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Monitoring of Rosmarinic Acid Accumulation in Sage Cell Cultures using Laccase Biosensor

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ABSTRACT:

Introduction – A recently developed laccase based biosensor is used for polyphenols determination from *in vitro Salvia* cultures, the results being expressed as rosmarinic acid equivalent content.

Objective – The aim of this work was to use a previously developed laccase biosensor for the determination of total phenolic content from *in vitro* cultivated *Salvia*, and to support the biosensors further application for the assessment of polyphenols metabolites.

Methodology – The biosensor was constructed by drop casting 3 μL of laccase solution and stabilisation with 0.1 % Nafion solution onto a DropSens carbon screen-printed electrode. Electrochemical measurements were carried out in a 0.1 mol/L phosphate buffer (pH 4.50), the applied working potential being −30 mV versus reference electrode.

Results – The response of the biosensor developed was characterised in terms of repeatability, accuracy and precision; the limit of detection was 7.5×10^{-7} mol/L, the limit of determination was 9.5×10^{-7} mol/L, and linear response range for rosmarinic acid was 1×10^{-6}–10^{-5} mol/L.

Conclusion – A stable, sensitive and simple biosensor based on laccase–nafion was used for monitoring the total polyphenolic content from two *in vitro* cultivated plants. The biosensor response was free of electrochemical interferences and of possible interferences from growth media constituents, demonstrating a high sensitivity for rosmarinic acid determination in cell culture suspensions.

Keywords: amperometric biosensor; nafion; polyphenol oxidase; rosmarinic acid; synchronised cell cultures

Introduction

Nowadays, when the main attention of researchers is focused on public health and the use of plants as sources of active principles, it is necessary to produce and properly analyse dietary products containing high amounts of natural metabolites (Balsano and Alisi, 2009; Chen and Luo, 2010; Das *et al*., 2010). Different active principles from plants are known as health promoters, having the following activities: anti-cancer, anti-lipidemic, anti-cholesterol, anti-microbial, anti-bacterial, anti-fungal, anti-viral, anti-hypertension, anti-inflammatory and anti-oxidant activity, etc. Since the anti-oxidant concept is very comprehensive, including in most cases even other effects (e.g. anti-inflammatory or anti-cancer), an increased interest has arisen in assessing the main class of anti-oxidants occurring in plant sources (fruits, leafs, roots), vegetables and medicinal plants. Amongst the ubiquitous occurring anti-oxidants, the polyphenolic compounds have to be mentioned. Polyphenols from plants are either wild species or '*in vitro*' synthesised secondary metabolites. The increase in the production yield may occur via chemical elicitation or as result of oxidative stress applied to '*in vitro*' cultivated plants (Litescu *et al*., 2010). Rosmarinic acid, for example, is rapidly accumulated during growth phase of sage plants subjected to oxidative light stress (Ruffoni *et al*., 2010). Plant cell cultures represent a good alternative source of phenolic metabolites to whole plants, as they are independent of geographic location and other environmental factors affecting the content of synthesised active principles, and providing the advantage of potential harvesting under the same conditions, being generated on a continuous year-round basis.

The use of sage plants as a source of important cardio-protective, anti-hypertension and anti-microbial phenolic anti-oxidants is widely recognised, especially after the alcoholic extraction of the active principles (Cahoon *et al*., 2003). In our particular case the cell growth aimed to improve the synthesis of rosmarinic acid, because rosmarinic acid, as a secondary metabolite, is resourceful. It is used in medical applications due to its anti-microbial, anti-viral, anti-oxidant, anti-inflammatory and immunomodulatory properties (Petersen and Simmonds, 2003; Zheng and Wang, 2001; Yamada *et al*., 2006; Peng *et al*., 2007; Komes *et al*., 2011).

The most common methods used for the determination of polyphenolic compounds are chromatography (HPLC and GC) (Helmja *et al*., 2009; Viñas *et al*., 2009) and spectrophotometry (Paixão *et al*., 2007). The necessity of simpler and user-friendly techniques has led to these techniques being replaced by bioanalytical tools. During recent years many polyphenol oxidase-based sensors have been reported in the literature for the determination of phenolic or polyphenolic compounds because of good selectivity, low cost and proven potential for miniaturisation and automation (Di Fusco *et al*., 2010; Diaconu *et al*., 2010; Litescu *et al*., 2010).

