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Temperature dependence of carotenoids on C_{18}, C_{30} and C_{34} bonded stationary phases

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Abstract

The influence of temperature on the liquid chromatographic retention of carotenoids was assessed for C_{18}, C_{30} and C_{34} stationary phases. Linear or mostly linear van't Hoff plots were observed for all solutes on the C_{18} stationary phase, but solutes displayed a variety of retention behaviors in response to temperature on the C_{30} and C_{34} stationary phases. Bent carotenoids and non-planar PAHs exhibited unusual retention behavior on the long-chain phases, displaying increases in retention with increasing temperature in some regions of the temperature range studied. Chromatograms are shown that illustrate how these varying responses can be used to optimize carotenoid separations.

Keywords: Column temperature; Temperature effect; Stationary phases, LC; Carotenoids; Polynuclear aromatic hydrocarbons

1. Introduction

Carotenoids are tetraterpenes found in many forms in the plant kingdom. Both hydrocarbon (non-polar) and oxygenated (polar) carotenoids exist in addition to many *cis* and *trans* isomers of the various carotenoids. The separation and measurement of carotenoids have received considerable attention recently due to their nutritional and biological importance [1–5]. Some studies have linked consumption of β-carotene, lycopene and other carotenoids to decreased risks of cardiovascular disease [6–8] and certain kinds of cancer [9–15], although more recent data have introduced some doubt as to those conclusions [16–18]. Separation and measurement of the individual carotenoids present in complex natural samples are necessary for the study of their varying biochemical properties. These separations are usually performed using liquid chromatography (LC), although several papers describing the separation of carotenoids by supercritical fluid chromatography have appeared in recent years [19–21]. Many variables have been investigated in order to optimize these separations, including chromatographic mode, column type, mobile phase and temperature.

Some normal-phase LC separations of carotenoids have been accomplished using polar adsorbent stationary phases such as calcium hydroxide [22–24]. These phases give excellent separations, but their lack of commercial availability, extreme sensitivities to water content and temperature, and long equilibration times limit their practicality for most analyses. In reversed-phase LC (RPLC), the majority of carotenoid separations have been performed using C_{18} stationary phases [25,26]. Both monomeric and

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polymeric C_{18} phases [27] have been employed, although increased shape selectivity, i.e., ability to separate carotenoid isomers, has been demonstrated for polymeric C_{18} phases [28–31]. A substantial advance in RPLC of carotenoids was realized with the introduction of a polymeric C_{30} stationary phase in 1994 by Sander et al. [32–35]. Even more recently, Bell et al. [36] have synthesized an extended length ($>C_{34}$) stationary phase with improved selectivity for carotenoids. These latter two phases demonstrated that separations of large rigid molecules can be significantly improved by using stationary phases whose ligand chain lengths are equal to or exceed the molecular sizes of the solutes of interest.

A variety of mobile phases have been employed for reversed-phase LC of carotenoids, the majority being methanol or acetonitrile, modified with methylene chloride, chloroform, THF, methyl *tert.*-butyl ether or ethyl acetate. In general, methanol-based mobile phases appear to be preferred over acetonitrile based ones [34], although the use of some acetonitrile to alter selectivity is warranted in many cases [29,37]. Importantly, recoveries of carotenoids are also better with methanol-based systems [28,37]. Methylene chloride, THF and methyl *tert.*-butyl ether appear to be the best modifiers for optimizing selectivities as well as reducing analysis times. Methylene chloride, however, is often contaminated with trace amounts of HCl which has been implicated in poor carotenoid recoveries [38]. Finally, many methods for carotenoid separations call for the use of additives such as ammonium acetate or triethylamine to increase carotenoid recoveries or improve peak shapes [35,37].

In contrast to the effects of other variables, the effect of temperature on reversed-phase LC separations of carotenoids has received only minor attention [29–31,39]. For liquid chromatography in general, numerous studies have been published concerning the effects of temperature on chromatographic systems [40–50]. Despite this, appreciation of temperature as a variable for improving LC separations is still not widespread. Many studies of temperature effects are concerned with elucidating retention mechanisms and not necessarily with improving separations. In fact, when temperature is controlled in LC methods, it is often merely held near ambient as a way of guaranteeing reproducibility of retention times.

Most studies examining the effect of temperature on carotenoid separations in RPLC have been concerned with the effect on α- and β-carotene retention on C_{18} columns [29–31]. These studies showed that capacity factors for these solutes decrease with increasing temperature. Slightly non-linear van't Hoff plots indicated that a change in the nature of the solute–stationary phase interaction may take place for some solutes. Some researchers attribute this to a phase transition that may occur in these phases [30,31]. Significant differences in selectivity, however, were not observed as a result of these changes.

