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Analytical Methods Section

Further observations on problems associated with the analysis of carotenoids by HPLC—2: Column temperature

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Using a chromatographic system which has previously been shown to give excellent separation of lutein, zeaxanthin, β-cryptoxanthin, lycopene and α- and β- carotene, changes in ambient temperature caused dramatic differences in the chromatographic response of carotenoids. This study demonstrates the effect of column temperature on the elution time and elution profile and the importance of temperature control in reducing analytical variation. The optimum resolution was achieved at 20–22·5°C.

INTRODUCTION

We have recently proposed a chromatographic procedure for the separation of carotenoids (Scott, 1992) which has the potential for application to the analysis of both foods and blood serum. During studies of factors that might affect the chromatographic response, and hence contribute to analytical variation and inaccuracies in the quantitative determination of carotenoids, it has been apparent that variation in ambient temperature causes dramatic changes in elution times and resolution.

This paper describes the use of a thermostatically controlled column water jacket and the effect of temperature on chromatographic response.

MATERIALS AND REAGENTS

A reference standard mixture comprised of lutein, zeaxanthin, β-cryptoxanthin, echinenone, lycopene, α-carotene and β-carotene was prepared in the mobile phase from stock solutions after evaporation under N_2.

An extract of a dried food mix containing sweetcorn, tomatoes and carrots was prepared as described below. The mobile phase consisted of HPLC grade, acetonitrile/methanol/dichloromethane (75:20:5, v/v/v) containing 0·1% B.H.T. (1·5 ml min^{-1}).

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HPLC

The HPLC system comprised a dual-piston solvent delivery pump (Pharmacia-LKB, Uppsala, Sweden) equipped with helium sparging. The column system consisted of a 5-μm Spherisorb ODS 2 metal-free guard column with a metal-free 5-μm Spherisorb ODS 2 (100 mm × 4·6 mm) cartridge column (both Alltech Associates, Carnforth, UK) connected to a 5-μm Vydac 201 TP 54 (250 mm × 4·6 mm) analytical column (Separations group, Hesperia, CA, USA), modified by the replacement of metal frits with 'biocompatible' teflon frits. The column temperature was controlled to ±0·1°C, using a column water jacket (Alltech Associates, Carnforth, UK) with a thermostatically controlled water bath (Grant Instruments, Cambridge, UK).

Peak responses were measured at 450 nm using a UV/VIS monitor (LKB) with a computer-controlled data handling system (Pye-Unicam, Cambridge, UK) which permitted manual manipulation of peak integration. Solutions were injected via a Model 7125 Rheodyne syringe-loading sample injector, fitted with a 50-μl loop (Rheodyne, Cotati, CA, USA)

EXPERIMENTAL

The column temperature was varied between 15 and 30°C and the elution profile of the standard working solution monitored. In addition, a reference 'food mix' (consisting of sweetcorn, tomato and carrot) was extracted with a mixture of THF (tetrahydrofuran)/

methanol without subsequent saponification and the elution profile assessed at 20, 22·5 and 25°C.

RESULTS AND DISCUSSION

The column and solvent systems described above have been shown previously (Scott, 1992) to give excellent separation of a standard carotenoid mixture and a reference food mix.

Within a laboratory it is quite possible that the ambient temperature can vary considerably. Figure 1 indicates that temperature has a very large effect, not only on elution time but also on the elution profile of a standard carotenoid mix. The data reported here show that there is a reduction of about 1 min in elution time for every 1°C rise in temperature. At the time this study was conducted we were also investigating the suitability of echinenone as as internal standard and this is included in the carotenoid standard mix and added to the food mix. The food mix does not contain a source of β-cryptoxanthin.

At 15°C the total elution time was around 35 min (β-carotene, 31 min). Lutein and zeaxanthin and α- and β-carotene were well separated, but β-cryptoxanthin and echinenone are poorly separated. *Trans*-lycopene eluted immediately prior to α-carotene with the probability that any other lycopene isomers present would co-elute with α-carotene. At 20°C the total elution time was reduced by about 7 min (β-carotene, 24 min). Again there was relatively poor separation of β-cryptoxanthin and echinenone, but the separation of lycopene and α-carotene was considerably improved.

