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Short Communication

Application of a commercially available derivatization instrument and commonly used reagents to HPLC on-line determination of antioxidants

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ABSTRACT

This study demonstrates the potential of a commercially available derivatization instrument coupled with HPLC for separation and on-line determination of antioxidants detected with widely used screening reagents—DPPH (2,2-diphenyl-1-picrylhydrazyl), ABTS (2,20-azinobis-(3-ethylbenzthiazoline-6-sulpho-nic acid)) or FCR (Folin–Ciocalteu's phenol reagent). As a result of optimization, key parameters of the analytical procedure were established: concentrations of derivatization reagents 1.5 mmol/L and 2.1 mmol/L in methanol for DPPH and ABTS, respectively, and 40% (v/v) FCR solution in water; reactor temperatures 130 8C for ABTS and FCR and 50 8C in the case of DPPH. In order to validate these procedures, Trolox equivalent antioxidant capacities (TEACs) for standard antioxidants obtained by post-column derivatization (on-line) and typical batch colorimetric methods (off-line) were compared; the corresponding measurements were found to be strongly correlated (Pearson coefficient: 0.973, 0.922, 0.853 for DPPH, ABTS and FCR, respectively). The results obtained for standard antioxidants and real fruit sample—aronia extract confirm the applicability of the proposed system to the on-line detection of antioxidants. Its great advantage compared to current routine methods is that it provides both chromatographic profiles and corresponding fingerprints of antioxidants (including unknown ones) along with quantitative determination of antioxidative potential—total, and those exhibited by individual compounds.

Keywords: Antioxidants, HPLC post-column derivatization, ABTS, DPPH, Folin–Ciocalteu reagent, High performance liquid chromatography, Aronia, Food analysis, Food composition

1. Introduction

The growing awareness of the importance of redox homeostasis for human health has generated interest in endogenous and dietary antioxidants and in consequence, the need for their quantitative and qualitative determination. As a response, over the past two decades, a number of analytical methods measuring antioxidative activity have been developed, most of which are based on the ability of an antioxidant to quench free radicals by hydrogen donation. Two assays that utilize stable model free radicals, ABTS$^{\bullet}$ (2,2′-azinobis-(3-ethylbenzothiazoline-6-sulphonate)) and DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), have gained the highest popularity. Initially, these were mainly spectrophotometric batch tests enabling the evaluation of antioxidant potential of all kinds of matrices such as plasma, raw vegetables and fruits, as well as processed foods and beverages (Huang et al., 2005; Moon and Shibamoto, 2009). Such commonly used assays are well suited for the determination of antioxidant activity of individual pure compounds as well as total antioxidative potential of antioxidants in mixtures or complex matrixes. In the latter case(s) however, assessing the contribution of individual antioxidants in the mixture is a difficult task.

More recently, some of the principles of ABTS and DPPH assays have been modified for on-line, post-column coupling with high performance liquid chromatography (HPLC) (Exarchou et al., 2006; Bartasiute et al., 2007; Milasiene et al., 2007; Kool et al., 2007; Niederlander et al., 2008; Shi et al., 2009). The on-line methods aim not only at the rapid measurement of antioxidative activity but also at the profiling of antioxidants in complex mixtures following their chromatographic separation from the matrix. In the most investigated approach, the solution of DPPH or ABTS radical is added post-column to the HPLC flow. Antioxidants present in a sample are detected by a decrease in absorbance at visible wavelengths due to the conversion of these radicals to their non-coloured reduced forms. So far, 'home-made' purpose-built devices have been used, usually consisting of reaction coils constructed from PEEK tubing fed by an additional syringe pump. The prototypical derivatization systems, even if very effective in a host laboratory, preclude however the interlaboratory standardization, hence are unsuitable for routine analyses of antioxidative

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properties as required by, say, the food industry. Nonetheless, they have demonstrated the potential of HPLC separation coupled with on-line detection of antioxidants in complex mixtures.

This study investigates the suitability of a commercially available derivatization instrument for post-column determining antioxidant compounds in routine HPLC analyses. This type of equipment automatically mixes the stream of effluent flowing from a chromatographic column with a stream of reagent solution. This is achieved with a 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 reach completion. In the quite frequent cases when the reaction is very slow at room temperature, the system can be heated. After leaving the reactor, the derivatives flow into the detector, where the absorbance of the effluent is measured on-line. Here we present an optimization of the working conditions of the commercially available instrument coupled with HPLC for the separation and determination of bioactive sample constituents with DPPH, ABTS and Folin–Ciocalteu reagent (FCR), routine, commonly used reagents that detect antioxidant compounds. With all these reagents, a reduction reaction leads to a significant shift in the UV–vis absorption spectrum of a compound absorption spectrum that can serve as a quantative measure of the antioxidative activity.

As far as we know, this is the first report on the application of a programmable commercial HPLC-coupled instrument for the on-line determination of antioxidants. The on-line application of FCR has not yet been described, either. In contrast to earlier reports, we propose here the qualitative/quantitative approach that can be used to monitor antioxidants for not only experimental purposes, but also routine production control under industrial settings.

