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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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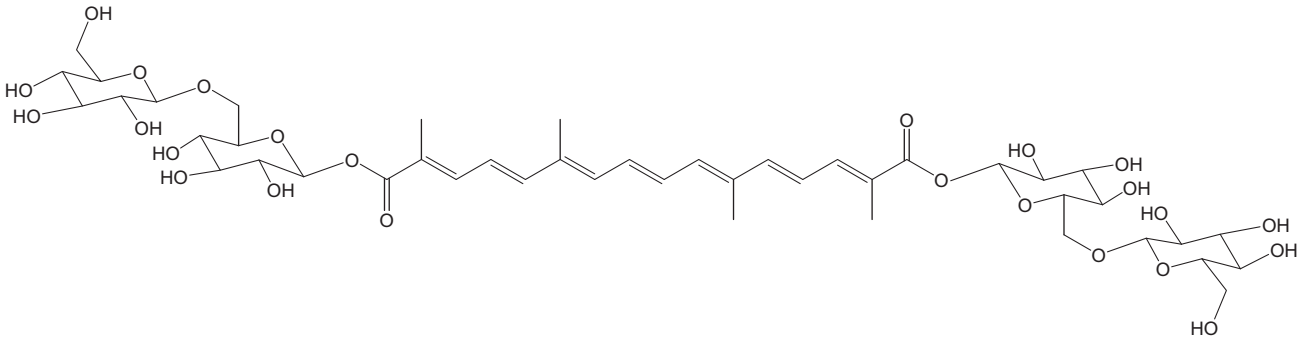

Fig. 1. Structure of crocin (syn. crocetin-di(β-D-gentiobiosyl) ester).

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

value. The MDA was calculated by multiplying the MDC with the injection volume (20 μL).

2.5. Preparation of green tea extract

The method of Ogawa et al. [17] was modified as follows. Dried green tea gunpowder (1 g) was added to 100 mL of initially boiling water and was then left standing without heating (i.e. similar to making tea at home). After cooling to room temperature, the leaves were removed by filtration through a filter paper and the remaining extract was passed through a 0.45 μm membrane filter.

2.6. Instrumental set-up and operating conditions

The instrumental set-up is presented in Fig. 2. The on-line HPLC system consisted of the following: an HPLC eluent pump (Gynkotek, model 480); a Spark Holland Basic Marathon autosampler with a 20 μL loop and a programmable diode array detector (DAD) (Gynkotek, UVD340S). Data were processed using Chromeleon Chromatography Data Systems software. For radical scavenging activity detection was used: an HPLC pump (Gynkotek, 300) for delivering the combined AAPH and crocin solutions and a Vis absorbance detector (Applied Biosystems, model 785A, Forster City, USA) equipped with a tungsten lamp connected to a recorder (Kipp & Zonen BD40; Delft, The Netherlands). Separations were carried out on an Alltima C18 HPLC column (5 μm, 250 mm × 4.6 mm i.d., Alltech Associates Inc.; Deerfield, IL).

Ascorbic acid was separated isocratically with MeOH—H_2O—H_3PO_4 (5:95:0.1, v/v). The detection was carried out at 270 nm. Rutin was separated isocratically with MeOH—H_2O—H_3PO_4 (45:55:0.1, v/v). The detection was carried out at 370 nm. Rosmarinic acid, EGCG (epigallocatechin gallate) and caffeic acid were separated isocratically with MeOH—H_2O—H_3PO_4 (50:50:0.1, v/v). The detection was carried out at 330 nm (rosmarinic acid), 220 nm (EGCG) and 340 nm (caffeic acid). Quercetin was separated isocratically with MeOH—H_2O—H_3PO_4 (60:40:0.1, v/v). The detection was carried out at 370 nm. Trolox was separated isocratically with MeOH—H_2O—H_3PO_4 (75:25:0.1, v/v). The detection was carried out at 290 nm. The tea extract was separated isocratically with MeOH—H_2O—H_3PO_4 (34:66:0.1, v/v) in 60 min. The detection was carried out at 225, 265, 280 and 340 nm. The flow rate was maintained at 0.7 mL/min during all above separations.

