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An on-line high performance liquid chromatography-crocin bleaching assay for detection of antioxidants

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ABSTRACT

An on-line HPLC (high performance liquid chromatography) method for the rapid screening of individual antioxidants in mixtures was developed using crocin as a substrate (i.e. oxidation probe) and 2,2′-azobis(2-amidinopropane dihydrochloride (AAPH)) in phosphate buffer (pH 7.5) as a radical generator. The polyene structure of crocin and AAPH-derived peroxyl radicals resemble the lipidic substrates and radicals found in true food more closely than the popular, albeit artificial, DPPH (1,1-diphenyl-2-picrylhydrazyl) and $ABTS^{\bullet+}$ (2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate)) do. After separation by a C18 (octadecyl silica) column and UV (ultraviolet) detection, antioxidative analytes react with peroxyl radicals at 90 °C and the inhibition of crocin oxidation (i.e. bleaching) is detected as a positive peak by an absorbance detector at 440 nm. The method is simple, uses standard instruments and inexpensive reagents. It can be applied for isocratic HPLC runs using mobile phases containing 10–90% organic solvent in water, weak acids or buffers (pH 3.5–8.5). With baseline correction, gradient runs are also feasible. The radical scavenging activity of several natural antioxidants and a green tea extract was studied. After optimisation of conditions such as reagent concentrations and flows, the limit of detection varied from 0.79 to 7.4 ng, depending on the antioxidant.

1. Introduction

A continued interest in the native presence of antioxidants in our food and the purposeful addition of natural antioxidants to food to prolong their shelf life [1–4] has led to a plethora of different methods for their analysis. Methods can be relatively simple and fast using artificial radicals such as DPPH and $ABTS^{\bullet+}$ [5], be of intermediate complexity making use of easy to oxidise natural lipidic substrates such as β-carotene, linoleic acid, eleostearic acid or safflower oil [5], or be performed in complex real food systems, e.g. sausages [6]. The latter type of assays is slow and expensive. Various papers summarising and comparing the different assays have appeared [5,7–10].

Over the last 13 years antioxidant assays have been coupled directly with HPLC to achieve high resolution screening (HRS) of antioxidants. In contrast to the off-line methods above, the on-line approach allows to link individual peaks, i.e. analytes, directly with antioxidant activity [11–13]. The forty or so on-line antioxidant applications have been reviewed [14]. Many individual antioxidants (phenolic compounds), plant extracts (culinary herbs, teas, medicinal plants) and complex food matrixes (vegetables, fruits, juices, meat, bread, milk, oil) have been successfully investigated. It is not unexpected that the most simple and rugged assays using DPPH and $ABTS^{\bullet+}$ radicals have found most widespread application [15,16]. A disadvantage of these stable radicals is that they do not occur in nature and they are radical and indicator at the same time, i.e. there is no real substrate. Reactive oxygen species (ROS, e.g. $O_2^{\bullet-}$) involved in the oxidation of real foods have also been used but met with little success due to their high instability [17–19]. Thus there is considerable scope for an on-line HPLC antioxidant activity assay that uses a substrate that is more related to polyunsaturated fatty acids, a radical that is more similar to the radicals encountered in real life oxidation processes than DPPH and $ABTS^{\bullet+}$, and is still sensitive and relatively easy to carry out. β-Carotene is an interesting substrate but its insolubility in aqueous media requires two phase systems which are difficult to combine with HPLC detection [5]. Similar to β-carotene, crocin (Fig. 1) possesses a polyene system that absorbs light in the visible light spectrum and is easily attacked by peroxyl radicals. Moreover, crocin is water soluble which allows for easy detection in reversed phase (RP) eluents. In recent years it has gained significant popularity as a probe in an off-line antioxidant assay, i.e.

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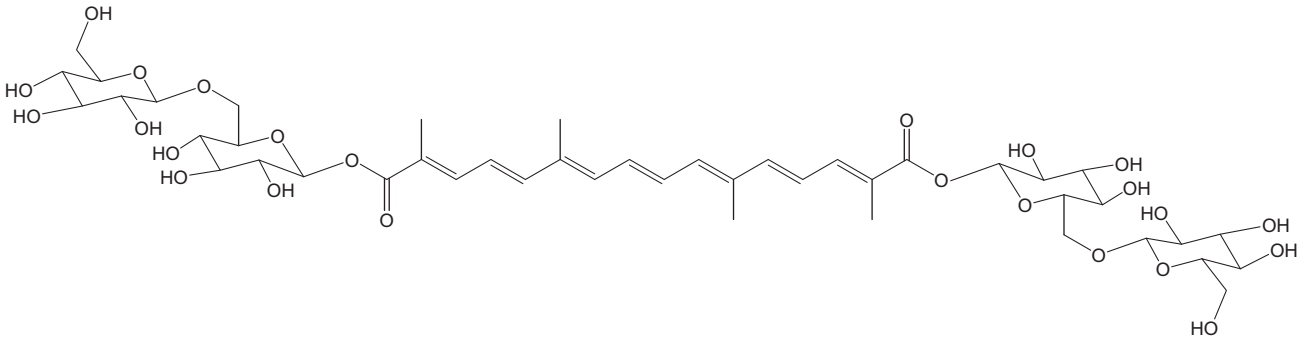

Fig. 1. Structure of crocin (syn. crocetin-di(β-D-gentiobiosyl) ester).

