

1 Rapid determination of *trans*-resveratrol in red wine by
2 solid-phase microextraction with on-fiber derivatization
3 and multidimensional gas chromatography – mass
4 spectrometry

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Abstract

There has been considerable public interest and a growing number of scientific studies linking certain phenolic compounds in grapes and wines particularly *trans*-resveratrol (TRA) to human health benefits. Typical TRA (*trans*-3,5,4'-trihydroxystilbene) concentrations in wine are very low. It is a polar compound with very low volatility, which makes it difficult to extract and to separate on a gas chromatography (GC) column without derivatization. In this study, a new method for trace analysis of TRA was developed using solid-phase microextraction (SPME) with on-fiber silylation derivatization. Multidimensional GC equipped with a heartcut valve and cryogenic focusing was coupled with a MSD and used for improved separations and analysis. The effects of SPME fiber selection, extraction time, temperature, and desorption time were investigated. The derivatization conditions, time/temperature and the volume of derivatization reagent were also optimized. The calibration curve was linear over the concentration range from 10 ng L⁻¹ to 5 mg L⁻¹, with a correlation coefficient of 0.9996 ~~0.9998~~. The average recovery of TRA in red wine was 83.6%±5.6% ~~91.7%±7.1%~~. The method detection limit (MDL) for TRA in 12.5% (v/v) ethanol:water solution in this study was 7.08 ng L⁻¹ whereas the MDL for TRA in pure water was 2.85 ng L⁻¹. ~~The method detection limit for TRA was 2.85 ng L⁻¹. The new method is superior in terms of sensitivity for TRA to all previously published methods.~~ The new method was used to test the TRA content in six selected Iowa red wine samples. Measured concentrations varied from 12.72 to 851.9 µg L⁻¹ ~~12.7 to 881.4 µg L⁻¹~~.

Keywords: *trans*-Resveratrol, *cis*-Resveratrol, ~~*Trans*-resveratrol, *Cis*-resveratrol~~, Solid-phase microextraction, Silylation, Multidimensional gas chromatography-mass spectrometry, Heartcut, Cryotrap, Red wine

1. Introduction

Resveratrol (3, 5, 4'-trihydroxystilbene, C₁₄H₁₂O₃) is a phytoalexin produced by plants in response to fungal infection [1] as well as to a variety of stress conditions, such as vicissitudes in climates, exposure to ozone, sunlight and heavy metal ions in soil [2]. Recently, resveratrol has attracted considerable public and scientific attention due to its beneficial effects on human health revealed by biological and clinical studies. These benefits include the antioxidative and anti-inflammatory effects [3], inhibition of human low-density lipoprotein oxidation [4,5], platelet aggregation [6], and the inhibition of the growth of a variety of cancer cells [3].

Resveratrol has been detected in many plant species [4]. ~~[7]~~. The main commercial source of resveratrol is the Japanese knotwood (*Fallopia japonica*) and Giant or Sakhalin knotwood (*Fallopia sachalinensis*). *Peanuts are another dietary source of resveratrol* [5]. However, grapes and grape products are considered the most important dietary sources [6] ~~[8,9]~~. Resveratrol is synthesized and concentrated especially in the grape skin, but not in the fruit flesh [7]. ~~[10,11]~~. Red wines are produced by fermenting grapes on skins as opposed to fermentation without grape skins that is typical for white wines. Thus, it is not surprising that the resveratrol content in red wines is much higher than in white wines [8], ~~[12-18]~~, regardless of winemaking techniques. Resveratrol exists in wine in two isomers, *trans*- and *cis*-. *trans*-Resveratrol ~~*Trans*-resveratrol~~ (TRA) has

69 been widely studied, although *cis*- isomer may also possess health-promoting properties
 70 [9]. ~~[19].~~ ~~*cis*-Resveratrol~~ ~~Cis-resveratrol~~ (CRA) is not a natural constituent of grape.
 71 However, since CRA has been detected in almost all wines analyzed so far, it is likely
 72 that CRA is derived from its *trans*- isomer during the wine-making process, storage in the
 73 bottle, or during analysis [10, 11]. ~~[20-22].~~ Resveratrol is becoming an important quality
 74 indicator of red wine and dietary supplements and it is possible that the TRA
 75 concentration could be used for marketing of wine and food products. Also, the
 76 knowledge of the TRA concentrations in wines could aid the optimization of viticultural
 77 practices and enological techniques targeting TRA level improvements [12] ~~[9,22-25].~~
 78 Interest in resveratrol have led to the development of various analytical methods
 79 for its measurement in wine. Methods developed for detection of resveratrol are mainly
 80 suitable for the analyses of the *trans*-form. Fewer methods are applicable for
 81 determination of both isomers. Sample preparation, such as liquid-liquid extraction
 82 ~~[12,21,22]~~ and solid-phase extraction ~~[9,25-28]~~ is usually required prior to the
 83 chromatographic separation due to the complex nature of the wine matrix. However,
 84 these conventional sample preparation procedures are time-consuming, labor-intensive
 85 and multi-stage operations and require the use of organic solvent and large sample
 86 volumes. As an alternative, solid-phase microextraction (SPME) integrates sampling,
 87 extraction, concentration and sample introduction into a single solvent-free step. The use
 88 of SPME results in a number of advantages by simplifying sample preparation, increasing
 89 reliability, selectivity, sensitivity and reducing the cost and time of analysis [13]. ~~[29].~~
 90 Recently, several methods for determination of TRA in wine based on SPME
 91 were developed. Luan et al. proposed the method using SPME with

bis(trimethylsilyl)trifluoroacetamide (BSTFA) on-fiber derivatization coupled with GC-MS for the analysis of TRA in wine [14], [30]. A linear concentration range over 10 ng L⁻¹ to 1 mg L⁻¹ with a detection limit of 5 ng L⁻¹ of TRA was reported, which is about 2000-times lower than that reported by Soleas et al., [12] [25] for the solid phase extraction (SPE) method. Shao et al., [15] [34] developed another method combining SPME-on-fiber derivatization with GC-MS and ~~the~~ comprehensive two-dimensional GC×GC – flame ionization detector (FID) for the determination of TRA in wines. Shao et al., [15] used two different derivatization reagents including acetic anhydride and BSTFA. The linearity range of TRA of the developed method based on acetic anhydride was 0.02 ~ 2 mg L⁻¹ without specifically reporting MDLs. Shao et al., [15] reported TRA content of five Australian red wine samples.