No report to date has examined the effects of temperature on the separation of carotenoids on stationary phases longer than C_{18}. Because C_{30} and longer phases provide significantly improved separations, and preliminary experiments indicated that temperature could be an important variable in carotenoid separations, a more in depth study was undertaken. In the present study, temperature effects on the retention of carotenoids on C_{18}, C_{30} and C_{34} columns during isocratic elution were studied. Both polar and non-polar carotenoids, including many *cis* isomers of α- and β-carotene were studied (Fig. 1). These *cis* isomers were not included in previous studies because they are not resolved on most C_{18} columns. This study was undertaken to investigate the effects of temperature on a variety of stationary phases and carotenoid solutes and to illustrate the utility of temperature as a chromatographic variable. It will be shown that different carotenoids, e.g., xanthophylls, all-*trans* non-polar carotenoids and *cis*, non-polar carotenoids, exhibit varying behaviors in response to temperature changes. These differences may be due to differences in retention mechanisms and/or differences in bonded phase chain mobility and conformation. In addition, some similar "irregular" temperature dependencies of polycyclic aromatic hydrocarbons (PAHs) on C_{30} columns will be discussed.

2. Experimental

2.1. Materials

Carotenoid solutes used in the present study were obtained from a variety of sources. All-*trans*-α- and

