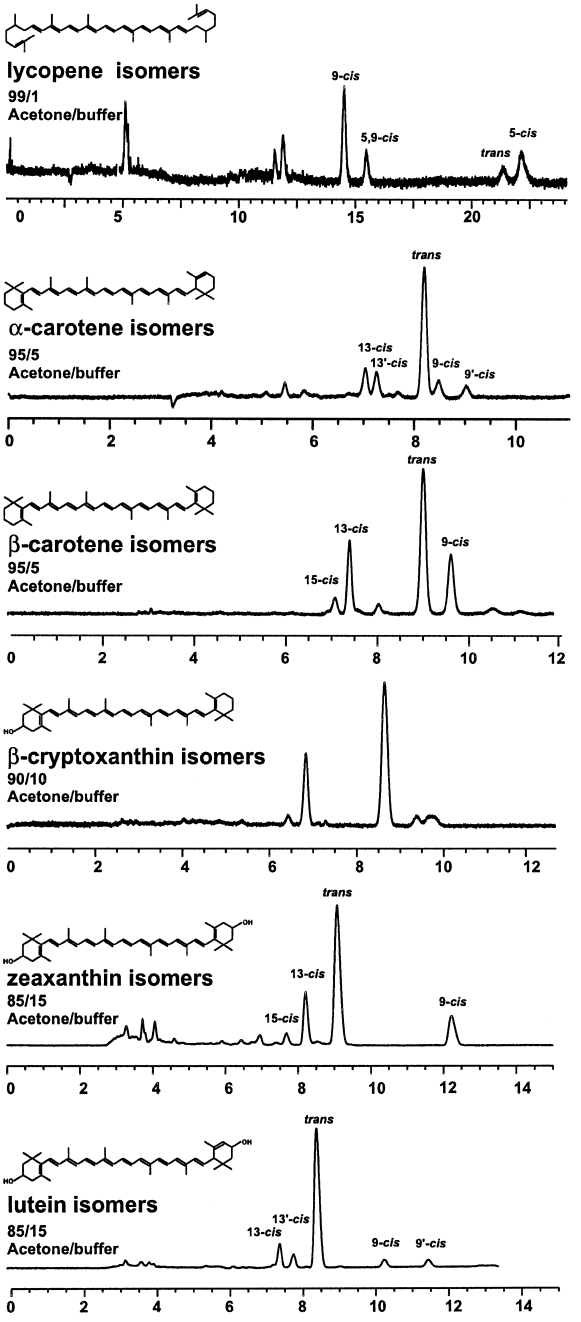

Fig. 3. Chromatograms of iodine-catalyzed, photoisomerized carotene standards, separated by capillary electrochromatography. Field strength: 30 kV; detection: 450 nm; time scale in min..

mended that column temperature be controlled to improve reproducibility. Temperature control may also prove useful for selectivity tuning in method development.

Several LC–MS applications have utilized C_{30} columns for the determination of carotenoids. Van Breemen presented the first application of electrospray mass spectrometry (ES-MS) of carotenoids following LC separation on a C_{30} column [37]. Trifluoroacetic acid and heptafluorobutanol were added to enhance ionization of nonpolar compounds such as β-carotene. Detection limits of 0.7 pmol were determined for lutein and β-carotene. Compared to absorbance detection, ES-MS provided about a 100-fold increase in sensitivity. In another approach, the suitability of atmospheric pressure chemical ionization (APCI) for carotenoid analysis was studied [38]. Experiments were performed in the positive and negative ion modes, and limits of detection for α-carotene and lutein were ≈1 to 13 pmol, which is slightly higher than the ES-MS measurements. More recently, LC–APCI-MS was utilized with a C_{30} column, for determination of retinol and derivatives in human serum [39]. In contrast to ES ionization, APCI produced a linear response over at least three orders of magnitude. LC–APCI-MS has also been used by Lacker et al. for the determination of various carotenoids in standard mixtures as well as a vegetable juice extract [40].

A mild and efficient ionization technique called coordination ion-spray (CIS) MS was introduced by Rentel and co-workers for the determination of a variety of unsaturated compounds, including tocopherols and carotenoids [41–43]. Silver perchlorate was added to the isomers after separation with the C_{30} column. It was found that all carotenoids and most of the tocopherols formed $M+Ag^+$ ions; additionally radical ions ($M^{\cdot +}$) appear in the mass spectra. Four tocopherol isomers and tocopherol acetate were separated in about 10 min (see Fig. 4).

2.2. Carotenoids in foods

Carotenes occur naturally in fruits and vegetables, predominantly in the *trans* form [44]. *cis* Isomers are formed during thermal processing. Retinol (vitamin

