Original Article

Optimization of Oleuropein Extraction from Organic Extracts Using a Microfluidic Device and Response Surface Methodology

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Abstract

Background and Aim: Pharmaceutical applications of natural materials from plants date back to ancient times. Oleuropein is one of the compounds that are found in olive leaves, fruit, bark and roots of *Olea europaea* (the olive tree) that cause the bitter taste of olive oil and fruit. The aim of this study was to develop and optimize the microfluidic device for the extraction of oleuropein into the aqueous phase using response surface methodology.

Materials and Methods: In this study, a microfluidic device was used for the extraction of oleuropein from the organic extract of olive leaves into the aqueous phase. The effects of main parameters on the extraction efficiency including temperature, flow rate ratio (ethyl acetate/water) and residence time were investigated and optimized by response surface methodology (RSM).

Results: The maximum extraction yield was obtained under the following conditions: deionized water as extractant phase, temperature of 40℃, flow rate ratio of 0.16 and residence time of 0.1010 min. The extraction yield of 70.93% was obtained under the above conditions with relative standard deviation of 2.0 %. The results of the analysis of variance indicate that unlike temperature, flow rate ratio and residence time are significant parameters. Moreover, square of temperature, flow rate ratio, residence time and interaction of flow rate ratio and residence time are significant factors. The extraction yield of the proposed microchannel was compared with conventional batch extraction method.

Conclusion: The results show that the proposed technique can successfully be applied to the extract of oleuropein from the organic phase into the aqueous phase.

Keywords: Extraction, Olive Leaves, Oleuropein, Microfluidic, Response Surface Methodology

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Introduction

Liquid-Liquid Extraction (LLE) techniques are used for several purposes including concentration and extraction of analytes and also the removal of interferences in different fields such as industrial, analytical and medicinal fields. The conventional LLE method suffers from a number of disadvantages such

as being time consuming. Moreover, it requires large amounts of solvent and also it is laborious. On the other hand, due to the small surface to volume ratio, mass transfer and extraction efficiency are low in conventional LLE method. Hence, the miniaturization of LLE method using microchannels can promote its application in various fields (1). A large surface to volume ratio and smaller molecular distance in microchannels than conventional LLE method lead to fast mass transfer (2). For these reasons, the volume of solvents, process time and cost could be decreased (3, 4). In recent years, the microchannel devices have been widely applied for different uses such as synthesis of organic molecules (5), synthesis of nanoparticles (6), kinetic studies (7, 8), emulsification (9), solvent extraction (10-12), DNA extraction (13), solid-phase extraction (14,15), laser reaction control (16), immunoassay (17), flow-injection analysis (18, 19), biofuel processes (20-22) and partition coefficient (log D) determination (23).

The application of LLE method using microfluidic devices has been reported for the extraction of analytes from the aqueous phase into the organic phase and vice versa. For example, the LLE extraction of uranium in HCl media by Aliquats 336 was performed using a Y–Y shaped microfluidic device (24). In this study, an extraction yield of 87% was obtained for an aqueous contact time of only 0.6 s in a microchannel volume of 320 nL. Furthermore, Xu *et al.* enhanced the mass transfer of succinic acid from *n*-butanol into the aqueous drops using a microfluidic device (25). Extraction of iron as Fe-bathophenanthroline disulfonic acid complex from the aqueous phase into chloroform is another example of microchannel application in the LLE method (10).

Pharmaceutical applications of natural materials from plants date back to ancient times (26). Oleuropein is one of the compounds that are found in olive leaves, fruit, bark and roots of *Olea europaea* (the olive tree) that cause the bitter taste of olive oil and fruit (27, 28). It belongs to a group of compounds that are referred to as polyphenols. During the exposure of enzyme, oleuropein decomposes to enolic acid and hydroxytyrosol. Oleuropein has beneficial properties in medical and food products including antibiotic (29), antimicrobial and antifungal (30, 31),

antimalarial (32), dairy products (33) food antioxidant (34, 35) and the prevention of Alzheimer (36). For these reasons, the separation and purification of oleuropein from inexpensive sources using economic methods is an essential procedure. The most common problem in the extraction process is the usage of toxic extractants such as the mixture of methanol-water (37) or methanol-hexane (38). The separation and purification of flavonoids and oleuropein from olive leaves using macroporous resins have already been reported (39). The use of resin is time consuming, expensive and involves various numerous steps. It seems necessary to find a simple, safe and cost effective method for the extraction of oleuropein into a safe medium.

