Flow Injection System with Fluorimetric Detection for Hydrogen Peroxide Scavenging Activity Evaluation of Several Synthetic Antioxidants

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A flow injection analysis (FIA) method with fluorimetric detection (FL) for the fast evaluation of the antioxidant activity of several synthetic compounds was developed. The method is based on the oxidation of homovanillic acid (HVA), in the presence of hydrogen peroxide (H_2O_2) and horseradish peroxidase (HRP), yielding a fluorescent dimmer compound. This reaction is inhibited by the presence of the substances with antioxidant properties due to the H_2O_2 consumption. The decrease in fluorescence intensity is proportional to the H_2O_2 scavenging activity. The method was optimized using Trolox as reference. The antioxidant activity of some commercially available and newly synthesized phenolic compounds was evaluated versus Trolox. The method is very sensitive, fast and has some advantages over the batch method, such as a detection limit almost 250 times lower (0.2 ppm Trolox), good reproducibility and lower reagents and sample consumption.

Keywords: flow injection analysis, fluorescence, synthetic antioxidants, homovanillic acid

Antioxidants are defined as any substance that, when present at low concentrations compared with those of an oxidizable substrate, significantly delay or prevent oxidation of that substrate [1]. In human beeing when the body's natural defense system against free radicals are overwhelmed and the concentration of antioxidants is decreasing, oxidative stress increases [2]. The most important group of antioxidants is represented by the phenolic compounds, which can be both of natural or synthetic origin. These antioxidants are commonly used as additives in a variety of products, like food products, to prevent oxidative degradation of fats and oils or as pharmaceuticals against the oxidative stress. The continuous discovery of new synthetic products with antioxidant activity is very important as well as their characterization regarding their antioxidant activity, bio-compatibility, etc.

In literature various methods for the antioxidant capacity evaluation based on different detection techniques, such as spectrophotometry [3-6], amperometry and voltammetry [7-13], chemiluminescence [14-20], fluorescence [21-23] or mass spectrometry [24, 25] are reported.

Taking into consideration the mechanism involved, the antioxidant capacity methods are divided in two major categories based on: hydrogen atom transfer (HAT) reactions and electron transfer (ET) reactions [26]. The antioxidant response of different radical or oxidant sources may be unlike. For example, compounds that are not particularly good quenchers of peroxyl radicals may be exceptional singlet-oxygen scavengers. When the antioxidant activity of different compounds is evaluated there is no single assay that can accurately reflects the both mechanisms. Antioxidant activity measured by an individual assay reflects only the chemical reactivity under applied specific conditions. For a complete characterization of the compounds antioxidant behaviour it is recommended to use simultaneously complementary methods, based on different principles.

HAT-based assays are based on competitive reaction kinetics (i.e., oxygen radical absorbance capacity assay (ORAC), the total radical-trapping antioxidant parameter assay (TRAP) and inhibited oxygen uptake assay (IOU)). The ET-based assays involve one redox reaction with the oxidant (i.e.,Trolox equivalent antioxidant capacity assay (TEAC), ferric ion reducing antioxidant parameter assay (FRAP), DPPH-based assay, copper (II) reduction capacity, and total phenolics assay by Folin-Ciocalteu (FC)) [27].

Several methods for antioxidant activity evaluation based on the hydrogen peroxide (H_2O_2) scavenging are already reported. One common method employs horseradish peroxidase which uses H_2O_2 to oxidize scopoletin into a nonfluorescencent product. In the presence of antioxidants, the oxidation is inhibited and this reaction can be fluorimetrically monitored [28]. Beside scopoletin, several other substrates were tested for the determination of the scavenging activity of H_2O_2 (*p*-hydroxyphenylacetic acid (*p*-HPA), homovanillic acid (HVA), *p*-hydroxyphenylpropionic acid (*p*-HPPA) and tyramine [27]. HVA has the same fluorescence intensity as HPA, but less then HPPA, and its fluorescent dimmer is more stable than scopoletin [28].