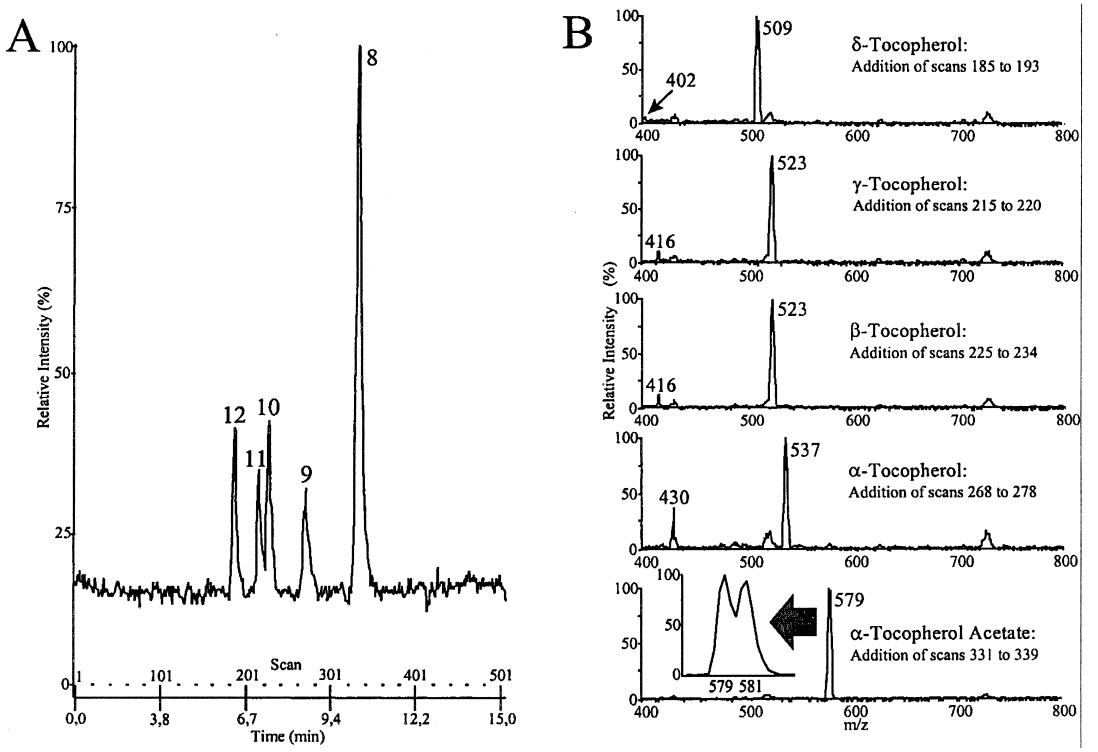

Fig. 4. Total ion chromatogram of tocopherol isomers separated on a C_{30} column with a 30 min linear gradient from acetone–water (90:10, v/v) to (100:0, v/v). Detection: Ag^+ enhanced electrospray mass spectrometry (from Ref. [43] with permission).

A) derivatives are formed from the metabolism of certain carotenoid isomers. The efficiency of this conversion depends on the form of the carotenoid and is referred to as provitamin A activity. Because the provitamin A activity of *cis* isomers is reduced by 50% or more compared with their *trans* counterparts [44], the capability for the determination of carotenoid isomers is required for the assessment of nutritional value of foods. Considerable effort has been directed toward the development of analytical methods for measurement of carotenoid isomers in foods.

Raw and thermally processed carrot samples were analyzed by Emenhiser et al. using a polymeric C_{30} column with mobile phase conditions optimized for carotenoid isomer standards [45] (Fig. 5). *trans* Forms of lutein and α- and β-carotene were found in raw carrot extracts. After thermal processing, samples were found to contain high levels of *cis* isomers, as expected as a consequence of thermal isomerization. Lessin et al. extended this work to include data for the effects of thermal processing on nine different fruits and vegetables, as well as vegetable soup [46].

Dachtler et al. utilized a polymeric C_{30} column with on-line solid-phase extraction, and NMR and absorbance detection for structural identification and quantification of lutein and zeaxanthin isomers in spinach [47]. By using on-line processes, potential sample degradation resulting from exposure to oxygen and/or light was minimized. Structures of the various isomers were elucidated by ^1H NMR, and resolution of the geometric isomers of lutein and zeaxanthin was achieved. Levels of lutein and zeaxanthin isomers ranged from 2 to 93 μg/g, with the highest levels representing *trans* carotenoids.

A polymeric C_{30} stationary phase was used in gradient elution capillary electrochromatography to separate an extract from a pilot batch of SRM 2383 Baby Food Composite [32]. This material was prepared by blending a variety of foods to produce a food matrix reference material with high levels of polar and nonpolar carotenoids from natural sources.

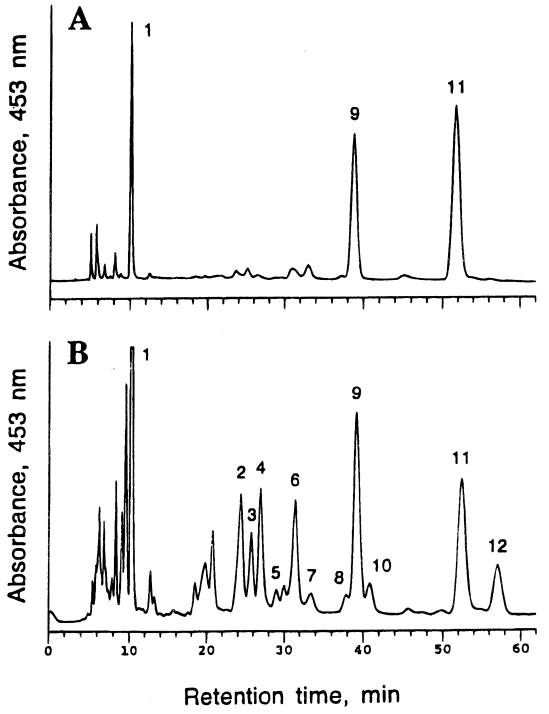

Fig. 5. Chromatograms for (a) raw and (b) thermally processed carrots, separated using a C_{30} column with a mobile phase of MTBE–methanol (11:89, v/v). Tentative peak identification: 1=*trans*-lutein; 2=13-*cis*-α-carotene; 3=*cis* α-carotene isomer; 4=13′-*cis*-α-carotene; 5=15-*cis*-β-carotene; 6=13-*cis*-β-carotene; 7–8=*cis*-β-carotene isomers; 9=*trans*-β-carotene; 10=9-*cis*-α-carotene; 11=*trans*-β-carotene and 12=9-*cis*-β-carotene (from Ref. [45] with permission).