The aim of this study was to develop and optimize the microfluidic device for the extraction of oleuropein into the aqueous phase using response surface methodology. The influences of effective parameters such as temperature, flow rate ratio, residence time and their interactions on extraction yield of oleuropein were investigated and explained.

Experimental

Preparation of Organic Extract
In order to carry out the extraction of oleuropein from olive leaves into the ethyl acetate, 10 g of air-dried and pulverized *Olea europaea* leaves were subjected to mechanical stirring for 24 h with 100 mL ethyl acetate. The supernatant phase was separated by filtration (Whatman filter paper). The obtained solution was used as the feed for microfluidic extraction.

Microfluidic Extraction
A schematic diagram of the proposed microfluidic device and process sequence has been shown in our previous report (40). Briefly, a T-shaped microchannel was used as the mixing chamber. The geometric dimensions of the T-shaped microchannel were 2.3 mm, 800 μm and 8.5 mm in outer diameter, inner diameter and length, respectively. In order to increase the length of contact time between the two phases (ethyl acetate and aqueous phase), a coil (outer diameter; 2.0 mm, inner diameter; 600 μm, length; 900 mm) was used in outlet stream. The syringe pumps were used as the driving force to divert feed in each inlet stream (ethyl acetate extract and aqueous phase). The T-shaped microchannel and coil were placed in a

water bath for temperature control. In the outlet stream, the two phases were separated according to their densities (aqueous phase at the bottom and ethyl acetate at the top of collector tube). The concentration of oleuropein in two phases was determined using high-performance liquid chromatography (HPLC) system. The results were reported in term of extraction yield percentage and were compared with that of batch process.

Materials and Methods

Oleuropein (purity≥ 98% by HPLC) was purchased from INDOFINE Chemical Company (Hillsborough, USA). Acetonitrile (HPLC grade), ethyl acetate, methanol, sodium hydroxide, dibasic sodium phosphate, potassium dihydrogen phosphate and orthophosphoric acid were purchased from Merck Chemical (Darmstadt, Germany). All solutions were prepared with deionized water from a Milli-Q system (Millipore, USA).

Samples
Olea europaea (variety Sevillana) leaves were collected from Agricultural Research Garden, Khorramabad, Iran. Before the extraction, the leaves were washed and dried, then milled, homogenized and kept at 4°C until analysis. The same sample was used in the whole optimization study.

Standard Solutions Preparation
A stock standard solution (4000 mg/L) was prepared by dissolving oleuropein in methanol. Working standard solutions at the concentrations of 500-2000 mg/L were prepared by diluting the suitable volume of the stock standard with deionized water. Oleuropein content (mg) in aqueous and organic phases were calculated by using calibration curve.

Chromatographic Conditions
The HPLC system (Shimadzu Corporation, Kyoto, Japan), which consisted of a quaternary pump (LC-10ATvp), UV-Vis detector (SPD-M10Avp), vacuum degasser and system controller (SCL-10Avp) was used. A manual injector with a 10 μL sample loop was applied for loading the sample. Class VP-LC workstation was employed to acquire and process chromatographic data. A reversed-phase C_{18} analytical column (Shim-Pack VP-ODS, 250 mm × 4.6 mm i.d., Shimadzu, Japan) was used. The mobile phase consisted of phosphate buffer (0.05

mol/L and pH 3 adjusted with orthophosphoric acid) and acetonitrile (70:30, v/v). Prior to the preparation of the mobile phase, buffer solution and acetonitrile were degassed separately using a Millipore vacuum pump. The UV detector was set at 254 nm. The chromatograms were run for 10 min at a flow rate of 1.0 mL/min at ambient temperature.

Experimental Design
In order to reach the optimum extraction conditions, the effect of various parameters such as pH, temperature, flow rate ratio and residence time were examined. A central composite design (CCD) was used to optimize and evaluate the experimental conditions of the microfluidic extraction technique. Our previous study indicated that pH could not have a significant effect on the extraction efficiency of target analytes (40). Therefore, temperature, flow rate ratio and residence time were selected as main variables. According to preliminary experimental results, low and high levels of main parameters including temperature (A), flow rate ratio (B) and residence time (C) were selected. The range and center point values of these parameters have been shown in Table 1. The extraction conditions were optimized using Design Expert software package (version 7.0.0). The extraction yield percentage of oleuropein was calculated according to the follow equation.

$$Yield \ (\%) = \frac{m_a}{m_t} \times 100 \qquad (1)$$

In which m_a is the oleuropein content (mg) in aqueous phase and m_t is the total content of oleuropein in ethyl acetate phase.

