Evaluation of output signals from CoulArray detector for determination of antioxidant capacity of apricots samples

Jiri Sochor[1], Petr Babula[2], Boris Krska[1], Ales Horna[3], Ivo Provaznik[4], Jaromir Hubalek[5], Rene Kizek[3*]

*(1)Department of Breeding and Propagation of Horticultural Plants, Faculty of Horticulture, Mendel University of Agriculture and Forestry in Brno, Valticka 337, 691 44 Lednice, Czech Republic; (2) Department of Natural Drugs, Faculty of Pharmacy, University of Veterinary and Pharmaceutical Sciences, Palackeho 1-3, CZ-612 42 Brno, Czech Republic; (3)Tomas Bata University, T.G. Masaryka 275, CZ-762 72 Zlin, Czech Republic; (4)Department of Biomedical Engineering, Brno University of Technology, Kolejni 4, CZ-61200 Brno, Czech Republic; (5)Department of Microelectronics, Brno University of Technology, Udolni 53, CZ-602 00 Brno, Czech Republic * kizek@sci.muni.cz*

In this study, we are interested in possibility of determination and expression of biological value of twenty perspective genotypes of apricot (Prunus armeniaca L.) breeding in Department of Breeding and Propagation of Horticultural Plants of Faculty of Horticulture, Mendel University, with aim of resistance against Plum pox potyvirus (PPV). Work was focused on photometric determination of antioxidant activity of studied samples and on verification and validation of HPLC technique with electrochemical detector (ED) for determination of total antioxidant capacity (TAC), which may represent suitable method for comparison of biological value of fruits.

1 Introduction

In fruits, a lot of structurally different phenolic compounds with antioxidant capacity occur [4]. Presently, interest in study of these natural compounds increases, especially because of connection between their uptake in nutrition and reduction of occurrence of lifestyle diseases, such as cancer or cardiovascular diseases[9]. In many experimental studies, antimutagenic, anticancerogenic and anti-inflammatory properties of these compounds have been confirmed. A lot of epidemiological studies indicate that regular consummation of fruits and vegetables may reduce risk of occurrence of some neurodegenerative diseases[4,5,7,9]. On total uptake of polyphenolic compounds, flavonoids (about two thirds) and phenolic acids (about one third) participate; other phenolic compounds form only negligible fraction[2]. Almost all of these phenolics present in fruits demonstrate antioxidant activity[8]. It was demonstrated that antioxidant activity varies in dependence on type of phenolic compound and that some types of phenolics demonstrate higher antioxidant activity in comparison with others[1-5]. It is supposed that on protective effect participates possibility of plant polyphenolics to scavenge reactive oxygen radicals which are able to generate highly reactive hydroxyl radicals[5,7].

Due to chemical variability of compounds with antioxidant capacity present in fruits, content of individual compounds is usually not known at all. Detection of biologically active compounds in biological matrix is usually very complicated, determined quantitative values are usually different and present compound/compounds responsible for antioxidant properties are not identified both using effective separation analytical methods and instruments. In addition, level of individual antioxidants present in fruits need not necessarily correspond to total antioxidant capacity [4].

Determination of total antioxidant activity/capacity is one of the possibilities how to express biological and nutrition value of fruits[2]. Main problem in this field is represented by absence of ideal analytical method and technique. Due to fact that antioxidants very easily

participate in redox reaction, they are very suitable for electrochemical detection[8].

2 Methods

Total antioxidant capacity was monitored using HPLC technique with coulometric detection, which represents one of the most sensitive detection techniques[1-5]. Concurrently, HPLC profile of fifteen flavonoids was detected; photometric determination of antioxidant activity was used as reference for corresponding correlation to our experiment.

For experiment, twenty genotypes (cultivars) of apricot (*Prunus armeniaca* L.) were used; their selection was connected with resistance against Plum pox potyvirus. Plants were cultivated in cadastre of village Lednice, climatic area T4. Fruits were collected in a state of consumption ripeness during July and first half of August 2009 and subsequently deep-frozen at -80°C. Representative samples (10 g) were taken away from individual fruits, transferred to mortar, deep-froze by liquid nitrogen and at 4°C homogenized with methanol (10 ml, 99% - w/w). Homogenized samples were quantitatively transferred to test tubes and at same laboratory conditions shaked for 30 min with subsequent sonification and centrifugation (Eppendorf 5804R, Germany) for 30 min at 16 400 rpm. Supernatants were filtered through membrane discs (0.45 µm, Metachem, Torrance, CA, USA). Filtrate (500 µl) was pipetted and diluted with methanol (500 µl).

