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The Use of Solid Phase Microextraction for the Determination of Polyphenolic Acids in Wine

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Abstract

A simple and efficient technique that does not require solvent and uses less operating time for the simultaneous determination of the phenolic compounds found in wine and fresh grapes by utilizing on-fiber derivatization after solid-phase microextraction (SPME) has been developed. A variety of different phenolic acids such as gentisic acid, caffeic acid, ferulic acid, and p-coumaric acid were used to investigate the scope and applicability of our methods. Compounds were first extracted on a relatively polar polyacrylate (PA)-coated fiber at room temperature for 60 min, followed by on-fiber silylation in a vial containing 20 μL of bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60℃ for 20 min. The identification of the polyphenolics was performed by GC-MS.

Keywords：on-fiber derivatization, SPME, phenolic acids, GC-MS

1. Introduction

Phenolic acids in red wine or fresh fruits have been shown to inhibit the *in vitro* oxidation of human low-density lipoprotein (LDL)[1], the role of these phenolics as natural antioxidants and free radical scavengers has attracted considerable interest[2-4]. In general, phenolic acids and their derivatives are widely distributed in plants[5-7]. There are many publications investigating the phenolic contents of grapes[8], fruits and wines[9, 10]. A number of analytical methods have been proposed for the separation and determination of these biologically active phenolic components in food. Most of these protocols are based on high-performance liquid chromatography (HPLC) techniques, with UV spectrophotometry or electrochemical detection (ED) methods[11-14], or coupled with coulometric detection method[15]. Leong and Shui developed a HPLC separation method with photo-diode array

detection for the simultaneous determination of organic acids and phenolic compounds in juices and drinks[16]. Careri and co-workers disclosed a particle-beam electron-impact mass spectrometry (PB-EI-MS) detection method for the HPLC analysis of phenolic acids[17]. Glowniak et al. have developed a procedure which combining solid-phase extraction and reversed-phase HPLC for the isolation, purification as well as qualitative and quantitative determinations of free phenolic acids in plants[18]. The SPE method also has been reported in separating and determining the flavonoids and other phenolic compounds in cranberry juice[19]. Fernandes et al. utilized a capillary zone and micellar electrokinetic capillary chromatographic technique for the determination of complex mixtures of flavonoids, cinnamic acids and simple phenolic acids[16, 20]. Gonzalez-SanJose and coworkers reported various applications of liquid chromatography-mass spectrometry for the analysis of phenolic compounds[21]. Other than using liquid chromatography, GC/FID and GC/MS were also employed for the identification of some monomeric and dimeric phenolic acids[22].

Phenolic acids have high melting point (about 200 °C), and decompose when heated above their melting point[23]. Consequently, trimethylsilyl (TMS) derivatives of phenolic acids are prepared for gas chromatography analysis[24,25]. Goldberg et al. have developed a gas chromatographic/mass spectrometric method preceded by solid phase extraction to simultaneously measure the concentrations of 15 phenolic components in wine[26]. Ng et al. used anion-exchange disk extraction and TMS derivatives for the determination of phenolic acids in distilled alcohol beverages[27]. An in-vial derivatization-extraction of phenolic acids and flavonoids in methanolic and aqueous plant extracts followed by GC-MS has been reported [28].

However, the extractions often demand laborious works and large consumption of solvent. The solid phase microextraction (SPME), developed by Pawliszyn and co-woekers[30-32], is a viable alternative to solvent extraction and offers a convenient, solvent-free and time-saving method which has been widely used[33-37]. To increase the recovery, SPME has been coupled with derivatization processes[38, 39]. Compared with direct SPME, the coupling of derivatization with SPME during sampling affords an improvement in selectivity and sensitivity of analysis[40]. SPME also has been applied to the analysis of the flavor and phenolic compounds in wine[41-43].