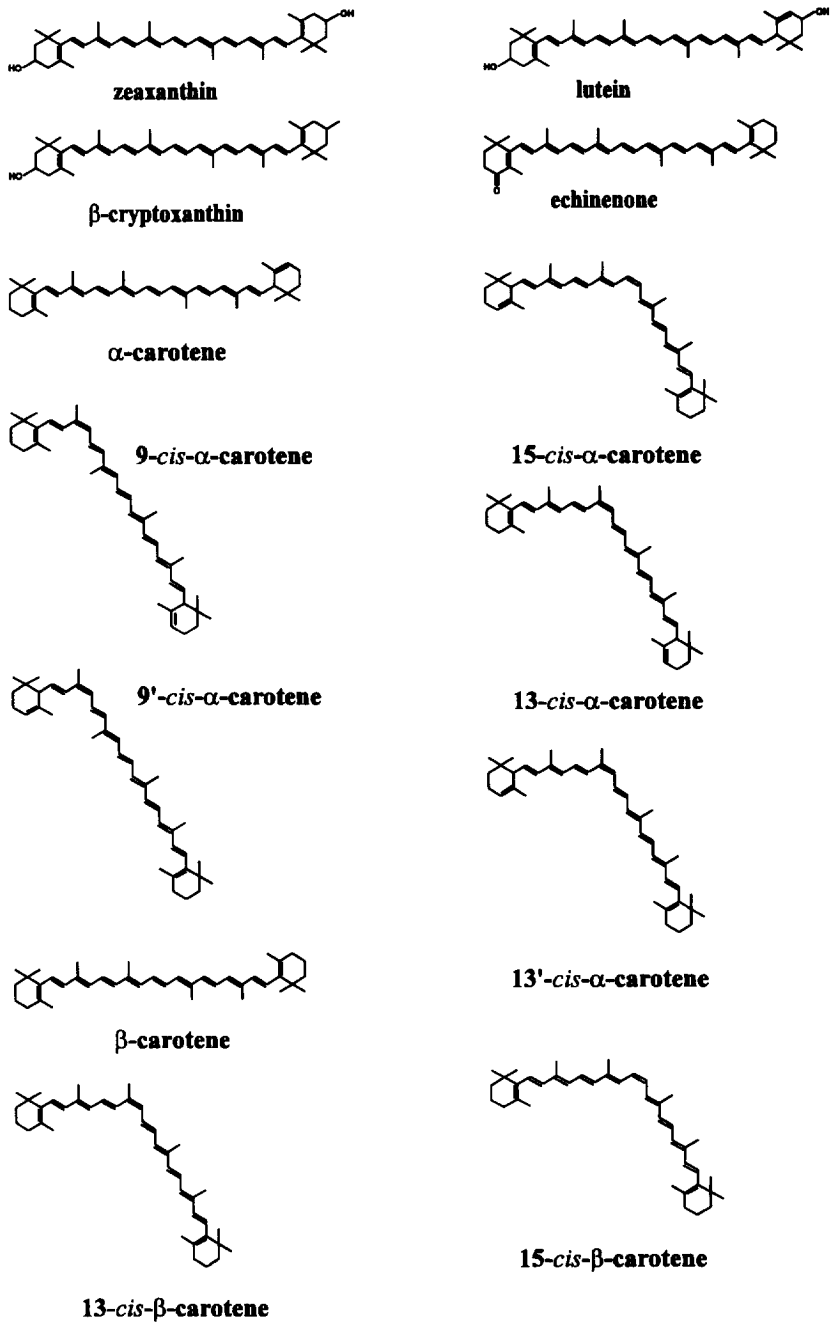

Fig. 1. Structures of carotenoids used in this study.

β-carotene were obtained from Sigma (St. Louis, MO, USA); lutein, zeaxanthin, echinenone and 15- and 13-*cis*-β-carotene were gifts from Hoffman-LaRoche (Nutley, NJ, USA and Basel, Switzerland); β-cryptoxanthin was obtained from Atomergic Chemetals (Farmingdale, NY, USA). *Cis* isomers of α-carotene were obtained by catalytic photoisomerization of an α-carotene standard according to a previously reported procedure [33,36,51]. The resulting mixture was injected without further purifica-

tion, and the isomer peaks were identified by comparison of the retention profiles with previous reports [22,33]. Standard Reference Material (SRM) 869, "Column Selectivity Test Mixture for Liquid Chromatography", was obtained from the NIST Standard Reference Materials Program (NIST, Gaithersburg, MD, USA). A commercial polymeric C_{18} column, Hypersil Green PAH (Shandon HPLC, Cheshire, UK), was used along with C_{30} [32] and C_{34} [36] columns that were prepared in our laboratory. All columns were 250 mm×4.6 mm I.D., and HPLC-grade solvents were used in all chromatographic separations.

2.2. Liquid chromatography

Separations of carotenoids were carried out using isocratic mobile phases of 100% methanol for the C_{18} column, and 95% methanol, 5% methyl *tert.*-butyl ether (volume percent) for the C_{30} and C_{34} columns. Separations of NIST SRM 869 were performed using isocratic mobile phases of 90% methanol, 10% water for the C_{18} column and 85% methanol, 15% water for the C_{30} column. The flow-rate in all cases was 2 ml/min, and the column temperature was controlled within ±0.1°C by a circulating water jacket. Carotenoids were detected at 450 nm and PAHs at 254 nm. Void volumes were determined by injecting acetone and detecting at 254 nm.

3. Results and discussion

The use of temperature in investigations of liquid chromatographic retention mechanisms is widespread. The thermodynamic relationship between the capacity factor (k') and temperature in Kelvin (T), as derived from free energy relationships is

$$\ln k' = -\Delta H/RT + \Delta S/R + \ln \phi \quad (1)$$

This is called the van't Hoff equation where ΔH is the enthalpy of transfer of the solute from the mobile phase to the stationary phase, ΔS is the entropy of transfer of the solute from the mobile phase to the stationary phase, R is the ideal gas constant, and ϕ is the volume phase ratio of the stationary and mobile phases. A plot of $\ln k'$ versus $1/T$ gives a slope of $-\Delta H/R$ and an intercept of $\Delta S/R + \ln\phi$. These plots can therefore be used to calculate enthalpies and entropies of transfer, although the latter can be difficult to determine owing to the non-trivial nature of calculating the phase ratio, especially for commercial columns and columns for which the nature of the ligand bonding is ambiguous. A linear van't Hoff plot is indicative of a ΔH that is invariant with temperature, and these have been reported for many solutes on both monomeric and polymeric C_{18} stationary phases [41,49,50,52]. Non-linear van't Hoff plots suggest a change in the nature of the interactions between the solute and the mobile phase or between the solute and the stationary phase or both. Because both ΔH and ΔS can be functions of temperature, non-linearity of van't Hoff plots may reflect a change in the relative contributions of these factors on retention. For example, in systems where the hydrophobic effect is the dominant force for retention, i.e., ones with highly aqueous mobile phases, it has been shown that at low temperatures, entropic contributions drive retention as both ΔH and ΔS are positive, while at higher temperatures, ΔH is negative and enthalpic forces contribute as well [47]. The changes in these relative contributions and possible changes in the retention mechanism are often thought to be related to conformational changes in the stationary phase. Some evidence for a phase transition in C_{18} stationary phases has been reported in the form of differential scanning calorimetry (DSC) and NMR data [40,45,46,53]. More generally, it can be said that lower temperatures induce greater rigidity and straightening of the ligand chains, while at higher temperatures, the chains are more mobile and disordered [53,54].

