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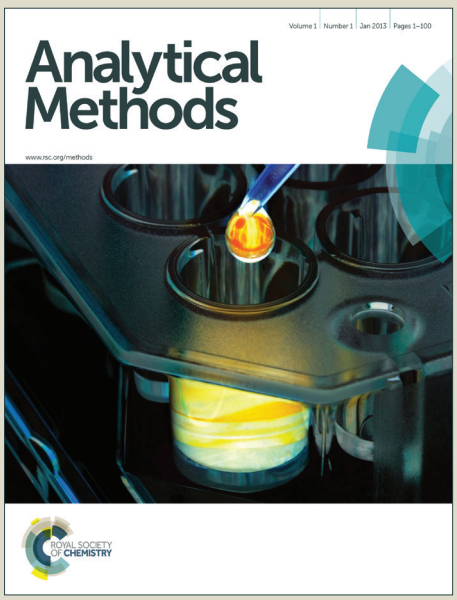

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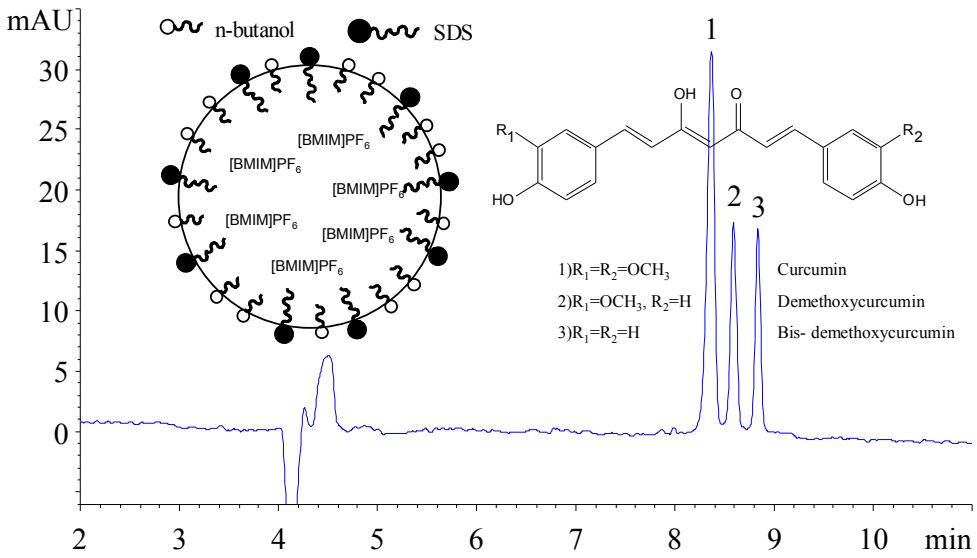
Graphical abstract



Determination of three curcuminoids in *Curcuma longa* by microemulsion electrokinetic chromatography with protective effect on the analytes

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Abstract

Alkaline degradation of curcuminoids was observed when they were exposed to alkaline solution, which are usually occur during capillary electrophoresis (CE) analysis with alkaline buffers. In the present study, the stability of curcuminoids (curcumin, demethoxycurcumin, bis-demethoxycurcumin) in different solutions (boric acid buffer, microemulsion with *n*-octane as oil phase+boric acid buffer, microemulsion with ionic liquid [BMIM]PF_6 as oil phase+boric acid buffer) were investigated. The results indicated that microemulsion systems have significant protective effect on the analytes even under alkaline conditions as compared to boric acid buffer only, while microemulsion with ionic liquid showed the best protecting effect. Therefore, a stable and rapid microemulsion electrokinetic chromatography (MEEKC) method with ionic liquid as oil phase was developed for simultaneous determination of the three curcuminoids in rhizome (*Jianghuang*) and tuberous root (*Yujin*) of *Curcuma longa*. Experimental parameters including the microemulsion compositions (surfactant, co-surfactant and oil phase), pH, concentration of borate buffer and capillary temperature were intensively investigated. Finally, the investigated curcuminoids were well separated within 9 min using the running buffer containing 100 mM SDS, 1.6 M *n*-butanol, 20 mM [BMIM]PF_6 in 10 mM borate buffer of pH 10.2. The developed method was fully validated (LOD, LOQ, intra-, inter-day precision and recovery) and successfully applied to determine the contents of investigated curcuminoids in four real samples (*Yujin* and *Jianghuang* from Chengdu and Leshan). All of the results showed that the MEEKC with ionic liquid as oil phase should be an ideal choice for the analysis of curcuminoids by CE.