Further improvement, but not baseline separation of β-cryptoxanthin and echinenone, was achieved at 22·5, 25 and 30°C. Elution times of β-carotene at these temperatures were around 21, 19 and 15 min, respectively. At 22·5°C there was still a good separation between lutein and zeaxanthin, but at 25 and 30°C the separation between lutein and zeaxanthin and α- and β-carotene became less satisfactory. Figure 2 shows a similar effect with the food mix (note that it does not contain β-cryptoxanthin). However, the peak eluting immediately prior to lutein (Fig. 2) at 20°C is not completely separated from lutein at 22·5°C and 25°C. There is a slightly better separation of β-carotene isomers at 22·5 and 25°C. Again, note the poorer separation of lutein and zeaxanthin above 22·5°C. The small peak occurring between lutein and zeaxanthin, is not resolved above 22·5°C. *Trans* lycopene and *trans* β-carotene are separated from their stereo-isomers at all three temperatures.

Also using a Vydac column (Separations Group, Hesperia, CA, USA) but with a mobile phase consisting of methanol and THF, 95:5 (v/v), Craft *et al.*, 1992 have also demonstrated that temperature affects both time of elution and resolution of carotenoids. Lycopene isomers are eluted after β-carotene with this mobile phase. They showed that at 15°C, echinenone and lycopene were more strongly retained with respect to

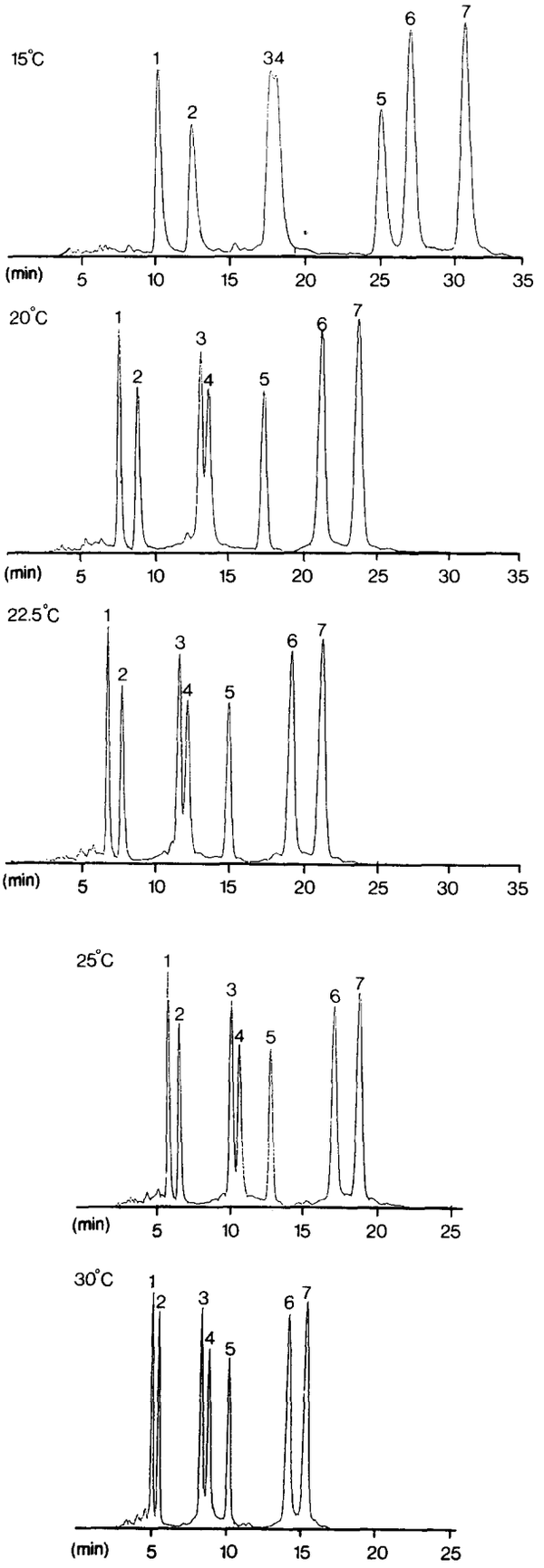

Fig. 1. The effect of five column temperatures on the separation of seven standard carotenoids. Chromatographic conditions are as described in the text. 1, Lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 4, echinenone; 5, lycopene; 6, α-carotene; 7, β-carotene.

