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On-line coupling of automatic solid-phase extraction and HPLC for determination of carotenoids in serum

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ABSTRACT

The automated method developed for the determination of carotenoids uses 200 μL of serum, which was mixed with 400 μL of tetrahydrofuran, vortexed for 1 min, settled for 10 min, centrifuged for 6 min and the supernatant injected into an automatic solid-phase extraction (SPE) system for cleanup–preconcentration. A 10% water–acetonitrile mobile phase at 1.5 mL min^{-1} eluted the retained compounds and transferred them on-line to a reversed-phase analytical column for individual separation of the target analytes. Visible detection was performed at 450 and 460 nm. The detection limits for the target analytes were between 3 and 30 ng mL^{-1}; the precision (expressed as relative standard deviation) ranged between 2.83 and 5.06% for repeatability and between 3.80 and 7.40% for within laboratory reproducibility. The total analysis time was 18 min. The proposed method is reliable, robust, and has an excellent potential for high-throughput use in both clinical and research laboratories.

1. Introduction

Carotenoids are fat-soluble pigments naturally present in plant chloroplasts and other photosynthetic organisms like algae, some types of fungi and bacteria. In the human organism, carotenoids, which are exclusively provided from the diet either free as esters or linked to proteins, are found in tissues and blood [1]. It is generally accepted that serum carotenoid concentrations are markers of recent fruit and vegetable intake [2], whereas tissue carotenoid levels are indicators of longer-term carotenoid consumption patterns [3].

In addition to provitamin A activity displayed by some carotenoids, several other biological activities of these compounds have been reported, including antioxidant capacity [4], which allows them to prevent chronic diseases by protecting against free radical damage [1]. Lycopene exhibits the highest antioxidant activity, followed by β-cryptoxanthin, β-carotene, lutein and zeaxanthin [5]. Oxidative stress has been related with diseases ranging from cancer [6] to age-related macular degeneration [7], even to the aging process itself [8]. Carotenoids have been reported to protect against oxidative stress and prevent cardiovascular diseases [4].

Epidemiological studies suggest that diet rich in vegetables containing β-carotene and other carotenoids decreases human lung cancer risk [9]; meanwhile low levels of carotenoids in placental tissue and maternal serum of women with preeclampsia suggest that oxidative stress or a dietary antioxidant influence might affect the pathophysiology of preeclampsia [10]. Also, carotenoids display other biological activities such as blue light filtering [4]. In fact, β-carotene is prescribed to ameliorate photosensitivity associated to erythropoietic protoporphyria and other light-sensitive diseases [11].

Individual separation and determination of carotenoids in biological samples is mainly carried out by HPLC with different types of detectors. A number of normal- and reversed-phase chromatographic methods have been described for individual separation of carotenes in serum using both isocratic [12] and gradient elution [13], the latter providing better resolution than the former. Concerning detection, the most widely employed technique has been ultraviolet–visible absorption, either conventional or photodiode-array detectors (DAD) [14]. More sensitive detectors (e.g. mass spectrometry – with atmospheric pressure ionization interfaces (APCI) or electrospray ionization interfaces (ESI) – and nuclear magnetic resonance spectroscopy) have also been used for carotenoids analysis. Nevertheless, these detectors are more expensive than absorption-based detectors and their use for routine analysis is more complicated [15,16].

The most common sample preparation procedure for subsequent determination of carotenoids in human serum involves a

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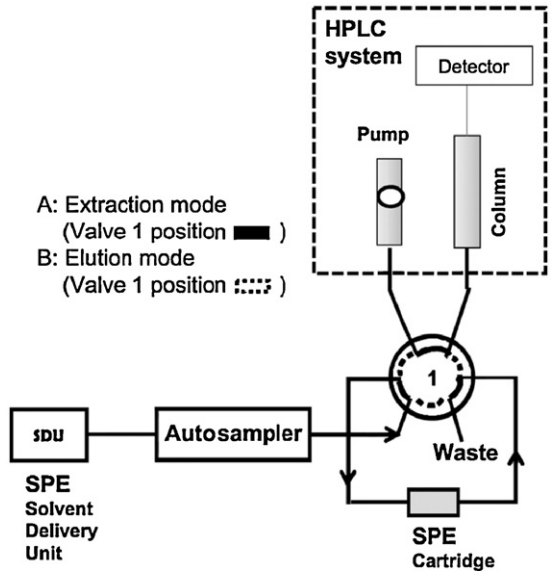

Fig. 1. Schematic diagram of the SPE–HPLC arrangement for automatic sample extraction – individual separation – determination. In the retention mode (valve 1 position ▬), an extraction cartridge is placed in the loop on the switching valve (1). The cartridge is washed and conditioned with liquid from the solvent delivery unit (SDU). An aliquot of the sample is led to the SPE cartridge by the loading solvent from the SDU. The analytes are retained and a washing solution is circulated through the cartridge to remove potential interferences. In the elution mode (valve 1 position —), the valve is switched to flush the analytes to the analytical column with the LC mobile phase. After the analytes have left the cartridge, the valve is switched again to the retention mode (A), and the extraction cycle repeated for a next sample, while the chromatographic step takes place.

step using ethanol for protein precipitation prior to extraction of the target analytes with an organic immiscible solvent (usually n-hexane) [17]. After centrifugation for phase separation, the hexane layer is evaporated to dryness under a nitrogen stream and the residue reconstituted with the appropriate organic solvent for injection into the HPLC system. This procedure is relatively tedious, complex, expensive, time-consuming, and with low precision and accuracy [4,17–20].