2. Materials and methods

2.1. Reagents and standards

HPLC grade and pure p.a. methanol were purchased from Chempur (Poland), formic acid (98–100%) from Merck (Germany). Water was purified using a Q_{PLUS}185 system from Millipore (USA). The following standards were used: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox, TRX), L-ascorbic acid (vitamin C, AA), gallic acid (GA), (±)-naringenin (NRG), (+)-catechin hydrate (CAT), resveratrol (RSV), luteolin (LUT), genistein (GEN), and rutin (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), cyanidine-3-O-galactoside chloride (CGA), chlorogenic acid (ChA), (−)-epicatechin (eCAT), morin (MOR), and cyanidin chloride (CCh) from Extrasynthese (France). The stock solutions of standards (4 mmol/L or 1 mmol/L in the case of Trolox and 1 mmol/L for the remaining compounds) were prepared in HPLC grade methanol; only vitamin C was dissolved in water. The derivatization agents used for the detection of antioxidants included 2,2′-azinobis(ethyl-2,3-dihydrobenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), both from Sigma–Aldrich, and Folin–Ciocalteu's phenol reagent (FCR) from Merck. The DPPH radical stock solution was prepared in methanol (5 mmol/L) immediately before the experiments and kept in a lightproof container. ABTS was dissolved in aq. $Na_2S_2O_8$ (2.45 mmol/L) to obtain a concentration of 7 mmol/L and left in dark at ambient temperature. Under such conditions, concentration of ABTS radical reaches maximum after 6 h, it is stable for more than two days. FCR was diluted with water before use.

2.2. HPLC conditions

An Agilent Technologies 1200 Series HPLC–DAD (high performance liquid chromatography–diode array detector) system (Agilent Technologies, USA) was employed throughout the study. Chromatographic separations were conducted on an Agilent Eclipse XDB-C18 column (4.6 mm × 150 mm, 5 μm particle size). The mobile phase consisted of 4.8% (v/v) aq. formic acid (solvent A) and HPLC grade methanol (solvent B). The flow rate was set at 0.7 mL/min and the injection volume of all samples was 2 μL. The composition of solvents for isocratic elution was 20% A and 80% B. During gradient elution the percentage of solvent B was increased from 10% to 55% in 40 min.

2.3. Post-column derivatization (PCD)

On-line post-column addition of DPPH, ABTS and FCR was performed using a commercially available derivatization instrument Pinnacle PCX (Pickering Laboratories Inc., USA) consisting of a pump delivery system and reactor that can be heated from 5 °C above ambient to 130 °C. The flow rate of the 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 was used. Chromatograms of products after DPPH, ABTS and FCR derivatization were recorded at 515 nm, 734 nm and 750 nm, respectively, using a multiple wavelength detector (Agilent 1200 Series MWD, USA).

2.4. Optimization of derivatization reagent concentration

The DPPH and ABTS stock solutions were diluted with methanol and FCR was diluted with water to concentrations ranging from 2.5% to 60% (v/v). The solutions of the reagents were delivered to the reactor using the PCD system. The reactor temperature was set at 30 °C. A standard solution of Trolox (4 mmol/L) was injected into the HPLC system and analysed in isocratic elution mode. The determination of Trolox peak area before derivatization was based on absorption measured at 290 nm (using DAD), and again after derivatization, as described above for each assay (using MWD).

2.5. The influence of temperature on derivatization efficiency

The derivatization efficiency was evaluated for six reactor temperatures ranging from 30 °C to 130 °C. The solutions of standards (TRX, AA, GA, NRG, CAT, RSV, LUT, GEN, CA, MYR, PHL, and CGA) were injected into the HPLC–PCD system, and resolution was conducted under isocratic conditions as specified earlier. The solutions of DPPH and ABTS at concentrations of 30% (v/v) stock in methanol and 40% (v/v) of FCR commercial solution in water were fed into the reactor using a syringe pump of the PCD system. The final concentrations of reagents in eluates were as follows: 5% for FCR, 0.19 mmol/L and 0.26 mmol/L for DPPH and ABTS, respectively. The peak areas of standards after derivatization were monitored for each reactor temperature.

2.6. The influence of temperature on stability of antioxidants

To test the stability of antioxidants the setting of HPLC–PCD system was rearranged. The PCD instrument was connected with HPLC system prior to the column and set initially at 30 °C (control run) then at 130 °C (stability test). The four mixtures of phenolic compounds at a concentration of 0.5 mmol/L for each standard were injected into the rearranged HPLC–PCD system. The resolution was carried out under the gradient conditions specified earlier. 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; Mixture IV: CGA, CGL, CCh, GEN, KAM. The detection of peaks during control run and stability test was performed at 270, 325, 380 and 525 nm. For each standard mixture, for both control run and stability test, the ratios between peak areas were calculated assuming that the area of the smallest peak equals 1.

2.7. Determination of Trolox equivalent antioxidant capacity (TEAC) using HPLC–PCD system

To generate the Trolox standard line, DPPH and ABTS (conc. 30%, v/v) and FCR (conc. 40%, v/v) were used. Derivatization was carried out at temperatures determined as optimal for each reagent: 50 °C for the DPPH radical, 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 as described before. For every derivatization reagent, the equation of the standard line *Trolox concentration = f(peak area)* was determined. These equations were used to calculate the TEAC values on the basis of peak areas of the standards and following derivatization at the appropriate temperature.

2.8. TEAC determination using colorimetric methods

The colorimetric determination of antioxidant activity was evaluated by the standard methods employing ABTS, DPPH and FCR indicators. The stock solutions of derivatization reagents were diluted before measurement as follows: DPPH with methanol until absorbance = 1.0 ± 0.02 at λ = 515 nm, ABTS radical solution with methanol to display absorbance of 0.7 ± 0.02 at 734 nm; commercial FCR with water (1:9, v/v). Absorbances were measured on a TECAN Infinite M200 spectrophotometer (Tecan Group Ltd., Switzerland) and all determinations were carried out in 48-well plates. DPPH solution (1 mL) was mixed with solutions of standards (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 measured after 10 min at 734 nm. The FCR solution (1 mL) was mixed with solutions of standards (0.1 mL) and the absorbance was measured after 10 min at 750 nm. In each case, Trolox solution served to generate the standard line (concentration range 0.2–4.0 mmol/L).

