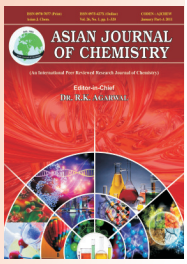
Asian Journal of Chemistry; Vol. 26, No. 16 (2014), 5058-5062

ASIAN JOURNAL OF CHEMISTRY

http://dx.doi.org/10.14233/ajchem.2014.16318



Determination of Four Flavonoids in *Cuscuta chinensis* Lam. by HPLC with Ultrahigh Pressure-Assisted Cloud Point Extraction

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Received: 10 September 2013; *Accepted*: 31 December 2013; *Published online*: 28 July 2014; AJC-15631

In this paper, a novel two-step extraction technique combining ultrahigh pressure-assisted micellar extraction with cloud point extraction was presented for the extraction and pre-concentration of four flavonoids (hyperoside, quercitrin, quercetin and kaempferol) from the seeds of *Cuscuta chinensis* Lam. (Tu-si-zi) for the first time. Various experimental conditions were investigated to optimize the extraction process. Under the optimal conditions, *i.e.*, 5 % (w/w) Triton X-100 solution, solid/liquid ratio of 1:30 (g/mL), with 100 MPa for only 1 min, the extraction efficiency of four flavonoids reached the highest value. The pre-concentration of four flavonoids by cloud point extraction were established as follows: the solution was incubated in a thermostatic water bath at 65 °C for 5 min and 15 % NaCl was added into the solution to facilitate the phase separation and increase the pre-concentration factor during the cloud point extraction process. The results indicated that the proposed method, combining two different and efficient techniques, offers satisfactory analytical features in terms of repeatability and reproducibility.

Keywords: Ultrahigh pressure extraction, Cloud point extraction, Flavonoids, Triton X-100, HPLC.

INTRODUCTION

Extraction is the first important step in the recovery and purification of active ingredients from plant materials and it determines the quality and credibility of the obtained results. Many techniques such as ultrasonic-assisted extraction (UAE)[1], microwave-assisted extraction (MAE)[2], supercritical fluid extraction (SFE)[3] and accelerated solvent extraction (ASE)[4] have been used to extract the active ingredients. These conventional extraction methods usually use large volume of toxic organic solvent and need heat processing or a long time for the extraction. Moreover, many natural products with low thermal stability may degrade and lose their biological activities during thermal extraction. Therefore some simple, no heat and environmental-friendly method is highly desirable.

The ultrahigh pressure extraction (UPE), as identified by US FDA[5], ranges from 100 to 800 MPa, has been widely used in extraction of chemical ingredients from plants or herbal materials, such as ginsenoside from ginseng[6], polyphenols from green tea leaves[7], salidroside from rhodiola sachalinensis[8] and flavonoids from litchi fruit pericarp[9]. All of these applications achieved high product yields with reduced processing time, energy and solvent consumption. The cloud point phenomenon of non-ionic surfactants occurs at a certain temperature and cause phase separation into phases: the large volume of aqueous and the small volume of surfactant-enrich phases[10]. The small volume of the surfactant-rich phase allows us to pre-concentrate the analytes[11,12]. This methodology offers the advantages of safety, low cost, ability to concentrate solutes, easy disposal of surfactant and low toxicity compared with classical organic solvents[13] and has been successfully used for the extraction and pre-concentration of compounds from plants, such as ginsenosides from ginseng[14], isoflavone daidzein from puerariae radix[15], aesculin and aesculetin from cortex fraxini[16], tanshinones from salvia miltiorrhiza bunge[10], glycyrrhizic acid and liquiritin from licorice root[17]. Therefore, the coupling of ultrahigh pressure-assisted extraction and cloud-point extraction could be an effective method for the rapid extraction and enrichment of active compounds from herbs without the organic.

Tu-si-zi, the seeds of *Cuscuta chinensis* Lam., is a widely used traditional Chinese herbal medicine[18]. The main active constituents of *Cuscuta chinensis* Lam. have been reported to be flavonoids. It has been not only used in improving and conditioning the liver and the kidney, but also been applied to improve sexual function and vision[19]. Many experiments have recently indicated that *Cuscuta chinensis* Lam. possessed pharmacological action on anticancer[20], immu-nostimulatory and antioxidant[21] activities.