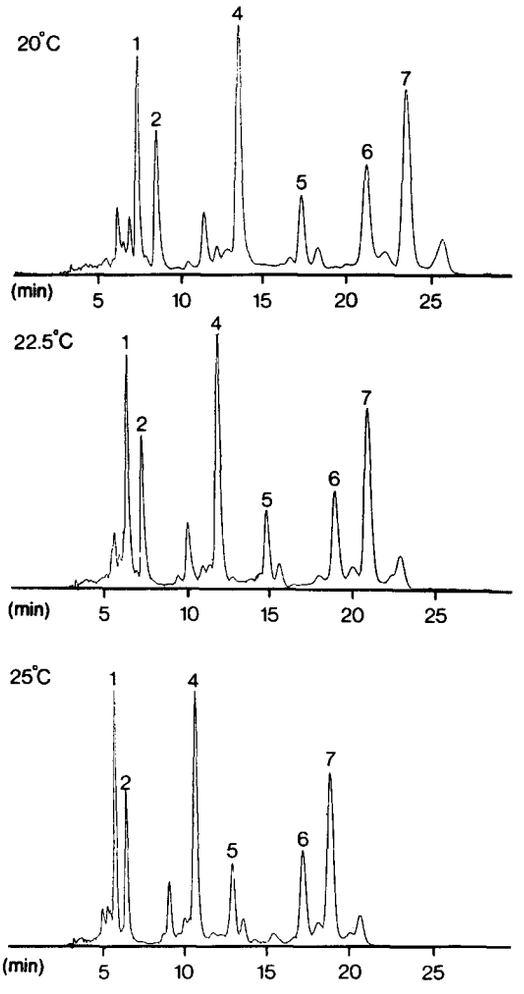

Fig. 2. The effect of three column temperatures on the separation of five carotenoids plus echinenone (internal standard) in an unsaponified 'food mix' containing sweetcorn, tomato and carrot. Chromatographic conditions are as described in the text. 1, Lutein; 2, zeaxanthin; 4, echinenone; 5, Lycopene; 6, α-carotene; 7, β-carotene.

β-carotene; echinenone and α-carotene were not baseline resolved. At 20°C, all carotenoids in the standard mixture were apparently well resolved, baseline separation existed between β-carotene and its geometric isomers, and there is a partial separation of the geometric isomers of lycopene. At 25°C and above, resolution decreased between lutein and zeaxanthin and between β-carotene and lycopene.

In many published procedures for the analysis of carotenoids in blood serum, there is no or poor resolution of lutein and zeaxanthin, and baseline separation of α- and β-carotene is not always achieved. Some systems do not separate the geometric isomers of lycopene and β-carotene. The system described here achieves a discrete separation of lutein, zeaxanthin, β-cryptoxanthin, lycopene and α- and β-carotene and the geometric isomers of lycopene and β-carotene.

With the particular analytical column used in this study, the most suitable temperature was around 20–22·5°C. Repeat analysis, over a period of several months at 22·5°C, of both the standard carotenoid solution and the food mix, gave reproducible elution times and resolution. However, as reported earlier (Scott 1992, Epler *et al.*, 1992), adequate separation of carotenoids and their isomers is complicated by differences in response between columns, not only on columns with different stationary phases, but also on columns with the same stationary phase. Thus, it may be necessary to reoptimise the temperature when a column is replaced. When a column with a different stationary phase is used, the effect of temperature should be assessed. In addition, although apparently good separation of this standard mixture is demonstrated, it must not be assumed that other carotenoids in unknown samples are necessarily separated from those shown in the figures. Canthaxanthin, for example, co-elutes with zeaxanthin at 22·5°C, but between lutein and zeaxanthin at 20°C. It is therefore important that the purity of a particular peak is validated by spectral analysis or other means.

The current interest in the influence of certain foods on human health, and in particular the antioxidant activity of carotenoids, increases the need for accurate qualitative and quantitative data on these vitamins in foods and blood. Scott (1992) has discussed some of the problems associated with the analysis of carotenoids. This study has demonstrated that the control of column temperature is an additional important factor in reducing analytical variation and increasing confidence in results.

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