An on-line post-column detection system for the detection of reactive-oxygen-species-producing compounds and antioxidants in mixtures

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Abstract Reactive oxygen species (ROS) can damage proteins, cause lipid peroxidation, and react with DNA, ultimately resulting in harmful effects. Antioxidants constitute one of the defense systems used to neutralize pro-oxidants. Since pro-oxidants and antioxidants are found ubiquitously in nature, pro-and antioxidant effects of individual compounds and of mixtures receive much attention in scientific research. A major bottleneck in these studies, however, is the identification of the individual pro-oxidants and antioxidants in mixtures. Here, we describe the development and validation of an on-line post-column biochemical detection system for ROS-producing compounds and antioxidants in mixtures. Inclusion of cytochrome P450s and cytochrome P450 reductase also permitted the screening of compounds that need bioactivation to exert their ROS-producing properties. This pro-oxidant and antioxidant detection system was integrated on-line with gradient HPLC. The resulting high-resolution screening technology was able to separate mixtures of ROS-producing compounds and antioxidants, allowing each species to be characterized rapidly and sensitively.

Keywords Pro-oxidant · Antioxidant · ROS · Bioactivation · On-line · Biochemical detection

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Introduction

The potentially toxic and beneficial properties of pro-oxidants and antioxidants have made them the focus of many studies. Pro-oxidants may represent a threat to health, whereas antioxidants may counteract these effects by scavenging pro-oxidants [1, 2]. Antioxidants are very important in industrial processes as well as in biological systems. They are known to possess anti-inflammatory [3], anti-cardiovascular disease [4], antineurogenerative [5], and anticancer properties [6]. Imbalances between pro-oxidants and antioxidants in favor of the pro-oxidants may result in oxidative stress, which in turn may result in oxidative damage [7] of cellular components in the form of lipid peroxidation, protein denaturation or DNA conjugation [8]. Oxidative stress has been associated with many diseases like cancer [6], post-ischemic and neural degradation [2], Parkinson's and Alzheimer disease [5], AIDS [9], and aging and cardiovascular diseases [4]. The metabolism of pro-oxidants by cytochrome P450s is another important process that can result in the formation of reactive oxygen species (ROS) [10, 11].

Because antioxidants may neutralize the potentially harmful effects of direct pro-oxidants or pro-oxidants formed upon bioactivation, much of the work done in this field has been directed at synthesizing antioxidants, in some cases organ- or tissue-specific ones [12–14]. In addition, the food industry pays a great deal of attention to antioxidants in foods. Phenols in tea [15], fish oils [16], curcumin in curry [17] and flavanoids in plants [18] are only a few examples of the large number of natural compounds that have been and are being studied for their positive, antioxidant-based, effects. When the pro-oxidant or antioxidant effects of natural extracts or synthetic compounds are investigated,

batch assay formats are normally utilized [19–21]. On-line assays that measure the pro-oxidant or antioxidant effects of compounds are also described [22–24]. However, when individual compounds in mixtures need be analyzed for their pro-oxidant or antioxidant properties using these techniques, cumbersome gradient HPLC separations are required before the purified compounds can be characterized. Moreover, care must be taken to ensure that the purified compounds are not oxidized or degraded in air before analysis. One strategy that could be employed to circumvent the need for this cumbersome purification and subsequent screening process is high-resolution screening (HRS), which stands for "on-line post-column biodetection after HPLC separation." HRS methodologies, which screen individual compounds in mixtures for affinity, have been developed for receptors (e.g., the estrogen receptor [25]), enzymes (e.g., cathepsin B [26], cytochrome P450s [27]), and antibodies (e.g., digoxin antibodies [28]). These HRS strategies have proven to be very useful for the rapid profiling and identifying of individual ligands in active mixtures, especially when HRS systems are run simultaneously with MS [29]. Moreover, the use of these fast HRS strategies can result in a reduced risk of the oxidation or degradation of sample components before analysis.

This paper presents the development and validation of a HRS-based on-line post-column detection system for the detection of ROS-producing compounds as well as antioxidants in mixtures. This so-called pro-oxidant and antioxidant detection (PAD) system is based on the oxidation of 4-hydroxyphenylacetic acid (4HPAA) by H_2O_2 in the presence of horseradish peroxidase (HRP) to a highly fluorescent dimer [30, 31]. Scheme 1 shows the general principles of the assay. H_2O_2 may be present as such or it may be formed from superoxide anion radicals (resulting from ROS-producing compounds) in the presence of superoxide dismutase (SOD). After optimization, the on-line detection system was validated with the well-known ROS-producing compounds
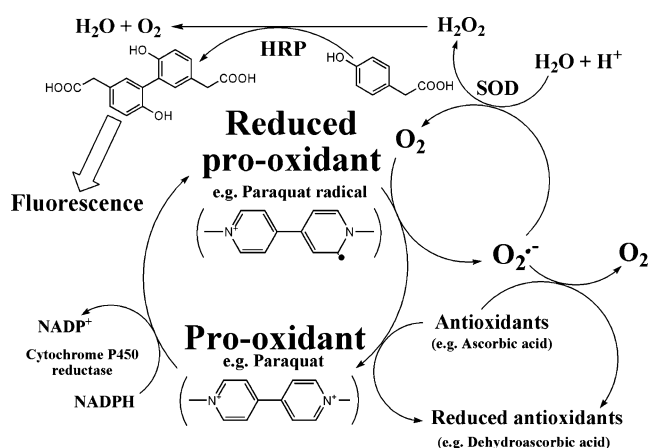
paraquat, menadione and duroquinone, and the antioxidants L-ascorbic acid and glutathione, in flow injection analysis (FIA) mode. Finally, the on-line PAD system was coupled to gradient HPLC and thus used in HRS mode to screen individual compounds in mixtures for their ROS-producing and/or antioxidant properties.

