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Utilization of inverted dispersive liquid–liquid microextraction followed by HPLC-UV as a sensitive and efficient method for the extraction and determination of quercetin in honey and biological samples

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ABSTRACT

A sensitive, rapid and efficient method for the extraction of quercetin as well as its determination in honey and biological samples was developed using inverted dispersive liquid–liquid microextraction (IDLLME) and HPLC-UV. The extraction method is based on the application of an extracting solvent lighter than water in the ternary component solvent (aqueous solution: extracting solvent: disperser solvent) system. The extraction parameters such as type and volume of extracting and disperser solvent, pH of sample, stirring rate and extraction time were optimized. Under the optimal conditions (extracting solvent: 100 μL 1-octanol; disperser solvent: 300 μL acetonitrile; pH of sample: 4.5 and stirring rate: 1000 rpm) a linear calibration curve was obtained in the range of 0.5–1000 ng mL^{-1} with $R^2 = 0.9993$ ($n = 10$). The limits of detection and quantification were 0.26 and 0.78 ng mL^{-1}, respectively. The extraction recovery was 97% and the preconcentration factor was 243. While the relative standard deviation for 25 ng mL^{-1} was 3.51 ($n = 5$), it was 2.12 ($n = 5$) for 500 ng mL^{-1} of quercetin. The method was successfully applied for the preconcentration and determination of quercetin in honey, urine and plasma samples.

1. Introduction

Flavonoids are the major secondary plant metabolites with antioxidant activity [1]. Because of its relative abundance in edibles including vegetables, especially onions (*Allium cepa*), fruits, tea and wine [2], quercetin is among the flavonoids attracting most attention. It has been reported to have biological properties that may play an important role in the prevention of human diseases, such as cancer, cardiovascular diseases, diabetes, ulcer, cataract and allergies [3–8]. Quercetin has a high propensity for electron transfers and is a free radical scavenger [9]. It may also be able to suppress the physiological reactions with heavy metals ions that are known to generate free radicals [10]; for this reason, many of the biological activities have been attributed to its antioxidant properties [11].

Nowadays, several kinds of honey are supplied in the markets that have to be checked in terms of quality, genuineness and labeling in order to inform consumers. The quality of honey is judged by botanical or floral origin and chemical composition [12]. Heretofore, the floral source of honey has been identified by the analysis of bee pollens present in honey matrices [13]. Tan et al. [14] suggested that a chemical approach might be more accurate and easily undertaken for the characterization of floral sources of honey. The analysis of phenolic compounds present in honey, for example represented by flavonoids, suggested the implementation of chemical approaches for the study of floral, geographical origin and quality [15]; for instance, it was found that quercetin is the useful flavonoid marker for sunflower honey [16]. Until now, numerous analytical methods such as capillary electrophoresis (CE) with ultra violet (UV) detection [17], gas chromatography (GC) with mass spectrometry (MS) detection [18], high performance liquid chromatography (HPLC) with UV [19], electrochemical [20] and MS [21] detection have been employed for the quantification of quercetin concentrations in the honey samples.

Quercetin mainly exists as glycosides, such as quercetin-4′-glucoside, quercetin-3-rutinoside (rutin) and quercetin-3-galactoside [22]. Investigations revealed that some quercetin glycosides could be absorbed in humans and the bioavailability of various quercetin glycosides is affected by their sugar moiety [23]; therefore, for the pharmacokinetic and bioavailability studies of flavonoid glycosides (such as rutin) possessing quercetin as aglycone, the estimation of quercetin in urine and plasma samples is imperative. Some of analytical methods suitable for the measurement of quercetin in biological fluids are consisted of: GC/MS [24], LC/MS [25], LC/MS/MS [26], HPLC with UV [27], fluorimetry [28], electrochemical [29] and MS [30] detection.

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Due to the complex matrix of the real samples and the low concentration of quercetin, making efforts to develop a simple and reliable method for preconcentration and determination of the quercetin is the main challenge and a very important step for the analysis of it. The preconcentration technique, which is commonly used to monitor quercetine in honey, urine and plasma, is solid phase extraction (SPE). The SPE procedures used were based on several solid sorbents such as Amberlite XAD, Bond Elut octadecyl C_{18}, Oasis HLB, Strata-X [31–34]. Up to our knowledge, the extraction of quercetin based on liquid–liquid extraction (LLE) [28] is very scarce. However, these methods are expensive, time consuming and labor intensive; thus, various microextraction techniques such as solid phase microextraction (SPME) [35], single drop microextraction (SDME) [36], hollow fibre–liquid phase microextraction (HF-LPME) [37], stir-bar sorptive extraction (SBSE) [38], cloud point extraction (CPE) [39] and dispersive liquid–liquid microextraction (DLLME) [40] have been masterminded to overcome LLE and SPE disadvantages and their use is becoming widespread.