The reaction coil was made of PEEK (polyetheretherketone) tubing of the following size: 10 m × 0.25 mm i.d. Detection of crocin bleaching was carried out at 440 nm. The UV detection wavelengths for the test compounds were chosen according to their characteristic absorbance maxima.

The off-line UV–vis (visible) measurements were carried out on a Carry 100 UV-Vis Spectrophotometer (Varian, Australia) at 440 nm. The data were processed using Cary WinUV software. Adjustment of pH was carried out with a Multical model 526 pH meter (Germany). The temperature in the coil and cuvette was controlled by an RC6 Lauda thermostatic bath (Germany).

2.7. Off-line kinetic studies

The method of Ordoudi and Tsimidou [20] was adjusted as follows. Crocin working solution was daily prepared in PBS from the crocin stock solution and kept at 0 °C. A certain volume of crocin working solution was diluted with methanol in a quartz cell (10 mm) to 3.0 mL (total volume) so that the absorbance value at 433 nm was 0.8 AU (absorption unit). The same volume of crocin working solution was then transferred into another cuvette and different amounts of antioxidants (50–500 μL of 0.5 mM methanolic solutions) were added. The reaction was started by the addition of AAPH (20–500 mM, 50–500 μL, $t = 0$ min). After immediate dilution to 3.0 mL (total volume) with 10 mM PBS or 84 mM phosphate buffer and stirring (2–3 s), the absorbance at 440 nm was read every 6 s for a period of 5–10 min. The temperature of the solutions before and after mixing was stabilised by a thermostatic bath.

2.8. On-line HPLC-CBA method

A certain volume of crocin stock solution was diluted with 84 mM phosphate buffer to give a final absorbance value at 433 nm of 1.13 AU. AAPH solution was prepared daily in 84 mM phosphate buffer to give a final concentration in the reaction coil of 0.355 mM. The two solutions were cooled on ice for 30–60 min. After mixing the solutions in a ratio of 1:1, membrane filtration took place and the final solution was kept at 0 °C during all the chromatographic runs ("crocin + AAPH solution"). The mobile phase composition for each analyte is given in 2.6. The flow rates of both pumps were 0.7 mL/min. The temperature in the reaction coil was 90 °C.

3. Results and discussion

3.1. Off-line experiments for determining initial conditions

The crocin bleaching assay, or alternatively crocin oxidation assay, was first introduced by Bors et al. in 1984 [22] and since then the method has been modified several times by other researchers. However the principle remained the same. As much as possible we tried to maintain the conditions used in earlier studies using crocin. Thus a standardised saffron extract rather than pure crocin (Fig. 1) and AAPH as radical initiator were used. According to the literature, peroxyl radicals $ROO^{\bullet}$ are generated at a steady, measurable rate for a given period of time by thermal decomposition of AAPH and diffusion-controlled reaction of the derived carbon-centred radicals $R^{\bullet}$ with oxygen [25]. The $ROO^{\bullet}$ subsequently oxidises the polyene skeleton of crocin yielding eventually epoxides and carbonyl-containing chain-cleavage products [26]. Similar to the β-carotene bleaching (i.e. oxidation) assays, the loss of conjugation is easily detectable by a Vis-detector at 440 nm. An added antioxidant will scavenge the radicals and thus retard the crocin bleaching/oxidation which is translated in a higher absorbance value at the end of the reaction monitoring period. The most important aspect of any on-line assay is that it is fast as to avoid chromatographic peak broadening and loss of resolution relative to the UV profile, i.e. preferably a significant effect should be measurable within 30 s. To gather some basic information on this assay, first off-line assays were performed in which the influence of the AAPH concentration (21, 42 and 83 mM final concentration in cuvette) and temperature (40, 60 and 70 °C) was investigated. The crocin concentration was expressed in AU and was ~0.9 AU in the final test solution at $t = 0$ min. Not surprisingly both higher AAPH concentrations and higher temperatures greatly increased the rate of crocin bleaching/oxidation (Fig. S1, supplementary data). At 70 °C and an AAPH concentration of 20.8 mM, the absorbance at 440 nm decreased by 85% after 30 s. The lower temperatures were not usable for an on-line assay due to an unacceptably long reaction time. Higher AAPH concentrations did give a faster reaction but have as disadvantages a high cost (100 mL of 83.3 mM AAPH stock solution would require already 2.26 g of AAPH), possible precipitation at higher organic modifier concentrations and possibly a lower sensitivity. We hypothesised that the effect of a low concentration of added antioxidant could be more sensitively measured with an initially low AAPH concentration and a very fast degradation reaction than with very high initial AAPH concentration and a slow degradation reaction.