In the present work, an ultrahigh pressure extraction-assisted micelle-mediated extraction with non-ionic surfactant (Triton X-100) and cloud point extraction pre-concentration of four flavonoids in Tu-si-zi were studied. The ultrahigh pressure extraction-assisted micelle-mediated extraction and cloud point extraction pre-concentration conditions were optimized. In comparison with conventional solvent extraction and concentration, the results indicated that this method is efficient, simple and environmental friendly.

EXPERIMENTAL

Dried seeds of *Cuscuta chinensis* Lam. were purchased from a local drug store. It was pulverized and sieved to generate samples with particle sizes up to 40 mesh screen. Authentic standards of hyperoside, quercitrin, quercetin and kaempferol (as shown in Fig. 1) were obtained from National Institute of the Control of Pharmaceutical and Biological Products (China). Tween 80, OP-10 and Triton X-100 were purchased from Tianjin Kemiou Chemical Co. Ltd. (Tianjin, China) and prepared in de-ionised water. HPLC-grade acetonitrile was obtained from Tedia (USA). All other reagents were of analytical grade.

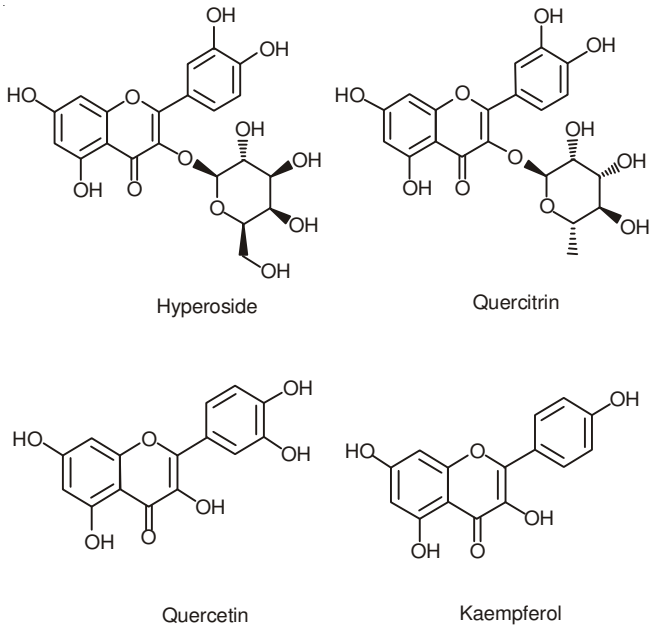

Fig. 1. Structure of four flavonoids (hyperoside, quercitrin, quercetin. kaempferol)

Ultrahigh pressure-assisted extraction was conducted with a High Hydrostatic Pressure Processor (HPP.L3-600, Huataisenmiao Biology Engineering Technology Co. Ltd., Tianjin, China). The pressure ranged from 100 to 600 MPa and the pressure precision was controlled at ± 5 MPa.

An Agilent 1120 HPLC G 4290A system, which was equipped with a solvent delivery pump, a UV detector, an on-line degasser, an automatic injector and an Agilent Chemstation for data treatment, was used for analysis of the samples.

Extraction of Tu-si-zi by ultrahigh pressure extraction: Sample powder (0.25 g) mixed with extraction solvent was poured into a sterile polyethylene bag. The bag was sealed by heating and subjected to ultrahigh pressure treatment. Then the system was subjected to an ultrahigh pressure for a given period. The type of surfactants (Triton X-100, OP-10, Tween 80), concentration of surfactants (1, 3, 5, 7, 9 and 12 %), solid/liquid ratio (1:10, 1:20, 1:30, 1:40, 1:50 g/mL), extraction pressure (100, 200, 300, 400, 500 MPa) and extraction time (1, 2, 3, 4, 5 min) were systematically studied. Following the ultrahigh pressure treatment the mixture was filtered through a syringe filter to remove the solid. The extraction solution was centrifuged at a speed of 2000 rpm for 4 min. Then the supernatant was filtered through 0.45 μm membrane and collected for cloud point extraction. The filtrate was injected into the HPLC for further analysis. All experiments were carried out in triplicate.

Traditional extraction methods: Heat reflux extraction (HRE) and ultrasonic-assisted extraction (UAE) were chosen as the traditional extraction methods compared with ultrahigh pressure extraction. 5 % (w/w) Triton X-100 solution was selected as solvent for two extraction methods. The dried plant sample (0.25 g) was weighed in a flask and then 10 mL extraction solvent was added. Heat reflux extraction was carried out at 100 °C for 1 h. Ultrasonic-assisted extraction was carried out at 30 kHz for 30 min. All experiments were carried out in triplicate.

