The Optimisation of Analytical Parameters for Routine Profiling of Antioxidants in Complex Mixtures by HPLC Coupled Post-column Derivatisation

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ABSTRACT:

Introduction – The wide application of natural and artificial antioxidants in the food, cosmetic and pharmaceutical industries as well as the recognition of the importance of food antioxidants for supporting human health have created a demand for reliable and industrially applicable methods of determining antioxidative activity. This requirement can be fulfilled with the recently proposed HPLC-post-column derivatisation approach, enabling rapid measurements of antioxidative potential along with profiling antioxidants in complex mixtures.

Objective – To employ the commercially available post-column derivatisation device for the on-line detection of antioxidants and to optimise analytical parameters enabling its application for routine profiling of antioxidants in complex mixtures such as those of plant or food origin.

Methodology – The mixtures of standards and fruit extracts were resolved using an optimised HPLC method followed by the on-line derivatisation of analytes in a PCX post-column derivatisation instrument. Such parameters as the type of indicator reagent (ABTS, DPPH, Folin-Ciocalteu), its concentration and the temperature of the derivatisation reaction were investigated. The determinations of the Trolox equivalents (TE) values were compared with those obtained by corresponding bulk spectrophotometric tests.

Results – The study confirms that the commercial HPLC-coupled post-column derivatisation instruments are suitable for routine on-line detection of antioxidants in complex mixtures and the determination of their TE values. The analytical parameters optimised here appear to represent a ready-to-use toolbox for the food and pharmaceutical industries, enabling the monitoring of bioactive substances along the production line and during storage, and the characterisation of plant material by creating chromatographic profiles supplemented with antioxidant fingerprints. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: post-column derivatisation; HPLC; fingerprints of antioxidants; antioxidant activity

Introduction

Natural and artificial antioxidants have been used in food production for decades. Until recently, their major role was to protect components against oxidative damage, an important issue in the preservation of food products, cosmetics and other consumer items (Tynek *et al.*, 2008; Li *et al.*, 2009; Tuckey *et al.*, 2009). In the past decade, the natural antioxidants have started to dominate the health food market, a phenomenon recognising the importance of redox homeostasis for human health (Valko *et al.*, 2007). This recognition has inspired the search for edible plants with high antioxidant potential, often referred to as "superfruits" or "superveg" and the design of food products and dietary supplements displaying high antioxidant potential. Although recent research has revealed that in the majority of cases it is not the antioxidative potential of the most prominent health-promoting antioxidant phytochemicals that is responsible for their biological benefits, the antioxidative potential remains, from the analytical perspective, the most convenient marker of bioactivity and a property that can be relatively easily tracked during food processing and the production of dietary supplements and pharmaceuticals. As a consequence, the rapid development of methods for assessing the antioxidant activity of purified compounds, mostly phytochemicals, but also complex mixtures obtained from natural sources, has taken place. Accordingly, many reviews and monographs have been published on the evaluation of antioxidant assays (Huang *et al.*, 2005; Moon and Shibamoto 2009). More recently, some of the

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principles of bulk spectrophotometric or electrochemical assays detecting substances with antioxidative properties have been modified for on-line, post-column coupling with high-performance liquid chromatography (HPLC; Dapkevicius *et al.*, 2001; Exarchou *et al.*, 2006; Bartasiute *et al.*, 2007; Kool *et al.*, 2007; Milasiene *et al.*, 2007; Niederländer *et al.*, 2008). The aim of such on-line approaches is not only the rapid measurement of total activity, but also the profiling of antioxidants in complex mixtures following their chromatographic separation from the matrix.

The studies by Koleva *et al.* (2000, 2001) pioneered the application of the two most popular colorimetric assays, ABTS and DPPH, for detecting antioxidative substances in HPLC eluates after the separation of phytochemicals in complex mixtures. These authors optimised several parameters such as stock concentrations of radicals, reaction times, choice of organic solvents and pH. Cardeñosa *et al.* (2002) extended the choice of chemical sensors of antioxidants by postulating the relatively stable phosphomolybdenum reagent for their detection. These investigations demonstrated the potential of the on-line approach; however, being based on prototypical self-made equipment, it has never reached beyond the purely experimental stage.

This study investigated the suitability of a commercial Pinnacle PCX post-column Derivatisation Instrument (PCX), the representative of this type of equipment, for determining antioxidant compounds in routine HPLC analyses. The PCX automatically mixes the stream of effluent flowing from a chromatographic column with a stream of reagent solution. This is achieved with a Pickering syringe pump that completes a filling cycle prior to the injection of a sample and delivers reagent during the run at a constant rate. The valve between the pump and reactor helps to regulate the reagent flow by opening key ports at the appropriate time. The mixture flows through a reactor to allow enough time for the chemical reactions to proceed with a satisfactory yield. After leaving the reactor, the derivatives flow into the detector, where the absorbance of the effluent is measured on-line. In the quite frequent cases when the reaction is very slow at room temperature, the system can be heated. As far as we know, temperature, a parameter that is crucial for the kinetics of chemical reactions, thus determines whether the derivatisation yield will be good enough to ensure appropriate reproducibility of the process, has not been optimised before. Only Cardeñosa *et al.* (2002) tried to use higher than ambient reactor temperatures during derivatisation with phosphomolybdenum reagent and Mnatsakanyan *et al.* (2010) heated the reaction coil to 60°C to enhance the sensitivity of detection of antioxidants following derivatisation with DPPH.

The aim of the present research was to find the conditions under which on-line antioxidant determination can be employed routinely in both research laboratories and industry. This was achieved using commercial equipment, optimal chromatographic conditions for resolving the major groups of antioxidants and finding the derivatisation reaction parameters that would guarantee satisfactory yields. Additionally, instead of "home-made" phosphomolybdenum reagent, we suggest using commercial Folin–Ciocalteu's solution, one of the most commonly used derivatisation reagents in bulk colorimetric determinations. Initially, the Folin–Ciocalteu reagent (FCR) was considered to exhibit selectivity towards polyphenols. Several studies documented, however, its reactivity towards antioxidants belonging to various classes, as well as other compounds, e.g. carbohydrates, nucleotide bases and ketones (Walker *et al.*, 2010). Despite this relatively broad reactivity, the determinations of Trolox equivalents (TE) values with the aid of FCR are very well correlated with those obtained by DPPH and ABTS assays. Therefore, this reagent is still the most frequently used in preliminary assessments of antioxidant potential of complex mixtures (Huang *et al.*, 2005; Kusznierewicz *et al.*, 2008). To verify the applicability of the proposed system, the antioxidant potentials measured after derivatisation as the TE values of individual phenolic compounds from standard mixtures and plant extracts separated by HPLC (on-line analyses) were compared with those obtained by standard bulk tests (off-line analyses).

