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CoulArray® Electrochemical Evaluation of Tocopherol and Tocotrienol Isomers in Barley, Oat and Spelt Grains

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This paper is dedicated to Professor Franco F. Vincieri for his 70th birthday.

Hexane extracts obtained from *Hordeum vulgare* L. (barley), *Avena sativa* L. (oat), *Triticum spelta* Schrank and *Triticum dicoccum* Schrank ex Schübler (spelt or emmer) whole grains, were examined for their tocochromanol (tocopherol and tocotrienol) content. The analyses were carried out on fatty extracts by means of HPLC coupled with a coulometric array electrochemical detector (ECD). Due to the specific high selectivity of the detector, the sample can be directly injected without any preliminary treatment (*e.g.* saponification). Eight tocochromanol isomers have been detected in barley grains. Different barley cultivars examined showed a tocochromanol content ranging from 1620 to 1852 ng/g caryopses. Oat grains contained *ca*. 45 ng/g caryopses and *Triticum* species *ca.*1070 ng/g caryopses. The results are considered in view of a potential use of vitamin E derivatives as human health enhancer and as sources of antioxidants for food lipid preservation.

Keywords: cereals, tocopherol, tocotrienol, vitamin E.

Cereal grains contain minor components, considered positive "nutrients" for human health. "Nutrients" may include a variety of minor food constituents such as vitamin E, carotenoids, flavonoids. In cereal grains minor components are mainly vitamin E derivatives. Natural tocochromanols (known as vitamin E) comprise two homologous series: the tocopherols with a saturated side chain, and the tocotrienols with an unsaturated side chain. The tocochromanol vitamin E homologues with the largest diffusion in nature are four tocopherols and four tocotrienols: α, β, γ and δ-tocopherol and α, β, γ and δ-tocotrienol [1]. A very high number of vitamin E publications have appeared over the past 40 – 50 years, but many literature data are specific for α-tocopherol. The other forms of natural vitamin E remain poorly understood. The abundance of α-tocopherol in the human body and the comparable efficiency of all vitamin E molecules as antioxidants led to neglect the non-tocopherol vitamin E molecules as topics for basic and clinical research. α-Tocotrienol, γ-tocopherol, and δ-tocotrienol have emerged as vitamin E molecules with functions in health and disease that are clearly distinct from that of α-tocopherol. At nanomolar concentration, α-tocotrienol, not α-tocopherol, prevents neurodegeneration [2].

Antioxidants protect key cell components by neutralizing free radicals before they can cause lipid oxidation or DNA damage. By reducing free radical attack, antioxidants break the chain reaction of lipid peroxidation (*chain-breaking antioxidant*) and they protect the cell membranes by lipid repair and lipid replacement. Epidemiological evidences indicate that diet-derived antioxidants, *e.g.*, vitamins A, C, and E, may be important in maintaining human and animal health [3]. More recent researches demonstrated that tocotrienols play a specific role which goes beyond their known vitamin E antioxidant activity [4a,4b]. Tocotrienols in animal cells inhibit cholesterol biosynthesis due to the 3 hydroxy-3-methyl glutarylCoA reductase enzyme (HMGR) suppression [4c,4d]. Tocotrienols possess antiproliferative and

apoptotic activities on normal and cancer human cells [4e,4f].

Currently tocochromanol analysis is undertaken by liquid chromatography (normal or reverse phase) usually coupled with UV (*e.g.* 292 nm) and/or a fluorescence detector (usually Exc = 295 nm, Em = 352 nm) at fixed wavelength or electrochemical detector at fixed oxidation / reduction potential [5]. In previous works we evaluated the vitamin E components in cereal grains using HPLC chromatographic separation coupled with different detectors. A fixed mode of detection appears not to be appropriate for the evaluation of the natural tocopherol and tocotrienol stereoisomers from plant material [6].

The method described below was developed around RP-HPLC coupled with a multichannel coulometric (or array) electrochemical detector (ECD) utilizing multiple sensors. Experimental resolution is increased by pre-screening of possible interfering solutes *via* their redox behavior. In a coulometric detector the carrier passes through a porous electrode, resulting in a large reactive surface and hence almost complete oxidation/ reduction that radically improves the sensitivity of the detector. The development of the coulometric electrode array detector constituted a major step towards improved selectivity and versatility. This method allows quantification of the tocochromanol in the crude extract, without pre-treatment of the sample (*e.g.* saponification) with good recovery and reproducibility.

