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Development and validation of a rapid resolution liquid chromatography method for the screening of dietary plant isoprenoids: Carotenoids, tocopherols and chlorophylls

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A rapid resolution liquid chromatography (RRLC) method was developed and validated for the simultaneous determination of nine carotenoids compounds (violaxanthin, lutein, zeaxanthin, β-cryptoxanthin, α-carotene, β-carotene, lycopene, phytoene, phytofluene), four tocopherols and four chlorophylls and derivates (chlorophylls and pheophytins). The methodology consisted in a micro-extraction procedure with or without saponification and subsequent analysis by RRLC. The limits of detection were <0.07 μg for carotenoids and tocopherols and <0.08 μg for chlorophylls and derivatives. The overall precision values (intra- and inter-day) were lower than 12% when samples were not saponified and <27.6%, when the saponification step was performed. The recovery of the method without the saponification step ranged from 92% to 107%, whilst that when saponification was carried out ranged from 60% for α-tocopherol to 82% for β-carotene. Finally, the applicability of the method was demonstrated by the identification and quantification of isoprenoids in different samples. The methodology is appropriate for the high-throughput screening of dietary isoprenoids in fruits and vegetables.

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1. Introduction

Many epidemiological studies indicate an inverse association between the consumption of appropriate amounts of fruits and vegetables and the risk of developing chronic diseases such as cardiovascular diseases, some forms of cancer and neurodegenerative disorders, among others [1–4].

This protective effect of diets rich in plant foods has been frequently associated to their content on vitamins, mineral, fiber and other compounds commonly referred to collectively as phytochemicals. These are secondary metabolites that plants produce for different purposes. For instance, they can be important for their survival, for example by intervening in their interaction with the environment, including other species. In humans, there is evidence that, among other actions, they can modulate metabolic pathways and have a beneficial effect in the context of the antioxidant defense [5]. Typical examples of dietary phytochemicals with interest in Food Science and Technology and Nutrition are carotenoids (CARS), chlorophylls (CHLS), tocopherols (TOCS) and phenolics, among others.

CHLS are essential molecules for life because of their roles in photosynthesis. Although their interest as colorants is undeniable, there are few scientific data about properties and actions that could be related to their possible impact on health. Some suggest that certain chlorophylls could exhibit antioxidant activity [6,7]. In other studies it is suggested that they may be beneficial against cancer [8,9]. In this context, the uptakes by human intestinal cultured cells have also been evaluated [10,11]. CARS are also essential pigments in photosynthesis and responsible for the color of a wide variety of fruit and vegetables [12,13]. They are beneficial to humans, since some of them exhibit vitamin A activity. Moreover their presence in plasma and human tissues is usually associated to a lower risk of developing certain diseases [12].

TOCS (α-, β-, γ- and δ-tocopherol) exhibit vitamin E activity. Traditionally they are regarded as lipid antioxidants that protect membrane lipids from the oxidative damage caused by reactive oxygen species [14]. However, non-antioxidant biological actions of these compounds have also been suggested [15,16]. Furthermore, their presence in foods could also be important to protect them from oxidation and increase their shelf life [17].

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Several methods have been described for the determination of TOCS, CHLS and CARS in plant material, either separately [18–20] or simultaneously [21–23], typically by reverse phase HPLC-DAD. Fraser et al. [22] described a method using a C30 column which enabled the simultaneous separation of CARS, TOCS, ubiquinones and plastoquinones in 60 min. Burns et al. [21] described an extraction and a HPLC method for the determination of CARS, TOCS and CHLS in fruits and vegetables in 40 min. Gleize et al. [23] described a method to analyze eleven CARS, retinol, α- and γ-tocopherol in foods, human plasma and human adipose tissue in 35 min using a C30 column.

The "traditional methods" mentioned above are appropriate for the detailed profiling of such compounds. More specifically the C30 column is very suitable for the separation of both geometrical (reviewed in [24], and even some optical CARS isomers [25]). However, due to its retention characteristics the HPLC methods that use it involve long analysis times and a higher use of solvents, with the associated negative consequences for the environment. Consequently, they are not the most appropriate for screening purposes. Recently, a clear improvement in chromatographic performance has been achieved by the introduction of rapid-resolution liquid chromatography (RRLC) or ultra-performance liquid chromatography (UPLC) that allows for a higher throughput of samples, often without compromising resolution, and a considerable saving of solvents. Although they are widely used in the pharmaceutical area, they are not as widespread for the analysis of food isoprenoids. Granado et al. [26] compared HPLC vs. UPLC methods for the simultaneous determination of biomarkers of the nutritional status. Furthermore, they proposed a method that allowed the separation of vitamin A, E, and D, and major plasma carotenoids (lutein, zeaxanthin, α-carotene, β-carotene, β-cryptoxanthin, lycopene) in 4.5 min. A recent study describes the analysis of a mixture of up to 16 CARS standards by UPLC–MS/MS in 12 min [27]. Similarly, Bohoyo-Gil et al. [28] proposed a methodology by UPLC for the determination of the seven carotenoids in honeybee pollen samples, pumpkin and nectarine flesh with satisfactory recoveries in 10 min.

