Solid Phase Microextraction with On-fiber Derivatization for the Determination of trans-Resveratrol in Iowa Red Wines

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Abstract
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Disciplines
Bioresource and Agricultural Engineering | Civil and Environmental Engineering | Food Science | Human and Clinical Nutrition

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Introduction

Resveratrol (3,4’-5-trihydroxystilbene, C_{14}H_{12}O_{3}) is a phytoalexin produced by plants in response to fungal infection (Delmas et al., 2006) as well as a variety of stress conditions, such as vicissitudes in climates, exposure to ozone, sunlight and heavy metal ions (Bavaresco et al., 2003). Recently resveratrol has attracted considerable scientific attention due to its beneficial effects on human health revealed by biological and clinical studies, such as the antioxidative and anti-inflammatory effects (Athar et al., 2007), inhibition of human low-density lipoprotein oxidation (Frankel et al., 1993; Teissedre et al., 1996), platelet aggregation (Schramm et al., 1997), and the growth of a variety of cancer cells (Athar et al., 2007).

Resveratrol has been detected from many plant species (Soleas et al., 1997), however, grapes and related products are considered the most important dietary sources of it (Goldberg et al., 1995; Mattivi et al., 1995). Resveratrol is synthesized and located especially in the grape skin but not in the fruit flesh (Creasy et al., 1988). It is not surprising that the resveratrol content in red wines is much higher than in white wines (Siemman et al., 1992), regardless of winemaking techniques. Resveratrol exists in wine in two isomers, trans- and cis-. Trans-resveratrol has being widely studied, although cis-isomer may also possess health promoting properties (Bertelli et al., 1996). Cis-resveratrol is not a natural constituent of grape, however, cis-resveratrol has been detected in almost all wines analyzed so far, it is likely that cis-resveratrol derived from its trans isomer during vinification (Jeandet et al., 1991).

There were various method developed for detection of resveratrol from wine. Sample preparation step is usually required prior to conduct the chromatographic separation due to the complex nature of the wine matrix, such as liquid-liquid extraction (Siemman et al., 1992) and solid phase extraction (Mattivi et al., 1995). However, the conventional sample preparation procedures are time-consuming, labor-intensive and multi-stage operations and required the utility of organic solvent and the large sample volume. SPME integrates sampling, extraction, concentration and sample introduction into a single solvent-free step (Kataoka et al., 2000). Recently, a few methods based on SPME were developed. Luan et al. proposed the method by employing SPME with BSTFA on fiber derivatization and coupled with GC-MS for the analysis of trans-resveratrol in wine (Luan et al., 2005). The method of the combination of SPME-on-fiber derivatization with GC-MS and the comprehensive two-dimensional GC×GC –FID for the determination of trans- resveratrol in wines was also developed by Shao et al. (Shao et al., 2003).

High performance liquid chromatograph (HPLC) is the most commonly used method coupled with UV detection (Goldberg et al., 1995) and coupled with mass spectrometry (Wang et al., 2002). However, the high resolution and great sensitivity make the GC method very attractive for the identification and quantification of resveratrol isomers in wines. The analysis times for methods using GC are much shorter than for those using LC. Most of GC methods have required derivatization with bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) prior to column separation detected by flame ionization detector or mass-spectrometry (Goldberg, et al., 1994).

The objective of this study was to the development and validation of an analytical method based on SPME and further multidimensional gas chromatographic analysis for the determination of trans-resveratrol in Iowa red wine.
Materials and Methods

