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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

linearly to 57% A, 40% B, 3% C in 3 min; then 30% A, 0% B and 70% C in 3 min; remained this composition for 6 min; and subsequently back to initial composition for 6 min followed by 7 min afterward equilibration. The pH values were adjusted using pH-meter (Jenway 3505).

3.2. Capillary electrophoresis (CE)

All separations were performed on an Agilent 1600 CE system (Agilent Technologies, Germany), equipped with a diode-array UV–Vis detection system (190 to 600 nm). CE Chemstation software was used for instrument control, data acquisition and data analysis. Bare fused-silica capillaries were obtained from Agilent Technologies with 64.5-cm total length, 56.0 cm effective length and 50.0 μm internal diameter. The applied voltage was maintained at 20.0 kV with controlled temperature at 25.0 °C to give current value of 15.0 μA. The CE sampling was performed by hydrodynamic injection at 50.0 mbar for 5.0 s; the detection wavelength was 280 nm. Before the injection into the CE system, each solution (running buffer and sample solutions) was subjected to filtration through a 0.45 μm membrane filter. New capillary was conditioned by rinsing with 1.0 mol L^{-1} NaOH for 40 min at 40.0 °C and water for 20 min at 25.0 °C. Flushing procedure was optimized to give precise analysis by flushing with 0.1 mol L^{-1} NaOH for 2 min, water for 2 min and then equilibrated with the running buffer for 5 min; and repeated between runs.

4. Results and discussion

The main difficulties in characterizing the flavone components in plant extracts are due to the existence of a large number of molecules belonging to such chemical class, often isomeric amongst them, that can be constituted by polymers, with high molecular weights. High-performance liquid chromatography (HPLC) has added a new dimension to the investigation of flavonoids in plant and food extracts. The extensive use of photo-diode array detection (PDA) in the analysis of flavonoids and phenolic acids can be attributed to the ability of collecting on-line spectra without using stopped-flow techniques. This has led to a considerable improvement in the HPLC analysis for identification purposes, and demonstrated the usefulness of qualitative information in phenolic analysis that is based on the absorption spectrum. However, the UV detection has the disadvantage of not being as sensitive or selective as fluorescence detection (FLD). In the analysis of phenolics, fluorescence detection offers major advantages over UV detection in terms of enhanced selectivity and sensitivity. Therefore, fluorescent detector (FLD) is the detector-of choice for the identification and separation of the hydroxyflavones- under investigation- in alfalfa plant.

4.1. Analytical separation of 5- and 7-HFs from alfalfa using HPLC-FLD

In order to have the chromatographic profile of alfalfa extract, optimization of the HPLC-FLD method has been assessed by checking many parameters viz excitation wavelength, resolution, pH, linearity, and recovery. To determine the excitation wavelengths; HPLC with diode array detector was carried out for both 5, 7-HFs. (Fig. 1A and B). 270 and 310 nm was used as excitation wavelengths for further fluorescence experiments of 5-HFand 7-HF respectively. Gradient excitation wavelengths at 310 nm for 7.5 min for the determination of 7-HF then at 270 nm for twelve min for the determination of 5-HF were applied and gave rise to good resolution for the analysis of the two flavones simultaneously. Since the pk_a of the monohydroxyflavones with different positions of hydroxyl group are ranged from 7 to 12 (11.44 for 5-HF and 8.5 for 7-HF) the protonated form of the HFs under investigation (pH 7) was chosen as it is useful from the biological point of

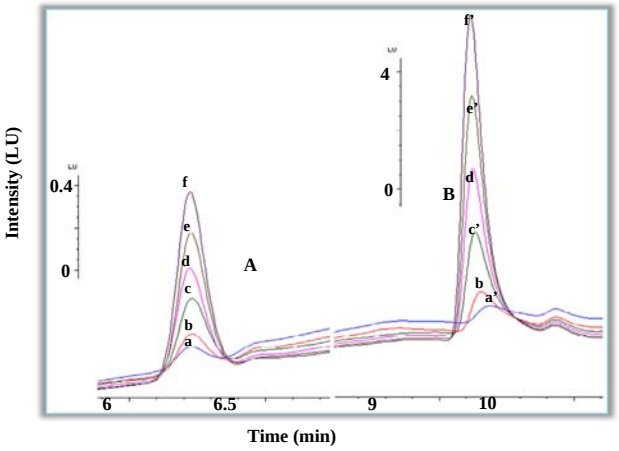

Fig. 2. Calibration chromatograms of: (A) 7-hydroxyflavone; a: 0.01, b: 0.02, c: 0.04, d: 0.06, e: 0.08, and f: 0.01 mg L^{-1} and (B) 5-hydroxyflavone; a′: 0.2, b′: 0.4, c′: 0.8, d′: 1.2, e′: 1.6 and f′: 2.0 mg L^{-1}.