Results and Discussion

The extraction yield of oleuropein from the ethyl acetate into the aqueous phase using microfluidic device was affected by various factors including temperature, flow rate ratio (ethyl acetate/water) and residence time. The effects of these parameters on extraction yield of oleuropein were considered by central composite design. Extraction yield data obtained from 20 experimental runs have been listed in Table 1. Typical chromatograms of the extracted oleuropein into the aqueous phase using microfluidic device and ethyl acetate extract have been indicated in Fig. 2.

Due to low toxicity and high ability of ethyl acetate to extract oleuropein more than flavonoids, it was selected as the organic phase for the preparation of crude extract from olive leaves (39). On the other hand, according to Food and Drug Administration (FDA) solvent classification, ethyl acetate is a low toxic solvent which can be used in food and pharmaceutical industries (41).
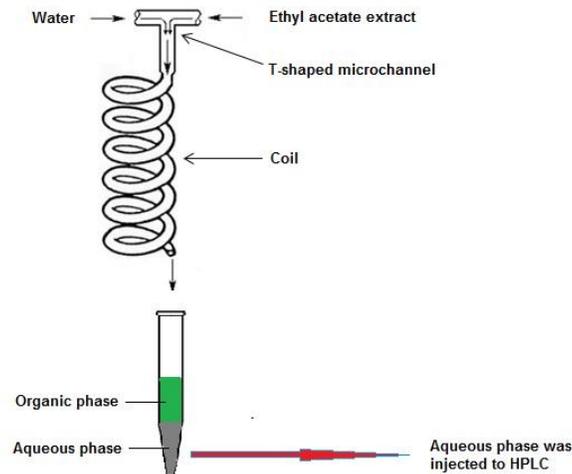
Figure 1. A schematic diagram of the microfluidic device. Ethyl acetate and water were delivered to T-shaped microchannel using two syringe pumps.
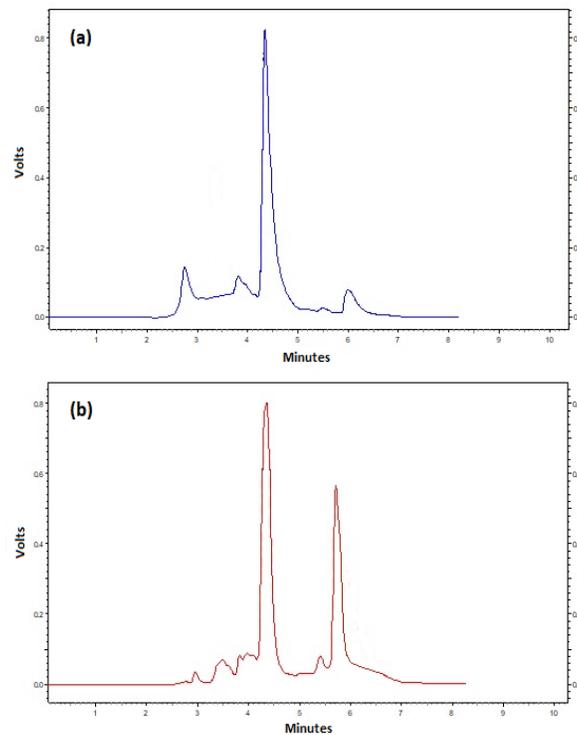
Figure 2. Typical chromatograms of extracted oleuropein into the aqueous phase by microchannel device (a) and ethyl acetate extract (b).
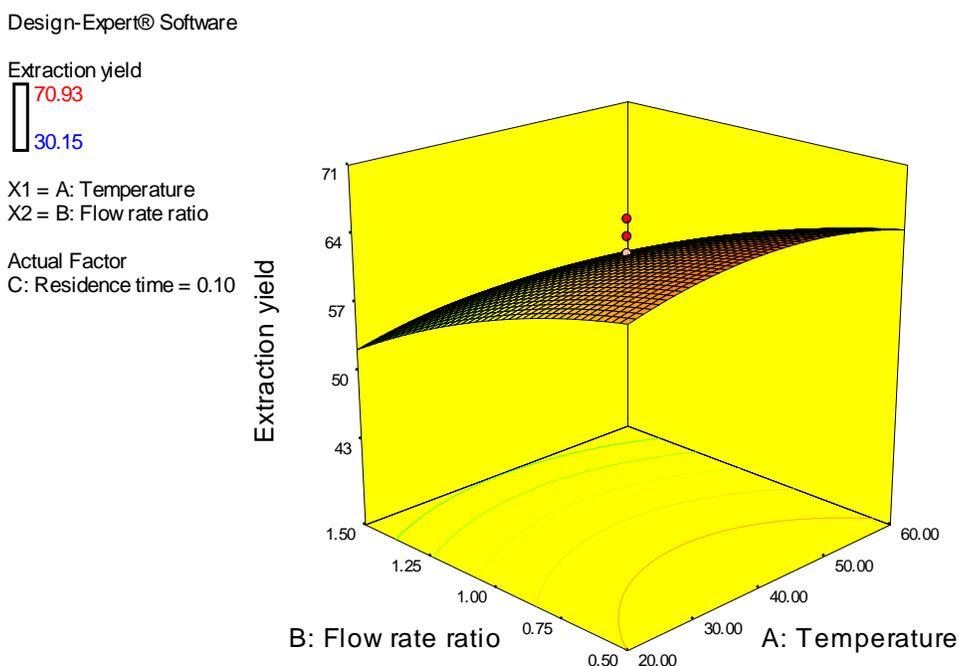
Figure 3. Response surface plot for extraction yield of oleuropein as a function of flow rate ratio and temperature.
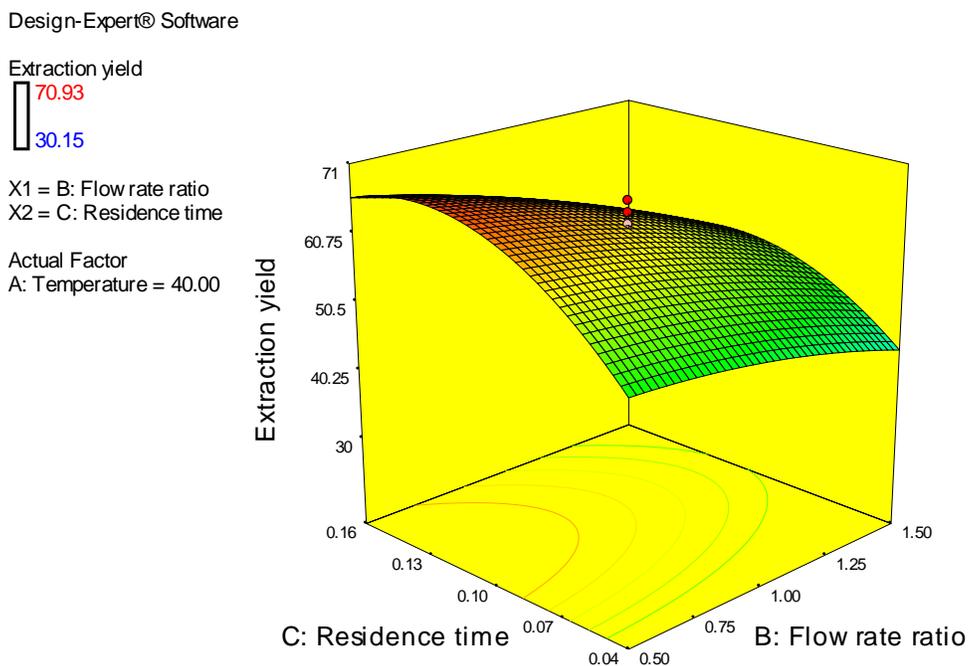
Figure 4. Response surface plot for extraction yield of oleuropein as a function of residence time and flow rate ratio.