Standard and solutions

The trans-resveratrol standard (3,4',5-trihydroxystilene, 99% GC) and bis(trimethylsilyl)-trifluoroacetamide (BSTFA, contained 1% trimethylchlorosilane) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. HPLC grade methanol was also obtained from Sigma-Aldrich. Cis-resveratrol was prepared from the trans-isomer by UV irradiation (2 hrs at 254 nm) and it was used for qualitative assessment only (Soleas, et al., 1995). The stock solution of 3 mg/mL trans-resveratrol was prepared by dissolving 0.03 g trans-resveratrol standard in 10 mL volumetric flask. The stock solution was sealed with parafilm and covered with aluminum foil to keep it from light and stored at 4 ºC until use. Standard solutions used in further studies were prepared fresh by diluting different amounts of the standard solution with pure water to the required concentrations. Ultrapure-grade water from a high purity water system (Culligan Water Conditioning, Lexington, KY, USA) with conductivity 18MΩ was used for developing the calibration curve. The external calibration standard solutions ranged from 10 ng/L to 5 mg/L and were made by dilution of the stock solutions in water using optimized DI-SPME conditions.

Multidimensional GC-MS system

Multidimensional GC-MS-olfactometry (MDGC-O) system (Microanalytics, Round Rock, TX, USA) built on a 6890N GC / 5973 MS platform (Agilent Inc., Wilmington, DE, USA) were used for all analyses. This system allows for the simultaneous identification and analysis of chemicals and corresponding odors. In this study, we only utilized the system for the chemical analysis. The system was equipped with two columns in series connected by a Dean’s switch. The non-polar pre-column was 12 m, 0.53 mm i.d.; film thickness, 1 µm with 5% phenyl methylpolysiloxane stationary phase (SGE BP5) and operated with constant pressure mode at 8.5 psi. The medium polar analytical column was a 30 m × 0.53 mm fused silica capillary column coated with 50% phenyl methylpolysiloxane stationary phase (SGE BP50) at a film thickness of 1 µm. The column pressure was constant at 5.8 psi. Both columns were connected in series. System automation and data acquisition software were MultiTrax™ V. 6.00 (Microanalytics, Round Rock, TX, USA) and ChemStation™ (Agilent, Santa Clara, CA, USA). The general run parameters used were as follows: injector, 280 °C; FID, 280 °C, column, 150 °C initial, 10 °C/ min, 300 °C final, 10 min hold; carrier gas, GC-grade helium. The GC was operated in a constant pressure mode where the mid-point pressure, i.e., pressure between pre-column and column, was always at 5.8 psi and the heart-cut sweep pressure was 5.0 psi. The FID connected to the pre-column was maintained at 280 °C with a H₂ flow rate of 35 mL/min, an air flow rate of 350 mL/min, and the makeup N₂ flow rate of 10 mL/min. The FID data acquisition rate was 20 Hz. Mass to charge ratio (m/z) range was set between 50 and 550. The MS was operated in the electron impact (EI) ionization mode with electron energy of 70eV. The MS ion source and mass filter temperature were held at 230 and 150 °C, respectively. Spectra were collected at 6 scans/sec and electron multiplier voltage was set to 1800 V. The selected ion monitoring mode of MS was normally chosen in quantitative trace analyses. The most abundant ion was generally monitored and quantified and the specific ion was used as the confirmed ion. Mass channels were m/z = 443, 444 and 445 for trans-resveratrol derivative with 50 ms dwell times. Ion m/z =444 was used for quantification for trans-resveratrol. The MS detector was auto-tuned every day. The solvent delay was set to 5 min to minimize the baseline shifting after a derivatizing reagent peak. The simultaneous acquisition of full scan and SIM mode was used for the same chromatographic run which allows for analyte confirmation and unknown identification while retaining the sensitivity and selectivity of target compound analysis by SIM. This helps to reduce the reporting of false positive results as the full scan data can be used to
confirm identity using library search techniques and gives the ability to have complimentary low level quantitative and qualitative data from the same injection.

MDGC equipped with a heart-cut valve and cryogenic focusing capacity and coupled with a mass spectrometric detector was made for a capable unit and permitted separation and identification of most compounds. Heart-cut valve based on Dean’s switch concept was located between the pre-column and analytical column. In such a dual column system, the heart-cut valve and cryogenical system was used to transfer and focus specific pre-separated retention regions with the compounds of our interest from the pre-column (and the entire sample matrix) to the analytical column. The heart-cut effluent was cryogenically focused onto the head of the second column by using a feature of this instrument that containing a spray nozzle that utilized liquid CO2 to provide the additional peak separation.