2.9. Stability of derivatization reagents in mobile phases of different compositions

DPPH and ABTS solutions (30%, v/v) and FCR solution (40%, v/v) were delivered to the reaction system at a constant rate of 0.1 mL/min. The reactor temperature of the derivatization instrument was set at 50 °C for DPPH and at 130 °C for ABTS and FCR solutions. Different proportions of solvent B (methanol) in the mobile phase (0–100%) were pumped through the HPLC–PCD system. The eluate was collected after each modification of mobile phase composition. The absorbance of the eluate samples was measured on a TECAN Infinite M200 spectrophotometer at 515, 734 and 750 nm for eluates containing DPPH, ABTS and FCR, respectively.

2.10. Sample collection and preparation

The chokeberry (*Aronia melanocarpa* E.), purchased from the local processing plant Fungopol (Poland), was lyophilized and ground. The freeze-dried powder (1 g) was extracted with methanol (3× 4 mL). The chokeberry extract was analysed using HPLC–PCD system with gradient elution and ABTS (conc. 30%, v/v) and FCR (conc. 40%, v/v) as derivatization reagents. The reactor temperature of the derivatization instrument was set at 130 °C.

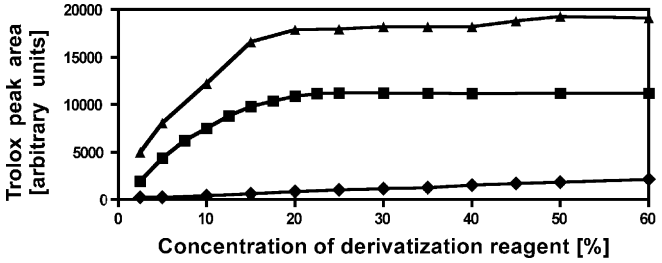

Fig. 1. Changes in Trolox peak area as a function of derivatization reagent concentration (■ – DPPH; ▲ – ABTS; ◆ – FC) determined using HPLC post-column derivatization. The points are means of three independent experiments; SD did not exceed 2% and 4%, respectively, in the case of ABTS and FCR or DPPH used as derivatization reagents.

3. Results and discussion

3.1. Optimizing the concentration of derivatization reagent

The reliable and reproducible on-line determination by post-column derivatization can be achieved provided two main requirements are fulfilled. Firstly, the equipment employed ensures stable conditions of the analysis with the composition of derivatization agents easy to control. Secondly, an analyte in the eluate leaving the chromatographic column reacts with the derivatization agent in a reproducible way, with maximum yield under conditions of optimized analytical procedure. Therefore, the first step in the described analysis of antioxidants was to establish the concentrations of ABTS, DPPH and FCR, ensuring that they were not limiting factors under the chromatographic conditions used in our laboratory for the efficient HPLC resolution of natural mixtures containing antioxidants. Fig. 1 shows the changes in Trolox peak area (2 μL, 4 mmol/L) depending on the derivatization reagent concentrations fed to the eluate. In the case of DPPH and ABTS radicals, there were no changes in Trolox peak area for concentrations exceeding 25% (v/v) of stock solution in methanol (Fig. 1). Therefore, for further analyses 30% (v/v) solutions of both radicals were chosen (corresponding to 1.5 mmol/L and 2.1 mmol/L for DPPH and ABTS, respectively). In the case of FCR, no such plateau was observed. However, the increase in FCR concentration above 50% (v/v) of commercial solution in water caused the salts in the HPLC–PCD system to crystallize, so in later experiments 40% (v/v) FCR commercial solution in water was used. In all determinations, the commercially available derivatization agents from internationally recognized companies were used (Sigma, Merck). In some earlier reports, phosphomolybdenum complex was proposed as an agent detecting antioxidants, but its solution had to be freshly prepared before use in a rather complicated way (Cardenosa et al., 2002), inevitably constituting additional source of errors, especially in changing pH of mobile phase.

3.2. Influence of reactor temperature on derivatization efficiency

Time and temperature are the most important factors affecting the yield of the derivatization reaction. The former is determined by the flow rate, which is adjusted to ensure the proper chromatographic resolution of analytes. In contrast, the application of professional, programmable device such as the one used in this study, makes it possible to precisely regulate the reactor temperature over a wide range. To determine the optimum derivatization temperature for each reagent investigated, a set of substances known to be potential antioxidants belonging to different classes of phenolic compounds was used, the most common antioxidants – vitamin C and Trolox – being included. All these test compounds might be expected to display different kinetic behaviours towards DPPH, ABTS and FCR. Fig. 2 presents

