A high-performance liquid chromatography method for determination of flavonoids in dipalmitoylphosphatidylcholine liposome solutions

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Un metodo de cromatografía líquida de alta resolución para la determinación de flavonoides en liposomas de dipalmitoilfosfatidilcolina

Un mètode de cromatografia líquida d'alta resolució per a la determinació de flavonoids en liposomes de dipalmitoilfosfatidilcolina

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RESUMEN

Se desarrolla un método de cromatografía de alta eficiencia (HPLC) para la determinación de cuatro flavonoides: rutina, morina, quercitina y 3-hidroxiflavona, incorporados a liposomas de dipalmitoilfosfatidilcolina (DPPC), que permite cuantificar su consumo después de reaccionar con oxígeno molecular singulete. El método HPLC emplea elución isocrática y detección con arreglo de diodos (DAD). La separación cromatográfica de estos componentes se realiza en una columna analítica C18 y se utiliza una mezcla de solventes formado por agua: acetonitrilo:ácido acético; 74,5:24,5:1 v/v. Esto permite obtener una buena resolución de los picos correspondientes a los cuatro flavonoides, sin interferencias de la matriz ni de los productos de reacción. La respuesta del método es lineal (r > 0,999) en un amplio rango de concentración y confiable, lo que permite estudiar la cinética de consumo de los flavonoides en reacciones con oxígeno molecular singulete en sistemas microorganizados de surfactantes lipídicos.

Palabras clave: **HPLC. Flavonoides. Liposomas. Oxígeno Molecular Singulete. Fosfolípidos.**

SUMMARY

A high-performance liquid chromatography (HPLC) method for the determination of four different flavonoids, rutin, morin, quercetin, and 3-hydroxyflavone in dipalmitoylphosphatidylcholine (DPPC) liposome solutions has been developed. The method allows to quantify their consumption upon reaction with singlet molecular oxygen. The actual HPLC method uses an isocratic elution and detection. The chromatographic separation of these components is achieved using a C18 analytical column with a water:acetonitrile:acetic acid mixture 74.5:24.5:1 v/v. The peaks for the four flavonoids are well resolved and free from matrix interference and reaction products. The method has been found to be linear (r > 0.999) over a wide concentration range and reliable to perform kinetic studies in which singlet molecular oxygen is involved and the time dependent consumption of flavonoids in a microorganized system composed by lipidic surfactants is monitored.

Key words: **HPLC. Flavonoids. Liposomes. Singlet Molecular Oxygen. Phospholipids.**

RESUM

Es desenvolupa un mètode de cromatografia d'alta eficiència (HPLC) per a la determinació de quatre flavonoids: rutina, morina, quercitina i 3-hidroxiflavona, incorporats a liposomes de dipalmitoilfosfatidilcolina (DPPC), que permet quantificar el seu consum després de reaccionar amb oxigen molecular singlet. El mètode HPLC empra elució isocrática i detecció amb arranjament de diodes (DAD). La separació cromatogràfica d'aquests components es realitza en una columna analítica C18 i s'utilitza una barreja de solvents formats per aigua: acetonitril:àcid acètic; 74,5:24,5:1 v/v. Això permet obtenir una bona resolució dels pics corresponents als quatre flavonoids, sense interferències de la matriu ni dels productes de reacció. La resposta del mètode és lineal (r > 0,999) en un ampli rang de concentració i fiable, el que permet estudiar la cinètica de consum dels flavonoids en reaccions amb oxigen molecular singlet en sistemes microorganitzats d'agents tensioactius lipídics.