Optimization of Extraction Process

Analysis of variance (ANOVA) results (Table 2) indicate that the proposed model for oleuropein extraction from ethyl acetate into the aqueous phase using microfluidic device is a quadratic model. R-squared value for this model is 0.9693 which shows
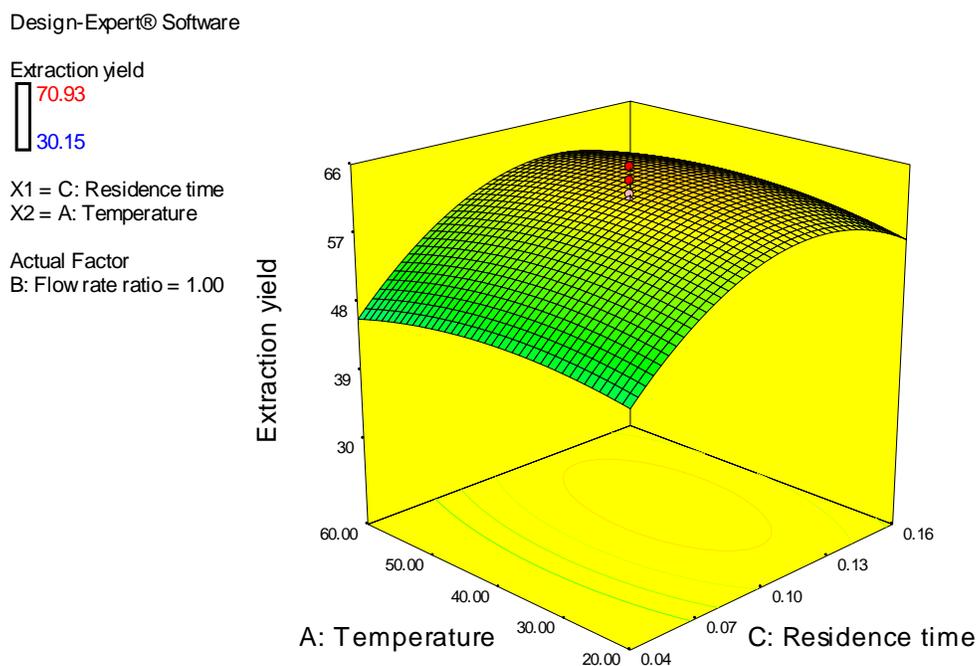
Figure 5. Response surface plot for extraction yield of oleuropein as a function of temperature and residence time.

Table 1: Central composite design with experimental conditions and extraction yield data.

Run	Day	A: Temperature (°C)	B: Flow rate ratio	C: Residence time (min)	Extraction yield (%)
1	1	60	1.50	0.0445	42.84
2	1	60	0.50	0.0445	47.41
3	1	20	1.50	0.1576	49.21
4	1	20	0.50	0.1576	61.93
5	1	20	0.50	0.0445	49.32
6	1	40	1.00	0.1010	61.05
7	1	60	1.50	0.1576	49.82
8	1	20	1.50	0.0445	44.39
9	1	60	0.50	0.1576	63.91
10	1	40	1.00	0.1010	62.18
11	1	40	1.00	0.1010	65.64
12	1	40	1.00	0.1010	63.89
13	2	40	0.16	0.1010	70.93
14	2	40	1.00	0.1010	60.62
15	2	40	1.84	0.1010	43.35
16	2	73.6	1.00	0.1010	55.82
17	2	40	1.00	0.0060	30.15
18	2	40	1.00	0.1961	48.23
19	2	40	1.00	0.1010	61.29
20	2	6.36	1.00	0.1010	54.96

the correlation between experimental and theoretical results (Table 2). Other statistical parameters of the model have been shown in Table 2. Model equation (2) shows, according to codded parameters, the

Table 2: ANOVA results of the proposed method.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Block	18.51	1	18.51			
Model	1857.31	9	206.37	31.59	<0.0001	significant
A-Temperature	0.024	1	0.024	3.723E-003	0.9527	
B-Flow rate ratio	500.72	1	500.72	76.65	<0.0001	significant
C-Residence time	372.42	1	372.42	57.01	<0.0001	significant
AB	0.13	1	0.13	0.020	0.8920	
AC	4.58	1	4.58	0.70	0.4243	
BC	37.45	1	37.45	5.73	0.0403	significant
A^2	70.10	1	70.10	10.73	0.0096	significant
B^2	36.30	1	36.30	5.56	0.0428	significant
C^2	906.44	1	906.44	138.76	<0.0001	significant
Residual	58.79	9	6.53			
Lack of Fit	46.48	5	9.30	3.02	0.1534	Not significant
Pure Error	12.32	4	3.08			
Cor Total	1934.61	19				
Std. Dev. [a]	2.56		R-Squared	0.9693		
Mean	54.35		Adj R-Squared	0.9386		
C.V. (%) [b]	4.70		Pred R-Squared	0.7390		
PRESS	500.07		Adeq Precision	19.475		

[a]Standard deviation, [b]Coefficient variation

mathematical relationship between the studied parameters and extraction yield of oleuropein.

