

ORIGINAL ARTICLE

Square wave voltammetry on screen printed electrodes: comparison to ferric reducing antioxidant power in plasma from model laboratory animal (Grey Partridge) and comparison to standard antioxidants

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Summary

Low molecular weight antioxidants (LMWAs) were assayed by square wave voltammetry (SWV) using screen printed electrodes. Standard antioxidants, i.e. uric acid, ascorbic acid, trolox and glutathione, were assayed in order to estimate the sensitivity and standard redox potentials of individual LMWAs. In another experiment, plasma from Grey Partridges was used as model real samples. Ferric reducing antioxidant power (FRAP) was used as a reference method. Two peaks in plasma samples were found by SWV and correlated to FRAP. The SWV peaks were successfully correlated to FRAP. The practical importance of SWV carried out on screen printed electrodes is discussed.

Key words: electrochemistry; antioxidant assay; oxidative stress; reactive oxygen species; avian blood; biochemistry

INTRODUCTION

Low molecular weight antioxidants (LMWAs) are chemical compounds which are easily oxidized and protect other molecules from oxidization (Podda and Grundmann-Kollmann 2001). Physiologically most relevant antioxidants include tocopherol, retinol,

ascorbic acid, glutathione, cysteine and uric acid (Finaud et al. 2006). In addition to the LMWAs, enzymatic antioxidants play an important role in the protection of the organism. The enzymes participating in LMWA reactivation, such as glutathione reductase, represent one group, and other enzymes catalyze the splitting of reactive oxygen species (ROS): superoxide dismutase splitting superoxide, or catalase splitting hydrogen peroxide. Enzymes catalyzing the reaction between ROS and LMWAs represent the last group participating in the protection against oxidative stress (Gilca et al. 2007), and glutathione peroxidase is a typical example of this group.

There are a number of reasons why levels of endogenous antioxidants may be increased. Oxidative stress burden is a common cause, perhaps as a consequence of exposure to drugs (Bandouchova et

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al. 2009). Stress accompanied by an increase in antioxidants is found in individuals exposed to toxins or infectious agents (Nemsadze et al. 2009, Pohanka et al. 2009a). The relationship between ageing and enzymatic antioxidants as well as LMWAs has also been extensively investigated (Khansari et al. 2009) and methods suitable for the fast and reliable assay of antioxidants have been established. Enzymatic antioxidants are assayed with respect to their catalytic activity or specific antibodies finding distinct regions in their structure, and LMWAs are typically assayed using their redox potency. Specific studies have been carried out on chromatographic devices where individual antioxidants can be distinguished, but the disadvantages of such techniques are the cost and time needed for each assay (Takemoto et al. 2009).

Probably two photometrical methods are most common for the estimation of antioxidant potency. The first is the oxygen radical absorbance capacity (ORAC), and the second is the ferric reducing antioxidant power (FRAP). ORAC is based on fluorescein damage and a decrease in fluorescence in the presence of the oxidant and its protection due to the antioxidant in the sample. The antioxidant potency is then recounted into a trolox equivalent (Prędka and Gronowska-Senger 2009). The FRAP method is a direct one. Antioxidants in the assayed sample reduce Fe^{III} to Fe^{II} . Accumulated Fe^{II} interacts with 2,4,6-tris(2pyridyl)-s-triazine (TPTZ) providing complex absorption at 593 nm. A selective method for assay of reduced glutathione (GSH) and cysteine is the Ellman method which employs 5,5'-dithiobis(2nitrobenzoic acid) (DTNB hereinafter) which in turn provides yellow 5-thio-2-nitrobenzoic acid (TNB). Voltammetric methods are considered a promising method of analysing LMWAs, and cyclic voltammetry, differential pulse voltammetry and square voltammetry (SWV) have been performed by many authors to measure LMWAs in biological matrices (Shohami et al. 1997, Chevion and Chevion 2000, Huang et al. 2004, Vacek et al. 2004, Adam et al. 2007, Pohanka and Stetina 2009).

The present study has two aims. The first is to estimate standard LMWAs in a sample using SWV and screen printed electrodes. The second aim is to perform SWV on real samples, i.e. plasma from the Grey Partridge. The suitability of SWV for the assay of LMWAs in plasma samples was confirmed by the standard FRAP test. Grey Partridges were chosen as an appropriate model allowing collection of blood samples in sufficient amounts and moreover, grey partridges are animals used as environmental models (Acevedo et al. 2007).