Introduction

Capillary electrophoresis (CE) has been widely applied to analyze curcuminoids using different modes including capillary zone electrophoresis (CZE) [1-3], micellar electrokinetic chromatography (MEKC) [4], microemulsion electrokinetic chromatography (MEEKC) [5]. However, alkaline degradation of curcuminoids was observed when they were exposed to alkaline solutions [6-8], which were usually used in CZE and MEKC analysis. Thumnoon Nhujak, et al [5] analyzed those curcuminoids by MEEKC with acidic buffer (pH 2.5), the alkaline degradation could be avoided to some extent, but the solubility of curcuminoids is very low under acidic and neutral solutions [5, 9]. Therefore, to develop a MEEKC method with protective effect on the curcuminoids under alkaline conditions should be of interest for analyzing those compounds.

Ionic liquids are molten salts which have strong dissolution ability for most organic and inorganic compounds, with very low volatility and high thermal stability and have been testified that they are more environmentally attractive than organic solvents [10-11]. Actually, ionic liquids have been used as the constituents (surfactant, co-surfactant or oil phase) to prepare microemulsion [12-17]. In this study, the stability of curcuminoids in different solutions [boric acid buffer, microemulsion with *n*-octane as oil phase+boric acid buffer, microemulsion with ionic liquid ([BMIM]PF_6) as oil phase+boric acid buffer] were preliminarily investigated by measuring the UV absorbance at 420 nm (Fig.1). Compared with the background electrolyte, the microemulsion could significantly suppress the alkaline degradation of curcuminoids. The protective effect of microemulsion may due to the inclusion of analytes into the oil phase, which is similar to that of cyclodextrin [9, 18]. Furthermore, it was reported that the stability of curcuminoids could be greatly increased by the presence of some reducing agents like GSH,

ascorbic acid [6, 7]. Therefore, the microemulsion with [BMIM]PF_6 had a better protection effect (even under alkaline conditions, Fig. 1) on curcuminoids than the microemulsion with octane as oil phase, which may due to the reducibility of [BMIM]PF_6 [19-20]. Herein, microemulsion with [BMIM]PF_6 as oil phase is undoubtedly a good choice for MEEKC analysis of curcuminoids.

Curcuma longa is widely used as a traditional Chinese medicine, natural pigment and spice [21]. Its rhizome (*Jianghuang*) and tuberous root (*Yujin*) are reported to mainly contain three curcuminoids, namely curcumin, demethoxycurcumin and bis-demethoxycurcumin (Fig.2). Curcumin possesses multiple pharmacological activities, including cholesterol-lowering [22], antidiabetic [23], anti-inflammatory [24], anti-oxidant [25], antimicrobial [26], immune activation [27] and antidepressant [28] activity. Demethoxycurcumin is reported to own practically the same activity as curcumin [29], while different from the cholesterol-lowering activity of the former two curcuminoids, bis-demethoxycurcumin is reported to have strong choleretic effect [30]. Therefore, analysis of those three curcuminoids in *C. longa* is helpful for its quality control and pharmaceutical studies.

In the present study, a MEEKC method with protective effects even under alkaline conditions on curcuminoids was developed by using [BMIM]PF_6 as oil phase, and the developed method was fully validated and applied to determine those curuminoids in *C. longa*.

Materials and methods

Chemicals and reagents

Sodium Dodecyl Sulfonate (SDS), *n*-octane and *n*-butanol were purchased from Chongqing Chuandong Chemical (Group) Co., Ltd. (Chongqing, China). Sodium hydroxide and boric acid were obtained from Chengdu Kelong Chemical Works (Chengdu, China), 1-Butyl-3-methylimidazolium hexafluorophosphate

([BMIM]PF_6) was purchased from Lanzhou Institute of Chemical Physics (Lanzhou, China). All reagents above were of analytical grade, and reverse osmosis (RO) water used throughout was prepared by an AKWL-IV-16 water purification system (Chengdu Tang's Kangning Science and Technology Development Co., Ltd., Chengdu, China).

