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Effective Sample Preparation of Polyphenols in Wine Using Deep Eutectic Solvent-based Dispersive Liquid–Liquid Microextraction HPLC-UV Determination

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Polyphenols are phytochemicals that exist in grapes and are beneficial to human health. In this study, resveratrol, oxyresveratrol, and piceatannol in wine were extracted by deep eutectic solvent dispersive liquid–liquid microextraction (DES-DLLME), and a method was established for quantifying these polyphenols by high-performance liquid chromatography-UV/Vis (HPLC-UV/Vis). Several parameters pertaining to sample extraction, clean-up, and concentration were optimized and verified with central composite design (CCD) using Design Expert 11. The optimized sample preparation parameters are as follows: the DES extraction solvent, tributylmethylammonium chloride/decanoic acid (1:3 M ratio); basic solvent, 1.3 mL of 5% potassium bicarbonate; volume of acetic anhydride, 250 μL; derivatization time, 5 min; dispersive solvent, methanol; ratio of extraction and dispersive solvents, 1:5.5; and salt, 1.0 g. Chromatographic separation by HPLC/UV–Vis was performed on an ACME C18 (4.6 mm id × 150 mm length, 5 μm particle size) column in gradient elution mode using water and 70% methanol. Under the established extraction and HPLC-UV conditions, the limit of detection (LOD) and limit of quantitation (LOQ) of the three analytes in spiked samples ranged from 1.69 to 2.53 μg/L and 5.64 to 8.42 μg/L, respectively. Recovery studies were performed in low, medium, and high concentration ranges to establish a calibration curve, and the accuracy and precision in the working range were 95.1–108.0% and 1.3–6.7 RSD%, respectively. The calibration curves for quantitative analysis were obtained in the concentration ranges 5.6–56.4, 8.3–82.6, and 8.4–84.2 μg/L, with correlation coefficients (r^2) ranging from 0.9947 to 0.9967. The proposed method was applied to the determination of polyphenols in wine samples.

Keywords: Deep eutectic solvent, Dispersive liquid–liquid microextraction, Polyphenol, Wine, HPLC-UV

Introduction

Interest in human health has increased as the quality of life has increased in recent decades. Foods consumed by humans can sustain good health, but people have also expended considerable effort to maintain healthy lives and prevent aging. Accordingly, interest in nutrients with biological activity has increased, as have healthy eating habits.[1]

Polyphenols are contained in various nutrients exhibiting biological activity. They are characterized by a variety of structures with two or more acidic hydroxyl groups on the aromatic rings (Figure 1), and it has been reported that, the greater the number of hydroxyl groups in the molecule, the stronger the antioxidant properties and the greater the protection against free radicals. In this group, oxyresveratrol, piceatannol, and resveratrol belong to the stilbene family and are known to suppress cardiovascular disease and inhibit brain degeneration and tumors.[1,2] Resveratrol is produced when plants are exposed to bacteria, fungi, and UV irradiation and are physically damaged.[3–5] It is found in peanuts, raspberries, blueberries, and so on. In particular, it has a high concentration in grapes and foods using grapes,[6] and it has a higher concentration in red wine grapes than it does in white wine grapes.[7] When carbonate is present in the winemaking process, the content of resveratrol may be reduced relative to that in wine produced in the traditional manner.[8]

Several methods have been reported for the quantitative analysis of stilbenes with high polarity. Analyses using high-performance liquid chromatograph-ultraviolet detection (HPLC-UV),[9–11] high-performance liquid chromatograph-florescence detection (HPLC-FLD),[12–14] and liquid chromatography-mass spectrometry (LC–MS) have been utilized.[15–17] The use of gas chromatography has the disadvantage that derivatization is necessary because the analytes are not sufficiently volatile, but it enables detection at lower concentrations than does liquid chromatography.[18,19]

Stilbenes exhibit low concentrations in foods, and the matrix of food components is complex, so pretreatment is essential for analysis. Classical extraction methods such as liquid–liquid microextraction (LLE) require a long extraction time and use large volumes of harmful organic

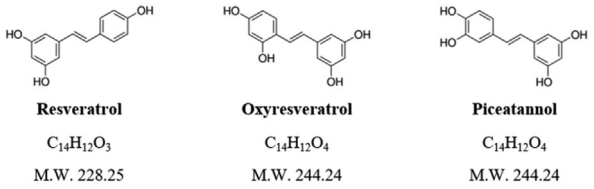

Figure 1. Chemical structure of polyphenols.

solvents, which can harm the environment and the human body.[6] To overcome this disadvantage, many different extraction methods have been developed and applied. It has been reported that a quick and effective pretreatment method may be applied to use a microextraction method and reduce the amount of solvent while enhancing selectivity. Applied microextraction methods include solid-phase microextraction (SPME),[16,17] single drop microextraction (SDME),[20] dispersion liquid–liquid microextraction (DLLME),[2] and stir bar adsorption extraction.[21] Although it is possible to reduce the amount of toxic organic solvents used by applying a microextraction method, the methods still involve exposure to harmful solvents. To eliminate this, methods using supercritical fluids (SFs)[22] and ionic liquids (ILs)[23] as extraction solvents have been proposed. However, since supercritical fluids require specific equipment to maintain the supercritical state, existing extraction methods cannot be replaced by SFs immediately. ILs have been touted as environmentally friendly solvents that replace toxic organic solvents, but questions have arisen as to whether they are truly environmentally friendly; some ILs have exhibited environmental toxicity, high cost, and limited biodegradability.[24–26] Therefore, in this study, a dispersion liquid–liquid microextraction method (DLLME) is used to enable desirable extraction and concentration performance, even with small amounts of extraction solvent. The toxic organic liquids normally used as extraction solvents have been replaced with a deep eutectic solvent (DES), so that an effective and environmentally friendly analysis method for polyphenols may be established.