Mots clau: **HPLC. Flavonoids. Liposomes. Oxígen Molecular Singlet. Fosfolípids.**

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1. INTRODUCTION

Flavonoids play a critical role in vegetal biology. They are widely distributed in fruits and vegetables and in plant food products, such as tea, coffee, cocoa, beer, wine among others[1, 2]. As a large group of bioactive chemicals, they have diverse biological functions. Flavonoids are essential to plant life controlling the auxin levels, which regulate plant growth and differentiation. Flavonoids in plants also act as antifungal and germicide, conferring coloration that can contribute to the pollination phenomena, fixing metals as iron and copper and making food unpalatable to predators. Structurally, flavonoids are diphenylpyranes (C6-C3-C6) consisting of two aromatic rings linked through three carbons forming an oxygenated heterocycle. Flavonols and flavones are flavonoids of particular importance. Foods containing high levels of such flavonoids show considerable antioxidant activity and free radical scavenging ability[3]. It was also indicated from epidemiological studies that their consumption reduces the risk of cancer and cardiovascular diseases[4-9]. The wide distribution of flavonoids in nature and the therapeutic importance of their use have promoted the development of a large number of detection and quantitation analytical methods pairing with an explosive increase of studies evaluating their biomedical properties. The qualitative and quantitative determination of flavonoid derivatives in natural matrices is a difficult task even for modern analytical techniques. Up to now, analysis of flavonoids is carried out by thin-layer chromatography[10-12], gas chromatography[13,14], capillary electrophoresis[15-20], electrochemical measurements[21-22] and high-performance liquid chromatography (HPLC)[23-33]. On the other hand, flavonoid derivatives are very efficient singlet oxygen quenchers in the lipidic environment of biological systems because they react very rapidly with singlet oxygen, however, to measure critical kinetic parameters accounting for flavonoid derivative reactivity, it is necessary to monitor flavonoid concentration changes in these complex systems. Absorption spectroscopic analysis of these flavonoids derivatives in microheterogeneous systems such as dipalmitoylphosphatidylcholine liposomes, is unviable because they present wavelength transitions non-distinguishable from absorptions of the bulk system components. Therefore, only liquid chromatography and/or capillary electrophoresis methods could lead to a robust protocol allowing separation of the flavonoid from the surfactant employed in preparing the organized aggregate and subsequent flavonoid derivative quantitation. In spite of this profusion of HPLC methods for quantifying flavonoids derivatives in a large number of different matrices we have not found in the literature a reliable protocol for analysis of flavonoids derivatives in systems mimicking biological membranes such as the lipidic bilayer of dipalmitoylphosphatidylcholine liposomes.

In this report, we present a reversed phase assay for the separation and simultaneous determination of four different flavonoids, rutin, morin, quercetin and 3-hydroxyflavone (Fig. 1) in a dipalmitoylphosphatidylcholine (DPPC) liposome matrix. The assay is robust and accurate requiring only routine laboratory equipment such as an HPLC with a diode array detector.

2. EXPERIMENTAL DETAILS

Chemicals

All solvents and reagents used were of reagent grade, spectroscopic or HPLC quality. Water was purified and deionized using a Waters Milli-Q system. 1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC), quercetin dihydrate, rutin monohydrate, morin dihydrate and 3-hydroxyflavone (Sigma) were used as received.

2.2. Liposome preparation[34, 35]

Blank multilamellar large liposomes, MLVs, were prepared by the thin layer evaporation method. In a typical experiment, 74.10 mg of dipalmitoylphosphatidylcholine were dissolved in a small amount of chloroform. The solution was put in a small round-bottomed flask, the organic solvent was evaporated under nitrogen stream and the dry lipid films were maintained 2 h under reduced pressure to remove solvent traces. Films were hydrated by adding an appropriate amount of 100 mM phosphate buffer pH 7.4, heated at a temperature 10 °C above the phospholipid gel-liquid crystalline phase transition temperature, to yield 10 mM phospholipid concentration, while shaking in vortex mixer. The phospholipid-buffer mixture was heated and shaked by short periods (four to six intervals of 1 min), until homogeneous milky suspensions were obtained. Then, the homogeneous suspension was carefully frozen using a liquid nitrogen bath for 5 min and thawed in a water bath kept at 60 °C for the same period of time. This cycle was repeated 5 times. MLVs can be frozen and stored at –22 °C and just thawed before the extrusion procedure. To obtain large unilamelar liposomes (LUVs), MLVs suspensions were repeatedly extruded (10 times) through a polycarbonate filter (pore size 200 nm) using a 10 mL Lipex extruder (Northern Lipids Inc.). During this process, the temperature of the extruder was maintained at 60 °C. The LUVs obtained were stored at 5 °C.