Extraction yield = +62.21 + 0.042 * A - 6.06 * B + 5.22 * C - 0.13 * A * B + 0.76 * A * C - 2.16 * B * C - 2.21 * A^2 - 1.59 * B^2 - 7.93 * C^2 (2)

ANOVA results indicate that the flow rate ratio (B), residence time (C), BC interaction, A^2, B^2 and C^2 are significant model terms and the lack of fit was not significant at the 5% level (P > 0.05). The results indicate temperature (A), AB and AC interactions do not have significant effects on the extraction yield of oleuropein using the microfluidic system.

The influences of studied parameters including temperature, flow rate ratio and residence time on extraction yield of oleuropein are shown in three-dimensional (3D) response surface graphs. Three-dimensional graphs reflect the effects of two variables on the response value, while the other variables were kept at zero level.

In order to investigate the temperature effect, the microchannel (T-shaped microchannel and coil) was placed in a water bath at different temperatures according to experimental runs. As observed from Figs. 3 and 5, temperature variation does not have a significant impact on the extraction yield of oleuropein. The reason for this phenomenon can be the high contact surface between the two phases in microchannels. On the other hand, due to high contact surface between the two phases in microchannels, mass transfer occurred easily and no driving force is required.

The effects of different flow rates of input streams at the constant total flow rate were examined according to experimental design runs. Figs. 3 and 4 indicate the effect of flow rate ratio (rate of ethyl acetate to water) on the extraction yield of oleuropein. In conventional LLE method, increasing the ratio of aqueous phase to organic phase raises the extraction yield. Hence, the maximum extraction yield was observed at minimal ratio of organic phase to aqueous phase. As indicated in Table 1, in line with conventional LLE method, the highest extraction yield was obtained in minimum flow rate ratio (run 13). Fig. 3 shows that the extraction yield decreased at higher flow rate ratio (>1). At higher flow rate ratios the amount of extractant phase compared to organic phase was

Table 3: Results of microfluidic and batch extraction.

Method	Yield (%) ± RSD (%)		
	50 (mL)[a]	100 (mL)[b]	200 (mL)[c]
Batch	52.6 ± 2.0	50.1 ± 1.5	49.3 ± 2.5
Microfluidic		65.4 ± 1.2	

[a] Total volume including 25 mL ethyl acetate and 25 mL water.
[b] Total volume including 50 mL ethyl acetate and 50 mL water.
[c] Total volume including 100 mL ethyl acetate and 100 mL water.

reduced which in turn increased the extraction yield. The effects of residence time on the extraction yield have been shown in Figs. 4 and 5. Residence time and its interaction with flow rate ratio are significant parameters in the proposed model (Table 2). It is expected that mass transfer from the organic phase into the aqueous phase increases with the increased contact time between these two phases. Figs. 4 and 5 show the increase of residence time up to 0.1 min lead to increase of extraction yield with more slope and then decreased slowly.

Models Evaluation

Under the adjusted conditions (temperature; 40 °C, flow rate ratio; 1.0 and residence time; 0.1 min), the maximum predicted extraction yield of oleuropein by the proposed quadratic model is equal to 65.0%. In order to investigate the reliability of the model, a verification test was carried out under the adjusted conditions. The mean extraction yield of oleuropein for three measurements was 65.2%. Clearly, the obtained experimental result is in agreement with the predicted response.

The Comparison of Microfluidic and Batch Extraction Efficiencies

In order to compare the microfluidic and batch processes, three different volumes including 25, 50 and 100 mL of ethyl acetate extract were mixed with the same volumes of water and were subsequently stirred for 45 min at 40 °C. The extraction process using microfluidic device was achieved under the following conditions: flow rate ratio: 1, temperature: 40 °C and residence time: 0.1010 min. The results have been indicated in Table 3. As it can be observed, the extraction yield by microfluidic device was approximately 15% more than batch process extraction. On the other hand, the required time for the batch extraction (45 min) is higher than microfluidic extraction (0.1293 min) which indicates

the time saving in microfluidic device.

Conclusion

In this study, oleuropein was extracted from ethyl acetate into the aqueous phase using a microfluidic device. The mass transfer between the two immiscible phases (ethyl acetate and water) was intensively increased. Extraction process is a continuous extraction method which provides advantages such as environmentally friendly and high efficiency compared to conventional techniques. In order to find the effect of the main parameters and their interactions, optimization was achieved by a central composite design. The maximum extraction yield of 70.93% was obtained under the follow conditions: deionized water as extractant phase, temperature of 40 °C, flow rate ratio of 0.16 and residence time of 0.1010 min. The results of the analysis of variance show that the temperature had not a significant effect on the extraction efficiency. The obtained results revealed that the extraction yield of oleuropein using the microfluidic device was improved compared to the batch process. Moreover, due to facility in scale-up, this method is expected to be used in mass production.