HVA, which represents an important metabolite of dopamine in the brain [31], reacts with hydrogen peroxide in the presence of horseradish peroxidase, yielding a fluorescence dimmer (λ_{ex} 312nm, λ_{em} 420nm). This reaction can be used for the determination of the reactive oxygen species (ROS) production rates in different biological systems [32]. Literature data illustrate a batch method for the determination of the hydrogen peroxide

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scavenging activity of several well known antioxidants (trolox, ferulic acid, caffeic acid, pyrogallol, etc) and tea infusions using the homovanillic acid oxidation reaction [33]. The aim of the present work was the adapting of this method to a flow injection analysis (FIA) system for the hydrogen peroxide scavenging activity determination of several synthetic antioxidants. The method is based on the oxidation of the monomer HVA to its fluorescent biphenyl dimmer, in the presence of hydrogen peroxide and horseradish peroxidase. When an antioxidant compound is injected into studied FIA system, it consumes the hydrogen peroxide and a decrease of the fluorescent intensity, proportional to the antioxidative activity, is observed. The FIA method has the following advantages: better reproducibility, low reagents and sample consumption, high sample throughput.

Experimental part

Reagents and chemicals

The following reagents were used: homovanillic acid, (Sigma), horseradish peroxidase (Sigma), hydrogen peroxide (Merck), $Na_2HPO_4 \cdot 2H_2O$ (Riedel-de Haën), KH_2PO_4 (Riedel-de Haën), methanol (Merck), 2,2-dyphenil-1,1-pycrylhydrazil - free radical form (DPPH*) (Sigma).

Several compounds were evaluated for the antioxidant activity (fig 1):

- commercially available: trolox (Fluka), 2,6 -di-*tert*-butylphenol (**1**) (Fluka); 2,4-di-*tert*-butylphenol (**6**) (Fluka);

- laboratory synthesized: N,N-dimethyl-3,5-di-*tert*-butyl-4-hydroxybenzylamine (**2**); 3,5-di-*tert*-butyl-4-hydroxybenzyl hydrazine (**3**); 1-(3',5'-di-*tert*-butyl-4'-hydroxybenzyl)-3,5-dimethylpyrazole (**4**); 1-(3',5'-di-*tert*-butyl-4'-hydroxybenzyl)-3-methylpyrazol-5-one (**5**); 1-(3',5'-di-*tert*-butyl-4'-hydroxybenzyl)-3,5-diphenylpyrazole (**7**); 4,4'-(hydrazine-1,2-diylidenebis (methanylylidene))bis(2,6-di-tert-butylphenol) (**8**). Stock solutions of 1000 ppm of these synthetic antioxidants were prepared in methanol.

The aqueous solutions were prepared daily in double distilled water.

Equipment

The flow injection system is presented in figure 2 and consists of a peristaltic pump with 4 channels, tygon pump tubes (1.42 mm i.d.), a 6-ways injection valve Rheodyne type, model 5051, Jasco FP-6500 spectrofluorimeter and an Alineinc FluoroVette flow cell, 1 mm optical path, specially designed for FIA –fluorescence determinations. All the tubes used in the FIA system were PTFE tubes, 0.5 mm i.d.

The absorbance measurements for the DPPH standard method were recorded on a Biomate 3 (Thermo) UV-VIS spectrophotometer.