DLLME has been developed by Assadi and co-workers [40] in 2006. DLLME is a new mode of LLE in miniaturized levels, which in comparison with the older classical methods, its consumption of organic extracting solvent is significantly lower and the obtained enrichment factor is much higher. DLLME employs a mixture of a high density extracting solvent, a water-miscible and polar disperser solvent. After a rapid injection of an appropriate mixture of extracting and disperser solvents into the aqueous sample a cloudy solution is formed. The contact area between the extracting solvent and the sample solution is extremely large; thus, the extraction equilibrium is obtained rapidly. After centrifugation, the sedimented phase is settled at the bottom of the conical test tube.

However, despite several advantages of DLLME, there is not any report about preconcentration of flavonoides by DLLME technique. Primarily, our investigations showed that the conventional extracting solvents used in DLLME such as dichloromethane, chloroform and carbon tetrachloride cannot extract quercetin. Furthermore, due to the centrifugation, there were some problems with gathering the extraction products from the bottom of the conical test tube because of the presence of the interfering materials in the biological samples. In 2009, Farajzadeh et al. [41] developed inverted dispersive liquid–liquid microextraction (IDLLME). In spite of the DLLME, IDLLME employs an extracting solvent with lower density than water and after centrifugation the extraction product is settled at the top of the sample.

For the first time, extraction of quercetin, one of the most important flavonoids, was developed using IDLLME-HPLC-UV which showed sufficient specificity and simplicity of operation for the measurement of quercetin in honey, human plasma and urine.

2. Experimental

2.1. Chemicals

Quercetin was purchased from Sigma–Aldrich (Steinheim, Germany). Methanol, acetonitrile, acetone (HPLC-grade) and phosphoric acid were purchased from Merck (Darmstadt, Germany). xylene, *n*-hexan, toloen, 2-ethylhexanol and 1-octanol were obtained from Aldrich (Milwaukee, WI, USA). Capsule of Geriatric Pharmaton manufactured by Bioggio-Switzerland on behalf of Boehringer Ingelheim International GmbH, Germany. The water used for mobile phase was double distilled deionized which was produced by a Milli-Q system (Millipore, Bedford, MA, USA,). A stock standard solution of quercetin ($100\,mg\,L^{-1}$) was prepared in methanol. The working solutions were prepared by appropriate dilution of the stock solution with double distilled/deionized water. All of the standard solutions were stored in the dark at 4 °C and pH 2 and brought to ambient temperature just prior to use.

2.2. Instrumentation and operating condition

The chromatographic analysis was performed on an HPLC system equipped with model 1525 binary solvent pump and a model 2487 dual λ absorbance detector set at 370 nm, all from Waters (Waters Assoc. Milford, MA, USA). The injector was a Reodyne, model 7725i (Cotati, CA, USA) fitted with a 20 μL loop. Separation was done by an isocratic elution on a C_{18} (250 mm × 4.6 mm, 10 μm) column from Dr. Maisch GmbH (Beim Brueckle, Germany). Mobile phase was a mixture of methanol and 0.3% phosphoric acid (58:42, v/v) with flow rate of $1.0\,mL\,min^{-1}$. Adjustment of pH was done by model 3030 Jenway pH meter (Leeds, UK). A Hettich Rotanta centrifuge model MIKRO 22R (Kirchlengern, Germany) was used to accelerate the phase separation.

2.3. Hydrolyzing the real samples

As mentioned above, quercetin mainly exists as glycosides [22]. The honey, urine and plasma samples contain the quercetin glucoronides (Fig. 1b) and free quercetin (Fig. 1a). In this study, the whole free quercetin was analyzed so the flavone glycosides were hydrolyzed before the analysis. According to the method of Wang et al. [42] for the hydrolysis of quercetin glucoronides, 1 ml of 25% HCl was added into 20 g of honey and 4.0 ml of urine and plasma samples and was mixed well for 30 min at 80 °C water bath; finally, the sample solution was diluted in 100 mL volumetric flask. Then, 20 mL of deluted and hydrolyzed sample was applied to IDLLME as described in Section 2.4.

