약학박사학위논문

Development of dispersive liquid-liquid microextraction methods for the analysis of polar and nonpolar components in liquid samples

액체 시료중 극성 및 비극성 물질 분석을 위한 분산 액체-액체 미량추출법의 개발

2017년 2월

서울대학교 대학원

약학과 약품분석학전공

LI JING

Development of dispersive liquid-liquid microextraction methods for the analysis of polar and nonpolar components in liquid samples

액체 시료중 극성 및 비극성 물질 분석을 위한 분산 액체-액체 미량추출법의 개발

지도교수 권 성 원

이 논문을 약학박사 학위논문으로 제출함

2017년 2월

서울대학교 대학원

약학과 약품분석학전공

이 정

이정의 박사학위논문을 인준함

2017년 2월

위 원 장 박 정 일 (인)

부 위 원 장 양 현 옥 (인)

위 원 권 성 원 (인)

위 원 김 유 선 (인)

위 원 홍 순 선 (인)

Abstract

This study describes a novel sample preparation method for simultaneous identification of 10 phenolic acids from wine using ion pair dispersive liquid-liquid microextraction based on the solidification of a floating organic droplet (IP-DLLME-SFO). For the first time, the ion-pairing technique combined with DLLME-SFO was applied for the detection of phenolic acids. The IP-DLLME-SFO dramatically enhanced the extraction efficiency for very polar phenolic acids, such as gallic acid and protocatechuic acid, which could not be extracted by DLLME-SFO in the absence of an ion-pairing reagent. The effects of the parameters that can affect the extraction efficiency were systematically investigated, including the type and concentration of ion-pairing reagent, type and volume of extraction and dispersive solvents, extraction time, sample pH, and ion strength. And the optimized microextraction conditions yielded high enrichment factors. The combined application of IP-DLLME-SFO and superficially porous particle (SPP) column provided a sensitivity of analysis method, which can separation 10 phenolic acids with similarity chemical structures in 40 min.

The method was validated in terms of linearity, sensitivity, precision and recovery. The coefficient of determination (R^2) was higher than 0.994 for all calibration curves. The method linearity was constructed in the range of 0.01 – 15 μg/mL, and the sensitivity expressed as limit of detection was as low as 10 ng/mL. The intra- and inter-day precisions were below 7.95 % and 9.33 %, respectively. With a simple dilution, the measured absolute recovery values of around 81.5 ~ 109% were obtained for the compounds, indicating that the extraction efficiency was very high. The method that we developed was successfully applied to the analysis of commercial wine samples with no significant matrix effect, revealing different levels

of phenolic acids among these products. The result suggest that the combined use of DLLME-SFO and SPP column may be applicable to the analysis of various polar and non-polar compounds in liquid sample with complex matrices.

Keywords: DLLME-SFO; ion pairing; polar; phenolic acids; wine; Core–shell particle column; HPLC

Student Number: 2011-23014

Contents

List of figures

List of tables

List of Abbreviations

IP: ion-pairing technique

DLLME: dispersive liquid–liquid microextraction

DLLME-SFO: dispersive liquid-liquid microextraction based on the solidification of a floating organic droplet

IP-DLLME-SFO: ion pair dispersive liquid-liquid microextraction based on the solidification of a floating organic droplet

LLE: liquid-liquid extraction

IP-SAME: ion pair-based surfactant-assisted microextraction

IP-SA-DLLME: ion pair-based surfactant assisted DLLME

LC: liquid chromatography

HPLC: high performance liquid chromatography

UHPLC: ultrahigh performance liquid chromatography

HPLC-UV: high performance liquid chromatography with ultraviolet detector

SPE: solid-phase extraction

LPME: liquid-phase microextraction

SPME: solid-phase microextraction

TEAB: tetraethylammonium bromide

TPAB: tetrapropylammonium bromide

TBAB: tetrabutylammonium bromide

THPAB: tetra-heptylammonium bromide

THAB: tetrahexylammonium bromide

LOQ: limit of quantification

RSD: relative standard deviation

LOD: limit of detection

OAD: orthogonal array design

HSD: honest significant difference

MS: mass spectrometry

MS/MS: tandem mass spectrometry

ACN: acetonitrile

RP: reversed Phase

SPP: superficially porous particle

EF: enrichment factor

ANOVA: analysis of variance

S.E.M.: standard error of the mean

GC: gas chromatography

GC–MS: gas chromatography–mass spectrometry

ND: not detected

1. Introduction

A new liquid phase microextraction technique, dispersive liquid–liquid microextraction (DLLME), has been extensively explored in recent years because of its simplicity, rapidity, convenience, and low cost [3]. However, the DLLME technique is limited in its application to the extraction of polar compounds or organic acids because their polar ionizable groups restrict transfer to water-immiscible solvents. To solve this problem, some successful methods for extraction of polar compounds have been developed. For example, in liquid-liquid extraction [4], extraction of ionizable compounds into an organic phase was made possible using an ion- pairing agent, resulting in improved recovery and selective isolation from complex matrices [5, 6]. Similarly, the ion-pairing technique was applied to LPME, as exemplified by ion pair-based surfactant-assisted microextraction (IP-SAME) of fluoroquinolones [7] and ion pair-based surfactant assisted DLLME (IP-SA-DLLME) of heavy metals [8]. In view of those studies, we employed a modified version of DLLME based on the solidification of floating organic droplets (DLLME-SFO) in the current study and first proposed to apply IP-DLLME-SFO coupled to LC using a core–shell particle column for the analysis of polar compounds in a liquid sample.

Among numerous polar compounds, phenolic acids are one of the most important classes of organic acids because they are common constituent in honey, fruits, vegetables and plants. Additionally, phenolic compounds are usually responsible for wine color and contribute to the bitter flavour of wine [9, 10]. Their antioxidant properties are beneficial for overall human health, due to their scavenging of reactive free radicals that are associated with the pathophysiology of various diseases, such as inflammatory and degenerative diseases [11]. The potential of phenolic compounds as preservatives in winemaking has been confirmed [12]. Phenolic acids are a predominant subclass of phenolic compounds comprising almost a third of phenolic acids in plants [13]. They are naturally present in red and white wine as free acids, glycosylated derivatives, or esters of tartaric, quinic, and shikimic acids [14].

Different numbers and positions of hydroxyl groups on the aromatic ring of phenolic acids produce a variety of similar chemical structures (Fig.1), contributing to the complexity of phenolic acid analysis [15]. The analysis of phenolic acids in wi-

ne requires pretreatment, including the clean-up and preconcentration of wine sample, because the wine matrix is very complex, and the concentration levels of phenolic acids in wine are quite low.

Among numerous methods developed for the analysis of phenolic acids in wine samples [10, 16, 17], liquid chromatography [18] has predominantly been used, due to its high accuracy and sensitivity. Before chromatographic analysis, pre-treatment steps such as extraction and concentration are usually required because wine matrices are very complex and the levels of phenolic acids in wines are low. The most frequently used sample preparation techniques are liquid–liquid extraction [4] [10, 18-20] and solid-phase extraction (SPE) [17, 21, 22]. However, LLE usually requires a large volume of organic solvents and is very time-consuming. The organic solvent precipitation method is generally non-specific, though the procedure is very simple. Although SPE consumes much less solvent and time than LLE, it still involves the consumption of expensive SPE cartridges. Alternatively, a number of microextraction methods such as liquid-phase microextraction (LPME) and solid-phase microextraction (SPME) have been developed for more sensitive and environmentally-friendly analysis of phenolic acids [17, 22, 23].

The goal of this study was to develop a new, eco-friendly analytical method to rapidly and efficiently determine the levels of various phenolic acids in wine. Recently, a novel liquid-phase microextraction (DLLME), has become very popular [21]. This method is based on a ternary component solvent system in which the appropriate mixture of dispersive and extraction solvents are injected into the aqueous sample. After violent agitation, a cloudy solution was formed that indicated the extraction solvent was dispersed into the aqueous sample as very fine droplets. The analytes transferred into the organic solvent from the aqueous solution, and an organic phase containing highly concentrated target compounds was formed after centrifugation. Thus, the target compounds could be easily transferred by a syringe for analysis [8]. Classic DLLME uses either high-density extraction solvents such as chloroform, carbon tetrachloride, and chlorobenzene or low-density solvents such as n-hexanol, n-hexane, cyclohexane, and dibutyl ether in addition to specially shaped extraction tubes to facilitate transfer and prevent the evaporation of the extracted phase.

In a modified DLLME method based on the solidification of floating organic droplets (DLLME-SFO), the easy and reliable collection of the extraction phase is possible without a special extraction tube because of the use of low-density solvents with melting points close to room temperature [16]. This technique has mainly been applied to samples consisting of simple matrices, and its use with complex matrices such as biological samples, or other food samples.

We employed DLLME based on the solidification of floating organic droplets (DLLME-SFO) in the current study because it can reduce matrix effects and facilitate the selective collection of the organic phase free from matrix interferences, as we found in our previous studies [24, 25]. IP extraction has long been used in combination with various sample preparation techniques such as SPE [26-29], solid-phase microextraction (SPME) [30], single-drop microextraction (SDM) [31-34], hollow-fiber liquid-phase microextraction (HF-LPME) [35], supported liquid membrane, SLM and LLE using a water immiscible organic (extraction) solvent such as chloroform30–32 and a water-miscible organic (extraction) solvent such as ethyl acetate, acetonitrile, acetone or methanol for the selective extraction of various ionizable organic compounds. For the quantitative determination of several polar organic compounds, to date the method has been reported for the simultaneous residual analysis of polar [36]. Therefore, in the current study, a novel sample preparation technique is proposed based on IP-DLLME-SFO.

In the method that we developed, 10 phenolic acids were effectively extracted and concentrated by IP-DLLME-SFO, followed by a rapid LC analysis using a column packed with sub-3 mm core-shell particles. Various parameters affecting the extraction efficiency of the technique as well as experimental parameters influencing the separation efficiencies of the target analytes were investigated so as to establish the optimum conditions. The applicability of the proposed analytical method has also been experimentally evaluated by applying it in the analysis of different wine samples of varying phenolic acids. To the best of our knowledge, this study is the first application of IP-DLLME-SFO coupled to LC using a core- shell particle column for the analysis of phenolic acids in wine.