Experimental section

Materials

L-ascorbic acid, L-glutathione (reduced; GSH) and 4-hydroxyphenylacetic acid (4-HPAA) were purchased from Aldrich (Zwijndrecht, The Netherlands). Tween 20, menadione, polyethyleneglycol 6000 (PEG6000), polyethyleneglycol 3350 (PEG3350), methylviologen (paraquat dichloride), peroxidase (horseradish, type I; HRP), and superoxide dismutase (from bovine erythrocytes; SOD) were purchased from Sigma (Zwijndrecht, The Netherlands). β-Nicotinamide adenine dinucleotide phosphate tetra sodium salt (NADPH) was from Applichem (Lokeren, Belgium). Methanol (MeOH) and isopropanol (IPA) were purchased from Riedel de Haën (Seelze, Germany). Acetonitrile (ACN) was from Baker (Deventer, The Netherlands). The MeOH, ACN and IPA were of HPLC reagent grade. Rat liver microsomes (β-NF induced) were prepared as described elsewhere [32]. In short: livers from β-NF induced rats were homogenized at 4 °C in two volumes of 50 mM potassium phosphate buffer (pH 7.4) with 0.9% sodium chloride using a Potter-Elvehjem (Sigma) homogenizer. The homogenate was centrifuged for 20 min at 12.000×g, and the supernatant obtained was further centrifuged for 60 min at 100.000×g. The resulting pellet was washed twice and subsequently resuspended in 50 mM potassium phosphate buffer (pH 7.4), 0.9% sodium chloride, and 25% glycerol, and stored at −80 °C. The protein concentration in the microsomes was determined as 13.1 mg/ml. Protein concentrations were determined with the standard Bio-Rad (Hercules, CA, USA) protein assay based on the method of Bradford.

Instrumentation

A Gilson 234 auto injector (Villiers-le-Bel, France) equipped with a Rheodyne (Bensheim, Germany) six-port injection valve (injection loop, 50 μl) was used for sample injections. A Knauer K-500 HPLC pump (Berlin, Germany) was used to deliver the injected samples into the on-line PAD system in FIA mode. Two Knauer K-500 HPLC pumps were used to deliver the cofactors, substrate and enzymes by means of superloops (SL-A and SL-B) (50 ml, Pharmacia, Peapack, NJ, USA), which were kept on ice, into the PAD system.

Scheme 1 Schematic representation of the principles of the biochemical assay

Prior to detection via an Agilent 1100 (Waldbronn, Germany) series fluorescence detector (λ_{ex} 320 nm; λ_{em} 409 nm), a knitted reaction coil (0.25 mm i.d.; 1.59 mm o.d.; internal volume of 75 μl) positioned in a Shimadzu CTO-10AC column oven (Duisburg, Germany), was used to perform the enzymatic reaction on-line. To reduce pump pulsing, flow restrictors were inserted between the pumps and the superloops. The flow restrictors were made in a similar way to the ones used by Kool et al. [27].

Pro-oxidant and antioxidant assay optimization

The initial optimization of the biochemical assay for the PAD system was performed off-line on a Shimadzu RF-1501 spectrofluorometer (λ_{ex} 320 nm; λ_{em} 409 nm), before the biochemical assay was transferred to the on-line format for further optimization. All measurements were performed in triplicate at 37 °C in quartz cuvettes with total volumes of 2 ml. Different concentrations of enzymes (rat liver microsomal cytochrome P450s and cytochrome P450 reductase, SOD and HRP), cofactor NADPH, and 4-HPAA were tested. Blocking reagents and detergents (that can improve the resolution of the PAD system) [27] and organic modifiers (that are necessary when the PAD system is operated in on-line gradient HPLC mode) were investigated as well. Potassium phosphate buffer (50 mM; pH 7.8) was used in all experiments. Initial conditions were rat liver microsomes (50 μg/ml), NADPH (40 μM), 4-HPAA (1 mM), SOD (10 U/ml), and HRP (10 U/ml). Reactions were started after 5 min of pre-incubation with the addition of paraquat (70 μM). The different parameters were optimized in the abovementioned order. When the

optimal (or best compromise) concentration of every parameter was subsequently used in the optimization of the next parameter. After the optimization process, the optimized conditions were used in the PAD system in FIA and HPLC mode.