Derivatization on solid phases has implemented a new method to alleviate the problems posed by interferences and the extra steps associated with classical derivatization[44]. Pawliszyn et al. analyzed the anatoxin-a in aqueous samples by solid-phase microextraction coupled to HPLC with fluorescence detection and on-fiber derivatization by dropping or

spraying the fluorogenic derivatizing reagent onto the fiber containing extacted analytes[45]. Campins-Falco *et al*. reported the analysis of methylamine by SPME and HPLC after on-fibre derivatization with 9-fluorenylmethyl chloroformate[46]. Tsai and Chang applied SPME with on-fiber derivatization to analyze aldehydes in water by HSSPME extraction of the aldehydes in water sample with PFBHA loaded fiber, followed by GC/MS analyses of oximes formed[47]. Rodriguez *et al*. employed SPME followed by on-fiber silylation to determine the anti-inflammatory drugs in water samples using MTBSTFA as the derivatizing reagent[49]. However, the determination of the phenolic components in wine by derivatization-SPME has yet to be established.

The application of microwave energy in promoting silylation reactions has been developed in our previous work[29]. Six antioxidatively active phenolic components in wines and fruits were used in the model study. The extracted solution from SPE was evaporated to dryness on a rotary evaporator followed by further drying under microwave irradiation (600 W, 30 s). The resultant residue was dissolved in pyridine and treated with *bis*(trimethylsilyl)acetamide (BSA) while irradiated with microwave using high power for 30 s.

With the end of monopoly of wine and tobacco by the government, private wineries have flourished in Taiwan recently producing a very wide range of different wines. A lot of them are claiming better anti-oxidative effects of their products over their competitors' ones. Consequently, there is an urgent need for a quick and reliable method for analyzing these wine products to guard customers' rights. Inspired by this need of a rapid and practical analytical method for the isolation and identification of the phenolic components in wine, we have studied the on-fiber derivatization- SPME technique for the isolation and analysis of these compounds with significant saving on sampling time and high sensitivity for minor components in complex matrix.

2. Experimental

2.1. Materials

Gentisic acid (GeA), caffeic acid (CaA), ferulic acid (FeA), *p*-coumaric acid (*p*-CoA), and the derivatization agent bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Aldrich (Steinheim, Germany). All HPLC-grade organic solvents, hydrochloric acid, and sodium chloride were purchased from Merck (Darmstadt, Germany). A manual SPME fiber holder and two types of SPME fibers, polyacrylate (PA, 85 μm) and StableFlex

polydimethylsiloxane-divinylbenzene (PDMS-DVB, 65 μm), were purchase from Supelco (Bellefonte, PA, USA).

2.2. Analytical procedure

2.2.1. Solution preparation

Standard stock solutions (1000 μg mL^{-1}) of each phenolic acid were prepared in ethanol and were stored in a refrigerator.   Working solutions were prepared by mixing each of the standard stock solutions with ultra pure water.   The pH of solution was adjusted to below 2.0 with 1 M hydrochloric acid and saturated with sodium chloride (0.3 g mL^{-1}).

2.2.2. SPME

A 3.0 mL sample solution in a 4 mL vial was extracted by direct immersion of SPME fiber using a magnetic stirrer provided constant agitation during extraction.   The extraction was carried out at room temperature for 60 min.   After the extraction is completed, the fiber was dipped into ultra pure water with stirring for 20 sec.   Then any trace of water on the fiber was wiped out by a soft tissue.

2.2.2. On-fiber silylation

Following the extraction step, the SPME needle was pierced through the Teflon-backed silicone septum into the headspace of a 2 mL vial containing 20 μL of BSTFA with a magnetic stir bar, partially embedded in a sand bath at 60 °C, and the fiber was exposed in the headspace for derivatization for 20 min.   After derivatization, the SPME fiber was inserted into the GC injection port to achieve the thermal desorption.

2.3. GC/MS parameters

Gas chromatography-mass spectrometry was performed on a Hewlett-Packard 6890 gas chromatograph, interfaced to a HP 5973 MSD.   Gas chromatographic separation was conducted using a DB-5MS capillary column (30 m x 0.25 mm i.d., 0.25 μm film thickness) in splitless injection mode.   Carrier gas was He (purity 99.995%) at 1.0 mL/min flow rate. The initial oven temperature was 80°C, held for 1 min, the temperature was raised to 240°C at

a rate of 15°C/min, held for 5 min, then the temperature was raised to 280°C at a rate of 20°C/min, and finally, held for 5 min, the total elution time was 23.67 min. The injection-port was set to 280°C. For SPME analysis a Supleco 0.75 mm i.d. GC inlet liner was used. SPME samples were injected by exposing the fiber in the hot injector of GC for 4 min and the chromatogram was then acquired.