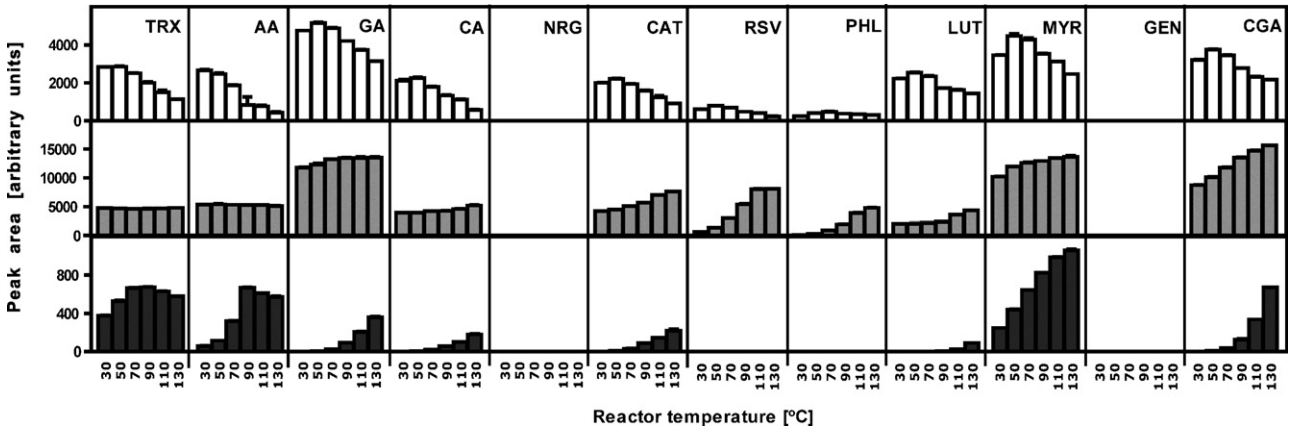

Fig. 2. Influence of reactor temperature on peak areas for several known phytochemicals determined using HPLC post-column derivatization system with □ – DPPH, ▩ – ABTS or ■ – FC as derivatization reagents. The values are means ± SD of three independent experiments; SD values were lower than 10% when DPPH or FCR were used as derivatization reagents, and did not exceed 5% in the case of ABTS derivatization. TRX – Trolox, AA – ascorbic acid, GA – gallic acid, CA – caffeic acid, NRG – naringenin, CAT – (+)catechin hydrate, RSV – resveratrol, PHL – phloretin, LUT – luteolin, MYR – myricetin, GEN – genistein, CGA – cyanidin-3-O-galactoside.

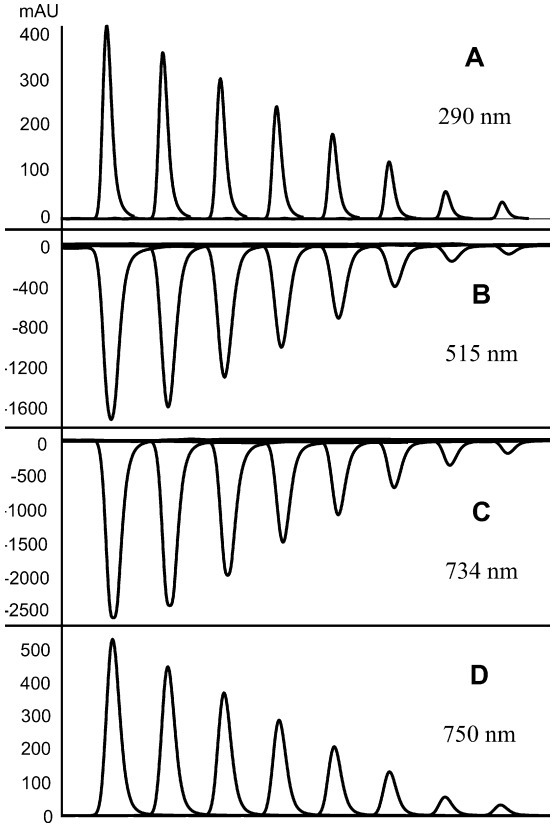

Fig. 3. Combined plot of profiles obtained for different concentrations of Trolox (0.6–4 mmol/L) sequentially injected (2 μL) and resolved under isocratic conditions. The chromatographic profiles were monitored spectrophotometrically before derivatization (A) and after derivatization with DPPH (B), ABTS (C) and FC (D) reagents. Derivatization was carried out at temperatures determined as optimal for each reagent: 50 °C for the DPPH, 130 °C for ABTS and FCR.

the areas of peaks detected for antioxidants studied after derivatization at different reactor temperatures. In the case of derivatization with DPPH, the peak areas of most compounds were largest when this process took place at 50 °C. Rising temperature during the reaction with ABTS was associated in almost all cases with increasing peak area. Only for Trolox and vitamin C, no temperature influence was observed. The effect of FCR derivatization depended on the substance; peaks were largest at 130 °C for phenolic compounds, but at 90 °C for Trolox and vitamin C. Fig. 3 shows the chromatograms of solutions containing declining concentrations of Trolox obtained before and after derivatization under the optimized conditions specified in the caption to this figure. The post-column detection of the reduction of DPPH and ABTS radicals in relation to Trolox content is reflected by the negative UV–vis chromatograms at 515 nm and 734 nm, respectively (Fig. 3B and C). In the case of derivatization with FCR, Trolox, like compounds containing active hydroxyl group(s), reacts with FCR to form a coloured complex, which appears as a positive chromatogram at 750 nm (Fig. 3D).

3.3. The influence of temperature on stability of antioxidants

The results presented in Fig. 2, where occasionally after derivatization either decline of peak areas or the lack of appearance of chromatographic peaks corresponding to some test antioxidants was observed, raised doubts about their thermal stability. To investigate such a possibility, the HPLC–PCD system was rearranged as depicted in Fig. 4. The normal flow of sample injected to the system initially passes the column, then enters DAD detector followed by PCD system (Fig. 4A). During tests of thermal stability of the antioxidants, the injected analyte first encountered the reaction coil of PCD device before passing through the column and flowing via DAD detector (Fig. 4B).