Curcumin, demethoxycurcumin and bis-demethoxycurcumin with the purity higher than 99% were purchased from Chengdu Must Bio-technology Co., Ltd. (Chengdu, China). The rhizome (*Jianghuang*) and tuberous root (*Yujin*) samples from the same plant of *C. longa* were collected from Chengdu and Leshan, Sichuan province, respectively. The voucher specimens of *C. longa* were deposited at the Department of Pharmaceutics, School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China.

Apparatus and procedures

All CE separations were performed on an Agilent 7100 3D CE system (Agilent Technologies, Palo Alto, CA, USA) equipped with a DAD and an Agilent ChemStation software. The uncoated fused-silica capillary (Hebei Yongnian Ruifeng Chromatographic Implements, Hebei, China) was of 50 μm i.d and had a total length of 54 cm (46 cm effective length). All the analyses in the present study were performed on the same capillary. The UV-2450 UV-visible spectrophotometer (Shimadzu UV-2450 spectrophotometer, Tokyo, Japan) was used for measurement of UV absorbance. The KQ-100B ultrasonic cleaner (Kunshan ultrasonic instruments Co., Ltd., Jiangsu, China) was applied for the preparation of microemulsion and samples. A Delta 320 pH meter (Mettler-Toledo Instruments, Shanghai, China) was used for measuring the pH of running buffer.

The running buffer was prepared by adding of 100 mM SDS, 1.6 M *n*-butanol and 20 mM [BMIM]PF_6 to 10 mM borate buffer of pH 10.2 (adjusted by 1 M NaOH

solution). The mixture was then ultrasonicated for 20 min. The freshly prepared microemulsion running buffer was stood for at least one hour at ambient temperature (about 25 °C) prior to use.

Curcumin, demethoxycurcumin and bis-demethoxycurcumin were accurately weighted and dissolved in ethanol to prepare the mixture of standard solutions (about 1.5 mg mL^{-1}), then the solutions were further diluted to different concentrations by adding desired amount of microemulsion. For preparation of sample solution, a desired amount (1.0 g) of crude material powders was extracted with 10 mL absolute ethanol in a 50 mL flask by ultrasonication for 40 min. After extraction, the extract was cooled down to the room temperature (25 °C), and made up the lost weight with absolute ethanol, then centrifuged for 5 min at 4.2×10^3 *g*, and the supernatant was filtered through a 0.45 μm nylon membrane (Auto science instrument Co., Ltd., Tianjing, China) before use.

The capillary was flushed daily with 0.1 M sodium hydroxide for 15 min, followed by RO water for 5 min and finally with running buffer for 15 min. Between consecutive analyses, the capillary was rinsed with 0.1 M sodium hydroxide (2 min), then with RO water (2 min), and finally with the running buffer (5 min). The operating conditions were: injection pressure 50 mbar; injection time 3 s and detection wavelength at 420 nm.

Results and discussion

Optimization of separation conditions

Effect of SDS concentration

It is known that the concentration of SDS has a remarkable effect on the separation achieved in MEEKC for it has effect on the oil droplet charge and size, the level and direction of the EOF, and the level of any ion-pairing of microemulsion droplets with charged solutes [31]. Different concentrations (60, 80, 100, 120, 140,

and 160 mM) of SDS were investigated by using resolution (*Rs*) of curcumin *vs* demethoxycurcumin (*Rs1*), demethoxycurcumin vs bis-demethoxycurcumin (*Rs2*) and the retention time of bis-demethoxycurcumin (RT_b) as markers (the other conditions were: 1.4 M *n*-butanol, 20 mM [BMIM]PF_6, pH 10.0, 20 mM borate buffer, 20 kV and 25 °C). As shown in Fig.3A, the migration time of analytes increased with the increase of concentration of SDS. When the concentration of SDS was higher than 120 mM, some baseline drift was observed due to the high level of current. Therefore, in order to achieve better separation with good repeatability in a shorter analysis time, 100 mM of SDS was used in the subsequent experiments.

