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Simultaneous quantitation of 5- and 7-hydroxyflavone antioxidants and their binding constants with BSA using dual chiral capillary electrophoresis (dCCE) and HPLC with fluorescent detection

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ABSTRACT

In this article we present two novel uses of the sensitive techniques HPLC fluorescence and dCCE for both the quantitation and binding studies of the 5- and 7-HFs extracted from the plant Alfalfa with Albumin. Ultrasonic extraction method as an extra energy source is used to enhance the extraction efficiency and speed up. The two antioxidants could be easily separated and quantified after a 10.0-min run time. Multiple calibration curves for their analysis exhibited consistent linearity and reproducibility in the range of 0.20–2.00 mg L^{-1} for 5-HF ($r > 0.9979$) and 0.01–0.10 mg L^{-1} for 7-HF ($r > 0.9999$). Limits of Detection were 0.500 μg L^{-1} and 0.025 μg L^{-1} for 5-HF and 7-HF respectively. Lower Limits of Quantification were 131.600 μg L^{-1} for 5-HF and 6.579 μg L^{-1} for 7-HF. Inter-assay imprecision was $< 10\%$ for both flavones. Mean recovery was 104.76% (range 90%–110%) for 5-HF and 93.18% (range 90%–110%) for 7-HF. Since the intermolecular hydrogen atom transfer in the excited triplet state as well as in the excited singlet state might play an important role in the quenching process of photo-excited molecules in biological systems, the binding constants of these HFs with serum albumin have been also estimated to be 1.910–2.019 × 10^5 L mol^{-1} and 2.390–2.500 × 10^5 L mol^{-1} for 5-HF and 7-HF respectively.

1. Introduction

5-and 7-hydroxyflavone (5HF and 7HF) are found in nature [1–3]. The properties of these flavones have been studied from different aspects; anti-oxidant properties against superoxide anion (O_2^-) [4], stability against photo-irradiation [5] and excited state proton transfer reactions [6–9]. Plant physiologists believe that flavonoids which possess the 5-OH group can act as photo-protectors, in which the excess light energy from the sun can be converted to heat [6]. Therefore, studying the deactivation process of flavonoids is intriguing from a biochemical point of view, as well as, from the photochemical aspect. Photophysical properties of 5HF show two strongly separated bands in their fluorescence spectrum which due to Excited State Intramolecular Proton Transfer (ESIPT) reaction [10–12]. In other publication [13], these photophysical properties through the proton-transfer fluorescence have been revised; they exhibited one emission band centered at ca. 700 nm. A small photoreaction quantum yield of 10^{-5}–10^{-6} denotes the great photostability exemplified by 5HF. In the presence of Al(III) ions, 5-hydroxyflavone (5HF) through a complexation reaction in MeOH, dual fluorescence is shown and it can be characterized by a newly developed peak at 554 nm upon excitation at 363 nm [14]. 7HF, which serves as a simple representative for naturally occurring flavones of therapeutic importance [15], undergoes photo-induced excited state proton transfer (ESPT) fluorescence with a large Stokes shift. 7-HF has therapeutic importance, its vasorelaxing properties have been reported [16]. ESPT fluorescence of 7-HF has been also investigated in considerable details [17–20].

Flavonoids have attracted a great interest as potential therapeutic drugs [21] against a wide range of diseases. While the antioxidant activity of these natural phenolic compounds is well known, their binding to DNA characteristics is not fully understood despite the fact that many of them exert their biological effects by reversibly binding to nucleic acids. Micro-DSC has been used to determine the temperature dependence of the heat capacity of the process of thermal denaturation of DNA in solutions containing 5-, and 7-hydroxyflavones [21].

Fluorescence spectroscopy [22] is a powerful tool for the binding studies with proteins since it allows nonintrusive measurements of substances in low concentration under physiological conditions. The first report on the interaction of 7HF with human serum albumin was studied via electronic absorption, steady-state and time resolved fluorescence and induced circular dichroism

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techniques [23]. Proteins including enzyme are frequently the targets for therapeutically active flavonoids of both natural and synthetic origin, therefore studies on the interaction of flavonoids with serum albumins are particularly notable since serum albumins play a critical role in the transport and disposition of flavonoids they increase their bioavailability. There have been several studies on fluorescence quenching of proteins induced by flavonoids and other polyphenols [24–30]. Monohydroxyflavones exert inhibition in the growth of cancer cell [31]. Many articles are found in the literature dealing with quantitation and separation of polyhydroxyflavones, however none or very little exist for the monohydroxyflavones. Recent studies, both in vivo and in vitro, have established that flavonoids are effective powerful antioxidants against a wide range of free radical mediated and other diseases including various types of cancers, tumors, diabetes mellitus, atherosclerosis, ischemia, neuronal degeneration, cardiovascular ailments, and AIDS [32–34].