EXPERIMENTAL

Animal manipulation, blood collection and processing

Healthy Grey Partridges (*Perdix perdix*) kept at the Game Bird Farm of the Veterinary and Pharmaceutical University Brno, Czech Republic were employed in the study. Partridges were supplied with a feeding mixture and drinking water *ad libitum*. The animals used in experiment were a sub-group of another experiment and the blood used was an aliquot. This approach allowed a saving not only in costs but also in the number of animals needed for experimental purposes. The whole experiment was permitted and supervised by the ethical committee of the Veterinary and Pharmaceutical University Brno.

Blood was collected from the ulnar vein, taken into tubes with heparin, and centrifuged at $2,000\times g$ for 5 minutes. The plasma was separated and used immediately for the assay of LMWAs, or stored at $-80\text{ }^{\circ}\text{C}$. The frozen plasma was processed within one month in order to avoid spontaneous degradation of antioxidants. The pH value of plasma samples was 7.4 as confirmed by a standard pH selective potentiometric electrode.

Ferric reducing antioxidant power assay

Minor modifications in methods referred to in Chen et al. (2007) and Szydłowska-Czerniak et al. (2008) were made to estimate the FRAP. 2,4,6-tris(2pyridyl)-s-triazine (TPTZ) containing reagent solution was prepared shortly before measurement. The mixture was composed of 2.5ml of 10 mM TPTZ in 40 mM HCl mixed with 20 mM FeCl_3 dissolved in another 2.5 ml of water. Finally, 25 ml of 0.1 M acetate buffer of pH 3.6 was added. The mixture was heated to $37\text{ }^{\circ}\text{C}$ for 10 minutes. After that, 30 μl of a plasma sample or phosphate buffered saline (as blank; abbreviated PBS) were added into 200 μl of TPTZ containing reagent solution and, as the last step, 1 ml of distilled water was injected into the solution. The mixture was incubated for 10 minutes and centrifuged at $10,000\times g$ for another ten minutes. Supernatant absorbance was measured at 593 nm against the fresh blank.

Square wave voltammetry assay

Square wave voltammetry (SWV) was used to estimate LMWAs in plasma without any pre-treatment of samples. Screen printed sensors (BVT, Brno, Czech Republic) containing platinum working (diameter 1 mm, dot shaped), platinum auxiliary (circle shaped) and silver covered with silver chloride reference (circle shaped) electrodes were used throughout experiments. The electro-

chemical assay was processed by an electrochemical analyser (EmStat, PalmSens, Houten, Netherlands). The electrochemical strips were washed by ethanol prior to use and the strips were used in a disposable way in order to avoid hysteresis. The potential range of SWV was 0–1 V with potential step and amplitude of 10 mV and frequency of 1 Hz. The strips were fixed horizontally and electrodes were covered with 20 μ l of the analyzed plasma sample. Experimental curves were processed using the PSLite software (PalmSens).

RESULTS AND DISCUSSION

Assay of standard antioxidants

In the first phase of the study, the suitability of SWV on screen printed electrodes for standards of antioxidants was examined. The antioxidants ascorbic acid, glutathione and uric acid were chosen as they are typically widely found in the body. Trolox was chosen as a water soluble derivate of tocopherol (Olsewska and Michel 2009). The example of real voltammograms is clearly depicted as Fig. 1. The antioxidants diluted in phosphate buffered saline (PBS) were adjusted in a wide range covering at least the expected physiological concentrations in the blood or plasma. The regression of SWV versus standard antioxidants is depicted as semi logarithmic calibration in Fig. 2.

The most relevant value able to provide qualitative information of the sample analyzed was considered to be the peak position. The limit of detection (LOD) and correlation for individual assays are taken for other valuable information. The applied voltage for maximal oxidation of the selected antioxidants can be divided into two basic intervals. The lower one is up to 500 mV and ascorbic (344 ± 42 mV) as well as uric (455 ± 29 mV) acids are contributors to that. Trolox (670 ± 23 mV) and glutathione (764 ± 17 mV) are oxidized at a higher applied voltage. Though trolox and glutathione peak positions would be considered as overlaid, the interference in a biological sample is not really anticipated since trolox is a man made derivative of poorly soluble tocopherol occurring typically in cell membranes *in vivo* (Polyzos et al. 2007).