Laccase immobilisation as a biorecognition element has been attempted by different methods such as: cross-linking with polyazetidine prepolimersolid (Di Fusco *et al*., 2010) or with glutaraldehyde (Gamella *et al*., 2006), entrapment into chitosan

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(Ruffoni *et al*., 2010; Xu *et al*., 2010), immobilisation in titania gel matrix (Kochana *et al*., 2008) and covalent immobilisation on modified polyethersulfone (PES) membrane (Gil *et al*., 2011). The use of hydrophobic ionic liquids (ILs) derived from the bis (trifluoromethylsulphonyl)imide anion associated with different imidazolium cations was reported when a laccase biosensor was designed, optimised and applied for highly sensitive rosmarinic acid determination from plant extracts (Franzoi *et al*., 2009; Brondani *et al*., 2011). Laccase-based biosensors developed using redox mediators were studied for the detection of phenolic compounds in must and wines (Mousty *et al*., 2007; Montereali *et al*., 2010). A complex bienzymatic sensor for the determination of total polyphenolic content has been developed in our laboratory (Diaconu *et al*., 2011).

In this context we report here the use of a previously developed simple laccase–nafion-based biosensor developed by enzyme immobilising by adsorption on a working electrode surface and stabilisation with a nafion membrane (Litescu *et al*., 2010). In the present work the focus is to provide information for very low concentrations of secondary polyphenolic metabolites and to monitor their accumulation during *in vitro* plant growth. Rosmarinic acid was used as a polyphenolic substrate for the laccase-based biosensor because it is the major component from *in vitro* cultivated medicinal plants of interest – *Salvia* (*maxima* and *verde*). The two genotypes of *S. maxima* and *S. verde* were cultivated as described previously for *Salvia officinalis* (Ruffoni *et al*., 2010) and the biosensor developed was used in assessing the polyphenolic content of *Salvia* extracts, both from mother cells and synchronised cells. The synchronised cells were the cells of the same size, obtained subsequent tospecific filtration (specific pore-size membrane, appropriate for plant cell dimensions) that have grown in unperturbed, steady-state culture.

Experimental

Materials

Laccase from *Trametes versicolor* was supplied by Sigma-Aldrich, Steinheim, Germany (EC 1.10.3.2, activity: 26 U/mg, according to the supplier), and its activity was spectrometrically checked each time prior to biosensor preparation using the unspecific, but sensitive substrate, 2,2′-azinobis-(3-ethylbenzothiazoline- 6-sulphonate) diammonium salt. Nafion perfluorinated, 5% in alcohol, rosmarinic acid, potassium chloride, sodium citrate, sodium and potassium phosphate salts (for buffer preparation), methanol, ethanol, 2,2′-azinobis-(3-ethylbenzothiazoline- 6-sulphonate) diammonium salt were purchased from Sigma-Aldrich. Phosphate buffer system was selected as the buffer solution (pH 4.50), 0.1 mol/L prepared in Milli-Q ultra pure water. *Salvia maxima*, *Salvia verde* extracts, mother cells and synchronised cells were provided by CRA-FSO Research Unit for Floriculture and Ornamental Plants, Sanremo, Italy.

Apparatus

For electrochemical determinations a PG580 Uniscan potentiostat (Uniscan Instruments Ltd., Buxton, United Kingdom) was used, the electrochemical cell being DropSens 110 C-working electrode carbon screen-printed (WE: C-SPE). The UV–vis spectrophotometric measurements were carried out on V 230 Jasco spectrometer (JASCO Germany GmbH, Gross-Umstadt, Germany).

Chromatographic determinations were carried out on a LC-DAD/MS system (Shimadzu, Kyoto, Japan) consisting of Shimadzu liquid chromatography pumps LC-20ADsp, equipped with an analytical RP-18 column (Synergy Fusion, 250 × 4 mm i.d., 3 mm; Merck), a Shimadzu SPD-M20A photodiode array detector and an ion trap Shimadzu LC-MS 2010 mass spectrometer. The ESI (electrospray ionisation) mode was employed for polyphenols analysis. Specific mass tuning was performed prior to HPLC-MS analysis using appropriate mass standards.

