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Aqueous Two-Phase Extraction of Polyphenols Using a Microchannel System – Process Optimization and Intensification

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Summary

Polyphenols are one of the most numerous and widespread groups of compounds in the plant world. Nowadays, organic solvents such as methanol, ethanol, acetone, dimethylformamide, ethyl acetate and diethylether are mainly used for the extraction of polyphenols. These solvents require special process conditions and special care in the disposal of the used solvents. In this paper, the extraction of polyphenols from the model solution was performed using the aqueous two-phase system which contains 80–90 % water and represents low burden on the environment. The aqueous solution of gallic acid (GA) was used as a model solution of polyphenols. The extraction was performed in the aqueous two-phase system containing $PEG_{6000}/H_2O/(NH_4)_2SO_4$ in a macroextractor (V=10 mL) and microextractor (V=14 μL). The influence of the process parameters, the concentration of gallic acid, pH and composition of the aqueous two-phase system was investigated in order to maximize the partition coefficient. The method of multifactor experimental planning was used to optimize the extraction process and the results were statistically analysed using the evolutionary operation method (EVOP). Optimal operating conditions of the extraction process were pH=6.50, γ_{GA}=4.50 g/L, the mass fraction of polyethylene glycol (PEG) w_{PEG}=0.1037 g/g and the mass fraction of ammonium sulphate (AMS) w_{AMS}=0.0925 g/g. Under these conditions the maximal partition coefficient of K=5.54 and the extraction efficiency of E=89.11 % were achieved and successfully applied for total phenol extraction from white wine in the macro- and microextractor. Approximately the same partition coefficients and extraction efficiency were achieved in the microextractor within a 60-fold shorter residence time.

Key words: polyphenols, gallic acid, aqueous two-phase systems, multifactor plan of experiments, evolutionary operation (EVOP), microextractor

Introduction

Polyphenols constitute one of the most numerous and ubiquitous groups of plant metabolites (*1*) and currently, more than 8000 phenolic compounds are described (*2*). They have several industrial applications, such as additives in the production of paints, paper, cosmetics, medicine and food industry (*1*). Extraction is one of the most common methods used for the isolation of polyphenolic compounds and it is mainly performed with organic solvents (methanol/hydrochloric acid, hexane/butanol, benzene, ethyl acetate/etoxyethane) (*3*). These extraction procedures are efficient but the extracts are not safe for human consumption due to the potentially toxic effect of residual solvents (*4*), so a great effort is being made to invest into and develop new separation techniques.

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The aqueous two-phase system (ATPS) and extraction (ATPE) have been recognized as superior and versatile, preparative and analytical tools for the downstream processing of biomolecules (*5*). They are formed in general when two incompatible polymers like polyethylene glycol (PEG) and dextran are mixed. The phase system can also be obtained by mixing certain polymers (*e.g.* PEG) with lyotropic salts (*e.g.* Na_2HPO_4) or detergents (*5*) where each phase generally contains 80–90 % (by mass) water (*6*). Comparing the results obtained in PEG/dextran and PEG/salt systems for similar experiments, generally higher extraction yield and selectivity were observed in PEG/salt systems (*7*). The technique is well suited for handling of both soluble and insoluble matter and it is easy to scale it up (*8*). Its other advantages are high capacity, biocompatible environment, low interfacial tension of phases, high yield, low process time and energy, which could easily be obtained by varying experimental conditions like pH, ionic strength and polymer molecular mass (*6*,*9*). Up to now, several different ATPS processes have been developed for different biological products such as cells, viruses, RNA, plasmids, monoclonal antibodies and proteins (*6*), but not for polyphenols.

Another new approach in the extraction of polyphenols is the application of microtechnology, which offers potential benefits due to the well defined high specific interfacial areas available for heat and mass transfer, which increases transfer rates and enhances yield, selectivity and process control (*10*). According to TeGrotenhuis *et al.* (*11*), who demonstrated one of the first extraction processes in microchannels, when microchips are applied for solvent extraction, two immiscible fluids flow through thin channels that are smaller than the normal mass transfer boundary layer. In this way, in each phase mass transfer resistance is reduced and consequently the mass transfer rate is significantly enhanced. Microextractions have been identified as a promising technique for the separation of different chemical and biochemical products (*12–17*).