Effect of *n*-butanol concentration

n-butanol was reported to stabilize the microemulsion and affect the selectivity of the analysis [32]. Furthermore, the concentration of co-surfactant could change the viscosity of the solution, and therefore affected the EOF. In the present study, different concentrations (1.2, 1.4, 1.6, 1.8 and 2.0 M) of *n*-butanol were investigated (the other conditions were: 100 mM SDS, 20 mM [BMIM]PF_6, pH 10.0, 20 mM borate buffer, 20 kV and 25 °C). As it was shown in Fig.3B, with the concentration of *n*-butanol increased from 1.2 M to 1.6 M, both the resolution of analytes and migration time increased, which may be caused by the increased viscosity of *n*-butanol. While further increase of *n*-butanol concentration led to the decrease of resolution and migration time. The excess *n*-butanol in the aqueous phase might be adsorbed and shield the negative-charged micelle, consequently increased the viscosity of micelle and shortened the migration time [33]. So 1.6 M *n*-butanol was selected for its best separation efficiency (*Rs1* > 1.35 and *Rs2* > 1.56).

Effect of [BMIM]PF_6 concentration

Different concentrations of [BMIM]PF_6 (0, 10, 20, 30, 40 and 50 mM) were compared (the other conditions were: 100 mM SDS, 1.6 M *n*-butanol, pH 10.0, 20 mM borate

buffer, 20 kV and 25 °C). The results (Fig.3C) indicated that the concentrations of BMIM]PF_6 had not significant effect on the separation of analytes, and the best resolution (*Rs1* > 1.40 and *Rs2* > 1.62) for analytes can be obtained at the concentration of 20 mM. Furthermore, when the concentration of [BMIM]PF_6 increased from 0 mM to 20 mM, the cation of [BMIM]PF_6 could shield the anion of SDS, so the EOF increased and migration time of analytes shortened. However, if the concentration of [BMIM]PF_6 increased from 20 mM to 50 mM, the adsorption effect of cation of [BMIM]PF_6 to capillary wall played the dominant role and led to the prolonging of migration time of analytes. Hence, 20 mM [BMIM]PF_6 was used.

Effect of pH, buffer concentration and temperature

As one of the most important parameters in MEEKC separation, pH is directly related to the degree of solute ionization and EOF velocity [34]. In this study, different pH (9.0, 9.5, 10.0, 10.2, 10.5, 11.0) were evaluated (the other conditions were: 100 mM SDS, 1.6 M *n*-butanol, 20 mM [BMIM]PF_6, 20 mM borate buffer, 20 kV and 25 °C). The results in Fig.3D showed that pH played a crucial role on the separation. With the pH increased from 9.0 to 11.0, both *Rs1* and *Rs2* increased accordingly, when the pH reached at 10.2, those three analytes could be baseline separated (*Rs1* > 1.8, *Rs2* > 2.3). Therefore, pH 10.2 was chosen to achieve acceptable resolution within a short migration time (11.65 min).

It has been shown previously that using low-ionic-strength (5 - 10 mM) borate or phosphate buffers generated relatively low currents and high EOF value [16]. In present study, different concentrations (5 - 50 mM) borate buffer were investigated (the other conditions were: 100 mM SDS, 1.6 M *n*-butanol, 20 mM [BMIM]PF_6, pH 10.2, 20 kV and 25 °C). The results (not shown) revealed that baseline separation could be achieved only when the concentration of borate buffer was above 10 mM. In general, use of high buffer concentrations should be prevented because the high electric current generated led to low efficiency and poor reproducibility [32]. So 10

mM borate buffer was used for its good separation within the shortest analysis time. Furthermore, the effect of temperature was also investigated. The migration time decreased with the temperature increased. Finally 25 °C was used for its best separation efficiency ($Rs1 > 1.7$, $Rs2 > 2.1$, $RT_b < 8.9$ min).