DESs are systems formed from a eutectic mixture of Lewis or Brønsted acids and bases that can contain various types of cationic and/or anionic species.[26] The DES thus formed gives a eutectic with a melting point lower than those seen for the individual compounds. DESs can easily be prepared by mixing a hydrogen bond donor (HBD) and a hydrogen bond acceptor (HBA), and they exhibit desirable characteristics such as low conductivity, low vapor pressure, high thermal stability, and high viscosity.[27] In this study, a DES was prepared by mixing an organic salt (hydrogen bond acceptor) and a hydrogen bond donor. To prepare a hydrophobic DES, quaternary ammonium salts and decanoic acid, which contains a long alkyl chain, were used, and the successfully prepared DES was used as an extraction solvent.

Table 1. HPLC-UV conditions for analysis of polyphenols.

Parameters	Conditions		
Column	ACME C_{18}, 4.6 × 150 mm, 5 μm		
Mobile phase	A: 70% Methanol B: Water		
Gradient	Time	A(%)	B(%)
	0	10	90
	2	10	90
	12	40	60
	35	100	0
Flow rate	1 mL/min		
Injection volume	5 μL		
Wavelength	304 nm		

Experimental

Reagents and Materials. *Trans*-Resveratrol, trans-oxyresveratrol, and trans-piceatannol were purchased from TCI (Seoul, Korea) and Toronto Research Chemicals (Toronto, ON, Canada), respectively. The standard materials were dissolved in methanol to prepare a solution with a concentration of 1000 μg/mL and stored in a refrigerator. The stored solution was diluted with methanol when used. Decanoic acid was used as an HBD, and ultra-high purity reagent was obtained from JUNSEI (Tokyo, Japan). Tributylmethylammonium chloride (N4441—Cl), methyltrioctylammonium chloride (N8881—Cl), methyltrioctyl ammonium bromide (N8881—Br), and tetraoctylammonium bromide (N8888—Br) were used as HBA, and potassium hydrogen phosphate, potassium carbonate, and potassium bicarbonate were used as derivatization catalysts; all were purchased from Sigma-Aldrich (St. Louis, MO). Acetic anhydride was used as a derivatization reagent, and methanol, ethanol, acetone, and acetonitrile were used as dispersion solvents, and all were HPLC grade reagents obtained from Deoksan Co. (Gyeonggi, Korea). For studies of salting effects, sodium sulfate obtained from Daejeong (Gyeonggi, Korea) was used. Purified water was passed through a Synergy UV system (Millipore S.A.S, Molsheim, France), and ultrapure water exhibiting a specific resistance of 18.2 MΩ·cm was used.

Table 2. Factors and their levels for the central composite design.

	Level		
Factor	Low (−1)	Medium (0)	High (+1)
(x_1) Volume of base solution (mL)	0.7	1.0	1.3
(x_2) Volume of acetic anhydride (μL)	250	400	550
(x_3) Ratio of Ext. and Disp. solvent	1: 2.5	1:4	1: 5.5
(x_4) Salt (Na_2SO_4, g)	1.0	1.5	2.0

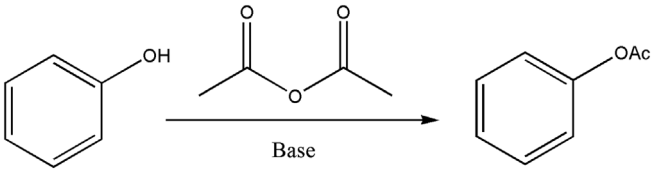

Figure 2. Acetylation mechanism of phenolic compound.

Apparatus and Instrumentation. The centrifuge used was an MF80 instrument made by Hanil (Seoul, Korea), and Design Expert 11 software from Stat Ease (Minneapolis, MN) was used for statistical analysis. A vortex mixer from Vision Scientific (Bucheon, Korea) was used for mixing samples, and test tubes for centrifugation were manufactured by Falcon (TX).

HPLC Analysis. HPLC/UV–Vis experiments were performed on an 1100 series HPLC system from Agilent (Palo Alto, CA). An ACME C18 column from Phase Analytical Technology, LLC (State College, PA), was used for the simultaneous analysis of three polyphenols by liquid chromatography. The length and inner diameter of the column were 150 mm and 2.1 mm, respectively, and the particle size was 5 μm. For the HPLC mobile phase, a gradient elution method was used in which the ratio of water and 70% methanol was maintained at 90:10 for the initial 2 min, followed by 60:40 at 12 min, and 0:100 at 35 min. The flow rate of the mobile phase and the injection volume were 1 mL/min and 5 μL, respectively, and the detection wavelength was 304 nm (Table 1).

Design of Experiments. Design of experiments was used to develop experimental parameters, with the goal of obtaining maximum information by identifying the main variables affecting the experiment and minimizing the number of experiments. In this study, the optimal value for each factor was selected by the one variable at a time (OVAT) method that optimizes one experimental factor individually. The OVAT method can easily be used to optimize each experimental factor by fixing the other factors but has the disadvantage that it is impossible to consider the effects of those other experimental factors. Therefore, the optimization process was performed again by applying the central composite design (CCD) method and using Design Expert 11. The main experimental factors for considering the interaction between experimental variables are (1) volume of derivatization catalyst (X_1), (2) volume of derivatization reagent (X_2), (3) ratio of extraction solvent and dispersion solvent (X_3), and (4) the amount of salt (X_4) (Table 2), and these were applied to the CCD method.

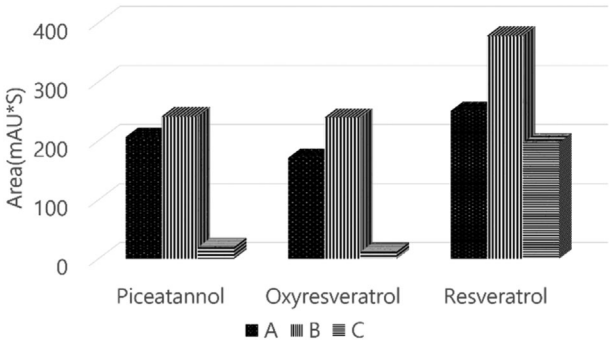

Figure 3. Effect of base solvent type on peak area of polyphenols: (a) potassium phosphate dibasic, (b) potassium bicarbonate, (c) potassium carbonate.