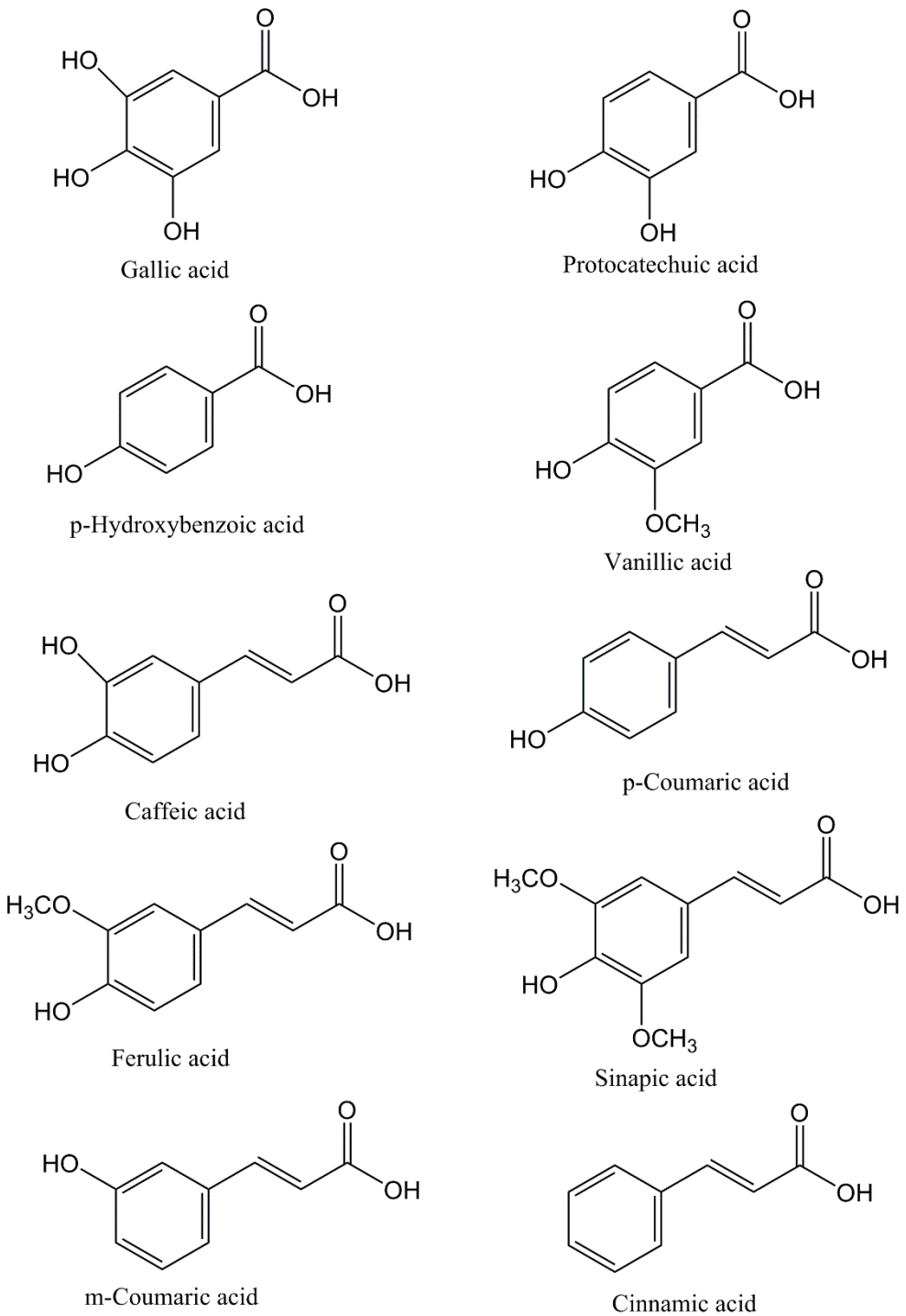


Figure 1. The chemical structures of phenolic acids

2. Materials and methods

2.1. Reagents and chemicals

Phenolic acids (gallic acid, p-hydroxybenzoic acid, vanillic acid, caffeic acid, p-coumaric acid, ferulic acid, sinapic acid, cinnamic acid) were purchased from Sigma–Aldrich (St. Louis, MO). Tetraethylammonium bromide (TEAB), tetrapropylammonium bromide (TPAB), tetrabutylammonium bromide (TBAB), tetra- heptylammonium bromide (THPAB), tetrahexylammonium bromide (THAB), protocatechuic acid, trans-m-coumaric acid, 1- undecanol, and 2-dodecanol were obtained from TCI (Tokyo, Japan). All other regents were purchased from Sigma–Aldrich unless otherwise noted. LC-grade acetone, water, methanol, and acetonitrile were obtained from Duksan Chemical Co. Ltd. (Ansan, Korea).

2.2. Instrumentation

Chromatographic analysis was performed using a Flexar FX-10 UHPLC system (PerkinElmer, Shelton, CT) with a Flexar FX PDA (PerkinElmer, Shelton, CT). The detection wavelength was 280 nm. An Agilent Poroshell EC-C18 column (2.1 - 150 mm) packed with 2.7 μm core–shell particles was used for chromatographic separation of the 10 phenolic acids. A gradient elution was carried out using a binary mobile phase composed of eluent A (water with 0.1% formic acid) and eluent B (acetonitrile with 0.1% formic acid). The linear gradient program was as follows: 0–1 min, 10% B; 1–25 min, 10–15% B; 25–35 min, 15–50% B; 35–36 min, 50– 100% B; 36–46 min, 100% B. After each run, the gradient was held at 100% B for 12 min for column washing and then returned to 10% B for 10 min for column equilibration. The column temperature was kept at 30℃. The flow rate was 0.2 mL/min and the

injection volume was 3 μL.

2.3. Preparation of standard solutions and blank, spiked, and real samples

Stock solutions of phenolic acids were prepared in methanol and working solutions were freshly made by appropriate dilution of the stock solutions in methanol. All the wine samples were purchased from local markets in Korea. The wine samples were filtered through a 0.2 μm hydrophobic filter (Toyo Roshi Kaisha, Ltd., Tokyo, Japan) and stored at 4℃ until use. Optimization studies for extraction conditions were performed using pure water spiked with standards. Spiked wine samples used for method application and method validation were prepared as follows: a fresh standard solution was evaporated in a glass test tube under a stream of high-purity nitrogen, and blank wine was added to the tube to produce a wine sample at the desired concentration of phenolic acids. To reduce sample matrix effects, red and white wine samples were diluted with water by ten-fold and five-fold, respectively, before processing using the microextraction proce-dure described below.

2.4. DLLME-SFO procedure

A total of 1.5 mL diluted wine (1.0 mL of wine + 0.5 mL of water) or water was mixed with 1.0 mL of 50 mM sodium phosphate buffer (pH = 6.0) and 2.5 mL of 10 mM THAB to produce a final 5.0 mL solution containing 5 mM THAB. Five hundred microlitres of a mixture of 1-dodecanol (extraction solvent) and methanol (dispersive solvent) mixed at 1:9 (v/v) were rapidly injected into the solution using a 1.0-mL gastight Hamilton syringe, forming a stable, cloudy solution. Fine droplets of the organic phase containing the ion pairs of phenolic acids and THAB accumulated at the surface of the sample solution after centrifugation at 3500 g for 5 min. The glass

tube was placed on ice for 10 min, and then the solidified droplet was quickly transferred to a 200 μL Eppendorf tube. After thawing, the droplet was centrifuged (10,000 g, 3 min), and 30 μL of the upper layer were diluted to 90 μL with methanol. Three microlitres of the diluted solution were directly injected onto the LC (Fig.2).

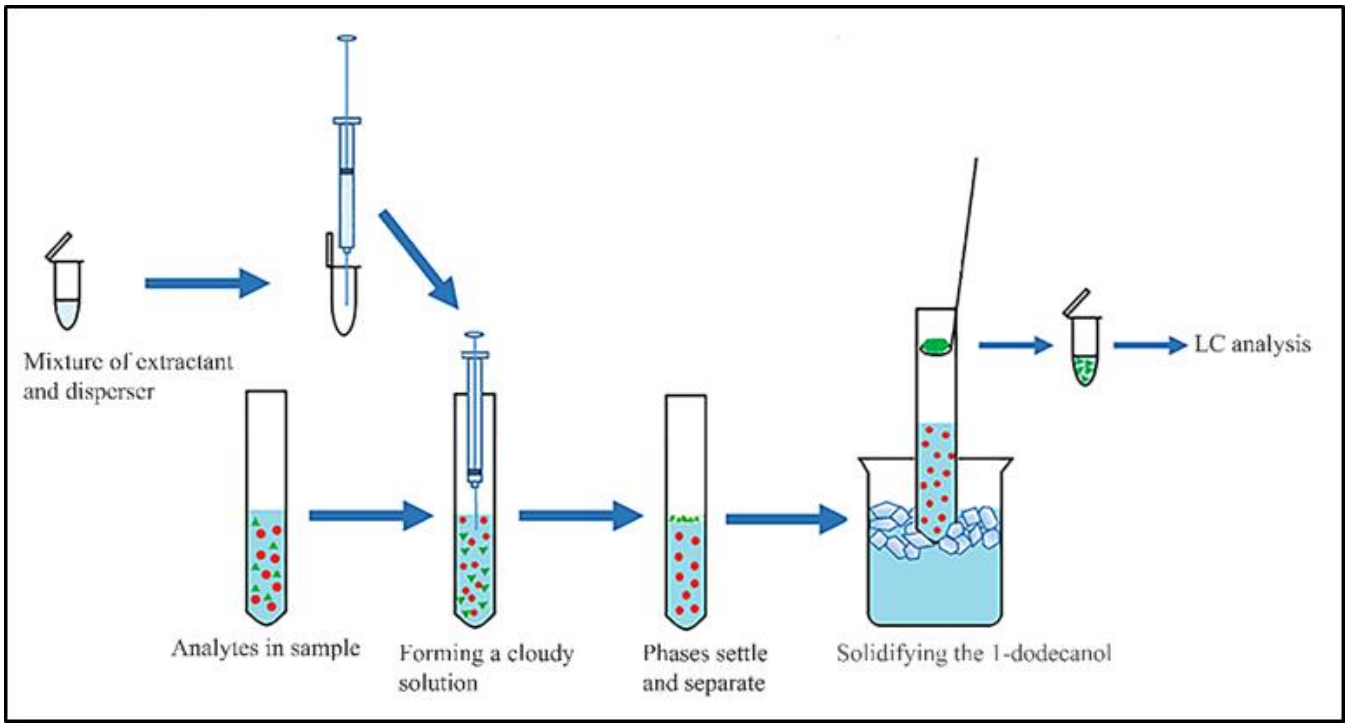


Figure 2. Schematic illustration of the experimental procedure

2.5. Validation

According to the ICH harmonized tripartite guideline, the method validation was evaluated for linearity, limit of detection [37], limit of quantification (LOQ), precision and recovery. Six concentration points for each of the phenolic acids were used for the linearity study. The limit of quantification (LOQ) was calculated as the minimum concentration that could be accurately and precisely qualified (RSD < 15%) and has been included as the lowest level in the calibration curves. The limit of detection [37] was defined as a peak height that is higher than three times the baseline noise. Intra- and inter-day precision was studied with quality control samples at three concentration levels, and the data were calculated as the relative standard deviation (RSD). The intra-day samples were measured as three replicates in one day, and inter-day variation was measured on three separate days (n = 3). The recovery of the method was determined by spiking wine samples with three levels of standard solution, and the results were calculated as:

$$100 \times \frac{(\text{amount found in the spiked sample} - \text{amount found in the sample})}{\text{amount added}}$$

2.6. Statistics

The extraction method was optimized by orthogonal array design (OAD) and analysis of variance (Ivanova-Petropulos et al.) using SPSS software (SPSS 23.0, SPSS Inc., Chicago, IL).

All available data were expressed as the mean ± standard deviation for each set of comparison experiments. Significant differences were evaluated by analysis of variance [38] followed by Tukey's honest significant difference (HSD) post hoc test. A probability level equal to or less than 0.05 ($p \leq 0.05$) was considered to be significantly different.

3. Results and discussion

3.1. Chromatographic separation on a core–shell particle column

Chromatographic separation of phenolic acids with similar structures usually requires a long analysis time [39, 40] using a conventional RP column without the assistance of a buffered mobile phase, unless the extracted ion chromatogram is provided by MS/MS. However, our aim is to employ conventional HPLC-UV equipment for effective separation of 10 phenolic acids in a shorter time. In the current study, a column packed with sub-3 μm core–shell particles was employed for the analytical task, in an attempt to reduce analysis time while maintaining good separation. Core–shell particle columns can provide a conventional LC system with a separation efficiency comparable to UHPLC, but the system pressure is much lower than UHPLC [41]. During the analysis, the system pressure was maintained below 3800 psi (262 bar). For simplicity, a binary elution system consisting of pure water and pure ACN was first tested, but the chromatographic peak shapes and separation were not good. To restrain ionization and improve the peak shapes, the conditions for the chromatographic separation have been optimized; a gradient elution of water and acetonitrile containing 0.1% and 0.2% formic acid has been considered necessary for a good response on a core–shell particle column in a narrow-bore diameter (2.1 mm). Addition of formic acid significantly improved the separation, and the peak shape and resolution improved with increasing concentrations of formic acid. Considering the negative effects of formic acid such as shortening of column life, 0.1% was chosen as the final formic acid concentration.

Here we must emphasize the important role of injection volume with regard to column efficiency during phenolic acid separation. The effect of injection volume in

the presence of the SPP column in this method was also evaluated. The peaks tailed and broadened, leading to loss of efficiency with increasing volume of injection. The best peak shapes were obtained with a 3-μL injection volume. Finally, the effect of column temperature was evaluated; we tested temperatures from 25 to 45 ℃. Although column temperature was not as significant as other factors, the best peak shapes for most compounds were achieved at 30℃. The resulting analysis conditions yielded a baseline separation of the ten phenolic acids within 40 min and required smaller volumes of solvents and smaller sample amounts compared to conventional columns (Fig. 3).