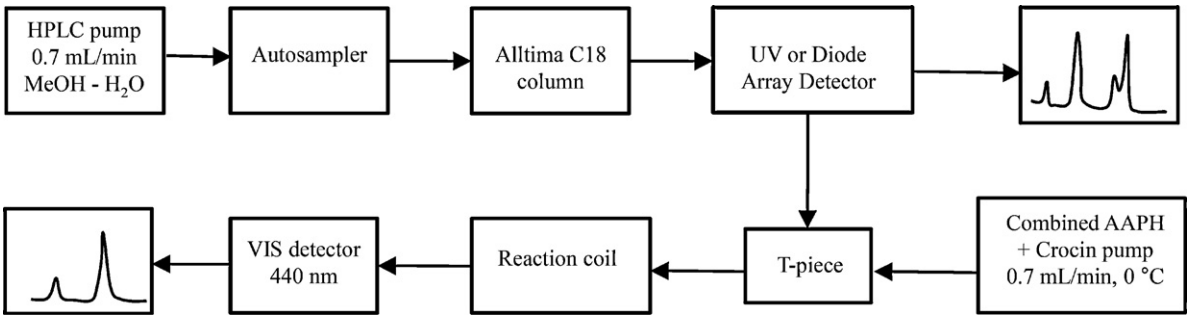

Fig. 2. Instrumental set-up for the HPLC on-line crocin bleaching detection of radical-scavenging compounds.

Next a model antioxidant (caffeic acid, 12.5 or 50 μM) was included to see at which concentrations an effect on the bleaching/oxidation rate could be measured. Also the influence of pH (6.4, 7.4, 8.4) and the organic modifier in the HPLC solvent (methanol or tetrahydrofuran (THF) or acetonitrile) at 12.5, 25 and 50% were investigated. As the on-line assay to be developed would use a C18 column, an organic solvent will inevitably be present in the reaction coil. The effect of pH (7.4 ± 1) on the bleaching rate in the presence of an antioxidant was marginal at reaction times below 1 min (results not shown) so in future experiments it was kept at the almost neutral, physiological pH of 7.4. On the other hand, higher concentrations of methanol significantly inhibited the rate of bleaching. For instance when the concentration of methanol increased from 12.5 to 50% while keeping the AAPH and caffeic acid concentration constant, the time to oxidise 50% of the originally present caffeic acid increased from ~80 to ~130 s (Fig. S2, supplementary data). This phenomenon will cause baseline drift with gradients. Crocin is soluble in water and polar organic solvents, but AAPH is only soluble in water. Most likely in water, methanol concentrates around the non-polar polyene part of crocin (Fig. 1) through hydrophobic interactions and thus shields it from the more polar radical initiator. Replacing methanol by acetonitrile or THF gave similar results so this protective effect appears to be a general effect of organic solvents. Methanol was used in all future experiments as mobile phase eluent.

It can be concluded that at 50% of methanol – a frequently encountered organic modifier percentage in RP-HPLC – there is a relation between the inhibiting effect on crocin bleaching and antioxidant concentration (Fig. S2, supplementary data, curves 3, 4 and 6). However to be usable in an on-line assay the rate of oxidation needed to be increased at least 5-fold and also the sensitivity needed to increase significantly.

Next it was investigated whether the formation of peroxyl radicals and thus the crocin oxidation, could be accelerated. AAPH initially decomposes relatively slowly in two carbon-centred radicals R•, which react rapidly with oxygen to form peroxyl radicals [25]. To this purpose the solution was sparged with pure oxygen during 15 min prior to the addition of crocin. However no change in reaction rate was observed. In a second experiment most oxygen was removed from the reaction medium by sparging with helium. Also this did not affect the reaction rate. Apparently the oxygen supply was not limiting (fast reaction of carbon-centred radical with the diradical oxygen) and/or R• also played a role in the initial phase of crocin oxidation. Anyhow the oxygen concentration plays no critical role and from this perspective the assay is robust.