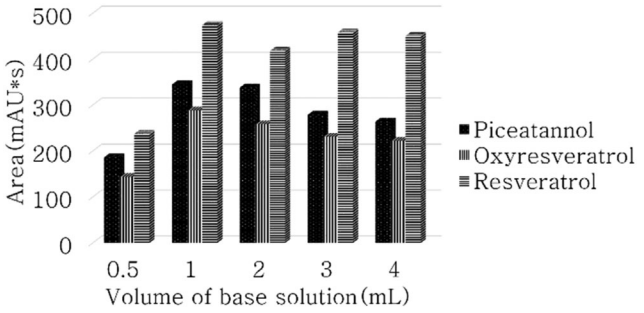

Figure 4. Effect of base solvent volume on peak area of polyphenols.

Sample Preparation. The experimental procedure for the DLLME is as follows. The 100 μL of DES as an extraction solvent and 550 μL of dispersion solvent were added rapidly to the aqueous solution sample resulting after the derivatization of the analyte with a base solvent as the catalyst and acetic anhydride as derivatization reagent, and 1.0 g of sodium sulfate was added. The solution was then gently shaken for 10 s. After centrifugation for 10 min at a speed of 4000 rpm, the layers were separated, and 20 μL of the extraction solvent comprising the upper layer was removed and injected into the HPLC instrument.

Results and Discussion

Optimization of Derivatization. A derivatization process is required for the effective extraction and chromatographic analysis of polyphenols contained in aqueous solution. Through derivatization, hydrophilic properties can be reduced to improve separation in a reversed-phase

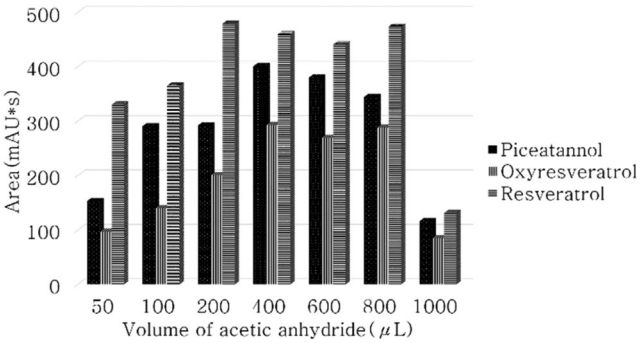

Figure 5. Effect of acetic anhydride volume on peak area of polyphenols.

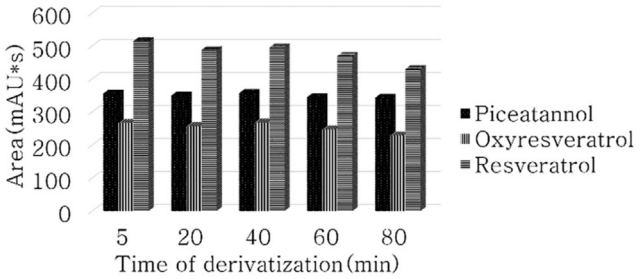

Figure 6. Effect of derivatizing time on peak area of polyphenols.

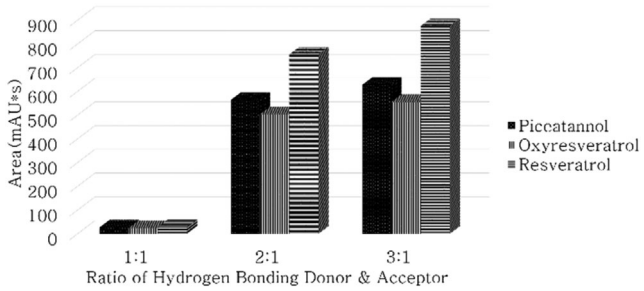

Figure 8. Effect of ratio (1:1, 2:1, and 3:1) of hydrogen bond donor to hydrogen bond acceptor on peak area of polyphenols.

chromatography system and to increase the extraction efficiency of hydrophobic extraction solvents.[2] For effective derivatization, it is necessary to adjust the pH and establish basic sample conditions. In basic solution, the hydroxyl hydrogen of the polyphenols is easily dissociated, and acetic anhydride, a derivatization reagent, is used to acetylate the site (Figure 2). The type and volume of the derivatization catalyst, volume of the derivatization reagent, and derivatization time were considered as parameters in the derivatization process, and their effects were investigated.

Type of Catalyst. Dibasic potassium hydrogen phosphate, potassium carbonate, and potassium bicarbonate (all at 5% (w/v) concentration) were compared as basic catalysts for derivatization. The conditions for selecting an optimized derivatization catalyst are as follows: the volume of derivatization catalyst used was 2.0 mL, the volume of derivatization reagent (acetic anhydride) used was 800 μL, the derivatization time was 5 min, the volume of extraction solvent (N8881-Cl:DecA [1:2]) used was 100 μL, the volume of dispersion solvent (acetone) used was 400 μL, and the amount of salt (sodium sulfate) used was 1.5 g. Figure 3 shows a graph comparing the integrated areas of the chromatograms with the type of derivatization catalyst used. Since the use of potassium bicarbonate resulted in the largest peak area, potassium bicarbonate was selected as the derivatization catalyst.