Cloud point extraction procedure: After ultrahigh pressure extraction and centrifugation, the supernatant was transferred into a 10 mL centrifuge tube. An appropriate amount of sodium chloride was added to the sample solution. Then the sample solution was kept in a thermostatic water bath at a fixed temperature until the solution completely separated into two phases. After centrifugation at a speed of 2000 rpm for 4 min, the aqueous phase was sucked out using a syringe and the sticky surfactant-rich phase was left in the tube. 1 mL of the surfactant-rich phase was transferred and diluted to 10 mL with ethanol to lower the viscosity of the surfactant-rich phase. After filtration through a 0.45 μm membrane, the aqueous phase and surfactant-rich phase were injected into the HPLC for analysis, respectively. All experiments were carried out in triplicate.

HPLC analysis: The HPLC analyses were accomplished with an Inertsil ODS-SP C_{18} column (250 × 4.6 mm) at 350 nm and column temperature of 25 °C. The mobile phase, a solution of acetonitrile and 0.1 % (v/v) phosphoric acid ingradient elution mode (0-30 min, 5-30 % acetonitrile; 30-35 min, 30-50 % acetonitrile; 35-36 min, 50-100 % acetonitrile; 36-45 min, 100 % acetonitrile), was set at a flow-rate of 1 mL/min and the injection volume was 10 μL.

RESULTS AND DISCUSSION

To optimize the ultrahigh pressure extraction-assisted micellar extraction of four flavonoids from Tu-si-zi, a number of experiments under different conditions were performed, such as the kinds of surfactants, concentration of the surfactant solution, extraction pressure, extraction time and liquid/solid ratio. The influence of each individual factor was investigated by mono-factor experiments. The sum of each flavonoid contents was used as the marker for evaluation of extraction efficiency. The experiment procedures and results were presented as follows.

Selection of surfactants: Compared to traditional solvents, such as ethanol and methanol, Triton X-100 solution showed higher extraction efficiency. Compare with the micelle extracted amounts of four flavonoids in 5 % (w/w) of Tween 80, Triton X-100 and OP-10 solutions, Triton X-100 solution was proven to be the best (Fig. 2a). This can be explained by more thorough diffusion of the surfactant solution into the solid matrix[17]. Surfactants increase the mass-transfer coefficient during desorption of soluble ingredients from the plant matrix to water. At the same time the use of Triton X-100 solutions instead of organic solvents for sample preparation was proven to be safer, less expensive and more environmental friendly.

Effect of surfactant concentration: In order to screen the optimal extraction solvent, the effect of Triton X-100 concentration, ranging from 1 to 12 % (w/w), was carried out. As shown in Fig. 2b, with the increasing of Triton X-100 concentration from 1 to 5 % (w/w), the extraction yields of four flavonoids noticeably increased from 3.69 to 4.95 g/mL. Further increase of Triton X-100 concentration did not increase the extracted amounts. Therefore, 5 % Triton X-100 was used as the solvent

Effect of extraction pressure: A group of samples were extracted accordingly to the previously optimized conditions at different control pressure, ranging from 100 to 500 MPa and the results were summarized in Fig. 2c. Ultrahigh pressure can increase the rate of mass transfer and enhance both solvent penetration into the solid material and the release of intracellular product by disrupting the cell walls. As shown in Fig. 2c, when the extracting pressure achieved at 100 MPa, there was enough solvent enter cells and the compounds permeated out to the solvent and the highest yield of four flavonoids content was obtained. Thus, 100 MPa was used for the subsequent study.

Effect of extraction time: The effect of extraction time on the extraction efficiency of four flavonoids was studied by varying the extraction time from 1 to 5 min. It was evident from Fig. 2d that the extraction efficiency of the four flavonoids remained approximately constant when the extraction time increased. This can be explained as follows: under high pressure, the solvent will permeate very fast through the broken membranes into cells and the mass transfer rate of solute or the rate of dissolution is very large. Therefore, 1 min was sufficient for the process of ultrahigh pressure extraction.

Effect of solid/liquid ratio: The solid/liquid ratio was also an important factor with respect to increasing the extracted amount of four flavonoids. Excessive solvents not only increase the extract efficiency, but also create unnecessary waste. As shown from Fig. 2e, the optimum solid/liquid ratio was 1:30 (g/mL).