Acknowledgment

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Assmann N, Ładosz A, Rudolf von Rohr P. Continuous micro liquid-liquid extraction. Chem Eng Technol. 2013;36:921-36.

2. Mary P, Studer V, Tabeling P. Microfluidic droplet-based liquid-liquid extraction. Anal Chem. 2008;80:2680-7.

3. Faryadi M, Rahimi M, Safari S, Moradi N. Effect of high frequency ultrasound on micromixing efficiency in microchannels. Chem Eng Process. 2014;77:13-21.

4. Wang X, Saridara C, Mitra S. Microfluidic supported liquid membrane extraction. Anal Chim Acta. 2005;543:92-8.

5. Noël T, Kuhn S, Musacchio AJ, Jensen KF, Buchwald SL. Suzuki–Miyaura cross-coupling reactions in flow: multistep synthesis enabled by a microfluidic extraction. Angew Chem. 2011;123:6065-8.

6. Liang Y, Chu G, Wang J, Huang Y, Chen J, Sun B, et al. Controllable preparation of nano-CaCO$_3$ in a microporous tube-in-tube microchannel reactor. Chem Eng Process. 2014;79:34-9.

7. Rupp M, Ruback W, Klemm E. Octanol ethoxylation in microchannels. Chem Eng Process. 2013;74:19-26.

8. Rupp M, Ruback W, Klemm E. Alcohol ethoxylation kinetics: proton transfer influence on product distribution in microchannels. Chem Eng Process. 2013;74:187-92.

9. Souilem S, Kobayashi I, Neves MA, Sayadi S, Ichikawa S, Nakajima M. Preparation of monodisperse food-grade oleuropein-loaded W/O/W emulsions using microchannel emulsification and evaluation of their storage stability. Food Bioprocess Technol. 2014;7:2014-27.

10. Tokeshi M, Minagawa T, Kitamori T. Integration of a microextraction system on a glass chip: ion-pair solvent extraction of Fe (II) with 4,7-Diphenyl-1,10-phenanthrolinedisulfonic acid and tri-n-octylmethylammonium chloride. Anal Chem. 2000;72:1711-4.

11. Tokeshi M, Minagawa T, Kitamori T. Integration of a microextraction system: Solvent extraction of a Co–2-nitroso-5-dimethylaminophenol complex on a microchip. J Chromatogr A. 2000;894:19-23.

12. Priest C, Zhou J, Klink S, Sedev R, Ralston J. Microfluidic solvent extraction of metal ions and complexes from leach solutions containing nanoparticles. Chem Eng Technol. 2012;35:1312-9.

13. Pan X, Zeng S, Zhang Q, Lin B, Qin J. Sequential microfluidic droplet processing for rapid DNA extraction. Electrophoresis. 2011;32: 3399-405.

14. Wang Z, Jemere AB, Harrison DJ. Integrated electrokinetic sample fractionation and solid-phase extraction in microfluidic devices. Electrophoresis. 2012;33:3151-8.

15. Kutter JP, Jacobson SC, Ramsey JM. Solid phase extraction on microfluidic devices. J Microcolumn Sep. 2000;12:93-7.

16. Tanaka Y, Slyadnev MN, Hibara A, Tokeshi M, Kitamori T. Non-contact photothermal control of enzyme reactions on a microchip by using a compact diode laser. J Chromatogr A. 2000;894:45-51.

17. Sato K, Tokeshi M, Odaka T, Kimura H, Ooi T, Nakao M, Saito T, Kitamori T. Integration of an immunosorbent assay system: analysis of secretory human immunoglobulin A on polystyrene beads in a microchip. Anal Chem. 2000;72:1144-7.

18. Sato K, Tokeshi M, Kitamori T, Sawada T. Integration of flow injection analysis and zeptomole-level detection of the Fe (II)-o-phenanthroline complex. Anal Sci. 1999;15:641-6.

19. Sorouraddin HM, Hibara A, Proskrunin MA, Kitamori T. Integrated FIA for the determination of ascorbic acid and dehydroascorbic acid in a microfabricated glass-channel by thermal-lens microscopy. Anal Sci. 2000;16:1033-8.