Procedure

It was used the system presented in figure 2. Sample volumes of 250 µL were injected in the carrier flow (sodium

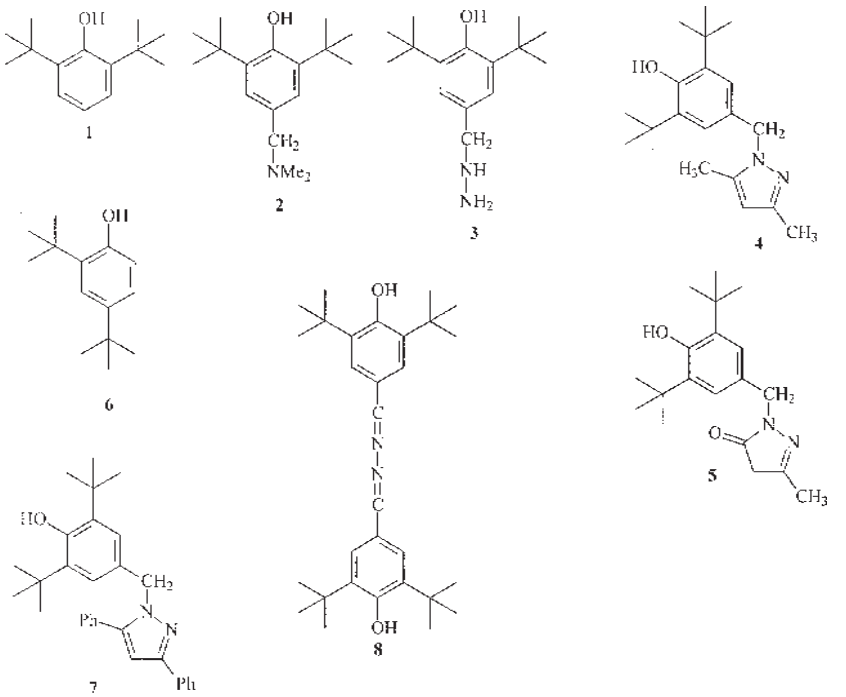

Fig. 1. Chemical structure of the compounds tested regarding their antioxidant activity. Compound **1**: 2,6 -di-tert- butylphenol; Compound **2**: N,N-dimethyl-3,5-di-tert-butyl-4-hydroxybenzylamine; Compound **3**: 3,5-di-tert-butyl-4-hydroxybenzyl hydrazine; Compound **4**: 1-(3',5'-di-tert-butyl-4'-hydroxybenzyl)-3,5-dimethylpyrazole; Compound **5**: 1-(3',5'-di-tert-butyl-4'-hydroxybenzyl)-3-methylpyrazol-5-one; Compound **6**: 2,4 -di-tert-butylphenol; Compound **7**: 1-(3',5'-di-tert-butyl-4'-hydroxybenzyl)- 3,5-diphenylpyrazole; Compound **8**: 4,4'-(hydrazine-1,2-diylidenebis(methanylylidene))bis(2,6-di-tert-butylphenol)

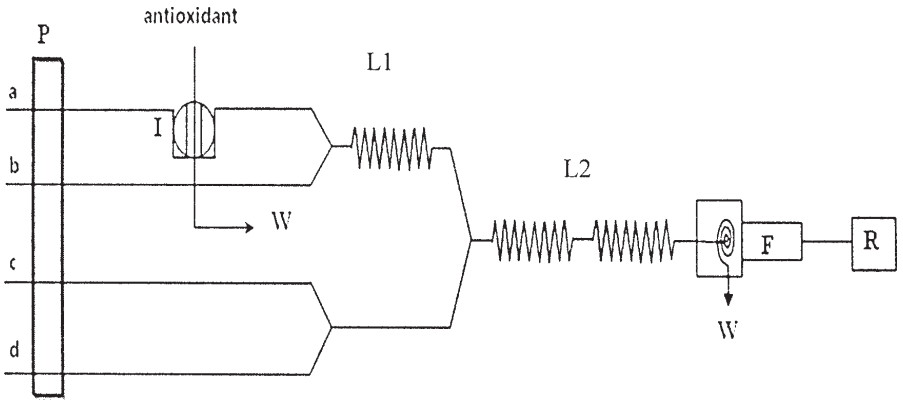

Fig. 2. Schematic FIA system used for the antioxidant activity evaluation. a. sodium diphosphate/ potassium monophosphate buffer solution, 25 mM, pH 8; b. hydrogen peroxide solution, 100 µM; c. homovanillic acid solution, 1 mM; d. horseradish peroxidase solution, 0.25 U mL^{-1}; P - peristaltic pump; I – injection valve; L1- reaction loop 1; L2 – reaction loop 2; W – waste; F – fluorimeter; R – recorder