3.2. Flow injection analysis (FIA) experiments for optimal AAPH concentration and temperature

The initial on-line experiments were carried out without HPLC column, i.e. in flow injection analysis (FIA) mode, to simplify the method development. AAPH concentrations in the reaction coil of 85.7, 42.9, 21.4, 10.7, 8.6, 4.3 and 2.1 mM and temperatures of 60, 70 and 80 °C were tested. This allowed comparison with the off-line results (*vide supra*) as well as testing of the hypothesis that lower AAPH concentrations in combination with higher temperatures are possible. The effect was evaluated in terms of signal to noise ratio (S/N) of an injected amount of caffeic acid as model antioxidant (20 μL of 100 or 500 μM solution). The flow rates were: carrier flow: 0.2 mL/min, crocin pump: 0.6 mL/min and AAPH pump: 0.6 mL/min. These initial experiments provided the proof of principle that (1) caffeic acid could be detected and (2) high temperatures along with low AAPH concentrations can be applied, resulting in similar or lower noise levels compared to the initial on-line conditions with high AAPH concentrations. These results indicated the possibility of using AAPH coil concentrations of 1 mM or even lower, at 90 °C. Lower concentrations were subsequently investigated. An AAPH coil concentration of ~0.35 mM at 90 °C provided the best compromise between rate of oxidation, S/N ratio and dynamic range. Dynamic range is defined as the difference between the baseline level when no antioxidant is present (maximum oxidation) and the baseline level when no AAPH is present (no radical-induced oxidation). At an AAPH coil concentration of 0.35 mM and 90 °C, the baseline fall was ~160 mAU (absorbance ~285 mAU for crocin only, ~125 mAU for crocin and AAPH).

3.3. Optimisation of on-line experimental set-up

Further optimisation was carried out in the HPLC on-line system which is schematically depicted in Fig. 2. The first issue was improving sensitivity by decreasing – under all conditions – the relatively high baseline noise of ~10 mAU. This baseline noise level was caused by (1) minor temperature fluctuations (±1 °C) of the water bath causing acceleration or slowing down of crocin oxidation in the reaction coil; (2) minor fluctuations in the ratio between crocin and AAPH flows; (3) formation of nitrogen or air bubbles in the reaction coil; and (4) a difference between mobile phase and sample solvent composition. The following measures were taken to remedy these problems: (1) use of a higher quality thermostatic bath; (2) premixing of crocin and AAPH solutions at 0 °C and pumping the combined, stable solution with one pump; (3) vacuum filtration of HPLC eluent and "AAPH + Crocin" mixture plus helium sparging of the "AAPH + Crocin" mixture during runs; (4) preferably dissolving the sample in HPLC eluent. All these measures reduced the baseline noise to 0.3–0.5 mAU, i.e. they led to an overall 25-fold increase of the S/N ratio.

Next four different ratios (0.27, 0.56, 1.0, 1.8) of HPLC column flow versus combined AAPH-crocin flow were investigated in terms of the minimum detectable concentration (MDC) of caffeic acid (Table S1, supplementary data). Best results were obtained at the ratios of 0.56 and 1.0. To allow the use of HPLC columns of 3.0 and 4.6 mm i.d., a ratio of 1:1 was chosen (total

Table 1
Minimum detectable amounts (MDA) of several hydrophilic antioxidants in the HPLC-CBA, HPLC-ABTS, HPLC-DPPH, and HPLC-CL on-line systems. Different isocratic conditions have been used in the different assays.

Compound	HPLC-CBA (ng)	HPLC-CL (ng) [11,19]	HPLC-ABTS (ng) [16]	HPLC-DPPH (ng) [15]
Caffeic acid	0.79	–	–	–
Ascorbic acid	7.4	0.18	0.43	3.9
Rosmarinic acid	1.8	0.43	0.43	0.07
Trolox	6.9	5.2	0.21	1.0
Quercetin	4.6	4.5	0.58	0.93
Rutin	6.0	15	0.55	4.0
EGCG	0.92	–	–	–