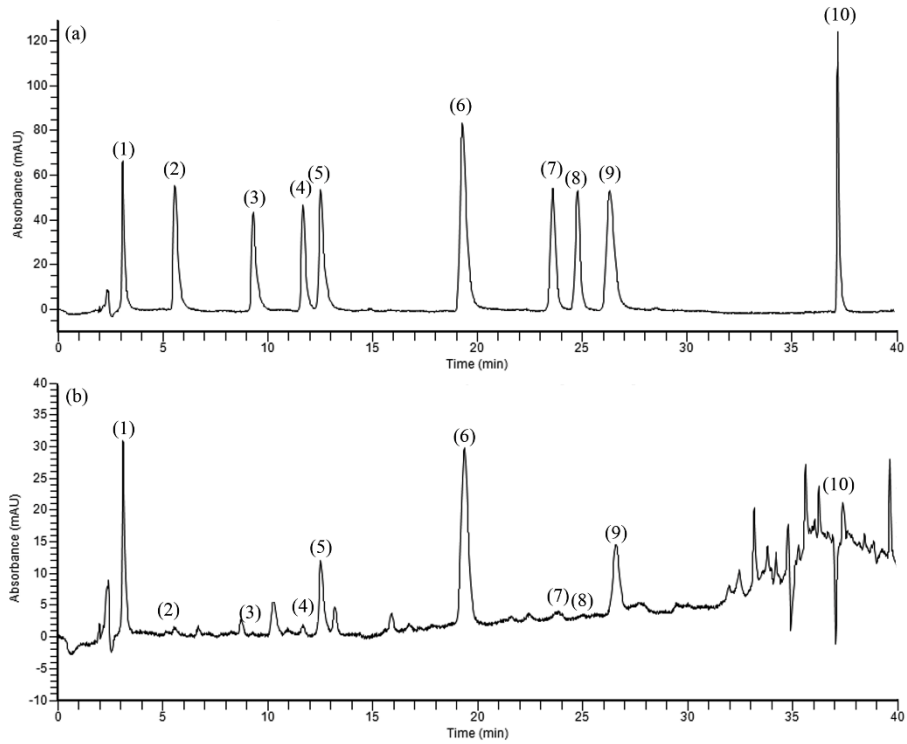


Figure 3. Chromatograms of phenolic acid standards (a) and real red wine sample (b) analyzed by the IP-DLLME-SFO method coupled with a core–shell column followed by HPLC-UV. Peak identification: 1, gallic acid; 2, protocatechuic acid; 3, 4-hydroxybenzoic acid; 4, vanillic acid; 5, caffeic acid; 6, p-coumaric acid; 7, ferulic acid; 8, sinapic acid; 9, m-coumaric acid; 10, cinnamic acid.

3.2. Selection of the type and concentration of ion-pairing reagent

Using traditional DLLME methods, the extraction efficiencies for phenolic acids were generally low, that is, phenolic acids could not be extracted with a satisfactory extraction efficiency once the concentration was higher than 5 μg/mL. Moreover, gallic acid and protocatechuic acid could not be extracted in any acidic sample with pH ranging between 2.0 and 6.0.

The low extraction efficiency was assumed to be due to the polar ionizable groups of phenolic acids. An ion-pairing reagent can form less polar ionic pairs with phenolic acids and consequently can facilitate the transfer of phenolic acids to the organic phase. Hence, six different quaternary ammonium ion-pairing reagents, TEAB, TPAB, TBAB, TBAI, THAB, and THPAB, were compared under the same experimental conditions in an aqueous solution of phenolic acids containing 5 mM of an ion-pairing reagent. Given that the pKa values of the phenolic acids were approximately 4.0 ± 1.0, the pH of test solutions was fixed at 6.0, which can keep the phenolic acids mostly in the ionized form [11]. The phenolic acids were extracted using a mixture of 1-dodecanol and methanol. As a result, the two ion-paring reagents with long carbon chains, THAB and THPAB, exhibited the highest extraction efficiencies, while the phenolic acids were barely extracted with the other reagents. THAB was selected as the ion-pairing reagent for further study because it is easier to obtain than THPAB.

The effects of THAB concentration were investigated over the range of 1–20 mM. The enrichment factor (EF), which is calculated as C_c/C_0 (where C_c is the analyte concentration in the collected phase, and C_0 is the analyte concentration in the initial aqueous phase), was used as a measure of the extraction efficiency. As shown in Fig. 4, while extraction conditions: 4 mL sample volume, 1 mL 50 mM phosphate buffer (pH = 6.0), 50 μL extraction solvent volume, 450 μL dispersive solvent volume (n =

3). THAB concentration at 5 mM was taken as 100%. Comparisons were made using ANOVA. Bar mean = S.E.M. *$p \leq 0.01$, **$p \leq 0.05$. The extraction efficiency increased with an increasing concentration of THAB up to 5 mM, the concentration recommended by the manufacturer, after which the efficiency decreased. Here, error bars gave a general idea of how to measure precision and how far the true value differed from the reported value. In this study, we chose the standard error of the mean to show the spread in values along with the p-value of the difference in sample means (n = 3).

The following figures all used error bars to indicate uncertainty in the data. In addition, high concentrations of THAB made it difficult to transfer solidified drops because the drops melted very quickly during transfer. As a result, 5 mM THAB was chosen for subsequent experiments.

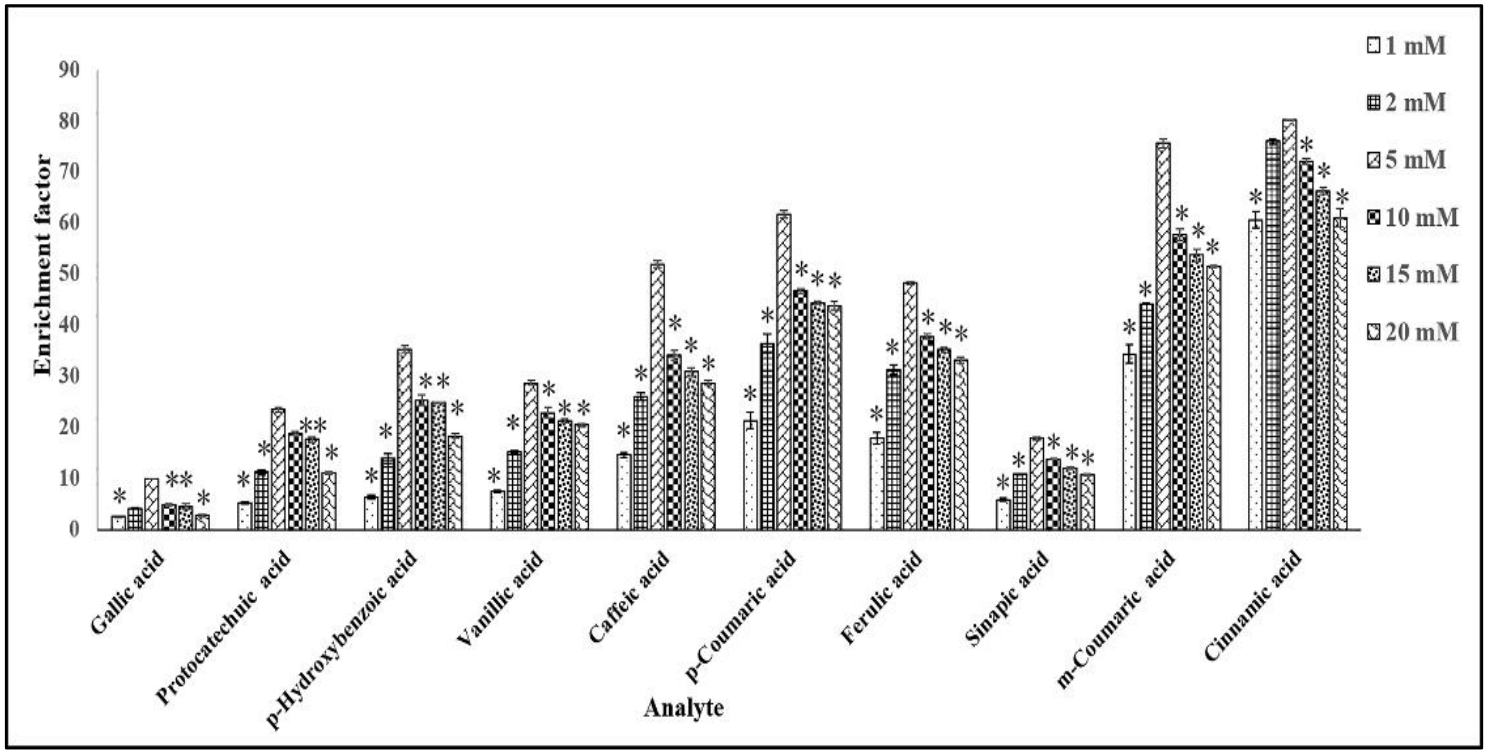


Figure 4. Effects of THAB concentration on the extraction efficiencies for phenolic acids.

3.3. Effect of sample pH on extraction efficiency involving THAB

Sample pH can affect the ionization state of both phenolic acids and THAB and, consequently, the formation of ion pairs between them. Although pH 7.5 is recommended by the manufacturer, extractions were performed over a wide range of pH values, to identify the optimal sample pH. In this work, 50 mM phosphate buffer was applied to adjust the pH to between 3.0 and 9.0. EFs of all the phenolic acids generally increased as the pH increased from 3.0 to 6.0 and then decreased as the pH reached 7.0 (Fig. 5. Extraction conditions were the same as in Fig. 4 except for the sample pH). Hence, the pH of the sample solution was adjusted to 6.0 for subsequent extractions.

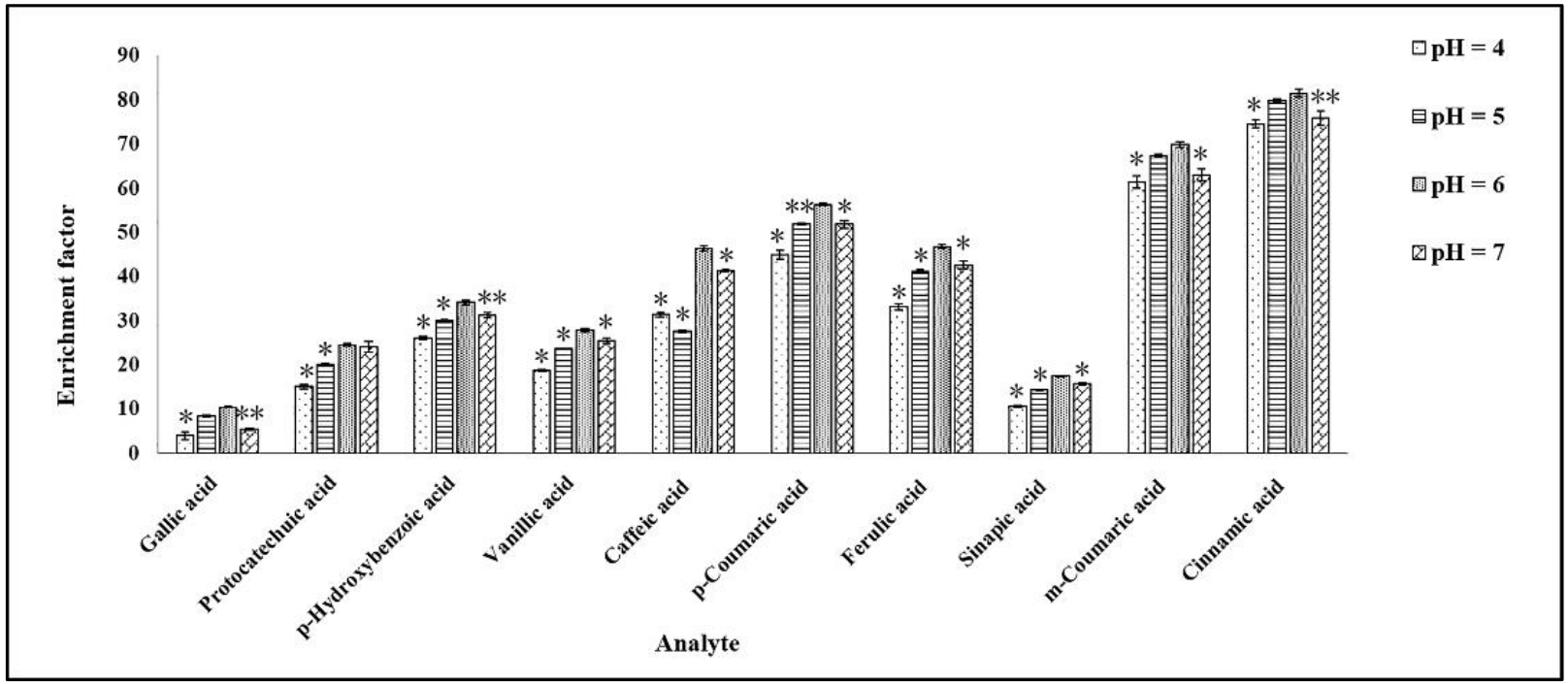


Figure 5. Effects of sample pH on the extraction efficiencies for phenolic acids.