diphosphate/potassium monophosphate buffer solution, 25 mM, *p*H 8 – flow ***a***). Flow ***b*** consisted in a 100 μM hydrogen peroxide solution, flow ***c*** in a 1 mM homovanillic acid solution and flow ***d*** in a 0.25 U mL^{-1} horseradish peroxidase solution. The total flow rate was 0.2 mL min^{-1}. Flow ***a*** mixed flow ***b*** in reaction loop L1, while flow ***c*** mixed flow ***d***. The two resulting flows were then mixed in another reaction loop, L2, before reaching the fluorescence flow cell. After injecting the analyzed sample, inverse peak shaped signals were obtained. At least three determinations were carried out for each analysed sample. The PMT voltage of the fluorimeter was set to 500V, the excitation wavelength to 315nm, while the emission was measured at 425 nm.

Results and discussion

The proposed method is based on the reaction presented in figure 3. The reaction occurs between homovanillic acid and hydrogen peroxide in the presence of horseradish peroxidase inside the reaction loop L2. Subsequently, the formed fluorescent dimmer of the homovanillic acid reaches the flow cell, where the fluorescence signal is measured by the fluorimeter. The flow injection analysis flow rate is set in such a way that the dimmer formation occurs inside L2. The fluorimeter records a constant and high fluorescence signal, which represents the baseline. Upon injecting the antioxidant compound, the hydrogen peroxide is partly or totally consumed inside the reaction loop L1. The percentage of hydrogen peroxide that is consumed is proportional to the antioxidant compound concentration and activity. Therefore, inside reaction loop L2, the hydrogen peroxide concentration is lower than in the absence of the antioxidant compound, and as a result the concentration of the fluorescent dimmer newly formed inside reaction loop L2 will be lower and inverse peak shaped signals are obtained (fig.4).

Method optimization

In order to determine the hydrogen scavenging activity using the proposed FIA system, the following parameters have been studied and optimized: HVA concentration, HRP concentration, H_2O_2 concentration, the *p*H of the carrier flow solution and the sample volume. The dispensable plastic PTFE flow cells used in these determinations are sensible to high flow rates, therefore the total flow rate of the system was set to a low flow rate (0.2 mL min^{-1}), and its influence was not studied. All parameters were optimized using trolox as a reference antioxidant compound, with concentrations between 2.5 and 5 ppm.

A study on the influence of the carrier *p*H on the FIA signals height and on the background value was carried on for *p*H values from 6.5 to 8 (data not showed). The highest peaks for trolox were obtained for the carrier pH value of 8, which was selected as optimum.

The H_2O_2 concentration was tested and its influence on the FIA signals is presented in figure 5. In the 50 to 200 μM range, the FIA peak heights were similar. A 100 μM H_2O_2 concentration was selected.

The HVA concentration influence was tested for the range 0.1 to 2.5 mM (fig. 6). For further studies a 1 mM concentration of HVA was selected as optimum as it generates the highest fluorescence signal decrease.

Figure 7 presents the HRP activity influence on the FIA peak height. As it can be observed, in the studied range the HRP activity does not significantly affect the peak height. In order to minimize the HRP consumption, a HRP activity of 0.25 U mL^{-1} was used for further experiments.