post-column flow 1.4 mL/min) in all further experiments. Also the effect of the absolute concentration of AAPH and crocin – keeping their ratio approximately constant – on the S/N ratio was investigated (Table S2, supplementary data). The AAPH coil concentration was varied by a factor of 4 (AAPH: 0.177, 0.355, 0.531 and 0.709 mM). The crocin absorbance values in the coil without AAPH being present were 0.14, 0.28, 0.42 and 0.50 AU. The optimum coil concentration and absorbance were found to be 0.355 mM for AAPH and 0.28 AU for crocin, respectively. Finally three different ratios (0.84, 1.27, 1.94) of AAPH concentration (in mM) and crocin concentration (expressed in AU) were investigated. Again the initially used concentrations appeared to be optimal. The MDA of this system for caffeic acid was approximately 0.8 ng (Table S3, supplementary data). Even at the detection limit, the method was very reproducible with a relative standard deviation of only 10%.

Other factors. The initial experiments showed that the methanol concentration had a significant effect on the crocin bleaching/oxidation rate. Thus the baseline was stable during isocratic runs, but in gradient mode the absorbance at 440 nm slowly increased with increasing methanol concentrations. This baseline drift could be largely corrected when automatic baseline subtraction was employed.

To prevent baseline disturbances through increasing or decreasing the rate of crocin bleaching, preferably the sample solvent composition should be the same as the HPLC mobile phase but this is true for most HPLC systems. Additionally, having a sample solvent stronger (i.e. more non-polar) than the eluent leads to lower sensitivity due to peak broadening.

3.4. Determination of minimum detectable concentrations and minimum detectable amounts

The sensitivity of the HPLC-CBA system varied less than one order of magnitude for seven structurally different antioxidants (Table 1, 2nd column). All values were in the low ng range. It should be noted that generic conditions were used in the HPLC-CBA system, i.e. for all compounds the same concentrations, flows and hardware was used and only the HPLC solvent varied. Certainly individual optimisation of parameters like reaction time (coil length), injection volume and temperature (rate of reaction versus compound stability) will affect the sensitivity levels and kinetic behaviour of the antioxidants in the system.

The MDAs of several tested antioxidants in other on-line HPLC systems than the CBA one, are also presented in Table 1. For most of the antioxidants tested, the HPLC-ABTS system shows the highest sensitivity. In the other three systems, the outcome varies with each antioxidant although the two systems making use of reactive oxygen species (HPLC-CBA and HPLC-CL (chemiluminescence)) appear slightly less sensitive. As MDA values are affected by many factors in all of these on-line systems, one should be cautious drawing too many conclusions. For a really good comparison the same HPLC pumps, column, elution conditions and Vis detector should be used. Each system has its strong and weak points. For example, the HPLC-DPPH and HPLC-ABTS systems have the advantage of simplicity and the disadvantage of using completely artificial model radicals. Moreover, the systems based on DPPH and CL methods suffer from big differences in sensitivity between individual antioxidants. The HPLC-CBA system described in this paper is less sensitive, but yields