In the first set of experiments, the four mixtures of standards analysed in the rearranged HPLC–PCD system were exposed to the temperature of 30 °C (Fig. 5A). During the next set of analyses, the temperature of the reaction coil was raised to 130 °C (Fig. 5B). In each case, the monitoring of peaks was carried out at wavelengths optimal for the detection of a given compound. As can be seen in Fig. 5, the chromatographic profiles obtained after heating the samples to 30 °C are virtually indiscernible from those detected when the temperature of reaction coil was set at 130 °C. This visual assessment is confirmed by the data presented in Table 1. In this table, for each mixture of standards, the ratios between peak areas corresponding to a given compound and the compound giving the weakest response are presented. These ratios calculated for analyses carried out for the reactor's temperatures 30 °C and 130 °C are basically identical, meaning that the amounts of analytes have not changed despite the brief (1 min) exposure to high temperature. Thus, both qualitative observations (chromatographic profiles) and quantitative results (peak area ratios) suggest that antioxidants can be safely detected by HPLC–PCD

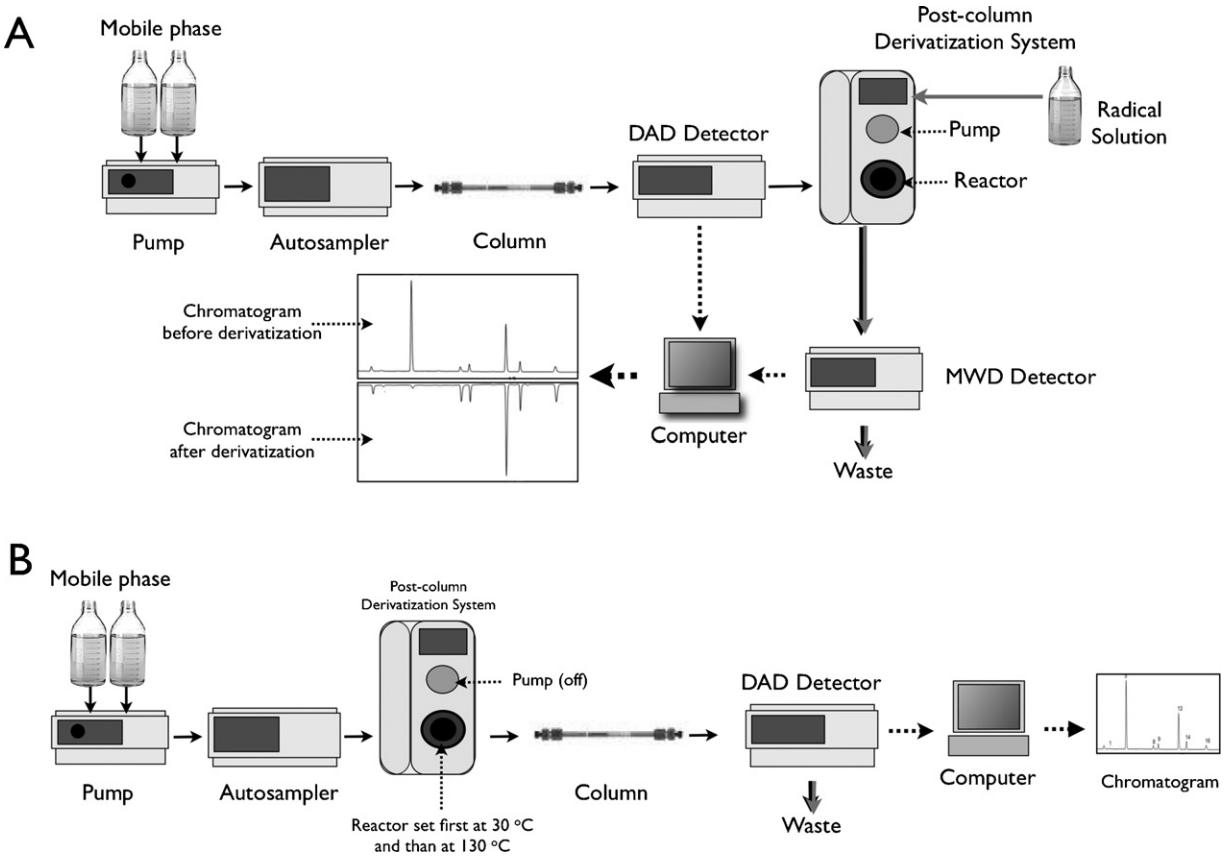

Fig. 4. The typical assembly of post-column derivatization system with HPLC used for on-line profiling of antioxidants (A); the rearranged connection of post-column derivatization system to HPLC (B) used in this study to test thermal stability of antioxidants.

system proposed here using elevated temperature to speed up derivatization process. However, it can happen that some compounds might be particularly vulnerable to thermal treatment and this should be checked before analysis.

3.4. Comparison of TEAC values

TEAC values calculated for the analysed standard antioxidants (1 mmol/L) obtained by post-column derivatization (on-line) and batch spectrophotometric methods (off-line) are listed in Table 2. Almost all the antioxidative activities determined by colorimetric tests are higher than those obtained with HPLC–PCD system, probably because of the longer time for the analysed compounds to react with the derivatization reagents in spectrophotometric tests (10 min) in comparison with the reaction coil (1 min). It is, however, very important to note that there is a strong correlation between TEAC values obtained from the corresponding measurements as shown by Pearson coefficient: DPPH $r = 0.973$; ABTS $r = 0.922$; FCR $r = 0.853$. Moreover, as can be seen in Fig. 2 and Table 2, two of the standards – NRG and GEN – that did not react with any of the derivatization reagents on-line regardless of conditions, did not give signal in colorimetric methods either. Only the results for RSV and PHL after FCR derivatization showed inconsistency: neither of these compounds reacted in the case of post-column derivatization. PHL gave no positive signal in the FCR colorimetric test either; in contrast, the antioxidative potential of RSV was readily measurable with all three batch tests. This could mean that acidic conditions in the eluate may influence the chemical properties of some compounds (Fig. 2, Table 2).