Based on the optimization, the best conditions for the analysis were: running buffer composed of 100 mM SDS, 1.6 M *n*-butanol, 20 mM [BMIM]PF_6 in 10 mM borate buffer of pH 10.2. Applied voltage was 20 kV and capillary temperature was 25 °C.

Method Validation

Linearity, LOD, and LOQ

Stock solution containing three standard compounds (curcumin, demethoxycurcumin and bis-demethoxycurcumin) was prepared and diluted to appropriate concentrations for the construction of calibration curves. At least five concentrations of the solution were analyzed in triplicates, and then the calibration curves were constructed by plotting the peak area of individual standard versus the concentration of each analyte. The limits of detection (LOD) and quantification (LOQ) under the optimum conditions were determined based on the signal-to-noise (S/N) ratio of 3 and 10 respectively. Table 1 shows the linear regression data, LOD and LOQ.

Precision and recovery

Intra- and inter-day variations were chosen to determine the precision of the developed assay. The known concentrations of the three investigated compounds were tested. For intra-day variability test, the mixed standards solutions were analyzed for six replicates within one day, while for inter-day variability test, the solutions were examined in duplicates for consecutive three days. Variations were expressed by relative standard deviation (RSD).

The recovery was preformed by adding a known amount of individual standards into a certain amount (0.50 g) of crude material (*Jianghaung* from Leshan) powder. The mixture was extracted and analyzed using the method mentioned above. Three replicates were performed for the test. Table 2 shows the precision and recovery results.

Determination of investigated compounds in four different samples

Under the optimum conditions, the ethanol extracts of the four samples (*Yujin* from Chengdu and Leshan, *Jianghuang* from Chengdu and Leshan) were analyzed. The electrochromatograms are shown in Fig.4. The identifications of compounds were done by comparing their retention time with those of standards, as well as adding the individual standard to the samples. By using the calibration curve of each standard, the contents of investigated compounds in those three TCMs were determined. The quantification results were shown in Table 3. Except for *Yujin* from Chengdu, all the analytes could be detected in other samples, while the content of bis-demethoxycurcumin in *Yujin* from Leshan was under the LOQ. Therefore, the contents of curcuminoids in the rhizome (*Jianghuang*) are significantly higher than in the tuberous root (*Yujin*) of *C. longa*. Furthermore, the content of curcumin was higher than that of demethoxycurcumin and bis-demethoxycurcumin in all samples.

Conclusion

An MEEKC method with protective effect on the analytes was developed to simultaneously determine the contents of curcumin, demethoxycurcumin and bis-demethoxycurcumin in *C. longa*. The curcuminoids are stable in the microemulsion system, which ensure the accuracy of the quantitative results of MEEKC method. Therefore, the developed MEEKC with ionic liquid as oil phase is an ideal method for the analysis of curcuminoids by CE. In addition, the developed

MEEKC method may also be applied in analyzing other analytes which are unstable during CZE analysis.

Acknowledgement

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Legend of Figures:

Fig. 1

The stability tests of three curcuminoids dissolved in 20 mM boric acid (◆), 3.3% SDS (w/v), 6.6% *n*-butanol and 0.8% *n*-octane in 20 mM boric acid buffer (■), and 3.3% SDS (w/v), 6.6% *n*-butanol and 0.8% [BMIM]PF_6 in 20 mM boric acid buffer (▲), detected by UV absorbance (420 nm).

Fig. 2

Chemical structures of three curcuminoids.

Fig. 3

Effects of SDS concentration (A), *n*-butanol concentration (B), [BMIM]PF_6 concentration (C) and buffer pH (D) on the *Rs1* (◆), *RS2* (■) and *RTb* (▲)

Fig. 4

Electrochromatograms for the mixture of standards (A), ethanol extracts of *Jianghuang* from Chengdu (B), *Yujin* from Leshan (C) and *Jianghuang* from Leshan (D).