Volume of Base Solvent. If the volume of the derivatization catalyst solution used is small, it may not have an appropriate catalytic effect, whereas, when the volume is large, the total volume of the sample increases and the extraction efficiency may decrease. Therefore, it is important to determine the optimal volume. Figure 4 shows a graph comparing the areas of chromatograms resulting from the volume of the potassium bicarbonate which is the derivatization catalyst. The volume of the derivatization catalyst was varied within the range of 0.5–4 mL to select the optimal volume of the derivatization catalyst. The other conditions were as follows: the derivatization catalyst used was 5% (w/v) potassium bicarbonate, the volume of derivatization reagent (acetic anhydride) used was 800 μL, the derivatization time was 5 min, the volume of extraction solvent (N8881-Cl:DecA [1:2]) used was 100 μL, the volume of dispersion solvent (acetone) used was 400 μL, and the amount of salt (sodium sulfate) used was 1.5 g.

Amount of Acetic Anhydride. When the amount (volume) of the derivatization reagent is small, derivatization does not proceed completely, resulting in loss of sample. If the amount of the reagent is large, the total volume of the sample increases, and this may decrease extraction efficiency. Therefore, it is important to determine the appropriate amount to achieve the optimal derivatization efficiency. To select the amount of the derivatization reagent, the volume was varied within the range of 50–1000 μL, the type and volume of the derivatization catalyst were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively, the derivatization time was 5 min, the volume of extraction solvent (N8881-Cl:DecA [1:2]) used was 100 μL, the volume of dispersion solvent (acetone) used was 400 μL, and the amount of salt (sodium sulfate) used was 1.5 g. Figure 5 shows a graph comparing the areas of the chromatogram peaks to the volumes of the derivatization reagent used,

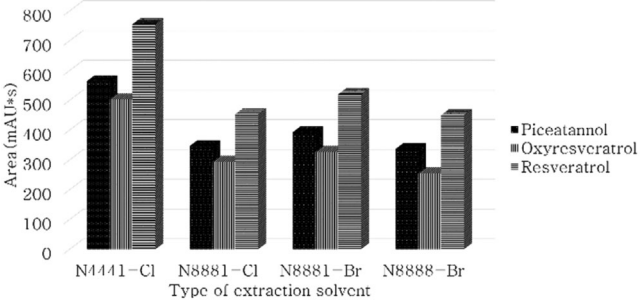

Figure 7. Effect of extraction solvent type on peak area of polyphenols.

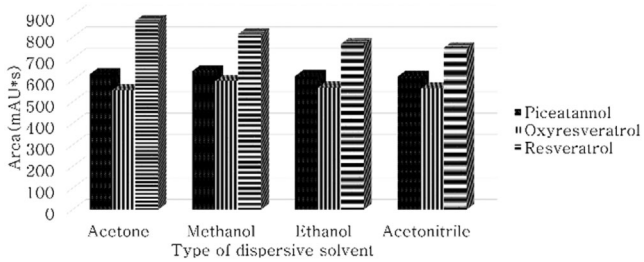

Figure 9. Effect of dispersive solvent type on peak area of polyphenols.

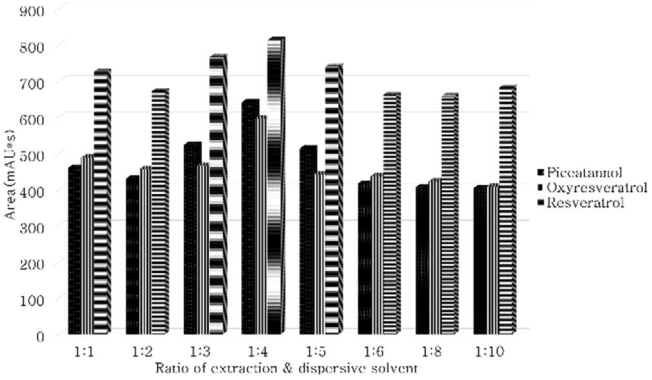

Figure 10. Effect of ratio of extraction to dispersive solvent on peak area of polyphenols.

and the optimal value for the volume of the derivatization reagent is found to be 400 μL.

Derivatization Time. If the derivatization time is insufficient, the analytes may not be derivatized completely and sample loss results. If the derivatization time is too long, the time required for sample preparation will increase unnecessarily. Therefore, it is important to shorten the sample preparation time by determining the required derivatization time. The derivatization time was probed by changing the time within the range of 5–80 min, the type and volume of the derivatization catalyst were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively, the amount of the derivatization reagent used was 400 μL, the volume of extraction solvent (N8881-Cl:DecA [1:2]) used was 100 μL, the volume of dispersion solvent (acetone) used was 400 μL, and the amount of salt (sodium sulfate) used was 1.5 g. Figure 6 shows a graph comparing the areas of the chromatogram peaks to the derivatization times; the change with derivatization time was not very great, so the derivatization time was set to 5 min for limiting the time of the experiments.

Synthesis of Deep Eutectic Solvent. To synthesize the optimal DES for extracting polyphenols, optimization was performed by comparing four HBA candidates. With decanoic acid chosen as HBD, tributylmethylammonium chloride (N4441—Cl), methyltrioctylammonium chloride (N8881—Cl), methyltrioctylammonium bromide (N8881—Br), and tetraoctylammonium bromide (N8888—Br) were used as

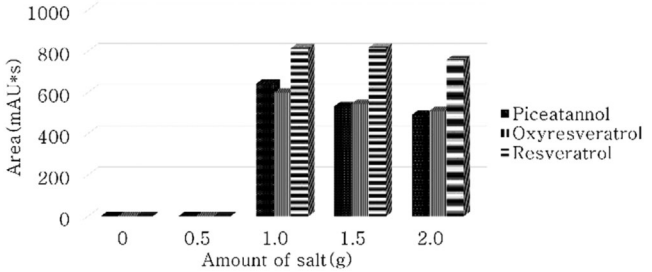

Figure 11. Effect of salt amount on peak area of polyphenolstes.