Experimental

Chemicals and reagents

HPLC-grade methanol and pure p.a. methanol were purchased from Chempur (Poland), formic acid (98–100%) was obtained from Merck (Germany). Water was purified with a Q_{PLUS}185 system from Millipore (USA). The following reagents and compounds were used: 2,2′-azinobis (ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), both from Sigma-Aldrich (USA); FCR and sodium persulphate from Merck (Germany). The following standards were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), gallic acid (GA), (±)-naringenin (NRG), (+)-catechin hydrate (CAT), resveratrol (RSV), luteolin (LUT), genistein (GEN) and rutin hydrate (RUT) from Sigma-Aldrich (USA); caffeic acid (CA), myricetin (MYR), protocatechuic acid (PCA), ferulic acid (FA), sinapic acid (SA), apigenin (API), cyanidin-3-glucoside (CGL) and kaempferol (KAM) from Fluka (USA); phloretin (PHL), cyanidin-3-*O*-galactoside chloride (CGA), chlorogenic acid (ChA), (–)-epicatechin (eCAT), morin (MOR) and cyanidin chloride (CCh) from Extrasynthese (France). Individual stocks of standard solutions at a concentration of 1 mg/mL were prepared in HPLC-grade methanol. For LC analysis and for colorimetric assays, the stock solutions were respectively diluted with HPLC-grade methanol or pure p.a. methanol to the required working concentrations.

Sample collection and preparation

Two kinds of fruits were used in the study: sloes, the fruit of the blackthorn tree (*Prunus spinosa*), and mirabelle plums (*Prunus domestica* var. *syriaca*), obtained from a local plant processing factory. The fruits were stoned, lyophilised and ground. One gram of each of the freeze-dried, powdered fruits was then extracted with 4 mL of methanol. Extraction was sonication-assisted for 10 min, after which the solids were centrifuged (5 min, 490***g***) and the supernatant collected. The extraction and the subsequent steps were repeated three times, then the supernatants were pooled.

HPLC conditions

An Agilent Technologies 1200 Series HPLC-DAD system (Agilent Technologies, USA) was employed throughout the study. Chromatographic separations were performed on an Agilent Eclipse XDB-C_{18} column (4.6×150 mm, 5 µm). For analysis with post-column derivatisation using ABTS and FCR, a mobile phase containing 4.8% (v/v) formic acid in water (solvent A) and 100% methanol (solvent B) was used. The solvent composition for gradient elution for the determinations with both ABTS and FCR was as follows: the percentage of solvent B was increased from 10 to 65% over 50 min. In the case of derivatisation with DPPH, two kinds of mobile phase were used. One of them contained 1% (v/v) formic acid in water (solvent A) and 100% methanol (solvent B),

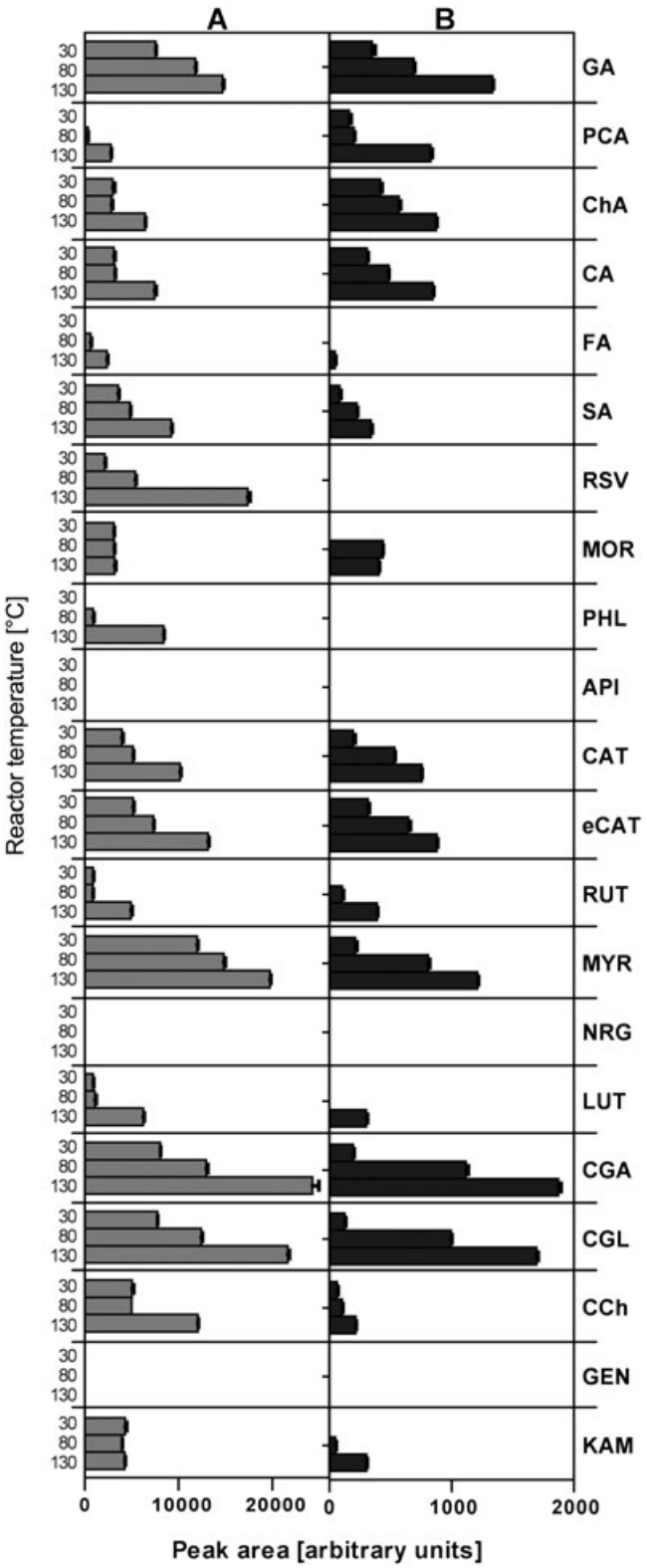


Figure 1. Influence of reactor temperature on peak areas after post-column derivatisation of known antioxidants belonging to different classes of phenolic compounds. The HPLC resolution was obtained using gradient mode and post-column derivatisation carried out with (A) - ABTS or (B) - FCR, as described in Experimental. The values are the means of three determinations±SD.