2.3. Preparation of reagents

Phosphate buffer (100 mM, pH 7.4) was typically prepared by mixing 100 mL of 0.1 M KH_2PO_4 and 78.2 mL of 0.1 M NaOH and adjusting pH to 7.4. The clear solution was filtered through a 0.45 μm nylon membrane filter and stored at 5 °C and used for 1-2 weeks. Standard stock solution of flavonoids in ethanol were prepared by weighing on a microbalance approximately 5 mg of flavonoid, dissolving in 3 mL of solvent in an ultrasonic bath at room temperature to get a clear solution and adjusting the volume (5-25 mL) with solvent. Calibration standards were prepared in ethanol-phosphate buffer pH 7.4 (4:3 v/v) by further diluting the standard stock solution. Flavonoid-liposome solutions standards were prepared by addition of small volumes of a standard stock solution of flavonoid in ethanol to 0.7 mL of 10 mM DPPC liposome solution. The mixture was homogenized in a vortex shaker and heated in a bath at 43 °C by 30 min. Then the solution was slowly cooled up to 20 °C and stored.

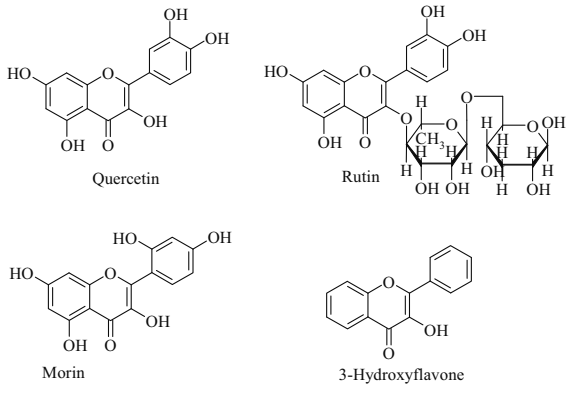

Figure 1. Structure of studied flavonoids.

2.4. High-performance liquid chromatography

The HPLC system Waters consisted of a Waters 600 controller, helium degasser, column thermostat, quaternary pump and a Waters 996 photodiode array detector. Chromatographic analysis was performed using a ODS Hypersyl (5 μm-particle size, 20 cm × 4.6 mm i.d.) column from Hewlett Packard. Also, a Chromolith RP-18e (2 mm macropore, 10 cm × 4.6 mm i.d.) column from Merck was tested. All experiments were carried out at column temperature of 25 °C. Isocratic elution at a flow-rate of 1 ml /min, was the most convenient method to achieve optimal separation between flavonoids and DPPC. The mobile phase consisted of a 75.5:23.5:1 v/v solution of water-acetonitrile-glacial acetic acid. These conditions were held for 50 min, the time required for flavonoid elution. After this time, a 60:40 v/v solution of methanol-isopropyl alcohol was added to the mobile phase for 2 min until 100% was reached. These conditions were held for 20 min to elute the DPPC. Initial conditions then were restored in 2 min and maintained during 10 min before a new injection, giving a total run time of 80 min. The diode array detector was operated at 370 nm with 4 nm bandwidth. The injection volume was set at 20 μL.

2.5. Preparation of flavonoid loaded liposome samples for HPLC analysis

Samples of flavonoid loaded liposomes for HPLC injections were prepared by diluting 300 μL of the liposome solution in 400 μL of ethanol in a conical plastic tube. The mixture was stirred in a vortex mixer for 2-3 min, allowed to rest for 10 min, and then centrifuged at 4000 g for 30 min. The supernatant was centrifuged again at 4000 g for 30 min and finally separated and employed for HPLC injections.