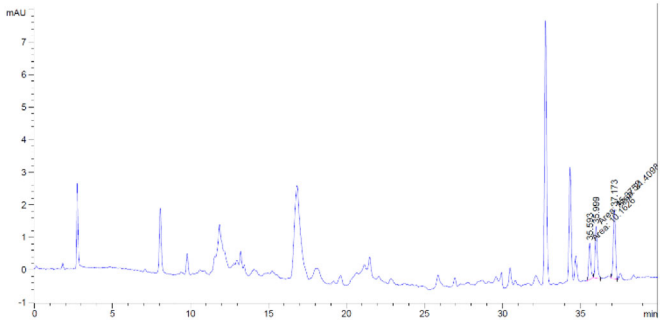

Figure 12. The HPLC chromatogram from the spike white wine (LOQ concentration level).

hydrogen bond acceptors (HBA). The molar ratio of the hydrogen bond donor and the hydrogen bond acceptor was fixed as 2:1. The derivatization catalyst and volume were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively, the amount of the derivatization reagent (acetic anhydride) was 400 μL, the derivatization time was 5 min, the volume of the DES as an extraction solvent was 100 μL, the volume of the dispersion solvent (acetone) was 400 μL, and the amount of salt was 1.5 g. Figure 7 shows a graph comparing the extraction efficiencies with the combination of hydrogen bond donor and hydrogen bond acceptor by using the areas of peaks in the chromatograms. Among the four hydrogen bond acceptors, tributylmethylammonium chloride, which has the lowest hydrophobicity, resulted in the greatest chromatogram peak area for the resulting extraction solvent (Figure 7).

Ratio of Hydrogen Bond Donor to Hydrogen Bond Acceptor. Although DESs are prepared using the same hydrogen bond donor and hydrogen bond acceptor, they have different properties when the molar ratio of the donor and acceptor is varied. Therefore, the extraction efficiency was studied by changing the ratio of the hydrogen bond donor (decanoic acid) and the hydrogen bond acceptor (N4441—Cl) within the range 4:1 to 1:4, and the type and volume of the derivatization catalyst were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively. The amount of

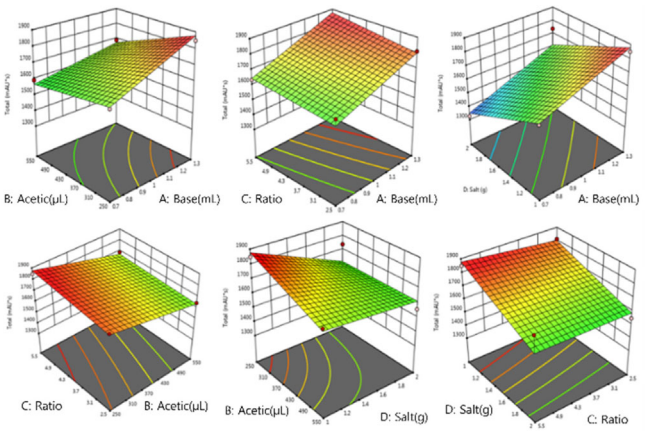

Figure 13. Response surface plots.

Table 3. Optimized DLLME conditions by using RSM.

	A (mL)	B (μL)	C	D (g)
High	1.3	250	5.5	2.0
Optimized	1.3	250	5.5	1.0
Low	0.7	550	2.5	1.0

Table 4. LODs and LOQs for polyphenols (μg/L).

Compounds	LOD	LOQ
Resveratrol	1.69	5.64
Oxyresveratrol	2.48	8.26
Piceatannol	2.53	8.42

the derivatization reagent (acetic anhydride) was 400 μL, the derivatization time was 5 min, the volume of the extraction solvent was 100 μL, the volume of the dispersion solvent was 400 μL, and the amount of salt was 1.5 g. When the molar ratio of the hydrogen bond donor and the hydrogen bond acceptor was 4:1, it exists as a solid at room temperature and could not be used as an extraction solvent (Figure 8). The highest extraction efficiency was obtained when the molar ratio was 1:3.

Optimization of Dispersive Liquid–Liquid Microextraction. After synthesis of the DES solvent suitable for extracting polyphenols, the solvent was used in the dispersion liquid–liquid microextraction (DLLME) method. The parameters for optimizing the extraction efficiency in the method were (1) the type of dispersion solvent, (2) the volume ratio of the extraction solvent and the dispersion solvent, and (3) the amount of salt. Therefore, experiments were undertaken to optimize these parameters.

Type of Dispersive Solvent. In the DLLME, the dispersion solvent must be miscible with both a hydrophilic sample and a hydrophobic extraction solvent, and, when added to an aqueous solution sample and an extraction solvent system, the extraction solvent must be dispersible into fine droplets. Extraction solvents dispersed in fine droplets can increase extraction efficiency due to increased surface area. It was confirmed that the DES prepared above mixed well with methanol, ethanol, acetone, and acetonitrile, as well as with aqueous solutions. Therefore, the extraction efficiency was compared by selecting the various solvents as a dispersion solvent. If the polarity of the dispersion solvent is too large or too small, it is difficult to disperse the extraction solvent into an aqueous solution.

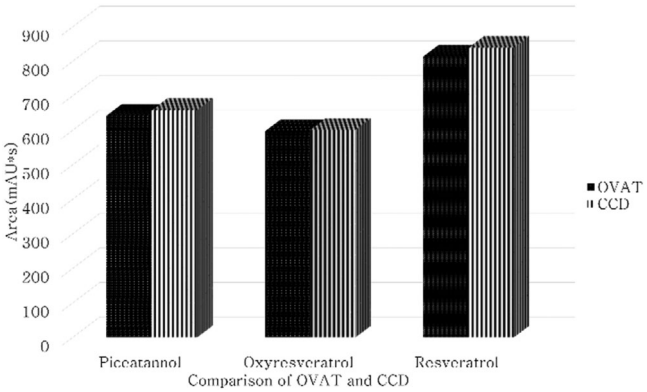

Figure 14. Comparison of OVAT and CCD.