3.4. Selection of extraction and dispersive solvents for DLLME-SFO

Based on our previous experience [24, 42], DLLME-SFO using a low-density solvent allowed for easier and more reliable collection of the extraction phase from complicated matrices such as biological fluids and food samples. Therefore, three extraction solvents (1-dodecanol, 2-dodecanol, and 1- undecanol) were tested. The solvents 1-octanol and 1-decanol were excluded from testing because their low melting points (-16 and 6.4 ℃ for 1-octanol and 1-decanol, respectively) made it difficult to solidify droplets using a simple ice bath. Three dispersive solvents, methanol, acetonitrile, and acetone, were combined with the three extraction solvents, resulting in nine combinations as follows: methanol/1-dodecanol, methanol/ 2-dodecanol, methanol/1-undecanol, acetonitrile/1-dodecanol, acetonitrile/2-dodecanol, acetonitrile/1-undecanol, acetone/1-dodecanol, acetone/2-dodecanol, and acetone/1-undecanol. These solvent mixtures were composed of 50 μL of extractant and 450 μL of dispersive solvent. As shown in Fig. 6, while extraction conditions were the same as in Fig. 4 except for the types of extraction and dispersive solvents, methanol/1-dodecanol exhibited exceptionally high extraction efficiency compared to the others.

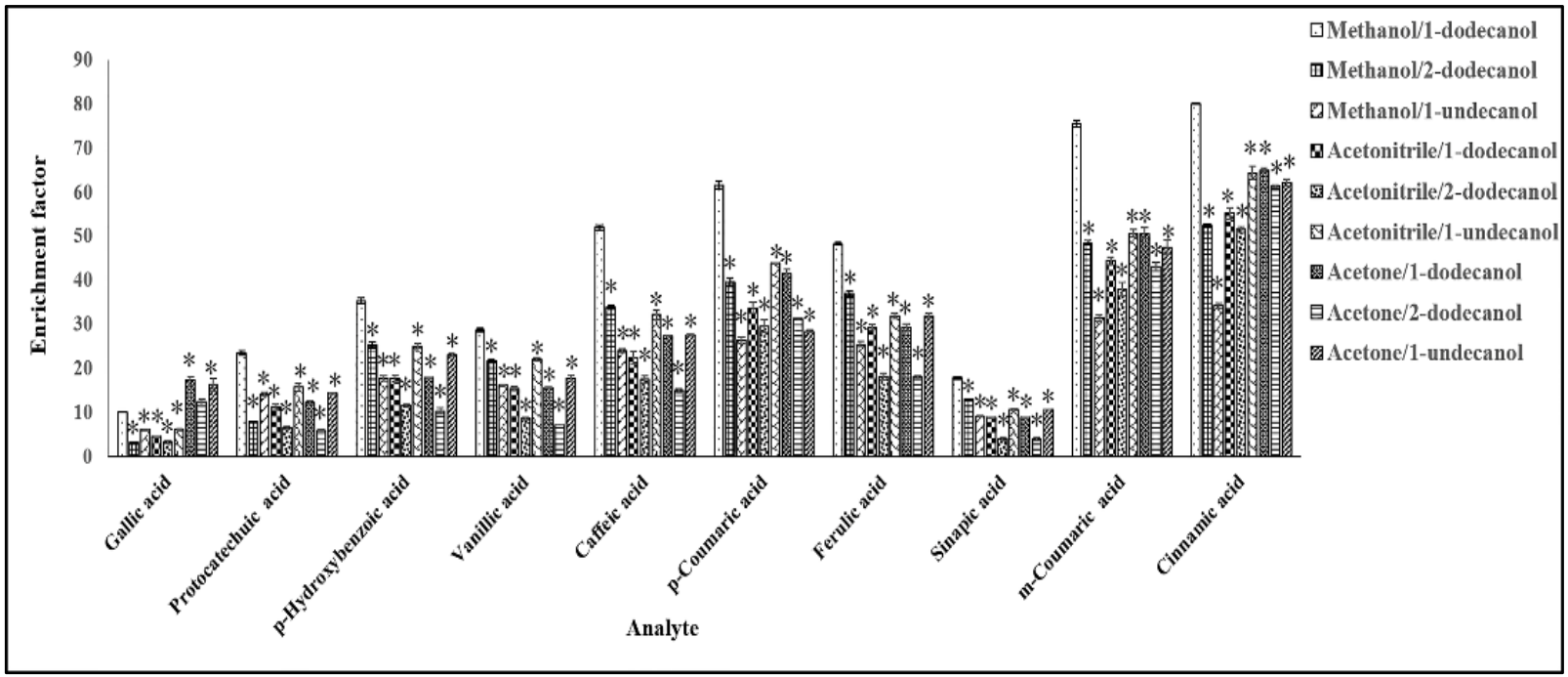


Figure 6. Selection of extraction and dispersive solvents.

3.5. Optimization of volume of the extraction and dispersive solvents

With 1-dodecanol and methanol selected as extraction and dispersive solvents, respectively, their volumes were optimized. First, while the volume of the mixture of 1-dodecanol and methanol was fixed at 500 μL, the volume ratio of 1-dodecanol to methanol was varied. As a result, the lowest volume ratio (1- dodecanol/methanol = 1:9) yielded the highest EF values for all tested compounds (Fig. 7. Extraction conditions were the same as in Fig. 6 except that the solvent mixture was composed of 1-dodecanol and methanol). Then, the effect of the solvent mixture volume on the extraction efficiency was investigated by varying the mixture volume between 250 μL and 1000 μL at a fixed volume ratio of 1:9. Although the highest extraction efficiency was acquired at the lowest mixture volume of 250 μL (Fig. 8. The volume ratio of 1-dodecanol to methanol was fixed at 1:9 (v:v). Other conditions were the same as in Fig. 7), it was difficult to collect the organic phase reliably, and the recovered volume of the droplets was not consistent under these conditions. Thus, 500 μL was selected as the optimized volume, providing a compromise between reproducibility and method sensitivity. The final optimized extraction mixture condition was composed of 450 μL methanol and 50 μL 1- dodecanol.

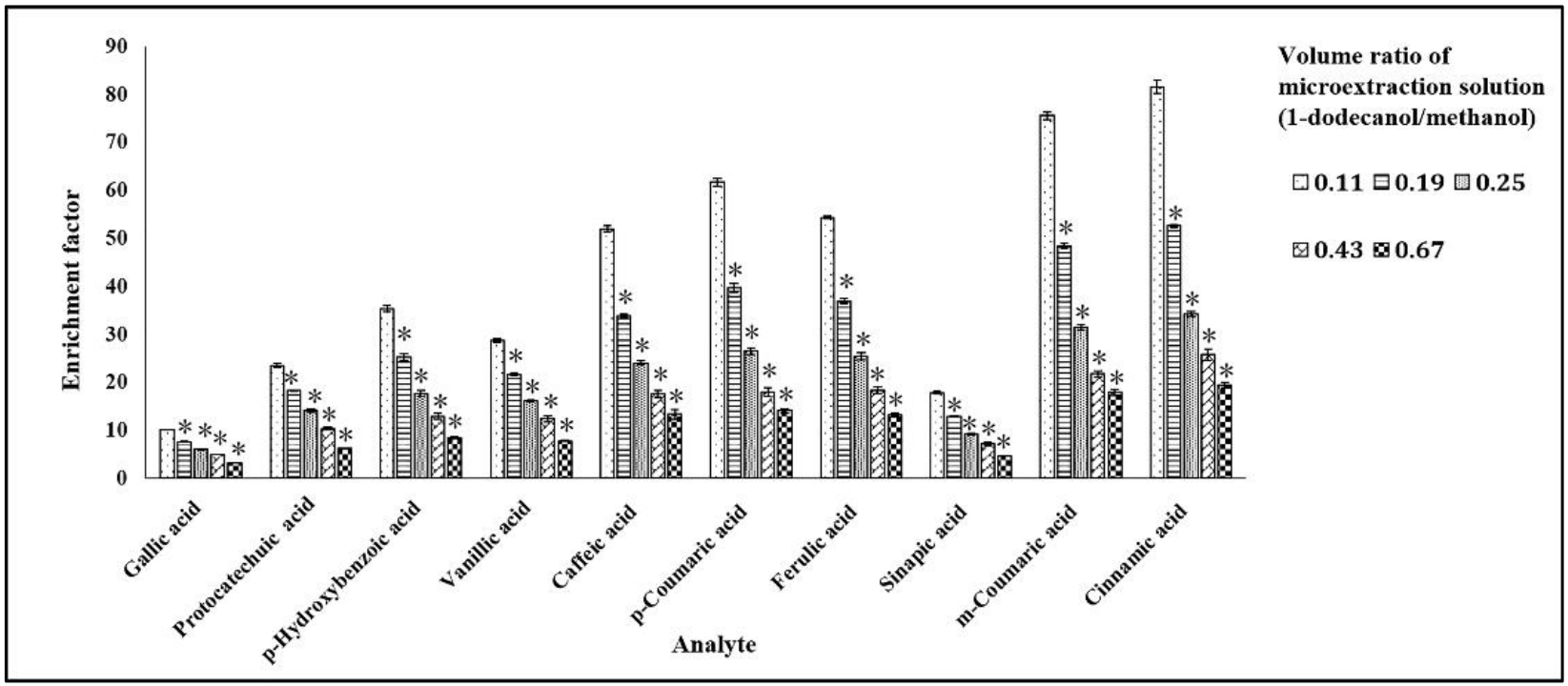


Figure 7. Effects of volume ratio of extraction solvent to dispersive solvent on the extraction efficiencies for phenolic acids.

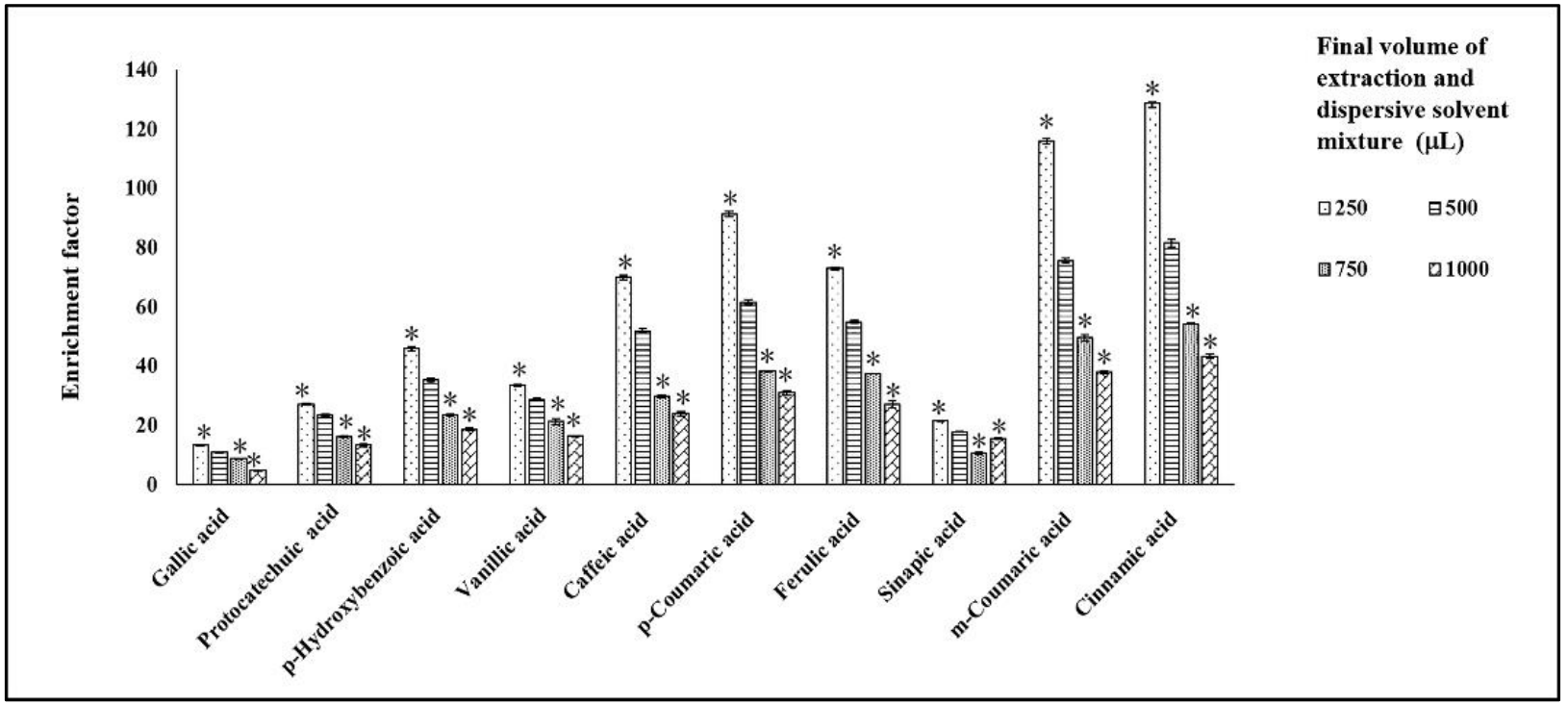


Figure 8. Optimization of the final volume of extraction and dispersive solvent mixture.