Fig.1:hybrid LE-9299

Fig.2:hybrid LE-2527

Fig.3:hybrid LE-985

Fig.4: hybrid LE-889

Determination of antioxidant activity using FRAP method: working FRAP solution was prepared by mixing of 10 volume parts of acetate buffer (300 mM, pH 3,6) with 1 volume part of TPTZ solution (10 mM 2,4,6- tripyridyl-S-triazine dissolved in 40 mM HCl) and with 1 volume part of $FeCl_3$ solution (20 mM). Absorbance was measured at wavelength λ=593 nm.

Determination of antioxidant activity by ABTS test: 54,9 mg of $ABTS^{\bullet}$ were dissolved in 20 ml of phosphate buffer (pH 7,0; 5 mM) and activated to cation of ABTS+ radical by addition of 1 g of MnO_2 under intermittent stirring with time of activation of 30 min. Solution was subsequently diluted by phosphate buffer to absorbance (t_0) 0,500 ± 0,01. Absorbance of solution was measured at wavelength λ=734 nm.

Determination of antioxidant activity by DPPH test: for measurement, technique according Parejo et al. was used[11]. Methanolic solution of $DPPH^{\bullet}$ with absorbance (t_0) 0,200±0,01 was prepared. Absorbance was measured at wavelength λ=515 nm.

Spectrophotometric determination of antioxidant activity: in 2 ml of solution of corresponding radical in cuvette (10 mm), absorbance was determined using automated VIS spectrophotometer (BS 200, Mindray, China) in time t_0. Reagents and samples were stored in cooled carrousel at 4 °C and automatically pipetted into plastic cuvettes (Mindray, China) and subsequently homogenized with real samples (5 µl). Changes in absorbance were measured

for 21 min at 16-second intervals from mixing of reagents at above mentioned wavelengths and optical trace 5 mm; incubation proceeded at 37°C.

HPLC-ED system consisted of two chromatographic pumps, Model 582 ESA (ESA Inc., Chelmsford, MA) (working range 0.001-9.999 ml min^{-1}) and reversed chromatographic column Zorbax SB C18 (150 × 4.6; size of particles 5 μm, Agilent Technologies, USA). For UV, UV detector Shimadzu (Model 528, ESA, USA) was used. For electrochemical detection was used twelve-channel detector CoulArray (ESA, USA) with flow analytical cell (Model 6210, ESA, USA). Flow cell contains planar glassy carbon electrode, hydrogen-palladium electrode as reference electrode and carbon electrode as auxiliary electrode. Sample was injected automatically by the use of autosampler (Model 542, ESA, USA), which has incorporated thermostatted place for column. Column was thermostated to 30°C. Output data were processed by application CSW 32 software (Version 1.2.4, Data Apex, Czech Republic).

Chromatographic conditions were optimized according to following parameters: volume of applied standard mixtures and real samples 30 μl, mobile phase A consisted of formic acid (0.2 %, v/v), mobile phase B was acetonitrile. Profile of gradient was linearly increased from 12 to 22 % for B (v/v) (0 - 20 min), to 50 % B (20 – 25 min), to 55 % (25 – 30 min). Flow rate was 0.8 $\mu l.min^{-1}$. Electrochemical detector scanned responses at potentials -80, 0, 80, 160, 240, 320, 400, 480, 560, 640, 720 and 800 mV. Resulting detection was expressed in microcoulombs.

Mathematical and statistical analysis of experimental data was carried out in package MATLAB®, Version 7.9.0.529 (R2009b).

3 Results and discussion

Due to composition of complexes of antioxidants in given biological material, it was necessary to choose set of suitable methods. A lot of authors use only one method for determination of antioxidant activity/capacity. Due to provision of objectivity of obtained results as well as for comparison of individual methods, we used concurrently four methods – method based on ability of antioxidants to extinct synthetic radicals of DPPH, FRAP technique, which is based on reduction of ferric complex to ferrous by action of antioxidants, TEAC method based on evaluation of amount of substance Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-karboxylic acid) corresponding to antioxidant activity of sample, and HPLC technique with electrochemical detection for determination of total antioxidant capacity.