Figs. 2–4 show van't Hoff plots for a variety of carotenoids on C_{18}, C_{30} and C_{34} stationary phases, respectively. For the dihydroxy polar carotenoids, lutein and zeaxanthin, on the C_{18} phase (Fig. 2), the plots are linear with approximately the same slopes. Echinenone and β-cryptoxanthin also give linear plots with only slightly different slopes. The non-polar carotenoids, α- and β-carotene, have slightly non-linear lines that flatten as the temperature is increased. The plots of the 13- and 15-*cis* isomers of β-carotene are linear but their slopes differ from that of β-carotene such that at high temperatures, these

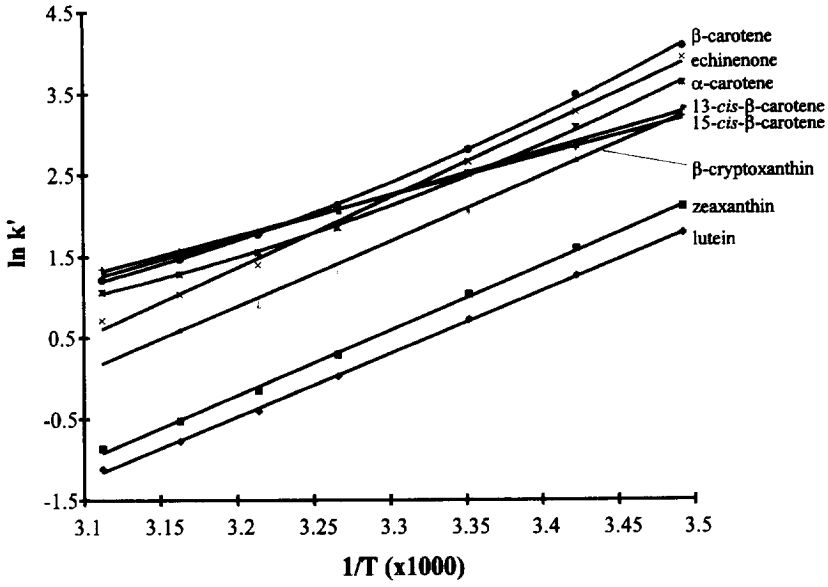

Fig. 2. Van't Hoff plots of selected carotenoids on a C_{18} stationary phase. Conditions are described in Section 2.2.

isomers elute after β-carotene. It should be noted, however, that column selectivity toward these isomers and β-carotene is quite small on C_{18} stationary phases at elevated temperatures.

For the C_{30} and C_{34} stationary phases, temperature has a distinctly different effect on the retention of some carotenoids. For example, for lutein and zeaxanthin, the van't Hoff plots are non-linear and appear to flatten out and diverge from one another as the temperature is decreased. It is believed that the

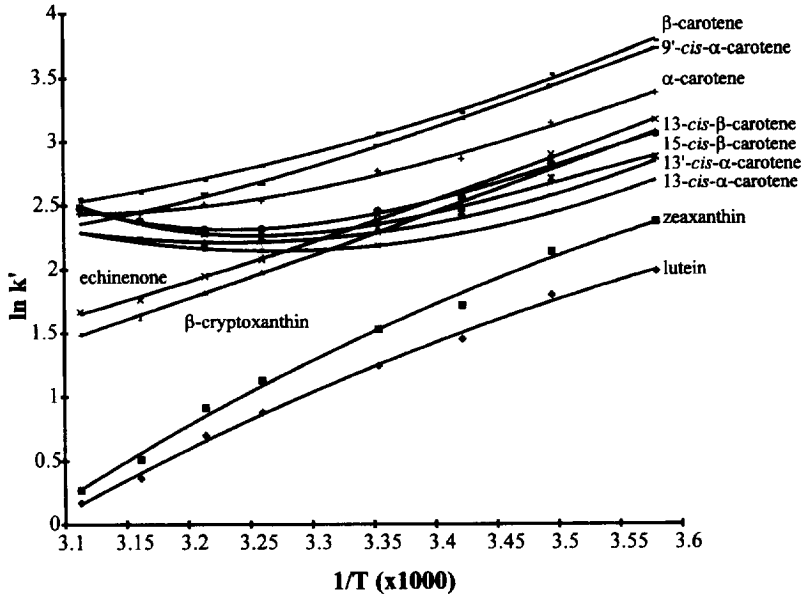

Fig. 3. van't Hoff plots of selected carotenoids on a C_{30} stationary phase. Conditions are described in Section 2.2.