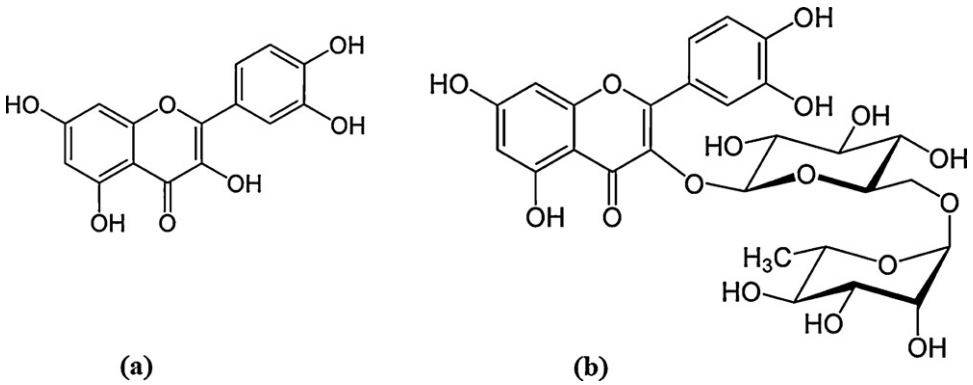

Fig. 1. Molecular structures of free quercetin (a) and rutin (b).

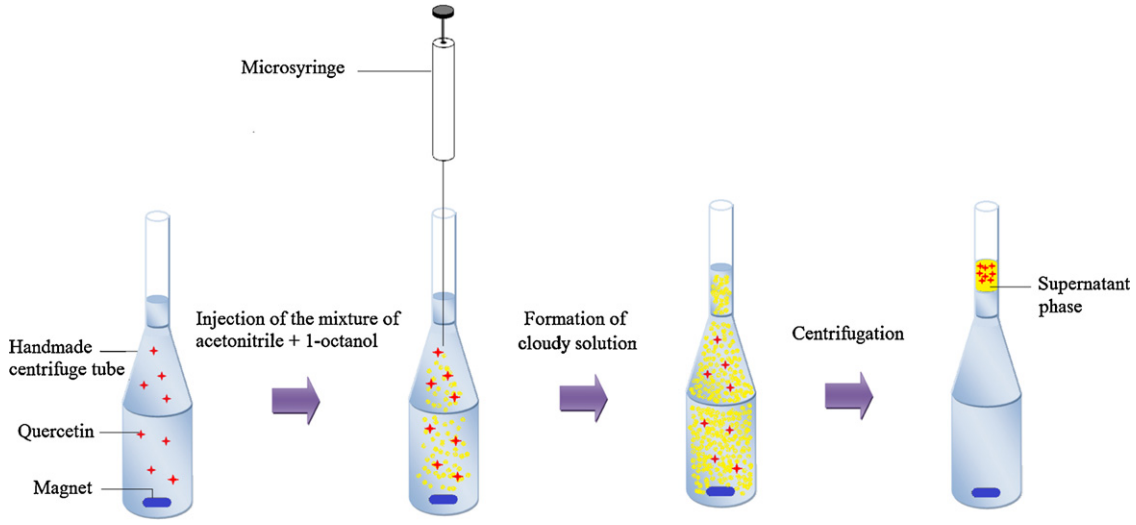

Fig. 2. Schematic diagram of IDLLME steps.

2.4. Inverted dispersive liquid–liquid microextraction procedure

A 20 mL of sample solution containing 0.1 mg L^{-1} of quercetin was placed in the handmade centrifuge tube (Fig. 2) with narrow neck (~4 mm i.d.) which was specially designed for ease of withdrawing supernatant phase. A mixture of 300 μL acetonitrile (as disperser solvent) and 100 μL 1-octanol (as extracting solvent) was injected into the sample solution using 1.0 mL syringe, rapidly and accompined by vortex mixing at 1000 rpm stirring rate, so that a cloudy mixture was formed. The cloudy solution was centrifuged for 5 min at 5000 rpm. Accordingly, after centrifugation the extraction product was settled at the top of the sample and in the neck of the handmade centrifuge tube (about 80 ± 2 μL) as supernatant phase. Finally, this product was injected to the HPLC using 20 μL sample loop. All the experiments were performed in triplicates and average of the results was reported.

3. Results and discussion

To obtain good sensitivity, precision and selectivity for extraction and determination of quercetin, the various experimental parameters which influence the efficiency of IDLLME procedure including extracting and disperser solvents as well as their volume, pH of the solution and stirring rate of stirrer were optimized using one variable-at-a-time optimization method.