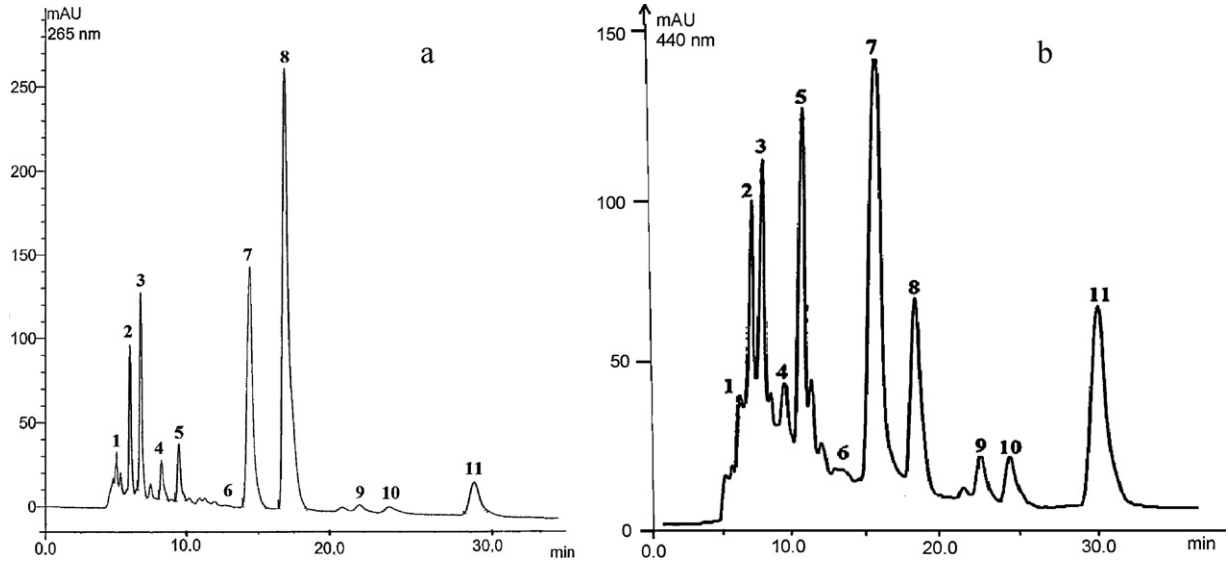

Fig. 3. Chromatograms with (a) UV (265 nm) and (b) CBA (440 nm) detection of an aqueous extract of green tea. Chromatographic conditions: Alltima C18 HPLC column (5 μm, 250 mm × 4.6 mm i.d.); flow rate 0.7 mL/min, injection 20 μL, isocratic conditions, MeOH—H_2O—H_3PO_4 (34:66:0.1).

positive peaks and uses an oxidation substrate that resembles the naturally found lipidic ones.

3.5. Suitability of the method for quantitative determinations

Different concentrations of caffeic acid were prepared and sequentially injected under isocratic conditions using the optimised parameters. The calibration curve was found to be linear (R^2 = 0.996) over the concentration range 0.8–12.8 μM of caffeic acid as a gradual increase in concentration of caffeic acid resulted in a proportional increase in the corresponding positive peak height (Fig. S3, supplementary data). Concentrations of 100 μM are still measurable, i.e. they fall within the dynamic range of 160 mAU.

3.6. Application of the on-line HPLC-CBA to a tea extract

Finally the on-line HPLC-CBA method was tested with a green tea extract. In Fig. 3 the UV and CBA chromatograms at 265 and 440 nm respectively, are shown. The retention time of EGCG, which is an important constituent of tea leaves, was 16 min (peak 7). According to the CBA chromatogram, EGCG is the major antioxidant present in tea. By a comparison of the two chromatograms, it is clear that the method has significant selectivity. As shown in the UV chromatogram at 265 nm, peak 8 (epicatechin) corresponds to the most abundant constituent of the green tea extract. This constituent is also a major antioxidant although not as potent as EGCG in this assay (Fig. 3). The observed selectivity is very comparable to that of the on-line HPLC-UV-CL system which also makes use of reactive oxygen species (ROS) [18]. Based on the obtained data, the peak broadening and the sensitivity are adequate for using the CBA in an on-line HPLC method. The positive peaks in the post-column chromatogram make it easier to compare UV and antioxidant profiles than for the other existing on-line antioxidant methods. Thus intrinsically the method is suitable for separation and antioxidant activity evaluation of plant extracts. More detailed comparisons are necessary to determine its true usefulness.

4. Conclusion

A rapid HPLC-CBA system for on-line detection of antioxidants was developed and optimised. It detects antioxidants by their inhibitory effect on the bleaching/oxidation of crocin. The limit of detection for seven different antioxidants varied from 0.79 to 7.4 ng. Advantages of this method over existing on-line HPLC methods for high-resolution screening (HRS) of antioxidants are: (1) it makes use of a reactive oxygen species (peroxyl radicals), (2) it makes use of a polyunsaturated substrate, (3) it is simpler to apply than other on-line assays using reactive oxygen species, and (4) it detects positive peaks in the post-column chromatogram. Disadvantages are: (1) it is slightly less sensitive than other on-line antioxidant assays, (2) due to the effect of the organic modifier in the HPLC eluent on the crocin bleaching rate, gradient runs are only possible after baseline correction, and (3) a reaction coil temperature of 90 °C is necessary to achieve significant oxidation of crocin within 30 s. The method can be applied for the direct screening of very polar to moderately polar compounds and plant extracts. Further research is needed to determine its usefulness for structure–activity-relationships.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2012.03.026..

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