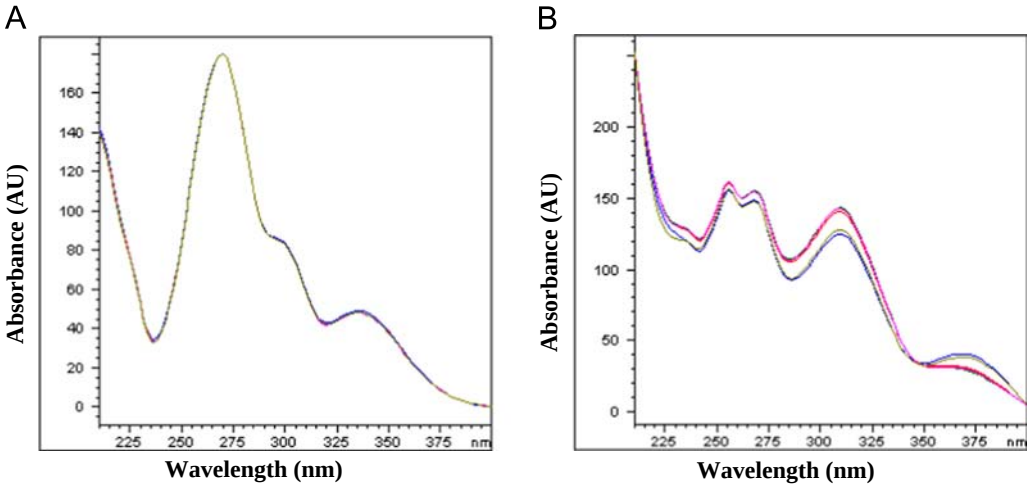

Fig. 1. A 5-hydroxyflavone absorption spectrum. B 7-hydroxyflavone absorption spectrum.

view and to avoid the column poisoning. Between several tested buffers as mobile phase, phosphate buffer gives rise to better separation and resolution. Linearity of the method was established by a series of standard mixtures of 5-HF 0.20–2.00 mg L^{-1} for 5-HF and 0.01–0.10 mg L^{-1} and 7-HF in methanol as analyzing in triplicate. Calibration curves (Fig.2) were then acquired by plotting their peak area of the analytes against their respective concentrations. Zero regression was applied, the slope (a) and the correlation coefficient (r) were determined Fig. 3A and B.

The suitability of the proposed method to recover the hydroxyflavone under investigation from the Alfalfa was then checked; spikes of these flavones into the alfalfa matrix have been added. The recovery was then calculated and found in the range of 90%–110% in a good agreement with the ICH guidelines for method validation Protocols [42].

4.2. Application in alfalfa sample

Under the forgoing experimental conditions; fluorescent chromatograms for the extracts of ground alfalfa whole plant, Leaves, Stems, and alfaalfa from Imtenan (commercial nutrient) were obtained as shown Fig. 4. Table 1 summarizes the concentrations found in each matrix. The highest content of the HFs is found in the leaves.

4.3. Analytical separation of 5- and 7-HFs using dCCE–diode array detector (DAD)

Many chiral selectors were used to achieve CE separations of the HFs, viz, ß-CD, HP-ß-CD, Succinyl-ß-CD and Carboxymethyl-ß-CD.

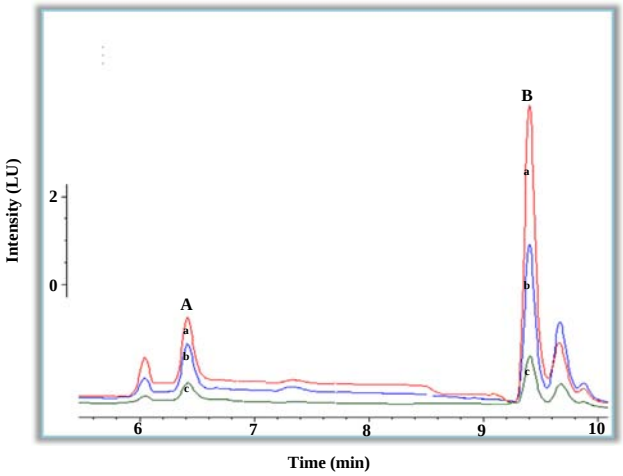

Fig. 4. HPLC fluorescence of A: 7-HF and B: 5-HF in (a) alfalfa plant grinding, (b) leaves of alfalfa plant, and (c) stems of alfalfa plant.