The parameters used for selecting the optimal dispersion solvent are as follows: the type and volume of the derivatization catalyst were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively, the amount of the derivatization reagent was 400 μL, the derivatization time was 5 min, the volume of the extraction solvent was 100 μL, the molar ratio of the hydrogen bond donor and the hydrogen bond acceptor was 3:1, the volume of the dispersion solvent was 400 μL, and the amount of salt was 1.5 g. The dispersion solvent showing the optimum extraction efficiency was identified from the area of a peak in the chromatogram, and the use of methanol resulted in the largest peak area evenly in three polyphenols (Figure 9).

Ratio of Extraction and Dispersive Solvents. When the volume of the dispersion solvent is small, the hydrophobic extraction solvent cannot disperse adequately in the aqueous solution, and, when the volume is large, the analyte may be more soluble in the aqueous solution than it is in the extraction solvent. Therefore, it is important to

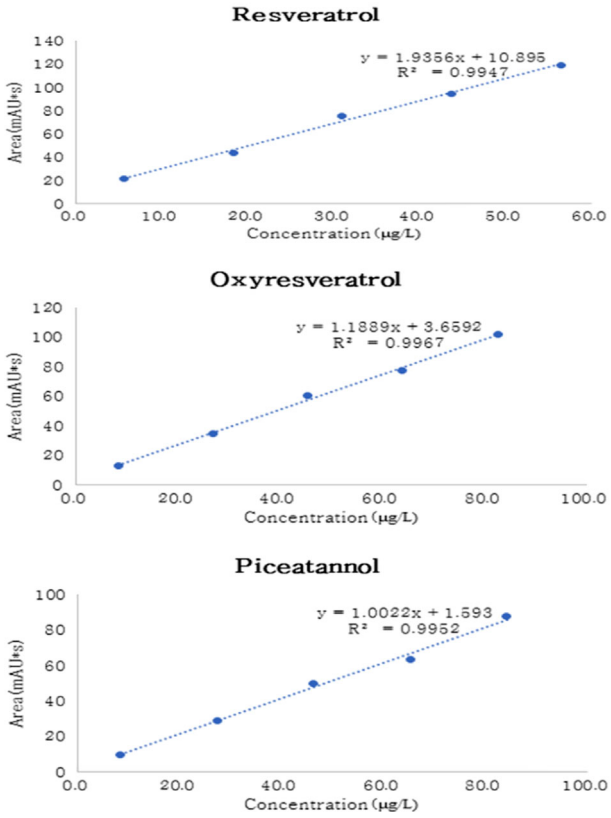

Figure 15. Calibration curves for determination of polyphenols.

Table 5. Working range, linear equation and r^2.

Compounds	Working range(μg/L)	Linear equation	r^2
Resveratrol	5.6–56.4	y = 1.9356x + 10.895	0.9947
Oxyresveratrol	8.3–82.6	y = 1.1889x + 3.6592	0.9967
Piceatannol	8.4–84.2	y = 1.0022x + 1.593	0.9952

determine the appropriate ratio of extraction solvent and dispersion solvent. To determine this optimal ratio for extraction solvent and dispersion solvent (methanol), ratios of the two solvents were varied within the range 1:1 to 1:10, and the other parameters were set as in the experiments described above. The best extraction efficiency (maximum chromatographic peak area) was obtained when the ratio of the extraction solvent to the dispersion solvent was 1:4 (Figure 10).

Amount of Salt. The salting-out effect can reduce the solubility of the analyte in the aqueous solution sample and increase the solubility of the analyte in the extraction solvent, and this is accomplished with a salt that is more soluble in aqueous solution than is the analyte. However, the addition of salt increases the ionic strength, so the extraction efficiency does not always increase. This is attributed to changes in the physical properties of the Nernst diffusion film, which reduce the rate at which the analyte diffuses into the extraction solvent.[28] To optimize the amount of salt (sodium sulfate) used, extraction efficiencies were compared by adding salt in amounts ranging from 0 to 2.0 g. The type and volume of the derivatization catalyst were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively. The volume of derivatizing reagent was 400 μL, the derivatization time was 5 min, the volume of the extraction solvent was 100 μL, the volume of the dispersion solvent was 400 μL, and the molar ratio of the hydrogen bond donor and the hydrogen bond acceptor was 3:1. When the amount of salt was less than 1.0 g, the extraction solvent did not separate well from the aqueous solution sample, and, when it was 1.0 g, the best extraction efficiency resulted (as determined by the area of the peak in the chromatogram) (Figure 11).

As such, the optimal derivatization conditions for the phenolic compounds present in the aqueous solution and the optimal extraction conditions by DLLME are as follows: the type and volume of the derivatization catalyst were 5% (w/v) potassium bicarbonate and 1.0 mL, respectively, the volume of derivatizing reagent (acetic anhydride) was 400 μL, the derivatization time was 5 min, the volume of the DES as an extraction solvent was 100 μL, the volume of the dispersion solvent was 400 μL, the molar ratio of the hydrogen bond donor (decanoic acid) and the hydrogen bond acceptor (tributylmethylammonium) was 3:1, and the amount of salt (sodium sulfate) was 1.0 g. Figure 12 shows the HPLC chromatogram analyzed after spiked with three polyphenol standards at the level of LOQ concentration in 10% blank white wine and extracted by the established method. Piceatannol, oxyresveratrol, and resveratrol were detected at 35.59, 35.99, and 37.17 min, respectively.