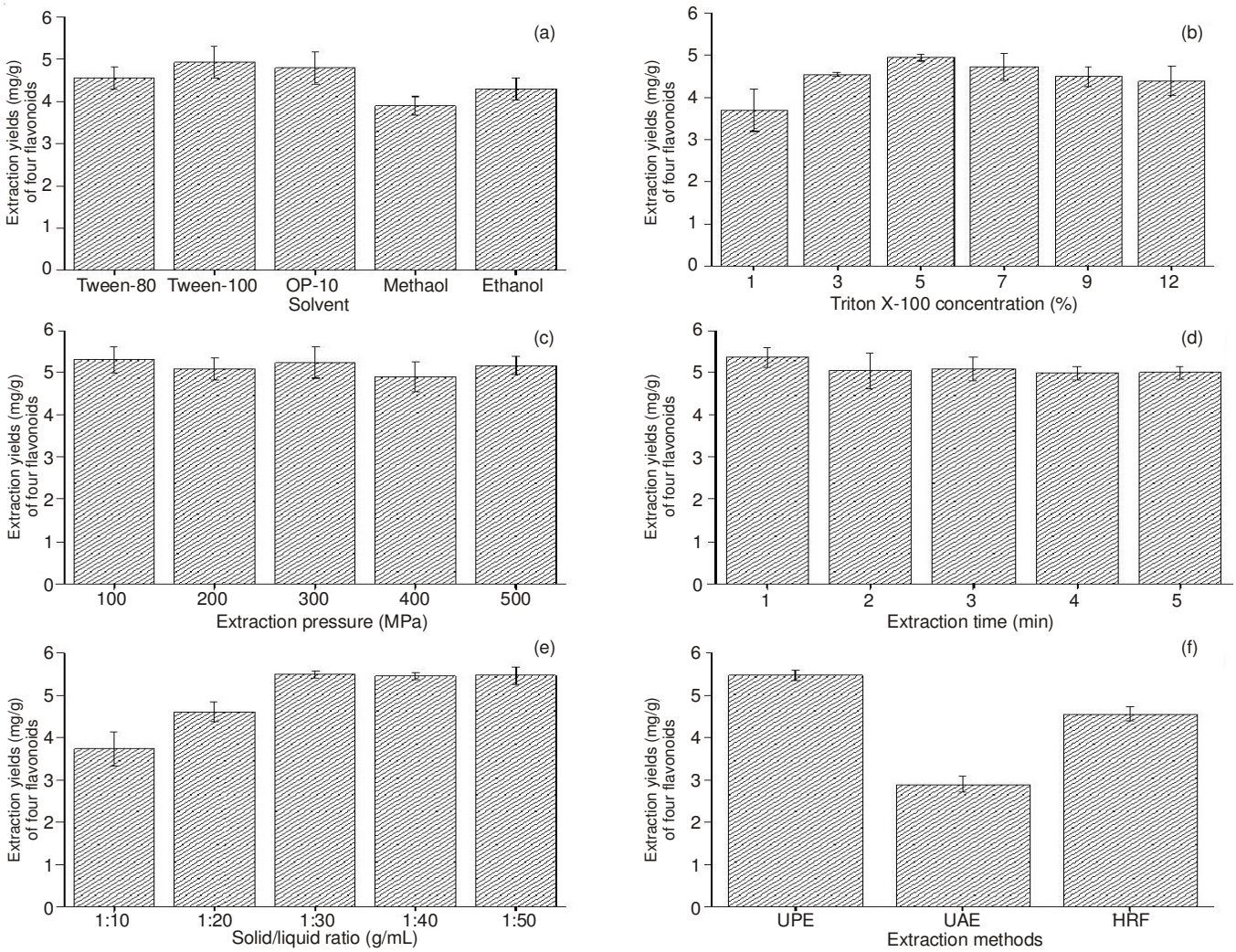

Fig. 2. Effects of different solvent (a), Triton X-100 concentration (b), extraction pressure (c), extraction time (d), solid solvent ratio (e), extraction methods (f) on the extraction yields of four flavonoids

Comparison with other extraction methods: To further investigate the advantages of ultrahigh pressure extraction method, parallel experiments were carried out with ultrasonic-assisted extraction and heat reflux extraction. From Fig. 2f, it is apparent that the ultrahigh pressure extraction method had the highest extraction yields. While the extraction time of ultrahigh pressure extraction, ultrasonic-assisted extraction and heat reflux extraction was 1 min, 30 and 60 min, respectively. Therefore, ultrahigh pressure extraction can greatly reduce the extraction time and have higher product yield in this system.