Tocopherol and tocotrienol components were evaluated in barley (*Hordeum vulgare* L.) and oat (*Avena sativa* L.) whole grains : two cereals largely employed as human and animal food. The tocopherol and tocotrienol content was also monitored in spelt grains: *Triticum spelta* Schrank and *Triticum dicoccum* Schrank ex Schübler [syn. *Triticum turgidum* L. subsp. *dicoccon* (Schrank) Thell] two species of *Triticum* genre used by ancient Romans as main source of starch but is now being reintroduced in the mediterranean area as a nutraceutical.

The plant material was processed in order to obtain the oily fraction. *A. sativa* had a higher lipid content (1.3 - 1.9% ± 0.021 dry wt.) than *H. vulgare* or *T. spelta* and *T. dicoccum* (0.75 - 0.90 % ± 0.011 dry wt.).

The raw oily fraction was monitored for its tocopherol and tocotrienol content. A RP-HPLC method coupled with an electrochemical coulometric array detector was developed. The array of electrodes facilitated determination of the optimal range of the applied voltage for the electrochemical detection. In each analysis eight chromatograms at eight cell potentials, were simultaneously monitored. The phenolic functional group can almost always be oxidized by an electrochemical HPLC detector within the usable potential range employing a porous graphite electrode at pH ranges of the mobile phases normally associated with reverse phase chromatography. Tocopherols and tocotrienols exhibited the same electrochemical characteristics and reached a maximum response at 400 mV voltage (electrode N° 3). The simultaneous separation and detection of tocopherols and tocotrienols was achieved. Tocotrienol standards are found to be composed of two isomers, at least, that may be chromatographically separated and revealed by electrochemical detector as, *i.e.*, α-tocotrienol-(stereoisomer form 1) and α-tocotrienol-(stereoisomer form 2) : that means that standard tocotrienols are synthetic compounds. It is noteworthy that the multi-electrode electrochemical detection method can discriminate between tocochromanol isomers inside the plant extract in comparison with the standard isomers. Two analytes may be separated due to differing redox properties even though they present similar chromatographic properties. The plants studied appear to produce only one α-tocotrienol-(stereoisomer form 2), only one β-tocotrienol form. Barley, oat and spelt produce only the stereoisomer form 2 as defined by the CoulArray® detector. It is noteworthy that the Ratio Accuracy is very high, ranging for each isomer from 0.933 and 1.00 (** $P < 0,01$), either for the standard and for plant extract. The separation of two positional isomers, γ- and δ- homologues proved to be problematic due to the same substituents on the chroman ring both for tocopherols and tocotrienols.

The method's linearity was tested *via* calibration with five concentration points for each analyte. The concentration range was from 0.050 μg/mL to 10.00 μg/mL of standard tocols and each concentration level was assayed in triplicate. The calibration curves were linear with correlation coefficients ($r2$) of more than 0.995. The terms of calibration curves expressed by the formula $y = ax + b$ for each analytical are reported in Table I. The limit of quantification (LOQ) was 0.002 μg/mL for each standard compounds.

Table 1: Calibration curve data and precision of the used method.

Compounds	a ± SD	Compounds	a ± SD
α-tocopherol	0.0688 ± 0.0171	γ-tocopherol	0.0662 ± 0.0208
α-tocotrienol	0.0429 ± 0.0101	δ-tocotrienol	0.1557 ± 0.0357
β-tocopherol	0.1065 ± 0.0260	γ̃-tocopherol	0.1295 ± 0.0409
β-tocotrienol	0.2335 ± 0.0726	δ̃-tocotrienol	0.0378 ± 0.0111

y = peak height and x = concentrations (μg/mL)

The tocochromanol components were quantified as a part of the total lipid fraction. The barley cultivars produce the highest level of total tocochromanols in a range from 1620 to 1852 ng ± 1.687 /g caryopses. There appears to be wide variation in tocopherol and tocotrienol composition across the grains and in total eight tocochromanols have been detected. The α-tocotrienol-(stereoisomer form 2) and β- and γ-tocotrienol-(stereoisomer form 2) are the main components in barley cultivars.

Avena and *Triticum* species had a poor pattern of tocochromanols. In *Avena* var. "Weibull" and var. "Solva" tocochromanols were undetectable. In *Avena* var. "Angelica" and *Avena* obtained from the local market the α-, β- and γ̃- isomers either of tocopherols and tocotrienols were the main components (26 - 28 ng/g caryopses). Other tocochromanols were in negligible quantities or undetectable. *Triticum* species had only the α-, β- and γ- isomers either of tocopherols and tocotrienols. The β- and γ- isomers were the main components (697 - 731 ng ± 2.42 g/caryopses).