Pro-oxidant and antioxidant detection system in flow-injection analysis mode

A schematic view of a similar on-line detection system to the PAD system used in FIA mode is shown and described elsewhere [27]. The main difference between the PAD system used in FIA mode and that used in HPLC mode is the replacement of a carrier solution used in FIA mode with a gradient reversed-phase HPLC system. The general scheme for a PAD system coupled on-line to gradient reversed-phase HPLC (described in the next paragraph) is shown in Fig. 1, which also shows the schematics of the PAD system in general. The continuous mixing of enzymes (cytochrome P450s and cytochrome P450 reductase, HRP and SOD) from SL-A and cofactors/substrate (NADPH/4-HPAA) from SL-B with a carrier solution in a reaction coil is the basic principle of the PAD system (when used in FIA mode). After mixing in a knitted reaction coil, ROS formed from pro-oxidants (after cytochrome P450/cytochrome P450 reductase-mediated bioactivation) are converted by SOD to (relatively stable) H_2O_2. The subsequent H_2O_2-dependent conversion of non-fluorescent 4-HPAA to its fluorescent dimer by HRP yields a spectroscopic handle for the efficient measurement of ROS formation. Thus, the ROS formed by eluting compounds give rise to a temporary increase in the formation of fluorescent product, which is
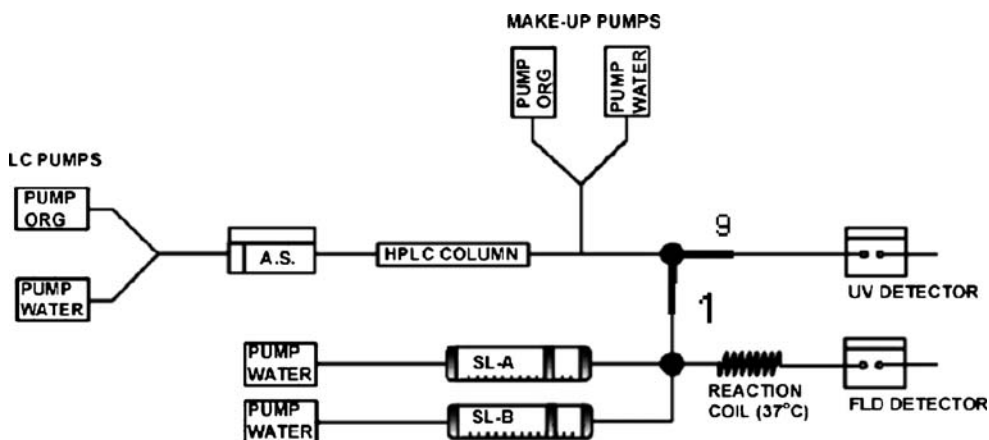

Fig. 1 Schematic view of the PAD system used in HPLC mode. Superloop A (SL-A) and superloop B (SL-B) are used to deliver enzymes and substrates to the reaction coil, respectively. ROS-producing pro-oxidants and antioxidants are introduced into the system by a gradient reversed-phase HPLC system. Antioxidants and ROS-producing compounds temporarily alter the amount of fluorescent

product formed, and this change is detected with a fluorescence (FLD) detector. After HPLC, the make-up pumps produce a counteracting gradient, resulting in a PAD-compatible constant organic modifier concentration. The effluent is then split 1:9 (90% to UV detection and 10% to CYP EAD). *AS*, autosampler

seen in the PAD system as a peak. When SL-B also contains a ROS-producing compound, continuous oxidation of the 4-HPAA results in an elevated fluorescent baseline. In this situation, injection of antioxidants causes a temporary decrease in 4-HPAA oxidation, resulting in a negative peak in the PAD system. Thus, the system is sensitive to both ROS-producing compounds and antioxidants.

Liquid chromatography coupled to the pro-oxidant and antioxidant detection system

A general scheme for the PAD system coupled on-line to gradient reversed-phase HPLC is shown in Fig. 1. Gradient HPLC separations were performed using a 30 mm length × 2 mm i.d. stainless steel column (Luna 3 μ C18(2), Phenomenex, Torrance, CA, USA). When the PAD system was operated in HPLC mode, two pumps were used to control the LC gradient and two pumps were used directly after the HPLC column to compensate for the increased concentration of organic modifier (during the gradient) before the delivery of the effluent to the PAD system. The following gradient was used for the HPLC separations: an initial flow rate of 300 μl/min for 3 min at H_2O:MeOH (95:5); a decreasie in the flow rate gradient for 6 min to 150 μl/min H_2O:MeOH (5:95); 14 min with H_2O:MeOH (5:95) at a flow rate of 150 μl/min. Thereafter, the column was re-equilibrated to the starting conditions in 5 min. To maintain a constant concentration of MeOH after the HPLC column, a second gradient with an increasing flow-rate was included in the system, after HPLC separation, with an initial flow-rate of 700 μl/min H_2O:MeOH (4:1) for 4.5 min. An increase in the flow-rate gradient for 6 min to 850 μl/min H_2O:MeOH (100:0) was then followed by a post-gradient flow-rate of 850 μl/min H_2O:MeOH (100:0) for 14 min. Finally, re-equilibration to starting conditions was performed in 5 min. The H_2O and MeOH of the increasing flow-rate gradient contained 100 mg/L Tween 20. The final constant flow-rate was 1000 μl/min, with a MeOH concentration of 15% in H_2O. This flow was connected to a T-piece and split 1/9 with a flow splitter. 90% was directed to the UV detector, while 10% was pumped into the PAD system. For HPLC analysis, all tested compounds were dissolved in 30% MeOH in water.