3.5. Stability of derivatization reagents

To evaluate the possible application of HPLC coupling to post-column derivatization device in the case of gradient elutions, the stability of DPPH, ABTS and FCR in an eluate with changing composition has to be ascertained. We investigated the influence of the expected proportions of methanol, water and formic acid on eluate absorbance following derivatization during effective HPLC separation of natural mixtures containing antioxidants, e.g. products prepared from anthocyan-rich fruits. The results (Fig. 6) show that in different mixtures containing the mentioned solvents, ABTS was the most stable of the three reagents studied. This accords with the results of Koleva et al. (2001) who suggested that an HPLC mobile phase containing up to 100% organic solvent (methanol or acetonitrile) and with pH reduced to 3 could be used in both isocratic and gradient runs with ABTS as a derivatization reagent.

The usability of ABTS for on-line detection of antioxidants was shown for different kinds of samples, for example extracts of coffee (Stalmach et al., 2006), herbs (Exarchou et al., 2006) or berries (Borges et al., 2010). 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 aq. formic acid); the application of this reagent to HPLC post-column derivatization under the gradient conditions typically used for the resolution of antioxidants in plant material is therefore troublesome.

The various conditions and applications of post-column derivatization using DPPH radical have been recently reviewed (Niederlander et al., 2008; Shi et al., 2009). Most experiments

involving on-line post-column antioxidant detection with DPPH radical used no acid (Wu et al., 2008; Mnatsakanyan et al., 2010) or very small fraction of acid in mobile phase (Dapkevicius et al., 2001; Exarchou et al., 2006; Perez-Bonilla et al., 2006). The highest reported amount of acid in mobile phase was about 2% (Bandoniene and Murkovic, 2002). Koleva et al. (2000) had earlier indicated that a highly acidic system (pH 2.2) caused a drastic reduction in DPPH absorbance: HPLC gradients with a non-acidic mobile phase consisting of 10–90% organic solvent in water could be applied on-line without significant changes in DPPH absorbance. But without an acidic component in the mobile phase, the resolution of many important phenolic analytes found in food