20. Bedekar AS, Feng JJ, Krishnamoorthy S, Lim KG, Palmore GTR, Sundaram S. Oxygen limitation in microfluidic biofuel cells. Chem Eng Commun. 2007;195:256-66.

21. Santacesaria E, Di Serio M, Tesser R, Tortorelli M, Turco R, Russo V. A simple device to test biodiesel process intensification. Chem Eng Process. 2011;50:1085-94.

22. Santacesaria E, Di Serio M, Tesser R, Turco R, Tortorelli M, Russo V. Biodiesel process intensification in a very simple microchannel device. Chem Eng Process. 2012;52:47-54.

23. Alimuddin M, Grant D, Bulloch D, Lee N, Peacock M, Dahl R. Determination of log D via automated microfluidic liquid-liquid extraction. J Med Chem. 2008;51:5140-2.

24. Hellé G, Mariet C, Cote G. Liquid–liquid extraction of uranium(VI) with Aliquats 336 from HCl media in microfluidic devices: Combination of micro-unit operations and online ICP-MS determination. Talanta. 2015;139:123-31.

25. Xu JH, Tan J, Li SW, Luo GS. Enhancement of mass transfer performance of liquid–liquid system by droplet flow in microchannels. Chem Eng J. 2008;141:242-9.

26. Pérez-Bonilla M, Salido S, Van Beek TA, Linares-Palomino PJ, Altarejos J, Nogueras M, et al. Isolation and identification of radical scavengers in olive tree (Oleaeuropaea) wood. J Chromatogr A. 2006; 1112: 311-8.

27. Soler-Rivas C, Espín JC, Wichers HJ. Oleuropein and related compounds. Sci Food Agric. 2000;80:1013-23.

28. Andrewes P, Busch JLHC, Joode TD, Groenewegen A, Alexandre H. Sensory properties of virgin olive oil polyphenols: identification of deacetoxy-ligstroside aglycon as a key contributor to pungency. J Agric Food Chem. 2003;51:1415-20.

29. Tranter HS, Tassou SC, Nychas GJ. The effect of the olive phenolic compound, oleuropein, on growth and enterotoxin B production by Staphylococcus aureus. J Appl Bacteriol. 1993;74:253-9.

30. Aziz NH, Farag SE, Mousa LA, Abo-Zaid MA. Comparative antibacterial and antifungal effects of some phenolic compounds. Microbios. 1998;93:43-54.

31. Capasso R, Evidente A, Schivo L, Orru G, Marcialis MA, Cristinzio G. Antibacterial polyphenols from olive oil mill waste waters. J Appl Bacteriol. 1995;79:393-8.

32. Caniato R, Puricelli L. Natural antimalerial agents (1995-2001). Crit Rev Plant Sci. 2003;22:79-105.

33. Zoidou E, Magiatis P, Melliou E, Constantinou M, Haroutounian S, Skaltsounis AL. Oleuropein as a bioactive constituent added in milk and yogurt. Food Chem. 2014;158:319-24.

34. Bouaziz M, Sayadi S. Isolation and evaluation of antioxidants from leaves of a Tunisian cultivar olive tree. Eur J Lipid Sci Tech. 2005;107:497-504.

35. Jemai H, El Feki A, Sayadi S. Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. J Agric Food Chem. 2009;57:8798-804.

36. Dai Q, Borenstein AR, Wu Y, Jackson JC, Larson BE. Fruit and vegetable juices and Alzheimer's disease: the kame project. Am J Med. 2006;119:751-9.

37. Savournin C, Baghdikian B, Elias R, Dargouth-Kesraoui F, Boukef K, Balansard G. Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in oleaeuropaea leaves. J Agric Food Chem. 2001;49:618-21.

38. Şahin S, Bilgin M, Dramur MU. Investigation of oleuropein content in olive leaf extract obtained by supercritical fluid extraction and soxhlet methods. Separ Sci Technol. 2011;46:1829-37.

39. Li C, Zheng Y, Wang X, Feng S, Di D. Simultaneous separation and purification of flavonoids and oleuropein from Oleaeuropaea L. (olive) leaves using macroporous resin. J Sci Food Agric. 2011;91:2826-34.

40. Naleini N, Rahimi M, Heydari R. Oleuropein extraction using microfluidic system. Chem Eng Process. 2015;92:1-6.

41. Rostagno MA, Prado JM. Natural Product Extraction: Principles and Applications, Published by The Royal Society of Chemistry, Thomas Graham House, Science Park, Milton Road, Cambridge CB4 0WF, UK, 2013.