Droplet Counter Current Chromatography of the Carotenoids of Parsley *Petroselinum crispum*

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Key Words

Droplet counter current chromatography
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Summary

The application of droplet counter current chromatography to the separation of the carotenoids of parsley, β-carotene, lutein, violaxanthin and neoxanthin, is described. The solvent system consists of petroleum spirit bp 40–60°C : acetonitrile : methanol in the proportions 50 : 10 : 40. The upper layer is used as the mobile phase and the lower layer as the stationary phase.

Introduction

Carotenoids are sensitive to air, light, heat and a variety of chemical reagents and do not tolerate acidic conditions [1]. This sensitivity limits the separation methods which can be used. However, a number of techniques including liquid chromatography, thin-layer chromatography, and high pressure liquid chromatography have been successfully applied [2–5]. An alternative approach based solely on partition chromatography, the Craig counter current apparatus, was utilised by Curl who achieved satisfactory separations even in such complicated situations as those found with the paprika carotenoids [6, 7]. The Craig equipment is bulky, requires large amounts of solvent and is liable to a variety of mechanical faults and because of this the method has largely fallen out of use. Recently, droplet counter current chromatography (DCCC) which is based on the same principle, liquid-liquid partition, has been developed and applied with success to a wide range of more polar organic compounds [8, 9]. The method does not appear to have been applied in the carotenoid field.

The solvent systems normally used for DCCC [8, 9] have been designed for polar compounds and are not appropriate for carotenoids. Although water : methanol : petroleum spirit bp 40–60°C systems are frequently used for the partition of carotenoids [3, 10] they do not give stable droplet conditions in our experience. However, we have recently shown that petroleum spirit bp 40–60°C : acetonitrile : methanol (PAM) mixtures are good developing solvents for the reversed phase TLC of carotenoids [11]. Using these solvents in the proportions 50 : 10 : 40 gave two phases which provided stable droplet conditions using the upper layer as the mobile phase and the lower as the stationary phase. This system allowed the separation of 10mg of parsley carotenoids in the course of a single separation lasting under 2 days. The compounds isolated, β-carotene, lutein, violaxanthin and neoxanthin (See Figure 1), represent the full polarity range normally found in carotenoids.

Experimental

Carotenoid Extraction

The carotenoid extract was obtained in accord with established procedures [12]. Leaves and stems (100g, wet weight) of curly-leaved parsley, *Petroselinum crispum*, were macerated in a Waring Blender containing acetone (200ml). The extract was filtered and the filtered mass reextracted in the same way with acetone and then acetone : methanol (1 : 1). The combined extracts were taken to dryness under reduced pressure and saponified for 3h in a mixture consisting of sodium dried diethyl ether (100ml) and methanol (100ml) containing NaOH (10g). Aqueous sodium chloride solution (5%, 200ml) was added and the carotenoids extracted with several portions of ether (3 × 200ml). The combined ether extracts were washed to neutrality with aqueous sodium chloride (5%, 3 × 200ml) and finally twice with distilled water (2 × 200 ml). The washed extract was then taken to dryness under reduced pressure, estimated quantitatively in ether assuming an extinction coefficient of 2,300, and again taken to dryness prior to dissolution in the mobile phase for DCCC.

Droplet Counter Current Chromatography (DCCC)

DCCC was performed using a DCC Model A chromatograph from Tokyo Rikakikai, Tokyo, Japan. The instrument was operated using 72 capillaries (40cm × 2mm) connected in series. Analytical grade solvents were used as received in preparing the solvent system, PAM (50 : 10 : 40). The upper

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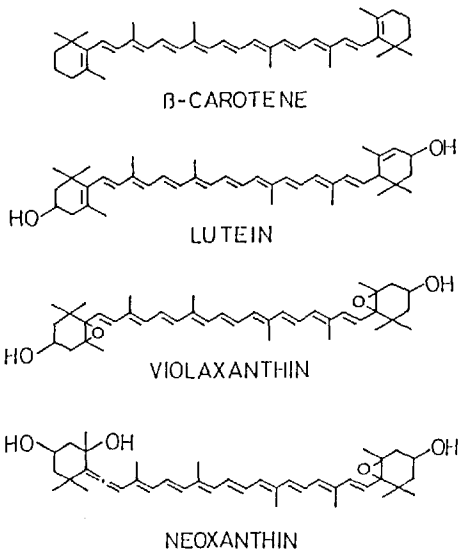

Figure 1
Structures of carotenoids found in curly-leaved parsley.

layer was used as mobile phase, while the lower layer provided the stationary phase. A mobile phase flow-rate of 36ml/h was maintained throughout. Individual fractions of 5ml were collected by means of an automatic fraction collector (ISCO, Model 1850).