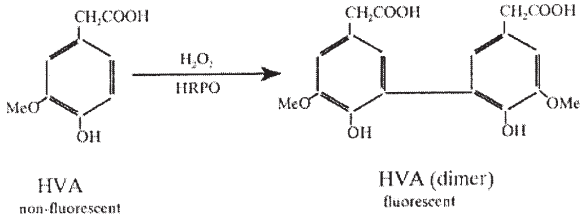

Fig. 3. Homovanillic acid oxidation reaction by hydrogen peroxide, in the presence of horseradish peroxidase

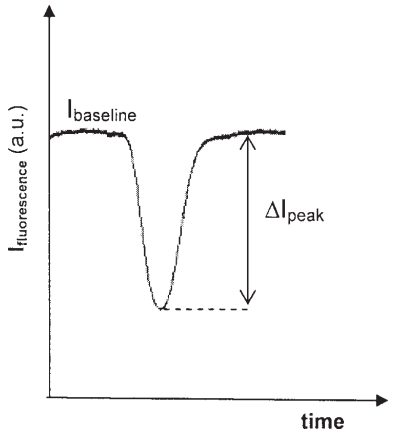

Fig. 4. Inverse FIA peak shape obtained for antioxidant activity evaluation using the FIA-CL proposed system

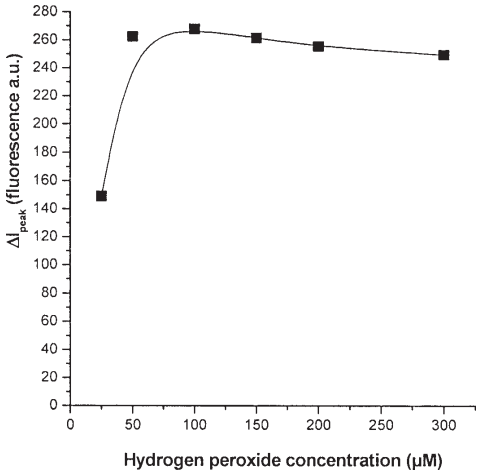

Fig. 5. Hydrogen peroxide concentration influence. HVA concentration 2.5 mM, HRP activity 1 U mL^{-1}, carrier *p*H 8, total flow rate 0.2 mL min^{-1}, trolox concentration 5 ppm, sample volume 250 μL

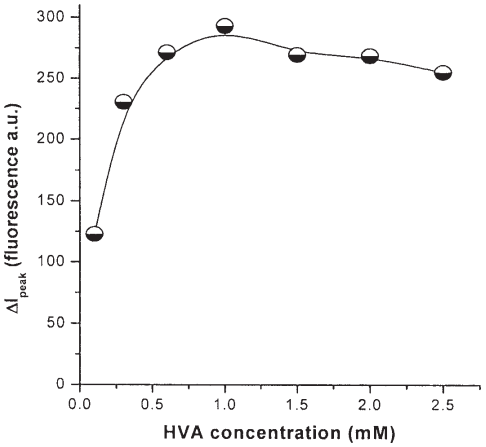

Fig. 6. Homovanillic acid concentration influence. HRP activity 1 U mL^{-1}, H_2O_2 concentration 100 μM, total flow rate 0.2 mL min^{-1}, trolox concentration 5 ppm, sample volume 250 μL

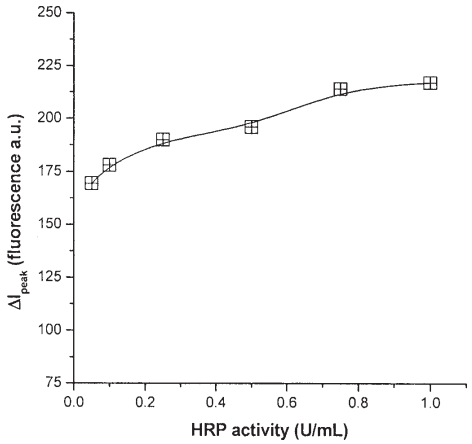

Fig. 7. Horseradish peroxidase activity influence. HVA concentration 1 mM, carrier *p*H 8, H_2O_2 concentration 100 μM, total flow rate 0.2 mL min^{-1}, trolox concentration 5 ppm, sample volume 250 μL