The extraction of bioactive compounds is mainly optimized by using response surface methodology, an effective technique for analyzing interactions among factors, exploring the relationships between the response and the independent variables, and optimizing the processes or products where multiple variables may influence the outputs (*18*,*19*). On the other hand, the evolutionary operation (EVOP) is a simple optimization technique, also based on statistical methods, which helps in finding optimal process conditions. Besides simplicity, the method offers a clearly defined decision-making procedure that makes it easy to optimize simultaneously the effects of two or more parameters to achieve maximal or minimal process response. Optimization is made sequentially, meaning that each experimental phase requires results (effects, change in the mean effect, standard deviation and error limits) from the previous one until the optimum is achieved (*20–22*).

The objective of the present study is to investigate the applicability of the aqueous two-phase systems for polyphenol extraction. Effects of phase composition, pH and polyphenol concentration on the partition behaviour were studied and optimized using the EVOP method. Continuous extraction of the polyphenol model solution in a microchannel was performed in order to replace the generally applied batch extraction process and to demonstrate the applicability of microchannel devices for ATPS extraction of polyphenols.

Materials and Methods

Chemicals

Polyethylene glycol 6000 (PEG_{6000}) was purchased from Merck (Darmstadt, Germany). Potassium dihydrogen phosphate (KH_2PO_4) and disodium hydrogen phosphate (Na_2HPO_4) were from Kemika (Zagreb, Croatia). Ammonium sulphate ($(NH_4)_2SO_4$) and gallic acid ($C_7H_6O_5$) were procured from Acros (Geel, Belgium) and methanol (CH_3OH) was procured from Sigma (Steinheim, Germany). The Folin-Ciocalteu reagent was purchased from Fluka (Buchs, Switzerland).

Concentration of stock solutions of PEG_{6000} and ammonium sulphate were 400 g/L (η_{PEG}=28.365 g/ms and η_{AMS}=1.741 g/ms). Distilled water was used to prepare the stock solution of gallic acid with an accurately known concentration of 80 g/L. Different stock solutions of phosphate buffer (c=0.5 mol/L) were used to prepare the aqueous two-phase system at different pH in the range of 6–7.5.

Apparatus

A microextractor set-up (Fig. 1) was used that consisted of two borosilicate glass microchips connected in a

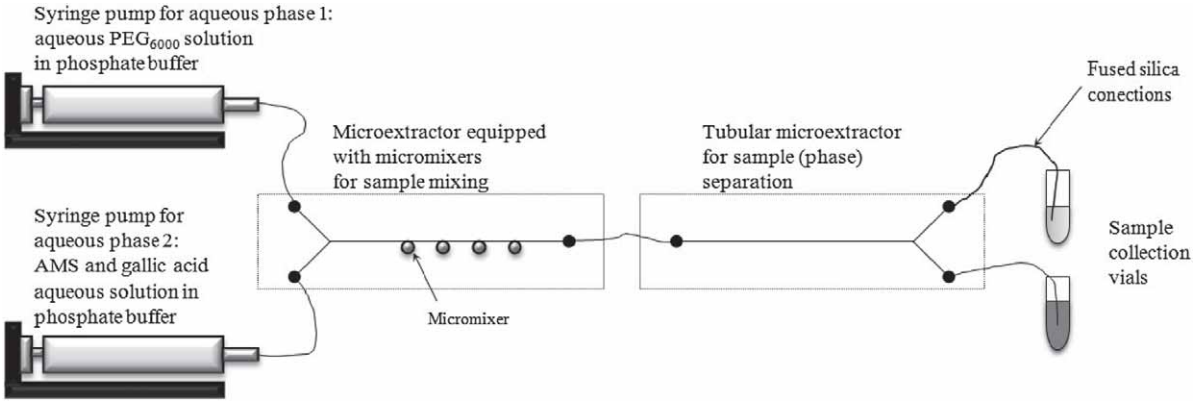

Fig. 1. Schematic diagram of a reaction system used for phenolic extraction in microextractor

series. The first microchip was equipped with swirl micromixers for mixing at high Reynolds number (Re>50) with a microchannel width×depth of 200×150 μm and a total internal volume of 1 μL. It is followed by a tubular microchip with the microchannel width×depth of 200×150 μm and a total internal volume of 13 μL.