Alfalfa plant *lucerne*, or *Mendicago sativa* is rich in vitamins, minerals and other nutrients [35] that play a vital role in the strength and growth of our bones and in the maintenance of a healthy body. It can be taken in the form of seeds, leaves or tablets. For centuries, Native Americans planted alfalfa seeds to use it as flour or to boil its leaves and eat them like greens. It is a "super" anti-oxidant rich in chlorophyll and trace minerals. It alkalizes and detoxifies the body; acts as a diuretic, is anti-inflammatory, and anti-fungal. It lowers cholesterol levels; balances blood sugar and hormones; aids the digestive system; improves skin texture [36]. Alfalfa is also been used by the Chinese [37] since the sixth century to treat several health conditions. Alfalfa maintains the integrity of prostate tissue in men and protects from tumors. Simple monohydroxy substituted flavone derivatives have also been found to possess significant antioxidant effects [15,38–40]. For a biological system, to avoid DNA damage, it is important to have functions which efficiently quench the excited states; especially the excited triplet state which has biradicaloid properties and it usually lives longer than the excited singlet state. If the flavones under investigation have a high affinity to serum albumin, this may alter the structure of serum albumin. The change of affinity binding of flavones to serum albumin will result in the variation of the level of the flavone, which is closely related to the flavone's therapeutic effect [41].

Therefore, the aim of the present work is to analytically separate and quantify the antioxidants 5- and 7-HF from the economic source of Alfalfa, and determine in vitro abilities to bind the serum albumin.

2. Materials and methods

2.1. Alfalfa sample

Alfa Alfa Green Blend (α–α) was purchased from Imtenan, Nutria, Egypt. Alfalfa Plants were obtained from farmers near Assiut.

2.2. Materials

5-Hydroxyflavone (5-HF) and 7-Hydroxyflavone (7-HF) were obtained commercially from Alfa Aesar (Ward Hill, MA, USA). Sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate anhydrous were from Merck (Darmstadt, Germany). Methanol (MeOH), acetonitrile (ACN) HPLC grade, Hydrochloric acid and Bovine Serum Albumin (BSA) were purchased from Sigma (St. Louis, Mom USA).

2.3. Preparation of solutions

5-HF and 7-HF (1500 μg/mL) stock standard solutions were accurately prepared in 10 mL measuring flask (class A, ± 0.008 at 20 °C) by dissolving the appropriate weight in methanol. Stock standard solution was transferred into PTFE-sealed screw-cap bottles and stored at −8 °C until the required analysis. Working solutions of 5-HF and 7-HF were prepared daily by appropriate dilution from stock standard solution in phosphate buffer. Phosphate buffer solution (20 mmol L^{-1}) for HPLC was prepared by weighing an appropriate amount of disodium hydrogen phosphate and adjusting pH to 7.0 using sodium dihydrogen phosphate.

Buffers were prepared with ultra-pure, Type I, water (Millipore, Milli Q Gradient, USA). For CE, phosphate buffer solution (67 mmol L^{-1}) was prepared by weighing an appropriate amount of disodium hydrogen phosphate and adjusting pH to 7.4 using sodium dihydrogen phosphate. BSA stock solution (1000 μg mL^{-1}) was prepared in 5 mL phosphate buffer.

Acetanilide (Ac 500 μg mL^{-1}) stock solution was prepared in 5 mL phosphate buffer pH 7.4

2.4. Preparations for binding constants studies

For CE binding studies, the concentrations of 5-HF and 7-HF were fixed at 876 μmol L^{-1} and 430 μmol L^{-1} respectively while that of BSA was varied from 5 to 30 μmol L^{-1}. Acetanilide (Ac 500 μg mL^{-1}) stock solution was added into each sample solution and used successfully as EOF marker without any difficulty for its solubility in the electrolyte. Injected sample solutions were filtered through 0.22 μm syringe filters degassed and sonicated for 10 min prior to their application onto the CE system.

For HPLC binding studies, since bovine serum albumin (BSA) has no fluorescence and we must follow the change in the fluorescence intensity with varying the concentration, therefore BSA concentration was fixed and the concentrations of 5-HF and 7-HF have been varied in the range 1.0–10.0 μmol L^{-1}.

2.5. Sample preparation and extraction procedure

Fresh Alfalfa plant was collected from Assiut farms and dried in the air then at 60 °C for 4 h in an oven and gently pulverized, an accurate weight amount of the powder 5 g was refluxed with 50 mL of methanol/HCL 10% for 6 h in an 80 °C water bath. After cooling, the mixture was filtered through a 0.45 μm membrane filter and the residue was washed twice with 10 mL of methanol. The extract and washings were combined and concentrated to about 40 mL under vacuum and then diluted to 100 mL in a volumetric flask with methanol.

3. Apparatus

3.1. High performance liquid chromatography-fluorescent detector (HPLC-FLD)

High performance liquid chromatography analysis was carried out using Agilent HPLC 1200 Series (USA) system consisting of degasser, quaternary pump and Fluorescence detector. HPLC chemstation software was used for instrument control, data acquisition and data analysis. The hydroxyflavones were eluted using a Zorbax Eclipsed XDB-C18 column (150 × 4.6 mm I.D × 5 μm particle size) and detected at 270 nm excitation and 670 nm emission with a flow rate of 1.0 mL min^{-1}. The column temperature was set at 30 °C. The elution was carried out with the gradient program began with 80% of 20 mM phosphate buffer (pH 7.0) (A), 5% Methanol (B) and 15% Acetonitrile (ACN) (C) changed