The composite was extracted and saponified using a modification of the procedure of Hart and Scott [48,49]. Using a mobile phase gradient consisting of acetone and a borate buffer, excellent separations of lutein, zeaxanthin, β-cryptoxanthin, and isomers of β-carotene and lycopene were achieved in 30 min.

Citrus fruits contain a very large number of carotenoids, estimated in one study to exceed 115 components [50]. The analysis of orange juice for carotenoid content is of considerable commercial interest. Quantitative determination of total carotenoids, as well as levels of specific constituents (e.g., phytofluene and ξ-carotene), has been suggested as a way of determining the country of origin for juices. Carotenoid measurements may also prove useful in the determination of possible food adulteration (e.g., lycopene as an indicator of adulteration with pink grapefruit juice). Rouseff et al. utilized a polymeric C_{30} column to separate 39 carotenoids in saponified orange juice [51]. In these separations, β-carotene was the last identified component to elute. Identifications were tentatively made for 20 of the components based on literature spectral data. Careful choice of detection wavelengths permitted quantification of certain components even when complete chromatographic resolution could not be achieved. Mouly et al. used a similar chromatographic method to quantify 18 polar and nonpolar carotenoids in 23 orange juice samples [52] (see Fig. 6). Overall separation profiles were very similar to those reported by

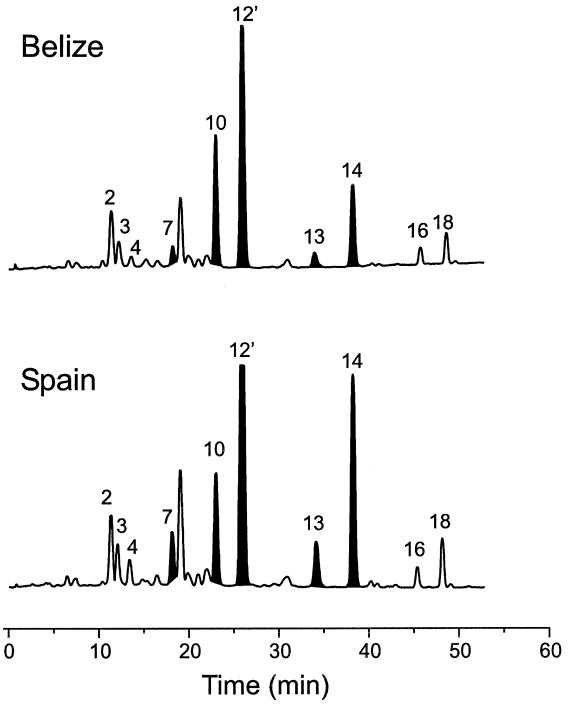

Fig. 6. Carotenoid profiles for pure Valencia orange juice from (a) Spain; and (b) Belize. Detection: 486 nm. Peak identification: 2=antheraxanthin; 3=*cis*-antheraxanthin; 4=neoxanthin; 7=*cis*-violaxanthin; 10=lutein; 12′=isolutein+zeaxanthin; 13=α-cryptoxanthin; 14=β-cryptoxanthin; 16=α-carotene; 18=β-carotene (modified from Ref. [52] with permission).

Rouseff. Total carotenoids were higher in juices obtained from Spain (17.0±5.0 mg/l) than from Belize (4.8±1.0 mg/l).

2.3. Carotenoids in other plants

High levels of carotenes are also found in plants not normally thought of as foods. For example, high levels of lutein are found in marigold flowers. Marigold extracts are commonly added to poultry feed supplements as a natural pigmenting agent. Delgado-Vargas and Paredes-López determined *cis* and *trans* isomers of lutein in saponified marigold extracts using the C_{30} column, and achieved baseline resolution of the components [53,54]. The primary constituent was identified as *trans*-lutein, but significant levels of 13- and 13′-*cis*-lutein and 9- or 9′-*cis*-lutein were also found. *cis*-Isomers of lutein esters were also identified in unsaponified extracts. A more complex separation resulted for the separation of saponified marigold carotenoids extracted from a poultry feed supplement [45].