The LOD is another important analytical parameter. The best limit of detection was achieved for trolox and glutathione. Uric acid was found in levels approximately two to three times higher than the above mentioned. The poorest limit of detection was found for ascorbic acid. The limits of detection were low enough to recognize the physiological

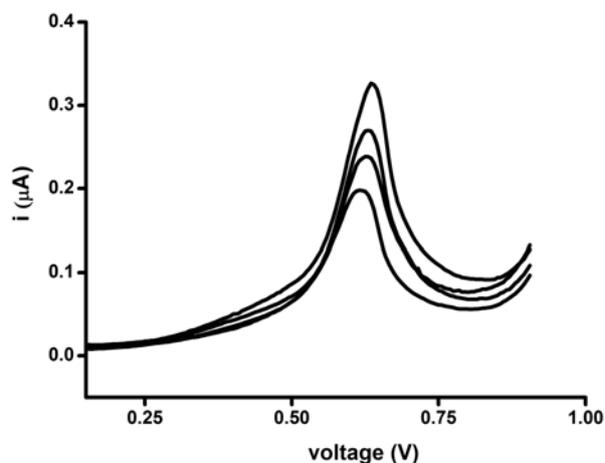


Fig. 1. **Real voltammograms achieved by SWV.** The curves represent assay of the antioxidant trolox in concentrations 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} mol/l.

values of the antioxidants. Correlation coefficients confirmed the analytical suitability of SWV for assay. They varied from 0.931 (ascorbic acid) to the highly significant 0.997 (uric acid). The SWV is definitely responsible for the change in the antioxidants although the curves were shaped in a different grade when compared with each other. According to the data included, the SWV on screen printed electrodes was confirmed as a suitable tool for assay of antioxidants.

Antioxidants levels in biological matrices represented by the Grey Partridge plasma

The Grey Partridge is a model organism frequently used in environmental studies. The plasma samples were collected as mentioned in the experimental section and assayed by both FRAP and SWV in order to assess suitability for clinical analysis. The typical peak heights and positions are clearly shown in the Fig. 3. There are two peaks found at positions 476 ± 43 and 745 ± 35 mV. According to the optimisation and finding of standard antioxidant redox peaks, glutathione can be identified as an important contributor to the second (*b*) peak (745 ± 35 versus the standard value 764 ± 17 mV), whereas uric acid mainly seems to be forming the first (*a*) peak (476 ± 43 versus the standard value 455 ± 29 mV). The peaks *b* and peak of glutathione respectively peak *a* and uric acid are significantly overlaid and can be identified accordingly.

The plasma samples were repeatedly analysed by both FRAP and SWV. Peaks *a* as well as peaks *b* achieved in the correlation between FRAP and SWV

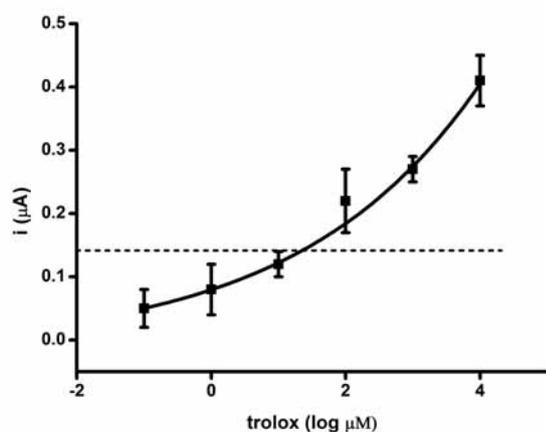
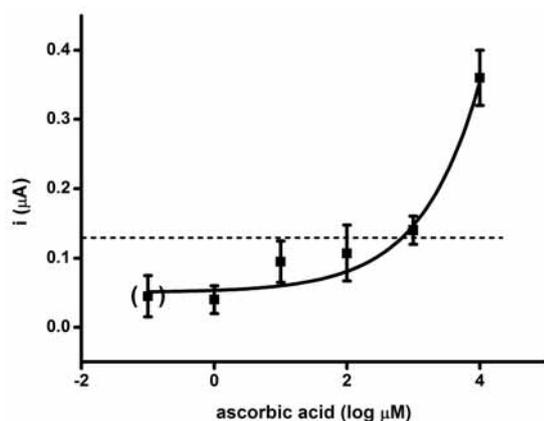
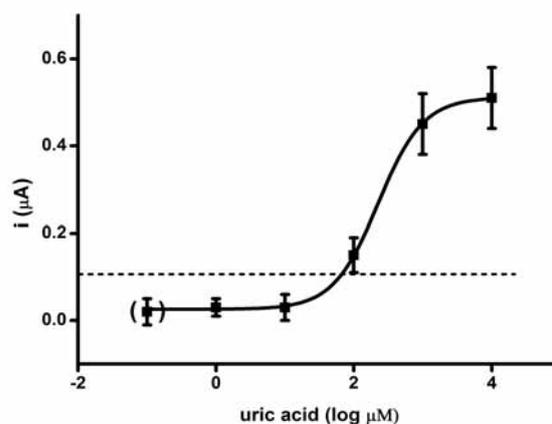
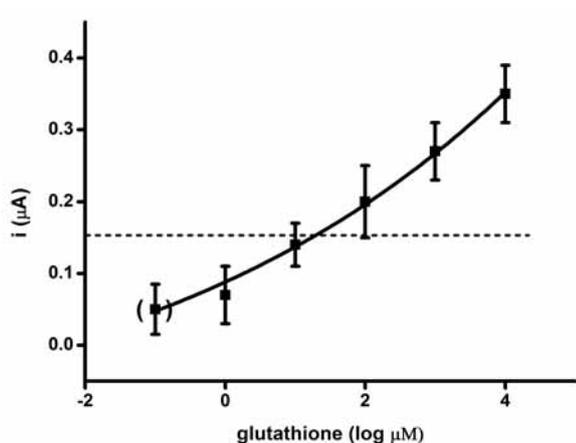