Relative antioxidant activity determined photometrically was expressed as percentage of absorbance decrease and subsequently calculated to equivalent content of Trolox. Each sample was tree times evaluated with average relative error 1.23 %. In each method, calibration curve of dependence of absorbance (increase/decrease) on concentration of Trolox was constructed.

For determination of total antioxidant capacity, HPLC-ED technique with gradient elution was used for determination of flavonoids in plant samples. Based on differences of signal areas, comparative series of determined fruits, where differences in contents of antioxidant flavonoids are expressed as relative % and interpreted as relative antioxidant capacity. We also determined total antioxidant capacity of fruits of individual apricot cultivars related to content of major represented antioxidant compounds; quantification of present antioxidants with their participation on TAC was also possible.

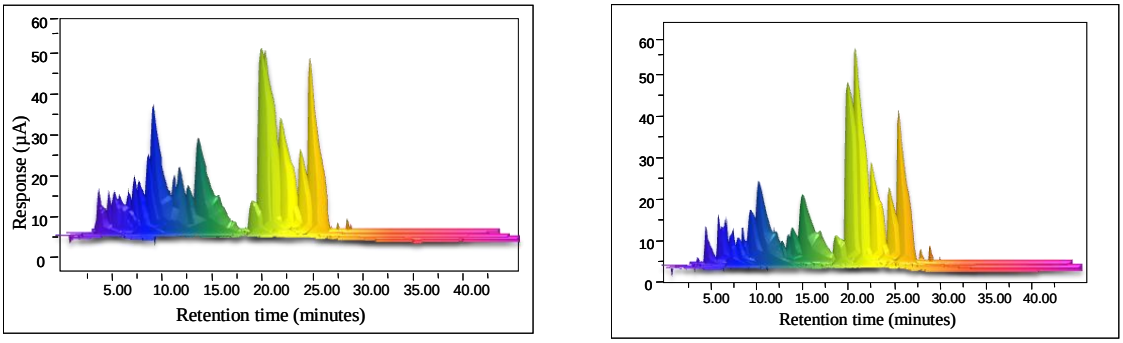
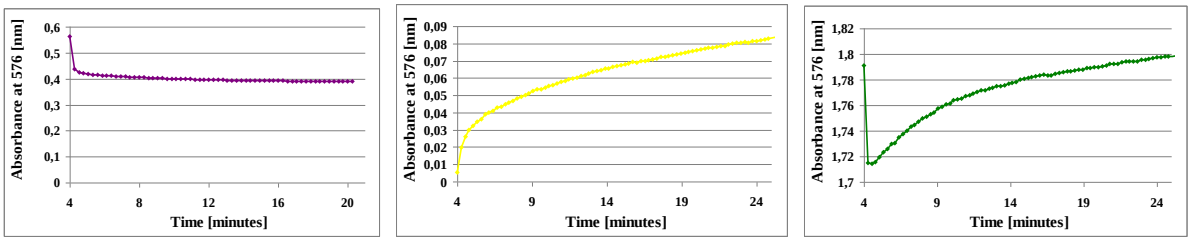


Fig. 5: Output of CoulArray detector with twelve working electrodes in the case of cultivars LE-9299 and LE-859.

Coulometric detection, especially in multichannel modification, brings many advantages in comparison with common amperometric arrangement[1-5]. HPLC/ED enabled monitoring of reaction kinetics at applied potential, evaluation of structures of antioxidant complexes and their total antioxidant capacity. Values TAC obtained using HPLC-ED were in correlation with photometric determination in the case of TEAC (r = 0.91), and also DPPH test (r = 0,89).

It is well known that compounds without electrochemical activity do not demonstrate antioxidant activity; contrariwise compounds with low half-wave oxidation potentials have most likely antioxidant activity[8]. Antioxidant activity was expressed and percentage of decrease of absorbance of initial solution. Values of antioxidant activity varied from 0.16 to 1.30 in the case of FRAP, from 0.05 to 0.45 for TEAC and from 0.11 to 1.64 for DPPH test of mmol. l^{-1} of Trolox equivalent. Between TEAC and DPPH, significant reciprocal correlation (r = 0,964) was found.