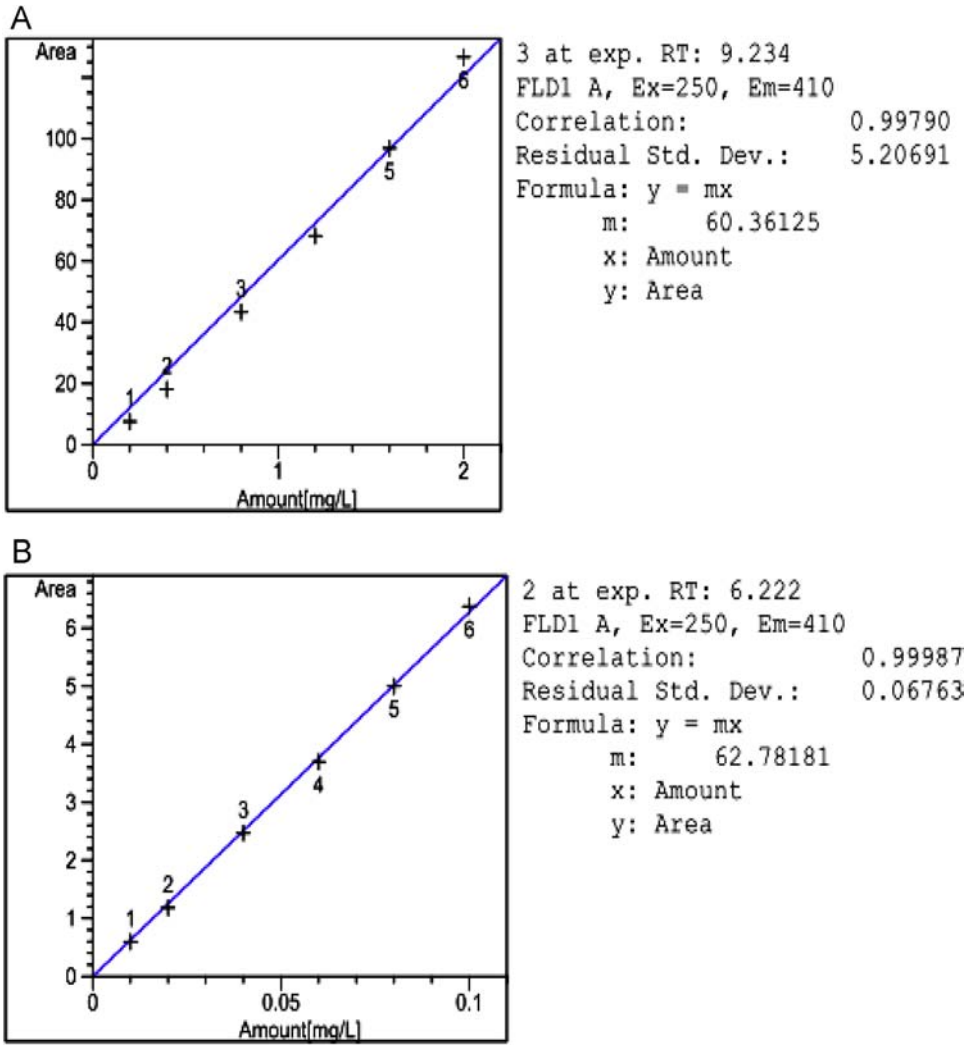

Fig. 3. Linearity of A: 5-HF and B: 7-HF.

Table 1
5- and 7-HF concentrations obtained from the analysis of different Alfalfa matrices using HPLC-FLD.