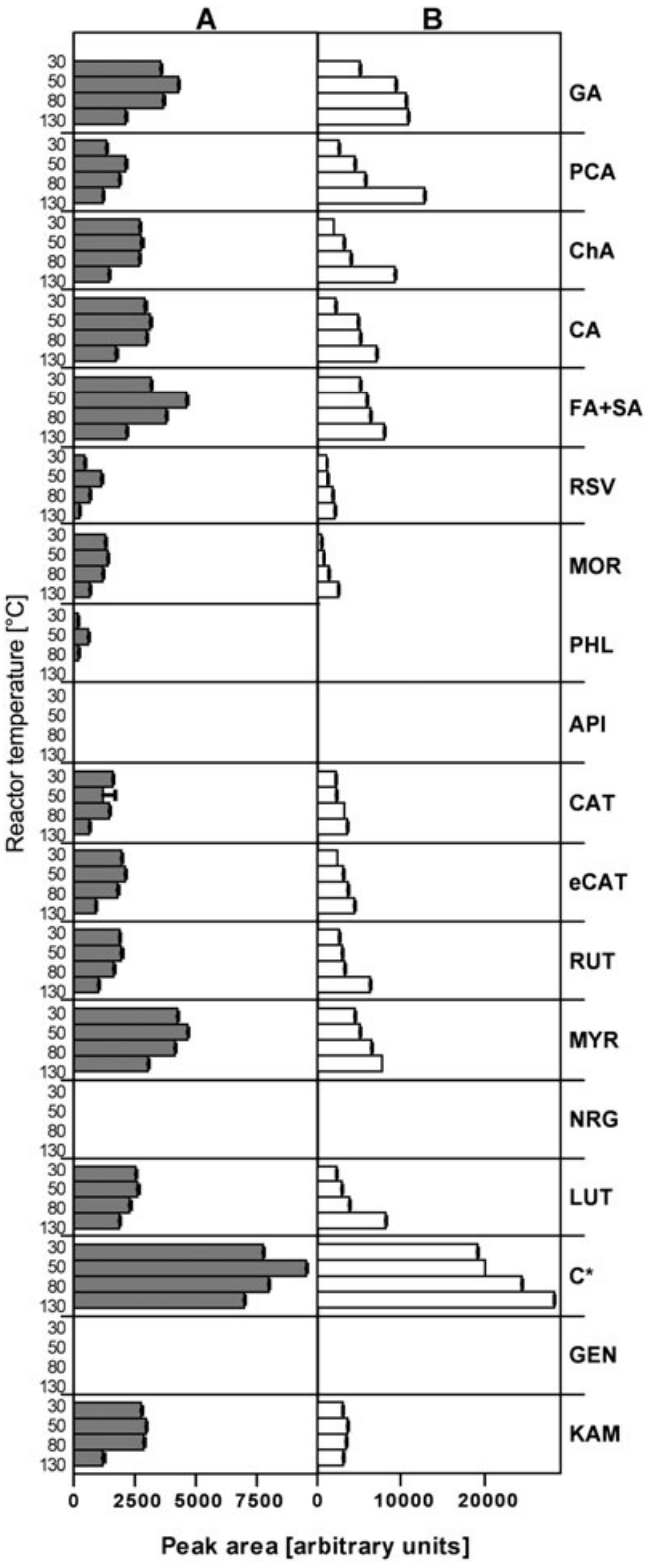


Figure 2. Influence of reactor temperature on peak areas after post-column DPPH derivatisation of several known antioxidants belonging to different classes of phenolic compounds. HPLC resolution carried out in gradient mode using either (A) - acidified mobile phase or (B) - unacidified mobile phase. The values are the means of three determinations±SD.

Table 1. Summary of the optimal derivatisation conditions proposed

	ABTS	DPPH		FCR
Wavelength (nm)	734	515		754
Flow of reagent (mL/min)	0.1	0.1		0.1
Concentration of reagent[a]	2.1 mmol/L in methanol	0.15 mg/mL in methanol		40% (v/v) in water
Flow of mobile phase (mL/min)	0.7	0.7		0.7
Content of organic solvent in mobile phase (%)	0–100	30–100	10–100	0–80
Concentration of formic acid in mobile phase (%)	≤5	≤0.3	0	≤5
Temperature of reactor (°C)	130	50	130	130
Isocratic elution	+	+	+	+
Gradient elution	+	± base line drift	+	+

[a]Concentration of derivatisation reagent is suitable for determination of compounds with antioxidant capacity not higher than 0.008 µmol of Trolox per injection (4 mM Trolox, 2 µL injection), higher antioxidant activity of analytes causes truncation of peaks.

while the other contained pure water (solvent A) and 100% methanol (solvent B). The composition of the gradient elution solvents for the determinations involving DPPH derivatisation was as follows: the percentage of solvent B was increased from 30 to 65% over 50 min. The mobile phase flow rate in all analyses was set at 0.7 mL/min, and the injection volume of all samples was 2 µL.

Post-column derivatisation conditions

On-line post-column addition of ABTS, DPPH and FCR was performed using a Pinnacle PCX Derivatisation Instrument (Pickering Laboratories, Inc., USA) consisting of a pump delivery system and reactor that could be heated from 5°C above ambient to 130°C. The flow rate of the individual reagents was set at 0.1 mL/min. In all experiments, the 0.5 mL (PTFE, 0.25 mm, 10 m) coil available as a standard part of the Pinnacle PCX Derivatisation Instrument was used. Chromatograms of products after ABTS, DPPH and FCR derivatisation were registered at 734, 515 and 750 nm respectively, using a multiple wavelength detector (Agilent 1200 Series MWD, USA). The derivatisation reagents were prepared as follows: ABTS was dissolved in aqueous sodium persulphate (2.45 mmol/L) to obtain a concentration of 7 mmol/L. The mixture was stored in the dark at room temperature for 12 h and before use was diluted with methanol to the stock concentration of 30% (v/v). The working solution of DPPH (0.15 mg/mL in methanol) was prepared just before analysis. Commercially available FCR was diluted with water to a concentration of 40% (v/v).

The influence of temperature on derivatisation efficiency

The derivatisation efficiency was calculated for reactor temperatures set at 30°C, 80°C and 130°C in the case of ABTS and FCR and 30, 50, 80 and 130°C for DPPH. The four mixtures of phenolic compounds at a concentration of 0.5 mmol/L for each standard were injected into the HPLC–PCX system, and resolution was carried out under the gradient conditions specified earlier. The peak areas of standards after derivatisation were monitored for each reactor temperature at 734, 515 and 750 nm for ABTS, DPPH and FCR, respectively. The standard mixtures used consisted of: mixture I, GA, PCA, ChA, CA, FA, SA; mixture II, RSV, MOR, PHL, API; mixture III, CAT, eCAT, RUT, MYR, NRG, LUT; and mixture IV, CGA, CGL, CCh, GEN, KAM.