1, curcumin 2, demethoxycurcumin 3, bis-demethoxycurcumin U, unknown

Table 1 Linear regression data, LOD and LOQ of three curcuminoids

Analytes	Calibration curves	Linear range (μg mL^{-1})	R^2	LOD (μg mL^{-1})	LOQ (μg mL^{-1})
Curcumin	y = 331.00x - 4.21	41.87-670.00	0.9962	9.30	25.04
Demethoxycurcumin	y = 565.12x + 0.91	25.63-410.00	0.9961	3.80	12.50
Bis-demethoxycurcumin	y = 584.75x + 7.45	25.63-410.00	0.9978	3.20	11.00

Table 2 Intra- and Inter-day precision and recovery of the investigated compounds

Analytes	Intra-day (*n* = 6, RSD %)	Inter-day (*n* = 6, RSD %)	Recovery (*n*=3, %)
Curcumin	3.56	3.97	98.87
Demethoxycurcumin	4.90	5.86	95.54
Bis-demethoxycurcumin	3.45	4.20	103.42

Table 3 Contents (mg g^{-1}) of investigated compounds in four samples

Analytes	*Yujin* (CD[a])	*Jianghuang* (CD)	*Yujin* (LS)	*Jianghuang* (LS)
Curcumin	-[b]	9.52	1.02	7.93
Demethoxycurcumin	-	2.27	0.12	1.78
Bis-demethoxycurcumin	-	2.73	+[c]	1.80

a, CD, Chengdu, LS, Leshan; b, under the limit of detection; c, under the limit of qualification