Optimization of pre-concentration conditions: After the micelle-mediated extraction process, the flavonoids and surfactant formed micelles. Upon appropriate alteration of the conditions such as temperature or addition of salts, the pre-concentration of the flavonoids by cloud point extraction (CPE) was systemically studied. In this paper, the effect of sodium chloride concentration (5-20 %, w/w), equilibration temperature (55-85 °C) and time (5-25 min) on the performance of the cloud point extraction has been investigated. The cloud point extraction efficiency (CPEE) and the pre-concentration factor (CF)[16] were evaluated as follows:

$$\text{CPEE}\ (\%) = \frac{\text{amount of analyte determined after CPE}}{\text{amount of analyte determined before CPE}} \times 100\ \%$$

$$\text{CF} = \frac{\text{volume of the extraction solvent}}{\text{volume of the obtained surfactant} - \text{rich phase}}$$

Effect of sodium chloride concentration: It has been reported that the addition of electrolytes may decrease the cloud point temperature and facilitate the separation of the two phases[21]. In this study, different concentrations of sodium chloride ranging from 5 to 20 % (w/w) were investigated in 70 °C water bath for 15 min. The results indicated that when the concentration of sodium chloride was 5 %, the extraction solution could not be divided into two separate phases. From Fig. 3a, it was apparent that cloud point extraction efficiency was increased up to sodium chloride concentration of 15 % and remained constant above that. At the same time, the volume of the surfactant-rich phase decreased when the amount of salt added increased. Considering cloud point extraction efficiency and pre-concentration factor, a concentration of 15 % (w/w) sodium chloride should be chosen for cloud point extraction.

Effect of equilibrium temperature: The dependence of the recovery of hyperoside, quercitrin, quercetin and kaempferol on the equilibration temperature were shown in Fig. 3b. When the temperature was under 60 °C, the solution could not be divided into two separate phases and with the equilibrium temperature increasing from 65 to 85 °C, the cloud point extraction efficiency of each compound decreasing slightly or remaining approximately constant. This phenomenon might be caused by the flavonoids instability at relatively high temperatures. In addition, the value of pre-concentration factor was maintained at 13. Based on these results, 65 °C was selected as the equilibrium temperature.

Effect of equilibrium time: The effect of the equilibrium time from 5 to 25 min on cloud point extraction efficiency of each flavonoid was investigated (Fig. 3c) and meanwhile the equilibration temperature was kept at 65 °C and the salt concentration was 15 %. As shown in Fig. 3c, longer equilibrium times did not have significant effects upon cloud point extraction efficiency and pre-concentration factor. Therefore, 5 min was chosen as the optimum equilibration time for this work.

Optimal condition: Based on the above discussion, 5 % of Triton X-100 solution was used to extract four flavonoid from Tu-si-zi by ultrahigh pressure extraction with an extraction pressure of 100 MPa in 1 min and a solid/liquid ratio of 1:30 (g/mL). The extraction efficiency of four flavonoids was 5.47 ± Ó 0.21 mg/g. Then, the extract was filtrated and cloud point extraction process was employed. After equilibrium 5 min at 65 °C and adding 15 % sodium chloride, the cloud point extraction efficiency of hyperoside, quercitrin, quercetin and kaempferol were 92.98, 0.21, 96.92, 0.16, 98.56, 0.19, 99.08 % and 0.12 %, respectively. The cloud point extraction efficiency increased with the decreasing of the polarity of flavonoids. This phenomenon indicates that cloud point extraction method is more suitable for hydrophobic compounds than for hydrophilic ones. The HPLC chromatograms of four flavonoids in optimal condition as well as the extracted and pre-concentrated ones were shown in Fig. 4.

Method validation: Peak area was used for the quantification of the extracted hyperoside, quercitrin, quercetin and kaempferol. Calibration graphs were obtained by plotting the peak area (y) *versus* concentration (x). Calibration curves were shown in Table-1.