3.6. Salt effect on IP-DLLME-SFO

Because ionic strength can influence the extraction efficiency, various concentrations of salt (NaCl) were examined, ranging from 0% to 15% (w/v). EF values decreased sharply with the addition of NaCl (Fig. 9); similar observations were made in the literature on IL-DLLME methods [2, 11, 16, 43]. Extraction conditions: 4 mL sample of aqueous solution, 1 mL 50 mM phosphate buffer (pH = 6.0), 50 μL extraction solvent, 450 μL dispersive solvent (n = 3). After the addition of NaCl, the sample was vortexed for approximately 1 min prior to extraction. As a result, salt was not considered for further optimization procedures.

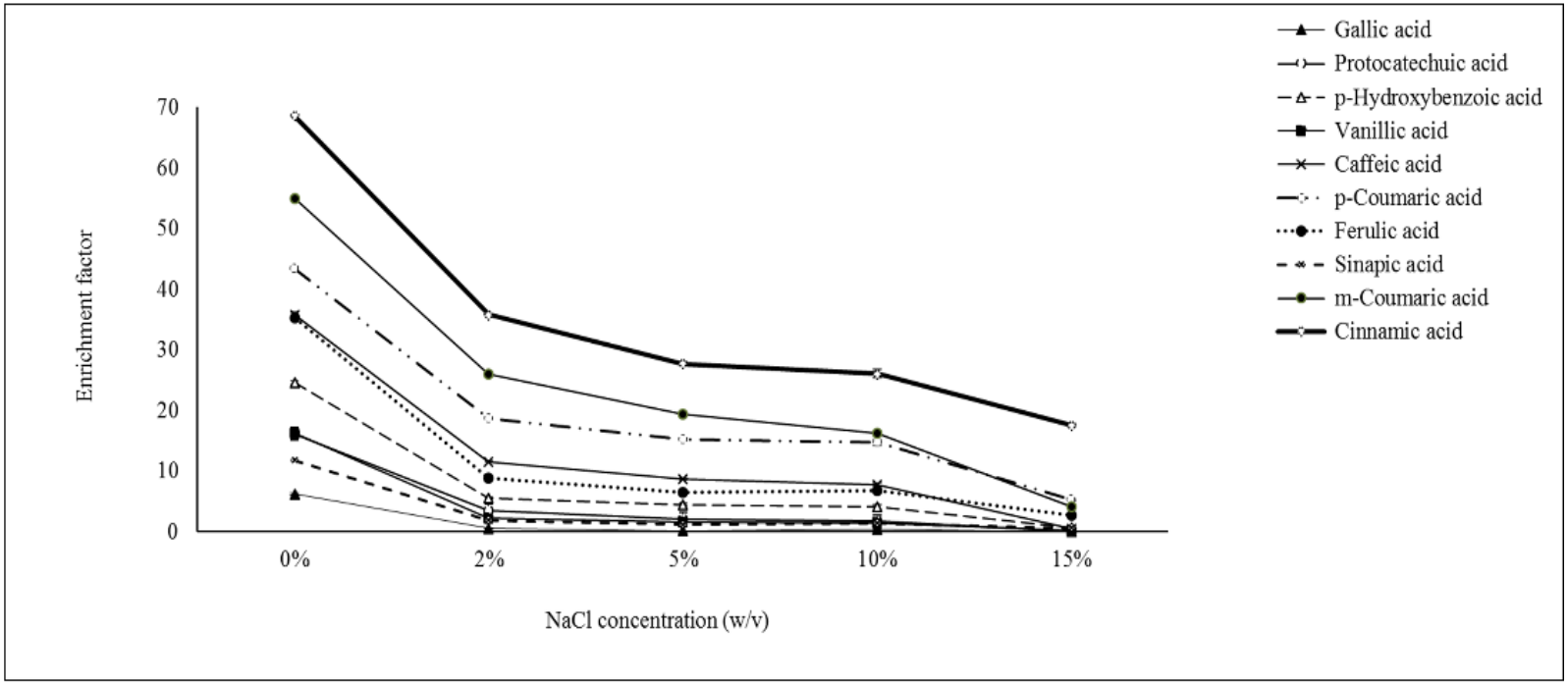


Figure 9. Effects of NaCl concentration on the extraction efficiencies for phenolic acids.

3.7. Facilitation of extraction and optimization of the extraction time

During DLLME-SFO, extraction can be facilitated by additional ultrasonic radiation or vortexing. In addition, the extraction efficiency can be affected by extraction time, which is defined as the time between the injection of the extraction solvent mixture and the centrifugation of the mixture [44]. However, in our study, the extraction efficiency did not change with extraction time nor was the extraction efficiency affected by additional ultrasonic radiation or vortexing (Fig. 10). Extraction conditions were the same as shown in Supplementary Fig. 9 except that no salt was added. For the final optimized conditions, the sample was centrifuged immediately after injection of the mixture of extractant and disperser.

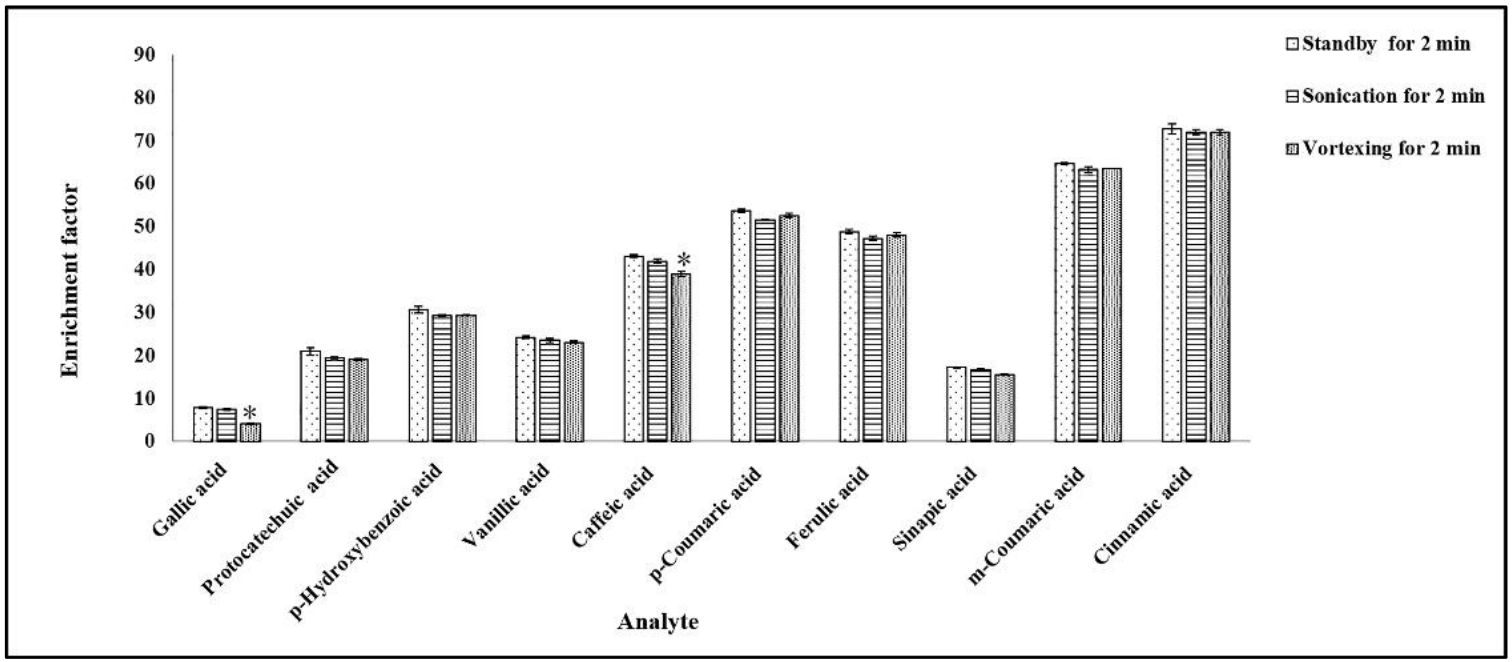


Figure 10. Comparison of extraction efficiencies for DLLME-SFO with or without the assistance of sonication or vortexing.

3.8. Method validation

Method validation was performed in terms of linearity, sensitivity, precision, and absolute recovery. The calibration curves were constructed using six triplicate data points. Because the detection responses differed depending on the analyte, concentration ranges from 0.01 to 15 μg/mL were used. The calibration curves were linear over the tested range, with correlation coefficients (r^2) higher than 0.994 (Table 1). The limit of detection [37], which was obtained by injecting a series of extracted solutions until the peak height was at least three times the baseline noise, ranged between 0.005 and 0.1 μg/mL (Table 1). The limit of quantification (LOQ), which was determined as the minimum concentration for precise quantification (RSD < 15%), was included as the lowest level in the calibration curves [25]. The current method was more sensitive than the LLE method coupled to LC analysis [45], in which the lowest LOD was 0.03 μg/mL and the SPE method coupled to LC analysis [19], in which the LOD obtained was 10-fold higher than the current method.

The precision of the developed method was evaluated at three concentration levels and expressed as the relative standard deviation (RSD%). The intra-day precision was determined for one day (n = 3), and inter-day precision was measured on three separate days (n = 3 × 3). As displayed in Table 2, the intra- and inter-day precision was below 7.9% and 8.8%, respectively, which was much lower than the RSD% achieved by the SPE method (18.7%) [19].

Relative recovery was estimated by comparing the peak areas of standards in spiked wine samples with the peak areas of standards in water at three concentration levels. As shown in Table 2, the relative recoveries were close to 100% for all phenolic acids tested. Taken together, the method validation results indicated that the proposed method was reproducible and sensitive for quantifying phenolic acids in wine samples.

Table 1. Linear ranges, correlation coefficients (R^2) and sensitivity of the developed method.

Analyte	Linearity range [a]	R^2	LOQ [a]	LOD [a]
Gallic acid	0.30 - 15.00	0.994	0.30	0.10
Protocatechuic acid	0.15 - 9.00	0.999	0.15	0.075
p-Hydroxybenzoic acid	0.10 - 6.00	0.998	0.10	0.05
Vanillic acid	0.10 - 6.00	0.999	0.10	0.05
Caffeic acid	0.04 - 2.40	0.998	0.04	0.02
p-Coumaric acid	0.04 - 2.40	0.999	0.04	0.02
Ferulic acid	0.04 - 2.40	0.999	0.04	0.02
Sinapic acid	0.10 - 6.00	0.998	0.10	0.05
m-Coumaric acid	0.02 - 1.20	0.998	0.02	0.01
Cinnamic acid	0.01 - 0.60	0.998	0.01	0.005

[a] μg/mL

3.9. Application of the developed method to wine sample analysis

The developed method involving IP-DLLME-SFO coupled to LC using a core–shell column was applied to the determination of phenolic acids in 10 real wine samples composed of seven red wine samples (R1–R7) and three white wine samples (W1–W3). Quantification of the phenolic acids in the wine samples was successfully performed (Table 3). Gallic acid was the dominant phenolic acid in all tested samples, at concentrations between 4.1 and 108.9 mg/mL, and its level was much higher in red wine samples than in white wine samples, which agrees with previous results [10, 39]. Showing no difference in levels between red wine and white wine samples, caffeic acid was also detected in all the samples, but at lower levels (0.4–3.9 mg/mL) than gallic acid. Protocatechuic acid, p-hydro-xybenzoic acid, and cinnamic acid were detected in nine samples. In GC–MS analysis of white wine samples [7], similar levels were observed for protocatechuic acid and p-hydroxybenzoic acid, of 0.44–5.75 mg/mL. Ferulic acid, sinapic acid, and m-coumaric acid were not detected in the red wine samples, except for R1 and R7 that contained m-coumaric and sinapic acid.