High-performance liquid chromatography (HPLC) coupled with UV detection [16], [15,18,32,33], fluorescence detection [17], [34,35], electrochemical detection [18] [36] and with mass spectrometry [19] [37,38,39] can also be used to quantify resveratrol in wine. The lowest method detection limit for HPLC method was about 0.1 µg L⁻¹ [17] [35] without derivatization. Capillary electrophoresis (CE) has been used in several investigations for the determination of resveratrol as well [20]. [40–42]. Typical CE-based methods for measuring resveratrol in wine could detect resveratrol at 45 to 228 µg L⁻¹ [20]. [42]. However, high resolution and very good sensitivity make the GC method with derivatization very attractive for the identification and quantification of resveratrol isomers in wines. The analysis times for methods using GC are typically much shorter than for those using LC. Most of GC-based methods require derivatization with BSTFA prior to separation and detection by ~~on~~ a FID or a MSD [14, 15]. [26,30,31,43,44].

However, compared with conventional single-column GC separations, additional selectivity can be provided by multidimensional gas chromatography (MDGC)–MS.

To date, two main types of MDGC: conventional MDGC with a Dean's switch and heartcut capability and the comprehensive two-dimensional GC×GC have been used. For high-throughput applications, comprehensive GC×GC is likely to be a better choice for separation since it gives a greater peak capacity and is less time consuming (i.e., one run can provide a complete chromatogram of the entire sample). However, in applications where a specific compound is of interest, conventional MDGC could be more useful. The conventional MDGC techniques have already been used in areas such as environmental analysis [21], [45] biochemical studies [22], [46–48], food science [23], [49,50], wine industry [24] [51] and livestock, poultry, and insect odors [25–29]. [52–55].

The objective of this study was the development and validation of an analytical method based on on-fiber derivatization SPME and multidimensional gas chromatographic analysis for the determination of *trans*-resveratrol in selected Iowa red wines.

2. Materials and Methods

2.1. Standard and solutions

The TRA standard (*trans*-3,5,4'-trihydroxystilbene, 99% GC-grade) and BSTFA (containing 1% trimethylchlorosilane) were purchased from Sigma-Aldrich (St. Louis, MO, USA) and used without further purification. Ethanol Methanol (HPLC-grade) was also obtained from Sigma-Aldrich. *cis*-Resveratrol was prepared from the *trans*- isomer by UV irradiation (2 h hrs at 254 nm) and it was used for qualitative assessment only [43].

The stock solution of 3 mg mL⁻¹ TRA was prepared by dissolving 0.03 g TRA standard in 10 mL of ethanol in a volumetric flask. The stock solution was sealed with Parafilm, covered with aluminum foil, and stored in the dark at 4 °C until use. Standard solutions used for the optimized SPME extraction conditions were prepared freshly by diluting different amounts of stock solution with pure water to the required concentrations. Ultrapure-grade water from a high purity water system (Culligan Water Conditioning, Lexington, KY, USA) with 18 MΩ·cm resistivity was used for developing the calibration curve. The external calibration standard solutions ranged from 10 ng L⁻¹ to 5 mg L⁻¹ and were made by dilution of the stock solutions in 12.5% (v/v) ethanol:water solution using optimized direct SPME immersion conditions.

2.2 Multidimensional GC-MS system

A multidimensional GC-MS-olfactometry (MDGC-O) system (Microanalytics, Round Rock, TX, USA) built on a 6890 GC / 5973N MS platform (Agilent Inc., Wilmington, DE, USA) was 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., 1 μm film thickness, with 5% phenyl methylpolysiloxane stationary phase (SGE BP5). The medium-polarity analytical column was a 30 m × 0.53 mm fused silica capillary column coated with 50% phenyl methylpolysiloxane stationary phase (SGE BP50) with film thickness of 1 μm. 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 heartcut sweep pressure was 5.0 psi. System automation and data acquisition software were MultiTrax™ V. 6.00 (Microanalytics, Round Rock, TX, USA) and ChemStation (Agilent, Santa Clara, CA, USA), respectively.

The general run parameters used were as follows: injector, 280 °C; FID, 280 °C, column, 150 °C initial, 10 °C min⁻¹, 300 °C final, 10 min hold; carrier gas, GC-grade helium. The FID system connected to the pre-column was maintained at 280 °C with a H₂ flow rate of 35 mL/min, an airflow 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 70 eV. The MS ion source and mass filter temperature were held at 230 and 150 °C, respectively. Spectra were collected at 6 scans sec⁻¹ and electron multiplier voltage was set to 1800 V.

The selected ion monitoring mode (SIM) of MS was chosen for quantitative trace analyses. The most abundant ion was generally monitored and quantified while the specific ions were used for confirmation. Mass channels were m/z = 443, 444 and 445 for the TRA derivative with 50 ms dwell times. Ion m/z = 444 was used for the quantification of TRA. The MS detector was auto-tuned every day. The solvent delay was set to 5 min to minimize the baseline shifting after the elution of the derivatizing reagent peak. The simultaneous acquisition of full scan and SIM mode was used. This allowed for analyte confirmation and identification of unknowns while retaining the sensitivity and selectivity of target compound analysis by the SIM. Simultaneous SIM and full scan reduced reporting of false positive results. The full-scan data were used to

confirm analyte identity using library search techniques and enabled complimentary low level quantitative and qualitative data analysis from the same injection.

The MDGC equipped with a heartcut valve and cryogenic focusing extends the separation power on a single GC column. The heartcut valve based on Dean's switch concept was located between the pre-column and analytical column. In such a dual column system, the heartcut valve and cryogenic cooling system was used to transfer and focus specific pre-separated GC retention time regions with the target compounds from the pre-column (and the entire sample matrix) to the analytical column. Transfer of only selected compounds to the analytical column was done to improve the quality and sensitivity of chemical analyses by reducing the background from the sample matrix [27]. [54]. The heartcut effluent was cryogenically focused onto the head of the analytical column by using a spray nozzle with liquid CO₂ to provide additional peak separation. The cryotrap was cooling the short section of the outside of the front of analytical column and was maintained at -40 °C when the cryotrap was activated.

2.3 Analytical procedure

The manual SPME holder and three different SPME fibers including 100 µm polydimethylsiloxane (PDMS), 85 µm polyacrylate (PA) and 65 µm polymethylsiloxane/divinylbenzene (PDMS/DVB) were purchased from Supelco (Bellefonte, PA, USA). New fibers were conditioned before the first use according to the manufacturer's instructions. Direct immersion extraction was carried out for the sampling of TRA from standard solution and from wine samples. A certain volume of the standard solution was added into 4 mL amber sample vials (from Supelco) sealed with a PTFE-

coated silicon septum, phenolic screw-caps and prefilled with a PTFE-coated stir bar (12.7 mm × 3.2 mm, Fisher Scientific, Pittsburgh, PA, USA) and with 3 mL of pure water.