Results and discussion

The primary aim of this study was to develop and validate a HRS-based on-line post-column detection system for the detection of ROS-producing compounds and antioxidants in mixtures. The biochemical assay is based on the oxidation of 4-hydroxyphenylacetic acid (4HPAA) to a highly fluorescent dimer (see Scheme 1). After optimiza-

tion, the on-line detection system was validated using the well-known ROS-producing compounds paraquat, menadione, and duroquinone, as well as the antioxidants L-ascorbic acid and glutathione, in flow injection analysis (FIA) mode. The on-line PAD system was then coupled to gradient HPLC and this was used in HRS mode to screen for individual compounds in mixtures based on their ROS-producing and/or antioxidant properties.

Optimization of pro-oxidant and antioxidant detection assay

The optimization of the biochemical assay for the PAD system was conducted first in an off-line batch format before the optimized biochemical assay was transferred to the PAD system in FIA and HPLC modes. Paraquat was used as a model ROS-producing compound. The following parameters were optimized: enzyme concentrations (rat liver microsomes, SOD, and HRP), cofactor NADPH and substrate 4-HPAA, blocking reagents PEG3350 and PEG6000, the detergent Tween 20 (which can improve the resolution of the on-line PAD system) and organic modifiers (that are necessary when the on-line PAD system is operated on-line in gradient HPLC mode). All optimizations were performed without the presence of paraquat as the continuous ROS-producing compound in SL-B. However, paraquat (0.036 mM) was added to the SL-B in the final optimized system in order to permit measurements of both pro-oxidants and antioxidants using the on-line PAD system.

First, the concentration of cytochrome P450s/cytochrome P450 reductase-containing rat liver microsomes was evaluated. Insertion of this important, mainly hepatic, biotransformation system permits the reductive bioactivation of compounds that need to be bioactivated before they can exhibit their ROS-producing effects (e.g., paraquat [33]). For the liver microsomes, it was found that higher concentrations resulted in an increase in fluorescence signals up to at least 150 μg/ml (microsomal protein concentration). This is due to increased cytochrome P450s/cytochrome P450 reductase-mediated redox cycling [33]. Since the use of high concentrations of rat liver microsomes increases the risk that on-line biochemical detection systems will become clogged [27], a concentration of 50 μg/ml rat liver microsomes was used in subsequent experiments. For SOD, concentrations higher than 14 U/ml did not increase the fluorescence signal significantly. Therefore, this SOD concentration was used in subsequent experiments. For HRP, the same effect was observed at a concentration of 18 U/ml, and so this concentration was employed from then on. Increasing the concentration of 4-HPAA led to the increased formation of fluorescent dimer, up to a concentration of 1.2 mM, after which the additional increase in sensitivity was counteracted by an increase in noise. A 4-HPAA concentration of 1.2 mM was therefore used in later

experiments. An NADPH concentration of greater than 44 μM did not increase the assay performance significantly and so this concentration was adopted. For the polymers PEG3350 and PEG6000, concentrations of up to 5 mg/ml did not produce significant differences in assay performance. Since a PEG6000 concentration of 1 mg/ml is sufficient to reduce possible peak broadening in on-line biodetection systems [27], this concentration was in subsequent experiments. Detergents, such as Tween 20, can be used to prevent (membrane-bound) enzymes from adhering to reaction coils in on-line biodetection systems, thereby resulting in reduced peak broadening [27]. Solubilization of membrane-bound enzymes, such as cytochrome P450s, can occur at high detergent concentrations and will inactivate the microsomes under the present conditions. A Tween 20 concentration of 100–200 mg/l showed minimal (5–20%) enzyme denaturation. These Tween 20 concentrations are known to efficiently reduce peak broadening when used in cytochrome-P450-containing on-line biodetection systems [27]. A Tween 20 concentration of 100 mg/l was therefore used from then on. Organic modifiers may be useful for preventing enzymes and lipophilic compounds from adhering to the walls of reaction coils, thus preventing peak broadening [27]. Moreover, when on-line biodetection systems are operated in the HPLC mode, organic modifiers are automatically introduced via the HPLC gradient. When testing MeOH, ACN, and IPA in the off-line biochemical batch assay format, they were tolerated up to concentrations of 10%, resulting in approximately half the fluorescence signal compared to that obtained when organic modifiers were not used.