3. Results and Discussion

3.1. Microextraction conditions

Four antioxidatively active phenolic components of wines and fruits, namely gentisic acid, caffeic acid, ferulic acid and p-coumaric acid were used in the model study. Several variables have been examined to determine their roles in extracting phenolic components: the duration of extraction, addition of salt, solution pH, and the adsorption capability of different fibers, such as polyacrylate (PA) fiber and polydimethylsiloxane-divinylbenzene (PDMS-DVB).

3.1.1 Extraction time

For the isolation of the phenolic compounds from the sample matrix, the sample solution was placed in a small vial (4 mL) and then the SPME fiber was immersed in the aqueous solution with stirring for a period of time for completing the extraction procedure. Fig. 1 showed the results of peak area versus extraction time up to 2 h on a standard solution with concentration of 10 $\mu g \ mL^{-1}$ for each of the phenolics. Except for gentisic acid, the extraction of the phenolic acids approached to equilibria after 60 min. Therefore, 60 min was chosen as the optimum extraction time.
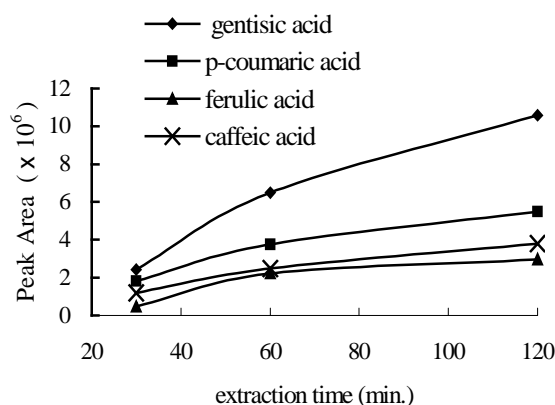

Fig. 1. Extraction efficiency as a function of time at room temperature; Samples were adjusted to pH < 2 and 300 mg of sodium chloride per mL added.

3.1.2 Sample pH and addition of sodium chloride

The effect of sample pH on extraction was examined at two different levels between pH < 2 and 3.88 (The pH of the mixed standard solution without adjustment).   In general, higher extraction efficiency is realized while the sample pH is maintained below the pK_a of the phenolic analytes.   As shown in Fig. 2, at low pH, significant improvement in the peak area was achieved for gentisic acid as compared to almost no analyte extracted at pH 3.88.   The peak area for the other three phenolic acids changed very little at the two different pH.   The reason for this observation is because of the pK_a of gentisic acid is 2.95, however the pK_a of caffeic acid, ferulic acid and p-coumaric acid are above 4.0.   Therefore, acidification of the sample solution with 1 M HCl is preferred for further experiments.
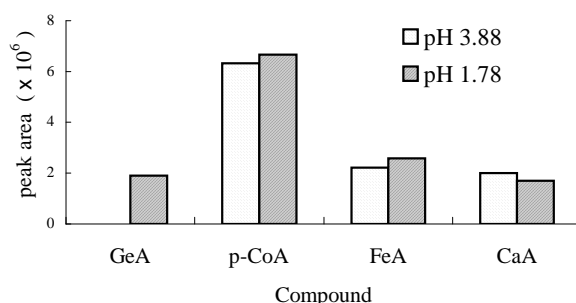

Fig. 2. Comparison of SPME responses obtained at pH 3.88 and 1.78 with salt addition.