Sample preparation

Depending on the type of sample analysed (*Salvia* cell suspension or *Salvia* callus), different sampling steps were used:

(1) Fresh plant material – cell suspensions, the optimum sample treatment was the following: a mixture of 1.5:1 (v/v) cell suspension:extraction solvent (ethanol:water (70:30, v/v)) was obtained, then a mechanical cell crush was performed; the crushed mixture was vigorously vortexed for 10 min, followed by a complex filtration on a Polytetrafluoroethylene filter (PFTE) 0.45 μm.
(2) Fresh plant material – callus, a ratio volume of 300:1 plant material: extraction solvent (ethanol 70%) was obtained and followed by mechanical crushing of the plant material; the crushed mixture was vigorously vortexed for 10 min, followed by a complex filtration on a PFTE filter 0.45 μm.

After accomplishing these steps, the filtered extract was diluted to 1:5 (v/v) with buffer (pH 4.50), followed by a second dilution with buffer, corresponding to a 1:1 (v/v) ratio. These multiple dilution steps were necessary in order to avoid laccase inhibition by ethanol. The dilution obtained was further analysed using the laccase–nafion-based biosensor.

Electrode modification and electrochemical measurements

The laccase–nafion-based biosensor was developed according to a previously reported method (Litescu *et al*., 2010). Briefly, a stock solution of known activity of laccase was prepared and 3 μL of solution were drop-casted onto the working electrode of DropSens carbon screen-printed electrodes and then allowed to dry with forced air. Nafion 0.1% (v/v) was used as an immobilisation membrane. Finally, the resulting biosensor had 300 mIU/electrode of laccase and 0.1% nafion. The biosensors were kept at 4°C for 12 h prior to the first use and stored on a desiccating layer, at the same temperature, based on the previously proved storage stability (to avoid enzyme leakage due to surface biosensor moistening).

Chronoamperometric measurements were carried out in a 0.1 mol/L phosphate buffer (pH 4.50), the applied working potential being −30 mV versus an Ag/AgCl reference electrode (the value of the applied potential was optimised as reported elsewhere; Diaconu *et al*., 2010). It should be mentioned that the biosensor response with respect to polyphenols accumulation is an important step in deciding the elicitation process sequences. When an *in vitro* culture is stressed (either by light or by chemical elicitors) it is expected to achieve a higher synthesis rate with respect to a certain secondary metabolite. According to the level of concentration reached in polyphenolic secondary metabolites the elicitation process may continue or stop. The use of a simple analytical tool as a biosensor provides the information about accumulated polyphenolic content in a few minutes, making the decision on the elicitation sequences easier.

In our case the *Salvia officinalis* cultures were stressed by light and the secondary metabolite of interest was rosmarinic acid. Accordingly, rosmarinic acid was used as a significant substrate because it is the major component found in the samples analysed (data were confirmed by chromatography and mass spectrometry screening). Consequently, all results were addressed as total phenolic content and expressed as rosmarinic acid equivalent content (RAEC).

HPLC–DAD–ESI/MS analysis

The determination of rosmarinic acid content from two types of *Salvia*, namely *S. maxima* and *S. verde*, was performed by HPLC and enzyme biosensor. The *Salvia* sample was extracted in methanol, filtered (0.45 μm PTFE filter) and injected (20 mL) into the LC –DAD –ESI/MS system. The mobile phase consisted of 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B), and the linear gradient applied was: 0–15 min

10–75% B and then conditioning to the initial step 10% B for 10 min. The flow rate was 0.2 mL/min with the analysis performed at 22°C, the analytical method being fine-tuned on negative ion mode. The calibration was performed using rosmarinic acid solution (peak area versus concentration). The amount of polyphenolic content from real samples was calculated by interpolation of the peak area to the calibration graph, using the peak ascribable to rosmarinic acid according to the specific retention time because, as previously mentioned, the main accumulated secondary metabolite was rosmarinic acid.

Results and Discussion

As previously stated, the aim of this work was to determine total polyphenolic content from *in vitro* cultivated plants using a previously developed laccase–nafion-based biosensor and to compare the results with the those obtained using the LC–DAD–ESI/MS method. The detection principle of polyphenols using a laccase biosensor is given in Fig 1.

Analytical performance of the laccase–nafion-based biosensor

Rosmarinic acid is used as the phenolic substrate of interest, due to the fact that cell cultures were submitted to an UV highlight elicitation procedure in order to attempt the normalisation of the polyphenolic content to rosmarinic acid. In order to achieve the highest sensitivity of the laccase-based biosensor for rosmarinic acid determination, pH 4.50 and the applied potential of −30 mV versus Ag/AgCl were selected as optimal parameters for the subsequent experiments. The optimised biosensing performance for rosmarinic acid is given in the Table 1.