The microchip was equipped with micromixers, used for phase mixing and had two Y-shaped inlets, so fluids could be injected separately, and one outlet that was connected by a fused silica connection (375 μm o.d., 150 μm i.d., Micronit Microfluidics B.V., Enschede, the Netherlands) to the inlet of a tubular microchip used for phase separation. Even though this type of micromixer is usually used when working with higher Reynolds number flows, it can be used efficiently at lower values (Re<50). Mixing is achieved by inertial force that is used to fold the flow. Double swirl chamber geometry is used when the fluid spirals down rapidly reducing the mixing length. The tubular microchip was equipped with two outlets used for sample collection. Microchips were placed into a stainless steel holder, which provided a leak-free connection (Micronit Microfluidics B.V.). Two syringe pumps (PHD 4400 Syringe Pump Series, Harvard Apparatus, Holliston, MA, USA) equipped with high pressure stainless steel syringes (8 mL, Harvard Apparatus) were used for solution supply. Fluid flow in the microreactor was observed by using a microscope (Motic B1-220A binocular, Wetzlar, Germany) at magnifications of 40× and 100× (eyepiece magnification=10×; objective magnification=4× and 10×).

Total phenolic (TP) content

The total phenolic (TP) content was determined by the Folin-Ciocalteu method (*23*) adapted to the microscale. Briefly, aliquots of 0.1 mL of samples and standards were mixed with 0.1 mL of the Folin-Ciocalteu phenol reagent. A volume of 0.1 mL of the saturated sodium carbonate solution was added to the mixture and made up to 2 mL with distilled water. After incubation in the dark (t=25 °C, time=2 h), the absorbance of the reaction mixture was measured at 725 nm (double-beam UV spectrophotometer, UV-1601, Shimadzu Corporation, Kyoto, Japan). The calibration curve was prepared using the standard solution of gallic acid (1-100 mg/L, R^2=0.9984).

Aqueous two-phase extraction of polyphenols in a batch macroextractor

Aqueous two-phase partitioning batch experiments were performed at 25 °C by mixing the determined volume of the phase-forming polymer stock solution with stock solutions of salt and a stock solution of gallic acid according to the EVOP factorial design technique. Finally, the phosphate buffer solution was added to obtain the final volume of 5 mL. The content was mixed using a laboratory shaker (Vibromix 313 EVT, Tehtnica, Železniki, Slovenia) at 600 rpm for 10 minutes in a 10-mL vial. After the two phases were separated by centrifugation at 4500 rpm (Universal 320R, Hettich Zentrifugen, Tuttlingen, Germany) samples were carefully withdrawn from the top (extract) phase and from the bottom (rafinate) phase and analysed for total phenolic content. Each experiment was done in duplicate and at 95 % confidence, the interval results had no statistical difference. The partition coefficient, K, was calculated as a ratio between equilibrium concentrations of polyphenols in the extract and in the rafinate phase. Extraction efficiency, E, was calculated as a ratio between the amount of polyphenols in the top (extract) phase and the total polyphenol content.

Aqueous two-phase extraction of polyphenols in a continuous microextractor

The aqueous PEG_{6000} solution (w_{PEG}=0.1037 g/g, η_{PEG}=7.496 g/ms) in the phosphate buffer (pH=6.5, c=0.5 mol/L) was fed from one inflow (Fig. 1), and AMS (w_{AMS}=0.0925 g/g, η_{AMS}=1.063 g/ms) and gallic acid (γ_{GA}=4.5 g/L) aqueous solution, also in the phosphate buffer (pH=6.5, c=0.5 mol/L), from another inflow in a microchip equipped with micromixers to ensure effective mixing of the two phases for the continuous extraction with the microextractor set-up. The inlet streams were fed in the first microchip at different flow rates to ensure equal volume distribution in the microchannel (PEG:AMS=1:3), in the range of 2.5-200 μL/min. In the second (tubular) microchip, the separation of phases took place. Outflows from the second microchip were gathered in separate vials.

The volumes of the collected phases in the macro- and microextractor systems were noted and total phenolic content was analysed according to the method described previously. All experiments were carried out at 25 °C in triplicate and the average values are reported (at 95 % confidence, the interval results have no statistical difference).

Results and Discussion

Experimental optimization of the aqueous two-phase extraction in the batch system

The phase diagram and the thermodynamic parameters of the aqueous two-phase system consisting of PEG_{6000} and ammonium sulphate were determined previously and published elsewhere (*24*). It was assumed that the presence of different concentrations of gallic acid and pH used in the experimental optimization do not have any influence on the thermodynamic equilibrium of the system. PEG_{6000} and ammonium sulphate were replaced with the tie-line length (TLL) (*25*) to reduce the number of parameters of the mass fraction. The TLL relates to the mass ratio between the phases. If two points of binodal curve, the top and the bottom phase composition, of a particular mixture are known, the distance between them is the tie-line length and may be calculated using Eq. 1:

$$\mathrm{TLL} = \sqrt{(w^{E^*}_{AMS} - w^{R^*}_{AMS})^2 + (w^{E^*}_{PEG} - w^{R^*}_{PEG})^2} \qquad /1/$$

The total mixture composition (w_{PEG}, w_{AMS}) was always prepared at the critical point of the binodal curve at which volumes of the extract and the rafinate phase theoretically become equal.