Although *trans* isomers are the predominant form of carotenoids in most plant extracts, very high levels of *cis* isomers of β-carotene are known to occur in extracts of *Dunaliella* algae [1,32,45]. Levels of 9-*cis*-β-carotene differ, depending on growth conditions, and can exceed the level of the *trans* form. Van Heukelem et al. have investigated the use of polymeric C_{30} columns for the separation of various carotenoids [36] and chlorophyll pigments [55]. The combined use of temperature and solvent strength was studied for method optimization. In a subsequent study, Van Heukelem evaluated 11 reversed-phase columns for their utility in separating algal pigments [56]. C_8, C_{14}, C_{18}, and C_{30} stationary phases were included in the study. Separations of 28 pigment standards were optimized with the use of chromatographic modeling software. Although better separations of many classes of isomers are usually possible at ambient or subambient conditions, Van Heukelem demonstrated the best separation within the temperature range 45–55°C. Excellent overall separations of the pigments were possible with the C_{30} column; however, certain important components coeluted under the optimized conditions. Separation of these critical components could be achieved by altering column temperature and mobile phase conditions, but with poorer resolution of the remaining constituents. Schmid and Stich also evaluated a C_{30} column for possible application to marine plankton samples [57]. Although certain difficult-to-resolve compounds were separated (e.g., lutein and zeaxanthin; chlorophyll C_1 and chlorophyll C_2), other important pigments were not separated with the chosen mobile phase. Wiltshire et al. used a C_{30} column for characterization of particulate matter in estuarine environments [58]. Various pigments were measured as a way of monitoring transport and dynamics of the material.

2.4. Carotenoids in human serum

The effectiveness of C_{30} columns to resolve isomer mixtures of carotenoids and other nutrients in foodstuffs suggests that clinical measurements may also benefit from the use of these columns. The development of analytical methods may involve different approaches to sample extraction, clean-up, and detection, but the chromatographic conditions are similar.

Sharpless et al. compared the use of C_{18} and C_{30} columns in the measurement of carotenoids in SRM 968b and SRM 968c fat-soluble vitamins, carotenoids, and cholesterol in human serum (see Fig. 7a) [59–61]. Mobile phase conditions were developed separately for each column, and the resulting methods provided separations of carotenoids that differed in elution order and resolution. Measurements from both methods were used in the certification of the SRMs. Emenhiser et al. demonstrated the separation of polar and nonpolar carotenoids in human serum extracts, using a polymeric C_{30} column [45]. Extracts were first separated into two fractions with an alumina solid-phase extraction cartridge. One fraction contained polar carotenoids and lycopene, and the other fraction, other nonpolar carotenoids (e.g., α- and β-carotene). The first fraction was separated under isocratic conditions optimized for xanthophylls. This extract was also separated under stronger mobile phase conditions intended to resolve lycopene isomers. The second fraction was separated under intermediate isocratic conditions for best resolution of *cis* isomers of α- and β-carotene isomers.

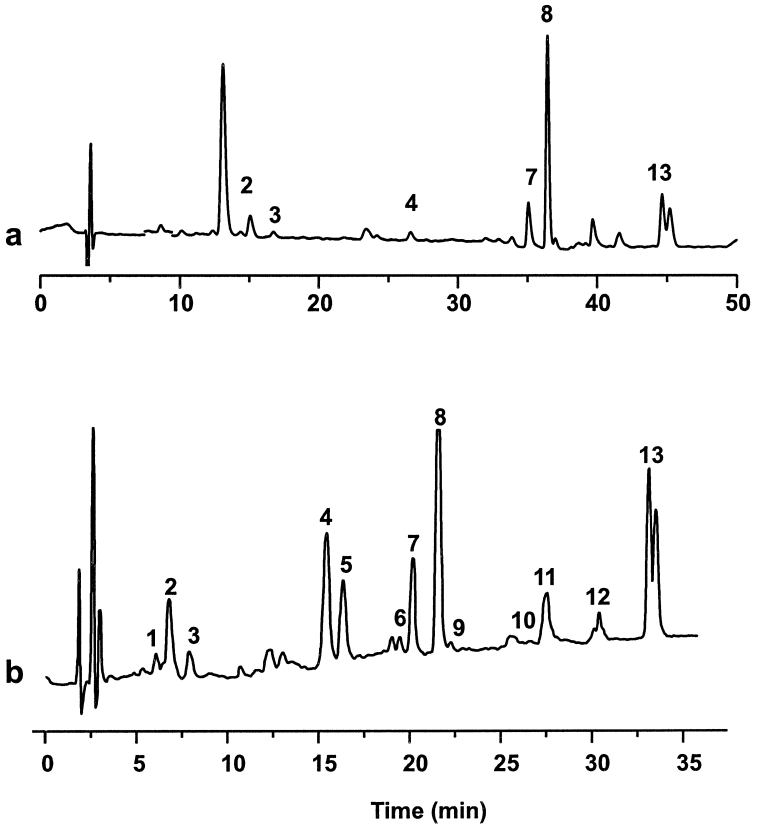

Fig. 7. Separation of the major carotenoids in human plasma. (a) Level II of SRM 968c fat-soluble vitamins, carotenoids, and cholesterol in human serum; (b) elevated levels of carotenoids after ingestion of a diet rich in fruits and vegetables. Peak identification: 1=lutein isomer; 2=lutein; 3=zeaxanthin; 4=β-cryptoxanthin; 5=echinenone (internal standard); 6=13-*cis*-β-carotene; 7=α-carotene; 8=*trans*-β-carotene; 9=ζ-carotene; 10=15-*cis*-lycopene; 11=13-*cis*-lycopene; 12=9-*cis*-lycopene; 13=*trans*-lycopene (from Refs. [61,62] with permission).