The effects of several parameters on the efficiency of the microextraction was investigated using spiked aliquots of working solution in pure water (at 10 µg L⁻¹ level) and fixing the on-fiber derivatization conditions. Resveratrol with three hydroxyl groups is a very polar compound and it has a low vapor pressure (1.24·10⁻⁹ mmHg, at 25 °C; 1 mmHg = 133.322 Pa ~~1.24E-09 mm-Hg~~, at 25 °C) and has good water solubility (16.9 mg L⁻¹ at 25 °C) [29]. ~~[56]~~. Thus, the direct immersion sampling with SPME was carried out for the entire study. Sample agitation increased the rate of resveratrol extraction onto the fiber coating, and a constant rapid agitation speed of 500 rpm was applied for all the experiments in this study. ~~However, care must be taken with the direct immersion extractions of red wine. Wine is an acidic aqueous ethanol solution with aroma compounds where ethanol content is approximately 12.5%. Organic acids, colloids, polyphenols, mineral salts and sugars constitute about 2% of wine composition [33]. With direct immersion extractions, the PA SPME fiber was directly exposed in the liquid phase of red wine. Thus, the matrix of wine could build up sugar and colloid coating on the SPME fiber causing irreversible adsorption. As a result, these potentially interfering compounds could still be absorbed in the SPME fiber even after relatively long thermal desorption at the GC injection port. We observed that the color of the surface of PA fibers became gradually dark yellow/brown and fibers were significantly less efficient in extractions after ~30 injections. Thus, the SPME fiber was replaced after 30 direct extractions from red wine in this study.~~

Unless specified otherwise, all of the optimization experiments were performed in triplicate. For the final, optimized extraction conditions, the 85 μ m PA SPME fiber was immersed in the stirred liquid sample (at 500 rpm) for 30 min at room temperature.

2.4 Derivatizations of TRA and CRA

Resveratrol is a low vapor pressure and a very polar compound with the CRA less polar than the TRA. Derivatization can increase the volatility and/or reduce the polarity of some of analytes and therefore can improve extraction efficiency, selectivity and detection. There are three different derivatization procedures that are currently used in SPME including direct derivatization, derivatization on the SPME fiber and derivatization in the GC injection port [30]. ~~[57]~~ On-fiber SPME derivatization was used in this research. The BSTFA was employed as derivatizing reagent for resveratrol. On-fiber silylation was conducted after the direct extraction with SPME. Any residual drop of water attached to the fiber needle after direct extractions was removed by a soft tissue after completion of the extraction step. The SPME fiber with extracted compounds including resveratrol isomers was transferred to a sealed headspace of 4 mL vial where it was exposed to the derivatizing reagent in the vapor phase. The 4 mL vial was prefilled with 5 μ L BSTFA. Resveratrol absorbed on the SPME fiber was then derivatized with the BSTFA vapor that was at equilibrium in 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 $^{\circ}$ C for 10 min. A new vial containing a fresh aliquot of BSTFA was used in each experiment. Wine samples were analyzed with the similar procedure described above.

Compared to conventional SPE, the SPME with on-fiber derivatization has several advantages [31]. [58]. First, the relatively hydrophobic SPME fiber resists polar matrix interferences found in wine better than the silica-phase extraction. Also, on-fiber derivatization was conducted in the vapor of the derivatizing reagent instead of the pure liquid or a solution, which should favor desirable kinetics and regioselectivity for the derivatizing reaction. Finally, SPME with on-fiber derivatization eliminated the removal of the derivatizing reagent step that is needed with SPE. This, in turn, reduced a possible source of sample loss.

2.5 *Linearity, repeatability and the method detection limit of the analytical method*

The new method repeatability was estimated at seven different TRA concentrations prepared in 12.5% (v/v) ethanol:water solution: 10 and 100 ng L⁻¹, 1, 10, and 100 µg L⁻¹ and 1 and 5 mg L⁻¹. All tests were conducted using 3 replicates. The exception was the lowest concentration of 10 ng L⁻¹ in 12.5% (v/v) ethanol:water solution that was analyzed in 7 40 replicates for the estimation of method detection limits (MDLs). The MDL was also estimated at 10 ng L⁻¹ in pure water with 10 replicates. Data were analyzed and compared using means and relative standard deviations (RSDs). All analyses were based on manual SPME injections.

The US U.S. Environmental Protection Agency (USEPA) methodology for estimation of MDLs was used [32]. [59]. 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 [32]. ~~[59]~~. The MDLs for TRA was estimated using equation 1:

$$\text{MDL} = s \times t_{(n-1, 1-\alpha)} \quad (1)$$

where:

n = number of replicate spike determinations at 1 to 5 times greater than 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-α (equal to 99%) confidence level.

When n = 7 and 10, α (defined as the level of significance) = 0.01, then t = 2.821 for 7 replicates and t=3.14 for 10 replicates.

2.6 Wine samples

Six Iowa red wine samples were obtained from the cooperating local wineries. All of the collected samples were in the original marketed bottles and were refrigerated until the time of analysis. All wines were from 2006 vintage. The ethanol content was measured for selected Iowa red wines and the average ethanol content was 12.5% (v/v).

2.7 Method recovery assays

The recovery experiments were performed using spiked standard resveratrol solutions in red wine samples at three different concentrations, i.e., 1000 µg L⁻¹, 60 µg L⁻¹ and 10 µg L⁻¹. The spiked wine samples were then analyzed by following the optimized extraction method described above.

3. Results and Discussion

3.1 Isolation of resveratrol isomers with MDGC-MS system

It is well-known that wine is a very complex matrix where more than 680 constituents [33] [60] have been found belonging to different chemical groups of compounds with different polarities. Resveratrol is a very polar compound and it exists as *trans*- and *cis*- isomers, both present in wines. The polarity poses challenges for the separation and quantification of resveratrol isomers in the complex wine matrix. Figures 1 and 2 illustrate the use of multidimensional GC-MS for the separation and quantification of resveratrol in red wine. Figure 1 shows a typical FID chromatogram of resveratrol-BSTFA derivatives separated from red wine using only the first GC column. The entire sample was separated on the pre-column without heartcut or cryotrapping. However, the TRA-BSTFA-derivative was not completely separated from baseline and eluted tightly with the adjacent peaks and with relatively high background. Then, the multidimensional GC mode with the heartcut valve and cryogenic cooling was used for the transfer and focusing of the resveratrol-BSTFA derivatives from the pre-column to the analytical column for improved isolation and separation. Heartcuts were cryogenically focused at the front of the second column, which resulted in enhanced sensitivity and narrow peak widths (Figures 2A and 2B). The rest of the sample (i.e., prior and post heartcuts) were sent from the Dean's switch to the FID (Figure 2A). In this context, the ability to obtain cleaner mass spectrum and higher quality peaks for the selected region cut from the complex wine matrix was the goal. This approach is more 'mechanistic' (peaks are visually separated) compared with the comprehensive GCxGC where sophisticated software is needed to deconvolute separate peaks eluting from a

shorter second column and [resulting from](#) faster run-times. This intrinsic difference between separations is the key for achieving such low MDL for *trans*-resveratrol in this research. The same objective is more challenging to achieve with a shorter second column and faster run-times used in comprehensive GC×GC for the whole sample. Compared with comprehensive GC×GC [34], ~~[61]~~, the MDGC could obtain a cleaner mass spectrum for TRA and achieved lower method detection limits. Furthermore, selected ion monitoring mode (SIM) coupled with MDGC was used in this study to provide additional ‘mass-based separation’ of target analyte. This resulted in improved quantitative results compared with those obtained by Shao et al., (2003) where the derivatized analyte could have been interfered with by co-eluting peaks by using a FID [15]. This study is a novel application of heartcut two-dimensional GC-GC/MS in wine. It also [illustrated](#) the advantages of MDGC over comprehensive GC×GC relative to focusing on the quantification of specific compound in a very complex matrix. Similar applications were successfully used in our previous work with insect volatiles [27] as well as other work with essential oil, wine, beverage and fragrance products [34-36].