The tie-line length, pH and gallic acid (GA) concentration were investigated and used in the optimization of partitioning by the EVOP method. The first experimental conditions were selected according to the personal

observation of the process (data not shown). Coded experimental conditions A1 and A6 in Table 1 (first experimental plan) present the initial process conditions, namely pH=7, γ_{GA}=5 g/L and TLL=10, which is equivalent to $w_{PEG6000}$=0.1018 g/g and w_{AMS}=0.0915 g/g. Each analysed parameter had a higher (+) or lower magnitude (–) in comparison with the initial process conditions: level region (0), namely the pH in the range of ±0.5, γ_{GA} in the range of ±0.5 g/L and TLL in the range of ±1, respectively (experimental conditions A2–A5 and A7–A10, Table 1). Two series of measurements were performed for each point of the experimental plan and partition coefficients were calculated (Fig. 2). The differences and average values of partition coefficients for the first experimental plan were calculated accordingly (Table 1). In Table 2 the value of effects and error limits for the first experimental plan are shown.

Based on the results given in Table 2 for the first experimental plan, it is obvious that the change in the mean effect is small and negative (–0.154), the effects of pH (–0.971) and of gallic acid concentration (–0.679) are negative, while the effect of TLL (0.057) is small and positive in comparison with error limits (±0.676). The effects of pH and gallic acid concentration are great compared to the error limit, and coded experimental conditions A1 and A6 cannot represent the optimum of the process. According to the decision making rules of the EVOP method (*21*), if the effect is positive and great and the change in the mean effect is small, levels of corresponding parameter(s) should be increased, while if the effect is negative and the change in the mean effect is small, the corresponding parameters should be decreased. Based on this, in the next (second) experimental plan (Table 1) pH and gallic acid concentrations were decreased, while TLL was increased in each point of the experimental plan for the same range used in the first experimental plan.

The new (second) experimental plan for the two measurement series with calculated differences and average values is shown in Table 1, while the corresponding effects and error limits are given in Table 2. The partition coefficients for the second experimental plan are shown in Fig. 2b. Generally, the partition coefficients of the second experimental plan were higher than those obtained in the points of the first experimental plan. Change in the mean effect remains small and negative (–0.218). Effect of pH (–0.200) was negative, while the effects of TLL (0.472) and gallic acid concentration (0.361) were both positive. All effects in the second experimental plan were significantly lower than the error limit (±0.988; Table 2). Following decision making rules of the EVOP method, having in mind small and negative changes in the mean effect, the magnitude steps for all investigated process parameters were decreased twofold. Coded experimental conditions A1 and A6 of the second experimental plan

Table 1. Experimental conditions and results of average partition coefficient for the first, second and third experimental plan

Conditions I	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
TLL	10	9	11	9	11	10	9	11	9	11
γ(gallic acid)/(g/L)	5	4.5	4.5	5.5	5.5	5	5.5	5.5	4.5	4.5
pH	7	6.5	7.5	7.5	6.5	7	6.5	7.5	7.5	6.5
K(cycle I)	3.01	4.84	2.86	2.50	2.26	3.99	3.19	2.59	2.16	3.28
K(cycle II)	3.03	3.82	2.83	2.25	3.26	2.77	2.74	2.66	2.31	4.53
Difference (cycle I-II)	–0.02	1.02	0.03	0.25	–1.00	1.22	0.45	–0.07	–0.15	–1.25
Average *K*	3.02	4.33	2.85	2.38	2.76	3.38	2.96	2.62	2.24	3.91
Conditions II	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
TLL	11	10	12	10	12	11	10	12	10	12
γ(gallic acid)/(g/L)	4.5	4.0	4.0	5.0	5.0	4.5	5.0	5.0	4.0	4.0
pH	6.5	6.0	7.0	7.0	6.0	6.5	6.0	7.0	7.0	6.0
K(cycle I)	2.57	3.89	3.43	2.92	4.36	4.36	3.59	4.00	2.95	2.84
K(cycle II)	3.67	1.27	2.43	3.41	3.41	3.56	2.95	2.94	3.26	4.61
Difference (cycle I-II)	–1.10	2.62	1.00	–0.52	0.95	0.80	0.64	1.06	–0.31	–1.77
Average *K*	3.12	2.58	2.93	3.16	3.86	3.96	3.27	3.47	3.10	3.73
Conditions III	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10
TLL	11.5	11	12	11	12	11.5	11	12	11	12
γ(gallic acid)/(g/L)	4.75	4.5	4.5	5	5	4.75	5	5	4.5	4.5
pH	6.25	6	6.5	6.5	6	6.25	6	6.5	6.5	6
K(cycle I)	3.32	3.26	2.83	3.68	3.49	2.60	2.76	2.57	4.62	3.16
K(cycle II)	3.99	4.44	3.87	3.11	3.29	1.52	3.13	2.85	6.47	3.21
Difference (cycle I-II)	–0.67	–1.18	–1.04	0.57	0.20	1.18	–0.37	–0.28	–1.85	–0.05
Average *K*	3.65	3.85	3.35	3.39	3.39	2.06	2.94	2.71	5.54	3.18