DPPH test LE-9299 FRAP LE-9299 TEAC LE-9299

Fig. 6: Changes of absorbance at photometric determination of antioxidant activity in the case of LE-9299 cultivar.

Our results demonstrate variation of antioxidant capacity in dependence on type of phenolic compounds present in fruits, as well as variation between individual types of phenolic compounds – some types of phenolics demonstrate higher antioxidant in comparison with others[10]. It is supposed that on protective effect participates possibility of plant polyphenolics to scavenge reactive oxygen radicals which are able to generate highly reactive hydroxyl radicals[4-7]. Due to direct connection between antioxidant activity/capacity and ability of compound to be oxidized/reduced, it means due to ability to provide signal in electrochemical detection[5,8], connection of selective and very sensitive electrochemical detection with high performance liquid chromatography (HPLC-ED) enabling simultaneous separation of many constituents in one run, represents ideal analytical tool for solving of these very difficult questions.

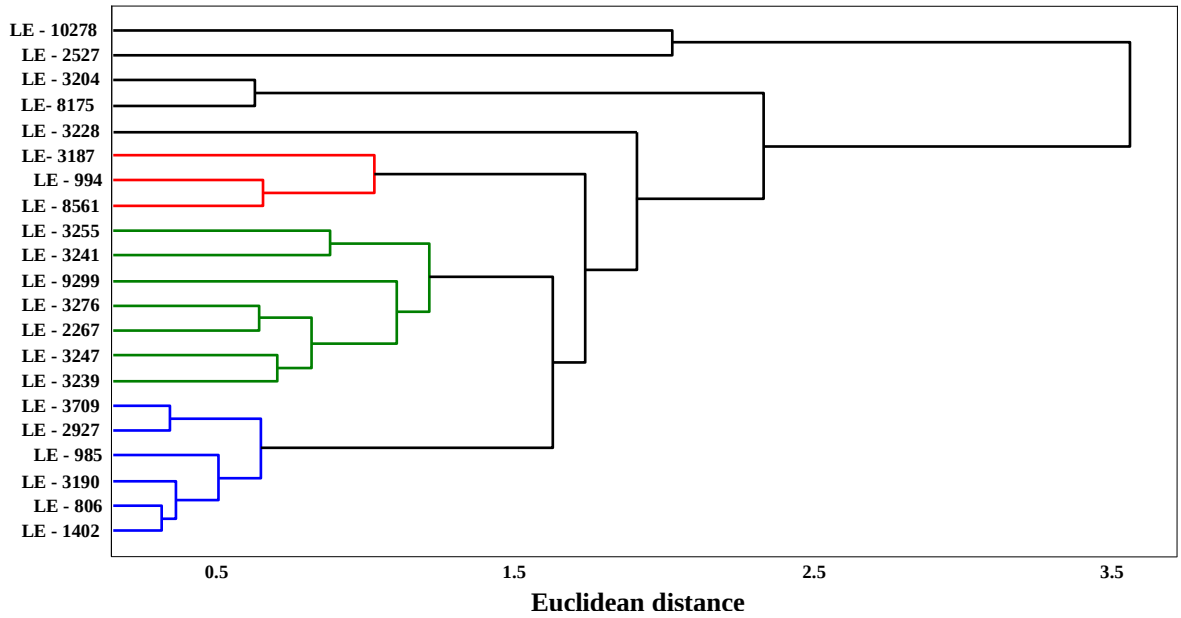


Fig. 7: Dendrogram of hierarchical cluster of 15 antioxidants developed on base of nearest neighbor method related to values of antioxidant capacity.

At determination of antioxidant capacity of 21 apricot genotypes, we were aimed at fact, whether this set can be divided into sub-sets, which would be internally homogenous, but respectively heterogenic. Due to this reason, we used hierarchical cluster analysis. For clustering, classical Euclid's metrics was used; for calculations of new clusters, nearest neighbor method was used. The help of Cophenetic Correlation Coefficient (CPCC) can express consequent conformity between characteristics of objects and final clustering process. There is connection between value of this coefficient and loss of information rising in process of aggregation. In practice, value of $CPPC \geq 0.8$ is considered to be sufficient. In our case, *CPPC* was 0,924, which means that distortion caused by dendrogram is acceptable. Output of this analysis consists in fact that 21 apricot cultivars can be divided into four groups.