Figures 2B and 2C show the synchronous total ion chromatogram (TIC) and the SIM chromatogram, respectively, of two heartcuts of CRA- and TRA-BSTFA derivatives from red wine samples. Precise heartcut times of two resveratrol isomers were determined by injecting reference standards and ensuring that only the CRA- and TRA-BSTFA derivatives and co-eluting matrix were heartcut to the analytical column. As a result, there were no interference peaks and the target analytes had a clean mass spectrum and low background, especially for the SIM mode (Figure 2C). Thus, the

multidimensional GC significantly improved the separation of target compounds and was used for the methods development.

The simultaneous acquisition of full scan (TIC) and SIM data illustrated in Figures 2B and 2C allowed for identifications of unknown compounds while retaining the sensitivity and selectivity of target compound analysis by SIM. ~~Figures 3A, B, C and D show the comparison of total ion mass spectrum and selected ion mass spectrums of CRA and TRA-BSTFA derivatives from red wine.~~ Both of CRA- and TRA-BSTFA derivatives were confirmed with pure standards. The *cis*-isomer derivative eluted approximately 3 min earlier than the *trans*- resveratrol derivative. ~~It is evident that~~ The mass spectrum of CRA-BSTFA derivative is identical with the same molecular ions and characteristic abundance ratio (~~Figures 3B and 3D~~) as that of TRA-BSTFA. The molecular ion ($m/z = 444$) was in both cases the major ion with a relative abundance of 100%. Therefore, $m/z = 444$ ion was used for quantitative analysis in SIM mode. The M-H ($m/z = 443$) and the C-isotope ($m/z = 445$) ions were used as qualifiers.

3.2 Optimization of SPME extraction conditions

Selection of a suitable SPME coating is one of the most important steps in the development of a SPME method. Two kinds of fibers including (a) absorptive 100 μm PDMS and 85 μm PA and (b) adsorptive 65 μm PDMS-DVB were tested. The 100 μm PDMS fiber coating is non-polar and was found to be less efficient for the resveratrol-BSTFA derivatives than polar 85 μm PA fiber coating. Furthermore, the PDMS coating was found to swell in the vapor of BSTFA, which eventually damaged the fiber coating. This observation is consistent with Shao et al., [15]. ~~[31]~~. The PDMS-DVB fiber coating

was also considered because of the benzyl group in DVB polymer, which might favor the extraction of resveratrol with two benzyl rings due to the π - π interactions. However, the results showed the extraction efficiency for 85 μ m PA fiber was still approximately 10 times greater than that of 65 μ m PDMS/DVB. Therefore, the polar 85 μ m PA was used for subsequent studies. Caution must be taken when the PA fiber is used for direct extractions and on-fiber silylation. It was found that the coating of the new fiber was unstable for the first one or two derivatizations. A significant decrease of extraction efficiency after approximately 30 extraction-derivatization cycles was also observed.

An extraction time profile for TRA-BSTFA at a fixed concentration of 10 μ g L⁻¹ is shown in Figure 3.4. The extraction time varied from 5 min to 90 min for determination of the optimum extraction time. All the extractions were conducted at room temperature. It was consistently observed that the extraction of TRA-BSTFA reached equilibrium at 30 min. Hence, 30 min extraction time was used for the subsequent experiments.

The effect of temperature on TRA-BSTFA extractions is summarized in Figure 4.5. Extraction temperature varied from room temperature (22 °C) to 60 °C. The sensitivity decreased proportionally with the increase in temperature. This was due to the decrease of fiber-sample partitioning coefficient with the increasing temperature. The apparent decrease of TRA-BSTFA with increasing extraction temperature was -0.0148 °C⁻¹. The sample recovery was also investigated between room temperature and 60 °C. The recovery of TRA-BSTFA derivatives was greater at higher temperature (50 °C). However, slight losses were observed at 60 °C and even greater at 70 °C. This may be due to the derivatives, which are generally more volatile and could be desorbed from the fiber

at high temperatures [31]. [58]. Few researchers have reported that the PA fiber coating may be damaged by high derivatization temperatures [14]. [30]. Thus, the optimum temperature of room temperature was used for the subsequent experiments.

Ethanol is one of the major constituents of wine and concentration typically varies from 10 to 15%. The effect of ethanol on the extraction efficiency was investigated in this study. Standard $10 \mu\text{g L}^{-1}$ TRA was prepared with 10%, 20% and 50% ethanol in pure water, respectively. Figure 5 6 indicates that the extraction efficiency of TRA-BSTFA decreased proportionally with increasing ethanol content. The apparent rate of extracted TRA-BSTFA decrease with % ethanol increase was $-0.0127 \text{ \% ethanol}^{-1}$. However, the apparent decrease of the extraction efficiency for the ethanol content $< 20\%$ was not significant rather insignificant. This finding is consistent with Luan et al. [14]. [30]. In summary, the optimized SPME extraction conditions used in this study were: $85 \mu\text{m}$ PA fiber, 30 min direct extraction time from wine at room temperature (22°C) and 500 rpm stirring, 10 min desorption time at 280°C .

3.3 Optimization of the on-fiber silylation

Two additional factors that affect to the performance of the on-fiber derivatization were investigated including derivatization time, temperature and the dose of BSTFA. Figure 6 7 shows the effect of derivatization time on the derivatization of TRA. Based on this experiment, it was determined that 20 min derivatization was adequate and was used for all experiments in this study. Longer derivatization times did not yield more of the TRA-BSTFA derivative. The apparent reaction rate for the 0 to 20 min derivatization range was 0.0323 min^{-1} . The effect of BSTFA dose was also tested. Various volumes of

derivatization reagent from 1 μL to 100 μL were used to explore the effects on the derivatization efficiency. The greatest derivatization efficiency was obtained with 5 μL of BSTFA in equilibrium with 4 mL vial headspace at room temperature for 20 min.