Analyte	Alfalfa plant grinding (mg kg^{-1})	Leaves of alfalfa plant (mg kg^{-1})	Stems of alfalfa plant (mg kg^{-1})	Alfaalfa from imtenan (mg kg^{-1})
5-HF	3.710 ± 0.031	2.721 ± 0.029	0.663 ± 0.032	2.730 ± 0.044
7-HF	0.261 ± 0.024	0.178 ± 0.040	0.144 ± 0.021	0.218 ± 0.038

The separation of 5-HF from the neutral marker has been established in the presence of Succinyl-ß-CD. Simultaneous separation of the two hydroxyflavones is achieved in the presence of dual CD system consisting of 10 mmol L^{-1} HP-ß-CD and 2.5 mmol L^{-1} Succinyl-ß-CD in the presence of 5% Methanol (MeOH) to slow down the EOF therefore improve resolution. Optimization of the proposed method was further checked by studying other parameters viz. temperature, voltage, injection time and injection pressure, higher efficiency and better resolution are obtained with field strength as high as possible, optimum was achieved at 20 kV applied voltage. Hydrodynamic injection, 50 mbar for 5 s and capillary length of 64.5 cm × 50 μm i.d. (56 cm effective length) and DAD detector, 280 nm, 25 °C gave rise to good resolution Fig. 5. The reproducibility of migration time and integrated area was determined for run-to run and day-to-day with 45.6 μg mL^{-1} and 220 μg mL^{-1} for 7-HF and 5-HF respectively.

The suggested analytical method was validated according to the International Conference on Harmonization (ICH) of Technical Requirements for the Registration of pharmaceuticals for Human Use [42] developed a consensus text on the validation of analytical procedures with respect to certain parameters such as specificity/selectivity, linearity, practical quantitation limit (PQL), limit of detection (LoD), precision, accuracy, sensitivity, and recovery.

4.4. Method validation

The linearity of the method was established by a series of standard mixtures of 5-HF (5.0–250 μg mL^{-1}) and 7-HF (0.50–160 μg mL^{-1}) in methanol and analyzed in triplicate. Calibration curves Fig. 6A and B were then acquired by plotting their response ratios (ratios of the peak area of the analytes to migration time) against their respective concentrations. Zero regression was applied and slope (a) and correlation coefficient (r) were determined. Sensitivity of the method was expressed as the slope of the linearity.

Precision tests were performed to determine both intra-day and inter-day variations (as percent relative standard deviation % RSD) in migration/retention time and peak area. Intra-day precision was carried out by repeating 7 runs within the same day and inter-day precision by repeating measurements within three consecutive days (seven runs per day) and calculated as follows:

$$\mathrm{RSD} = (s/m)X100\% \quad (1)$$

where: s = standard deviation, and m = mean of replicate measurements. The intra- and inter-day assays were less than 10% in every case (Table 2).

Method accuracy has been determined from the analysis of a laboratory fortified blank, and matrix spikes. Accuracy is then measured by measuring the recovery of the analytes from the matrices.

$$\%R = 100 \times \frac{(S-U)}{C_{sa}} \quad (2)$$

where %R = percent recovery, S = measured concentration in spiked aliquot, U = measured concentration in unspiked aliquot, C_{sa} = actual concentration of spiked added. The acceptable range of %R lies between 91 and 105% Table 2 in a good agreement with the ICH guidelines [42].

The degree of bias caused by expected sample component and common interferences is determined by measuring the analyte with and without anticipated interferences through accuracy and precision calculations. LoD is then calculated using equation LoD = 3.3 σ/S, where σ is the standard deviation of the response and S is the slope of the calibration plots and cited in Table 2. PQL is determined taking into consideration both accuracy and precision of the method and cited in Table 2.

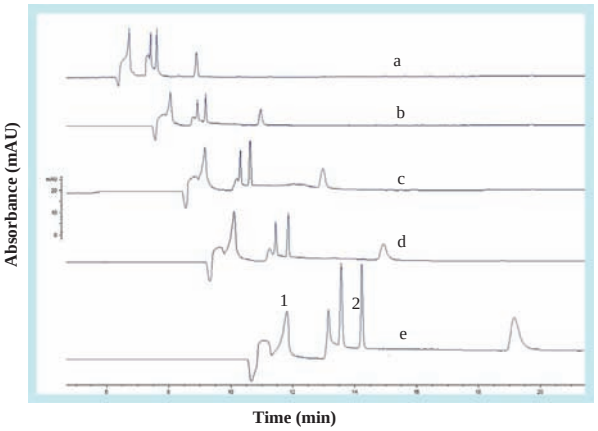

Fig. 5. Effect of borate buffer concentration (pH 9.0) in the presence of 10 mmol L^{-1} HP-ß-CD and 2.5 mmol L^{-1} Succinyl-ß-CD BGE: (a) 25, (b) 50, (c) 75, (d) 100, and (e) 150 mmol L^{-1} on the electropherogram of HFs mixture, 1: 5-HF (220.0 μg mL^{-1}) and 2: 7-HF (45.60 μg mL^{-1}), 20 KV, 25 °C, 280 nm, and 50 mbar for 5 s.