3.1. Optimization of IDLLME

In order to obtain the optimized extraction condition, extraction recovery (*ER*) was used to evaluate the optimum condition. *ER%* was defined as the percentage of the total analyte (n_0) extracted into the supernatant phase (n_{sup}). Accordingly, calculation of the extraction recovery, as analytical response, was carried out using the following equation:

$$ER\% = \frac{n_{sup}}{n_0} = \frac{C_{sup} \times V_{sup}}{C_0 \times V_{sam}} \times 100 \quad (1)$$

where C_{sup} and C_0 are the concentrations of analyte in supernatant phase and initial concentration of analyte in aqueous sample, respectively. C_{sup} is determined from a calibration curve which was obtained using direct injection of standard solutions. V_{sup} and V_{sam} are the volumes of supernatant phase and aqueous sample, respectively.

The preconcentration factor (*PF*) was defined as the ratio between the analyte concentration in the supernatant phase (C_{sup}) and the initial concentration of analyte (C_0) in the aqueous sample, as follows:

$$PF = \frac{C\,sup}{C0} \quad (2)$$

Combination of Eqs. (1) and (2) gives:

$$ER\% = PF \times \frac{V_{sup}}{V_{sam}} \times 100 \quad (3)$$

3.1.1. Selection of disperser and extracting solvent

To obtain a good extraction recovery for IDLLME of quercetin, the selection of an appropriate solvent is very important. The extracting solvent has to meet two properties to extract the analytes efficiently: having lower density than water and having low solubility in water. Hence, xylene (density, 0.86 g mL^{-1}), *n*-hexan (density, 0.65 g mL^{-1}), toloen (density, 0.87 g mL^{-1}), 2-ethylhexanol (density, 0.83 g mL^{-1}) and 1-octanol (density, 0.82 g mL^{-1}) were considered for this purpose.

In order to choose disperser solvent in IDLLME, the miscibility in organic phase (extracting solvent) and aqueous phase (sample solution) is a key factor, which can disperse extracting solvent into very fine droplets in aqueous phase. Acetonitrile, acetone and methanol were compared as disperser solvent in the extraction of quercetin. For obtaining good efficiency, all combinations using xylene, hexan, toloen, 2-ethylhexanol and 1-octanol (250 μL) as extractant with acetone, acetonitrile, methanol (400 μL) as dispersive solvent were tried. As results shown in Fig. 3 indicates, acetone as the disperser solvent and 1-octanol as the extracting solvent provided maximum extraction recovery of 93% (extraction recovery percents were inserted at the top of the each column.). Therefore, we selected acetonitrile/1-octanol as a suitable set for subsequent experiment.

3.1.2. Effect of extracting solvent volume

To consider the effect of the extracting solvent volume on extraction recovery, different volumes of 1-octanol were tested. Therefore, the volume of disperser solvent (acetonitrile) was fixed at 400 μL and the volume of 1-octanol was changed from 100 to 350 μL. According to Table 1, it is clear that the volume of the supernatant phase increases by increasing the volume of 1-octanol, whereas extraction recovery is slightly constant which indicates the high distribution coefficients of quercetin in these conditions

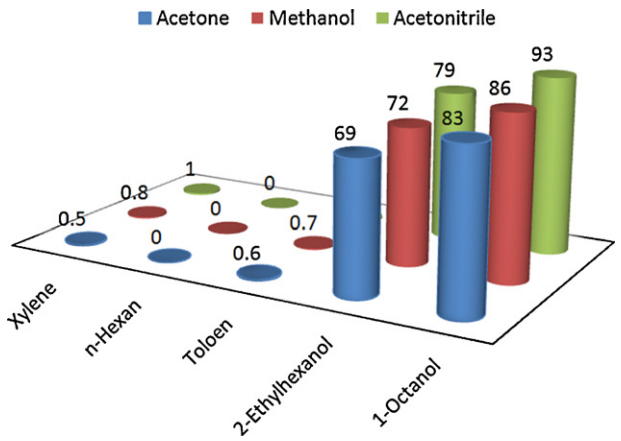

Fig. 3. Effect of the kind of extracting and disperser solvent on extraction recovery of quercetin using IDLLME. *Extraction conditions*: aqueous sample volume, 20 mL; disperser solvent volume, 400 μL; extracting solvent volume, 250 μL; pH of sample solution, 5.5; stirring rate: 750 rpm (extraction recovery percents were inserted at the top of the each column).

Table 1
Effect of extracting solvent volume on supernatant phase volume, ER (%) and PF.

Extracting solvent volume	Supernatant phase volume	*ER* (%)	*PF*
100	82	93	227
150	130	91	140
200	192	93	97
250	225	93	83
300	278	92	66
350	335	94	56

Extraction conditions: water sample volume, 20 mL; disperser solvent (acetonitrile) volume, 400 μL; extracting solvent, 1-octanol; pH of sample solution, 5.5; stirring rate: 750 rpm.