Carotenoid Analysis

Individual fractions were analysed by thin-layer chromatography [11] on RP-18 (Merck nr 15423) plates using PAM (25:25:50) as developing solvent. Relative amounts of the individual compounds in each fraction were determined by densitometry with a Quick-Scan R & D densitometer (Desaga/Helena) using a 445nm filter. The individual carotenoids are known to have approximately the same absorbance at this wavelength.

Carotenoids were identified by visible light absorption spectroscopy, mass spectrometry and co-chromatography (TLC) with genuine compounds extracted from established sources [13]. Identification of the carotenoids was in accord with results previously found for parsley.

Results and Discussion

The hydrocarbon nature and consequent lack of polarity of carotenoids imposes considerable constraints on the type of solvent systems available for chromatography. Such limitations are of great importance in DCCC where there is a requirement for two immiscible layers of not too dissimilar polarity. The partition between the phases formed by water:methanol:petroleum spirit bp 40–60°C mixtures is a well-established structural indicator in carotenoid work [3, 10]. Initial attempts to apply this and similar systems to the DCCC of carotenoids proved fruitless, since it was impossible to obtain stable droplet conditions over a period of time with the apparatus available. Reversed phase TLC of carotenoids is successful using a wide range of PAM mixtures [11]. It was thus decided to attempt to use such a solvent system for the semi-preparative DCCC of carotenoids.

Curly-leaved parsley (*Petroselinum crispum*) is a readily available source for the carotenoids of photosynthetic tissue. Somewhat surprisingly, the initial investigation [14] has been largely ignored and it thus seemed appropriate to combine reinvestigation of this convenient source with the application of DCCC to the carotenoid field. Using solvent proportions lying within the non-miscible part of the PAM system phase diagram [11] and examining the TLC Rf-values according to the suggestion of Hostettmann [9] it was found that a variety of compositions might be useful. Ultimately, a system consisting of PAM in the proportions 50:10:40 was chosen. Even when the upper layer was used as the mobile phase extended times were required for elution. In order to minimise the time needed, and thus possible decomposition, the total flow path was reduced and the rate of throughput increased relative to normal operation. The results for the DCCC of a parsley extract containing 10mg of carotenoid are given in Figure 2. The main carotenoids, β-carotene, lutein, violaxanthin and neoxanthin, were well separated in 36h. Two minor components eluted between β-carotene and lutein. These latter had nonaene chromophores on the basis of visible light absorption and corresponded in polarity to monohydroxy-β-carotene, but were not further investigated. Since β-carotene is the least polar carotenoid normally found and neoxanthin among the most polar, the full range of carotenoid polarity is covered by this system. Where difficulties occur due to the presence of several carotenoids of similar polarity, separation should thus be possible by increasing the number of capillaries used.

The advantages of DCCC for carotenoid work lie in that the compounds are exposed to neither adsorption pheno-

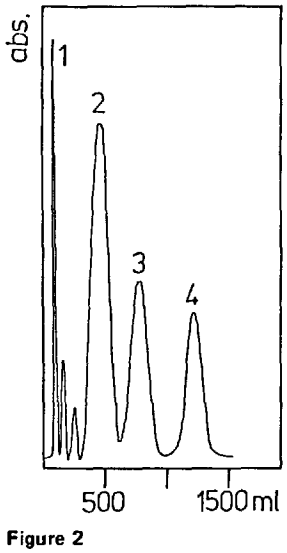

Figure 2
Chromatogram showing the results of the DCCC of 10mg of carotenoid extract from curly-leaved parsley. Mobile (upper) and stationary (lower) phases were obtained by mixing petroleum spirit bp 40–60°C:acetonitrile:methanol (PAM) in the proportions 50:10:40. Flow rate: 36ml/h. Peak identities: **1** = β-carotene; **2** = lutein; **3** = violaxanthin; **4** = neoxanthin.

mena nor extraneous chemical or physical influences in the closed system. A single separation lasting 1–2 days suffices to separate 10mg of carotenoid which is a considerable amount of pigment in this field.

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