3. RESULTS AND DISCUSSION

The HPLC method described here was developed for flavonoid quantitation following the FDA guidelines[36]. Linearity, accuracy, precision, and method quantitation and detection limits were tested to ensure method suitability for quantitation of flavonoids included in DPPC LUVs. The near UV detection wavelength set at 370 nm was considered a good compromise between the sensitivity of the compound of interest and eventual interferences present in our kinetic experiments: photooxidation products generated in sensitized reactions, the sensitizer present in the same experiments, and the stability of the baseline. After several trials with mixtures of different compositions of water-methanol-glacial acetic acid and water-acetonitrile-acetic acid, the later was found to give the best compromise between resolution and analysis time. In addition, we tried different water-acetonitrile proportions. With water rich elution mixtures (> 80%) the retention time for quercetin increased considerably being close to 45 min, and the signal showed a noticeable tailing. In the opposite composition extreme, with acetonitrile > 90%, quercetin eluted with the solvent front. As a result, a mobile phase formed by a 75.5 : 23.5 : 1 v/v solution of water-acetonitrile-glacial acetic acid was preferred. Figure 2 shows that the isocratic elution described in Section 2.4 provides a good separation of the flavonoids from DPPC with retention times of 4.38, 13.03, 19.74 and 41.79 min, for rutin, morin, quercetin and 3-hydroxyflavone, respectively, without interference of Rose Bengal (RB) employed as sensitizer or of the photo-oxidation products. Chromatographic performance data for a typical run are presented in Table I.

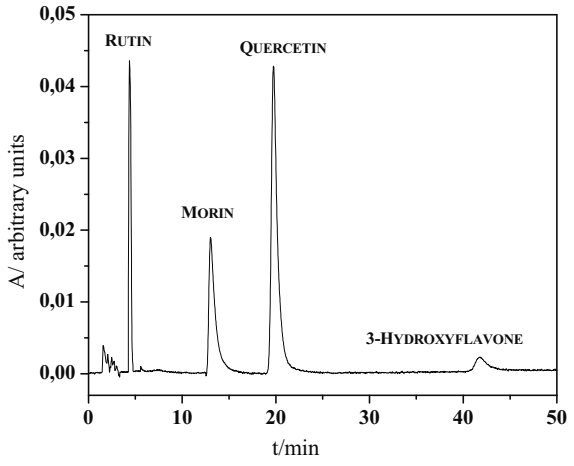

Figure 2. Chromatogram of a sample containing 26.3, 14.5, 29.0 and 30.6 μg mL^{-1} of rutin, morin, quercetin and 3-hydroxyflavone, respectively, in 10 mM DPPC LUV's.

TABLE I

Chromatographic performance data of the method.

Analite	Retention time/min	Tailinga	Retention Factor[b]	Resolution between critical band pair
Rutin	4.38	1.37	1.05	9.55
Morin	13.03	2.94	5.10	5.58
Quercetin	19.74	1.89	8.25	9.77
3-Hydroxyflavone	41.79	1,62	18.58	

[a]Tailing is defined as $W_{0.05}/2t_w$, where $W_{0.05}$ is peak width at 5% of peak height (min) and t_w is distance between peak front and peak retention measured at 5% of the peak height (min).
[b]Retention factor is defined as $(t_R - t_0)/t_0$, where t_R is retention time of peak (min) and t_0 is void time (min). Void time = 2.135 min for the method.

Resolution of 2.0 or greater is desired for critical band pair. Critical resolution of 9.55, 5.58 and 9.77 were observed between rutin-morin, morin-quercetin and quercetin-3-hydroxyflavone peaks, respectively. Tailing factors for rutin, quercetin and 3-hydroxyflavone are near to 1.5. Morin shows a larger tailing value, close to 3, which increases slightly the quantitation standard errors. However due to the larger values of the resolution between critical band pairs the overall quality of the method is not affected. Retention factor in the range of $0.5 < k' < 20.0$ is desired to clearly separate the first peak from void time and to avoid a higher retention time for the last band. Retention factors of 1.05, 5.10, 8.25, and 18.58 (with solvent front as unretained compound) were found for rutin, morin, quercetin and 3-hydroxyflavone, respectively, indicating a very good separation of rutin peak from void time and between successive peaks corresponding to the flavonoids errors. However the retention factor for 3-hydroxyflavone is close to 20.0, increasing the analysis time. We attempted to improve the retention factors of the flavonoid peaks by varying the mobile phase composition and changing the chromatographic column. However the results found employing a large set of mixtures, in which the water-acetonitrile relative volumes were varied, were not good. As

