Ionic Liquid-Based Cloud-Point Extraction of Quercetin for Its Sensitive HPLC–UV Determination in Juice Samples

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An ionic liquid-based cloud-point extraction (IL-CPE) method was developed for the extraction of quercetin in juice samples before its determination by high-performance liquid chromatography (HPLC). 1-Butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF6]) was used as the ionic liquid. The cloud-point extraction parameters such as sample pH, extraction temperature, extraction time, amount of ionic liquid, extraction volume, and salt concentration were carefully studied and optimized for the achievement of maximum extraction recovery. Under the optimized conditions, i.e., 20 min heating at 40 °C, 100 μL IL volume, pH 2.0, and no salt addition, a mean recovery of 92.5% and an enrichment factor of 20 were obtained for quercetin. Relative standard deviation of the method was 3.76% for 6 replicates, and the calculated detection limit (3σ) of quercetin was 0.002 mg L−1. The method, coupled to HPLC was successfully applied to the sensitive determination of quercetin in apple and grape juice samples with quantitative recoveries.

Keywords: Apple juice, grape juice, ionic liquid, cloud-point extraction, HPLC

Introduction

Quercetin is among the most attracting flavones, a subclass of flavonoids, distributed extensively in the plant kingdom [1]. It is known as a free radical scavenger [2] with substantial antioxidant activity [3]. It is believed that this important flavonoid plays an important role in the prevention of human diseases such as cancer, cardiovascular diseases, diabetes, ulcers, and allergies [4–7]. Several analytical methods such as gas chromatography–mass spectrometry [8], liquid chromatography–tandem mass spectrometry [9], high-performance liquid chromatography (HPLC) [10, 11], and fluorimetry [12] have been used for the determination of quercetin in natural samples. However, due to the complex matrix of real samples and the low concentration of quercetin, an extraction or preconcentration step is often required before its final determination [13–15]. The classic sample preparation methods are liquid–liquid extraction (LLE) and solid-phase extraction (SPE) techniques [16–18]. However, LLE and SPE are usually expensive and time-consuming methods and require large quantities of samples and toxic organic solvents.

Cloud-point extraction (CPE) is a fast, simple, green, and efficient separation and extraction technique that avoids the use of large quantities of organic solvents and provides high enrichment factors [19–21]. This technique is based on formation of a turbid solution by increasing the temperature or increasing additives in aqueous solutions of non-ionic surfactants [22].

Recently, the use of room temperature ionic liquids (ILs) as alternatives to surfactants or organic solvents has been reported in CPE [23] methods. ILs are less toxic than organic solvents and have excellent physiochemical properties such as low vapor pressure, good thermal stability, and tunable viscosity [24, 25]. ILs result from the combination of organic cations and anions and are usually liquids at room temperature [26].

In the present study, an ionic liquid-based cloud-point extraction (IL-CPE) method coupled to HPLC–UV is proposed for the determination of quercetin in juice samples. The possibility of using an ionic liquid for the extraction of quercetin in the CPE method is studied, and the advantages and disadvantages of the method are discussed. To the best of our knowledge, this is the first study on the use of IL-CPE to the extraction and enrichment of flavonoids.

Experimental

Reagents and Standards. Quercetin, methanol (HPLC grade), acetonitrile, acetone, and phosphoric acid were purchased from Merck (Darmstadt, Germany). 1-Butyl-3-methylimidazolium hexafluorophosphate ([BMIM][PF6]) was obtained from Kima-Elixir (Tehran, Iran). Double distilled water was used throughout. Stock standard solution of quercetin (1000 mg L−1) was prepared in methanol. The working solutions were prepared by appropriate dilution of the stock solution with double distilled water. All of the standard solutions were stored in the dark at 4 °C.