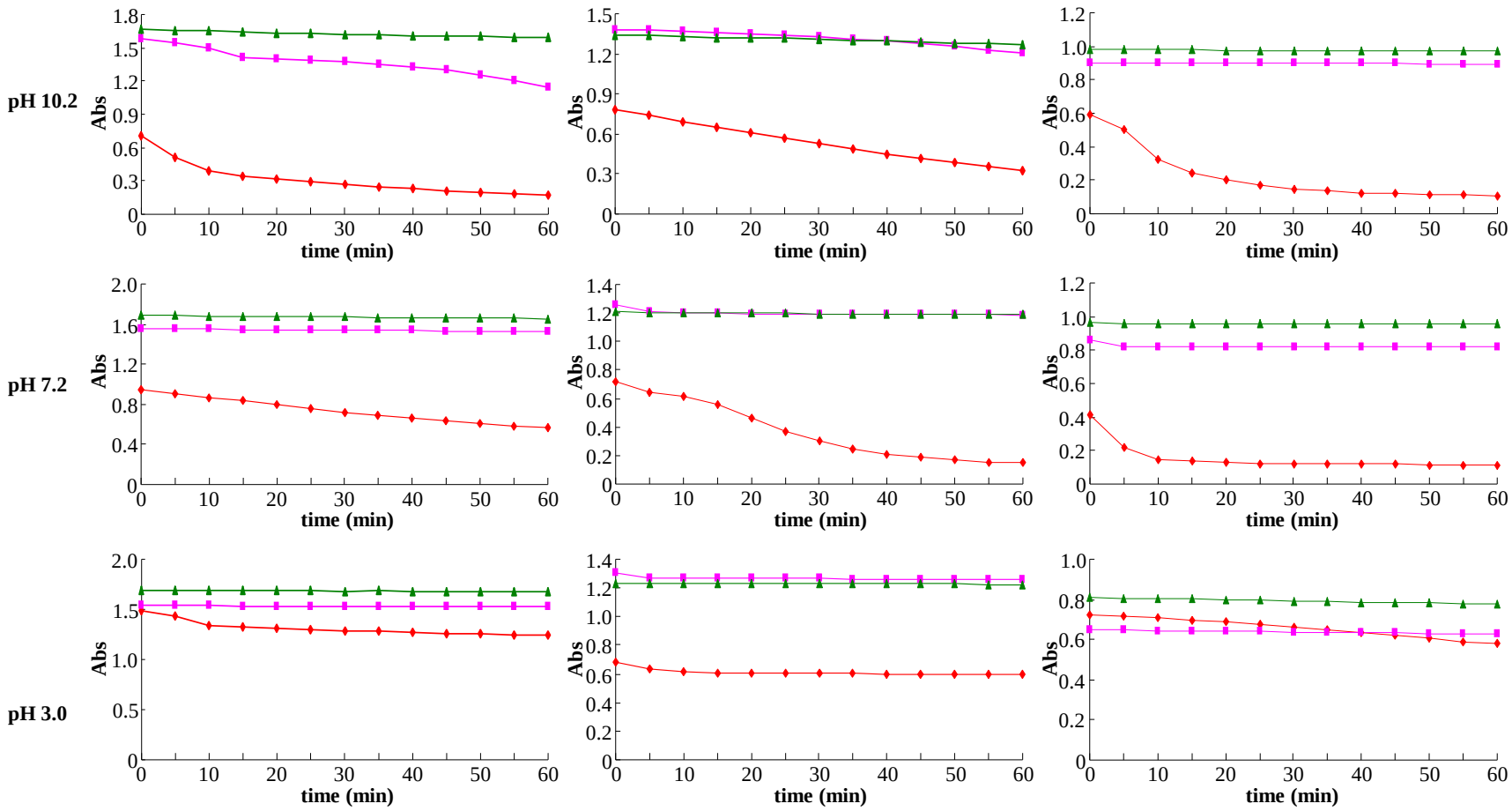

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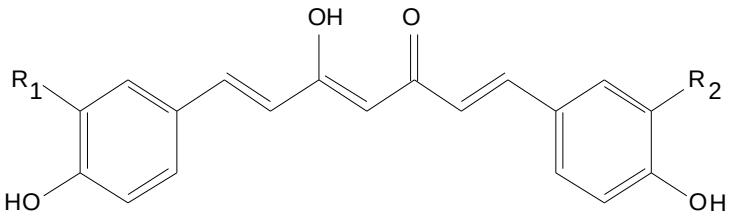
Fig. 1