3.4 Method validation

The optimized procedure was applied for the validation of the developed analytical method including linearity, detection limit, repeatability and recovery. The linearity of the method was evaluated by preparing calibration standards with seven different TRA concentrations in 12.5% (v/v) ethanol:water solution. Each concentration was conducted in triplicate. The calibration curve was linear over the concentration range of 10 ng L^{-1} to 5 mg L^{-1} , with $R^2 = 0.9996$. The linear regression equation was as follows:

$$y = 55131x - 1\text{E}+06, y = 52973x - 727040,$$

where y and x are the peak area counts and concentrations ($\mu\text{g L}^{-1}$) of standard TRA solutions, respectively.

The method detection limits (MDLs) were estimated based on the experiment with 7 10 replicate direct SPME extractions of standard TRA in 12.5% (v/v) ethanol:water solution at the lowest concentration (10 ng L^{-1}) using equation 1. The MDL for TRA in ethanol:water solution in this study was 7.08 ng L^{-1} whereas the MDL for TRA in pure water was 2.85 ng L^{-1} . Thus, SPME coupled with heartcut MDGC/MS method presented in this study is superior in terms of sensitivity for TRA to all previously published methods. Such a low MDL is likely due to the reduction of interferences with the introduction of narrow heartcuts and cryotrapping in multidimensional mode. The analytical column was only separating a very small portion

of the total sample at one time. The separation based on MDGC of the specific region enabled isolation of the target compounds from the interference background and resulted in a cleaner mass spectrum and furthermore, it improved the MDL for the target compound. In order to further assess the new method and to estimate the MDL, the recovery of 10 ng L⁻¹ TRA was investigated by spiking the TRA standard solution in 12.5% (v/v) ethanol:water solution. The RSD of 7 replicates was 15% and the average recovery of 10 ng L⁻¹ TRA in 12.5% (v/v) ethanol:water solution was 78.2%.

The repeatability of the optimized direct SPME on fiber silylation and MDGC-MS method for TRA in 12.5% ethanol:water solution, expressed as relative standard deviation (RSD, %, n = 3), ranged from 3.5 to 8.9% 1.3 to 6.7% at seven different concentrations including 10, 100 ng L⁻¹, 1, 10, 100 µg L⁻¹ and 1, 5 mg L⁻¹.

3.5 Analysis of wine samples and the recovery assay for trans-resveratrol in selected Iowa red wines.

Six selected Iowa red wine samples were analyzed using the optimized analytical method developed in this research (Table 1). These wines were from three winemakers and represented five different varieties with the same 2006 vintage. Reported RSD (%) are for three replicate samples from the same bottle of wine. The average amount of TRA was 206.70 µg L⁻¹. Lamuela-Raventos et al., (1997) reported resveratrol content in red US wines below 1 mg L⁻¹ [37] [62] and being significantly lower compared to Italian, French and Spanish wines [38]. [16]. The highest average level of TRA was found in wines made from Pinot Noir grown in France (5.4 ± 1.2 mg L⁻¹) [8]. [63]. For the selected few wines analyzed in this work the amount of TRA varied greatly from one wine variety

to another, i.e., from 12.72 to 851.9 $\mu\text{g L}^{-1}$ 12.7 to 881.4 $\mu\text{g L}^{-1}$. It is interesting to note that the amount of TRA varied from 181.7 to 12.72 $\mu\text{g L}^{-1}$ 202.67 to 12.7 $\mu\text{g L}^{-1}$ for the same wine variety originating from a different winemaker, e.g., Frontenac from winery B and winery A, respectively. This finding suggests that wine-making techniques, such as increased temperature, high levels of SO_2 and/or decreased pH results in higher levels of TRA in red wine [64]. This finding is consistent with McMurtey (1997) [39] [65] who also reported that a number of factors such as climate, geographical area of cultivation, growing conditions, wine-making techniques and storage conditions affect resveratrol content in wines within the same grape variety.

The ratios of *trans*- to *cis*-resveratrol from the selected red wines were also investigated in this study (Table 1). The peak area count of *trans*- and *cis*- isomers were used for the calculation of the ratio. The average ratio of *trans*- to *cis*- found in the six selected Iowa red wines was about 3.0. *Trans*- isomer content was greater for almost all of the selected wines except for Marechal Foch from winery C, for which the ratio of *trans*-/*cis*- was 0.66 0.22. The generally high ratio of *trans*- to *cis*- isomers supports the notion that *cis*-isomers could arise from light exposure of wine during the winemaking process or possibly from the light exposure of wine bottles during storage [40]. [66].

The recovery of the overall method was determined for the overall assay by analysis of three wine samples with low, medium and high concentrations of TRA supplemented with known concentrations of standard TRA. The summary of TRA recoveries for each concentration is presented in Table 2. The new method showed very good recoveries (from 72.7% to 94.7% 81.8% to 106.5%) with a RSD < 7.1%. The mean recovery was 83.6% 91.7%.

4. Conclusions

The following conclusions were drawn from this study:

- (1) Heartcut-based multidimensional GC-MS is a powerful approach to improve the separation of *trans*- and *cis*- resveratrol-BSTFA derivatives.
- (2) ~~The SPME coupled with MDGC-MS method is superior in terms of sensitivity of resveratrol detection to all previously published methods.~~ The MDL for TRA in ethanol:water solution in this study was 7.08 ng L⁻¹ whereas the MDL for TRA in water was 2.85 ng L⁻¹ with heartcut and cryotrap and SIM. ~~The MDL was as low as 2.85 ng L⁻¹ with heartcut and cryotrap.~~ The linearity was excellent between 10 ng L⁻¹ to 5 mg L⁻¹. The average recovery for *trans*-resveratrol in wine was 83.6% ~~91.7%.~~
- (3) ~~The new method can be used to determine both *trans*- and *cis*-resveratrol. This study focused on the more interesting *trans*- form. However,~~ The *trans*- to *cis*- ratio was investigated in this study. The average ratio of *trans*- to *cis*- found in the six selected Iowa red wines was approximately 3.0. ~~The *trans*-isomer *Trans*-isomer~~ content was predominant for five out of the six selected wines. The new method could also be used for resveratrol analyses in grape juice, jams and jellies, and other related products.
- (4) ~~There was a considerable variability in *trans*-resveratrol concentrations even in wines produced from the same grape variety, which is not unexpected since a number of factors such as climate, geographical area of cultivation, growing~~

503 ~~conditions, wine-making techniques and storage conditions affect *trans*-~~
504 ~~resveratrol content of wines.~~
505 ~~(5) Winemaking techniques may have important effects on the resveratrol content of~~
506 ~~wine. Further studies are needed to investigate the contribution of winemaking~~
507 ~~procedures to resveratrol content in wine.~~

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Figure captions

Figure 1 Separation of *cis*- and *trans*-resveratrol-BSTFA derivatives in GC-FID mode with no heartcut. Chromatogram (FID) of the red wine sample collected with 85 μm PA SPME fiber for 30 min direct extraction at room temperature.