Optimized conditions were derived from the abovementioned experiments performed in the off-line batch assay format and subsequently translated to the on-line PAD system in FIA mode. These final conditions were: a carrier solution consisting of 10% MeOH and 100 mg/l Tween 20 at a flow rate of 100 μl/min; SL-A containing potassium phosphate buffer (50 mM; pH 7.8), rat liver microsomes (50 μg/ml), HRP (18 U/ml), and SOD (14 U/ml), and SL-B with the same buffer containing PEG6000 (1 mg/ml), NADPH (44 μM), and 4-HPAA (1.2 mM). For continuous ROS production (resulting in a stable fluorescent baseline), paraquat was present in the optimized system in SL-B at a concentration of 0.036 mM. Both superloops had a flow-rate of 100 μl/min.

PAD system in flow-injection analysis mode

Before coupling the on-line PAD system to gradient HPLC, it was first evaluated and validated in FIA mode. Evaluation of the PAD system was done with three well-known model ROS-producing compounds (i.e., paraquat, menadione, and duroquinone), and two well-known model antioxidants (L-ascorbic acid and glutathione). First, paraquat was injected in triplicate at different concentrations (1.0, 0.25, 0.06, 0.015, 0.004, 0.001, 0 mM) into the PAD system in FIA mode. Figure 2a shows the resulting signals. The highest concentration of paraquat resulted in fluorescence quenching. An analogous FIA trace for the redox cycling compound menadione is depicted in Fig. 2b.

Figure 2c and d show the resulting PAD traces when L-ascorbic acid and glutathione (well-known antioxidants) were injected in triplicate in a dose-response manner, respectively. It was shown that ascorbic acid acted as a pro-oxidant compound at low concentrations (Fig. 2c). Pro-oxidant effects of ascorbic acid at low concentrations were previously demonstrated by Abudu et al. [34], who stated that ascorbic acid can undergo one-electron reduction to form an ascorbyl radical. Ascorbic acid can also switch from being an antioxidant to being a pro-oxidant in the presence of transition metals [35].

The direct antioxidant effect of L-ascorbic was also shown using the PAD system (without paraquat in SL-B) by first injecting paraquat and then injecting a mixture of paraquat and L-ascorbic acid. Figure 3 shows that the addition of L-ascorbic acid neutralizes the ROS formed from paraquat. The same effect is shown for menadione (Fig. 3).

To determine the effects of the test compounds paraquat, menadione, L-ascorbic acid, and glutathione on the fluorescence baseline, injections of different concentrations of the test compounds into the PAD system were performed without HRP and SOD in SL-A. Menadione and duroquinone caused fluorescence signals when injected in concentrations larger then 0.8 and 0.4 mM, respectively. This implies that these compounds can only be measured efficiently at these or higher concentrations if a parallel signal measuring their auto-fluorescence is employed, so that this can be subtracted from the PAD signal. In order to determine the relative pro-oxidant or antioxidant effects of the compounds, and to compare the off-line batch assay and the on-line assay (in FIA and HPLC mode) with each other, the fluorescence signals per mole of the test compound were determined from the data obtained in the off-line batch assay and the PAD system in FIA and HPLC modes (Table 1). When comparing the relative fluorescence signals per mole of test compound obtained using the off-line batch and both of the on-line systems, the relative fluorescence signals appeared to differ significantly for some compounds. This difference might be explained by the pre-incubation step, which was only performed for the off-line batch assays and not for the on-line PAD measurements. In contrast, for the off-line batch assay and both of the on-line PAD system formats, the measured pro-oxidant or antioxidant effects were comparable for all test compounds in terms of determinations of their individual pro-oxidant or antioxidant effects. Although the results obtained
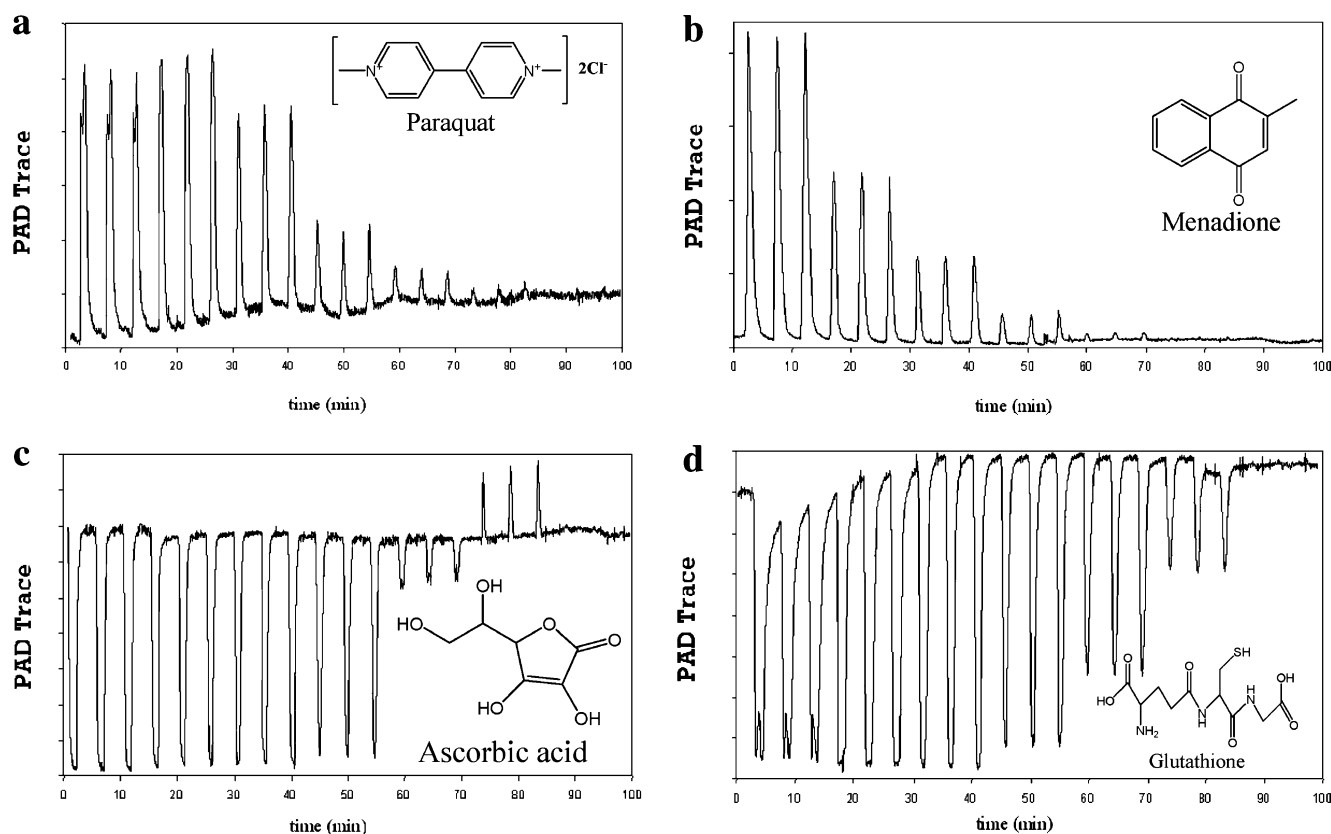
Fig. 2 Triplicate injections of a series of dilutions (dilution factor of 4) of different compounds into the PAD system used in FIA mode. a The pro-oxidant paraquat (starting with 1.0 mM). b The pro-oxidant menadione (starting with 0.15 mM). c The antioxidant ascorbic acid (starting with 1.0 mM). d The antioxidant glutathione (starting with 25 mM)