1)R_1=R_2=OCH_3 Curcumin

2)R_1=OCH_3, R_2=H Demethoxycurcumin

3)R_1=R_2=H Bis- demethoxycurcumin

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Fig. 2

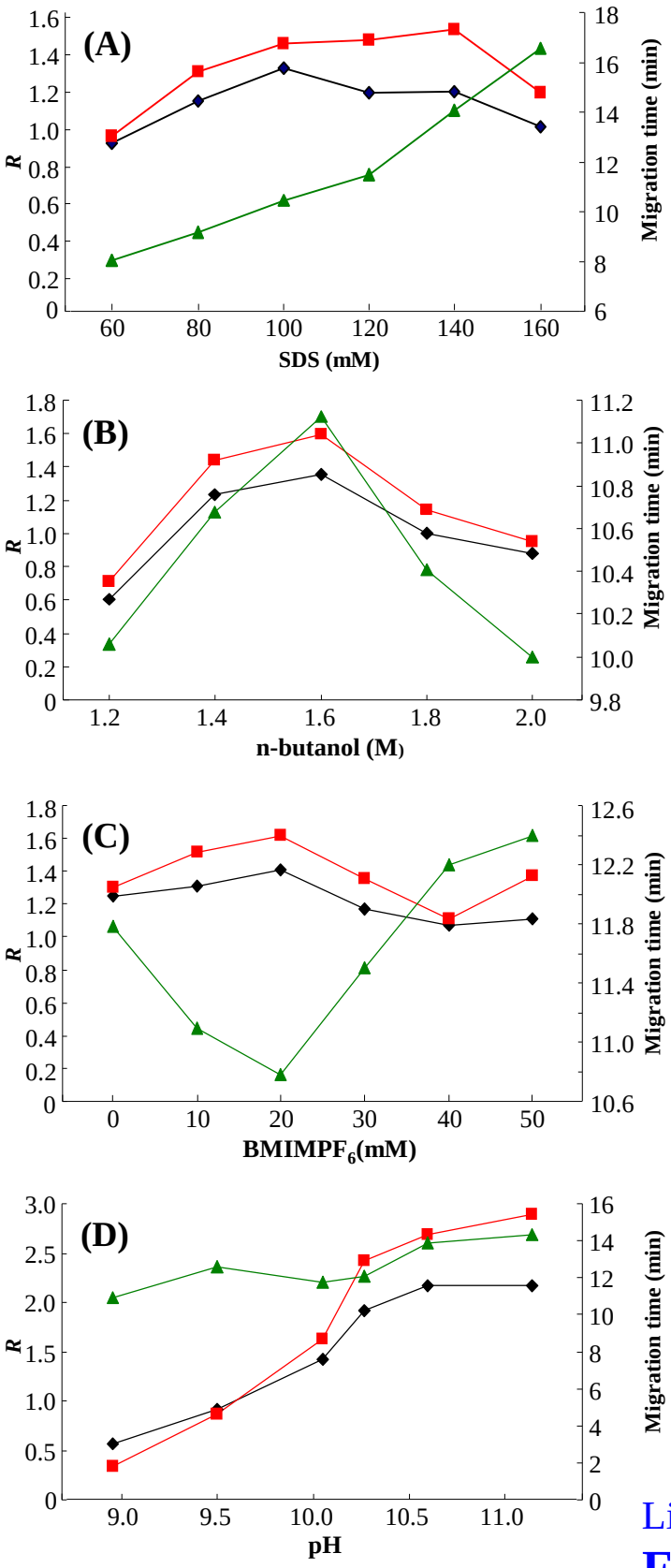

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Fig. 3

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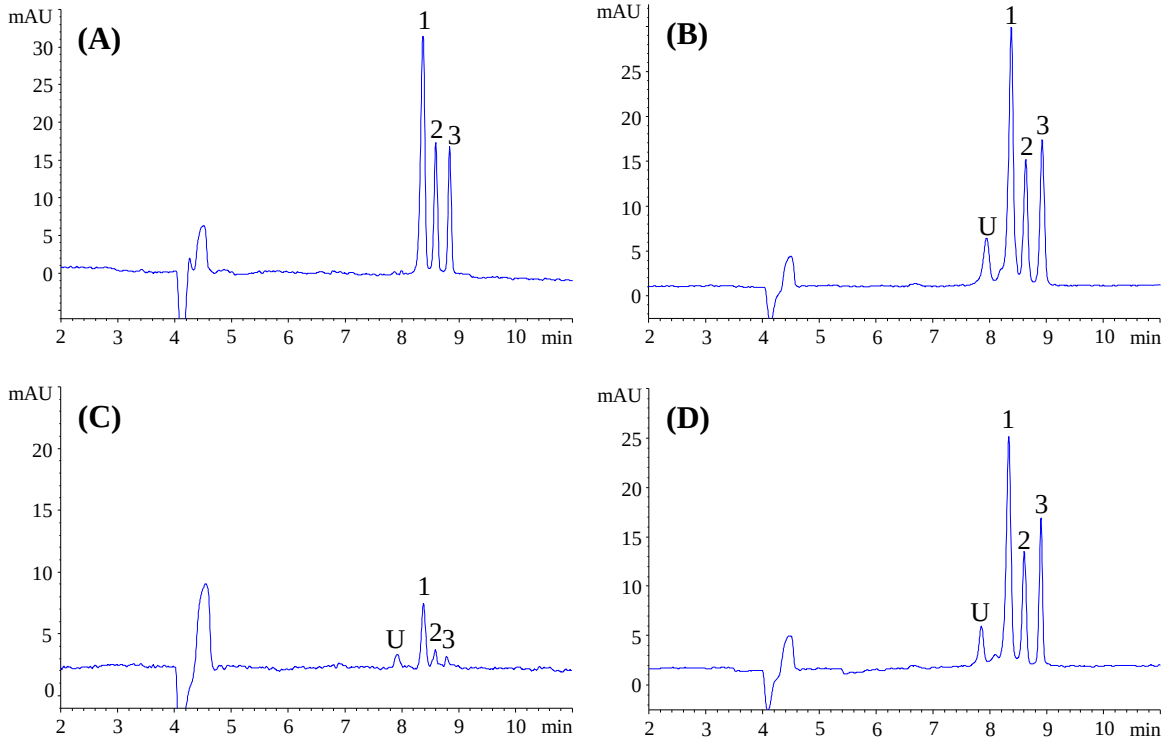

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Fig. 4