Figure 2 Separations in multidimensional GC-MS mode with cryotrap and heartcut between pre-column and analytical column: comparison of the FID chromatogram (part A), total ion chromatogram (part B) and selected ion chromatogram (part C) isolating *trans*- and *cis*-resveratrol-BSTFA derivatives from red wine samples with direct-SPME-MDGC-MS. Cryotrap range: 10.1 min -10.9 min; 12.7 min -13.4 min. Heartcut range: 10.2 min -10.9 min; 12.8 min - 13.4 min.

~~**Figure 3** Comparison of total ion mass spectrum and selected ion mass spectrum for *cis*- and *trans*-resveratrol-BSTFA derivatives from red wine sample.~~

Figure 3 4 Extraction time profiles using 85 μm PA fiber. Direct-SPME extraction at room temperature and 500 rpm stirring for the standard *trans*-resveratrol (at 10 $\mu\text{g L}^{-1}$) -BSTFA derivatives.

Figure 4 5 Effect of the extraction temperature on extractions of *trans*-resveratrol-BSTFA derivatives using 85 μm PA fiber and direct-SPME for 30 min and 500 rpm stirring.

Figure 5 6 Effect of ethanol content on the 30 min direct SPME extraction efficiency of *trans*-resveratrol (at 10 $\mu\text{g L}^{-1}$) -BSTFA derivatives at room temperature and 500 rpm stirring.

Figure 6 7 Effect of reaction time on derivatization of *trans*-resveratrol (at 10 $\mu\text{g L}^{-1}$) with BSTFA at room temperature.

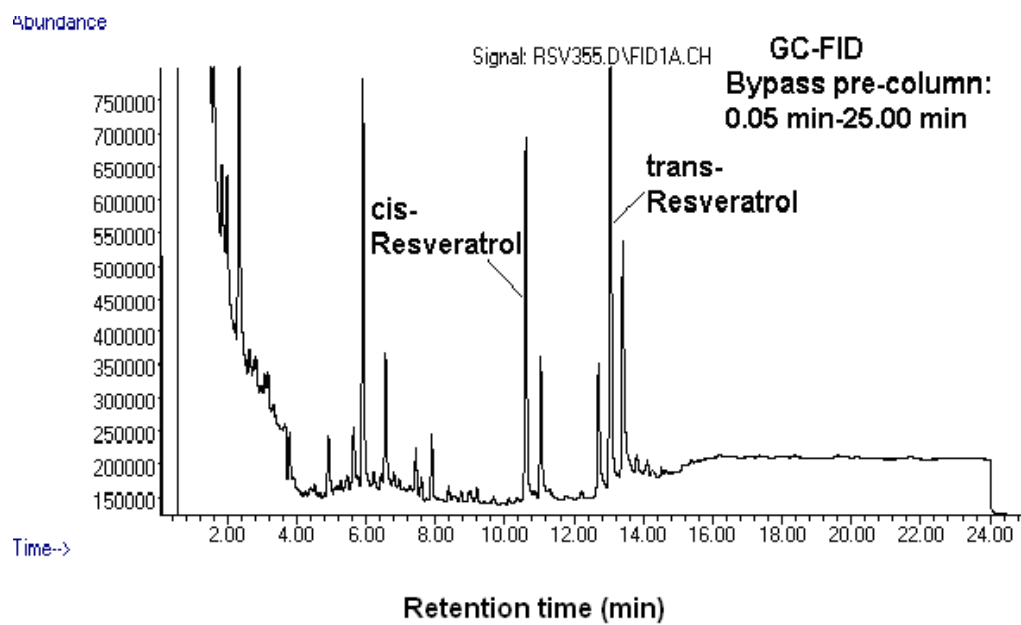


Figure 1 Cai et al.