The Michaelis–Menten kinetic analysis was carried out using the Lineweaver–Burk plot, and the K_m^{app} value obtained indicated a good bioaffinity of the immobilised enzyme to rosmarinic acid.

The operational stability of the developed biosensor response was calculated by 10 repeated measurements on the same day, using the same electrode in the optimum working conditions previously established and in the presence of the same concentration of rosmarinic acid (Fig 2). A relative standard deviation of 4.3% was obtained for the first eight measurements, followed by a decrease in current intensity for the last two measurements.

Long-term stability of the biosensor was assessed by taking repeated measurements every 2 weeks in the optimised

Table 1. Analytical performance characteristics

Equation of calibration curve	$Y = (43.97 \pm 5.43)\ X + (7.45 \pm 1.82)$
	$Y = nA$; X = [rosmarinic acid] (μmol/L)
Linear range	1×10^{-6}–10^{-5} mol/L
Response time	100 s
K_m	8.3×10^{-6} mol/L
LOD	7.5×10^{-7} mol/L (calculated as 3 S/N)

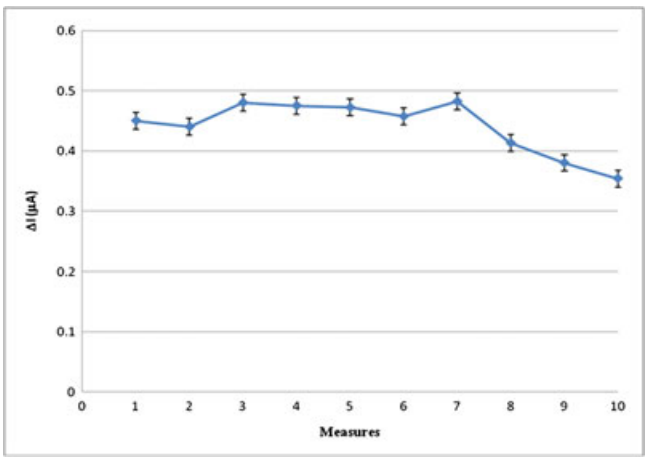

Figure 2. Operational stability of proposed biosensor using 10^{-5} mol/L rosmarinic acid in 0.1 mol/L phosphate buffer pH 4.50.

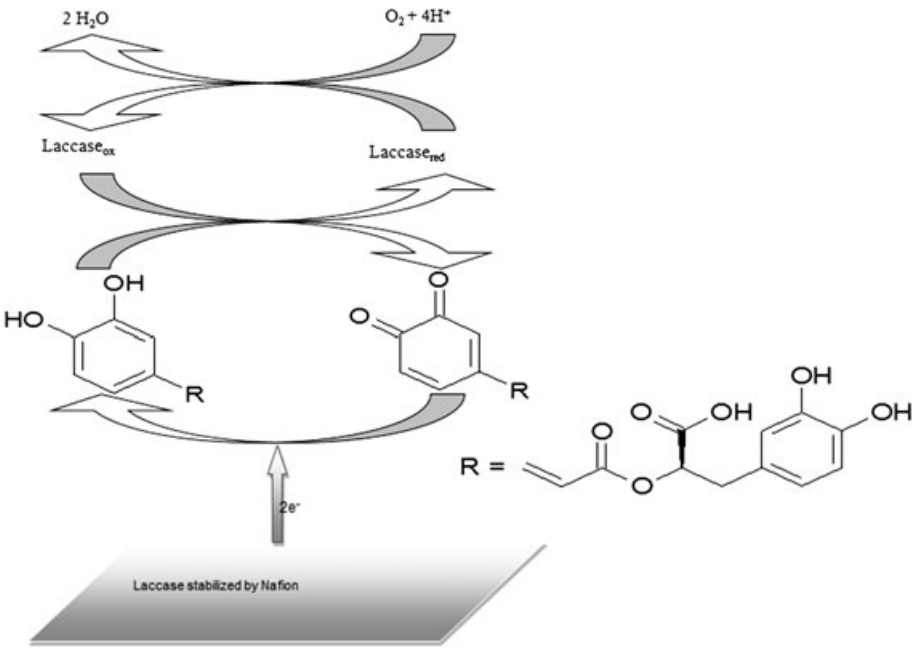

Figure 1. Schematic representation of the reaction involving rosmarinic acid and laccase immobilised onto the carbon screen-printed electrodes and stabilised by a nafion membrane.