Determination of the TE antioxidant capacity of standards and plant extracts using the HPLC–PCX system

DPPH, ABTS or FCR derivatisation reagents were used to generate the Trolox standard lines. Derivatisation was carried out at the temperatures determined as optimal for each reagent, i.e. 50°C for the DPPH radical, and 130°C for ABTS and FCR. Methanolic solutions of Trolox (concentrations 0.6–4 mmol/L) were injected into the HPLC system and analysed under isocratic conditions (40% of solvent A and 60% of solvent B). The equation of the standard line

$$\text{Trolox concentration} = f(\text{peak area})$$

was determined for each derivatisation reagent. These equations were used to calculate TE values from the peak areas of the standard phenolic compounds and analytes from plant extracts following derivatisation at the optimal reactor temperature.

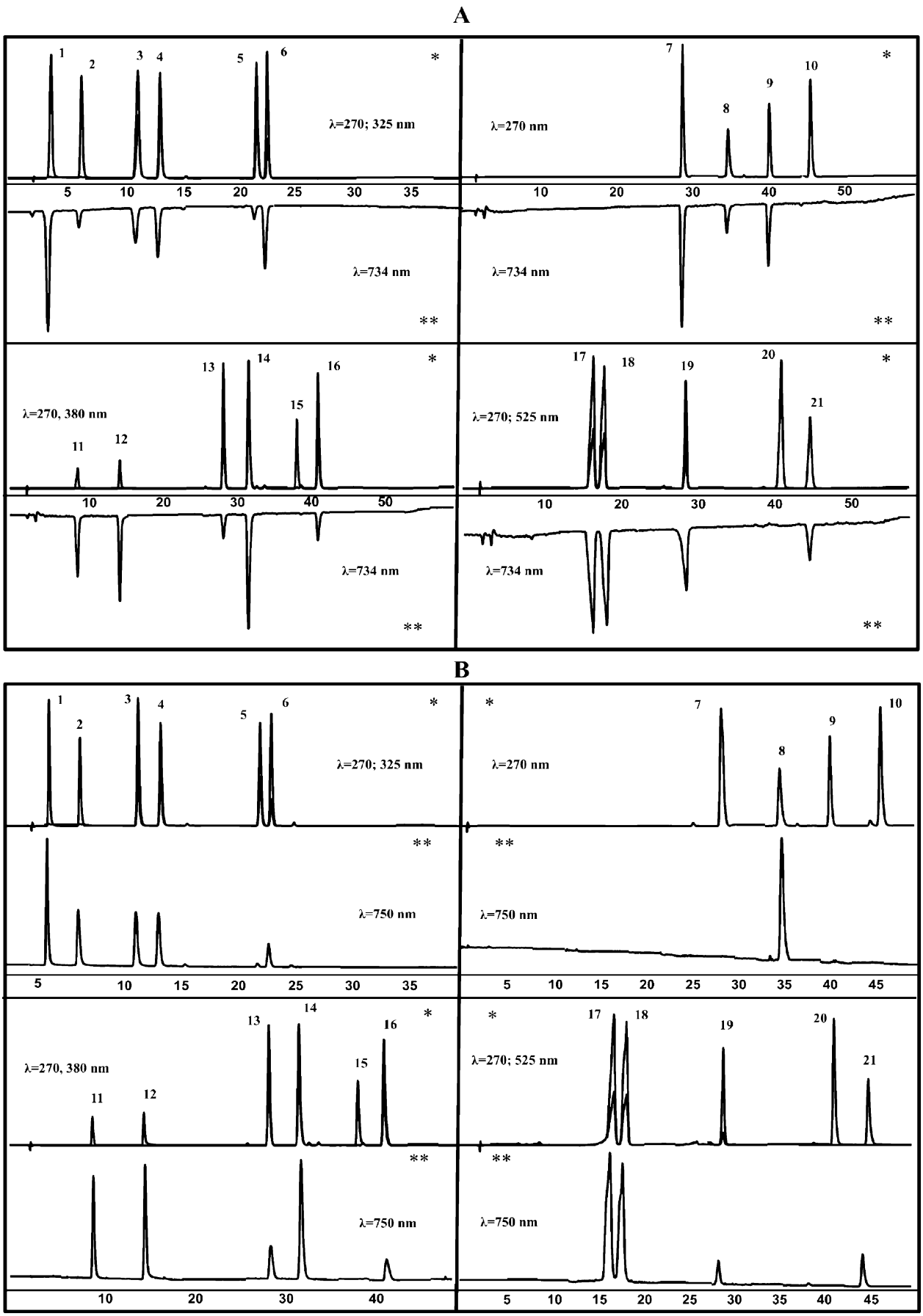
Determination of TE using colorimetric methods

Standard methods using ABTS, DPPH and FCR were used for the colorimetric determination of antioxidant activity. In each case, a Trolox solution served to generate the standard line (concentration range 0–4.0 mmol/L). The stock solutions of derivatisation reagents were diluted before measurements as follows: DPPH was diluted with methanol until the absorbance reached 1.0±0.02 at λ=515 nm; the ABTS radical cation solution was diluted with methanol to display an absorbance of 0.7±0.02 at 734 nm; the commercial FCR preparation was diluted with water (1:9 v/v). All determinations were carried out in 48-well plates at room temperature, and the absorbance was measured on a Tecan Infinite M200 spectrophotometer (Tecan Group Ltd, Switzerland). DPPH solution (1 mL) was mixed with standard solutions (30 µL), and the absorbance of the mixture was measured after 10 min at 515 nm. The ABTS solution (1 mL) was mixed with solutions of standards (10 µL) and the absorbance was measured after 10 min at 734 nm. The FCR solution (1 mL) was mixed with solutions of standards (0.1 mL) and the absorbance measured after 10 min at 750 nm.

Results and Discussion

Reliable and reproducible on-line determination by post-column derivatisation can be achieved provided that two main requirements