were kept as central points in the third experimental plan. In the third experimental plan the pH was reduced, while TLL and the concentration of gallic acid were increased around the central point for new smaller magnitude.

The new (third) experimental plan is shown in Table 1, and the corresponding effects and error limits are given in Table 2. The calculated partition coefficients for all points of the third experimental plan are shown in Fig. 2c. By analysing the obtained results, change in the mean effect is large and positive in comparison with the previous experiments. Despite the fact that pH is positive (0.407) and lower than the error limit (±0.705), the effects of TLL (–0.776) and gallic acid concentration (–0.872) were negative and insignificantly higher than the error limit (±0.705). According to the decision rules of the EVOP method, when change in the mean effect is large and effects of the investigated process parameters are small compared to the error limit, the optimum of the process was obtained. Therefore, optimal operating conditions of the extraction process were determined to be: pH=6.50, γ_{GA}=4.50 g/L, mass fraction of polyethylene glycol w_{PEG}=0.1037 g/g and mass fraction of ammonium sulphate w_{AMS}=0.0925 g/g (TLL=11). Under these conditions (coded experimental conditions A9) the maximal partition coefficient of K=5.54 (Fig. 2c) and extraction efficiency E=89.11 % were achieved.

Table 2. Calculation of the effects and error limits of the first, second and third experimental plan

Effect		1st experimental plan	2nd experimental plan	3rd experimental plan
Effect of pH		–0.971	–0.200	0.407
Effect of TLL		0.057	0.472	–0.776
Effect of γ(gallic acid)		–0.679	0.361	–0.872
Effect of pH and TLL		0.370	–0.409	–0.665
Effect of pH and γ(gallic acid)		0.608	–0.061	–0.522
Effect of TLL and γ(gallic acid)		–0.035	–0.013	0.655
Effect of pH, TLL and γ(gallic acid)		–0.148	0.252	0.100
Change in the mean effect		–0.154	–0.218	0.551
Standard deviation		0.673	0.984	0.702
Error limits for:	average	±0.952	±1.392	±0.993
	effect	±0.676	±0.988	±0.705
	change in the mean effect	±0.600	±0.877	±0.626