Within this context, the main aims of the present work were two:

1) To develop and validate a rapid and effective RRLC method for the simultaneous determination of dietary plant isoprenoids of interest in Food Science and Nutrition.
2) To apply it to their analysis in plant foods with different colors as a result of their CARS and/or CHLS contents.

2. Materials and methods

2.1. Chemicals and standards

The extraction solvents were of analytical grade. Hexane, acetone and dichloromethane were purchased from Carlo-Erba (Milan, Italy). The chromatographic solvents were methanol, acetonitrile, ethyl acetate (HPLC grade, procured from Merck, Darmstadt, Germany). Water was purified in a NANOpure® DIamond™ system (Barnsted Inc., Dubuque, IO). β-carotene, β-cryptoxantin and zeaxanthin were purchased from Sigma-Aldrich (Steinheim, Germany). Violaxanthin, α-carotene, lutein lycopene, phytoene and phytofluene were isolated from appropriate sources in accordance to standard procedures [29]. Chlorophylls a and b, and *trans*-β-apo-8′-carotenal were purchased from Sigma-Aldrich (Steinheim, Germany).

A mixture of tocopherols (α-tocopherol, β-tocopherol, δ-tocopherol and γ-tocopherol) was purchased from Calbiochem (Merck, Darmstadt, Germany). Pheophytins a and b were obtained from their respective chlorophyll counterparts by treating solutions of the latter with diluted HCl [30].

The concentrations of all the standards were determined spectrophotometrically using a HP-8453 UV–vis spectrophotometer. The values of molar absorptivity used to quantify the stock solutions, as well as the corresponding bibliographic references [31–34] are indicated in Supplementary material S1.

2.2. Samples

In order to assess the applicability of the method, twelve commercial fruits and vegetables were analyzed. The samples were categorized by their color, which was mainly due to their CARS and/or CHLS profiles. Specifically, orange/red (guava, watermelon and tamarillo), orange/yellow (carrot, pumpkin, papaya, physalis, corn, sweetpotato and nectarine) and green (broccoli and spinach) plant foods were considered. All the samples were acquired from local supermarkets. Upon arrival at the laboratory they were chopped and freeze-dried. In the case of the fruits, the peel and the seeds were previously removed.

2.3. Extraction procedure

Approximately, 10 mg of homogenized freeze-dried powder from the samples were used for the extractions. Due to the importance of sampling in the analytical procedures and the small amount of dried material used for this micro-extraction protocol, it was especially important to start from representative large quantities (ca. 250 g) of the material appropriately taken to ensure representativeness and homogenize it thoroughly upon freeze-drying to keep the representativeness. The powder was gently mixed with 1 mL of the MiliQ-water and then vortexed and centrifuged to remove the aqueous phase at 18,000 × *g* for 3 min. Subsequently, 1 mL of extracting solvent (hexane/acetone, 1:1 v/v) was added, the mixture was vortexed and then centrifuged for 3 min at 18,000 × *g*. After recovering the colored fraction, a further 500 μl of extracting solvent was added, and the mixture was vortexed and finally spun as explained before. These operations were repeated until color exhaustion. The pooled organic colored fractions were eventually evaporated to dryness in a vacuum concentrator at a temperature below 30 °C and stored under N_2 at −20 °C until analysis. The dry residue was re-dissolved in 100 μl of acetonitrile prior to their injection in the RRLC system.

The corn, nectarine, papaya, physalis, pumpkin and tamarillo extracts were saponified to hydrolyse the CARS esters. For this purpose they were redissolved in 500 μl of dichloromethane and treated with 500 μl of methanolic KOH (30%, w/v) overnight, under an atmosphere of nitrogen and at room temperature (25 °C). Finally, the saponified extracts were washed several times with water to remove any trace of base, concentrated to dryness and re-dissolved in 100 μl of acetonitrile prior their analysis by RRLC. All the samples were extracted in triplicate and injected three times.

2.4. Rapid resolution liquid chromatography conditions (RRLC)

The RRLC analyses were carried out on an Agilent 1260 system equipped with a diode-array detector, which was set to scan from 200 to 770 nm. A C18 Poroshell 120 column (2.7 μm, 5 cm × 4.6 mm) (Agilent, Palo Alto, CA) kept at 28 °C was used as stationary phase. The injection volume was set at 10–20 μL. The mobile phase was pumped at 1 mL/min and consisted of three solvents: solvent A, acetonitrile, solvent B, methanol and solvent C, ethyl acetate. The linear gradient elution was 0 min, 85% A + 15% B; 5 min, 60% A + 20% B + 20% C; 7 min, 60% A + 20% B + 20% C; 9 min, 85% A + 15% B; 12 min, 85% A + 15% B. The open lab ChemStation

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