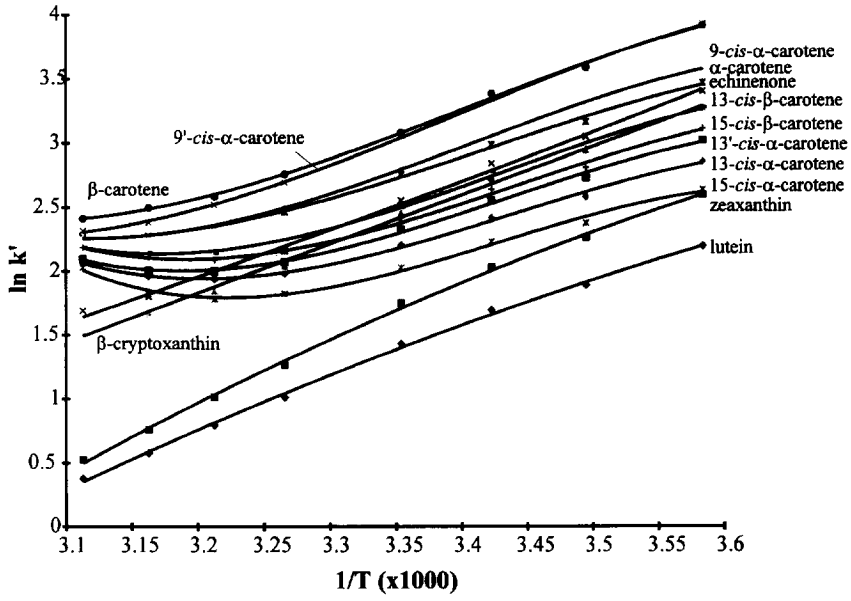

Fig. 4. Van't Hoff plots of selected carotenoids on a C_{34} stationary phase. Conditions are described in Section 2.2.

retention of these polar carotenoids involves interaction with silanols on or near the surface of the silica, and that retention results from a combination of mechanisms [32]. For the C_{18} stationary phase, ΔH is invariant with temperature, suggesting that the mechanism of retention is constant. The non-linear nature of the van't Hoff plots on the C_{30} and C_{34} stationary phases indicates temperature dependent changes in the solute–stationary phase interactions. The increased rigidity and order induced by the lower temperature may prevent the solutes from penetrating all the way to the surface and interacting with silanols, thus changing the relative contributions of solute–silanol and solute–bonded phase interactions to retention. In this case, each contributing mechanism would be governed by an equation of the form of Eq. (1), but the overall effect would not be so correlated. For the shorter C_{18} phase, this effect is not apparent in the temperature range studied here. However, on the C_{30} and C_{34} phases, the plots for the oxygenated carotenoids, echinenone and β-cryptoxanthin, are essentially linear with approximately equal slopes.

For all-*trans*, i.e., straight chain, non-polar carotenoids α- and β-carotene, the effect is nearly opposite. The deviation from linearity leads to a reduction in slope of the fitted line at higher temperatures. For these compounds, as has been observed in some previous studies, the deviation from linearity is slight and does not appear to have a significant effect on selectivity. These plots may be indicative of a continuous transition from liquid-like (i.e., disordered) properties at higher temperatures to solid-like (ordered) properties at lower temperatures, which does not alter the fundamental nature of the solute–stationary phase interaction.

A dramatic effect of temperature is seen for bent, i.e., *cis*, isomers of α- and β-carotene on C_{30} and C_{34} stationary phases. For 13- and 15-*cis*-β-carotene, and 13-, 13′-, and 15-*cis*-α-carotene, after an initial decrease, the capacity factors actually increase at temperatures above approximately 35°C. This means that at some point as the temperature increases, interaction of the solutes with the stationary phase increases. The cause of this change is not entirely clear, but is believed to be primarily a consequence of the solutes' greater accessibility to and overlap with the stationary phase. Solid-state NMR data for C_{30} interphases show that as the temperature is increased, the ligand chains of the phase become more mobile and more disordered with an increase in kinks and bends [55]. Generally, this kind of data is

used to explain why shape selectivities on C_{18} and C_{30} stationary phases increase with decreasing temperature [49]. The increased order and rigidity of stationary phases at lower temperatures induce preferential retention of long, narrow solutes, and partial exclusion of bent, bulky solutes. Conversely, the greater chain mobility and less rigid conformation of the stationary phases at higher temperatures may increase the contact area available for interaction by bulky solutes. In addition, the greater chain mobility may lower the barrier for bent solutes to penetrate the stationary phase.