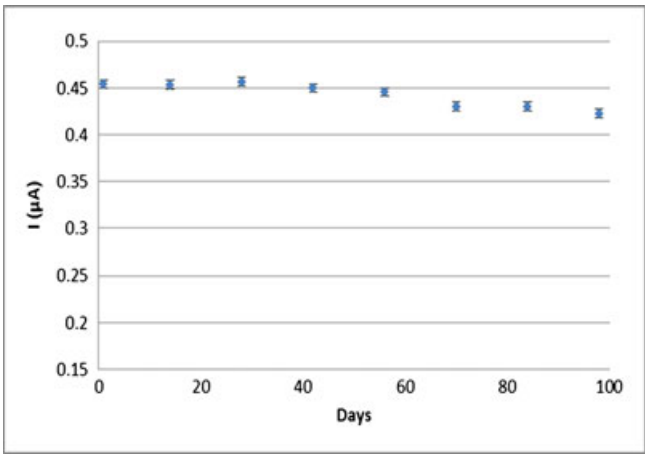

Figure 3. Life-time response of laccase–nafion-based biosensor for 10^{-5} mol/L rosmarinic acid in 0.1 mol/L phosphate buffer pH 4.50.

conditions, using the same concentration of rosmarinic acid, and storing the biosensor at 4°C. Fig 3shows the evolution of the response with time up to 90 days. It can be seen that over this period the biosensor maintains more than 85% of its initial response.

The developed biosensor reports appropriate sensitivities for phenolic secondary metabolites analysis from *in vitro* cultivated plants, even providing a high signal resolution, since it is free of chemical and electrochemical interferences. The linearity range, limit of detection (LOD) and Michaelis–Menten apparent constant are in agreement with the values reported in recent publications (Yi *et al*., 2001; Carvalho *et al*., 2003; Gomes *et al*., 2004; Santhiago *et al*., 2008; Franzoi *et al*., 2009).

Application for synchronised cell cultures

In order to prove biosensor practical suitability, the laccase-based biosensor was applied for the determination of the content of secondary metabolites expressed as rosmarinic acid equivalent content in suspension cultures. Determinations were performed both in mother cells (mother c.c.) and synchronised cells. Two methods were used to obtain synchronised cell culture suspension: without vacuum (synchr. cells) and with vacuum (vacuum synchr.). The cell culture suspensions without vacuum were synchronised on 500 μm via a specific size filter. The cell culture suspension named 500 μm-1 was obtained by direct filtration between mother cells and specific synchronised cells using the difference of pressure between the two containers (without vacuum applied), while the suspension named 500 μm-2 was obtained in the same way but from the 1:1 (v/v) dilution of mother cells suspension. Samples are collected via a derivative loop from the main containers with specific synchronised cell suspensions.

Determinations were performed in triplicate using three different samples, at initial moment ($t = 0$). The main part of the cells were synchronised at 500 μm via a specific size filter. The results obtained are given in the Table 2, proving biosensor suitability to polyphenolic metabolites content determination from cell suspensions.

A decrease in the polyphenolic content for synchronised cells can be observed in only 70% of mother cells, the rest proved a random response with respect to the main group. When vacuum was applied to synchronise the cell it is believed that some cells died during the process, thus the obtained low RAEC value. As can be noted from the CV (coefficient of variance) values the biosensor response is characterised by a good precision, and repeatability. Proving these two qualities on real samples is very important for industrial applications of the built biosensor, since

Table 2. Determination[a] of metabolite content from suspensions cell cultures using the laccase–nafion biosensor

Sample	Average concentration equivalent of rosmarinic acid (mol/L)	Coeffient of variation (CV)	Amount of polyphenolic content, RAEC (μg/g fresh material)
Mother c.c	8.19×10^{-2}	6.84×10^{-2}	29.56
Synchrised cells, 500 μm-1	7.74×10^{-2}	3.59×10^{-1}	27.92
Synchronised cells, 500 μm-2	3.97×10^{-2}	4.77×10^{-1}	14.35
Vacuum synchronised	2.07×10^{-2}	1.06×10^{-1}	7.46

[a]Determinations were performed in triplicate.