(100–350 μL extracting solvent volume). However, owing to the supernatant phase increases, preconcentration factor decreases with increasing the volume of 1-octanol. Therefore, in line with Table 1, at low volume of extracting solvent, high preconcentration factor and good recovery are obtained. Thereby, 100 μL of 1-octanol was chosen as optimum volume.

3.1.3. Effect of disperser solvent volume

To obtain optimized volume of disperser solvent, extractions were carried out by changing the volume of acetonitrile in the range of 100–500 μL. The represented results in Fig. 4, shows that with increasing the volume of acetonitrile, extraction recovery first increased till reached a maximum point at 300 μL and then became

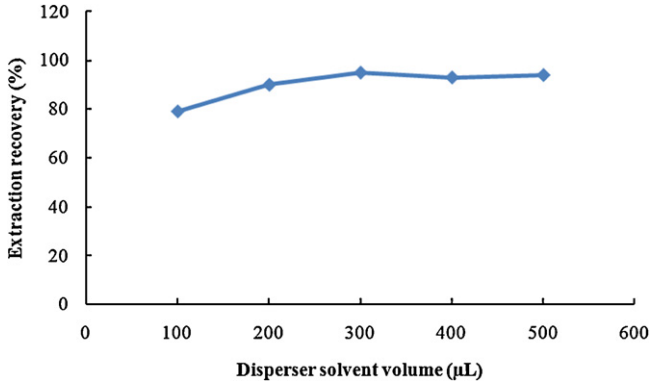

Fig. 4. Effect of disperser solvent (acetonitrile) volume on extraction recovery of quercetin using IDLLME. *Extraction conditions*: extracting solvent (1-octanol) volume, 100 μL; pH of sample solution, 5.5; stirring rate: 750 rpm.

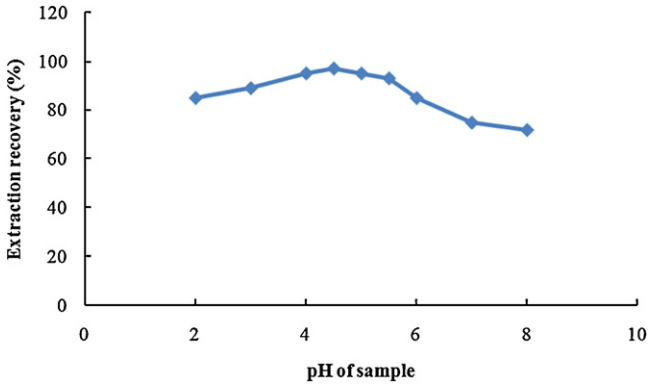

Fig. 5. Effect of pH of the sample solution on extraction recovery of quercetin using IDLLME. *Extraction conditions*: disperser solvent (acetonitrile) volume, 300 μL; extracting solvent (1-octanol) volume, 100 μL; stirring rate: 750 rpm.

almost fixed. It can be attributed to the fact that, at a lower volume of acetoneitrile consumption, cloudy state was not formed well and the extracting solvent (1-octanol) could not be well dispersed among aqueous solution in the form of very little droplet, which resulted in poor extraction recovery. Therefore, in the following experiments, 300 μL acetonitrile was used as optimal disperser solvent volume.

3.1.4. Effect of sample pH

pH of the sample is an important factor during liquid–liquid extraction (LLE) process involving analytes that possess an acidic or basic moiety. The ionic form of a neutral molecule formed upon deprotonation of a weak acid or protonation of a weak base normally does not extract through the organic solvent as strongly as its neutral form does. Thus pH should be adjusted to ensure that neutral molecular forms of the analytes are present prior to performing the microextraction step. In this step, effect of pH of the solution on the amount of extracted quercetin was investigated in the range of 2–8. Values of pH higher than 8.0 were not examined, because quercetin is a weak acidic compound which can be hydrolyzed at basic pH. As can be seen in Fig. 5, the best pH for extraction of quercetin is 4.5, that quercetin is completely in its molecular form.

3.1.5. Effect of stirring rate

In IDLLME, making a condition with stable cloudy solution is the most important goal of the analyzer to obtain a good extraction recovery. Investigation of the stirring rate of the stirrer in the range of 0–1000 rpm showed that at the rate of 750 rpm, this condition is provided and at the higher rate the variations of extraction recovery versus the stirring rate are not significant (Fig. 6). Hence, all the extraction experiments were performed at 1000 rpm stirring rate.