mentioned above, when water rich mixtures were employed (> 80% of water), the retention times for quercetin and 3-hydroxyflavone increased considerably, being close to 45 min and longer than 65 min, respectively. In addition, the signal showed a very noticeable tailing. With acetonitrile-rich mixtures (> 90% in acetonitrile) the retention factor and resolution decreased dramatically and rutin, morin and quercetin eluted with the solvent front. Using a Chromolith RP-18e column from Merck (2 mm macropore, 10 cm × 4.6 mm i.d.) with the optimized elution mixture, the chromatogram worsened considerably. Rutin eluted with the solvent front, morin and quercetin bands superimposed, and the 3-hydroxyflavone signal intensity decreased. No further attempts to improve the retention factor and resolution were performed with this column.

3.1. Linearity and range

Linearity, accuracy, precision, and method quantitation and detection limits were fully tested to ensure method suitability by using quercetin as target analyte, although similar results were obtained for rutin and morin. Linearity of the quercetin calibration standards was tested in the concentration range of 1.49-105.9 μg mL^{-1}. Calibration standards were prepared at various concentration levels.

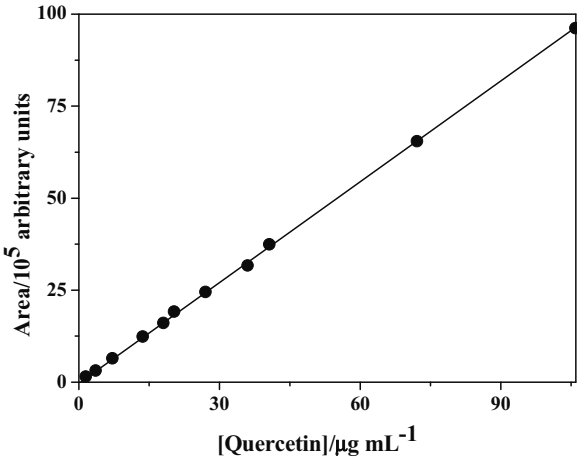

Figure 3. Response curve of quercetin in ethanol-phosphate buffer pH 7.4 (4 : 3 v/v).

It is clear from Fig. 3 that the curve is linear in this range of concentration and the correlation is suitable for quantitation. In our analytical conditions, the calibration curve shows a linear regression equation of y = 90770 x – 2681, where y is the peak area in arbitrary units and x is the quercetin concentration in μg mL^{-1}. The correlation coefficient (r) and the coefficient of determination (r^2) for quercetin were equal to 0.9998 and 0.9997, respectively. For the other flavonoids studied, the calibration curves show linear regression equations as well, namely y = 31089 x + 2243 (r^2 = 0.9997) and y = 38907 x - 20529 (r^2 = 0.9990) for rutin and morin, respectively, where x and y are defined as above. Typical chromatograms for quercetin standard solutions are shown in Fig. 4.