Table 3. Determination[a] of polyphenolic secondary metabolites in two callus samples

Method used	Limit of detection (mol/L)	Limit of quantification (10 × S/N) (mol/L)	Amount of polyphenolic content, RAEC (μg/g fresh material)	
			S. maxima	*S. verde*
HPLC–DAD–MS	3.4×10^{-7}	1.1×10^{-6}	103 ± 2	174 ± 2
Laccase–nafion biosensor	4.2×10^{-7}	1.3×10^{-6}	97.8 ± 8.2	162.2 ± 11.3

[a]Determinations were performed in triplicate.

the reliability of industrial application is strictly related to the robustness and accuracy of analytical tools used in cell growth monitoring. Moreover, as the final response with respect to polyphenol accumulation is provided in a maximum of 20 min, supporting a quick decision on the elicitation process, the use of biosensors in industrial application may be considered as a valid alternative for cell growth monitoring.

In addition, the validation of the biosensor response can be supported by comparing data obtained when the laccase biosensor was used in the determination of polyphenolic metabolites content with those obtained by HPLC–DAD–ESI/MS for two types of *Salvia* callus, *S. maxima* and *S. verde* provided by CRA-Sanremo, and given in the Table 3. The RAEC values obtained using the laccase biosensor are in agreement with those obtained by HPLC, the difference being ascribed to the fact that laccase is an enzyme with broad specificity. The similarity of the results was to some extent expected, because the growth elicitation was driven toward specific synthesis of rosmarinic acid. Fig 4depicts an example of results obtained via HPLC analysis.

Interferences study

As the applied potential is very close to 0, namely −30 mV versus Ag/AgCl, the electrochemical interferences are significantly eliminated. However, the growth media has been tested because the laccase biosensor is intended to be used in several possible applications where chemical interferences caused by the growth media could be a major drawback, namely in the growth phases of callus and suspension cultures. It is important to assess the possible interferences occurring because the extraction process may not avoid the passing of hydrosoluble components of the cultivation media used to the extract. Moreover, if the elicitation is not performed by light stress, but chemically, then an instrument able to respond fast, reliably and free of potential chemical interferences is desirable for large-scale measurement applications. Therefore, a mixture containing components of the growth media (sugars, thiamine, mineral salts, and growth elicitors) was tested first and a decrease of 10% from the biosensor response was determined (Fig 5). This was followed by the study of the interferences from a 'Murashige and Skoog' media (the media where the plants were cultured) and the study led to the conclusion that the biosensor is free from any chemical interferences in the experimental conditions tested. Since the decrease of the biosensor response with 10% was proved for more than 10 measurements, being a systematic error, the registered bias may be overcome by calculation. The current intensity value of the culture media is considered to be the steady-state current intensity, and the significant intensity value is the difference between the current intensity obtained for the cell suspension and the current intensity obtained for the culture media.

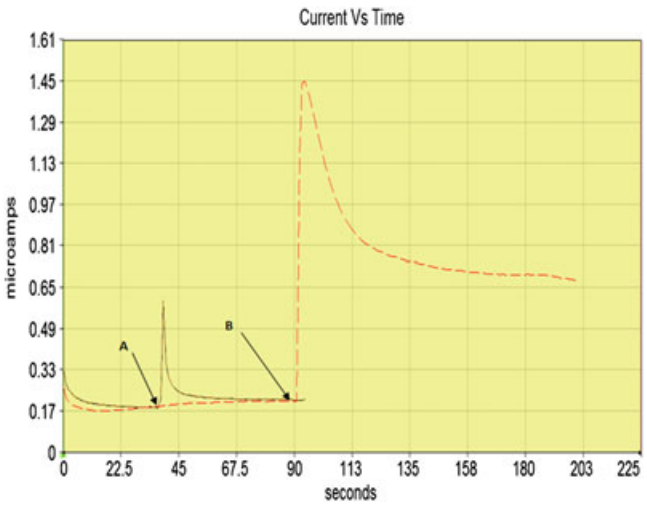

Figure 5. Interferences study. (A) Injection of components of the growth media (sugars, thiamine, mineral salts and growth elicitors). (B) Injection of 10^{-5} mol/L rosmarinic acid in 0.1 mol/L phosphate buffer pH 4.50.