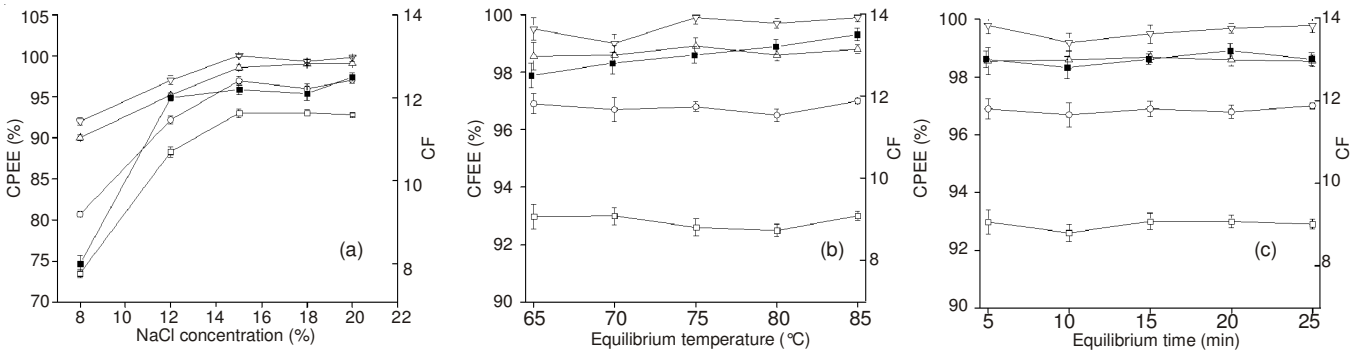

Fig. 3. Effect of sodium chloride concentration (a), equilibrium temperature (b), equilibrium time (c) on cloud point extraction efficiency and pre-concentration factor by cloud point extraction □: cloud point extraction efficiency of hyperoside ○: cloud point extraction efficiency of quercitrin △: cloud point extraction efficiency of quercetin ▽: cloud point extraction efficiency of kaempferol ■: pre-concentration factor value

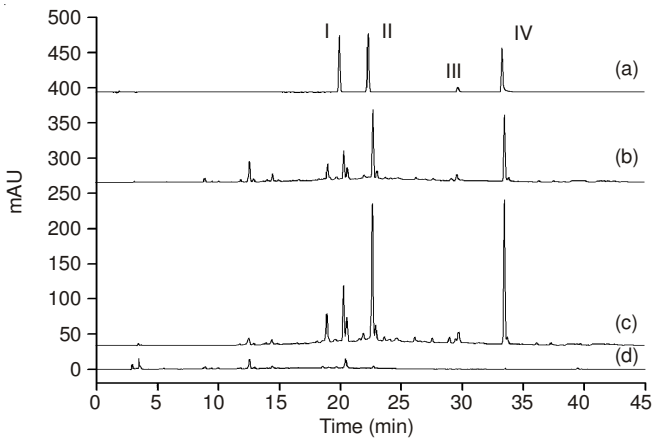

Fig. 4. HPLC chromatograms of four flavonoids standard solution (a), 1 mL of original extraction solution diluted to 10 mL (b), 1 mL of surfactant enrich phase after cloud point phase separation diluted to 10 mL (c), 5 mL of aqueous after cloud point phase separation diluted to 10 mL (d). I: hyperoside, II: quercitrin, III: quercetin, IV: kaempferol

TABLE-1
FEATURES OF HYPEROSIDE, QUERCITRIN, QUERCETIN AND KAEMPFEROL

Parameter	Linear range (μg)	Linear equation	Correlation coefficient
Hyperoside	0.0225-0.510	Y = 2694.75796x + 9.48637	0.9999
Quercitrin	0.0250-0.500	Y = 2898.42406x + 3.56627	0.9998
Quercetin	0.0032-0.064	Y = 2776.64263x - 2.83077	0.9996
Kaempferol	0.0250-0.500	Y = 2650.3215x - 26.22604	0.9993

The repeatability of the HPLC profile was determined by injecting the same sample five times on the same day. The RSD of retention time was 0.45 % for hyperoside, 0.27 % for quercitrin, 0.21 % for quercetin and 0.15 % for kaempferol, respectively. The RSD of peak area of hyperoside, quercitrin, quercetin and kaempferol was 1.89, 1.51, 1.02 and 0.95 %, respectively.

Conclusion

The ultrahigh pressure extraction-assisted micelle-mediated extraction was successfully applied to the extraction and pre-concentration of four flavonoids in Tu-si-zi. The process was only 6 min from extraction to pre-concentration. Compared with the other techniques, it was proven to be a high efficient and environment friendly method. Taking into account of the advantages of ultrahigh pressure extraction and cloud point extraction, these sample extraction and pre-concentration techniques are worth of further exploration.

ACKNOWLEDGEMENTS

Financial supports from the Scientific and Technological Development Project of Shandong Province (2011SJGZ19), Large Scientific Instruments and Upgrading Technology Research Project of Shandong Province (2012SJGZ01) and Shandong Analysis and Test Center Fund are gratefully acknowledged.

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