using the off-line batch format and both on-line formats were not similar, the comparable results still allowed the off-line batch assay to be transformed into both on-line assay formats. We can expect these relatively complex assay systems to give slightly different assay characteristics when performed under different assay conditions, like off-line batch or on-line assay conditions. Therefore, we can conclude that the off-line batch assay format can be transferred to the present PAD system used to screen for antioxidants and ROS-producing compounds.

Interday and intra-day variabilities were determined in the on-line PAD system in FIA mode as follows: intraday variability was determined by injecting paraquat (0.2 mM) in triplicate at 3.5-hour time intervals into the PAD system. The intraday variability was determined without changing the contents of the superloops. For the interday variability, paraquat (0.2 mM) was injected daily in triplicate for three days with fresh solutions in the superloops each day. Intraday variability was 3.3%±1.1% and interday variability 4.1%±0.6%, which are both within the ranges exhibited by

Fig. 3 Injections (triplicates) of different compounds into the PAD system in FIA mode (without the continuous addition of a pro-oxidant): 1) paraquat (0.05 mM); 2) paraquat (0.05 mM) and ascorbic acid (0.1 mM); 3) menadione (0.03 mM); 4) menadione (0.03 mM) and ascorbic acid (0.1 mM)
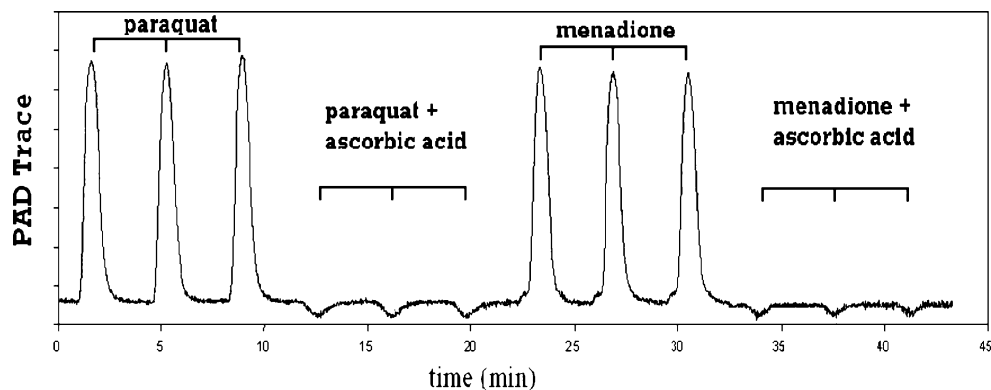
Table 1 Initial relative increases or decreases (in fluorescence units, FU) of different ROS-producing pro-oxidant compounds and antioxidants compared to paraquat for the PAD system used in FIA mode, in gradient HPLC mode and for a traditional batch assay

