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Electrochemical quantification of the structure/antioxidant activity relationship of flavonoids

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ABSTRACT

Ceric Reducing/Antioxidant Capacity (CRAC) is an electrochemical test that has recently emerged as an alternative to the spectrophotometric tests employed in the determination of antioxidant capacity. CRAC simply and rapidly quantifies the reducing capacity of antioxidant compounds based on the consumption of a standard oxidizer (Ce^{4+}). In this study, eight samples of flavonoids from three distinct groups were evaluated and showed the following antioxidant hierarchy: morin > kaempferol ≅ quercetin > fisetin > apigenin > luteolin > catechin > chrysin. This hierarchy is correlated with the behavior expected according to the structure/antioxidant activity relationship (SAR) of these polyphenolic compounds. Additionally, other correlations were established using SAR to explain the antioxidant behavior of the compounds with unrelated groups: OH(C2′C4′) > OH(C4′) ≅ OH(C3′C4′) > C2 = C3 + 4-oxo > OH(C3,C5) + 4-oxo > OH(C3) + 4-oxo > OH(C5) + 4-oxo > OH(C3,C5). Therefore, the use of these two tools together is very important for the study of the antioxidant behavior of flavonoids, contributing uniquely to the understanding of electronic transfer mechanisms involved in the antioxidant processes.

1. Introduction

Flavonoids are secondary metabolites consisting of a broad class of polyphenolic compounds with low molecular weight that share a common skeleton of phenyl-benzo-γ-pyran (C6-C3-C6), also known as the flavan nucleus, composed of two phenyl rings (A and B) connected through a pyran ring (C) (Fig. 1). These compounds are widely distributed in the leaves, seeds, bark and flowers of plants and are important because they show significant antioxidant properties [1,2].

Several of the biological actions of flavonoids are attributed its antioxidant properties, whether through their reducing capacity in itself or through a possible influence on the intracellular redox state. The precise mechanisms by which the flavonoids exert their beneficial effects or toxic actions on human health, however, are still unclear [2–4].

The advances already obtained highlight that the biochemical activity of flavonoids and their metabolites depends on the chemical structure and relative orientation of the substituents in the molecule, more commonly known as the structure/antioxidant activity relationship or simply SAR. Accordingly, the determination of the SAR has already been performed using liquid high-performance chromatography with electrochemical detection (HPLC-ED) [5] and spectroscopic identification [6]. The literature highlights the existence of three determining factors in the effective capacity for capturing/sequestering free radicals [7–9]:

(i) the presence of the catechol group (3′,4′-dihydroxy or OH (C3′C4′)) in ring B, which confers greater stability on the radical form by contributing to the delocalization of electrons;
(ii) a double bond conjugated with the 4-oxo (C2 = C3 + 4-oxo) function, which increases electron delocalization from ring B. The phenoxyl radicals produced are stabilized by the resonance effect of the aromatic nucleus; and
(iii) hydroxyl groups in positions C3 and C5, which promote the electron delocalization of the 4-oxo group to these two substituents (OH(C3,C5) + 4-oxo).

Consequently, the magnitude of the contributions of different substituents of the structure of polyphenols to antioxidant activity can be estimated. These substitution patterns of diverse flavonoids are generally studied using quantitative structure-property relationships (QSARs). QSARs represent an attempt at the physical or structural correlation of the descriptors of a set of structurally

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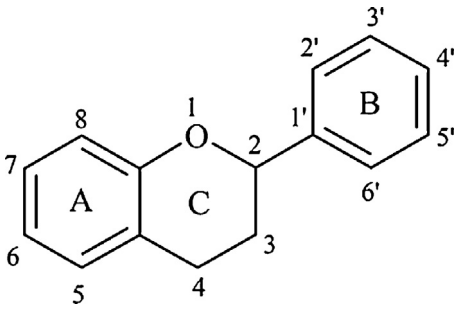

Fig. 1. General structure of the flavan nucleus shown by the flavonoids.

related compounds with their biological, pharmacological, toxicological or ecological activities, or even with their physical properties [10,11]. Molecular descriptors generally include parameters that represent electronic properties, hydrophobicity, topology and steric effects. The activities include measurements using chemical and biological assays. A crucial factor in QSAR is finding descriptors that are rich in information regarding a molecule or a fragment [12].

Additionally, there is a consensus that, due to their low reduction potential, flavonoids are thermodynamically capable of reducing radicals involved in biological oxidation processes, such as superoxide, peroxide, alkoxy and hydroxide, through the donation of electrons [2]. They are also capable of reducing oxidizing compounds employed in *in vitro* antioxidant tests. Therefore, total antioxidants potential has been already estimated potentiometrically, voltammetrically and chronoamperometrically [13–15]. One of these tests, known as CRAC (Ceric Reducing/Antioxidant Capacity), was developed by Ferreira and Avaca as an alternative to the chromogenic assays based on spectrophotometric detection. CRAC is an electrochemical test that determines the consumption of an oxidizing species (Ce^{4+}) using chronoamperometric detection for the direct quantification of the reducing power of the antioxidant sample [16,17].

Despite their emphasis in the scientific community, flavonoids still have a great deal of unexplored potential. These gaps are primarily due to the lack of studies that quantitatively correlate the mechanisms of action and the antioxidant capacity of these compounds to their chemical structure. Therefore, the objective of this study is to apply the CRAC assay to determining the antioxidant capacity of flavonoids and to correlate the experimental results with the structure/antioxidant activity relationship of each compound.