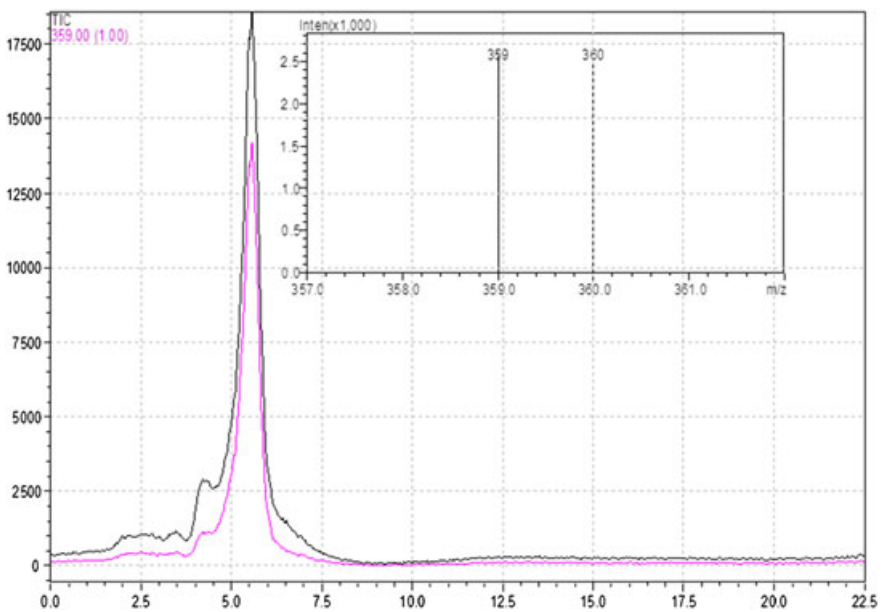

Figure 4. Mass spectrometry chromatogram (inset MS spectrum) for *Salvia verde* extract.

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References

Balsano C, Alisi A. 2009. Antioxidant effects of natural bioactive compounds. *Curr Pharm Design* **15**: 3063–3073.

Brondani D, Zapp E, Vieira IC, Dupont J, Scheeren CW. 2011. Gold nanoparticles in an ionic liquid phase supported in a biopolymeric matrix applied in the development of a rosmarinic acid biosensor. *Analyst* **136**: 2495–2505.

Cahoon EB, Hall SE, Ripp KG, Ganzke TS, Hitz WD, Coughlan SJ. 2003. Metabolic redesign of vitamin E biosynthesis in plants for tocotrienol production and increased antioxidant content. *Nat Biotechnol* **21**: 1082–1087.

Carvalho RM, Kubota LT, Rath S. 2003. Influence of EDTA on the electrochemical behavior of phenols. *J Electroanal Chem* **548**: 19–26.

Chen G, Luo J. 2010. Anthocyanins: Are they beneficial in treating ethanol neurotoxicity? *Neurotox Res* **17**: 91–101.

Das K, Tiwari RKS, Shrivastava DK. 2010.Techniques for evaluation of medicinal plant products as antimicrobial agent: Current methods and future trends. *J Med Plants Res* **4**: 104–111.

Di Fusco M, Tortolini C, Deriu D, Mazzei F. 2010. Laccase-based biosensor for the determination of polyphenol index in wine. *Talanta* **81**: 235–240.

Diaconu M, Litescu SC, Radu GL. 2010. Laccase–MWCNT–chitosan biosensor – A new tool for total polyphenolic content evaluation from *in vitro* cultivated plants. *Sensor Actuat B – Chem* **145**: 800–806.

Diaconu M, Litescu SC, Radu GL. 2011. Bienzymatic sensor based on the use of redox enzymes and chitosan–MWCNT nanocomposite. Evaluation of total phenolic content in plant extracts. *Microchim Acta* **172**: 177–184.

Franzoi AC, Dupont J, Spinelli A, Vieira IC. 2009. Biosensor based on laccase and an ionic liquid for determination of rosmarinic acid in plant extracts. *Talanta* **77**: 1322–1327.

Gamella M, Campuzano S, Reviejo AJ, Pingarron JM. 2006. Electrochemical estimation of the polyphenol index in wines using a laccase biosensor. *J Agric Food Chem* **54**: 7960–7967.

Gil DMA, Falé PLV, Serralheiro MLM, Rebelo MJF. 2011. Herbal infusions bioelectrochemical polyphenolic index: Green tea – the gallic acid interference. *Food Chem* **129**: 1537–1543.