3.2. Accuracy

Accuracy studies were performed to determine the closeness between the true concentration value and the experimental results. Samples of quercetin-loaded liposomes were prepared and treated as described in 2.5. Preliminary experiments showed a recovery in the order of 75%, therefore it was crucial to determine if this value remains constant across a wide range of concentrations. Considering that in our kinetic experiments we employ an initial quercetin concentration close to 21 μg mL^{-1}, we defined this value as the target concentration. In addition, we typically monitor the quercetin consumption no further than two lifetimes. Consequently, the recovery study was performed at five different concentration levels (16.6, 33.3, 100.0, 133.3 and 166.6%) of the target concentration, a sufficiently wide range to guarantee that all quercetin concentrations measured in our experiments are covered. For each level, six preparations were tested. Table II summarizes the results of the accuracy experiments. Although from the analytical point of view a recovery of 75% is not a very good value, an average recovery of 76.2% was observed for quercetin, which is within ± 2.0% of the normally accepted value. Similarly, recoveries of 81% and 72.5% at one target concentration (20 μg mL^{-1}) were found for rutin and morin. For 3-hydroxyflavone, with a target concentration in the order of 30 μg mL^{-1}, an unsatisfactory recovery below 60% was found. However, the results obtained are appropriate for kinetic studies. In these experiments, a constant recovery value in the whole concentration range is a necessary condition, given that under pseudo order conditions, the ratio between the concentration at time zero and concentration at time t is currently employed.

TABLE II

Summary of method accuracy results.

Recovery solution at target level (%)	Theoretical concentration (μg mL^{-1})	Recovered average concentration[a] (μg mL^{-1})	RSD[a] (%)	Analytical recovery[a] (%)
16.6	3.51	2.65 (0.055)	2.07	75.5
33.3	6.99	5.37 (0.035)	0.65	76.8
100.0	21.20	16.33 (0.089)	0.55	77.0
133.3	28.29	21.15 (0.056)	0.26	76.2
166.6	35.19	26.62 (0.058)	0.22	75.6

[a]Based on six data points. Standard deviation is given in parentheses.

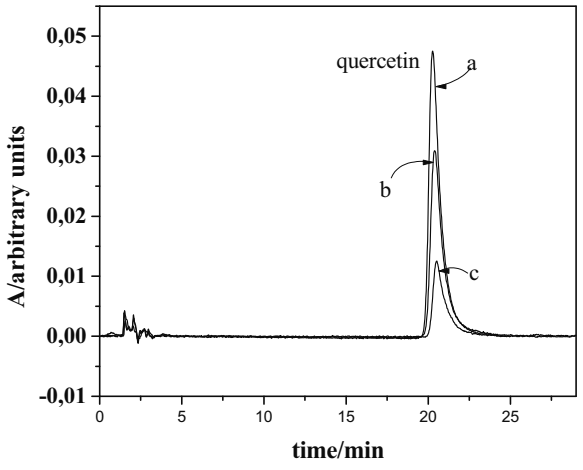

Figure 4. Chromatogram of quercetin standards in ethanol-phosphate buffer pH 7.4 (4:3 v/v). a) 27 μg mL^{-1}, b) 18 μg mL^{-1}, c) 7,17 μg mL^{-1}.

3.3. Precision

Instrument precision was performed as part of each sequence run at the beginning of the sequence. Ten injections of the target level of calibration standard were performed and the data were evaluated. Table III shows typical values obtained for a liposome preparation loaded with 7.25 μg mL^{-1} of quercetin. The mean retention time, area response, and the corresponding relative standard deviations are shown.

The relative standard deviations for both the retention times and area response are <1.50%, indicating sufficient instrument reproducibility for this method. For sample precision measurements, twelve replicate samples were prepared and analyzed on the first day.

TABLE III

Reproducibility of retention times and peak areas of the quercetin employed as standard compound.

Sample	Retention time/min	Area response/a.u.
1	19.87	505856
2	19.52	507633
3	19.67	497662
4	19.61	507661
5	20.24	503723
6	19.81	513841
7	20.26	512370
8	19.66	506850
9	20.21	515482
10	19.54	502825
Mean value	**19.83**	**507390**
R.S.D (%)	**1.4**	**1.06**

TABLE IV

Summary of method precision results.