Twenty-eight peaks were assigned, however most assignments were tentative because of the lack of individual standards or NMR data.

C_{30} columns have been successfully applied to the analysis of human serum and plasma in studies of response to dietary carotenoid intake. Yeum et al. measured three xanthophylls (lutein, zeaxanthin, and β-cryptoxanthin), and three hydrocarbon carotenoids (α-carotene, β-carotene, and lycopene) in plasma for correlation with dietary intake [62]. *trans* Forms of the carotenoids and several *cis* isomers were quantified. Echinenone is not normally found in plasma samples and was utilized as an internal standard. Good overall separation of the carotenoids was achieved in less than 35 min (see Fig. 7b).

It is interesting to compare the two chromatograms of serum extracts in Fig. 7. Fig. 7b resulted from a study in which participants consumed diets high in fruits and vegetables, and Fig. 7a resulted from the certification analyses of SRM 968c, in which the plasma donors were consuming a more ‘normal’ diet. Carotenoids were eluted in Fig. 7b in 35 min using a 15-cm column prepared by YMC (Wilmington, NC, USA) with 3-μm particle size and in Fig. 7a in 50 min using a 25-cm column with a 5-μm particle size (prepared at NIST). Both chromatograms were obtained using mobile phases consisting of methanol, methyl *tert*.-butyl ether, and water. Aside from the differences in run time and the presence of additional peaks in the serum of the person consuming the high-carotenoid diet, the chromatograms originating from columns prepared by two ‘manufacturers’ using

different particle-size silica and used in two laboratories' analyses using two different methods are remarkably similar.

2.5. Carotenoids in tissue

The use of an electrochemical array detector was demonstrated in conjunction with a C_{30} column for the analysis of carotenoids in extracts of mg-size cervical tissue samples [63]. In addition to the improvement in sensitivity, the electrochemical array detector can aid in the identification of carotenoids and distinguish between certain *cis* and *trans* isomers.

Lycopene isomers have been measured in human prostate tissue using a method developed with the C_{30} column [64]. Analytes were separated isocratically using a mobile phase composition of MTBE–methanol (38:62). Levels of *cis* isomers relative to *trans* isomers were higher within prostate tissue extracts than within various food extracts. A correlation between reduced prostrate cancer risk and dietary intake of foods rich in lycopene was suggested.

The progression of age-related macular degeneration of the eye has been shown to be influenced by the concentration of lutein and zeaxanthin isomers [65]. Dachtler et al. described methodology for the analysis of these isomers in bovine retina [66]. Samples were extracted and concentrated by on-line solid-phase extraction. Analytical separations were carried out with a custom synthesized C_{30} column, with isocratic elution using acetone–water (85:15). All of the major geometric isomers of lutein and zeaxanthin were identified in retina extracts (see Fig. 8). In a subsequent study, levels of *cis* and *trans* isomers were quantified [47].

2.6. Retinoids

As with carotenoids, retinol and retinyl derivatives can exist in several isomeric forms. Mixtures of *cis* and *trans* retinoids are difficult to separate by RPLC, particularly with monomeric C_{18} columns. Although C_{30} phases have not provided complete separation of retinoic acid isomers, better separations have been achieved compared with monomeric C_{18} phases [35]. Five retinoic acid isomers were identified using the C_{30} column, whereas only three different isomers could be separated and identified with a C_{18} column.

As with carotenoids, temperature has been shown to significantly influence C_{30} column selectivity for retinyl acetate isomers [35,67]. The elution order of *trans*-retinyl acetate and four *cis* isomers was observed to change over a temperature interval of 50°C. At temperatures >35°C the *trans* isomer is most strongly retained and at most three isomers are separated. At subambient temperatures, the separation is improved and four isomers are separated. In each case, the 9-*cis* isomer was observed to elute after the *trans* isomer. For these separations, structural assignment of the respective isomers was made by LC–NMR.

Retinyl derivatives have been studied in detail by Van Breemen et al. [38,39,68]. In a human serum sample, retinol, retinyl acetate, and retinyl palmitate were separated and identified using LC–MS after sample extraction in hexane. Detection limits of approximately 0.7 pmol have been determined and limits of quantitation were $\approx$5 pmol.

Roed et al. utilized a capillary column packed with C_{30} modified 5-μm particles to separate retinyl esters obtained from arctic seal liver extracts [69,70]. Separations were made by capillary electrochromatography with a nonaqueous mobile phase consisting of 2.5 mmol/l lithium acetate in *N,N*-dimethylformamide–methanol (99:1, v/v). Increased resolution of the retinyl esters was achieved with this system, even when compared with separations made with a more efficient column packed with a 3 μm C_{18} column. In subsequent work, a C_{30} capillary column was operated with pressure driven flow [71]. Temperature programming was used as a convenient alternative to gradient elution for control of selectivity and retention. Improved overall resolution of retinyl esters in seal liver extracts was demonstrated for a temperature programmed method compared with an isothermal method. On column focusing of very large injection volumes was utilized to achieve a concentration limit of detection of 2.7 pg/μl.