Instrumentation and Operating Condition. For spectrophotometric determinations, a Spekol, model 2000/1 (Germany), UV–visible instrument was used. A pair of quartz 350 μL cell (ES-quartz, model Q124, Spain) was utilized for absorbance determinations of test solutions. HPLC was performed with a Shimadzu, LC-10AD instrument comprising of two reciprocating pumps, a DGU-14A in-line degasser, a model CT10-10AC column oven, a high-pressure manual injection valve (with 20-L injection loop), and an SPD-10A UV–visible detector with the wavelength adjusted on 370 nm. Data acquisition and processing were performed with Class-vp v.R.6.1 software. Compounds were separated on a 25 cm × 4.6 mm i.d. RP-18 analytical column (Shim-Pack CLC-C18) packed with 5-m particles and equipped with a 1-cm guard column. The mobile phase was a 1:1 mixture of methanol and a 0.1 % phosphoric acid solution, delivered from separate pumps. A Hamilton, model wsc/4D all Pyrex was used for preparation of double-distilled water. For centrifugation of the extracts, a model 5810, Hamburg, centrifuge was used. A Memmert water bath, model TW14 (Germany), was used for temperature control of the samples.

Hydrolyzing the Real Samples. As mentioned above, quercetin mainly exists as glycosides. Apple and grape juice samples were received from Shadli Co. (Mashhad, Iran) and Sunich Co. (Saveh, Iran). The samples were hydrolyzed before the analysis. For the hydrolysis of juice samples, 2.5 mL of 25% HCl was added into 10.0 mL of the samples and the mixture...
was shaken for 45 min in an 80 °C water bath [13]. 2.5 mL sodium phosphate solution (0.05 mol L$^{-1}$) was added to the samples. The sample pH was adjusted on either 2 or 4, for the IL-CPE method, by dropwise addition of a 1.0 mol L$^{-1}$ sodium hydroxide solution. The mixture was stirred with a magnetic stirrer at room temperature for 5 min and diluted to 25 mL by water in a volumetric flask. The solution was filtered through a 0.45 μm membrane filter in order to remove suspended solids. Finally, 5 mL portions of the hydrolyzed samples were used for the CPE procedure.

The IL-CPE Procedure. In the IL-CPE method, 100 μL of the ionic liquid was added to 5 mL of the sample (pH 2.0) in a 15-mL screw cap conical tube. The mixture was shaken and then incubated in the water bath (40 °C) for a preset time (20 min). After that, the tube was taken out and cooled in an ice bath. Consequently, a cloudy solution was developed that was then centrifuged at 3000 rpm for 10 min for the phase separation. The supernatant solution was decanted, and the ionic liquid-rich phase was diluted with 100 μL methanol and directly injected into the HPLC–UV system for the analysis. A schematic representation of the steps of the method has been shown in Figure 1. During the optimization procedure, absorbance measurements at 370 nm were used instead of injection to HPLC.

Results and Discussion

Optimization of the IL-CPE Operation Parameters. The factors that affect the cloud-point extraction such as sample pH, extraction temperature, extraction time, amount of ionic liquid, and salting-out effect were investigated using spiked samples. A spectrophotometric method with absorption monitoring at 370 nm was used during the optimization procedure. Enrichment factor (EF) was the independent parameter to be optimized in order to improve the detection limit of the method. EF is defined as the ratio between the analyte concentration in the extraction phase and the original sample. Triplicate measurements under identical experimental conditions were performed, and the results were reported with standard deviations.

The effect of heating time on the IL-CPE was studied to achieve the maximum extraction efficiency. It was observed that, beyond 20 min, there was a decrease in the enrichment factor (Figure 2). Extraction temperature is a key factor for cloud-point extraction. In view of this, the effect of various temperatures was studied on the extraction efficiency of quercetin. For this purpose, the tubes were incubated for 20 min in a water bath at different temperatures, ranging from 30 °C to 70 °C. The maximum EF for quercetin was obtained at 40 °C, and no further improvement in the extraction efficiency was observed in higher temperatures (Figure 3).

Sample pH is an important factor during a CPE procedure for the analytes containing an acidic or basic group. Deprotonation of a weak acid or protonation of a weak base at extreme pH values increases the charge of the molecule and, therefore, decreases its extraction efficiency by an organic solvent. Thus, pH should be adjusted so to ensure that appropriate molecular forms of the analytes are present prior to performing the extraction. In this step, effect of pH of the solution on the amount of extracted quercetin was investigated in the range of 1 to 6. Higher pH values were not examined, because quercetin is a weak acidic compound which can be hydrolyzed at basic pH [13]. As can be seen in Figure 4, the best pH for extraction of quercetin is 2, in which quercetin is completely in its molecular form.