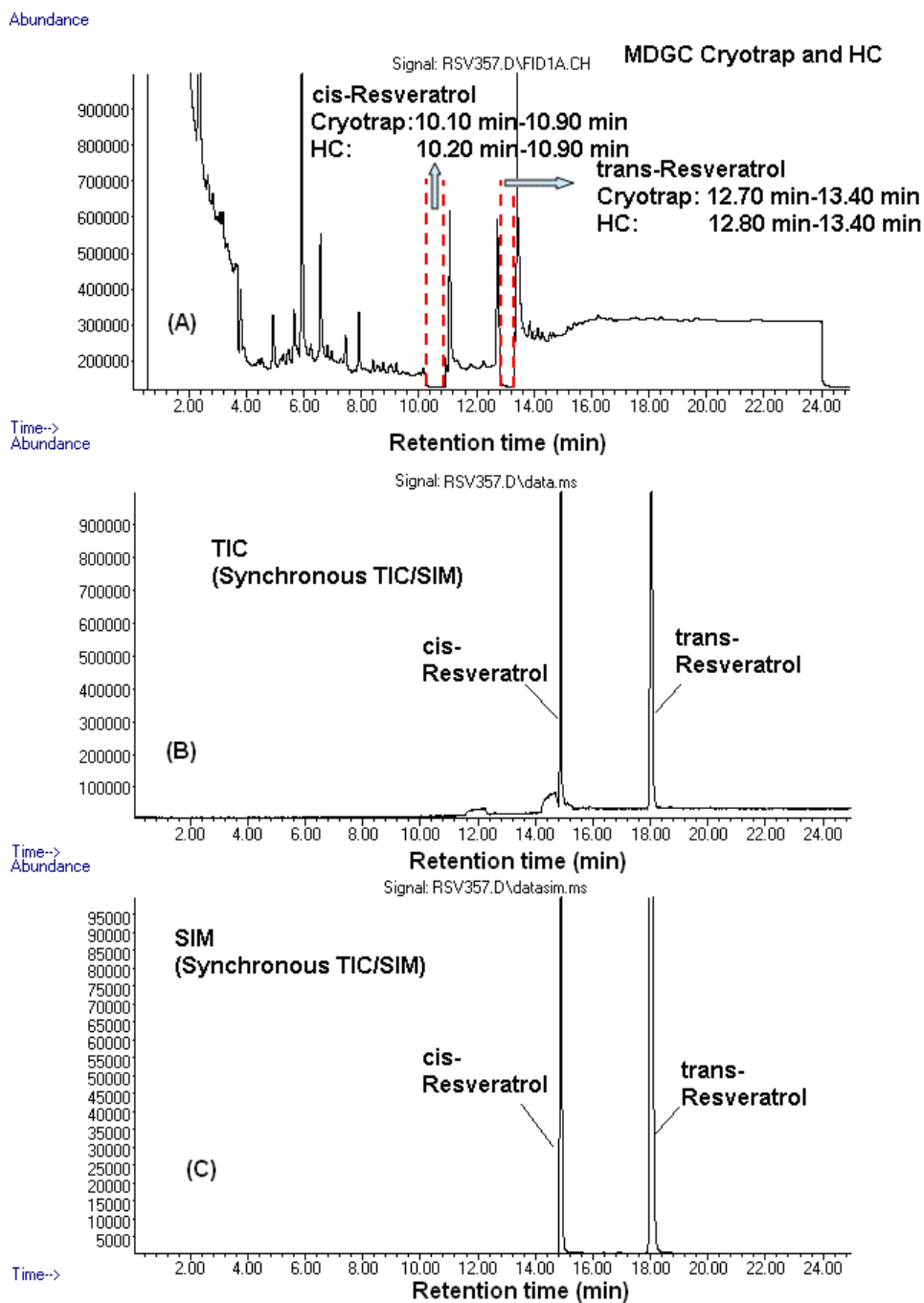


Figure 2 Cai et al.

Figure 3 Cai et al.

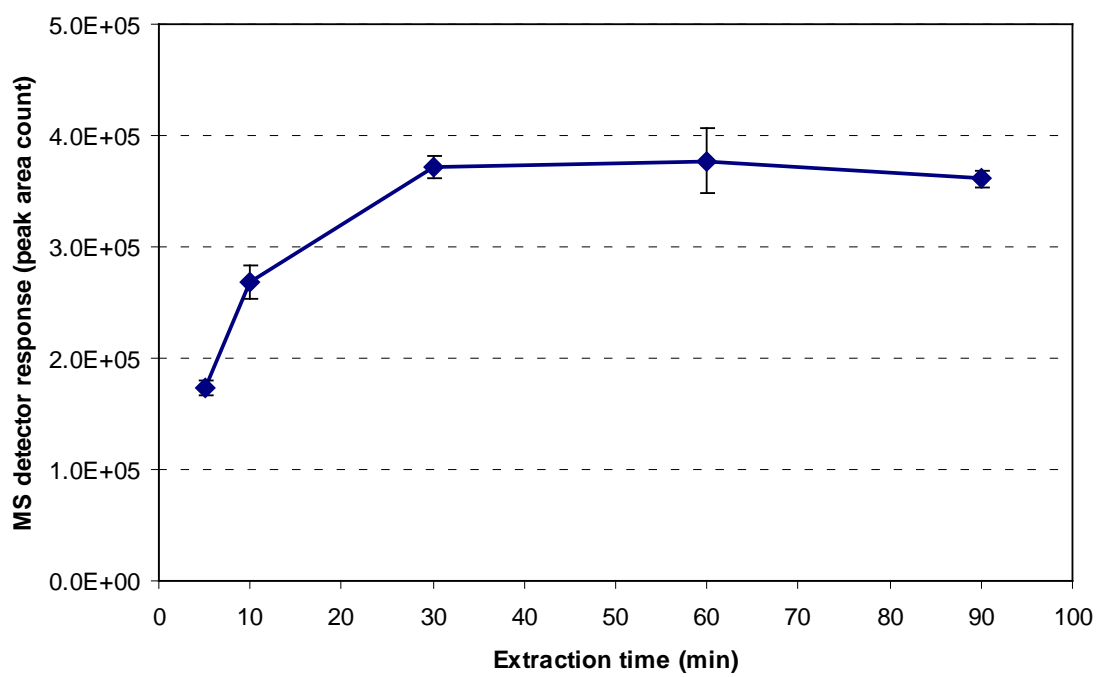


Figure 3 Cai et al.

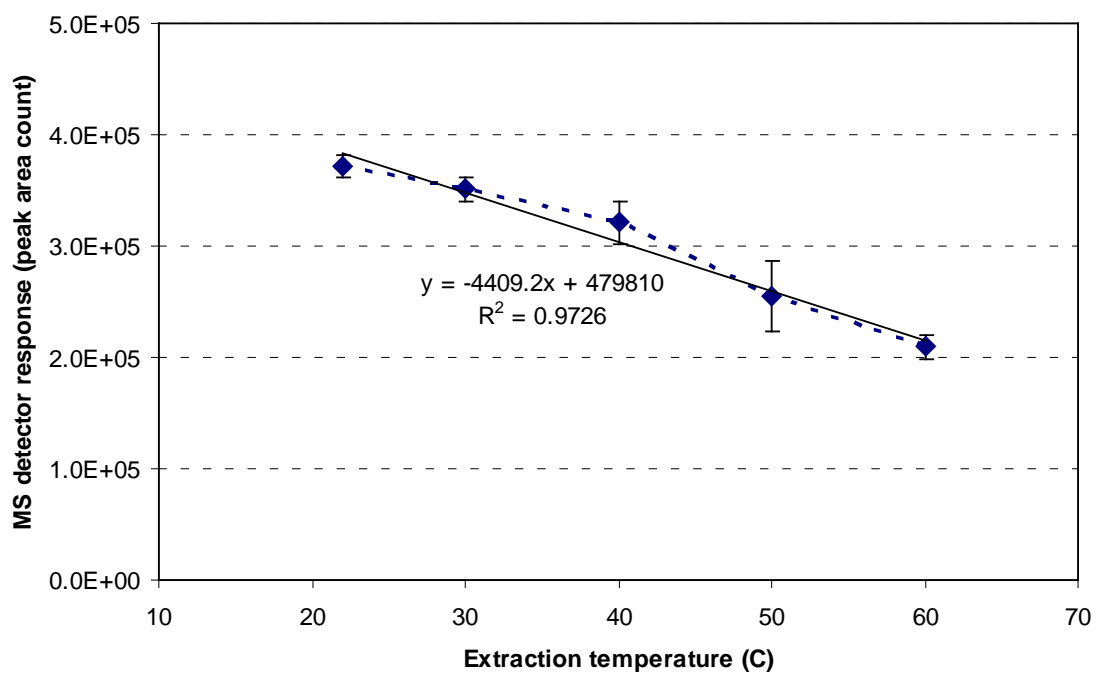


Figure 45 Cai et al.