2.7. Tocopherols and other vitamins

Tocopherol exists in four isomeric states, namely α-, β-, γ-, and δ-tocopherol. These isomers can be separated with normal-phase LC, but with reversed-

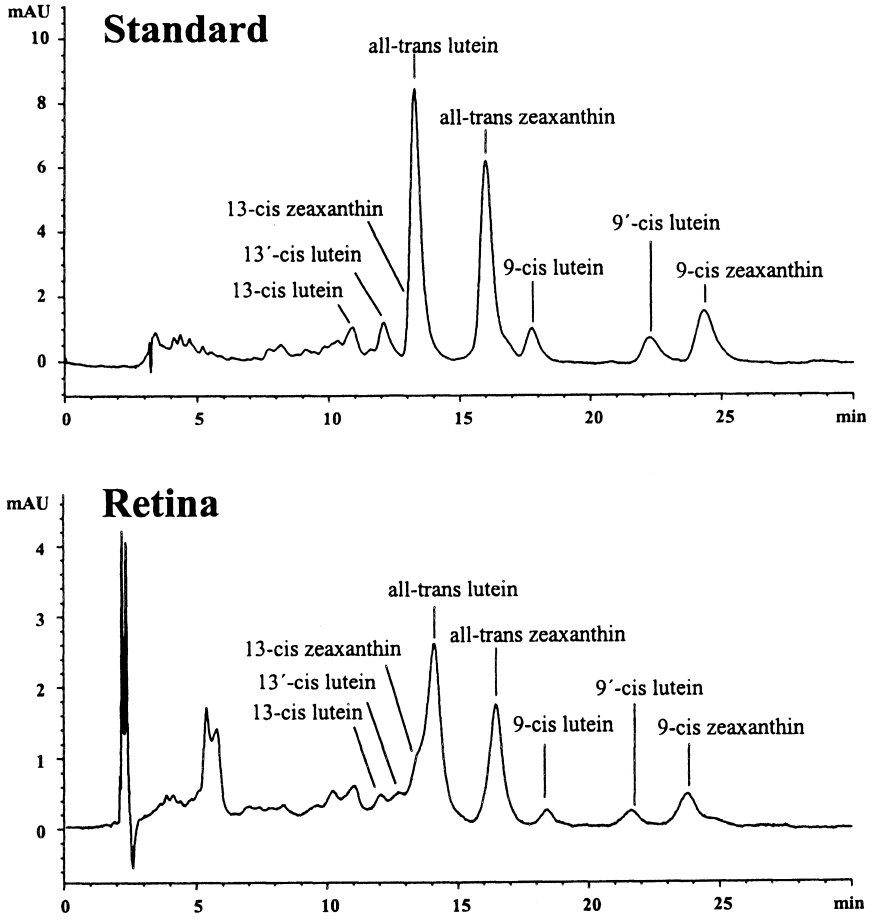

Fig. 8. Separation of a bovine retina extract with a C_{30} stationary phase (from Ref. [66] with permission).

phase LC and a C_{18} column, β- and γ-isomers coelute. Strohschein et al. demonstrated that separation of β- and γ-tocopherol is possible with a polymeric C_{30} column [26]. Separations were performed in less than 15 min using methanol as the mobile phase. Structural identification of the components was carried out by LC–NMR. Greater sample masses could be used with the polymeric C_{30} column than with C_{18} columns. This aspect of the polymeric C_{30} column was exploited to provide improved signal levels for on-line LC–NMR. Sample masses as large as 0.5 mg could be injected onto the column with only marginal loss in resolution.

Tocotrienols, tocoenol, and tocopherol extracted from Malaysian palm oil were also determined by LC–MS on the C_{30} column [41]. Unambiguous component identifications were made based on molecular mass, and further information on isomeric structure was obtained from LC–NMR. Both hyphenated methods were shown to be fast and powerful tools for analysis of plant extracts.

Recently, vitamin K_1 (phylloquinone) isomers in margarines and margarine-like products have been analyzed with the C_{30} phase [72]. *cis*/*trans* Isomers and a dihydro derivative of vitamin K_1 were well separated with the C_{30} column. Previously, separation of these compounds had not been achieved with conventional C_{18} columns. As with carotene isomers, the highest biological activity for vitamin K_1 is associated with the *trans* isomer. Less active *cis*-isomers are found in synthetic forms of vitamin K_1, and after exposure to light [73,74]. It was found

that liquid soybean oil or canola oil contain about 50–160 μg vitamin K_1 per 100 g. Less vitamin K_1 (<50 μg/100 g) is present in blends of sunflower and soybean oils. In general, higher-fat margarines contained more vitamin K_1 than their lower-fat counterparts.

3. Conclusions

Since their introduction 6 years ago, C_{30} columns have been widely used in the determination of carotenoids, retenoids, tocopherols, and related compounds with over 50 literature citations. The enhanced shape selective properties exhibited by these columns permit better separations of complex isomer mixtures than are typically possible with C_{18} columns, and measurements of individual geometric isomers are often feasible in complex, natural-matrix samples.