Figure 3. Combined plots of profiles before (*) and after (**) derivatisation obtained for four mixtures of standard phenolic compounds resolved under gradient HPLC conditions and post-column derivatised at 130°C with ABTS (A) or FCR (B) as derivatisation reagents. The samples analysed were mixture I: 1-GA, 2-PCA, 3-ChA, 4-CA, 5-FA, 6-SA; mixture II: 7-RSV, 8-MOR, 9-PHL, 10-API; mixture III: 11-CAT, 12-eCAT, 13-RUT, 14-MYR, 15-NRG, 16-LUT; mixture IV: 17-CGA, 18-CGL, 19-CCh, 20-GEN, 21-KAM.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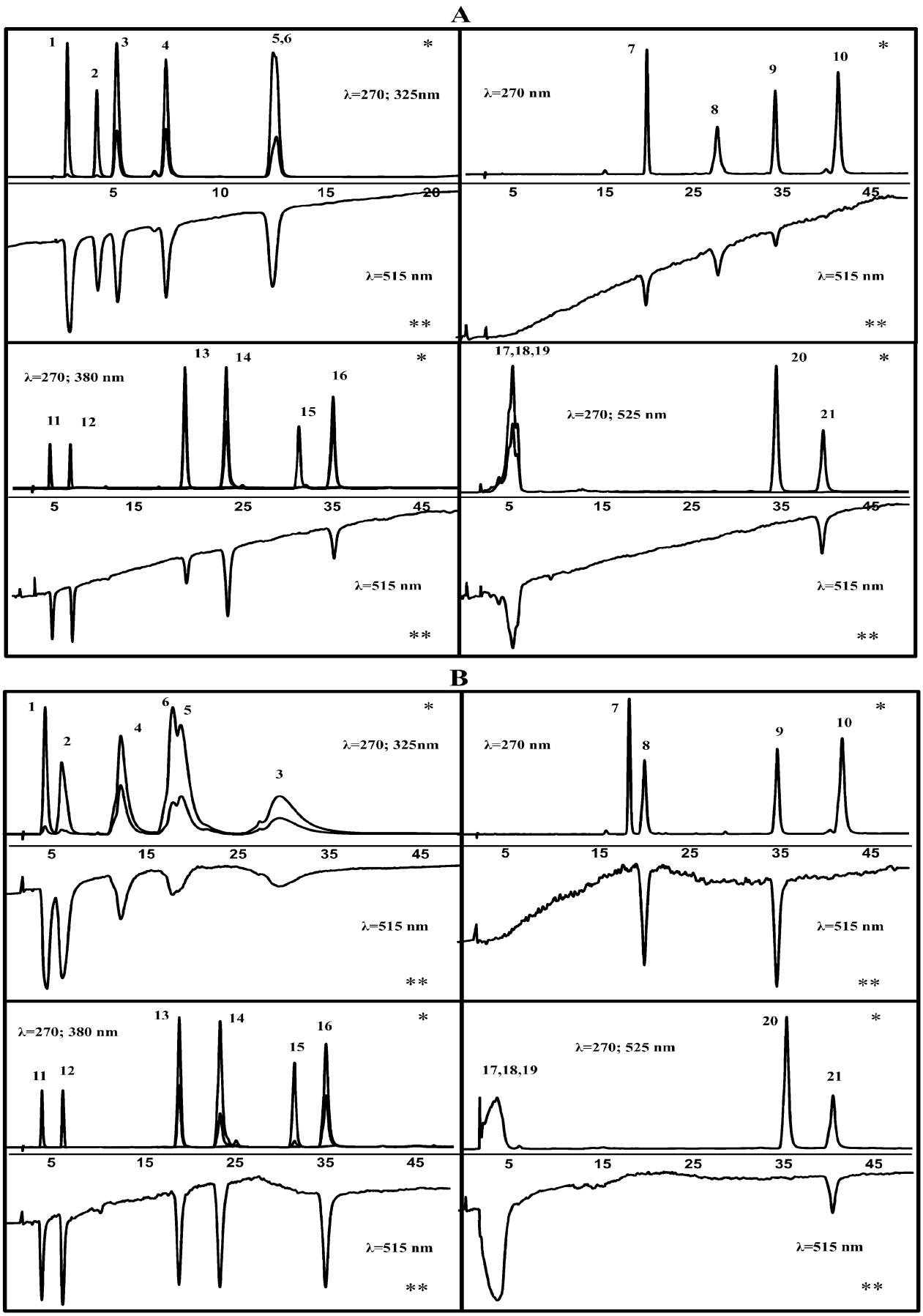
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are fulfilled. Firstly, the equipment employed must guarantee stable conditions of analysis, with the concentration of derivatisation agents being easy to control. In this study, the application of fully automated and programmable devices dedicated to such tasks enabled not only the convenient modification of HPLC and the derivatisation parameters, but also their stabilisation during the analytical procedure. Secondly, an analyte in the eluate leaving the chromatographic column must react reproducibly with the derivatisation agent to give a maximum yield when the conditions of the analytical procedure are optimal. The efficiency of any chemical reaction depends on three parameters: reagent concentrations, reaction time and temperature. Therefore, the elaboration of the method of antioxidant determination by post-column derivatisation using the PCX system began with the establishment of the favourable ranges of these parameters within the limits of the equipment's capabilities on the one hand and efficient HPLC separation of most common groups of natural antioxidants on the other.

Concentration of derivatisation reagents

The first step in this analysis of antioxidants was to establish the concentrations of ABTS, FCR and DPPH, to ensure that they were not limiting factors under the chromatographic conditions enabling the efficient HPLC resolution of natural mixtures containing antioxidants. Apart from the requirement of satisfactory separation of analytes, it was necessary to take into account other restrictions affecting the concentration of reagents, such as their solubility and stability in the eluate from HPLC, as well as the pressure limit of PCX (30 bar). To comply with the latter, we determined that, for a mobile phase flow rate of 0.7 mL/min, which still provided satisfactory separation, the highest possible eluate/reagent ratio could not exceed 7:1. Taking this into account, the concentration of stock solutions of reagents was adjusted based on 4 mmol/L Trolox, the concentration exhibiting an antioxidative activity comparable to that of most potent plant preparations (data not shown).

In the case of ABTS and FCR, the optimal concentrations of the solutions were 2.1 mmol/L and 40% v/v, respectively, corresponding to about 0.135 mg/mL and 5% v/v in the eluate for a mobile phase flow rate of 0.7 mL/min and a reagent flow rate of 0.1 mL/min. These concentrations are thus suitable for derivatising compounds with an antioxidant activity equal to or less than 4 mmol/L Trolox; the peaks of compounds with a greater activity will be "truncated". The two reagents, in the concentrations established and at a flow rate of 0.1 mL/min (eluate flow 0.7 mL/min), can be used in the gradient runs with a mobile phase containing 0–100 and 0–80% (v/v) methanol in aqueous formic acid (4.8% formic acid in water) in the case of ABTS and FCR, respectively. During derivatisation with FCR, methanol concentrations exceeding 80% (v/v) in the mobile phase caused the crystallization of salts in the HPLC–PCX system.