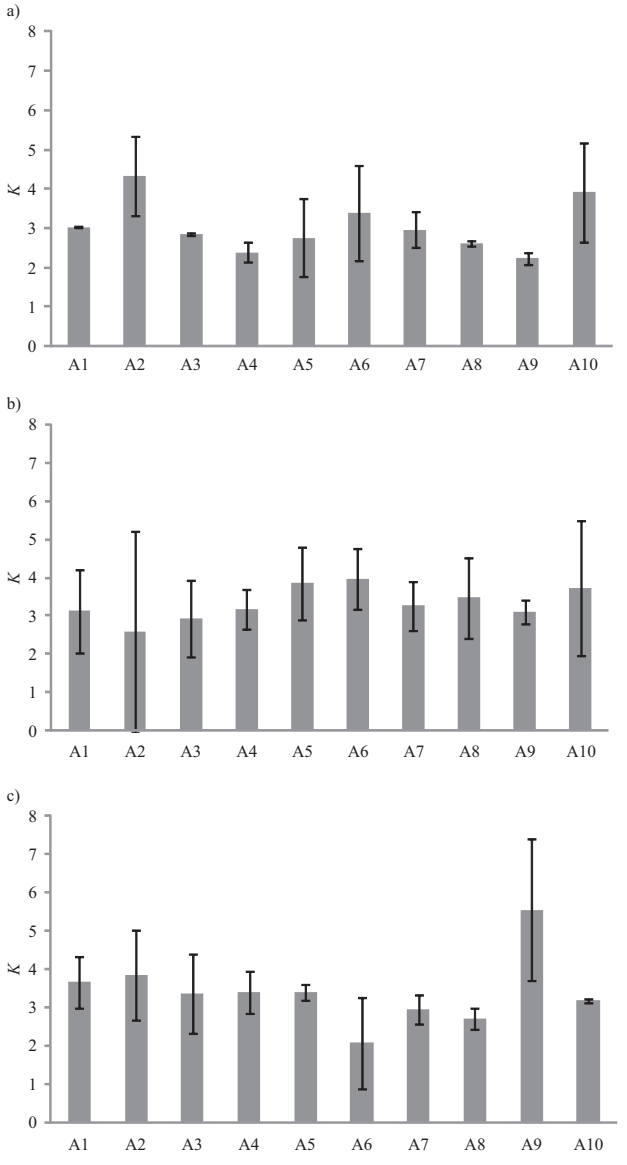

Fig. 2. Partition coefficients calculated for points of: a) first experimental plan, b) second experimental plan, and c) third experimental plan

Aqueous two-phase extraction in the continuous system

The aqueous two-phase extraction in the continuous system was performed under optimal process conditions estimated using the EVOP method. The microextractor set-up with a Y-shaped entrance and exit composed of the micromixer chip and tubular chip was used to test the applicability of microchannles for the extraction of polyphenols (Fig. 1). The experiments were performed at equal flow rates of both phases ranging from q_V=2.5 to 200 μL/min, which corresponds to the residence time in the microextractor set-up in the range of τ=2.6 to 208 s. Resulting Reynolds numbers were below 50, indicating laminar flow conditions for all investigated flow rates. At the inlet of the microchannel, the inlet streams were clearly separated (Fig. 3), indicating that parallel laminar low was established. This liquid-liquid interface parallel to the sidewalls enabled efficient phase separation at the Y-shaped end of the microchannel. As mentioned, under those conditions mass transfer resistances in each phase were reduced and consequently the mass transfer rate was significantly enhanced.

The results of the experiments in which the effect of residence time on the partition coefficient and extraction efficiency was investigated are shown in Fig. 4.

Increasing the residence time in the range from 2.60 to 10.41 s positively influences the achieved partition coefficients and extraction efficiency. Residence times higher than 20.83 s do not insure higher values of partition co-

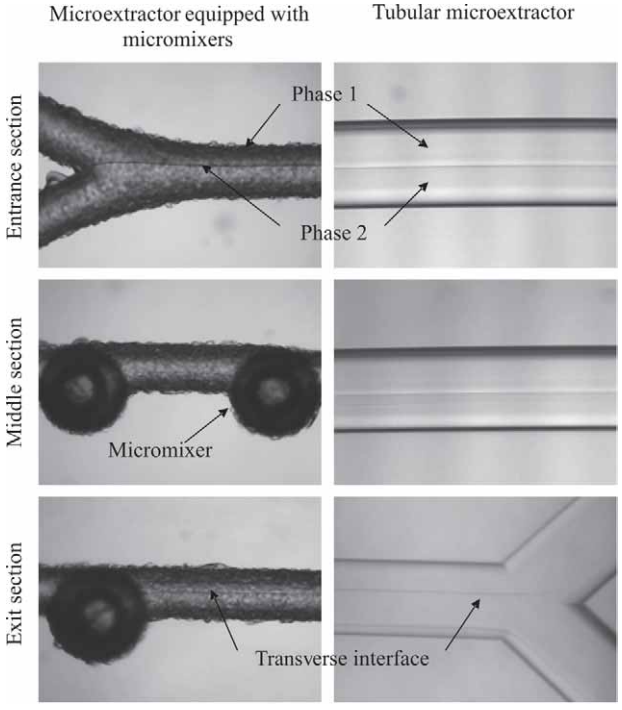

Fig. 3. Phase separation at stable laminar flow with the aqueous-aqueous interface formed in the middle of the microchannel at different positions across the microextractor equipped with micromixers and tubular microextractor. Aqueous PEG_{6000} solution flow rate of 10 μL/min, aqueous AMS solution flow rate of 30 μL/min