We have also observed unusual temperature effects for some PAHs on the C_{30} stationary phase. The top of Fig. 5 shows van't Hoff plots for the three components of the NIST SRM 869 "Column Selectivity Test Mixture for Liquid Chromatography" on a C_{30} stationary phase. This mixture, which is used to assess the shape selectivity of C_{18} columns,

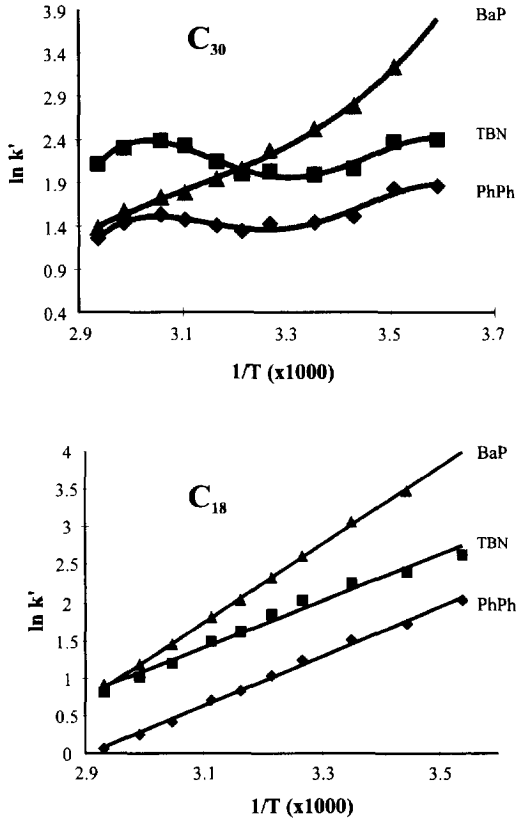

Fig. 5. Van't Hoff plots of the PAHs in NIST SRM 869 on C_{30} and C_{18} stationary phases. Conditions are described in Section 2.2.

consists of both planar and non-planar solutes [27,56]. For benzo[*a*]pyrene (BaP), a planar PAH, the van't Hoff plot is only slightly non-linear with behavior similar to that of the linear carotenoid, *trans*-β-carotene. The bulkier, non-planar PAHs, phenanthro[3,4-*c*]phenanthrene (PhPh) and tetrabenzonaphthalene (TBN), however, have van't Hoff plots that resemble those of the bent carotenoids with some differences. As the temperature is increased, the retention of the PAH solutes decreases and then increases before beginning to decrease again. Because the separation of the PAHs required a different mobile phase (85% methanol, 15% water) which allowed for the use of a wider temperature range, direct comparison of the PAH plots with the carotenoid plots is not possible. Nevertheless, the observation of some increase in retention for bulkier solutes with increasing temperature is consistent. Comparable temperature studies of SRM 869 on a C_{18} stationary phase yielded linear van't Hoff plots with a greater slope for BaP compared to those of PhPh and TBN (Fig. 5). This is similar to the behavior shown in Fig. 2 where the retentions of the bent solutes, 15- and 13-*cis*-β-carotene decreased more slowly than that of *trans*-β-carotene as the temperature was increased. These observations on C_{18} stationary phases are in agreement with an early study by Snyder [42] that showed that bulkier solutes gave increased retention (relative to other solutes) with increasing temperature. The differences between the retention of bulky and flat solutes in those systems were said to be "entropy dominated" [41] and the effect was expected to be more pronounced for highly ordered phases. Finally, in a new study aimed at predicting temperature effects on retention for many classes of solutes, Zhu and coworkers [57] have also observed non-ideal temperature dependencies for some PAHs on polymeric C_{18} stationary phases.

Jinno et al. [53] have seen a similar effect for bulky fullerenes on monomeric C_{18} and polymeric C_{30} phases. For C_{18} and C_{30} stationary phases, they showed that retention of C_{60} increased linearly with increasing temperature until a certain temperature (−30°C for C_{18} and +10°C for C_{30}) at which time the retention became essentially constant. The temperatures at which the changes occurred were associated with changes in the NMR spin–spin relaxation

times, which are related to the order and rigidity of the phases. However, for C_{18} stationary phases that consisted of ligands with phenyl substituents on the silicon of the silane, typical van't Hoff behavior was observed, i.e., a linear increase in ln k' with $1/T$. This was probably because the phenyl groups forced the ligand chains to be widely enough spaced for the large fullerene solutes to easily penetrate the stationary phase at all temperatures within the range studied.