Table 2. Intra- and inter-day precisions and relative recovery of the developed method at three different concentration levels.

Analyte	Intra-day (% RSD, n = 3)			Inter-day (% RSD, n = 9)			Relative recovery (%, n=3)		
	Low [a]	Medium [b]	High [c]	Low [a]	Medium [b]	High [c]	Low [a]	Medium [b]	High [c]
Gallic acid	4.20	3.41	3.26	8.75	8.34	5.23	92.4	95.6	101
Protocatechuic acid	2.11	3.14	2.11	8.85	3.54	3.98	77.2	83.6	79.9
p-Hydroxybenzoic acid	1.77	0.90	0.60	7.66	6.22	6.38	92.6	93.2	90.9
Vanillic acid	7.95	6.83	4.24	7.47	7.62	5.73	78.5	85.1	81.5
Caffeic acid	7.75	1.90	1.73	8.89	7.41	7.81	96.2	102	103
p-Coumaric acid	0.18	2.06	2.11	8.62	6.67	4.45	97.4	102	117
Ferulic acid	3.48	3.58	3.07	9.33	7.79	4.46	76.0	78.6	86.8
Sinapic acid	2.31	1.83	0.85	8.74	8.68	5.30	81.2	89.4	84.8
m-Coumaric acid	4.21	4.73	5.59	5.77	8.81	6.24	105	109	102
Cinnamic acid	5.73	4.32	2.39	4.29	5.88	3.08	87.1	95.6	105

[a] 1.0 μg/mL for gallic acid and protocatechuic acid; 0.5 μg/mL for p-hydroxybenzoic acid, vanillic acid and sinapic acid; 0.2 μg/mL for caffeic acid, p-coumaric acid and ferulic acid; 0.1 μg/mL for m-coumaric acid; 0.05 μg/mL for cinnamic acid.

[b] 3.0 μg/mL for gallic acid and protocatechuic acid; 1.5 μg/mL for p-hydroxybenzoic acid, vanillic acid and sinapic acid; 0.6 μg/mL for caffeic acid, p-coumaric acid and ferulic acid; 0.3 μg/mL for m-coumaric acid; 0.15 μg/mL for cinnamic acid.

[c] 6.0 μg/mL for gallic acid and protocatechuic acid; 3.0 μg/mL for p-hydroxybenzoic acid, vanillic acid and sinapic acid; 1.2 μg/mL for caffeic acid, p-coumaric acid and ferulic acid; 0.6 μg/mL for m-coumaric acid; 0.3 μg/mL for cinnamic acid

Table 3 Levels of phenolic acids determined in 10 wine samples.

Table 3. Levels of phenolic acids determined in 10 wine samples.

Analyte	Real wine samples									
	R_1	R_2	R_3	R_4	R_5	R_6	R_7	W_8	W_9	W_{10}
Gallic acid	53.8 [a]	59.1	39.2	39.2	49.6	43.8	108	4.11	10.3	7.29
Protocatechuic acid	ND [b]	4.44	3.45	3.46	1.79	2.04	2.07	0.75	1.66	1.88
p-Hydroxybenzoic acid	ND	1.77	1.28	1.28	2.21	2.35	1.07	0.51	1.87	3.89
Vanillic acid	1.05	2.21	ND	1.76	1.45	1.53	3.12	ND	ND	0.50
Caffeic acid	3.10	1.54	0.40	0.77	1.16	2.52	1.73	1.56	0.92	3.86
p-Coumaric acid	5.62	2.62	ND	0.46	1.58	1.55	4.30	1.91	1.28	2.18
Ferulic acid	ND	ND	ND	ND	ND	ND	ND	0.63	ND	0.82
Sinapic acid	ND	ND	ND	ND	ND	ND	2.00	1.47	0.84	ND
m-Coumaric acid	0.22	ND	ND	ND	ND	ND	ND	0.31	0.12	ND
Cinnamic acid	0.17	0.32	0.10	ND	0.32	0.40	2.49	0.11	0.07	0.18

[a] μg/mL.

[b] Not detected.

4. Conclusions

The sample preparation method that was developed based on IP-DLLME-SFO provided an efficient one-step operation enabling both sample clean-up and enrichment of a number of phenolic acids. In addition, the current analytical method employed for the first time a core–shell particle column to separate phenolic acids, and this separation scheme allowed for significantly reduced analysis time without the use of a complex buffered mobile phase. The current approach of combining IP-DLLME-SFO with LC using a core–shell particle column may be applicable for a rapid environmentally friendly, and efficient analysis of ionizable polar compounds in samples of complex matrices.

References

[1] A. Balinova, Strategies for chromatographic analysis of pesticide residues in water, Journal of Chromatography A, 754 (1996) 125-135.

[2] M. Rajabi, S. Asemipour, B. Barfi, M.R. Jamali, M. Behzad, Ultrasound-assisted ionic liquid based dispersive liquid–liquid microextraction and flame atomic absorption spectrometry of cobalt, copper, and zinc in environmental water samples, Journal of Molecular Liquids, 194 (2014) 166-171.

[3] M. Rezaee, Y. Yamini, M. Faraji, Evolution of dispersive liquid-liquid microextraction method, Journal of chromatography. A, 1217 (2010) 2342-2357.

[4] Z.Q. Zhang, N. Jaffrezic-Renault, F. Bessueille, D. Leonard, S.Q. Xia, X.J. Wang, L. Chen, J.F. Zhao, Development of a conductometric phosphate biosensor based on tri-layer maltose phosphorylase composite films, Anal Chim Acta, 615 (2008) 73-79.

[5] L. Xu, C. Basheer, H.K. Lee, Chemical reactions in liquid-phase microextraction, Journal of chromatography. A, 1216 (2009) 701-707.

[6] J.O. Fernandes, M.A. Ferreira, Combined ion-pair extraction and gas chromatography-mass spectrometry for the simultaneous determination of diamines, polyamines and aromatic amines in Port wine and grape juice, Journal of chromatography. A, 886 (2000) 183-195.

[7] B. Ebrahimpour, Y. Yamini, M. Moradi, Application of ionic surfactant as a carrier and emulsifier agent for the microextraction of fluoroquinolones, J Pharmaceut Biomed, 66 (2012) 264-270.

[8] S.M. Yousefi, F. Shemirani, Selective and sensitive speciation analysis of Cr(VI) and Cr(III) in water samples by fiber optic-linear array detection spectrophotometry after ion pair based-surfactant assisted dispersive liquid-liquid microextraction, Journal of hazardous materials, 254-255 (2013) 134-140.

[9] M.J.R. Vaquero, M.R. Alberto, M.C.M. de Nadra, Influence of phenolic compounds from wines on the growth of Listeria monocytogenes, Food Control, 18 (2007) 587-593.

[10] E. Porgalı, E. Büyüktuncel, Determination of phenolic composition and antioxidant capacity of native red wines by high performance liquid chromatography and spectrophotometric methods, Food Research International, 45 (2012) 145-154.

[11] W.L. Liu, Y.C. Ko, B.H. Hwang, Z.G. Li, T.C.C. Yang, M.R. Lee, Determination of perfluorocarboxylic acids in water by ion-pair dispersive liquid-liquid microextraction and gas chromatography-tandem mass spectrometry with injection port derivatization, Anal Chim Acta, 726 (2012) 28-34.
[12] A. Garcia-Ruiz, C. Cueva, E.M. Gonzalez-Rompinelli, M. Yuste, M. Torres, P.J. Martin-Alvarez, B. Bartolome, M.V. Moreno-Arribas, Antimicrobial phenolic extracts able to inhibit lactic acid bacteria growth and wine malolactic fermentation, Food Control, 28 (2012) 212-219.
[13] R.J. Robbins, Phenolic acids in foods: an overview of analytical methodology, Journal of agricultural and food chemistry, 51 (2003) 2866-2887.
[14] I. Mudnic, D. Modun, V. Rastija, J. Vukovic, I. Brizic, V. Katalinic, B. Kozina, M. Medic-Saric, M. Boban, Antioxidative and vasodilatory effects of phenolic acids in wine, Food Chemistry, 119 (2010) 1205-1210.
[15] C.D. Stalikas, Extraction, separation, and detection methods for phenolic acids and flavonoids, Journal of separation science, 30 (2007) 3268-3295.
[16] S. Dong, Q. Hu, Z. Yang, R. Liu, G. Huang, T. Huang, An ionic liquid-based ultrasound assisted dispersive liquid–liquid microextraction procedure followed by HPLC for the determination of low concentration of phytocides in soil, Microchemical Journal, 110 (2013) 221-226.
[17] M.N. Irakli, V.F. Samanidou, C.G. Biliaderis, I.N. Papadoyannis, Development and validation of an HPLC-method for determination of free and bound phenolic acids in cereals after solid-phase extraction, Food Chemistry, 134 (2012) 1624-1632.
[18] M. Garciafalcon, C. Perezlamela, E. Martinezcarballo, J. Simalgandara, Determination of phenolic compounds in wines: Influence of bottle storage of young red wines on their evolution, Food Chemistry, 105 (2007) 248-259.
[19] M. del Álamo, L. Casado, V. Hernández, J.J. Jiménez, Determination of free molecular phenolics and catechins in wine by solid phase extraction on polymeric cartridges and liquid chromatography with diode array detection, Journal of Chromatography A, 1049 (2004) 97-105.
[20] S. Malovana, D. Gajdosova, J. Benedik, J. Havel, Determination of esmolol in serum by capillary zone electrophoresis and its monitoring in course of heart surgery, Journal of chromatography. B, Biomedical sciences and applications, 760 (2001) 37-

43.
[21] M.I. Alarcon Flores, R. Romero-Gonzalez, A. Garrido Frenich, J.L. Martinez Vidal, Analysis of phenolic compounds in olive oil by solid-phase extraction and ultra high performance liquid chromatography-tandem mass spectrometry, Food Chem, 134 (2012) 2465-2472.
[22] A. Michalkiewicz, M. Biesaga, K. Pyrzynska, Solid-phase extraction procedure for determination of phenolic acids and some flavonols in honey, Journal of chromatography. A, 1187 (2008) 18-24.
[23] M. Becerra-Herrera, M. Sánchez-Astudillo, R. Beltrán, A. Sayago, Determination of phenolic compounds in olive oil: New method based on liquid–liquid micro extraction and ultra high performance liquid chromatography-triple–quadrupole mass spectrometry, LWT - Food Science and Technology, 57 (2014) 49-57.
[24] S. Jia, J. Li, S.R. Park, Y. Ryu, I.H. Park, J.H. Park, S.S. Hong, S.W. Kwon, J. Lee, Combined application of dispersive liquid-liquid microextraction based on the solidification of floating organic droplets and charged aerosol detection for the simple and sensitive quantification of macrolide antibiotics in human urine, J Pharm Biomed Anal, 86 (2013) 204-213.
[25] S. Jia, J. Li, N. Yunusova, J.H. Park, S.W. Kwon, J. Lee, A new application of charged aerosol detection in liquid chromatography for the simultaneous determination of polar and less polar ginsenosides in ginseng products, Phytochemical analysis : PCA, 24 (2013) 374-380.
[26] A. Balinova, Ion-pairing mechanism in the solid-phase extraction and reversed-phase high-performance liquid chromatographic determination of acidic herbicides in water, Journal of Chromatography A, 728 (1996) 319-324.
[27] M.C. Carson, Ion-pair solid-phase extraction, Journal of chromatography. A, 885 (2000) 343-350.
[28] U.V.R.V. Saradhi, S. Prabhakar, T.J. Reddy, M. Vairamani, Ion-pair solid-phase extraction and gas chromatography-mass spectrometric determination of acidic hydrolysis products of chemical warfare agents from aqueous samples, Journal of Chromatography A, 1129 (2006) 9-13.
[29] U.V.R.V. Saradhi, S. Prabhakar, T.J. Reddy, M.R.V.S. Murty, Gas