Unfortunately, the stability of the DPPH radical, and hence the absorbance of the eluate, depended strongly on the concentration of the mobile phase constituents (methanol and aqueous formic acid). The application of this reagent to HPLC post-column derivatisation under the gradient conditions typically used for the resolution of antioxidants in plant material seems therefore troublesome. Koleva *et al.* (2000) had earlier indicated that a highly acidic eluent (pH 2.2) caused a drastic reduction in DPPH absorbance, while HPLC gradients with a mobile phase consisting of 10–90% organic solvent in water could be applied on-line without significant changes in DPPH absorbance. In our investigations, DPPH at a concentration of 0.15 mg/mL in methanol and a flow rate set at 0.1 mL/min (eluate flow 0.7 mL/min, DPPH concentration in eluate ca 0.002 mg/mL) enabled gradient runs to be carried out with a mobile phase consisting of 35–100% methanol in water or in 1% formic acid. The mobile phase containing less than 35% methanol caused DPPH to precipitate in the HPLC–PCX system (data not shown). The various conditions and applications of post-column derivatisation using DPPH radical have been recently reviewed (Niederländer *et al.*, 2008; Shi *et al.*, 2009). All these reports illustrate that it is possible to establish analytical conditions that for specific samples can successfully combine efficient separation and post-column reaction with DPPH. However, for a routine protocol, applicable to a wide variety of plant samples, this reagent does not appear to provide any benefit. Its spectrum of reactivity overlaps with that of ABTS radical or is even narrower according to some reports (Exarchou *et al.*, 2006). Moreover, the MDCs (minimum detectable concentration) determined with ABTS radical have been also lower in the case of the majority of antioxidants studied (Koleva *et al.*, 2001). As mentioned above, DPPH radical stability is challenged by a number of factors, including the acidity of the mobile phase. The latter is especially important, as it precludes the application of post-column derivatisation in conjunction with IFU Method No. 71 (1998). This standardized method, which requires high concentration of formic acid (5–10%) in mobile phase, is often used in the analysis of food pigments and fruit juice adulteration (Sass-Kiss *et al.*, 2005; Stintzing *et al.*, 2006).

Duration of the derivatisation reaction

In the on-line post-column derivatisation methods, the duration of the reaction between eluate components and derivatisation reagents depends on the flow rate and the volume of the reaction coil. In this study, the 0.5 mL reaction coil (a basic item of PCX equipment) was used with the flow rate predetermined by optimal HPLC analysis conditions. The final flow rate of reagents in the reaction coil was typically 0.8 mL/min, which allowed about 1 min of reaction time. From our experience, we conclude that, in the case of the PCX system, the duration of the reaction can be manipulated only using different reaction coils, although we do not think this is worthwhile. As can be seen from the data presented, detection of antioxidants in the current PCX configuration is very efficient, with the peaks only slightly more diffuse in the profiles recorded by the second detector (after derivatisation) than by the first one (before derivatisation). Even if extension of the reaction coil prolonged the reaction time, thus increasing the derivatisation yield, this would make the peaks more diffuse or even cause them to overlap, especially in the case of complex natural plant

Figure 4. Combined plots of profiles before (*) and after (**) derivatisation obtained for four mixtures of standard phenolic compounds resolved under gradient HPLC conditions with an acidified mobile phase (A) and an unacidified mobile phase (B) and post-column derivatised with DPPH; reactor temperature set at 50°C or 130°C respectively. The samples analysed were mixture I: 1- GA, 2-PCA, 3-ChA, 4-CA, 5-FA, 6-SA; mixture II: 7-RSV, 8-MOR, 9-PHL, 10-API; mixture III: 11- CAT, 12-eCAT, 13-RUT, 14-MYR, 15-NRG, 16-LUT; mixture IV: 17-CGA, 18-CGL, 19-CCh, 20-GEN, 21-KAM.

Table 2. TE (mmole/L) determined for known standard phenolic compounds using HPLC - post column derivatisation system (on-line) or standard colorimetric tests (off-line)

		ABTS		FCR		DPPH		
Samples		On-line	Off-line	On-line	Off-line	On-line (acidified mobile phase)	On-line (not acidified mobile phase)	Off-line
Mixture I	GA	3.172±0.049	3.288±0.003	2.154±0.012	1.177±0.051	2.180±0.012	2.378±0.015	3.426±0.163
	PCA	0.414±0.014	0.813±0.049	1.548±0.024	0.697±0.012	1.399±0.032	2.759±0.011	1.114±0.004
	ChA	1.259±0.014	0.803±0.004	1.603±0.014	1.184±0.035	1.633±0.044	2.098±0.019	1.272±0.041
	CA	1.490±0.051	0.996±0.040	1.569±0.010	1.134±0.016	1.765±0.023	1.683±0.012	1.392±0.041
	FA	0.309±0.032	0.938±0.053	0.608±0.014	0.418±0.006	2.303±0.021	1.858±0.016	0.644±0.008
	SA	1.903±0.041	1.324±0.047	0.966±0.017	1.276±0.005			0.854±0.029
	Sum	8.548	8.162	8.448	5.887	9.281	10.777	8.702
	Total mixture I	—	9.556±0.061	—	6.595±0.087	—	—	8.844±0.287
Mixture II	RSV	3.795±0.102	2.236±0.012	n.d.	0.738±0.002	1.055±0.018	0.752±0.015	0.504±0.011
	MOR	0.496±0.044	1.016±0.064	1.041±0.007	0.789±0.004	1.150±0.006	0.827±0.005	1.092±0.025
	PHL	1.714±0.024	1.816±0.111	n.d.	n.d.	0.860±0.022		0.298±0.020
	API	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Sum	6.006	5.068	1.041	1.527	3.065	1.579	1.894
	Total mixture II	—	4.646±0.469	—	1.642±0.017	—	—	2.252±0.088
Mixture III	CAT	2.122±0.028	2.112±0.160	1.462±0.007	0.979±0.017	1.076±0.014	1.022±0.011	1.360±0.018
	eCAT	2.816±0.029	2.609±0.144	1.610±0.010	1.049±0.002	1.398±0.018	1.196±0.010	1.870±0.037
	RUT	0.908±0.042	0.809±0.074	1.024±0.008	0.753±0.001	1.340±0.034	1.538±0.010	2.046±0.049
	MYR	4.344±0.043	3.241±0.041	2.009±0.013	1.806±0.059	2.314±0.019	1.810±0.003	1.880±0.094
	NRG	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	LUT	1.210±0.019	1.135±0.033	0.919±0.015	0.774±0.027	1.578±0.033	1.886±0.008	2.246±0.207
	Sum	11.400	9.907	7.025	5.360	7.706	7.453	9.402
	Total mixture III	—	10.462±0.427	—	5.205±0.153	—	—	9.662±0.053
Mixture IV	CGA	3.927±0.021	3.360±0.013	2.834±0.036	2.339±0.034	3.917±0.015	2.469±0.002	2.145±0.135
	CGL	3.443±0.055	3.148±0.281	2.667±0.017	2.646±0.010			2.335±0.010
	CCh	1.712±0.019	2.526±0.335	1.283±0.007	1.576±0.087			2.715±0.105
	GEN	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	KAM	0.301±0.016	1.079±0.080	1.367±0.003	1.147±0.006	2.084±0.016	0.580±0.001	0.930±0.009
	Sum	9.384	10.113	8.152	7.707	6.000	3.050	8.125
	Total mixture IV	—	8.504±0.302	—	7.612±0.231	—	—	9.090±0.112
Plant extracts	Blackthorn	7.737	54.348±0.422	9.633	31.360±0.107	7.977	13.771	35.761±0.400
	Mirabelle plum	3.125	5.879±0.154	5.517	4.122±0.170	1.479	4.601	2.700±0.083

mixtures; the overall result of the analytical procedure would thus be worse.