Similar observations concerning retention changes for bulky solutes have been reported for PAHs. Sander and Wise [58] recently showed that for a variety of PAH solutes on C_{18} phases, retention increased with increasing surface coverage up to a maximum, at which point retention decreased. This was in agreement with the retention model of Dill [59] and the work of Sentell and Dorsey [60], and, significantly, showed that the maximum retention was reached at lower surface coverages for the bulkier, non-planar PAHs. Dill described this maximum as occurring because at this surface coverage, the "energetic cost" of creating a cavity for the solute in the stationary phase becomes large. It is probable that in our studies with bent carotenoids and PAHs on long-chain stationary phases, increasing the temperature helps to overcome this "cost."

While investigations of chromatographic retention mechanisms are both interesting and important, the goal of most analysts is improved separations. In the case of carotenoids, it is clear that temperature can be a significant factor in improving separations. Temperature control is crucial to the reproducibility of retention times which is vital to correct peak identification in complicated mixtures. In addition, because of the different behavior of various carotenoids, some separations may be optimized at temperatures above or below ambient.

It is interesting to use van't Hoff plots to determine a temperature at which a separation is optimized. The variety of solutes studied in this report provides a good example of this strategy. Because C_{30} stationary phases have obvious selectivity advantages over C_{18} phases and the slightly improved C_{34} stationary phases are not commercially available, a C_{30} stationary phase is used for this illustration. Figs. 6–8 display chromatograms of a variety of carotenoids on a C_{30} stationary phase at

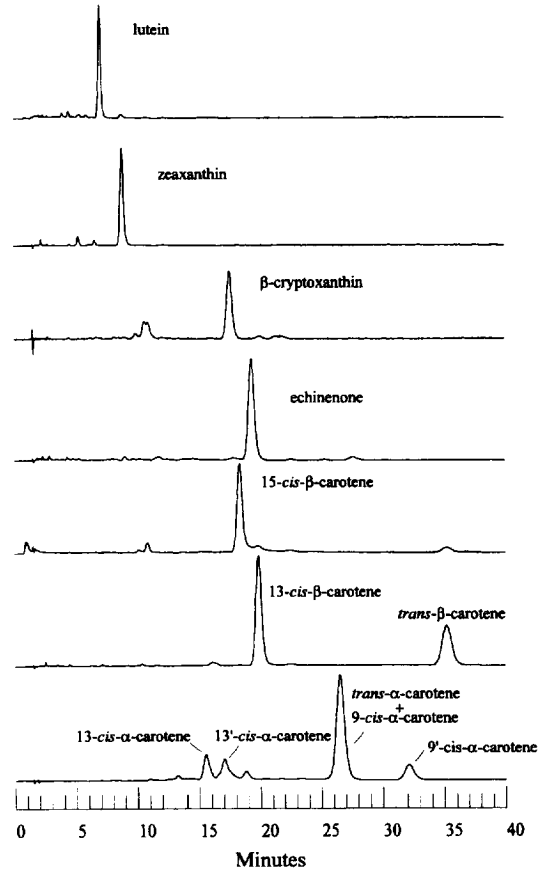

Fig. 6. Chromatograms of a variety of carotenoids on a C_{30} stationary phase at 25°C. Conditions are described in Section 2.2.

25°C, 13°C and 38°C, respectively. It can be seen that at 25°C, separations of related pairs such as lutein and zeaxanthin, and 15- and 13-*cis*-β-carotene, are good, but that several compounds are bunched together, including 13- and 15-*cis*-β-carotene, β-cryptoxanthin, echinenone, and some *cis* isomers of α-carotene. Lowering the temperature to 13°C increases the selectivity for many isomers including that of α-carotene and its 9-*cis* isomer, but 13-*cis*-β-carotene and β-cryptoxanthin coelute and the retention times become long. Raising the temperature to 38°C has two major effects. The first is some loss of selectivity, especially between 15- and 13-*cis*-β-carotene, which are now only partially resolved. Secondly, because at this temperature the different behaviors of the solutes in response to temperature begin to take effect, β-cryptoxanthin and echinenone