Pro-oxidant/ Antioxidant	FIA PAD system (FU/mol±SEM)	Detection limit for the FIA PAD system (nmol)	HPLC PAD system (FU/mol±SEM)	Detection limit for the HPLC PAD system (nmol)	Batch assay set-up (FU/mol±SEM)
Paraquat	1.00±0.08	0.07	1.00±0.33	0.9	1.00±0.05
Menadione	1.55±0.20	0.01	0.56±0.01	0.4	1.60±0.33
Duroquinone	0.13±0.02	0.04	0.36±0.19	1.3	0.16±0.07
Glutathione	-2.20±0.03	1.9	-0.67±0.18	8.0	-1.01±0.04
Ascorbic acid	-4.58±0.22	0.1	-1.35±0.13	0.2	-1.22±0.12

The detection limits of the PAD system used in the FIA and HPLC modes are also given

bioanalytical screening methods [27, 29]. Detection limits (Table 1) were determined by triplicate injections of a series of dilutions of every test compound. The detection limit was defined as the concentration of test compound that gave an average signal of three times the noise (S/N ratio=3).

The sensitivities obtained for the different compounds, which are also intrinsically determined by their pro-oxidant or antioxidant potencies, are also indicated in Table 1. These sensitivities were of the same order as those obtained with the off-line batch assay format (data not shown). Thus, the present PAD system provided a useful novel rapid screening tool for pro-oxidant and antioxidant activities of individual compounds in mixtures in this respect too.

On-line coupling of the PAD system to gradient HPLC

The PAD system in gradient HPLC mode was evaluated by analyzing the five test compounds after HPLC separation with a decreasing flow-rate gradient. The advantage of this decreasing flow-rate gradient lies with the initially high flow-rate through the column at low concentrations of organic modifier, which results in better eluting compound resolution at the start of the gradient. At higher concentrations of organic modifier, the flow rates are obviously gradually decreased (and the post-column counteracting flow rates are gradually increased) in order to obtain a continuous flow rate (1 ml/min) and concentration of organic modifier (of 10%) after mixing in the post-column counteracting gradient. This results in a constant flow rate and organic modifier concentration (after the 1:9 split) when entering the on-line PAD. The added value of this approach is that alterations in the chromatographic method can be made without much effect on the on-line PAD assay.

First, the individual test compounds were analyzed. The test compounds were injected (in triplicate) in five different concentrations prepared by serial dilution of 100 μl of stock solution with 300 μl MeOH (30% v/v). Typical superimposed chromatograms of menadione and L-ascorbic acid are shown in Fig. 4a and b, respectively. At high concentrations of L-ascorbic acid, two peaks were seen. This was the result of overloading the analytical column, as

additional mass spectrometry data showed that both peaks were from L-ascorbic acid (data not shown). The relative pro-oxidant or antioxidant response of every test compound in the PAD system used in gradient HPLC mode is shown in Table 1. The sensitivities obtained for the test compounds are also depicted in Table 1. When comparing the PAD system used in HPLC mode with the PAD system used in FIA mode and the off-line batch assays, it is seen that all three assay formats allow the detection of both pro-oxidants and antioxidants. The differences between the three assay formats probably result from different factors, such as the pre-incubation step applied in the off-line batch assay format and the peak broadening of compounds during the chromatographic separation. Another possible cause derives from minor pro-oxidant or antioxidant impurities in
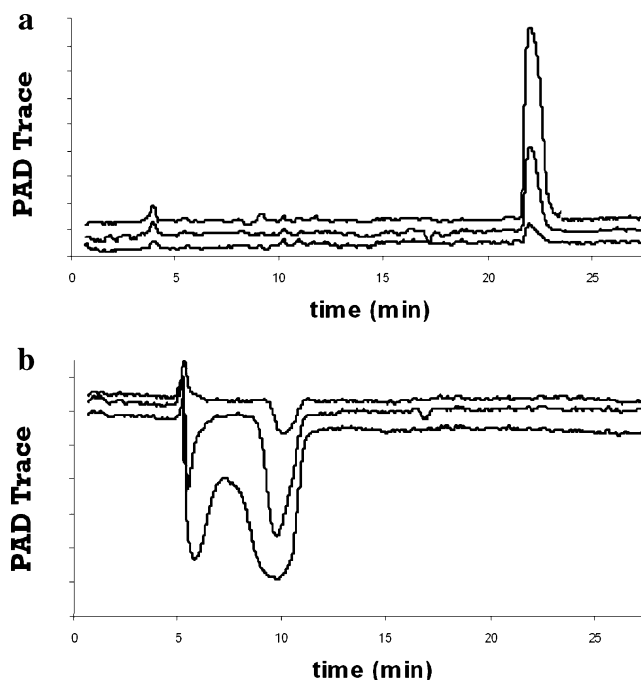

Fig. 4 a Superimposed PAD traces of menadione injected in different amounts into the PAD system in HPLC mode (12.5, 50 and 200 μM from *bottom* to *top* chromatogram, respectively). **b** Superimposed PAD traces of ascorbic acid injected in different amounts into the PAD system in HPLC mode (50, 200 and 800 μM from *top* to *bottom* chromatogram, respectively)

the test compounds. As these impurities would be separated from the test compounds in the on-line PAD system used in HPLC mode, they would only influence the results obtained in the off-line batch format and the on-line PAD system used in FIA mode. However, comparable results are still obtained (no more than threefold differences in measured pro-oxidant or antioxidant effects were obtained per mole of test compound) in the different experimental set-ups. Therefore, it can be concluded that the PAD system used in gradient HPLC mode can be easily applied to and is a sensitive method for the screening of individual compounds for their ROS-producing capacity and antioxidant potentials.