Central Composite Design. The optimized experimental factors obtained with the OVAT method are as follows: the derivatization catalyst was 5% (w/v) potassium bicarbonate, the volume of derivatization catalyst was 1.0 mL, the amount of derivatization reagent was 400 μL, the derivatization time was 5 min, the volume of the extraction solvent was 100 μL, the volume of the dispersion solvent was 400 μL, the ratio of hydrogen bond donor to hydrogen bond acceptor was 3:1, and the amount of salt was 1.0 g. To overcome the problems resulting from experimental factors that cannot be considered in the OVAT method, the optimization was performed again using the CCD method and the optimization values obtained from the OVAT method. The mutual effects of the four experimental factors were obtained from six response surface curves (Figure 13). As a result, the best result was obtained when the volume of the derivatization catalyst was 1.3 mL, the volume of the derivatization reagent was 250 μL, the volume ratio of the extraction solvent and the dispersion

Table 6. Accuracy and precision of polyphenols (n = 3).

Compounds	Concentration (μg/L)	Accuracy (%)	RSD (%)
Resveratrol	5.6	98.9	5.5
	31.0	108.0	4.6
	56.4	99.1	2.8
Oxyresveratrol	8.3	95.1	6.7
	45.4	105.6	4.3
	82.6	100.5	2.4
Piceatannol	8.4	99.0	6.1
	46.3	103.6	3.9
	84.2	102.0	1.3

Table 7. Intra- and inter-day accuracy and precision (n = 3).

		Intra-day		Inter-day	
Compds.	Conc (μg/L)	Accuracy (%)	RSD (%)	Accuracy (%)	RSD (%)
Resveratrol	5.6	95.9	2.3	104.5	4.0
	31.0	101.3	4.2	107.2	4.3
	56.4	91.2	3.2	96.6	3.5
Oxyresveratrol	8.3	86.1	4.1	97.8	4.7
	45.4	94.2	4.3	103.5	4.9
	82.6	87.3	3.3	96.7	3.7
Piceatannol	8.4	80.5	5.8	90.1	3.7
	46.3	91.1	5.0	100.5	4.4
	84.2	89.8	3.2	98.8	4.1

solvent was 1:5.5, and the amount of salt was 1.0 g (Table 3). Therefore, somewhat better results were obtained with optimization using the CCD method, as compared to those obtained with optimizations using the OVAT method (Figure 14).

Method Validation. Using the optimized experimental methods, detection limits, quantitation limits, calibration curves, accuracy, precision, and so on were probed with validation experiments described below.

LOD and LOQ: After assuming the limit of detection (LOD), seven spiked aqueous samples were analyzed under the optimized conditions and at an analyte concentration, approximately three times that of the assumed LOD. The standard deviation (σ) was calculated from the analytical results for each sample. Another spiked sample, with approximately twice the analyte level of the spiked sample, was also analyzed, and calibration curves were plotted. The slope (m) of each calibration curve was used to calculate the LOD and LOQ. The LOD and LOQ values were calculated from 3 σ/m and 10 σ/m, respectively. The LOD was required to have a S/N of 3 or higher, and the LOQ was required to have a S/N of 3 or higher and a relative standard deviation (RSD) of less than 15%. If these conditions were not satisfied, this experiment was performed with increased analyte concentration. As a result, the LOD and the LOQ exhibited ranges of 1.69–2.53 μg/L and 5.64–8.42 μg/L, respectively (Table 4). The LOD and LOQ in other literatures by HPLC-UV/Vis analysis was 0.4–950 and 1.13–3160 ug/L,[11,29–31] and our experimental results were similar of better than these results.

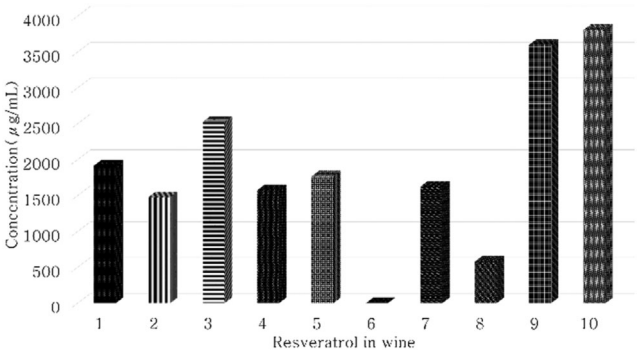

Figure 16. Concentration of resveratrol in real wine sample: Number 1–7 wines from Chile, number 8–10 wines from Australia.

Calibration Curves. The working range of the calibration curve spanned analyte concentrations from the concentration limit to a concentration more than 10 times that of the limit. The concentrations for the calibration curve ranged from 5.6 μg/L, which is the LOQ concentration of the analytes, to 84.2 μg/L, which is 15 times the LOQ concentration (Figure 15). The correlation coefficient (r^2) showed good linearity and ranged from 0.9947 to 0.9967 (Table 5).

Accuracy and Precision. After spiking three polyphenol standards for wine blank samples to establish a low concentration, a medium concentration, and a high concentration within the range of the calibration curve, accuracy, and precision determinations were performed for each concentration. The accuracy was expressed as a relative recovery and ranged from 95.1 to 108.0%, showing good accuracy (n = 3). The precision, which was expressed as a RSD, was determined 3 times per sample and showed good reproducibility, with variations ranging from 1.3 to 6.7% (Table 6).

To measure the errors observed within a day, three experiments for determining accuracy and precision were performed at different times; the relative recovery ranged from 80.5 to 101.3% and the RSD from 2.3 to 5.8% (Table 7). Accuracy and precision experiments were also carried out on multiple days, and the relative recovery ranged from 90.1 to 107.2% and the RSD from 3.4 to 5.0% (Table 7).

Real Sample Monitoring. Analysis of polyphenols contained in commercially available wines was performed using the experimental methods established herein. The experimental procedure for the real wine sample is as follows: 100 μL of extraction solvent (DES) and 550 μL of dispersion solvent (methanol) were added rapidly to the aqueous solution sample resulting after the derivatization of the analyte with a base solvent (5% (w/v) potassium bicarbonate) as the catalyst and acetic anhydride as derivatization reagent, and 1.0 g of sodium sulfate was added. The solution was then gently shaken for 10 s. After centrifugation for 10 min at a speed of 4000 rpm, the layers were

separated, and 20 μL of the extraction solvent comprising the upper layer was removed and injected into the HPLC instrument.