Addition of sodium chloride (300 mg/ml) to increase the ionic strength of the solution was investigated.   As shown in Fig. 3, an obvious increase in the peak areas of most of the analytes were produced, the salting out effect decreased the solubility of the analytes in solution and caused an improvement in sensitivity.   To reduce the interference of further derivatization with salt on the surface of fiber, the fiber was exposed in pure water for 20 sec after extraction.
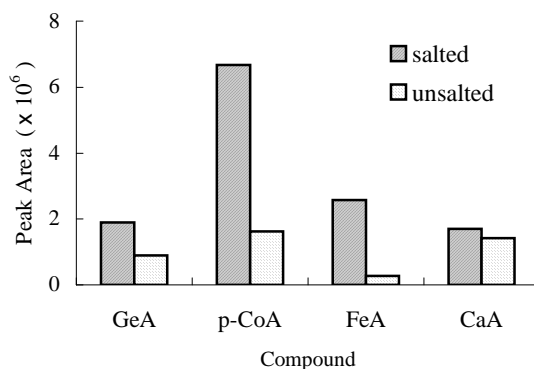

Fig. 3. Effect of addition of sodium chloride on peak areas at pH 1.78.

3.1.3 Fiber selection

Two different fibers were evaluated using the optimal sampling conditions (Section *3.1.1and 3.1.2*) to determine which fiber most effectively extracted phenolic acid compounds from sample solutions.   The results of the fiber screening were shown in Fig. 3. Higher extraction efficiency was obtained by the relatively polar PA coating for the polar phenolic acid compounds.   However, under this condition the PDMS/DVB fiber had an extremely low sorption capacity.   Hence the PA fiber was selected for further studies.
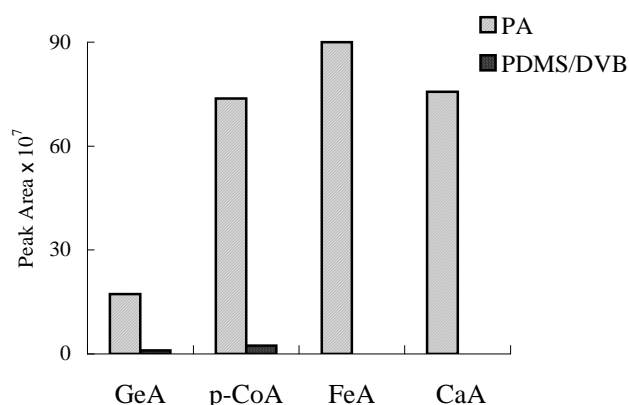

Fig. 4. Comparison of extraction efficiency obtained with two different fibers.

Consequently, on account of the foregoing experiments, the SPME conditions were selected as follows.   The pH of the sample solution was first adjusted to pH $<$ 2 by the addition of 1 *M* hydrochloric acid, and 0.9 g of sodium chloride was added to 3.0 mL of the acidified sample solution which was then extracted on polyacrylate (PA) fiber for 60 min at room temperature.

3.2. On-fiber derivatization conditions

In general, three different approaches have been utilized for the derivatization on solid phases[48], namely, in the sample matrix, on the fiber (after sampling), and in the GC injection port.

Since direct immersion of the fiber in the organic derivatizing agents will cause the damage of the coating on the fiber, an on-fiber headspace solid-phase microextraction derivatization procedure has been explored in order to prolong the life of the fiber.   Several variables have been examined to determine their roles in converting the polar phenolic components into more volatile analytes: the derivatization time and temperature, and the

amount of derivatizing reagent were studied.

3.2.1. Derivatization time and temperature

The effects of BSTFA derivatization time and temperature were examined. While the reaction was carried out at 80℃ for 10 min and 60℃ for 20 min, the peak area counts of the TMS derivatives reached the highest amount. However, the BSTFA will damage the fiber coating at elevated silylation temperature e.g. above 65°C. Hence, the derivatization condition was selected at 60℃ for 20 min.

3.2.2. Amount of derivatizing reagent

The influence of derivatizing reagents amount on the peak area for each selected phenolic acid was estimated. Sine the results of 20 μL and 60 μL of BSTFA were not significantly different, 20 μL of BSTFA was chosen to reduce the quantity of organic reagent used.

4. Conclusions

A simple, efficient, and highly selective method for the rapid determination of the phenolics in wine utilizing SPME and on-fiber derivatization procedure has been established.

Due to savings of the analysis time, elimination of solvent, and the advantage of avoiding possible contaminants from sample matrix, this method we have explored will find its applications not only in academic laboratories, but also will be a very useful and practical method in medical and food industrial routine analysis. Especially, it will serve the booming wine industry in Taiwan for providing reliable scientific data on the amounts of the antioxidants in and to support their claims for the quality of their products.

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