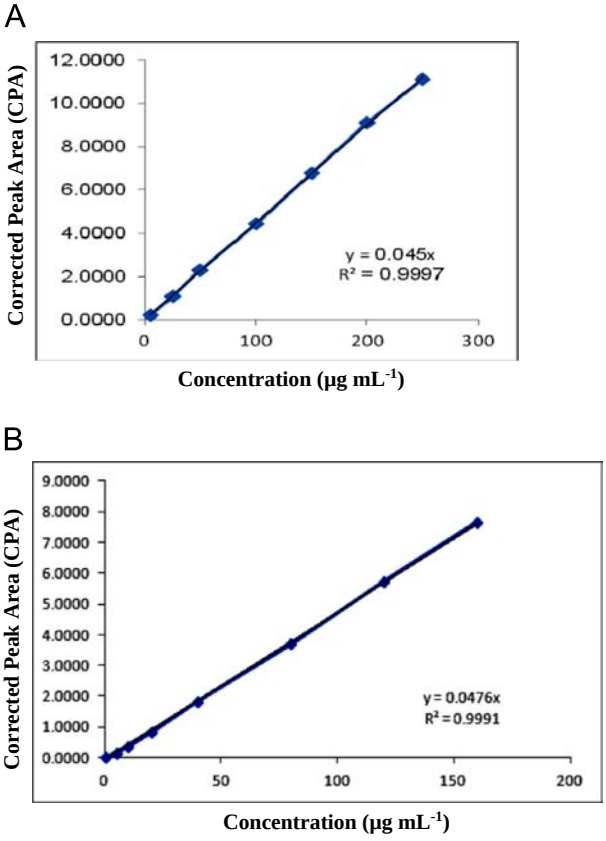

Fig. 6. A: calibration curve of 5-hydroxyflavone. B: calibration curve of 7-hydroxyflavone.

Table 2
Intra-day precision, Inter-day precision, recovery, limit of detection and practical quantitation limit for 5- and 7-hydroxyflavones.

Method	Analyte	Intra-day precision,% RSD ($n=7$)	Inter-day precision, % RSD ($n=21$)	Recovery (%)	Linearity (equation, R^2)	LoD ($\mu g\ mL^{-1}$)	PQL, ($\mu g\ mL^{-1}$)
dCCE	5-HF	3.692	3.983	100.558	$Y=0.0450X$, 0.9997	1.021	4.084
	7-HF	7.379	8.090	91.925	$Y=0.0476X$, 0.9991	0.203	1.017
HPLC-FLD	5-HF	7.850	9.192	104.758	$Y=60.36125X$, 0.9979	0.005	1.316
	7-HF	2.107	3.279	93.177	$Y=62.78181$, 0.99987	0.111	0.295