The 10 wines selected included nine red wines and one white wine, and seven were from Chile, and three were from Australia. Oxyresveratrol and piceatannol were not detected, and resveratrol was detected in the range of 0.00–4000 mg/L in all wines. Due to the small number of samples, no difference could be attributed to the country of origin or wine type (white vs. red wine) (Figure 16).

Conclusion

In this study, an eco-friendly extraction solvent applicable to DESME was synthesized, and the optimal conditions for the simultaneous analysis of three polyphenols (resveratrol, oxyresveratrol, piceatannol) contained in wine were established. This method was applied to the analysis of real samples. The LOD and LOQ for the three polyphenols ranged from 1.69 to 2.53 μg/L and 5.6 to 8.4 μg/L, respectively. The intra- and multi-day accuracies ranged from 80.5 to 101.3% and 90.1 to 107.2%, respectively, and the precisions ranged from 2.3 to 5.8 RSD% and 3.3 to 4.9 RSD%, respectively. The correlation coefficient (r^2) exhibited linearity in the range 0.9947 to 0.9967.

In this study, a deep eutectic solvent (DES) was used as an extraction solvent to establish a method that can effectively analyze three types of polyphenols as an environmentally friendly analysis method. In addition, in terms of LOD and LOQ, results were almost similar or better than those of other HPLC/UV–Vis analysis methods.

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References

1. M. Fabjanowicz, J. P. Wasylka, J. Namiesnik, *Trends Anal. Chem.* **2018**, *103*, 21.
2. T. R. Cabo, I. Rodriguez, R. Cela, *J. Chromatogr. A* **2012**, *1258*, 21.
3. G. Arslan, N. Yilmaz, *Asian J. Chem.* **2013**, *25*, 1225.
4. J. Burns, T. Yokota, H. Ashihara, M. Lean, A. Crozier, *J. Agric. Food Chem.* **2002**, *50*, 3337.
5. R. E. King, A. Joshua, A. Bomser, D. B. Min, *Compr. Rev. Food Sci. Food Saf.* **2006**, *5*, 65.
6. J. I. Cacho, N. Campillo, P. Vinas, M. Hernandez-Cordoba, *J. Chromatogr. A* **2013**, *1315*, 21.
7. L. Fremont, *Life Sci.* **2000**, *66*, 663.
8. B. Sun, A. M. Ribes, M. C. Leandro, A. P. Belchior, M. I. Spranger, *Anal. Chim. Acta* **2006**, *563*, 382.
9. J. A. Baptista, J. F. da, P. Tavares, R. C. Carvalho, *Food Res. Int.* **2001**, *34*, 345.
10. F. Y. Du, X. H. Xiao, G. K. Li, *J. Chromatogr. A* **2007**, *1140*, 56.
11. J. Goncalves, J. S. Câmara, *J. Sep. Sci.* **2011**, *34*, 2376.
12. L. Mercolini, R. Mandrioli, M. A. Raggi, *J. Pineal Res.* **2012**, *53*, 21.
13. P. Viñas, C. López-Erroz, J. J. Marín-Hernández, M. Hernández-Córdoba, *J. Chromatogr. A* **2000**, *871*, 85.
14. P. Viñas, N. Campillo, M. Hernández-Pérez, M. Hernández-Córdoba, *Anal. Chim. Acta* **2008**, *611*, 119.
15. J. B. Jean-Denis, R. Pezet, R. Tabacchi, *J. Chromatogr. A* **2006**, *1112*, 263.
16. F. S. Mirnaghi, F. Mousavi, S. M. Rocha, J. Pawliszyn, *J. Chromatogr. A* **2013**, *1276*, 12.
17. G. Sagratini, F. Maggi, G. Caprioli, G. Cristalli, M. Ricciutelli, E. Torregiani, S. Vittori, *Food Chem.* **2012**, *132*, 1592.
18. R. Montes, M. Garcia-Lopez, I. Rodriguez, R. Cela, *Anal. Chim. Acta* **2010**, *673*, 47.
19. O. Tokus, oglu, M.K. Unal, F. Yemis, *J. Agric. Food Chem.* **2005**, *53*, 5003.
20. P. Viñas, N. Martínez-Castillo, N. Campillo, M. Hernández-Córdoba, *J. Chromatogr. A* **2011**, *1218*, 639.
21. E. Baltussen, P. Sandra, F. David, C. Cramers, *J. Microcolumn Sep.* **1999**, *11*, 737.
22. A. Mouahid, C. Crampon, S.-A. A. Toudji, E. Badens, *J. Supercrit Fluids* **2013**, *77*, 7.
23. D. Zhao, Y. Liao, Z. Zhang, *Clean* **2007**, *35*, 42.
24. J. F. Brennecke, E. J. Maginn, *AIChE Jo* **2001**, *47*, 2384.
25. B. Tang, K. H. Row, *Monatsh. Chem.* **2013**, *144*, 1427.
26. E. L. Smith, A. P. Abbott, K. S. Ryder, *Chem., Rev.* **2014**, *114*, 11 060.
27. A. Abbott, G. Capper, D. Davies, R. Rasheed, V. Tambyrajah, *Chem Commun.* **2003**, *1*, 70.
28. E. Psillakis, N. Kalogerakis, *Trends Anal. Chem.* **2003**, *22*, 565.
29. C. Dominguez, D. Guillen, C. Barroso, *J. Chromatogr. A* **2001**, *918*, 303.
30. A. Aresta, P. Cotugno, F. Massari, C. Zambonin, *Food Anal. Methods* **2018**, *11*, 426.
31. C. Silva, J. Pereira, V. Wouter, C. Giro, J. Camara, *Talanta* **2011**, *86*, 82.