Fig. 2. Calibration curves for antioxidants glutathione, ascorbic acid, uric acid and trolox. The achieved current against antioxidants logarithmic concentration was extrapolated. The point in brackets represents control (phosphate buffered saline). Dashed lines indicate the limit of detection (signal to noise ratio equal to three). Error bars were calculated as standard deviation for quadruplicate.

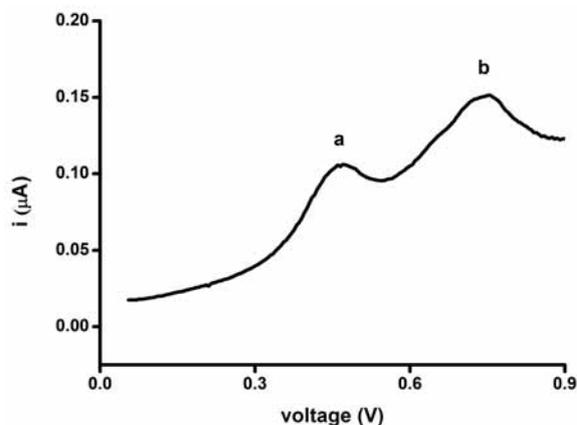


Fig. 3. Example of SWV for the Grey Partridge plasma sample. According to the text, the peaks are labelled as *a* and *b*.

are clearly depicted in Fig. 4. As seen on the calibration, the peak *a* achieved by SWV was found to be clearly correlated to the FRAP value of the same plasma samples. The correlation coefficient for five plasma samples, each assayed four times, was 0.903. In contrast, the peak *b* value was quite stable and the

Table 1. **Basic analytical parameters of antioxidants assay.** U represents peak position, LOD limit of detection, and R correlation coefficients of calibrations shown above.

	U (mV)	LOD (mol/l)	R
ascorbic acid	344±42	6.30×10 ⁻⁴	0.931
glutathione	764±17	2.82×10 ⁻⁵	0.993
trolox	670±23	2.47×10 ⁻⁵	0.988
uric acid	455±29	6.46×10 ⁻⁵	0.997

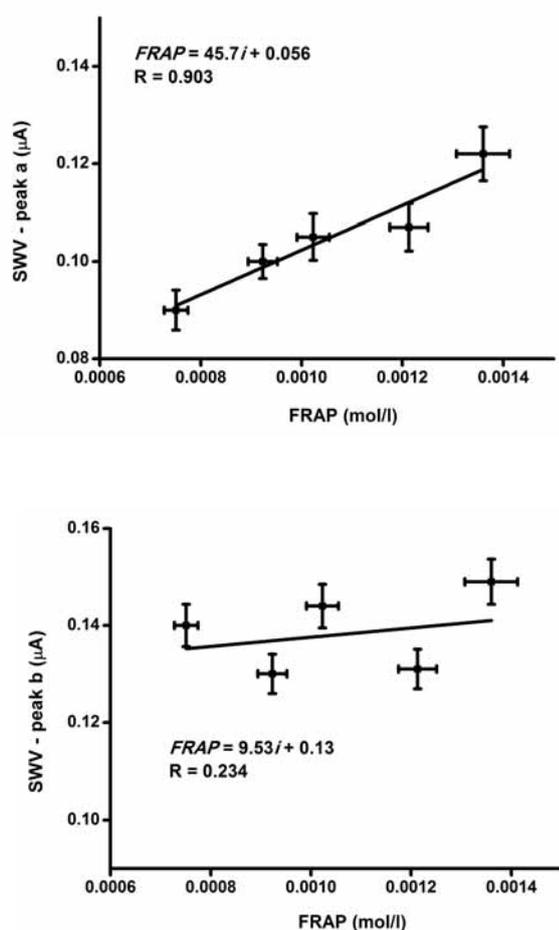


Fig. 4. **Correlation of SWV to FRAP.** Peaks a and b represent currents achieved at 476±43 and 745±35 mV, respectively. Error bars were achieved by tetraplicate measurement and indicate standard deviation.