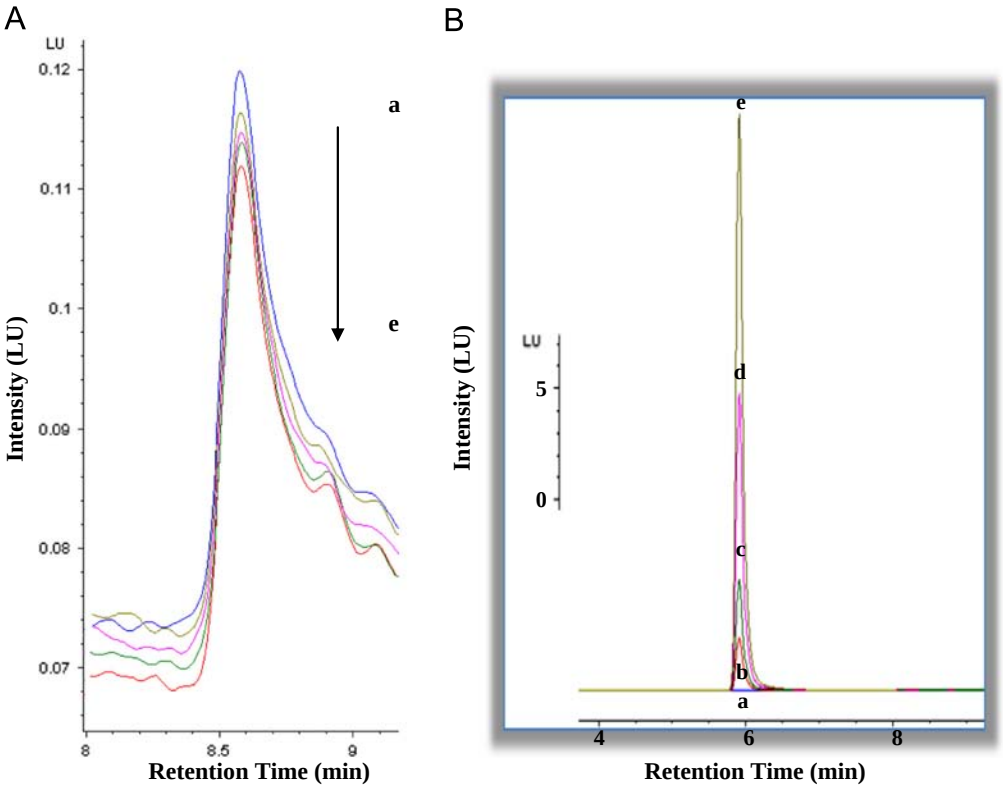

Fig. 7. A: effect of BSA on the Lu intensity of 5-HF: (a) 0, (b) 1.0, (c) 2.0, (d) 5.0, and (e) 10.0 μmol L^{-1}. B: effect of BSA on the Lu intensity of 7-HF: (a) 0, (b) 1.0, (c) 2.0, (d) 5.0, and (e) 10.0 μmol L^{-1}.

5. HPLC fluorescence measurements of the interaction between 5- and 7-HF and BSA

Quenching can occur by different mechanisms which are usually classified as dynamic quenching and static quenching. They can be distinguished by their differing dependence on temperature and viscosity, or preferably by lifetime measurements. Higher temperatures result in faster diffusion and hence larger extent of collision quenching. Higher temperatures will also result in the dissociation of weakly bound complexes, and hence lead to less static quenching.

In the presence of 5- and 7-HF individually at different concentrations, the fluorescence of 5-HF is quenched while enhancement in the fluorescence chromatogram is obtained for 7-HF (Fig. 7A and B). This may be attributed to increasing binding tendency of this flavone towards the serum albumin; therefore simulated peaks were used for the calculation of the binding constants. The equilibrium between free and bound molecules is given by the Lineweaver–Burk equation:

$$\log\ (F_0 - F)/F = \log\ K + n \log\ [HF] \quad (3)$$

where K and n are the binding constant and the number of binding sites, respectively. Appling this equation to the obtained fluorescence data yielded a linear plot from which the values of K and the number of binding sites are deduced. Therefore, the quenching data were analyzed according to the modified Lineweaver–Burk:

$$1/F_o - F = 1/F_o + 1/K.F_o.[Q] \quad (4)$$

where F_0 and F are the fluorescence intensities in the absence and presence of quencher respectively, K is the binding constant and [Q] is the quencher concentration. The binding constants are cited in Table 3.

Table 3
Binding constant of hydroxyflavones–BSA system.

Flavone	[a]Binding constant, ($\times 10^5$ L mol^{-1})	[b]Binding constant, ($\times 10^5$ L mol^{-1})
5-Hydroxyflavone	1.910 ± 0.065	2.019 ± 0.049
7-Hydroxyflavone	2.390 ± 0.051	2.500 ± 0.038

[a] CE technique,
[b] HPLC technique.

6. Binding constants studies of 5- and 7-HFs with bovine serum albumin (BSA) using capillary electrophoresis (CE)

Affinity capillary electrophoresis (ACE) is used advantageously for studying affinity interactions compared with other well-

established techniques [43,44]. Among the virtues that make ACE an attractive platform are low sample and ligand consumption, relatively short analysis times, high efficiency and suitability for probing high and weak affinity interactions. In ACE, separations can be performed in solution under physiological buffer conditions, it is – as a rule – possible to preserve the analyte in a native state and hence to maintain its molecular function. Therefore, ACE has been used quite broadly in analytical chemistry and the biological sciences, integrated in many functional biology studies.

There are a number of formats to measure binding parameters for affinity interactions [44–46]. ACE has been demonstrated to be a useful approach for the determination of binding constants based on mobility measurements [47]. In ACE, one of the two binding species is injected to form a narrow plug into the capillary filled with a buffer containing the other binding species at varying concentrations [46]. To reduce protein adsorption, we used the rinsing strategy. We also used small multivalent ions viz. phosphate under physiological conditions as a running electrolyte and solvent for flavones and protein to reduce the imprecision in measurements resulting from conductivity and/or pH inhomogeneities or sticking of the capillary wall. The mobility ratio of the flavones related to the mobility of a nonreacting neutral marker (Ac) is used to compensate for fluctuations of the EOF.