Gomes SASS, Nogueira JMF, Rebelo MJF. 2004. An amperometric biosensor for polyphenolic compounds in red wine. *Biosens Bioelectron* **20**:1211–1216.

Helmja K, Vaher M, Püssa T, Kaljurand M. 2009. Analysis of the stable free radical scavenging capability of artificial polyphenol mixtures and plant extracts by capillary electrophoresis and liquid chromatography–diode array detection–tandem mass spectrometry. *J Chromatogr A* **1216**: 2417–2423.

Kochana J, Nowak P, Jarosz-Wilkołazka A, Bieroń M. 2008. Tyrosinase/laccase bienzyme biosensor for amperometric determination of phenolic compounds. *Microchem J* **89** : 171–174.

Komes D, Belščak-Cvitanović A, Horžić D, Rusak G, Likić S, Berendika M. 2011. Phenolic composition and antioxidant properties of some traditionally used medicinal plants affected by the extraction time and hydrolysis. *Phytochem Anal* **22**: 172–180.

Litescu SC, Eremia SAV, Radu GL. 2010a. Methods for the determination of antioxidant capacity in food and raw materials. In: *Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors*. Book Series: Advances in Experimental Medicine and Biology Bio-Farms for Nutraceuticals: Functional Food and Safety Control by Biosensors, Vol. **698**. Landes Bioscience: Austin, Texas; 241–249.

Litescu SC, Eremia SAV, Bertoli A, Pistelli L, Radu G-L. 2010b. Laccase–nafion based biosensor for the determination of polyphenolic secondary metabolites. *Anal Lett* **43**: 1089–1099.

Montereali MR, Seta LD, Vastarella W, Pilloton R. 2010. A disposable laccase–tyrosinase based biosensor for amperometric detection of phenolic compounds in must and wine. *J Mol Catal B: Enzym* **64**: 189–194.

Mousty C, Vieille L, Cosnier S. 2007. Laccase immobilization in redox active layered double hydroxides: A reagentless amperometric biosensor. *Biosens Bioelectron* **22**: 1733–1738.

Paixão N, Perestrelo R, Marques JC, Câmara JS. 2007. Relationship between antioxidant capacity and total phenolic content of red, rosé and white wines. *Food Chem* **105**: 204–214.

Peng C-H, Su J-D, Chyau C-C, Sung T-Y, Ho S-S, Peng C-C, Peng RY. 2007. Supercritical fluid extracts of rosemary leaves exhibit potent anti-inflammation and anti-tumor effects. *Biosci Biotech Bioch* **71**: 2223–2232.

Petersen M, Simmonds MSJ. 2003. Rosmarinic acid. *Phytochemistry* **62**: 121–125.

Ruffoni B, Raffi D, Rizzo A, Oleszek W, Giardi MT, Bertoli A, Pistelli L. 2010. Establishment of *in vitro Salvia* cell biomass for the controlled production of antioxidant metabolites. *Acta Hort* **829**: 423–427.

Santhiago M, Peralta RA, Nevesb A, Mickea GA, Vieira IC. 2008. Rosmarinic acid determination using biomimetic sensor based on purple acid phosphatase mimetic. *Anal Chim Acta* **613**: 91–97.

Viñas P, Campillo N, Martínez-Castillo N, Hernández-Córdoba M. 2009. Solid-phase microextraction on-fiber derivatization for the analysis of some polyphenols in wine and grapes using gas chromatography–mass spectrometry. *J Chromatogr A* **1216**: 1279–1284.

Xu X, Guo M, Lu P, Wang R. 2010. Development of amperometric laccase biosensor through immobilizing enzyme in copper-containing ordered mesoporous carbon (Cu-OMC)/chitosan matrix. *Mat Sci Eng C-Mater* **30**: 722–729.

Yamada Y, Yasui H, Sakurai H. 2006. Suppressive effect of caffeic acid and its derivatives on the generation of UVA-induced reactive oxygen species in the skin of hairless mice and pharmacokinetic analysis on organ distribution of caffeic acid in ddY mice. *Photochem Photobiol* **82**: 1668–1676.

Yi H, Wu K, Hu S, Cui D. 2001. Adsorption stripping voltammetry of phenol at Nafion-modified glassy carbon electrode in the presence of surfactants. *Talanta* **55**: 1205–1210.

Zheng W, Wang SY. 2001. Anti-oxidant activity and phenolic compounds in selected herbs. *J Agr Food Chem* **49**: 5165–5170.