differences between glutathione levels in individual partridges were minimal. There was a low significance correlation to the FRAP; the correlation

coefficient was only 0.234. The reason for this effect could be the low fluctuation of the reduced glutathione in the plasma samples. The Ellman assay of thiols was carried out to confirm this (Navarová et al. 2008, Pašková et al. 2008). The pure DTNB was used as stated in the references with minor adaptation. The molar concentration of thiols was calculated from absorbance at 412 nm. The levels of thiols were 4.04–5.11×10⁻⁴ mol/l in the tested plasma samples. This fact confirms the suspicion that reduced glutathione is not strongly changed as indicated by SWV.

The currents found at peaks a and b were summarized and the sum of the current was plotted against FRAP values. As can be seen in Fig. 5, the sum of currents was well correlated to FRAP. The correlation coefficient was even higher when correlating the peak a to FRAP only. The value of correlation coefficient found was 0.971. It is probably due to the reduced glutathione impact on total antioxidant power of plasma. Though the reduced glutathione changed its level in a lower scale when compared to the other LMWAs, its total level is quite high and even low changes can cause the smoothening of the correlation introduced in Fig. 5.

SWV based on screen printed electrodes was found to be fully suitable for the fast and precise assay of LMWAs. Moreover, the assay is able to provide a simple distinction of the LMWAs into two basic groups including the most important antioxidants. The most relevant advantage of SWV is the fact that there is no need to use any reagents or pre-treatment of samples as typical for chromatography methods (Teixeira et al. 2009, Kurzawa 2010). SWV especially allows the process of samples without the extraction typical for the processing of biological matrices (Jin et al. 2010). Other interferences in plasma samples are not expected. Previous experiments aimed at the evaluation of direct oxidative stress markers and reduced antioxidants produce a lower potential (Pohanka and Stetina 2009); e.g. hydroperoxides and

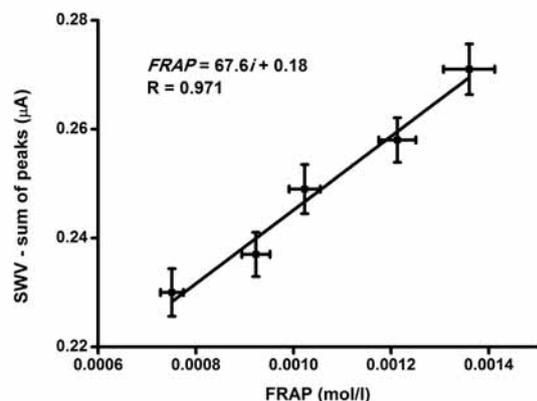


Fig. 5. Correlation of the sum of peak currents achieved by SWV to FRAP. Error bars were achieved by tetraplicate measurement and indicate standard deviation.

organic peroxides have a peak in the anodic range at a potential of around -1000 mV (Evans 1999).

The overall time needed per assay is lower than three minutes when compared to the approximately two hours of manipulation required for the FRAP assay. The minimal consumption of samples can be mentioned as another advantage and point to the suitability of screen printed and composite electrode based sensors for practical performance (Pohanka and Skládal 2008, Pohanka et al. 2008, 2009b, Freitas et al. 2009).

CONCLUSIONS

SWV based on screen printed electrodes was found suitable for the assay of low molecular weight antioxidants. The assay was successfully performed on standard antioxidants such as trolox, ascorbic acid, uric acid, and glutathione. Limits of detection and redox peaks for antioxidant standards were both investigated. The practical suitability of SWV was performed on real plasma samples. SWV fully corresponds to the standard FRAP assay. In comparison to the FRAP assay, LMWAs in plasma samples can be analysed by SWV without any reagents or pre-treatment of samples. The time needed for one assay is significantly shorter for SWV when compared with FRAP. The low consumption of the plasma sample and no sensitivity to interference of the other oxidative stress markers can be mentioned as other advantages.

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