The PAD system used in HPLC mode was also applied to the detection of individual antioxidants and ROS-producing compounds in mixtures. Typical PAD traces of two different mixtures that were injected and separated on HPLC are shown in Fig. 5a and b, respectively. Figure 5a shows that all three ROS-producing compounds—paraquat, menadione and duroquinone—were individually identified as oxidant species. The compounds in the second mixture, which contained two antioxidants and two ROS-producing compounds, were individually identified as antioxidants (L-ascorbic acid and glutathione) and pro-oxidants (menadione and duroquinone). Thus, mixtures in which ROS-producing compounds are present together with antioxidants could effectively be

measured individually with the present PAD system in gradient HPLC mode. When analyzing such mixtures with traditional off-line batch assay formats, antioxidants can counteract the effects of pro-oxidants thereby reducing or even totally removing ability to detect the pro-oxidants. The relatively low resolutions obtained in the PAD assay directly reflect the resolutions resulting from the chromatographic part of the total system. Since this study was a proof-of-principle study, we did not perform a thorough optimization of the chromatographic part of the system. The resolutions that can be obtained with the PAD part of the system (used in FIA mode) are shown in Fig. 2 and reflect the performance of the assay. Connecting the PAD part to a chromatographic separation system with higher resolution will obviously result in higher resolution, which may be needed when screening real life samples. The present PAD system in HPLC mode therefore opens up new avenues to the efficient and rapid screening of complex mixtures for individual pro-oxidant and antioxidant components.

Conclusion

This paper presents the development and validation of a HRS-based on-line post-column detection system for the detection of ROS-producing compounds as well as anti-oxidants in mixtures. Different parameters, such as sub-strate (4-HPAA) and enzyme concentrations, reaction time, temperature, additives, and organic modifier concentrations were first optimized for the PAD system used in FIA mode. Several ROS-producing compounds as well as antioxidants were successfully measured with the optimized system. The intraday and interday variabilities of the PAD system used in FIA mode were determined and found to be lower than 5%. Good sensitivities, at least comparable with similar off-line batch assay formats for individual compounds, were obtained. On-line coupling of the novel PAD system to gradient HPLC permitted the screening of individual compounds in mixtures for ROS-producing and antioxidant properties. It should be noted, however, that compounds that show fluorescence quenching or intrinsic fluorescence may interfere with the methodology. However, it may be possible to adjust the system so that another split directs some of the flow to a second on-line assay that measures these artifacts with a "negative control PAD system" in order to detect such interferences. This PAD system used in gradient HPLC mode is potentially of great value to drug discovery and toxicology and food research.
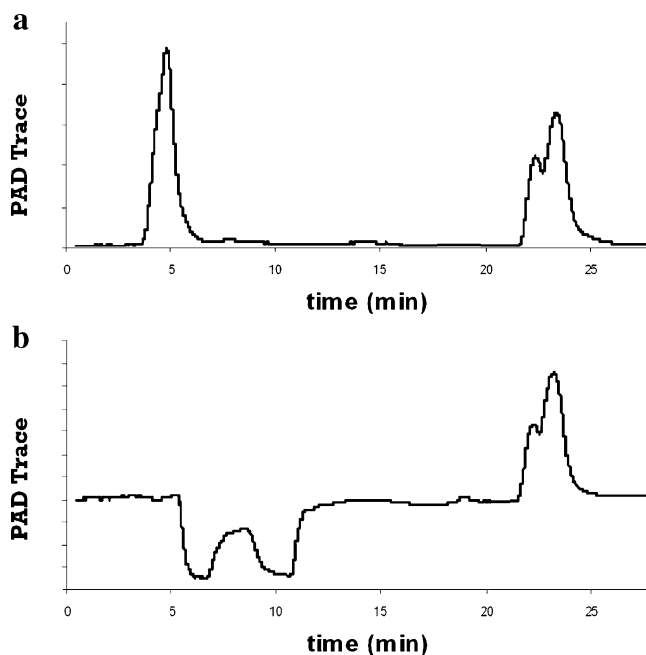

Fig. 5 **a** PAD trace for a mixture of three pro-oxidants injected into the PAD system in HPLC mode. Injected compounds are: paraquat (0.12 mM; 3.5 min), menadion (0.05 mM; 17.5 min), and duroqui-none (0.17 mM; 18.5 min). **b** PAD trace of a mixture of two pro-oxidants and two antioxidants injected into the PAD system in HPLC mode. Linjected compounds are: ascorbic acid (0.14 mM; 5 min), glutathione (0.4 mM; 8 min), menadion (0.05 mM; 17.5 min), and duroquinone (0.17 mM; 18.5 min)

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