Volume of the extraction phase is one of the important parameters to be considered while developing an analytical method which involves preconcentration. For this purpose, different volumes of the ionic liquid (50–250 μL) were used, keeping the other extraction parameters unchanged (Figure 5). Volumes less than 50 μL of the IL could not be used as it gets dissolved in the reaction mixture and does not form the distinct phase upon cooling. It was observed that EF increased sharply from 50 μL to 100 μL and moderately decreased when higher IL volumes were used. The decrease in higher IL volumes may be a result of dilution of the extraction phase and the corresponding decrease in the preconcentration factor. For further experiments, 100 μL of the ionic liquid was used (Figure 5).
Ionic strength of sample may influence the partition of analytes between aqueous phase and organic phase (the salting-out effect). The effects of the ionic strength on the extraction efficiency were examined by adding different amounts of sodium chloride (0–5% \(\text{w/v}\)) into the aqueous samples. The results indicated that the salt addition decreases extraction efficiency. This could be attributed to the fact that the dissolution of sodium chloride in water increases the viscosity of the solution, which reduces the diffusion rate of the target analyte into the extraction solvent [27]. Furthermore, the addition of salt enhanced the solubility of IL in water. According to these facts, the subsequent experiments were carried out without addition of salts.

Analytical Performances of the Methods. Using the optimized conditions for the IL-CPE method, i.e., 20 min heating at 40 °C, 100 \(\mu\text{L}\) IL volume, pH 2.0, and no salt addition resulted in a mean recovery of 92.5% for quercetin and an enrichment factor of 20. Relative standard deviation of the method for 6 repeated determinations of quercetin by HPLC was 3.76%. The detection limit of quercetin determination by this method was calculated from 3\(\sigma\) \((n = 20)\) to be 0.002 mg L\(^{-1}\). A six-point calibration curve was drawn for the quantitation of quercetin with the method that was linear over a range of 1 to 20 mg L\(^{-1}\) with an \(R^2\) value of 0.998.

Application of IL-CPE to Juice Samples. A simple spectrophotometric method was satisfactory for the quercetin analysis during the optimization procedure. However, for the study of real samples, there was a risk of interferences. Therefore, an HPLC method was used for the analysis of juice samples. Table 1 summarizes the results of the analysis of three juice samples obtained by the developed IL-CPE method. There was a similar correlation between the added and found results, which shows the capability of the method in the enrichment and analysis of the juice samples. Figure 6 indicates typical chromatograms of an apple juice sample obtained by the proposed method before and after the preconcentration. The high signal enhancement of quercetin after the enrichment is obvious.

Conclusion

An IL-CPE method was successfully developed for the extraction and preconcentration of quercetin in juice samples. The proposed method is simple, rapid, environmentally friendly, and efficient. In comparison with previously reported methods for the determination of quercetin, the proposed methods have better or comparable detection limits, linear ranges, recoveries, and precisions (Table 2).
Table 2. Comparison of the proposed IL-CPE method with some reported methods in the literature for the determination of quercetin

| Methods                              | Linear range (mg L\(^{-1}\)) | LOD (mg L\(^{-1}\)) | RSD% | Recovery% | Ref.     |
|--------------------------------------|-------------------------------|---------------------|------|-----------|----------|
| IL based pressurized liquid extraction LC | 0.01–0.5                     | 0.0038              | 5.7  | 93.7–105  | [14]     |
| Fluorescence probe                    | 0.86–9.5                      | 0.031               | 2.5  | 93.3–105  | [12]     |
| Inverted DLLME-HPLC                  | 0.0005–1                      | 0.0003              | 2.12 | 97        | [13]     |
| HPLC–UV                              | 0.2–50                        | 0.15                | 6    | —         | [11]     |
| Microextraction by packed syringe LC  | 0.05–5                        | 0.006               | 1.6  | 83.0–97.7 | [15]     |
| IL-CPE                               | 1–20                          | 0.002               | 3.76 | 100–108   | This paper |

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