3.1.6. Effect of extraction time

In miniaturized preconcentration methods such as SPME and LPME, extraction time is one of the most consequential parameters. The time of extraction is defined as an interval time between injection of the mixture of disperser solvent (acetonitrile) and extracting solvent (1-octanol), and before starting to centrifuge. In this work, effect of the extraction time was examined from 0 to 30 min. The obtained results showed that the variations of extraction recovery against the extraction time are not significant. In IDLLME, the surface area between the extracting solvent and the aqueous phase is significantly large so that the transfer of the analyte from the aqueous phase into the extraction phase is carried out quickly. Therefore, the time of extraction was very succinct because equilibrium state was obtained very fast. On the other hand, the

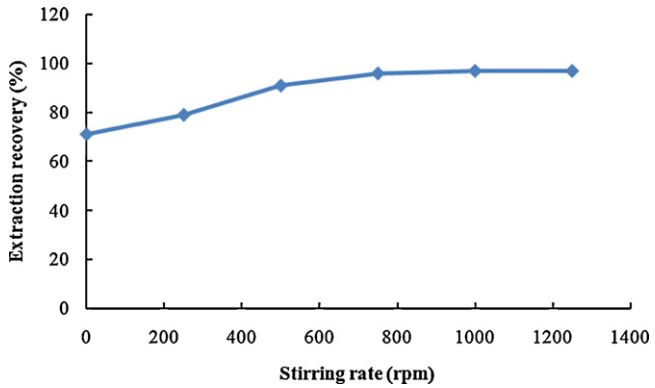

Fig. 6. Effect of pH of the sample solution on extraction recovery of quercetin using IDLLME. *Extraction conditions*: disperser solvent (acetonitrile) volume, 300 μL; extracting solvent (1-octanol) volume, 100 μL; pH of sample solution, 5.5.

most time consuming step in IDLLME is the centrifuging of sample solution in the extraction procedure, which is about 5 min.

3.2. Analytical performance of the IDLLME-HPLC for determination of quercetin

Under optimum condition, quantitative characteristics of the proposed method consisting linear range (LR), determination coefficient (R^2), limit of detection (LOD), limit of quantification (LOQ) and preconcentration factor (*PF*) were studied. For the purpose of quantitative analysis, a calibration curve for quercetin was obtained by spiking the standard directly into distilled water and extracting under the optimal conditions. Linearity was observed over the range of 0.5–1000 ng mL^{-1} of quercetin in the initial solution with a determination coefficient of 0.9993. The limit of detection and quantification, defined as LOD = $3S_b/m$ and LOQ = $10S_b$/m (where S_b is the standard deviation of blank and m is the slope of calibration graph after preconcentration.), were 0.26 and 0.78 ng mL^{-1} (n = 10), respectively. The relative standard deviation for 25 and 500 ng mL^{-1} of quercetin was 3.51 and 2.12 (n = 5), respectively. The *PF* defined as the ratio of the concentrations of analyte in the supernatant phase and in the aqueous sample solution (concentrations after and before preconcentration) was 243.

4. Analysis of real samples using IDLLME-HPLC

To evaluate the accuracy and applicability of the proposed method, the extraction and determination of quercetin in biological samples, i.e., honey, urine and plasma, were performed. All the samples were spiked with quercetin standard at four levels; subsequently, they were extracted using the IDLLME technique and finally the extracts were analyzed by HPLC method.

Honey samples were commercial one (Chashtgah, Shahdavaran Co.) and pure sun flower honey. pH of the 25 mL hydrolyzed honey sample (containing 5.0 g honey) was adjusted at pH 4.5 using concentrated HCl and NaOH; then it was stirred with a magnetic stirrer at room temperature for 5 min. The solution was filtered through 0.45 μm membrane filters (Millipore, Bedford, MA) in order to remove suspended solids. The measurement of quercetin was performed by standard addition method using calibration curves of 50–300 ng mL^{-1} spiked samples.

For determination of quercetin in human urine and plasma, a capsule (Geriatric Pharmaton) consist of 20 mg amount of rutin was orally administrated to a 30-year-old healthy male volunteer. The volunteer avoided from consumption of flavonoid containing foods (fruits, vegetables, wine and tea) 1 week prior to the study. Plasma sample (1.0 mL) was collected just before and at 6 h after administration, as well as urine sample. After hydrolysis and filtration, determination of quercetin was performed by standard addition method using calibration curves of 5.0–30 ng mL^{-1} and 0.5–3.0 ng mL^{-1} spiked samples for plasma and urine, respectively.