6.1. Optimization of ACE experimental conditions

The flavones/protein interaction studies are followed by either the addition of the flavone or the protein into the ACE running buffer. Protein interactions with the inner surface of the capillary may contribute in a change in mobility http://www.sciencedirect.com/science/article/pii/S0731708509007651 - bib26, therefore an EOF neutral marker is used to measure of the mobility shift. Acetanilide (Ac) was successfully used as EOF marker since this substance remained uncharged under the applied conditions without any difficulty for its solubility in the electrolyte.

The mobility shifts of flavones in the presence of bovine serum albumin (BSA) are studied by adding different concentrations of BSA to each flavone individually. Different mobilities from that in the absence of serum albumin are obtained. The adsorption of serum albumin onto the capillary inner wall results in width and tailing of peak, even low resolution and efficiency. To eliminate this, the serum albumin concentration was controlled lower than 30 μmol L^{-1}. Hereby, in the case that the adsorption of serum albumin onto the capillary inner wall was considered to be negligible. The concentrations of serum albumin were in the range of 0–30 μmol L^{-1}.

As the concentration of the analyte has a significant impact in ACE, generally higher concentration of analyte tends to lower the separation efficiency and the imprecision of the obtained data. 1 mmol L^{-1} flavone is found to be the maximum concentration to be used in order to get good precision in ACE measurements. The rinsing procedure has also great influence on the mobility study. Rinsing was efficiently completed by water for 2 min at 3000 mbar with running electrolyte for 3 min after each run. However, it was obvious that the mobility of analytes changed dramatically after 30 consecutive runs. The baseline noises could be due to the aging of small amounts of protein which may be adsorbed on the inner wall after 30 consecutive runs. These particles have been mechanically removed from the wall by flushing the capillary with buffer for 25 min under 3000 mbar (high streaming velocity). Therefore rinsing protocol was used in experiments with long period-continued measurements.

Among several tested buffers vis. borate, acetate, Tris–HCl, phosphate, the last one gave rise to good results, since it had no effect on the protein. Therefore, a 67 mmol L^{-1} phosphate buffer was used for all investigations. As the aim of this work was to determine the binding constant under the physiological conditions, a pH value of 7.4 was fixed in all measurements. Organic solvent has great influence since BSA may be denatured by increasing its content than 5%. Therefore, 5% methanol was fixed in all analyzes.

After applying the foregoing optimal experimental conditions, the migration of flavones was studied at different protein concentrations. There are several practical considerations limiting the choice of protein concentrations range for the estimation of ACE binding constant, the most effective parameters were the protein's solubility in the running electrolyte, the detector response at the selected wavelengths and the saturation of the flavones– protein complex. 67 mmol L^{-1} phosphate buffer was used without any problems with BSA, this presents the solubility limit of this protein. Optimal wavelengths of 210 and/or 280 nm gave rise to best detection for the flavones- Protein interaction.

6.2. Estimation of the binding constants for the binary system

The equation which relates the apparent binding constant to the electrophoretic mobility of free and uncomplexed solute is [44]

$$K[L] = \left(\frac{\mu_f - \mu_i}{\mu_i - \mu_c}\right) \quad (5)$$

where K is the binding constant, [L] is the equilibrium concentrations of uncomplexd ligand, and μ_f, μ_c are the electrophoretic mobilities of free and complexed solute; μ_i is the solute mobility measured at ligand concentration, [L]. The obtained electropherograms are shown in Fig. 8. As higher concentrations of protein were used, the extent of flavone–protein binding increased and a larger shift in mobility was observed. These shifts in mobility and their relation to binding affinity made such studies useful in the determination of flavone–protein equilibrium constants.

The electrophoretic mobility of 5-HF is increased accompanied with an increase in the broadness of the peak of 5-HF. The EOF remained relatively constant with increasing protein concentration in the buffer. Therefore, further ACE experiments were carried out up to 30 μmol L^{-1} BSA.