chromatographic-mass spectrometric determination of alkylphosphonic acids from aqueous samples by ion-pair solid-phase extraction on activated charcoal and methylation, Journal of Chromatography A, 1157 (2007) 391-398.
[30] R. Alzaga, P. Montuori, L. Ortiz, J.M. Bayona, J. Albaiges, Fast solid-phase extraction-gas chromatography-mass spectrometry procedure for oil fingerprinting - Application to the Prestige oil spill, Journal of Chromatography A, 1025 (2004) 133-138.
[31] Y.C. Fiamegos, A.P. Kefala, C.D. Stalikas, Ion-pair single-drop microextraction versus phase-transfer catalytic extraction for the gas chromatographic determination of phenols as tosylated derivatives, Journal of Chromatography A, 1190 (2008) 44-51.
[32] L. Xu, X.Y. Gong, H.K. Lee, P.C. Hauser, Ion-pair liquid-liquid-liquid microextraction of nerve agent degradation products followed by capillary electrophoresis with contactless conductivity detection, Journal of Chromatography A, 1205 (2008) 158-162.
[33] Z.H. Zhang, C.G. Zhang, X.L. Su, M. Ma, B. Chen, S.Z. Yao, Carrier-mediated liquid phase microextraction coupled with high performance liquid chromatography for determination of illicit drugs in human urine, Anal Chim Acta, 621 (2008) 185-192.
[34] Y.K. Park, W.Y. Chung, B. Kim, Y.S. Kye, M.S. Shin, D. Kim, Ion-Pair Single-Drop Microextraction Determinations of Degradation Products of Chemical Warfare Agents in Water, Chromatographia, 76 (2013) 679-685.
[35] J.M. Wu, H.K. Lee, Injection port derivatization following ion-pair hollow fiber-protected liquid-phase microextraction for determining acidic herbicides by gas chromatography/mass spectrometry, Anal Chem, 78 (2006) 7292-7301.
[36] A. Gure, N. Megersa, N. Retta, Ion-pair assisted liquid-liquid extraction for selective separation and analysis of multiclass pesticide residues in environmental waters, Anal Methods-Uk, 6 (2014) 4633-4642.
[37] D.N. Zhang, Y.L. Liao, J. Li, T.L. Wen, L.C. Jin, X.Y. Wang, J. Kolodzey, Effect of in-situ annealing on the structural and optical properties of GeSn films grown by MBE, J Alloy Compd, 684 (2016) 643-648.
[38] V. Ivanova-Petropulos, I. Hermosin-Gutierrez, B. Boros, M. Stefova, T. Stafilov,

B. Vojnoski, A. Dornyei, F. Kilar, Phenolic compounds and antioxidant activity of Macedonian red wines, J Food Compos Anal, 41 (2015) 1-14.
[39] M.Y. Choi, C. Chai, J.H. Park, J. Lim, J. Lee, S.W. Kwon, Effects of storage period and heat treatment on phenolic compound composition in dried Citrus peels (Chenpi) and discrimination of Chenpi with different storage periods through targeted metabolomic study using HPLC-DAD analysis, J Pharm Biomed Anal, 54 (2011) 638-645.
[40] H.K. Ju, E.J. Cho, M.H. Jang, Y.Y. Lee, S.S. Hong, J.H. Park, S.W. Kwon, Characterization of increased phenolic compounds from fermented Bokbunja (Rubus coreanus Miq.) and related antioxidant activity, J Pharm Biomed Anal, 49 (2009) 820-827.
[41] J.M. Cunliffe, T.D. Maloney, Fused-core particle technology as an alternative to sub-2-microm particles to achieve high separation efficiency with low backpressure, Journal of separation science, 30 (2007) 3104-3109.
[42] S. Jia, Y. Ryu, S.W. Kwon, J. Lee, An in situ benzoylation-dispersive liquid-liquid microextraction method based on solidification of floating organic droplets for determination of biogenic amines by liquid chromatography-ultraviolet analysis, Journal of chromatography. A, 1282 (2013) 1-10.
[43] J.N. Sun, J. Chen, Y.P. Shi, Multiple functional ionic liquids based dispersive liquid-liquid microextraction combined with high performance chromatography for the determination of phenolic compounds in water samples, Talanta, 125 (2014) 329-335.
[44] C. Zheng, J. Zhao, P. Bao, J. Gao, J. He, Dispersive liquid-liquid microextraction based on solidification of floating organic droplet followed by high-performance liquid chromatography with ultraviolet detection and liquid chromatography-tandem mass spectrometry for the determination of triclosan and 2,4-dichlorophenol in water samples, Journal of chromatography. A, 1218 (2011) 3830-3836.
[45] M.A. Rodriguez-Delgado, S. Malovana, J.P. Perez, T. Borges, F.J.G. Montelongo, Separation of phenolic compounds by high-performance liquid chromatography with absorbance and fluorimetric detection, Journal of Chromatography A, 912 (2001) 249-257.

Appendix

1. The principle of dispersive liquid-liquid microextraction (DLLME)

Dipersive liquid-liquid micro extraction is firstly a proposed in 2006, which is a novel sample-preparation technique offering high enrichment factors from low volumes of water samples. This method principle is based on a ternary component solvent system in which the appropriate mixture of dispersive and extraction solvents are injected into the aqueous sample. Usually, water-miscible solutions play as dispersive solvent, such as methanol, acetonitrile, acetone. Dispersive solvent can increase extraction solvent interface, so enhanced the extraction efficiency, enriched the analytes and got high recovery. After violent agitation, a cloudy solution was formed that indicated the extraction solvent was dispersed into the aqueous sample as very fine droplets. The analytes transferred into the organic solvent from the aqueous solution, and an organic phase containing highly concentrated target compounds was formed after centrifugation. Thus, the target compounds could be easily transferred by a syringe for analysis. Classic DLLME uses either high-density extraction solvents such as chloroform, carbon tetrachloride, and chlorobenzene or low-density solvents such as n-hexanol, n-hexane, cyclohexane, and dibutyl ether in addition to specially shaped extraction tubes to facilitate transfer and prevent the evaporation of the extracted phase,

The experimental procedure of classical DLLME is by rapidly injected into the mixture of water-miscible organic solvent (dispersive solution) and water-immiscible organic solvent (extraction solvent) using a 1.0 mL syringe in water sample, and then forming tiny organic droplets (a stable cloudy solution). Droplets of the organic phase containing the analytes separated after centrifugation. Then, droplet is easily transferred by a syringe for analysis.

(Reference [24])

2. DLLME-SFO

Based on the principle of the classic DLLME mentioned previously, a new method was introduced in recently. By using extraction solvents with low density and appropriate melting points, DLLME based on the solidification of a floating organic droplet (DLLME-SFO) was developed by Leong and Huang. The extract, which forms a layer on the top of aqueous sample, can be collected by solidifying it at low temperature. Meantime, very tiny particles in the system settle without interfering with the target analytes. So this method is more sensitive and accurate. It has been successfully used for extraction and pre-concentration of many trace substances from water samples. SFO method overcomes the aforementioned problems. This technique is easily carried out. The large contact surface between the sample and the droplets of extractant speeds up mass transfer, as fast as DLLME.

2.1. Requirements of SFO solvent

(a) Lower density than water;

(b) Low water solubility;

(c) Ability to form a cloudy solution in the presence of a disperser solvent when injected into a sample solution;

(d) Good extraction capability of the target compounds;

(e) Ability to form a stable two-phase solution;

(f) Good chromatographic behavior.

2.2. Physical properties of the extraction solvents evaluated for SFO method

The extraction solvents suitable for DLLME-SFO should meet several criteria, including low solubility in water, a lower density than that of water, and a melting point close to room temperature. In addition, low toxicity, a high affinity for the target compounds, and good chromatographic behavior are preferred. The dispersive solvents should be miscible with both water and the extraction solvent, with a cloudy solution forming upon the injection of a mixture of the dispersive and extraction solvents into an aqueous sample. Accordingly, in this study, 1-dodeca-nol, 2-dodecanol, 1-undecanol, and 1-decanol were tested as extraction solvents, while acetone, acetonitrile, and methanol were tested as dispersive solvents. The physical properties of the extraction solvents are listed in Table.

Extraction solvent	Melting point (◦C)	Density (g/mL)	Solubility in water (wt%)
1-Dodecanol	22-24	0.83	Insoluble (0.04)[a]
2-Dodecano	17-18	0.80	Insoluble (N.A.)[b]
1-Undecanol	16	0.83	Insoluble (0.051)[a]
1-Decanol	6.4	0.83	Insoluble (0.021)[a]

[a] Experimental value was obtained from (R. Stephenson, J. Stuart, Mutual binary solubilities: water−alcohols and water−esters, J. Chem. Eng. Data 31. 1986. 56 -70).

[b] Experimental value was not available.

3. SPP column

3.1. Concept

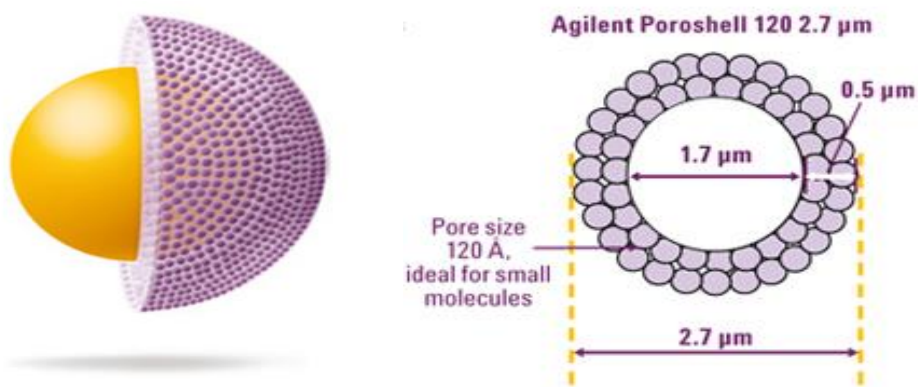
General Description Poroshell 120 SB-C18 is a superficially porous microparticulate (SPP) column packing. Superficially porous silica particles, such as Poroshell, have a solid silica core and a porous silica outer layer. A Stable Bond SB-C18 bonded phase is applied to the totally porous outer layer for this column. This type of particle provides high efficiency at lower pressures when compared to small, totally porous particles and is ideal for fast or high resolution separations of many types of analytes.

3.2. Column Chracteristics

The Poroshell 120 packing has a solid core of 1.7 µm in size with a porous outer layer 0.5 µm thick and a total particle size of 2.7 µm. The particles have a nominal surface area of 120 m^2/g and a controlled pore size of 120Å. The columns can be used up to an operating pressure of 600 bar (9000 psi). The uniform, spherical particles are ultrahigh purity (>99.995% SiO2) silica. This high purity silica is designed to reduce or eliminate strong adsorption of basic and highly polar compounds.

The Stable Bond SB-C18 bonded phase is made by chemically bonding a sterically-protected C18 stationary phase to the porous shell of the Poroshell 120 silica support. The densely covered, sterically protected, di-isobutyl-n-octadecylsilane stationary phase is chemically stable and gives long column life at low pH. Poroshell 120 SB-C18 is a reversed-phase packing that can be used for basic, neutral or acidic samples. It is particularly well suited for use with aggressive low pH mobile phases (for example, pH < 2, high ionic strength (> 25 mM), ion-pair additives, etc.) since the steric protection of the bonded phase resists degradation with such mobile phases. The recommended high temperature limit for this bonded phase is 90 °C at low pH. Column Characteristics

A typical Poroshell 120 SB-C18, 4.6 mm × 50 mm, 2.7 µm column is shown in Figure.



All points of con-nection in liquid chromatographic systems are potential sources of leaks. Users of LCs and UHPLCs should be aware of the toxicity or flammability of their mobile phases.

These Poroshell 120 columns are mechanically stable and have been tested to very high pressures to assure safe lab operation on a variety of LC and UHPLC instruments. The operating pressure limit for all 2.1-, 3.0- and 4.6-mm id columns is 600 bar (9000 psi). While the 2.1- and 3.0-mm id columns are safe to 1300 bar (20,000 psi) and the 4.6-mm id columns are safe to 1000 bar (16,000 psi), chromategraphic performance will be compromised if the 600 bar pressure limit is exceeded and the column may need to be replaced.