The effect of temperature on derivatisation efficiency

Temperature is the most important factor affecting the kinetics, and thus the completeness, of the derivatisation reaction within the analytical time period. Yet this parameter has very rarely been taken into consideration during previously reported on-line antioxidant determinations. As mentioned before, the PCX reactor temperature can be regulated over a wide range. To determine the optimum derivatisation temperature for each reagent investigated, a set of substances known to be potential antioxidants belonging to different classes of phenolic compounds was used. The stability of these compounds exposed to the highest temperature used (130°C) during passage through reaction coil was ensured beforehand (data not shown). Figure 1 presents the areas of peaks detected for phenolic compounds separated, then derivatised with ABTS or FCR at different reactor temperatures. Rising temperature during the reaction with both ABTS and FCR was associated in all cases with a larger peak area.

In the case of DPPH used for derivatisation, it turned out that HPLC gradient runs required a slightly different mobile phase composition. The aqueous component of the mobile phase (4.8% formic acid) was replaced with either 1% formic acid in water or water alone. The peak areas of phenolic compounds separated in an acidified or unacidified mobile phase determined after DPPH derivatisation for different reactor temperatures are presented in Fig. 2. This shows that the mobile phase composition alters the response of reagents to temperature. In the case of the acidified phase, the optimum temperature was 50°C, whereas without the addition of formic acid, the derivatisation yield increased over whole range of temperatures used. Table 1 contains a summary of optimised analytical parameters for all three reagents tested in the context of on-line post-column profiling of antioxidants.

Analysis of solutions of standard antioxidants under optimised conditions

To evaluate the efficacy of antioxidant determination using the PCX system under optimised conditions, four mixtures of standard phenolic compounds were analysed (mixtures I–IV as specified under Experimental). Figure 3 shows the chromatograms of these mixtures obtained before and after derivatisation with ABTS (Fig. 3A) and FCR (Fig. 3B) at 130°C. The post-column detection of the reduction of ABTS radicals in relation to the content of antioxidants is reflected by the negative UV–VIS chromatograms at 734 nm (Fig. 3A**). In the case of FCR derivatisation, compounds containing active hydroxyl group(s) react to form a coloured complex, which appears as a positive chromatogram at 750 nm (Fig. 3B**).

The profiles of antioxidants after ABTS and FCR derivatisation indicate almost the same substances. Contrary to the situation with ABTS, only RSV and PHL did not give a signal with FCR. API, NRG and GEN were not detected with either ABTS or FCR. The observed differences in peak heights between the profiles of antioxidants detected with ABTS and FCR probably reflect the different kinetic behaviour of the separated phenolic compounds towards these reagents.

Figure 4 presents chromatograms of four mixtures of standard phenolic compounds (composition as specified before) obtained during analyses with the use of DPPH as derivatisation reagent. The optimisation of analytical parameters to ensure proper derivatisation with DPPH resulted in the poor resolution of some polyphenols. Under these conditions, in which the content of formic acid and water in the mobile phase had to be lowered, the separation of anthocyanins particulary suffered (Fig. 4, compounds 17–19).

Determination of TE

Table 2 compares TE values calculated for the analysed standard phenolic compounds (0.5 mmol/L) based on peak areas obtained by post-column derivatisation (on-line) and batch spectrophotometric methods (off-line). The TEs of phenolic compounds obtained in on-line analyses are in many cases very similar to those determined in off-line analyses. However, often some values are higher or lower than the corresponding off-line results, which points to the differential influence of such parameters as time, temperature and reaction media on the derivatisation yield of different phenolics. It is, however, very important to note that there is a strong correlation between TEs obtained from the corresponding on/off-line measurements, as shown by the Pearson coefficient: ABTS, $r=0.919$; FCR, $r=0.895$; DPPH in acidified mobile phase, $r=0.851$. In the case of DPPH on-line derivatisation in an unacidified mobile phase, it was hard to calculate the TEs of individual phenolic compounds from HPLC profiles, because some of them were co-eluted (Fig. 4B). The Pearson coefficient for the latter on-line and off-line analyses was $r=0.667$.

The compounds with the highest antioxidant activity according to all the on-line and off-line methods were MYR, GA, anthocyanins and catechins. As can be seen from Figs 3 and 4 and Table 2, three of the standards – API, NRG and GEN – which did not react with any of the derivatisation reagents on-line regardless of conditions, did not give a signal in the colorimetric methods either. However, there are several articles in the literature demonstrating the antioxidant activity of these compounds (Romanová *et al.*, 2001; Russin *et al.*, 2006; Shohreh *et al.*, 2008; Jayaraman *et al.*, 2009). The definition of an antioxidant does not impose any limitation on the mechanism(s) of antioxidant action. Therefore, a dietary antioxidant can (sacrificially) scavenge reactive oxygen/nitrogen species (ROS/RNS) to stop radical chain reactions, or it can inhibit the reactive oxidants from being formed in the first place (preventive). Dietary antioxidants often broadly include radical chain reaction inhibitors, metal chelators, oxidative enzyme inhibitors and antioxidant enzyme cofactors. That is why the negative results of some methods do not necessarily preclude the antioxidant activity of certain compounds.

Only the results for RSV after FCR derivatisation were inconsistent, since this compound did not react during post-column derivatisation. In contrast, the antioxidative potential of RSV was readily measurable in all three batch tests. This suggests that acidic conditions in the eluate may influence the chemical properties of some compounds.

The analysis of fruit extracts under optimised conditions

Once the appropriateness of the PCX system parameters was verified with the aid of standard antioxidants, we applied our protocol to determine the antioxidant profile of natural plant mixtures. The derivatisation was carried out with FCR and ABTS

only; DPPH was not used due to its instability under optimised HPLC conditions. The samples chosen were methanol extracts of lyophilised sloes and mirabelle plums, as we knew these preparations contain a complex mixture of phytochemicals, many of which display antioxidant potential. The main compounds identified with such activity in the extracts were chlorogenic acid and its derivatives, and also catechins and anthocyanins (Fig. 5). Table 2 lists the TEs of the sloe and mirabelle plum extracts.