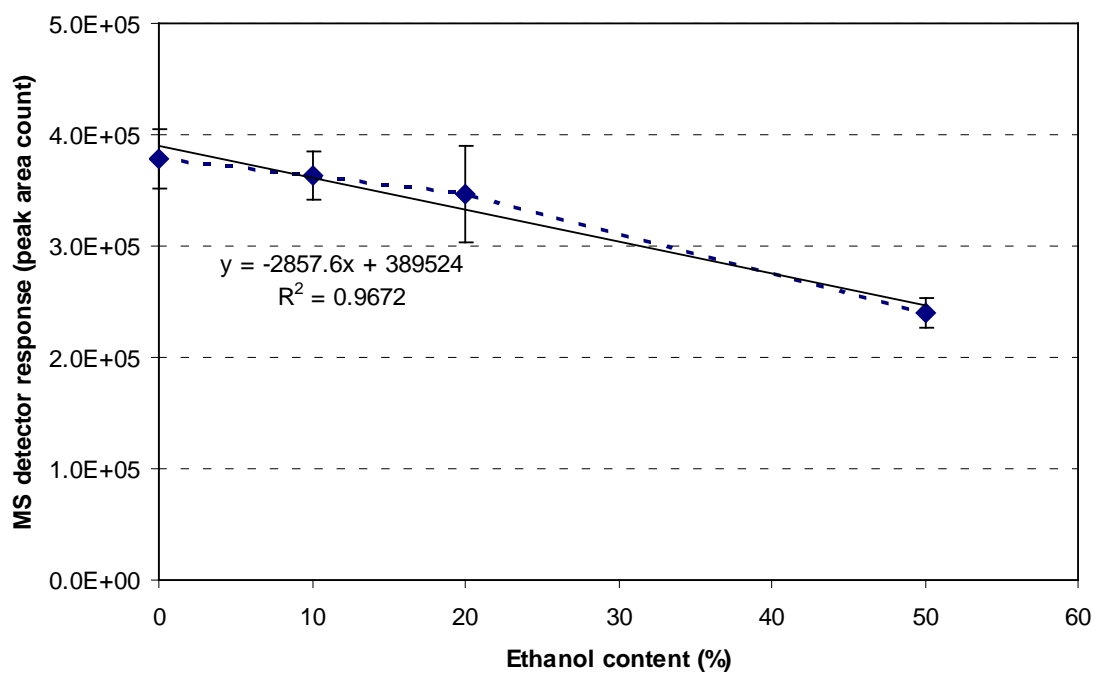


Figure 5 6 Cai et al.

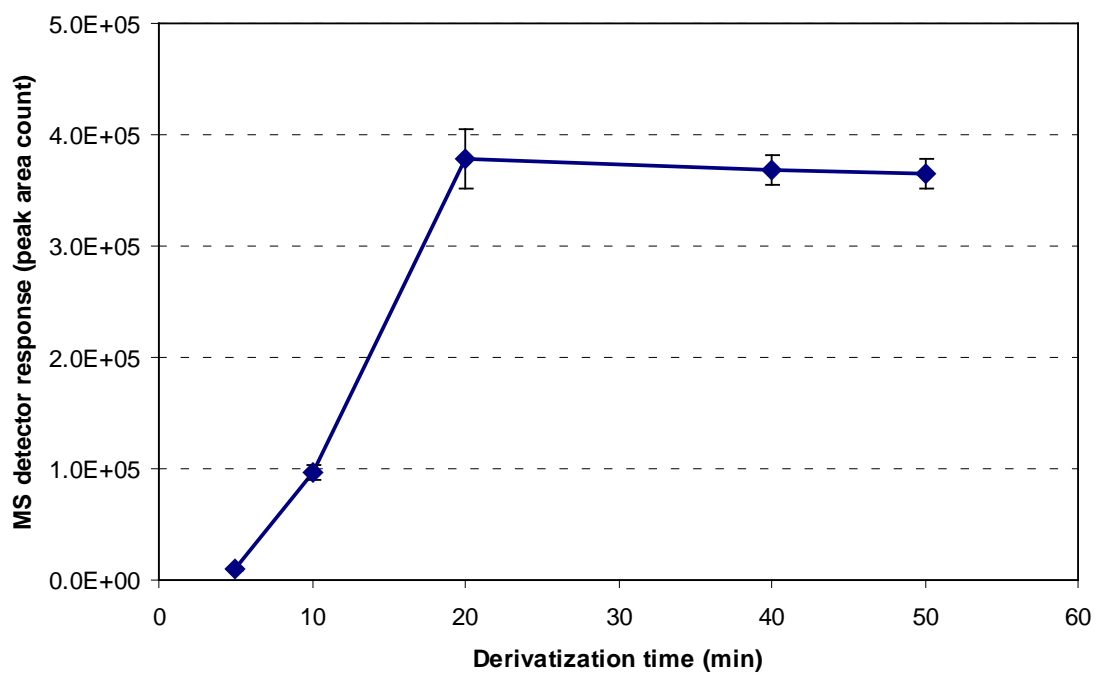


Figure 67 Cai et al.

Table 1 Measured concentrations of *trans*-resveratrol in six selected Iowa red wines.

Sample number	Winery	Variety	Vintage	trans-/cis-	RSD (% , n=3)	trans-Resveratrol ($\mu\text{g L}^{-1}$)	RSD (% , n=3)
1	A	FOCH	2006	6.67	8.8	53.34	3.0
2	A	St. Croix	2006	4.35	3	19.41	6.0
3	A	Frontenac	2006	1.56	7.8	12.72	9.2
4	B	Vincent	2006	2.63	10.0	851.9	5.2
5	B	Frontenac	2006	2.22	1.3	181.7	5.8
6	C	Marechal Foch	2006	0.66	1.7	59.10	6.3

Sample number	Winery	Variety	Vintage	Ratio <i>trans-/cis-</i>	RSD % (n=3)	<i>trans</i> - resveratrol ($\mu\text{g L}^{-1}$)	RSD % (n=3)
1	A	Foch	2006	6.67	8.8	58.40	3.0
2	A	St. Croix	2006	4.35	3	20.23	6.0
3	A	Frontenac	2006	1.56	7.8	12.70	9.2
4	B	Vincent	2006	2.63	10.0	881.40	5.2
5	B	Frontenac	2006	2.22	1.3	202.67	5.8
6	C	Marechal Foch	2006	0.66	1.7	64.85	6.3

Table 2 Recovery of the spiked *trans*-resveratrol from wine samples.

Winery	Wine sample	Found before spiking ($\mu\text{g L}^{-1}$)	Spiked ($\mu\text{g L}^{-1}$)	Total found after spiking ($\mu\text{g L}^{-1}$)	Recovery (%)	RSD (%; n=3)
A	Foch	53.34	60	96.97	72.7	5.1
A	Frontenac	12.72	10	22.19	94.7	4.7
B	Vincent	851.9	1000	1685.5	83.4	7.1

Winery	Wine sample	Found ($\mu\text{g L}^{-1}$)	Spiked ($\mu\text{g L}^{-1}$)	Total found ($\mu\text{g L}^{-1}$)	Recovery (%)	RSD(%; n=3)
A	Foch	58.40	60	107.40	81.8	5.1
A	Frontenac	12.70	10	23.40	106.5	4.7
B	Vincent	881.40	1000	1749.10	86.8	7.1