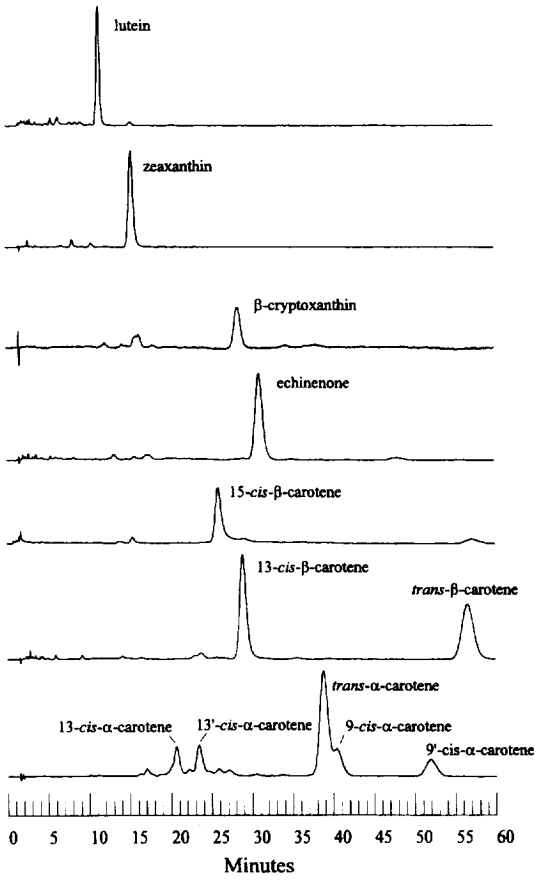

Fig. 7. Chromatograms of a variety of carotenoids on a C_{30} stationary phase at 13°C. Conditions are described in Section 2.2.

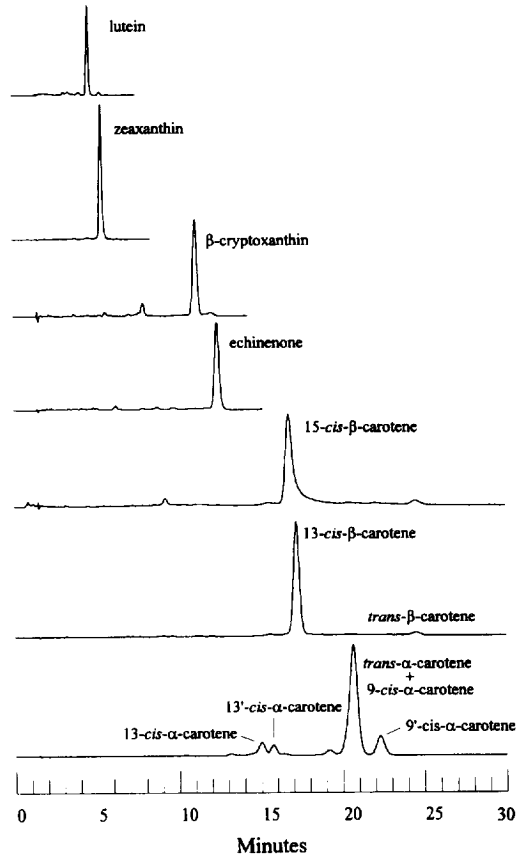

Fig. 8. Chromatograms of a variety of carotenoids on a C_{30} stationary phase at 38°C. Conditions are described in Section 2.2.

are completely removed from that portion of the chromatogram that overlaps with the retention times of many of the *cis* isomers of α- and β-carotene. In addition, at 38°C, there is greater column efficiency and the time for analysis is greatly reduced. In cases where the maximum selectivity for a particular set of carotenoids is necessary, lower temperatures may offer needed improvement. Conversely, in cases where optimal selectivity is not necessary or in cases where a sample contains different types of carotenoid solutes which may have different temperature dependencies, increased temperature may be warranted. Finally, some separations may benefit from temperature programming which may be able to combine these advantages in a manner analogous to mobile phase gradients.

4. Summary

Varying temperature dependencies were observed for a series of carotenoids on three different length alkyl-bonded stationary phases. These differences illustrate the potential of temperature as a useful parameter for optimizing chromatographic separations. An unusual phenomenon of increasing retention with increasing temperature was observed for bent carotenoids on long-chain stationary phases above 35°C. In addition, atypical temperature behaviors of some non-planar PAHs on a C_{30} column are comparable to those of bent carotenoids and suggest similar retention mechanisms. These behaviors are believed to be related to conformational changes in the stationary phases with temperature. Additional data on the physical nature of these

phases and on their conformational and chemical responses to solvents, solutes, and temperature would be helpful for a clearer understanding of their behavior under chromatographic conditions.

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