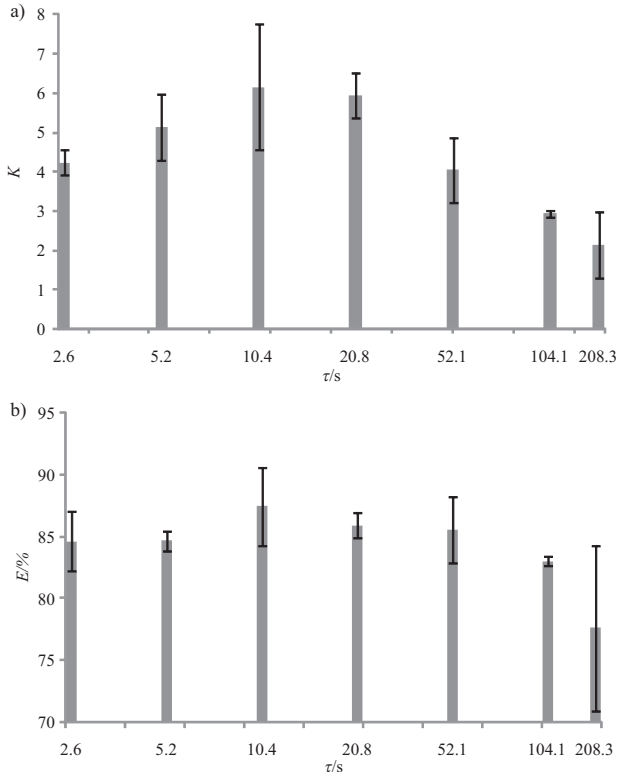

Fig. 4. Effect of residence time on: a) partition coefficient and b) extraction efficiency of polyphenols in experiments conducted using continuous microextractor

efficient nor extraction efficiency. This can be explained by the fact that longer residence time means lower flow rate (lower than 50 μL/min) that does not ensure effective mixing in the micromixer chip. For experiments performed at higher flow rates, the length of the tubular chip is not sufficient to ensure the effective separation of phases. The highest partition coefficient of K=6.1 was achieved for a residence time of only 10.41 s, which is significantly higher than in the experiment performed in the batch macroextractor. For the same residence time, the highest extraction efficiency of E=87.45 % was observed. Additionally, a higher partition coefficient was achieved in just 10.41 s in the continuous microextractor set-up, which is 60 times shorter in comparison with the extraction performed in the batch system (10 min).

Aqueous two-phase extraction of polyphenols from wine

In order to test the applicability of the aqueous two-phase extraction process in the extraction of polyphenols from real samples, experiments were performed with white wine (Graševina, Daruvar, Croatia, 2008) in the macro- and microextractor. Experiments were carried out under previously optimized process conditions in the batch system. As in previous experiments with a model solution of polyphenols, higher amounts of total phenols were detected in the top phase for both types of extractors. In the batch experiment with macroextractors, the partition coefficients of K=12.97 and the extraction efficiency of E=95.33 % were achieved after 10 min. Almost the same partition coefficient of K=12.80 and extraction efficiency of E=95.52 % were obtained in the continuous microextractor set-up for the residence time of only 10.41 s. This was an additional clear demonstration of both, the applicability of aqueous two-phase systems for the extraction of polyphenols from liquid sources and the potential of microchannels for efficient and fast extraction.

Conclusion

ATPS combined with microextractors could be a good process alternative for the extraction of polyphenols from liquid sources. Under optimal process conditions obtained during batch extraction: pH=6.50, w_{AMS}=0.0925 g/g, $w_{PEG6000}$=0.1037 g/g and γ=4.50 g/L, partition coefficient of K=5.54 and extraction efficiency of E=89.11 % were achieved. Approximately the same partition coefficient and extraction efficiency were achieved in the continuous microextractor in 60-fold shorter residence time in comparison with the experiment conducted in the macroextractor. The successful extraction of polyphenols from white wine was performed using the macro- and microextractor set-up.