To know the impact of carotenoids in human health, a precise method that improves the repeatability and reproducibility of the already existing methods and provides better throughput is required for application in systematic epidemiologic studies, routine analysis and, in general, in cases in which the determination of these compounds in a high number of samples is needed. Thereby, the aim of this research was to fill this gap with a method to determine the target carotenoids in human serum with minimum user involvement.

2. Experimental

2.1. Instruments and apparatus

The experimental setup used for the automatic determination of the target analytes in serum samples is shown in Fig. 1.

An Agilent liquid chromatograph (Model 1100, Pittsburgh, PA, USA) consisting of a quaternary pump, vacuum degasser, and a diode array detector (DAD) with a tungsten lamp was used for the analysis of the target analytes. Hyphenated solid-phase extraction (SPE) was performed with a Prospekt2 system (Spark Holland, Emmen, The Netherlands) and an autosampler (Midas) with a 500 μL sample loop. The Prospekt2 system comprises a unit for SPE cartridge exchange – automatic cartridge exchange (ACE) – and a high pressure syringe dispenser (HPD) for SPE solvent delivery. Peek tube of 0.25 mm i.d. (VICI, Houston, TX, USA) was used to connect all valves of the Prospekt2 unit and this with the analytical column. A cartridge packed with Hysphere CN-SE (silica-based cyanopropyl phase second edition, from Spark Holland) as sorbent material 10 mm × 2 mm and 7 μm particle diameter was used for SPE. The analytical column was a 4 μm Novapak C18 column, 100 mm × 3.9 mm i.d. (Waters corporation, Milford, MA, USA).

A centrifuge (Selecta, Barcelona, Spain) and a vortex (Ika-Works, Wilmington, USA) were also used.

2.2. Chemicals and standard solutions

Deionised water (18 mΩ) from a Millipore Milli-Q water purification system was used to prepare all aqueous solutions. Violaxanthin (viola), β-criptoxanthin (cripto), neoxanthin (neo), anteraxanthin (antera), lycopene (lyco), zeaxanthin (zea), mutatoxanthin (mutato), canthaxanthin (cantha), lutein (lut), β-carotene (β-caro) and α-carotene (α-caro) from Carotenature (Im Budler 8 CH-4419, Lupsingen, Switzerland) were used as standards. Methanol, ethanol, acetone, acetonitrile, tetrahydrofuran (THF), sodium dodecyl sulfate (SDS) from Scharlab (Barcelona, Spain), heptane, di-tert-butyl-4-methylphenol (BHT) from Panreac (Barcelona, Spain), ammonium formate from Sigma (Sigma–Aldrich, St. Louis, MO, USA) were used. All chemicals were HPLC grade and used without further purification.Stock standard solutions were prepared by dissolving 0.1 g of each analyte in 50 mL methanol in the case of viola, cripto, antera, zea, mutato, and lut, and in 100 mL in the case of neo. 50 mL ethanol was used to dissolve cantha; 100 mL tetrahydrofuran (THF) for lyc, and 200 mL THF for β-caro and α-caro. All stock solutions contained 30 mM BHT to prevent oxidation [21]. The concentration of the stock solutions were checked using the extinction coefficient of each standard. Standard working solutions were prepared by dilution of the appropriate volumes of the stock solutions in methanol to a final concentration of the analytes 0.5 μg mL^{-1}, and used for optimisation of both the chromatographic step and the preconcentration effect achieved by the automatic SPE step. Deionised water was used as loading solvent in the preconcentration step and as washing solution in the cleanup step. The initial chromatographic mobile phase was used for elution of the analytes from the cartridge. Then, methanol and deionised water were used to purge the tubing system of Prospekt2 at the end of the SPE step.

2.3. Preparation of serum samples

A total of 80 serum samples were collected from blood donors (age range: 18–65 yr, 48 men, 32 women). Venous blood was collected into a plastic Vacutainer® tube from Becton Dickinson (Franklin Lakes, NJ, USA) without additives (red top). The tube, without opening it to ambient air, was placed in ice or kept refrigerated until processing. Blood samples were processed – centrifuged at 2375 × *g* for 15 min at 4 °C – within 1 h after collection. Serum was collected, placed in plastic tubes and stored at or below −80 °C until analysis. Appropriate volumes of standard solutions of the analytes were added in the validation step.

For protein precipitation, the serum samples were equilibrated at room temperature for at least 30 min, then vortexed for 30 s. 200 μL of serum was pipetted into glass tubes and 400 μL of THF was added to each, then vortexed for 1 min, and allowed to settle for 10 min. The mixture was centrifuged for 6 min at 855 × *g*, and the supernatant transferred to an autosampler vial.

2.4. Proposed method

The supernatant from the previous step, transferred to an injection vial, was placed in the autosampler tray, thus being ready for analysis. 500 μL of the content of the injection vial was injected