For each concentration level, three replicate experiments with the whole analysis process were made and experimental results are shown in Table 1. Relative recovery (*RR*) was calculated as follows:

$$RR(\%) = \frac{C_{\text{spiked}} - C_{\text{unspiked}}}{C_{\text{added}}} \times 100 \quad (4)$$

where C_{spiked}, C_{unspiked} and C_{added} represent the concentration of the analyte after adding a known amount of standard to the real sample, the concentration of the analyte in the real sample and the concentration of a known amount of standard that was spiked in the real sample, respectively. In urine and plasma samples collected just before the intake of capsule, quercetin was not

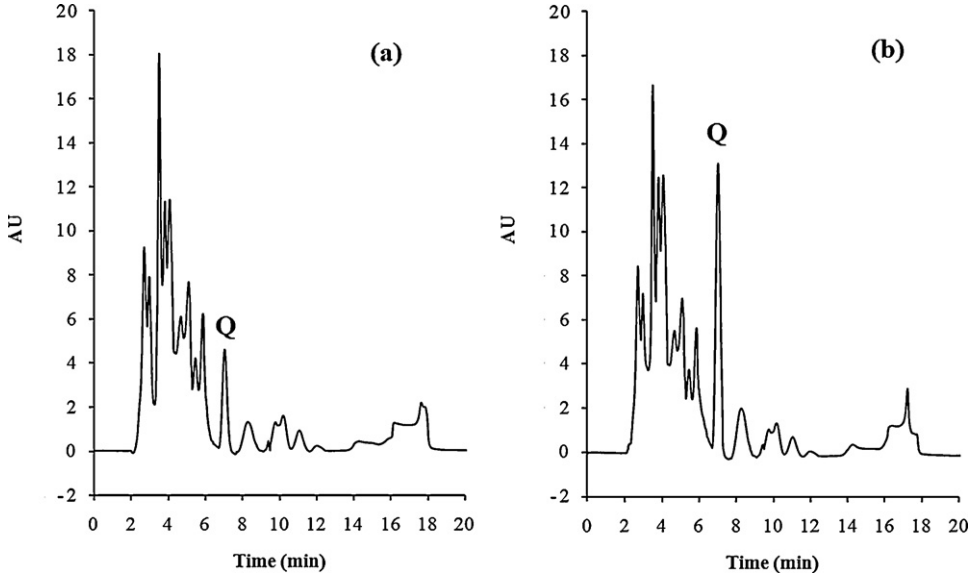

Fig. 7. Representative chromatograms of sun flower honey sample (a) and spiked sample of sun flower honey (b). Experimental details are described in the text. Q = quercetin.

Table 2
The application of presented method for determination of quercetin in the honey and biological samples ($n = 3$).

Samples	Quercetin added (ng mL^{-1})	Quercetin founded	Recovery (%) ± RSD (%)
Honey 1[a]	–	2.65[c]	–
	100	3.07	97.5 ± 3.1[e]
	300	4.08	98.3 ± 2.9
Honey 2[b]	–	3.33[c]	–
	100	3.75	97.9 ± 1.7
	300	5.93	98.2 ± 1.5
Plasma	–	11.70[d]	–
	10.0	21.07	97.1 ± 3.3
	30.0	40.62	97.4 ± 4.1
Urine	–	0.73[d]	–
	1.0	1.66	96.2 ± 4.9
	3.0	3.59	96.3 ± 4.6

[a] Commercial honey sample (Chashtgah, Shahdavaran Co.)
[b] Sun flower honey sample.
[c] μg g^{-1}.
[d] ng mL^{-1}.
[e] Mean value ± relative standard deviation.

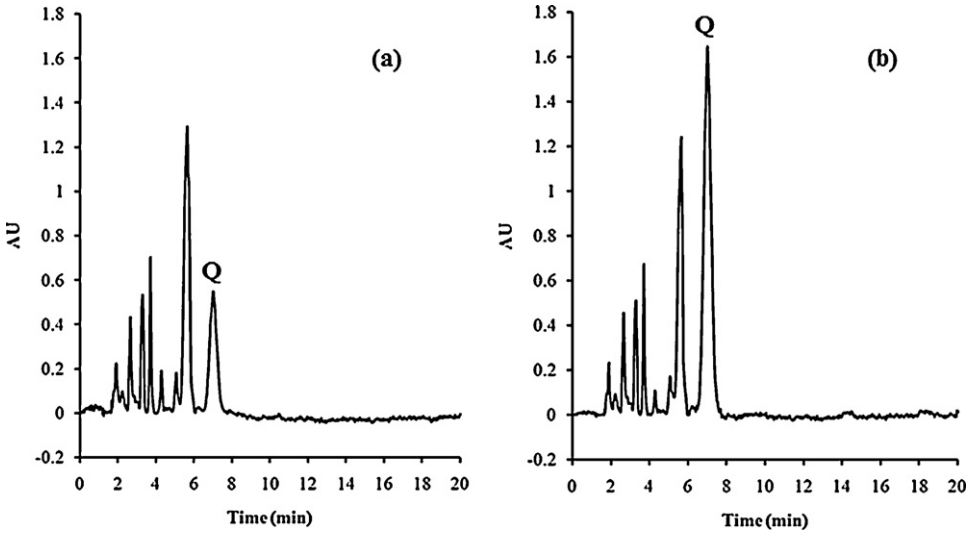

Fig. 8. Representative chromatograms of plasma sample (a) and spiked plasma sample (b). Experimental details are described in the text. Q = quercetin.