4 Conclusions

We developed suitable separation procedure and technique for determination of total antioxidant capacity in newly cultivated apricot cultivars. Antioxidant activity/capacity of our samples was determined, namely by the use of four different methods. Resulting correlation coefficients demonstrated statistically significant relation between antioxidant properties of monitored apricot cultivars. This correlation was achieved due to optimization of separation techniques and detection methods with a view to identification and quantitative determination of targeted molecules – flavonoids. Due to combination of features of individual techniques, we demonstrated relations between antioxidant characteristics in monitored apricot cultivars.

One of the quite factual outputs utilizable in practise will be registration of perspective cultivars with respect to their phenolic profile, which is expressed by antioxidant characteristics.

5 Acknowledgement

This work was financially supported by grants NAZV 91A032 a GAČR 102/08/1546

References

1. Adam V., Mikelova R., Hubalek J., Hanustiak P., Beklova M., Hodek P., Horna A., Trnkova L., Stiborova M., Zeman L., Kizek R. 2007. Utilizing of Square Wave Voltammetry to Detect Flavonoids in the Presence of Human Urine. *Sensors*; 7(10):2402-2418.
2. Beklová, M., Zítka, O., Gazdík, Z., Adam, V., Hodek, P., Stiborová, M., Horna, A., Kizek, R., 2008. Electroanalytical techniques for determination of flavonoids. *Toxicology Letters.* 2008. sv. 180, č. 1, s. 230. ISSN 0378-4274.
3. Diopan, V.; Babula, P.; Shestivska, V.; Adam, V.; Zemlicka, M.; Dvorska, M.; Hubalek, J.; Trnkova, L.; Havel, L.; Kizek, R. 2008. Electrochemical and spectrometric study of antioxidant aktivity of pomiferin, isopomiferin, osajin and catalposide. *J. Pharm. Biomed. Anal.* 2008, *48*, 127-133.
4. Gazdík, Z., Krška, B., Adam, V., Saloun, J., Řezníček, V., Horna, A., Kizek, R. 2008. Electrochemical determination of antioxidant potential of less common fruit species. *Sensors.* 2008. sv. 8, č. 11, s. 7564-7570. ISSN 1424-8220.
5. Gazdík, Z., Řezníček, V., Adam, V., Zítka, O., Krška, B., Matuskovic, J., Plšek, J., Saloun, J., Horna, A., Kizek, R. 2008. Utilizing of liquid chromatography with electrochemical detection for determination of antioxidants in less common. *Molecules*. 2008. sv. 13, č. 11, s. 2823-2836. ISSN 1420-3049.
6. Gillman M W, Cupples L A, Gagnon D, Posner B M, Ellison R C, Castelli W P, Wolf P A, 1995. Protective Effect of Fruits and Vegetables on Development of Stroke in Men. *JAMA* 1995, 273, 1113
7. Hodek P., Hanuštiak P., Křížková J., Mikelová R., Křížková S., Stiborová M., Trnková L., Horna A., Beklová M., Kizek R., 2006 Toxicological Aspects of Flavonoid Interaction with Biomacromolecules. *Neuroendocrinology letter*. 2006. sv. 27, č. 2, s. 14--17. ISSN 0172-780X.
8. Jirovský D, 2007. Vysokoúčinné separační techniky v analýze fyziologicky významných látek, Habilitační práce, PFUP, Katedra analytické chemie, Olomouc 2007, 14
9. La Vecchia C, Altieri A, Tavani A, 2001. Vegetables, fruit, antioxidants and cancer: a review of Italian studies, *European journal of nutrition* 2001, 40, 261
10. Rice-Evans, C. A.; Miller, N. J.; Paganga, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20:933–956; 1996.
11. Parejo I, Codina C, Petrakis C, Kefalas P. 2000. Evaluation of scavenging activity assessed by Co(II)/EDTA-induced luminol chemiluminescence and DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical assay. *J Pharmacol Toxicol Meth*. 2000;44:507-512.