**Analytical procedure**

The manual SPME holder and three different SPME fibers including 100 µm polydimethylsiloxane (PDMS), 85 µm polyacrylate (PA) and 65 µm polymethylsiloxane/divinyl benzene (PDMS/DVB) were purchased from Supelco. The new fibers were conditioned before the first use according to the manufacture’s instruction.

Direct immersion extraction was carried out for the extraction of resveratrol from standard solution or wine samples. A certain volume of the standard solution were added into 4mL amber sample vials (from Supelco) sealed with PTFE coated silicon septum and prefilled with a stir bar (12.7 mm × 3.2 mm, Fisher Scientific, Pittsburgh, PA, USA) and with 3 mL of pure water. Under the optimized extraction condition, PA SPME fiber was exposed into the stirred liquid sample (500 rpm) for 30 min at room temperature. After finishing the extraction step, the SPME fiber was exposed in the headspace of 4 mL vial prefilled with 5 µLBSTFA, where the resveratrol absorbed on the fiber was immediately derivatized with the BSTFA (1% TMCS) vapor arose from the bottom of the vial. After 20 min of derivatization, the fiber was retracted into the needle, pulled out from the vial and immediately inserted into the GC injection port at 280 ºC for 10 min. Wine samples were analyzed by following the similar procedure described above.

**Repeatability of the analytical method and Method detection limit**

The repeatability was calculated at different levels of concentration: 10, 100 ng/L, 1, 10, 100 µg/L and 1, 5 mg/L. All the concentrations were conducted 3 replicates except for 10 ng/L was 10 replicates. Data were analyzed and compared using means and relative standard deviation (RSD). The U.S. Environmental Protection Agency (USEPA) methodology for estimation of method detection limits (MDLs) was used (28). The MDLs were defined as the minimum concentration of a substance that can be measured and reported with 99% confidence when the analyte concentration is greater than zero and is determined from analysis of a sample in a given matrix containing the analyte (28). The MDLs for trans-resveratrol was estimated using equation 1:

\[
\text{MDL} = s \times t \left( n-1, 1-\alpha = 0.99 \right)
\]

where:

- n = number of replicate spike determinations at 1 to 5 times the estimated MDL,
- s = standard deviation of measured concentrations of n spike determinations,
- t = Student’s t value at n-1 degree of freedom and 1-\alpha (99%) confidence level.

When n = 10 and α = 0.01, then t = 2.821, and α = level of significance.

**Wine Samples**

Six Iowa red wine samples were obtained from the cooperating wineries. All of the collected samples were in the original bottles and were refrigerated until the time of analysis.
Results and Discussion

Figure 1 illustrated the procedure of on 85 µm polyacrylate SPME fiber derivatization. SPME fiber was first directly exposed in the red wine in order to adsorb/isolate high polar and less volatile compound-resveratrol from red wine. After sampling, SPME fiber was exposed in a sealed HS vial to the derivatizing reagent in the vapor phase for a given time. Then the SPME fiber loaded with resveratrol derivatives were analyzed by multidimensional GC-MS.

I. Fiber is immersed  
II. Fiber is exposed to vapor of derivatizing reagent.  
III. Fiber is desorbed in splitless GC inlet.

Figure 1 Direct immersion SPME extraction of trans-resveratrol in red wine, on-fiber derivatization and analysis of trans-resveratrol derivatives with MDGC-MS.

In this study, multidimensional GC-MS was employed for separation and quantification of resveratrol from red wine. The dual-column GC system equipped with a hear-cut valve and cryogenic system allows transferring and focusing a portion of the sample from the first column to the second column for further separation. Only the selected time region of samples, cut to the second column and freeze-trapped, is allowed to elute with prior and post cut sample portions sent to waste.