Sample	[quercetin]/mg mL^{-1} Day 1	Day 2
1	5.51	5.48
2	5.60	5.52
3	5.62	5.57
4	5.49	5.41
5	5.62	5.60
6	5.57	5.45
7	5.44	5.39
8	5.69	5.61
9	5.67	5.59
10	5.61	5.55
11	5.71	5.60
12	5.57	5.53
Mean value	**5.59**	**5.53**
R.S.D (%)	**1.25**	**1.38**

For each sample preparation two injections were performed. On the second day, the same analyst prepared a second set of twelve samples from the same sample vial and independently analyzed them on the same system. The results for quercetin are summarized in Table IV. The RSD values are found to be <1.4% on each day and between days 1 and 2. These results clearly indicate sufficient sample repeatability with this method.

3.4. Quantitation limit

There are at least four different ways to determine quantitation limit of analytes[21],signal-to-noise ratio (S/N) being one of the most commonly used procedures. We have used this procedure for determining the quantitation limit of quercetin. Samples were obtained according to the protocol described in Section 2.5 from DPPC liposomes loaded with 7.25 μg mL^{-1} of the analyte. Signal-to-noise ratios were determined until a minimum S/N ratio of 10 was achieved. Using this method, quantitation limits of 1.12, 2.48 and 1.19 μg/mL were observed for rutin, morin and quercetin, respectively. Similarly, detection limits (defined at signal to noise ratio of 3) of 0.25, 1.13 and 0.41 μg mL^{-1} were also determined for rutin, morin and quercetin, respectively.

3.5. A kinetic experiment

Several steady-state experiments allowed us to evaluate the method's applicability to the study of the kinetics of the reaction between singlet molecular oxygen and quercetin (or other flavonoids) in liposomes. Typically, experimental chemical reaction rate constants were determined in 10 mM DPPC liposome solutions loaded with quercetin using a 10 ml double-wall cell, light-protected by black paint. A centered window allows irradiation with light of a given wavelength using Schott cut-off filters. Circulating water maintained the cell temperature at 20 ± 0.5 °C. The irradiation of the sensitizer, RB, was performed with a visible, 35 W, halogen lamp. Time dependent quercetin consumption was followed taking 300 μL samples of the reaction cell at several times, treating them as described in Section 2.5, and analyzing them by HPLC. Fig. 5 shows the results obtained in a duplicated analysis. The plot in Fig. 5 indicates that the decrease in quercetin concentration with the reaction time follows first-order kinetics, as expected. An experimental rate constant of 5.32×10^{-4} s^{-1} was calculated from the slope of the linear fit. Results obtained with real samples account for the robustness and accuracy of the analytical method to quantify flavonoids in DPPC liposome solutions.

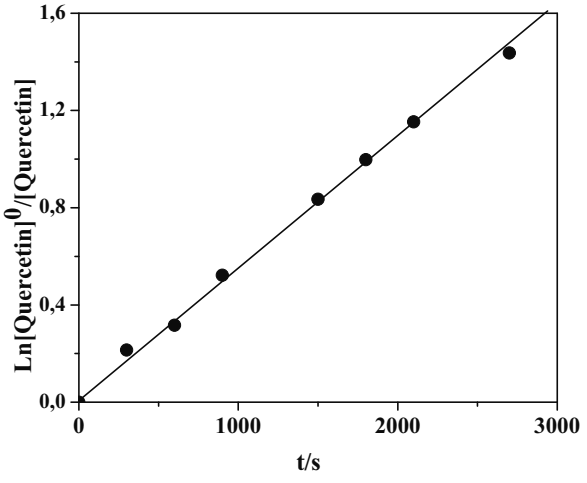

Figure 5. First-order plot (r^2 = 0.998) for the reaction between quercetin and singlet molecular oxygen in 10 mM DPPC liposomes employing RB as sensitizer. T = 22 °C.

4. CONCLUSIONS

A simple HPLC method for quantitation of flavonoids in DPPC liposome solutions has been developed. The method is simple, linear, precise, accurate, sensitive, and allows for the first time to quantify flavonoid derivatives in the presence of DPPC liposomes. In addition, this is a reliable method to perform kinetic experiments where the time dependent consumption of a flavonoid is monitored. These compounds are very efficient singlet molecular oxygen quenchers protecting biological membranes from the attack of reactive oxygen species.

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