Because of its small particle size, dry Poroshell packings are respirable. Columns should only be opened in a well-ventilated area, and opening the column will compromise column performance.

3.3. Application

Applications Poroshell 120 SB-C18 columns are designed for fast and high resolution separations of a wide range of small molecule analytes, including acidic, basic and neutral compounds. The unique, superficially porous particle and 2.7-µm particle size make this column ideal for fast separations at up to 40% to 50% lower pressures than sub 2-µm particles with similar (90% to 100%) efficiency. The columns can be used at high flow rates to achieve fast separations.

The 120 Å pore size means these columns are well suited for separations of peptides, such as those from a protein digest. These types of samples can be analyzed efficiently and with mobile phases containing additives such as TFA or formic acid for greater mass spectrometer compatibility. The Poroshell 120 SB-C18 bonded phase is ideal with a low pH mobile phase such as TFA. The sterically hindered bonded phase provides superior low pH lifetime, but this bonded phase is not endcapped to further reduce interactions with silanols. Therefore, for many basic compounds excellent peak shape will be obtained, but for some compounds the Poroshell 120 EC-C18, an endcapped packing may be a better choice for improved peak shape.

Alternatively, basic modifiers such as 20–30 mM triethylamine can be added to the mobile phase to improve peak shape. Poroshell 120 SB-C18 can also be used at 90 °C at low pH and is therefore a good choice for higher temperature separations at low pH. Elevated temperature may enhance or change selectivity and lower operating pressure.

(From: http://www.agilent.com/cs/library/datasheets/public/820302-002.pdf)

4. Principle and mechanisms of ion-pairing

Ion-pairing is a very useful analytical technique for the separation of charged molecules, which can be used for both positively and negatively charged analytes and is an advanced technique.

The animation below will explain the Principle and Mechanism. In this study the analyte is phenolic acid which is containing carboxyl group molecule and hence negatively charged. In regular extraction procedure, such a polar molecule would not be extracted by the non-polar organic solvents. In extraction solvent, an add-ition of ion-pairing agent applied a long carbon chain. Which is capable of inter-acting with the ionic group and make it more non-polar due to it's long chain of carbons. This interaction results in more interactions of the analyte with the extra-ctant phase and is hence more effecient. Better extraction efficiency would even-tually correspond to better enrichment.

Note-The ion pairing agent must be oppositely charged as compared to the analyte and must have good hydrophobicity. Other ion-pairing agents for negatively charged analytes such as carboxylates include other linear alkyl amines (e.g. pentylamine, heptylamine), tertiary alkyl amines (e.g. triethylamine, tetrabutylammonium). Ion-pairing agents for positively charged analytes such as amines includes sulfonates (e.g. n-pentanesulfonate, n-hexanesulfonate) and carboxylates (e.g. formic acid, trifluoroacetic acid).

(From: http://pharmaxchange.info/press/2013/01/principle-and-mechanisms-of-reversed-phase-ion-pairing-chromatography-with-animation/)

5. The influence of salt

The problem of the influence of salts on the activity coefficients of nonelectrolytes in aqueous solutions is of both fundamental and applied interest. Salt effect studies can provide considerable information of theoretical importance as to the complex interactions of ions and neutral molecules and as to the unique nature of water as a solvent. The data also have application to such related problems as kinetic salt effects and mechanisms of reactions, and they have a practical bearing on the separation of nonelectrolytes from water solutions by salting-out processes.

There have been a number of qualitative and quantitative theories of the salt effect, all with common underlying aspects but emphasizing different approaches to the problem. The discussion of the theoretical material presented in this review is in four sections which reflect the different approaches; this subdivision has been made primarily for convenience of presentation and is not intended to imply that there are sharp distinctions. Since detailed developments can be found in the original references, these sections will be restricted to a statement of fundamental ideas and final results.

According to this viewpoint the degree of salting out or salting in of a nonpolar solute is determined by the extent to which the solvent medium is compressed or loosened when ions are present. Salt effects on nonpolar nonelectrolytes merit separate discussion, since this class should be the simplest to interpret and gives a good reference point from which to consider the polar nonelectrolytes. The major role of a nonpolar solute is simply to occupy volume and thereby modify the ion-solvent interactions characteristic of a particular electrolyte solution. One of the objects of this section is to test the utility of this assumption.

In general, the degree of salting in of nonpolar solutes increases with ionic size. There are, however, several notable exceptions. One such is lithium ion, which invariably salts in much more than the larger sodium ion, and in fact gives results similar to rubidium ion. Two other cations which give large salting-out effects in relation to their sizes are ammonium ion and hydrogen ion.

For polar nonelectrolytes one would expect, just as with the nonpolar species, a salting-out contribution roughly proportional to the volume of the nonelectrolyte, a continuation of the specific effects characteristic of nonpolar molecules, and finally an increased salting in as the dipole moment of the molecule increases.

Salting in refers to the effect where increasing the ionic strength of a solution increases the solubility of some solute. This effect tends to be observed at lower ionic strengths. The solubility is a complex function of the physicochemical nature of the compound, pH, temperature, and the concentration of the salt used.

In summary, the high concentration of salt in the aqueous system lead to extra ion-pair reagent. This negative effect of high concentration of salt changed the physical properties of the aqueous system, which reduced the rate of diffusion of the ion pairs into extractant. Thus, at a high concentration of NaCl, the decrease of the solubility of the IP in water could be important in the extraction process, thereby reducing the extraction efficiency.

Reference from (F.A. Long, W.F. McDevit, Chem. Rev., 51, (119) 1952)

5. Validation of analytical procedures

According to the ICH harmonized tripartite guideline, the method validation was evaluated for linearity, limit of detection (LOD), limit of quantification (LOQ), precision and recovery.

5.1. Linearity

The linear relationship was evaluated across the range of the analytical procedure. It was demonstrated by spiked dilution of a standard stock solution in water, using the proposed procedure. The latter aspect was studied during investigation of the range.

Linearity was evaluated by visual inspection of a plot of signals as a function of analyte concentration. Test results have been evaluated by appropriate statistical methods. To obtain linearity between assays and sample concentration, the peak areas from results have been subjected to a logarithm transformation prior to the regression analysis. Data from the regression line itself may be help to provide mathematical estimates of the degree of linearity.

The correlation coefficient, y-intercept, slop of the regression line and residual sum of square have been submitted. A plot of the data was included. In addition, an analysis of the deviation of the actual data points from the regression line may also be helpful for evaluating linearity. For the establishment of linearity, a minimum of 5 concentrations was applied in experiment.

Range: The specified range is normally derived from linearity studies and depends on the intended application of the procedure. It is established by confirming that the analytical procedure provides an acceptable degree of linearity, accuracy and precision when applied to samples containing amounts of analyte within or at the extremes of the specified range of the analytical procedure.

5.2. LOD

The detection limit of an individual analytical procedure is the lowerst amount of analyte in a sample, which can be detected but not necessarily quantitated as an exact value. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and establishing the minimum concentration at which the analyte can be reliably detected. A signal-to-noise ratio between 3 or 2:1 is generally considered acceptable for estimating the detection limit.

The detection limit (DL) may be expressed as:

$$DL = \frac{3.3\ \sigma}{S}$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways, for example:

5.3. LOQ

The quantitation limit of an analytical procedure is the lowest amout of analyte in a sample, which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample, and is used particularly for the determination of the analytes in product. Determination of the signal-to-noise ratio is performed by comparing measured signals from samples with known low concentrations of analyte with those of blank samples and by establishing the minimum concentration at which the analyte can be reliably quantified. A typical signal-to-noise ratio is 10:1.

The quantitation limit (DL) may be expressed as:

$$DL = \frac{10\ \sigma}{S}$$

Where σ = the standard deviation of the response

S = the slope of the calibration curve

The slope S may be estimated from the calibration curve of the analyte. The estimate of σ may be carried out in a variety of ways for example：

5.4. Precision

The precision of an analytical procedure expresses the closeness of agreement (degree of scatter) between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions. Precision may be considered at three levels: repeatability, intermediate precision and reproducibility.

Precision should be investigated using homogeneous, authentic samples. However, if it is not possible to obtain a homogeneous sample it may be investigated using artificially prepared samples or a sample solution.

The precision of an analytical procedure is usually expressed as the variance, standard deviation or coefficient of variation of a series of measurements. Precision was considered at three levels: repeatability, intermediate precision and reproducibility.

Repeatability: Repeatability expresses the precision under the same operating conditions over a short interval of time. Repeatability is also termed intra-assay precision

Intermediate precision: Intermediate precision expresses within-laboratories variations: different days, different analysts, different equipment, etc.

5.5. Recovery

Absolute recovery was estimated by comparing the peak areas of standards i n spiked wine samples with the peak areas of standards in water at three co ncentration levels. The formula is used to calculate the recovery values as f ollow.

$$\text{Recovery\%} = \frac{(\text{amount found in spiked sample} - \text{amount found in sample})}{\text{amount spiked}} \times 100$$

(From: ICH harmonized tripartite guideline --- validation of analytical procedure)

Publications

[1] Shaodong Jia, Jing Li, So-Ra Park, Yeonsuk Ryu, Il Ho Park, Jeong Hill Park, Soon-Sun Hong, Sung Won Kwon, Jeongmi Lee. Combined application of dispersive liquid–liquid microextraction based on the solidification of floating organic droplets and charged aerosol detection for the simple and sensitive quantification of macrolide antibiotics in human urine. *Journal of Pharmaceutical and Biomedical Analysis*, 2013, 86, 204-213

[2] Jing Li, Shaodong Jia, Sang Jun Yoon, Seul Ji Lee, Sung Won Kwon, Jeongmi Lee. Ion-pair dispersive liquid–liquid microextraction solidification of floating organic droplets method for the rapid and sensitive detection of phenolic acids in wine samples using liquid chromatography combined with a core–shell particle column. *Journal of Food Composition and Analysis*, 2015, 45, 73-79

국문초록

본 연구에서는 와인에 포함된 10가지 페놀산의 검출을 위해 ion pair dispersive liquid-liquid microextraction based on the solidification of a floating organic droplet (IP-DLLME-SFO)를 이용한 새로운 분석법을 제시하였다. 기존에 있던 DLLME-SFO 방법을 ion-pairing technique 과 처음으로 접목하여 phenolic acid 를 분석하였다. 이 IP-DLLME-SFO 방법은 gallic acid 와 protocatechuic acid 와 같이 ion-pairing 없이는 DLLME-SFO 에서 추출되지 못하는 극성성분의 추출 효율을 증가시킬 수 있으므로 페놀산의 추출에 유용할 것이라고 생각했다. 따라서 DLLME-SFO 법의 개발에서 추출 효율에 영향을 미칠 수 있는 요소들을 분류법에 근거하여 최적화 하였다. 그러한 요소로써 ion-pairing reagent 의 종류와 농도, 추출 용매와 분산 용매의 종류와 농도, 추출 시간, 샘플의 pH, 이온 강도를 비교 분석하였다. 가장 높은 enrichment factor 를 갖는 조건으로 설정하였으며, 40 분 안에 10개의 phenolic acid 를 분리 검출할 수 있는 superficially porous particle (SPP) column 과 접목하여 고분해능, 고감도의 분석법을 갖추었다. 최적화된 분석법은 직선성, 검출한계, 재현성, 회수율 측면에서 validation 되었다. 모든 페놀산의 검량선은 결정 계수 (R^2) 0.994 이상을 가졌으며, 직선성의 한계는 0.01–15 μg/mL 의 범위로 나타났으며, 감도는 10 ng/mL 의 검출한계를 보여 분석법에 문제가 없음을 증명하였다. 또한 intra- 와 inter-day 정밀성은 각각 7.95 %와 9.33% 이하로 나타났고 회수율은 81.5 ~ 109 %의 범위에 속하는 것으로 나타났기에 재현성있는 분석법의 적용이 가능한것으로 확인하였다. 분석에 있어 매트릭스의 영향은 크게 관찰되지 않았다. 이렇게 개발된 분석법은 시판 중인 10가지 와인에 대한 페놀산의 분석에 실제 적용되었으며, matrix effect

없이 각 와인에 함유된 각기 다른 농도의 페놀산을 성공적으로 검출하였다.

주요어: DLLME-SFO; ion pairing; 극성; 페놀산; 와인; Core-shell particle Core–shell particle column; UPLC;

학 번: 2011-23014