Figure 2 Separations of cis- and trans-resveratrol in GC-FID-O mode with no heart-cut. Chromatogram (FID) for the red wine sample and collected with 85 µm Polyacrylate SPME fiber.
Figure 3. Separations in MDGC-MS-O mode with cryotrap and heart-cut between pre-column and analytical column: comparison of the FID chromatogram, total ion chromatogram and selected ion chromatogram isolating trans- and cis-resveratrol from red wine samples with direct immersion SPME-MDGC-MS. Cryotrap range: 10.10 min-13.40 min; Heart-cut time range: 10.20-13.40 min;

Figure 2 shows the typical FID chromatogram of resveratrol derivatives separation from wine under the first column GC condition, which the entire sample passed through the first column to FID with neither heart-cut nor cryotrapping. As can be seen, trans-resveratrol peak eluted tightly with the neighbor peaks with relative high background and it did not achieve completely the baseline separation. Figure 2 shows FID, total ion and selected ion chromatogram of resveratrol from red wine samples by MDGC with two hear-cuts and cryotrapping for isolation of cis- and trans- resveratrol, respectively. Precise heart-cut times of two resveratrol isomers were determined by injecting reference standards, ensuring that only the transfer of resveratrol
isomers and overlapping matrix components were delivered to the second column and detected by MSD. Figure 3 shows the primary separation resulting from the transfer of the heart-cuts and cryogenically being focused onto the head of the second column to provide additional peak separation. It also shows the total ion and selected ion chromatograms for resveratrol on the second column. As it observed, there is no other interference peaks but only resveratrol isomers with clean mass spectrum and low background. This clearly demonstrated the heart-cut MDGC is a powerful approach to improve the separation of selected region of the first column separation.

The calibration curve of quantification trans-resveratrol in wine based on direct immersion SPME extraction, on-fiber derivatization coupled with MDGC-MS was developed. The linear regression equation is as follows: \( y = 52973x - 727040 \), where \( y \) and \( x \) are the peak area counts and the concentrations of standard trans-resveratrol solutions, respectively. The correlation coefficient is 0.9998 and linearity ranged from 10 ng/L to 5 mg/L.

The MDLs were estimated based on the experiment with 10 replicate DI-SPME extractions of trans-Resveratrol at the lowest concentration (10 ng/L) of linearity range using equation 1. The MDL for trans-Resveratrol in this study is 2.85 ng/L. The concentrations of trans-resveratrol were determined in six Iowa red wine. There was a broad range in the concentration of resveratrol in the different wines analyzed from 881.4 µg/L to 12.7 µg/L. Within a variety, vintage, there were also large differences. Winemaking techniques probably make an important contribution to the resveratrol content of wine.

**Conclusions**

Heart-cut multidimensional GC is a powerful approach to improve the separation of selected portions of the primary separation to a secondary column for further analysis. MDGC can provide great peak capacity for the selected region.

1. Multidimensional GC-MS is a useful and practical tool for the determination of resveratrol in wine.
2. SPME coupled with MDGC/MS method presented in this study is superior in terms of sensitivity of resveratrol to all previously published methods. With heart-cut and cryotrap, we are able to quantitate trans-resveratrol as low as 2.85 ng/L.
3. Method described can be used to determine both trans- and cis-resveratrol. This study focused on more interesting trans– form. However, trans to cis ratio was investigated in this study. The average is 3.01, trans- isomer is predominated in most of the Iowa red wine samples investigated in this study.
4. The linearity was excellent up to concentrations of 5 mg/L.
5. Method could also be used for resveratrol analyses in grape juice, jams and jellies.
6. This is the first attempt concerning the concentration of trans-resveratrol in Iowa varietal red wines.
7. In agreement with some previous research, there was a considerable variability in resveratrol concentrations even in wines produced by the same grape variety which is not unexpected since a number of factors such as climate, geographical area of cultivation, growing conditions, wine-making techniques and storage conditions affect resveratrol content of wines.
8. Winemaking techniques may have important effects on the resveratrol content of wine. Further studies are needed to investigate the contribution of winemaking procedures to resveratrol content in wine.
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