Plotting the shifts in mobility ratio difference (R_i–R_f), R_i is the mobility ratio (Ac/HF) measured in the presence of protein and R_f

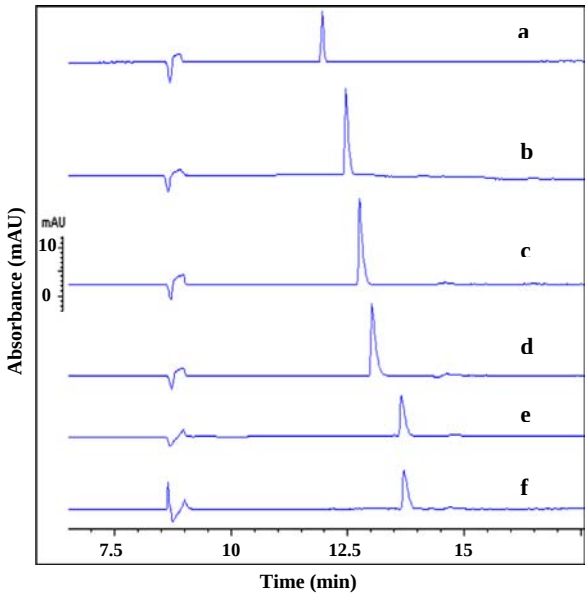

Fig. 8. Electropherogram of 7-HF-BSA in the presence of BSA (a) 0, (b) 5, (c) 7.5, (d) 10, (e) 20, and (f) 30 μmol L^{-1}. Background electrolyte, 67 mM phosphate buffer (pH 7.4); hydrodynamic injection, 50 mbar for 5 s; voltage, 20 kV; 280 nm; 25 °C.

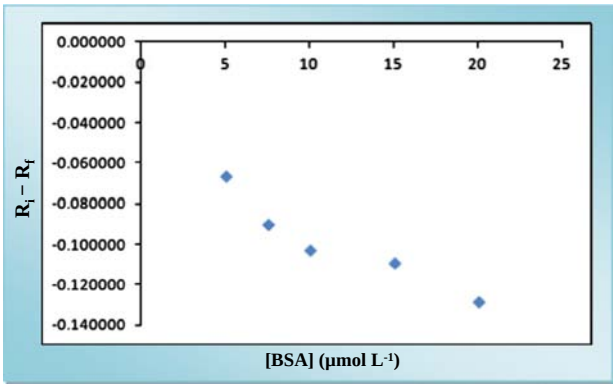

Fig. 9. Binding curve of 5-HF-BSA system.

is the mobility ratio (Ac/HF) measured in the absence of protein, versus the protein concentration results in Linear behavior up to 30 μmol L^{-1} BSA Fig. 9. From the above results, we can say that the main factor limiting the useful range of protein concentrations was the resolution obtained between the peak for the partially complexed flavone (R_i) and that for the free one (R_f). This item was capable of causing problems at either low or high protein concentrations, and was found to be a factor of the precision of the mobility measurements.

The analysis of data for the calculation of binding constants was achieved [44] by the nonlinear regression model using Excel Spreadsheets.

$$\text{Nonlinearregression}: \quad Kc(L) = \left(\frac{R_f - R_i}{R_i - R_c}\right) \tag{6}$$

A major advantage of the nonlinear regression method is the elimination of the cumbersome weighting procedure necessary in the statistical analysis of the linearized plots (x-reciprocal, y-reciprocal and double reciprocal). The binding constant was then calculated under the case of R_c equal to the mobility ratio of the flavones measured at saturated protein concentration (R_{sat}).The results are cited in Table 3. It was found that the calculated binding constant under the optimal parameters was very close to the values calculated previously by other techniques [28,48] (Table 3). The slight difference in the comparable values was due to the difference in temperature between the controlled temperature around the capillary and the internal temperature of the capillary. The temperature was controlled at 25 °C, the internal temperature was estimated to be 37 °C. Generally, nonlinear regression should provide the most accurate and precise (low standard deviation values) for the estimation of ACE binding constants than linear regressions following algebraic manipulation.

7. Conclusion

This work has shown an experimental evidence for the presence of hydroxyflavones in Alfalfa plant. These naturally occurring flavones interact with BSA. Two methodologies are employed for this purpose HPLC-FLD and d-CCE, this interaction may contribute to understand the mechanism of the action of these flavonoids. The obtained results by both techniques show a significant effect of the structure of the hydroxyflavones on the extent of the interaction. Since flavonoids-albumin binding is expected to modulate the bioavailability of flavonoids, the results may provide not only a deeper insight into the Serum Albumin-binding properties of flavonoids but also a useful guideline for the design of efficient serum albumin-binding agents for chemotherapy.

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