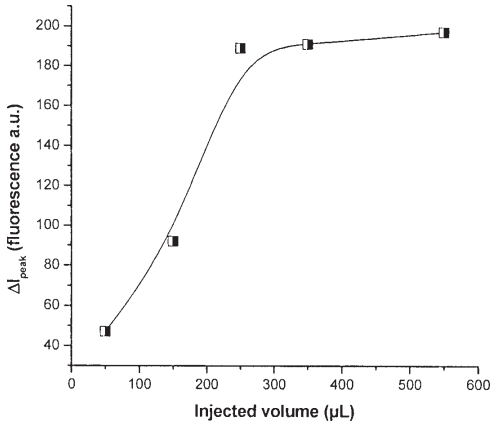

Fig. 8. Injected volume influence. HRP activity 0.25 U mL^{-1}, HVA concentration 1 mM, carrier *p*H 8, H_2O_2 concentration 100 μM, total flow rate 0.2 mL min^{-1}, trolox concentration 2.5 ppm

The influence of the injected volume of the sample is presented in figure 8. For a better optimization of this parameter, a lower trolox concentration (2.5 ppm) was used as higher concentrations, injected in large volumes, may consume all the hydrogen peroxide, and subsequently provide signals that drop close to zero. This should be avoided for a correct evaluation of the injected volume influence on the fluorescence signal. A 250 μL volume was selected for plotting the calibration graph, as bigger volumes did not generate higher fluorescence signals.

Method performances and antioxidant activity evaluation of synthetic antioxidants

A study regarding the reproducibility of the signal was carried on for a 2.5 ppm concentration of trolox, providing a 2.83% RSD value for 10 determinations. The method allowed the determination of trolox in the 0.65 - 5 ppm range, with a LOD of 0.2 ppm. The calibration graph for trolox done under the conditions presented in the procedure yielded the equation $\Delta I_{peak} = 73.19\ c + 6.14$, with a R^2 of 0.9942, where ΔI_{peak} is the FIA peak height (fluorescence arbitrary units) and c is the trolox concentration (ppm).

Several newly synthesized compounds were tested in regards of their antioxidant activity. In a previous paper, the synthesis of some hindered phenolic derivatives, namely 1-(3,5-dialkyl-4-hydroxybenzyl)-pyrazole and -pyrazol-5-one derivatives and their corresponding aroxyls, was described. Compound **2** was obtained by a Mannich reaction from compound **1**, and compound **3** was obtained by reaction of **2** with hydrazine hydrate according with literature data [36], while compounds **4**, **5** and **7** were synthesized by reaction of 3 with acethylacetone (4), ethyl acetoacetate (5) and dibenzoylmethane (7) [35] (scheme 1). The compound **8** is subject of unpublished results.

It was shown that these hindered phenol derivatives afforded with lead dioxide or lead tetraacetate in toluene persistent free radicals (aroxyls) that were studied by EPR (electron paramagnetic resonance) spectra. The persistence of these free radicals owing with the electron spin delocalization from oxygen atom (**A**) and carbon atom (**B**) that generates hiperconjugative limited structures (fig. 9), showed that these phenolic derivatives are good antioxidants.

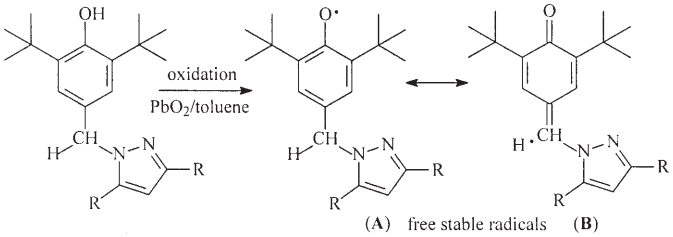

Fig. 9. Hiperconjugative limiting structures (**A**) and (**B**) of free radicals obtained by oxidation of hindered phenols

The total antioxidant activities of these commercially available and newly synthesized compounds using the developed FIA-FL method and comparing with the spectrophotometric method based on DPPH* radical scavenging were evaluated.