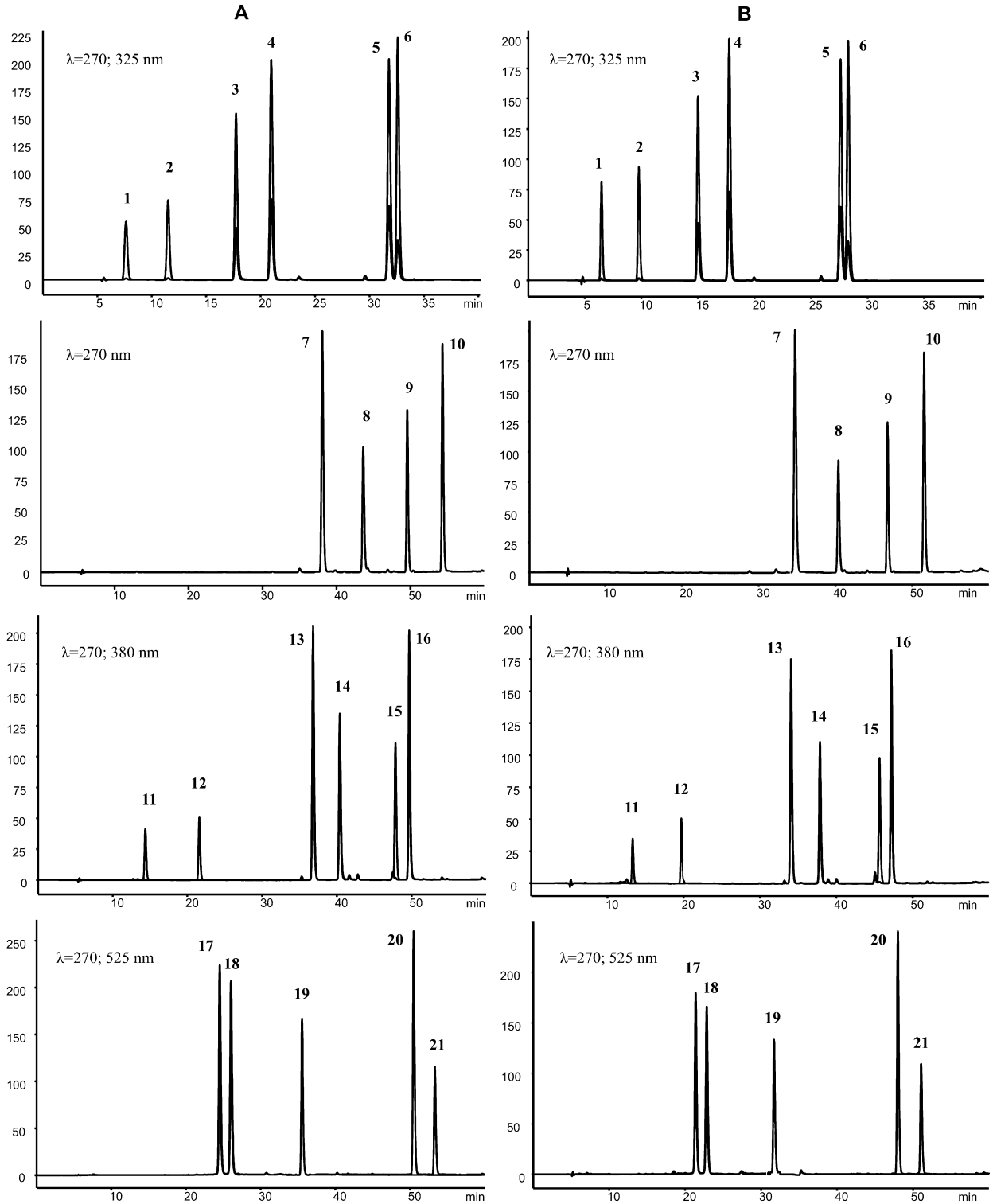

Fig. 5. Chromatograms obtained for four mixtures of standard phenolics analysed with the use of the rearranged HPLC–PCD system (as shown in Fig. 4B) and the reaction coil set at 30 °C (A) or 130 °C (B). The resolution was carried out under gradient conditions as specified in Section 2. The peak numbers in the chromatograms correspond to the following analytes: 1 – gallic acid, 2 – protocatechuic acid, 3 – chlorogenic acid, 4 – caffeic acid, 5 – ferulic acid, 6 – sinapic acid, 7 – resveratrol, 8 – morin, 9 – phloretin, 10 – apigenin, 11 – (+)catechin hydrate, 12 – (−)epicatechin, 13 – rutin, 14 – myricetin, 15 – naringenin, 16 – luteolin, 17 – cyanidin-3-O-galactoside chloride, 18 – cyanidin-3-O-glucoside, 19 – cyanidin chloride, 20 – genistein, and 21 – kaempferol.

Table 1
The comparison of peak area ratios calculated for compounds in each mixture of standard phenolics exposed to temperature of 30 °C (control run) or 130 °C (stability test) during analysis in a rearranged HPLC–PCD system.

	Compound[a]	λ [nm]	30 °C		130 °C	
			t_R [min]	Peak area ratio[b]	t_R [min]	Peak area ratio[b]
Mixture I	1 – GA	270	7.6	1	6.5	1
	2 – PCA	260	11.4	1.7	9.8	1.7
	3 – ChA	325	17.7	2.5	15.0	2.4
	4 – CA	325	29.9	3.6	17.8	3.3
	5 – FA	325	31.7	3.6	27.6	3.3
	6 – SA	325	32.5	3.7	28.3	3.4
Mixture II	7 – RSV	300	38.1	3	34.9	3
	8 – MOR	260	43.6	1	40.4	1
	9 – PHL	280	49.6	1.3	46.8	1.3
	10 – API	270	54.4	1.1	51.6	1.2
Mixture III	11 – CAT	280	14.4	1	13.3	1
	12 – eCAT	280	21.6	2	19.7	1.8
	13 – RUT	260	36.8	6.5	34.1	6.1
	14 – MYR	260	40.4	2.8	37.8	2.5
	15 – NRG	280	47.8	4.4	45.7	4.4
	16 – LUT	360	49.7	6.2	47.2	6.3
Mixture IV	17 – CGA	525	24.5	1.7	21.4	1.3
	18 – CGL	525	26.0	1.6	22.9	1.3
	19 – CCh	525	36.4	1.3	31.2	1.1
	20 – GEN	260	50.5	2.2	48.0	2.2
	21 – KAM	360	53.3	1	51.1	1

[a] The numbers correspond to peak numbers used on the chromatograms presented in Fig. 5; the abbreviations refer to the following compounds: GA – gallic acid, PCA – protocatechuic acid, ChA – chlorogenic acid, CA – caffeic acid, FA – ferulic acid, SA – sinapic acid, RSV – resveratrol, MOR – morin, PHL – phloretin, API – apigenin, CAT – (+)catechin hydrate, eCAT – (−)epicatechin, RUT – rutin, MYR – myricetin, NRG – naringenin, LUT – luteolin, CGA – cyanidin-3-O-galactoside chloride, CGL – cyanidin-3-O-glucoside, CCh – cyanidin chloride, GEN – genistein, and KAM – kaempferol.
[b] The ratios were calculated based on the results of a single experiment.

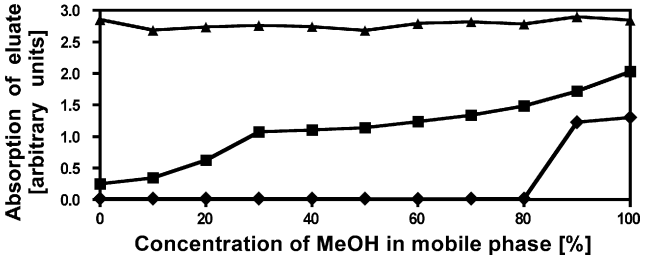

Fig. 6. Changes in eluate absorbance as a function of different proportions of methanol and aqueous formic acid in the mobile phase monitored at 515 nm, 734 nm and 750 nm, respectively for ■ – DPPH; ▲ – ABTS and ◆ – FCR derivatization reagents. The points represent results of a single experiment.