The TEs determined for the sloe extract both on-line and off-line were much higher than those of the mirabelle plum extracts (Table 2). However, the profile of antioxidants of the latter fruit was more diverse and included catechins (Fig. 5A). An interesting observation in the case of the sloe samples analysed here was that the total antioxidant activity determined by off-line methods was several times higher than the corresponding TE values derived from on-line methods (Table 2). No such differences were noted for the mixtures of standard phenolic compounds. Calculating total TEs in the on-line method involves summing the individual TE determinations for the separated compounds; off-line batch methods measure the antioxidant potential of the mixture. These divergent results can be explained by fact, suggested earlier, that the levels of single antioxidants in food do not necessarily reflect their total antioxidant capacity, which also depends on synergic and redox interactions among the different molecules present in, for example, food samples (Pellegrini *et al.*, 2003). Paulovicsova *et al.* (2008) noted that the high contents of vitamin C and anthocyanins in sloes are not necessarily responsible for the high antiradical activity of these fruits. These authors put more emphasis on the synergic effects of antioxidants (including vitamins with antioxidative properties, flavonoids, etc.). Thus, another benefit of on-line antioxidant activity determinations in conjunction with batch tests could be to indicate favourable interactions between constituents. Such knowledge could provide the background for designing compositions of phytochemicals with enhanced antioxidant potential.

To conclude, the results of this study confirm that the HPLC–PCX system, and therefore probably any post-column instrument, is readily applicable to the on-line detection of antioxidants in complex mixtures and the determination of their TE values. The methodology optimised here appears to be a promising tool for the food and pharmaceutical industries, enabling the monitoring of bioactive substances along the production line and during storage, and the characterisation of plant material by creating chromatographic profiles supplemented with antioxidant fingerprints. The HPLC post-column derivatisation systems can also be used for the direct calculation and comparison of the antioxidative efficacy of compounds, their mixtures and interactions. Moreover, as has been recently

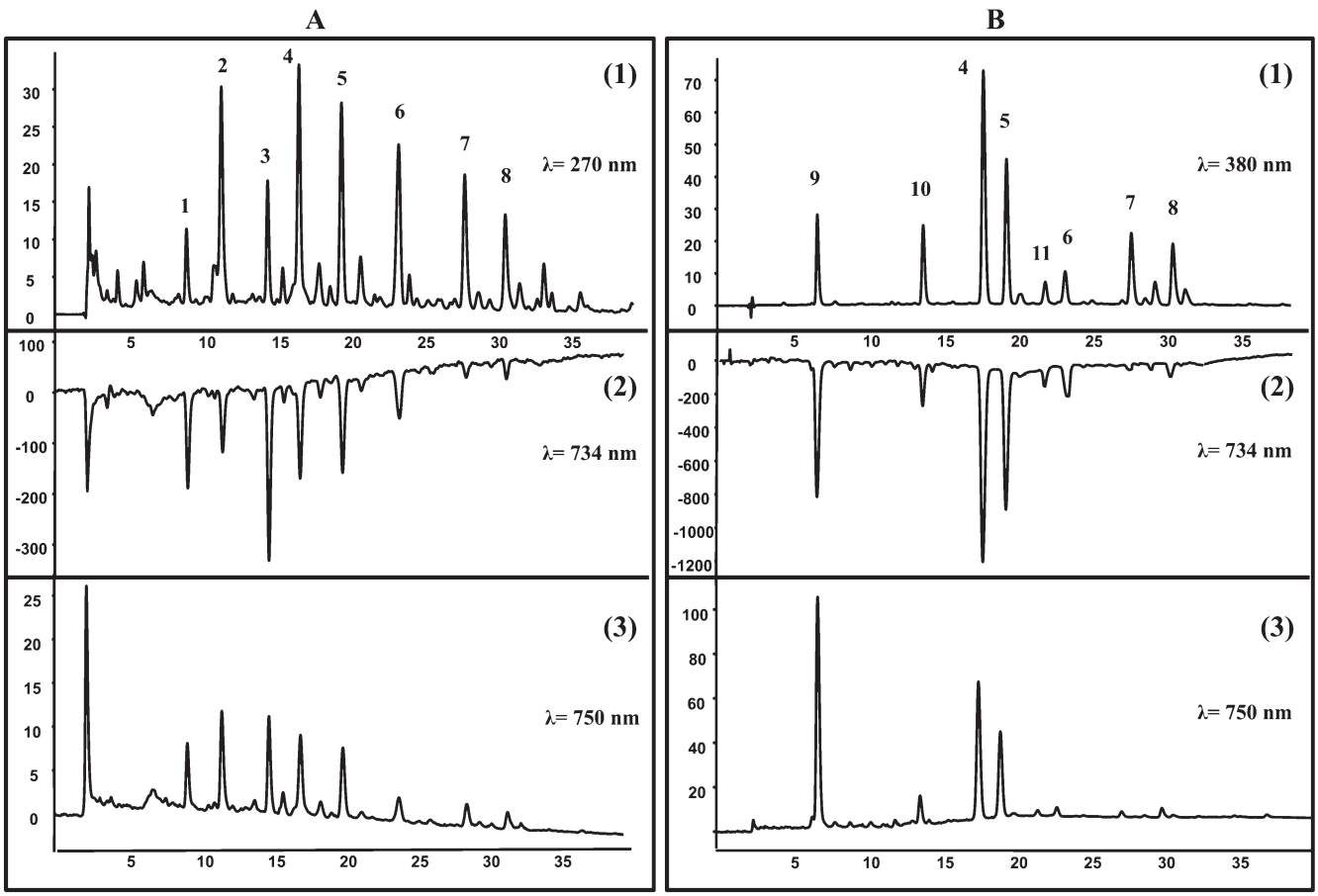

Figure 5. Combined plots of profiles before derivatisation (1) and after derivatisation with ABTS (2) or FC (3) reagents obtained for mirabelle plum (A) and sloe (B) extracts using the HPLC-postcolumn derivatisation system. The compounds identified include 1-catechin, 2-chlorogenic acid, 3-epicatechin, 4-cyanidine-3-O-galactoside, 5-cyanidine-3-O-glucoside, 6-cyanidine derivative, 7-rutin derivative, 8-morin, 9-chlorogenic acid derivative, 10-caffeic acid, 11-cyanidine derivative.

demonstrated, this system can be further enhanced. It is possible to split the eluate into two streams; one directed to PCX as described above, the other to MS (Borges *et al.*, 2010) or NMR (Exarchou *et al.*, 2006). Such a setup additionally enables the identification of detected antioxidants.

We believe that, by employing a commercially available, fully automated and programmable post-column derivatisation system, and by providing a set of tested parameters that enable the rapid detection of antioxidative substances in plant extracts or food products in the presence of many inactive constituents with minimum preparatory manipulation, we are proposing a ready-to-use method that can be routinely applied not only for experimental purposes, but also for regular production control in industrial settings.

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