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References

1. L. Bravo, Polyphenols: Chemistry, dietary sources, metabolism, and nutritional significance, *Nutr. Rev. 56* (1998) 317–333.
2. I.E. Dreosti, Antioxidant polyphenols in tea, cocoa and wine, *Nutrition, 16* (2000) 692–694.
3. K. Robards, M. Antolovich, Analytical chemistry of fruit bioflavonoids: A review, *Analyst, 122* (1997) 11R–34R.
4. H. Nawaz, J. Shi, G.S. Mittal, Y. Kakuda, Extraction of polyphenols from grape seeds and concentration by ultrafiltration, *Separ. Purif. Technol. 48* (2006) 176–181.
5. P.A. Albertsson: *Partition of Cell Particles and Macromolecules*, Wiley, New York, NY, USA (1986) p. 22.
6. A.M. Azevedo, P.A.J. Rosa, I.F. Ferreira, M.R. Aires-Barros, Optimisation of aqueous two-phase extraction of human antibodies, *J. Biotechnol. 132* (2007) 209–217.
7. S. Sharma, S. Teotia, M.N. Gupta, Bioconversion in an aqueous two-phase system using a smart biocatalyst: Casein hydrolysis by a α-chymotrypsin derivative, *Enzyme Microb. Technol. 32* (2003) 337–339.
8. C. Kepka, E. Collet, J. Persson, Å. Ståhl, T. Lagerstedt, F. Tjerneld, A. Veide, Pilot-scale extraction of an intracellular recombinant cutinase from *E. coli* cell homogenate using a thermoseparating aqueous two-phase system, *J. Biotechnol. 103* (2003) 165–181.
9. S. Chethana, C.A. Nayak, K.S.M.S. Raghavarao, Aqueous two phase extraction for purification and concentration of betalains, *J. Food Eng. 81* (2007) 679–687.
10. M.N. Kashid, D.W. Agar, Hydrodynamics of liquid-liquid slug flow capillary microreactor: Flow regimes, slug size and pressure drop, *Chem. Eng. J. 131* (2007) 1–13.
11. W.E. TeGrotenhuis, R.J. Cameron, M.G. Butcher, P.M. Martin, R.S. Wegeng, Microchannel devices for efficient contacting of liquids in solvent extraction, *Separ. Sci. Technol. 34* (1999) 951–974.
12. S.A. Bowden, P.B. Monaghan, M.J. Cooper, J. Parnell, R. Wilson, The liquid-liquid diffusive extraction of hydrocarbons from a North Sea oil using a microfluidic format, *Lab. Chip, 6* (2006) 740-743.
13. A. Hibara, M. Tokeshi, K. Uchiyama, H. Hisamoto, T. Kitamori, Integrated mutilayer flow system on a microchip, *Anal. Sci. 17* (2001) 89–93.
14. J.G. Kralj, H.R. Sahoo, K.F. Jensen, Integrated continuous microfluidic liquid-liquid extraction, *Lab. Chip, 7* (2007) 256–263.
15. H. Miyaguchi, M. Tokeshi, Y. Kikutani, A. Hibara, H. Inoue, T. Kitamori, Microchip-based liquid-liquid extraction for gas chromatography analysis of amphetamine-type stimulants in urine, *J. Chromatogr. A, 1129* (2006) 105–110.
16. K. Sato, M. Tokeshi, T. Sawada, T. Kitamori, Molecular transport between two phases in a microchannel, *Anal. Sci. 16* (2000) 455–456.
17. P. Žnidaršič-Plazl, I. Plazl, Steroid extraction in a microchannel system-mathematical modelling and experiments, *Lab. Chip, 7* (2007) 883–889.
18. B. Yang, X. Liu, Y. Gao, Extraction optimization of bioactive compounds (crocin, geniposide and total phenolic compounds) from gardenia (*Gardenia jasminoides* Ellis) fruits with response surface methodology, *Innov. Food Sci. Emerg. Technol. 10* (2009) 610–615.
19. K. Ghafoor, J. Park, Y.H. Choi, Optimization of supercritical fluid extraction of bioactive compounds from grape (*Vitis labrusca* B.) peel by using response surface methodology, *Innov. Food Sci. Emerg. Technol. 11* (2010) 485–490.
20. S. Negi, R. Banerjee, Optimization of amylase and protease production from *Aspergillus awamori* in single bioreactor through EVOP factorial design technique, *Food Technol. Biotechnol. 44* (2006) 257–261.
21. R. Banerjee, B.C. Bhattacharyya, Evolutionary operation as a tool of optimization for solid state fermentation, *Biochem. Eng. J. 13* (2003) 149–155.
22. B. Kar, R. Banerjee, B.C. Bhattacharyya, Optimization of physicochemical parameters for gallic acid production by evolutionary operation-factorial design technique, *Process Biochem. 37* (2002) 1395–1401.
23. V.L. Singleton, R. Orthofer, R.M. Lamuela-Raventós, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent, *Methods Enzymol. 299* (1999) 152–178.
24. S. Bogdan, D. Gosak, Đ. Vasić-Rački, Mathematical modeling of liquid-liquid equlibria in aqueous polymer solution containing neutral proteinase and oxytetracycline using artificial neural network, *Comput. Chem. Eng. 19* (1995) 791–796.
25. Z. Findrik, B. Zelić, Đ. Vasić-Rački, Model-based and experimental optimization using genetic algorithm, *Chem. Biochem. Eng. Q. 18* (2004) 105–116.