Acknowledgements

Financial support for MP from the Alexander-von-Humboldt foundation (Bonn, Germany) is gratefully acknowledged. Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

References

[1] L.C. Sander, K.E. Sharpless, N.E. Craft, S.A. Wise, Anal. Chem. 66 (1994) 1667.
[2] S.A. Wise, W.J. Bonnett, F.R. Guenther, W.E. May, J. Chromatogr. Sci. 19 (1981) 457.
[3] S.A. Wise, W.J. Bonnett, F.R. Guenther, W.E. May, in: A. Bjorseth, A.J. Dennis (Eds.), Polynuclear Aromatic Hydrocarbons: Chemistry and Biological Effects, Battelle Press, Columbus, OH, 1980, p. 791.
[4] K. Ogan, E. Katz, J. Chromatogr. 188 (1980) 115.
[5] R. Amos, J. Chromatogr. 204 (1981) 469.
[6] A. Colmsjo, J.C. MacDonald, Chromatographia 13 (1980) 350.
[7] L.C. Sander, S.A. Wise, Anal. Chem. 56 (1984) 504.
[8] L.C. Sander, S.A. Wise, in: R.M. Smith (Ed.), Retention and Selectivity Studies in HPLC, Elsevier, Amsterdam, 1994, p. 337, Ch. 10.
[9] K.S. Epler, L.C. Sander, R.G. Ziegler, S.A. Wise, N.E. Craft, J. Chromatogr. 595 (1992) 89.
[10] L.C. Sander, S.A. Wise, Anal. Chem. 59 (1987) 2309.
[11] K. Makino, H. Ozaki, H. Imaishi, T. Takeuchi, T. Fukui, Chem. Lett. (1987) 1251.
[12] I.Z. Atamna, G.M. Muschik, J. Liq. Chromatogr. 12 (1989) 2227.
[13] W. Poetter, S. Lamotte, H. Engelhardt, U. Karst, J. Chromatogr. A 786 (1997) 47.
[14] H. Ohta, K. Jinno, Y. Saito, J.C. Fetzer, W.R. Biggs, J.J. Pesek, M.T. Matyska, Y.L. Chen, Chromatographia 42 (1996) 56.
[15] J.J. Pesek, M.T. Matyska, E.J. Williamsen, M. Evanchic, V. Hazari, K. Konjuh, S. Takhar, R. Tranchina, J. Chromatogr. A 786 (1997) 219.
[16] J.J. Pesek, M.T. Matyska, S. Takhar, Chromatographia 48 (1998) 631.
[17] C.M. Bell, L.C. Sander, J.C. Fetzer, S.A. Wise, J. Chromatogr. A 753 (1996) 37.
[18] L.C. Sander, M. Pursch, S.A. Wise, Anal. Chem. 71 (1999) 4821.
[19] S.A. Wise, L.C. Sander, J. High Resolut. Chromatogr. Chromatogr. Commun. 8 (1985) 248.
[20] Z. Matus, R. Ohmacht, Chromatographia 30 (1990) 318.
[21] L.C. Sander, S.A. Wise, J. Chromatogr. 316 (1984) 163.
[22] M. Pursch, L.C. Sander, K. Albert, Anal. Chem. 71 (1999) 733A.
[23] K. Albert, T. Lacker, M. Raitza, M. Pursch, H.J. Egelhaaf, D. Oelkrug, Angew. Chem. Int. Ed. Engl. 37 (1998) 777.
[24] K. Albert, Trends Anal. Chem. 17 (1998) 648.
[25] M. Raitza, M. Pursch, S. Strohschein, L.C. Sander, K. Albert, GIT Fachz. Lab. 3 (1998) 237.
[26] S. Strohschein, M. Pursch, D. Lubda, K. Albert, Anal. Chem. 70 (1998) 13.
[27] M. Pursch, R. Brindle, A. Ellwanger, L.C. Sander, C.M. Bell, H. Haendel, K. Albert, Solid State NMR 9 (1997) 191.
[28] L.C. Sander, J.B. Callis, L.R. Field, Anal. Chem. 55 (1983) 1068.
[29] L.C. Sander, S.A. Wise, Anal. Chem. 61 (1989) 1749.
[30] C. Emenhiser, L.C. Sander, S.J. Schwartz, J. Chromatogr. A 707 (1995) 205.
[31] C. Emenhiser, G.E. Englert, L.C. Sander, B. Ludwig, S. Schwartz, J. Chromatogr. A 719 (1996) 333.
[32] L.C. Sander, M. Pursch, B. Märker, Anal. Chem. 71 (1999) 3477.
[33] S. Strohschein, M. Pursch, H. Handel, K. Albert, Fresenius J. Anal. Chem. 357 (1997) 498.
[34] C.M. Bell, L.C. Sander, S.A. Wise, J. Chromatogr. A 757 (1997) 29.
[35] M. Pursch, S. Strohschein, H. Handel, K. Albert, Anal. Chem. 68 (1996) 386.