In conclusion, this work focused on analysis of multicomponent matrices, *i.e*. a tocochromanol crude extract, utilising CoulArray® electrochemical detection delivering improved selectivity in detecting isomeric tocochromanols. At present we can confirm that barley grains are one of the best natural source of tocochromanols. According to literature data [7a], our findings demonstrate that oat grains have low tocochromanol content. It is noteworthy that, in order to prevent lipid oxidation, the plants mainly accumulate tocochromanols in oily seeds and fruits or in young tissues with active cell divisions [7b,7c]. From an health point of view there is a great interest in the natural forms of tocochromanols, because they are able to maintain a healthy cardiovascular system and satisfactory blood cholesterol levels, in comparison to synthetic sources [7d]. Evidence indicates that the antioxidant effect may differ between natural or synthetic tocochromanols (vitamin E) [7e,7f]. The difference in biological activities in stereoisomers seem to suggest that discrimination may occur by variation in absorption, uptake, and metabolism.

Experimental

Plant material: Caryopses of *Hordeum vulgare* L. var. "Fiore Nudo", var. "Proctor", var. "Nudinka", var."Trebbia" and *Avena sativa* L. var. "Angelica", var. "Weibull", var. "Solva" were obtained from the Experimental Institute for Cereal Research (Fiorenzuola d'Arda, Piacenza, Italy). *Avena sativa* L. whole grains purchased from local market were also analyzed. *Triticum turgidum* L. subsp. *dicoccon* (Schrank) Thell and *Triticum dicoccum* Schrank ex Schübler caryopses were a gift from the National Institute of Selected Seeds (Ente Nazionale Sementi Elette, E.N.S.E., Milano, Italy).

Extraction procedure and sample preparation: Whole grains (approx. 30 g dry wt.) were thoroughly minced (1-3 mm diameter) in a blender and then exhaustively extracted, 1h at RT with *n*-hexane (1:10 w/v) under stirring. This extraction was carried out three times per sample. The combined organic phases, concentrated *in vacuo*, were kept refrigerated (+4°C) until final preparation. Before the injection the samples were resuspended in 10 mL of mobile phase and diluted 1:10 again with the same phase, filtered with 0.22 μm cartridge filter (Millipore Corporation, USA) and 20 μL injected into the HPLC system.

Standard compounds and Calibration curve: Standard tocopherols and tocotrienols were from Merck, Darmstadt, Germany. The content of each ampoule of each isomer (about 20 mg) was dissolved in *n*-hexane and transferred to a volumetric flask (10 mL). The ampoule was rinsed two times with *n*-hexane to a final volume of 10 mL. One mL aliquots (*ca*. 2 mg of each isomer) were portioned in Eppendorf, the solvent evaporated (nitrogen stream) and stored under nitrogen – 20°C. The method was calibrated using five concentration levels of standards, each of which was injected in triplicate. The diluted tocochromanols (*n*-hexane) were employed in order to obtain a calibration curve covering the concentration ranges 0.050 μg/mL–10.00 μg/mL. The calibration curve for each compound was prepared from triplicate standard injections (20 μL) at concentrations: 0.050 μg/μL, 0.100 μg/μL, 1.000 μg/μL, 5.000 μg/μL, 10.000 μg/μL. Calibration curves and quantifications were generated directly by ESA CoulArray® software (Model 5600 CoulArray®).

HPLC multi-electrode electrochemical detection: A CoulArray® Model 5600 HPLC integrated

workstation was used consisted of two model 580 pumps, a high pressure gradient mixer, PEEK pulse damper and a model 540 autoinjector, CoulArray® thermostatic chamber, serial array of eight coulometric electrodes and Windows software for CoulArray® (ESA Inc., Chelmsford, MA, U.S.A.). The HPLC system consisted of a CoulArray® from ESA. The analytical column (250 x 4 mm i.d., 5 μm) was silica-based C18 material (Alltech, Deerfield, IL, U.S.A.). The detection system consisted of two coulometric array modules, each containing four electrochemical detector cells. The detectors, porous graphite working sensors with palladium reference and counter electrodes, were arranged in series after the analytical column. The entire rig was controlled by CoulArray® software.

Chromatographic separation: Crude hexane extract of whole grains was subjected to HPLC analysis under isocratic conditions. The mobile phase consisted of methanol:1,4 dioxane:water (86:10:4 v/v) + 75 mM $NaClO_4$. The flow rate was 1 mL/min. The cell potentials constituted an increasing array: 100mV at electrode No.1; 250 mV at electrode No.2; and so on: 400 mV, 550 mV, 700 mV, 850 mV, 1000 mV and 1150 mV at electrode No.8.

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