2. Experimental

2.1. Reagents and solutions

All of the solutions employed in the experiments were prepared from analytical grade reagents using ultrapure water (resistivity of 18.2 MΩ cm 25 °C) from a Milli-Q Plus purification system (Millipore). Stock solutions of flavonoids were used in the electrochemical assays: apigenin (4′,5,7-trihydroxyflavone – $C_{15}H_{10}O_5$), catechin ([(+)-cyanidol-3-(2 R,3S)-2-(4,4-dihydroxyphenyl)-3,4-dihydro-1(2H)-benzopyran-3,5,7-triol(+) trans-3,3,4,5,7-pentahydroxyflavone – $C_{15}H_{14}O_6$), chrysin (5,7-dihydroxyflavone – $C_{15}H_{10}O_4$), fisetin (3,3′,4′,7-tetrahydroxyflavone – $C_{15}H_{10}O_6$), kaempferol (3,4′,5,7-tetrahydroxyflavone – $C_{15}H_{10}O_6$), luteolin (3′,4′,5,7-tetrahydroxyflavone – $C_{15}H_{10}O_6$), morin (2′,3,4′,5,7-pentahydroxyflavone – $C_{15}H_{10}O_7$) and quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate – $C_{15}H_{10}O_7.2H_2O$); all from Sigma-Aldrich Chemical Co. with purity greater than 97%. The solutions were prepared in absolute ethanol at a final concentration of 5.0×10^{-3} mol L^{-1}.

To prepare the oxidizing solution for the CRAC assay, a stock solution of 1.0×10^{-3} mol L^{-1} $Ce(SO_4)_2.4H_2O$ (98%, E. Merck) in 0.5 mol L^{-1} H_2SO_4 (97.99%, Mallinckrodt) was used. The previously cited flavonoids were employed in the CRAC assay at a final concentration of 25×10^{-6} mol L^{-1}. Additionally, Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), a water-soluble analog of vitamin E (97%, Sigma-Aldrich Chemical Co.), was used as a standard for the CRAC assay at a final concentration of 25×10^{-6} mol L^{-1}.

2.2. Cells and electrodes

The voltammetric and chronoamperometric experiments were carried out using a model PGSTAT30 potentiostat/galvanostat from Autolab® (Utrecht, Netherlands) connected to a computer for data collection and analysis. The electrochemical experiments were carried out in one-compartment Pyrex® glass cell (30 mL) provided with three electrodes and degassing facilities for bubbling N_2. The reference system was the Ag/AgCl/3 mol L^{-1} KCl (0.22 V vs SHE) electrode and the counter one was a 2 cm^2 Pt foil. The working electrode was a boron-doped diamond (BDD) single-faced plate with an exposed area of 0.36 cm^2 and final boron content of the order of 8000 ppm. The BDD films were produced by Adamant Technologies SA, La Chaux-de-Fonds, Switzerland, on silicon wafers using the hot filament chemical vapor deposition (HF/CVD) technique. Before each voltammetric or chronoamperometric measurement the solution was deoxygenated by bubbling nitrogen gas up to 10 minutes. At the beginning of the experiment and between each measurement, the boron-doped diamond working electrode (8000 ppm of boron) was electrochemically treated according to Suffredini et al. [18] with a solution of 0.5 mol L^{-1} H_2SO_4 as a support electrolyte.

2.3. Ceric Reducing/Antioxidant Capacity Assay

As described by Ferreira and Avaca [16], CRAC is an electrochemical assay that uses chronoamperometry to directly quantify the antioxidant capacity of substances. CRAC uses an acid solution of cerium (IV) sulfate as an oxidizer and is therefore

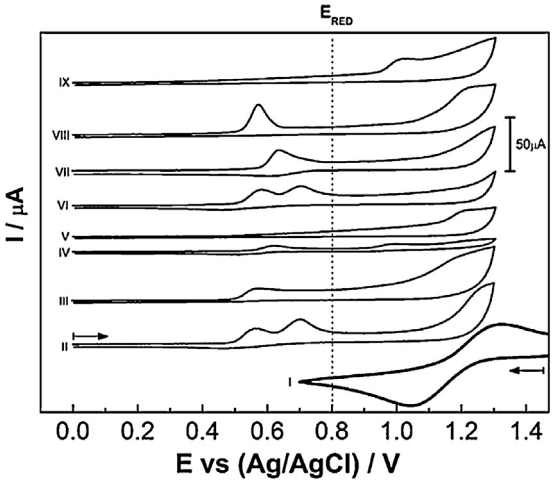

Fig. 2. Cyclic voltammograms of the (I) oxidizer (Ce^{4+} 1.0×10^{-3} mol L^{-1} in 0.5 mol L^{-1} H_2SO_4) and of the flavonoids: (II) quercetin, (III) morin, (IV) catechin, (V) chrysin, (VI) fisetin, (VII) luteolin, (VIII) kaempferol and (IX) apigenin, all at a final concentration of 25×10^{-6} mol L^{-1} in acid media (0.5 mol L^{-1} H_2SO_4); scan rate of 100 mV s^{-1}; (←) scanning direction for the oxidizer; (→) scanning direction for the antioxidants; (E_{RED} = 0.8 V) chronoamperometric step reduction.