[36] P.L. Zhu, J.W. Dolan, L.R. Snyder, N.M. Djordjevic, D.W. Hill, J.-T. Lin, L.C. Sander, L. Van Heukelem, J. Chromatogr. A 756 (1996) 63.
[37] R.B. Van Breemen, Anal. Chem. 67 (1995) 2004.
[38] R.B. Van Breemen, C. Huang, Y. Tan, L.C. Sander, A. Schilling, J. Mass Spectrom. 31 (1996) 975.
[39] R.B. Van Breemen, D. Nikolic, X.Y. Xu, Y.S. Xiong, M. van Lieshout, C.E. West, A.B. Schilling, J. Chromatogr. A 794 (1998) 245.
[40] T. Lacker, S. Strohschein, K. Albert, J. Chromatogr. A 854 (1999) 37.
[41] S. Strohschein, C. Rentel, T. Lacker, E. Bayer, K. Albert, Anal. Chem. 71 (1999) 1780.
[42] E. Bayer, P. Gfrorer, C. Rentel, Angew. Chem. Int. Ed. Engl. 38 (1999) 992.
[43] C. Rentel, S. Strohschein, K. Albert, E. Bayer, Anal. Chem. 70 (1998) 4394.
[44] L. Zechmeister, *cis–trans* Isomeric Carotenoids, Vitamins A, and Arylpolyenes, Academic Press, New York, 1962.
[45] C. Emenhiser, N. Simunovic, L.C. Sander, S.J. Schwartz, J. Agric. Food Chem. 44 (1996) 3887.
[46] W.J. Lessin, G.L. Catigani, S.J. Schwartz, J. Agric. Food Chem. 45 (1997) 3728.
[47] M. Dachtler, T. Glaser, K. Kohler, K. Albert, Determination and qunatification of lutein and zeaxanthin stereoisomers in spinach and in the retina with on-line LC–NMR coupling, Anal. Chem., 2000, submitted for publication.
[48] D.J. Hart, K.J. Scott, Food Chem 54 (1995) 101.
[49] K.E. Sharpless, M. Arce-Osuna, J. Brown Thomas, L.M. Gill, J. AOAC Int. 82 (1999) 288.
[50] I. Stewart, T.A. Wheaton, Phytochemistry 12 (1973) 2947.
[51] R. Rouseff, L. Raley, H.J. Hofsommer, J. Agric. Food Chem. 44 (1996) 2176.
[52] P.P. Mouly, E.M. Gaydou, J. Corsetti, J. Chromatogr. A 844 (1999) 149.
[53] F. Delgado-Vargas, O. Paredes-López, J. Sci. Food Agric. 72 (1996) 283.
[54] F. Delgado-Vargas, O. Paredes-Lopez, J. Agric. Food Chem. 45 (1997) 1097.
[55] P.L. Zhu, J.W. Dolan, L.R. Snyder, D.W. Hill, L. Van Heukelem, T.J. Waeghe, J. Chromatogr. A 756 (1996) 51.
[56] L. Van Heukelem, C.S. Thomas, Chromatography modeling software in pigment method development: an evaluation of eleven HPLC columns, Mar. Chem., 2000, submitted for publication.
[57] H. Schmid, H.B. Stich, J. Appl. Phycol. 7 (1995) 487.
[58] K.H. Wiltshire, L. Villerius, F. Schroeder, D.M. Paterson, Pigments in Particulate Matter — New Methods and Their Application in Estuarine Environments. American Society of Limnology and Oceanography, Feb. 10–14, 1997, Sante Fe, NM, 1997.
[59] J. Brown Thomas, M.C. Kline, S.B. Schiller, P.M. Ellerbe, L.T. Sniegoski, D.L. Duewer, K.E. Sharpless, Fresenius' J. Anal. Chem. 356 (1996) 1.
[60] K.E. Sharpless, J. Brown Thomas, L.C. Sander, S.A. Wise, J. Chromatogr. B 678 (1996) 187.
[61] Certificate of Analysis, SRM 968c Fat-Soluble Vitamins and Cholesterol in Human Serum. Office of Standard Reference Materials, NIST, Gaithersburg, MD, 1999.
[62] K.J. Yeum, S.L. Booth, J.A. Sadowski, C. Liu, G.W. Tang, N.I. Krinsky, R.M. Russell, Am. J. Clin. Nutr. 64 (1996) 594.
[63] M.G. Ferruzzi, L.C. Sander, S. Schwartz, Anal. Biochem. 256 (1998) 74.
[64] S.K. Clinton, C. Emenhiser, S.J. Schwartz, D.G. Bostwick, A.W. Williams, B.J. Moore, J.W. Erdman, Cancer Epidem. Biomarker Prev. 5 (1996) 823.
[65] R.A. Bone, J.T. Landrum, L.M. Friedes, C.M. Gomex, M.D. Kilburn, E. Denendez, I. Vidal, W. Wang, Exp. Eye Res. 64 (1997) 211.
[66] M. Dachtler, K. Kohler, K. Albert, J. Chromatogr. B 720 (1998) 211.
[67] S. Strohschein, G. Schlotterbeck, J. Richter, M. Pursch, L.H. Tseng, H. Handel, K. Albert, J. Chromatogr. A 765 (1997) 207.
[68] R.B. Van Breemen, C. Huang, Faseb J. 10 (1996) 1098.
[69] L. Roed, E. Lundanes, T. Greibrokkk., in: 21st International Symposium on Capillary Chromatography and Electrophoresis, 30 June, 1999.
[70] L. Roed, E. Lundanes, T. Greibrokk, Electrophoresis 20 (1999) 2373.
[71] P. Molander, S.J. Thommesen, I.A. Bruheim, R. Trones, T. Greibrokk, E. Lundanes, T.E. Gundersen, J. High Resolut. Chromatogr. 22 (1999) 490.
[72] K.K. Cook, G.V. Mitchell, E. Grundel, J.I. Rader, Food Chem. 67 (1999) 79.
[73] S. Hwang, J. Assoc. Off. Anal. Chem. 68 (1985) 684.
[74] H. Indyk, Milchwissenschaft 43 (1988) 8.