detected. Table 2 shows that with respect to the complexity of the matrices studied, the average result of three replicate analysis of each biological sample obtained by the proposed method are in satisfactory agreement (extraction recoveries between 96.2 and 98.3%) with the added amounts of quercetin standards, with RSD ($n = 3$) less than 5.0%. Figs. 7–9 show the chromatograms obtained from honey, plasma and urine samples by IDLLME-HPLC-UV, respectively.

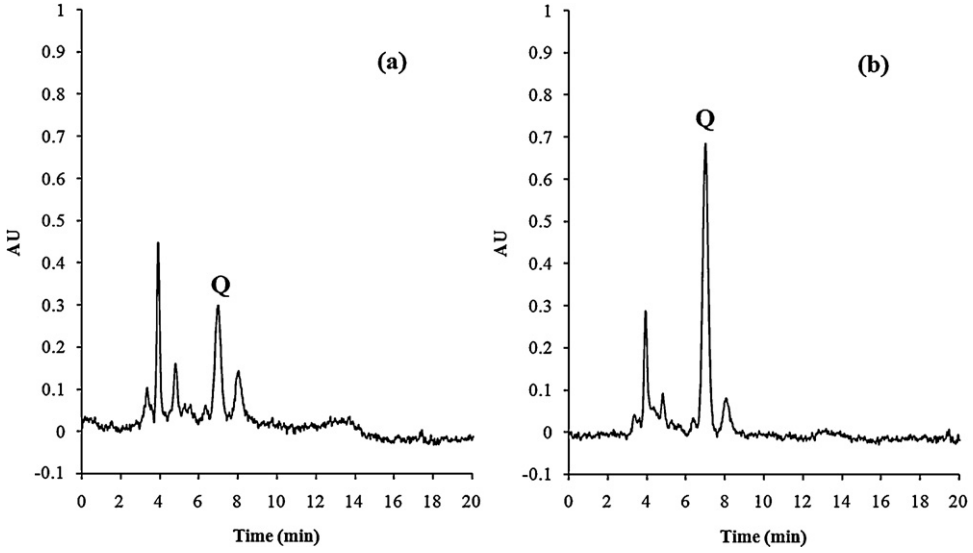

Fig. 9. Representative chromatograms of urine sample (a) and spiked urine sample (b). Experimental details are described in the text. Q = quercetin.

Table 3
Comparison of IDLLME with SPE.

Biological sample	Methods	LOD (ng mL^{-1})	LR (ng mL^{-1})	RSD%	References
Honey	SPE-HPLC-UV	60	–	<2.0	[33]
Urine	SPE-HPLC-UV	5	0.67–33.34	–	[27]
Plasma	SPE-HPLC-UV	0.35	4–700	<9.5	[43]
Honey, urine and plasma	IDLLME-HPLC-UV	0.26	0.5–1000	<5.0	Present study

5. Conclusion

In this paper, the new mode of DLLME technique, i.e., IDLLME coupled with HPLC-UV has been developed for rapid determination of quercetin, as one of the most abundant naturally polyphenolic compounds, in the biological samples. The proposed method permits the quantification of quercetin at trace levels; in comparison with SPE procedures previously reported [27,33,42] (Table 3), the proposed method shows lower LOD and wider linear range (about 10^4 magnitudes) for quantitative analysis of quercetin. The results demonstrate that regarding the complexity of the matrices studied, this analytical method has good recovery and reproducibility. Furthermore, in line with our previous work [34], SPE method is very time consuming toward the present work. The advantages of IDLLME method are simplicity of operation, rapidity, low cost, high recovery, high preconcentration factor and environmental benignity with wide application prospects in trace analysis. One of the best advantages of IDLLME in comparison with DLLME is the elimination of protein precipitation and centrifugation steps before the extraction process because in IDLLME the extraction phase is accumulated at the top of the sample solution after centrifugation. This analytical method is suitable for studying on pharmacokinetic and bioavailability of quercetin with sufficient specificity, simplicity and sensitivity, after consumption of foods and drugs containing this flavonoid. Also, it can be used in characterization of several types of honey based on their flavonoid marker compounds content.

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