Using the FIA-CL method, the H_2O_2 scavenging activity was evaluated. After injecting the samples, it was observed that only compounds 2, 3 and 5 generated a decrease of the fluorescent signal. Compounds 1, 4, 6, 7 and 8 did not show a change of the fluorescence signal. Several concentrations were tested for each of the studied synthetic antioxidant compound. The trolox equivalent was calculated with the formula:

$$\text{Trolox equivalent (\%)} = (c_{\text{equivalent trolox}} / c_{\text{compound}})\ 100$$

where:

$c_{\text{equivalent trolox}}$ is the concentration obtained from the trolox calibration graph by interpolating the fluorescence inhibition of the tested compound;

C_{compound} - the tested antioxidant concentration injected in the FIA-FL system.

The results were compared with those of the spectrophotometrical method (table 1) based on the DPPH* radical scavenging activity, by applying the method described [34], with some modification (the synthesized compounds were dissolved in a methanol-water mixture (1:1)). For the trolox calibration curve, different trolox solution with concentration varying in the range 1 – 5 ppm, all containing 20 ppm DPPH, were prepared in methanol-water mixture (1:1). After a 30 min reaction time, the absorbance of these mixtures was read at 517 nm. The antioxidant activity percentage was plotted against trolox concentrations, yielding a (%) DPPH* = 20.47 c – 5.59 equation, with R^2 of 0.9935, where (%) DPPH* represents the percentual DPPH radical scavenging activity and c is the trolox concentration (ppm).

All synthesized compounds were tested for DPPH* radical scavenging activity. In order to eliminate the absorbance of the DPPH* solution, a 20 ppm control solution of DPPH*, diluted in distilled water, was prepared.

Table 1
COMPARATIVE EVALUATION OF THE ANTIOXIDANT ACTIVITY FOR SEVERAL SYNTHETIC COMPOUNDS

compound	Trolox equivalent (%)	
	H_2O_2 scavenging activity (FIA-FL method)	DPPH* radical scavenging activity (spectrophotometric batch method)
1	n.d.[a]	23
2	3.3	12
3	6.0	49
4	n.d.	6.9
5	6.5	82
6	n.d.	10
7	n.d.	11
8	n.d.	23

[a] n.d.: non-detectable

The results presented in table 1 demonstrate that the tested synthetic compounds have a much higher DPPH* radical scavenging activity than a H_2O_2 scavenging activity.

Conclusions

A new and very sensitive system based on flow injection analysis technique with fluorescence detection (FIA-FL) for antioxidant activity evaluation in terms of hydrogen peroxide scavenging was optimized. This method has the advantages of having a good reproducibility, a detection limit 250 times lower (0.2 ppm) than of the reported batch fluorimetric method (0.2 mM ≈ 50 ppm) [33] and good linearity intervals (0.65 – 5 ppm) for trolox. The FIA-FL method has very low consumptions of reagents and sample (approx. 2 mL of reagents and 250 µL of sample per determination) and a higher throughput compared to the batch method (6 samples / h *vs*. 3 samples / h). Another advantage is the reduced contact of the analyst with the reagents due to the use of the flow injection analysis technique.

The hydrogen peroxide scavenging activity of several synthetic compounds was evaluated using the optimized FIA-FL method. The developed method may be used as a complementary one for the antioxidant activity evaluation together with the DPPH* radical scavenging method characterized by similar detection limit (0.33 ppm) and linearity interval (1 - 5 ppm) for trolox.

In conclusion, it can be stated that the developed FIA-FL system for H_2O_2 scavenging activity is useful to estimate the potential of different substances to act as antioxidants. The results may be corroborated with those obtained with methods based on other principles for antioxidant activity determination, like the DPPH* radical scavenging or TRAP, in order to have a more complete evaluation of the antioxidant potential.

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