crocin bleaching assay (CBA) [20–23]. The latter is based on competitive reactions between crocin and antioxidants towards peroxyl radicals, ROO•, that are derived by thermal decomposition of the water-soluble azo-initiator 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH). These reactions are generally consistent with the simple Stern–Volmer kinetic model:

$$\frac{V_0}{V} = 1 + \left(\frac{k_{AH}}{k_{\text{crocin}}} \times \frac{[AH]}{[\text{crocin}]}\right)$$

where [*AH*] and [crocin] are the concentrations of the tested antioxidant and crocin, k_{AH} and k_{crocin} the rate constants for reaction of ROO• with AH and crocin respectively, and V_0 and V the bleaching rates in the absence and presence of AH, respectively. The CBA was originally developed to kinetically evaluate the antioxidant activity of complex mixtures such as plant extracts, natural products and biological fluids [23,24] but can also be applied for screening purposes. In this contribution we report on the development and use of an on-line HPLC method based on the crocin bleaching assay for high resolution screening of antioxidants in mixtures.

2. Experimental

2.1. Solvents and chemicals

HPLC grade methanol and acetonitrile were obtained from Biosolve (The Netherlands). Diethyl ether stabilized with ethanol was purchased from Panreac Quimica S.A. (Barcelona, Spain). Analytical grade tetrahydrofuran was from Fisher Scientific (UK). Ultra high purity water (0.05 μS/cm) was produced using a Barnstead Easy Pure device (Thermo Scientific, USA). Before use in the HPLC system, all sample solutions and solvents were membrane-filtered (0.45 μm, type RC 55, Schleicher & Schuell, Dassel, Germany). During the HPLC runs, solvents were continuously degassed by sparging with helium.

The following reagents and compounds were used: AAPH (97%), quercetin (98%, HPLC), L-ascorbic acid, caffeic acid (99%) and Trolox (syn. 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%) from Sigma–Aldrich Chemie (Steinheim, Germany); rosmarinic acid (97%, HPLC) and rutin (purissimum) from Fluka (Buchs, Switzerland); epigallocatechin gallate (green tea extract, 90%, Teavigo™) from DSM (Switzerland); uracil (99+%) and H_3PO_4 (85%) from Acros Organics (USA); NaCl, KH_2PO_4, $Na_2HPO_4{\cdot}2H_2O$, and KCl used for the preparation of buffers from Merck (Darmstadt, Germany).

Saffron was donated to the Laboratory of Chemistry and Food Technology, Department of Chemistry, Aristotle University of Thessaloniki, Greece by the Saffron Cooperative of Kozani (Greece). Green tea gunpowder was from Taous, Majid S.A. (China).

2.2. Crocin stock solution

The method proposed by Ordoudi and Tsimidou [20] was further modified. Raw saffron (0.5 g) was defatted by washing thrice with diethyl ether (3× 15 mL each time for 5 min), and the residual adhering ether was evaporated under a nitrogen stream. Defatted saffron was suspended in 25 mL of methanol, stirred manually for 5 min, and filtered through paper filter (⌀ 70 mm, Schleicher & Schuell, Dassel, Germany). The filtrate was stored at −18 °C for a maximum of 1 month and used as the *crocin stock solution*. All the experiments were carried out without direct exposure to light.

2.3. Preparation of AAPH solution, buffers, and sample solutions

AAPH stock solutions of suitable concentration (0.01–500 mM) were daily prepared in 10 mM phosphate-buffered saline (PBS) (0.08% (w/v) NaCl, pH 7.4) or 84 mM phosphate buffer (pH 7.5). 10 mM PBS solution (pH 7.4) was made by dissolving NaCl (8 g), KCl (0.2 g), $Na_2HPO_4{\cdot}2H_2O$ (1.81 g) and KH_2PO_4 (0.24 g) in 1 L of distilled water. 84 mM phosphate buffer was made by dissolving $Na_2HPO_4{\cdot}2H_2O$ (15 g) and KH_2PO_4 (1.2 g) in 1 L of distilled water and bringing the pH to 7.5 with KOH. The solution was stored on ice (0 °C) in the dark before use. Crocin and AAPH solutions were mixed in a 1:1 ratio 30 min before HPLC runs, then membrane filtered and kept at 0 °C.

Stock solutions of antioxidants (10 mM) were prepared in methanol or water. Different dilutions of each antioxidant in eluent were prepared daily and injected under various chromatographic conditions.

2.4. Determination of sensitivity

For the determination of the minimum detectable concentration (MDC) several dilutions of each antioxidant were prepared and sequentially injected under isocratic conditions. The method of Koleva et al. [16] was used for the determination of the minimum detectable concentration (MDC, μmol/L) and the minimum detectable amount (MDA, ng), the coefficient $t = 1.725$ for $n = 21$ measurements of the blank signal with a confidence interval of 90% was used, and the MDC was calculated as:

$$MDC = -2t\sigma_{blank}$$

where σ_{blank} is the standard deviation of the blank signal and t is Student's t statistic [16]. The blank sample consisted of the HPLC mobile phase in combination with optimal AAPH and crocin concentrations, flow rates and temperature. The positive peak, that is, the increase in absorbance values at 440 nm due to a decrease in the rate of crocin bleaching/oxidation was considered detectable if its height exceeded the value corresponding to the calculated MDC