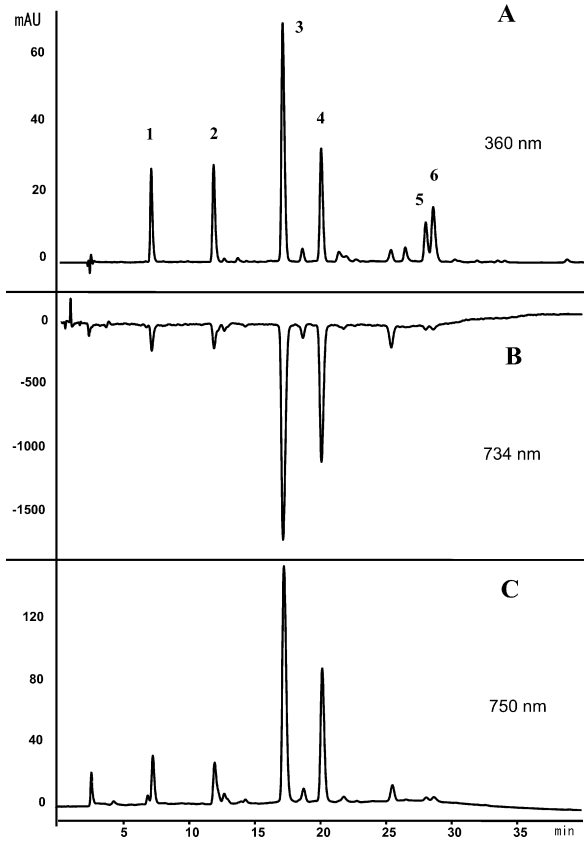

Fig. 7. HPLC–PCD profiles of chokeberry extract obtained before (A) and after derivatization with ABTS (B) or FC (C) reagents. The identified peaks include: 1–chlorogenic acid derivative, 2 – chlorogenic acid, 3 – cyanidine-3-O-galactoside, 4 – cyanidine derivative, 5 – hyperoside and 6 – rutin.

Table 2
Trolox equivalents antioxidant capacity (TEAC, mmol/L) determined for known phytochemicals using isocratic conditions of HPLC coupled with post-column derivatization (on-line) or standard colorimetric tests (off-line).

Standards	DPPH		ABTS		FC	
	On-line	Off-line	On-line	Off-line	On-line	Off-line
AA	1.268 ± 0.032	1.123 ± 0.299	1.122 ± 0.041	0.661 ± 0.391	1.012 ± 0.011	1.259 ± 0.038
GA	2.275 ± 0.019	3.345 ± 0.331	3.192 ± 0.056	3.205 ± 0.298	0.716 ± 0.017	1.520 ± 0.002
CA	1.189 ± 0.023	1.581 ± 0.072	1.149 ± 0.023	0.635 ± 0.257	0.519 ± 0.011	1.318 ± 0.077
NRG	nd	nd	nd	nd	nd	nd
CAT	1.169 ± 0.021	1.733 ± 0.057	1.738 ± 0.007	2.738 ± 0.414	0.567 ± 0.029	1.130 ± 0.067
RSV	0.639 ± 0.010	0.894 ± 0.005	1.856 ± 0.004	2.247 ± 0.370	nd	1.016 ± 0.027
PHL	0.492 ± 0.016	0.641 ± 0.020	1.046 ± 0.013	2.231 ± 0.051	nd	nd
LUT	1.294 ± 0.013	1.568 ± 0.011	0.935 ± 0.003	1.417 ± 0.208	0.409 ± 0.011	1.104 ± 0.004
MYR	2.019 ± 0.072	2.368 ± 0.254	3.218 ± 0.082	3.666 ± 0.244	1.626 ± 0.019	2.266 ± 0.125
GEN	nd	nd	nd	nd	nd	nd
CGA	1.749 ± 0.021	2.414 ± 0.227	3.703 ± 0.022	3.506 ± 0.662	1.137 ± 0.006	3.001 ± 0.139

TEAC values are means ± SD ($n = 3$), the reactor temperature was set at 50 °C for DPPH and 130 °C for ABTS and FCR derivatization reagents, nd – not detected (in on-line measurements LOD expressed as millimoles of Trolox per liter amounted to 0.2, 0.04 and 0.02 for DPPH, ABTS and FC, respectively, and in off-line tests LOD equalled to 0.1). AA – ascorbic acid, GA – gallic acid, CA – caffeic acid, NRG – naringenin, CAT – (+)catechin hydrate, RSV – resveratrol, PHL – phloretin, LUT – luteolin, MYR – myricetin, GEN – genistein, CGA – cyanidin-3-O-galactoside chloride.

products, epecially anthocyans, is markedly less efficient. Samples analysed in the mentioned publications did not contain anthocyans. The results with FCR as post-column derivatization reagent were more satisfactory (Fig. 6). A mobile phase containing 0–80% (v/v) methanol in aq. formic acid may be used in gradient runs without jeopardising this reagent's stability. Methanol concentrations exceeding 80% (v/v) in the mobile phase caused the salts in the HPLC–PCD system to crystallize.

3.6. Fruit extract analysis

To show the usability of the post-column derivatization system, the extract from chokeberry, one of the most known sources of health-promoting dietary antioxidants, was analysed using the HPLC–PCD system in gradient conditions with ABTS and FCR as derivatization reagents. The obtained chromatograms are presented in Fig. 7. The main compounds detected in the extract were chlorogenic acid and its derivative, cyanidin and quercetin derivatives. All these compounds react with ABTS as well as with FCR derivatization reagent, hence they contribute to antioxidant potential of chokeberry. However, as the comparison of chromatograms clearly demonstrates, the relative abundance of a given compound (measured as a peak area, Fig. 7A) was not necessarily paralleled by its share in antioxidative potential.

4. Conclusions

The results of this study confirm that the HPLC–PCD system, most probably not only the one used in this study but also any professional post-column instrument, is applicable to the on-line detection of antioxidants in complex mixtures and simultaneous determination of their TEAC values. The employment of a commercially available, fully automated and programmable post-column derivatization system in conjunction with commercial reagents renders this approach suitable for routine detection of antioxidative substances in plant extracts or food products in the presence of other constituents with minimum preparatory manipulation. The methodology described here thus appears to be a promising tool for the food and pharmaceutical industries. The great advantage of the proposed approach compared to current routine methods is that it provides both chromatographic profiles and corresponding fingerprints of antioxidants (including unknown ones) along with quantitative determination of antioxidative potential—total and those exhibited by individual compounds. Moreover, this versatility combined with the possibility of strict control of conditions of analytical procedure enables monitoring of bioactive substances along the production line and during storage, especially important in the case of plant-based products regarded as displaying health-promoting properties.

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