JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of 4-Ethylphenol and 4-Ethylguaiacol in Wines by LC-MS-MS and HPLC-DAD-Fluorescence

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Volatile phenols produced by *Brettanomyces dekkera* have been associate with off-flavors of wines. A versatile liquid chromatography-tandem mass spectrometry together with an HPLC-DAD-fluorescence methods were developed for the quantitation of two phenols, 4-ethylphenol (4EP) and 4-ethylguaiacol (4EG), in red and white wines. For LC-MS-MS analysis, fortified wines were directly injected after a dilution with methanol, and levels of phenols were measured by monitoring the multiple reaction (MRM) transitions of precursor ions mass charge (*m*/*z*) 121 → 106 for 4EP and (*m*/*z*) 151 → 136 for 4EG. Qualitative and quantitative confirmation data were acquired simultaneously by monitoring alternative MRM transitions following an external standard method. Calibration was linear over a working range of 10 and 5000 μg/L. Limit of determination (LOD) and limit of quantification (LOQ) were 10 and 50 μg/L for both 4EG and 4EP. HPLC analysis phenols were separated with a gradient system of acetonitrile−water and detected using a diode array detector (DAD) at 280 nm, and for the fluorescence analysis, excitation and emission wavelengths of 260 and 305 nm were used. Quantitative analysis of 4EP and 4EG was performed by the standard addition method to avoid matrix interferences. Calibration was linear over a concentration range from 10 to 5000 μg/L for HPLC-DAD, from 1 to 10 000 μg/L for 4EP, and from 10 to 10 000 for 4EG for fluorescence analysis. LOD and LOQ for the DAD analysis were 10 and 50 μg/L for both 4EG and 4EP. For fluorescence analysis, LOD and LOQ were 1 and 5 μg/L for 4EP and 10 and 50, respectively, for 4EG. The proposed methods can be easily used for the qualitative and quantitative determination of 4EP and 4EG in wines affected by microbial contamination with yeasts of the *Brettanomyces* genus.

KEYWORDS: Wine; 4-ethylphenol; 4-ethylguaiacol; HPLC-DAD; fluorescence; LC-MS-MS

INTRODUCTION

The hemiascomycete yeast *Dekkera bruxellensis*, also known as *Brettanomyces bruxellensis*, is the main cause of wine spoilage worldwide and causes severe economic losses within the wine industry (*1*). Wines contaminated by *D. bruxellensis* have a "Brett" character of smelling mousy, medicinal, of a barnyard, or of horse sweat (*2*). The mousy odor is the result of tetrahydropyridines synthesized by *D. bruxellensis* from lysine and ethanol (*3*), while medicinal or barnyard odors are caused by the low molecular weight volatile phenols 4-ethylguaiacol (4EG) and 4-ethylphenol (4EP), secondary metabolites produced by *D. bruxellensis* as a result of enzymatic conversion of vinylphenols derived from cinnamic acids naturally present in the grape must (*4*, *5*). When the total concentration of 4EP and 4EG is greater than 620 μg/L, the "Brett" or phenolic character of the wine becomes too pronounced for the wine to be acceptable (*6*). At less than 400 μg/L, it contributes favorably to the complexity of the wine aroma by imparting aromatic notes of spices, smoke, and leather (*7*).

Different analytical methods, most of them laborious and time-consuming, have been reported for the simultaneous determination of 4EG and 4EP in wines. Chatonnet proposed a gas chromatographic technique coupled with a flame ionization detector (FID) after a liquid−liquid extraction using dichloromethane. The proposed limit of detection of this method was 1 μg/L for both phenols (*8*). Monje et al. (*9*) and Martorell et al. (*10*) studied the determination of volatile phenols by headspace solid-phase microextraction. The detection limits for this GC-FID method were 1 and 2 μg/L for 4EG and 4EP (*9*), while the linearity range was 5−5000 μg/L.

Other gas chromatographic procedures and methods were performed with mass spectrometry detectors (*11*), GC-olfactometry (*12*, *13*), stir bar sorptive extraction (SBSE) coupled with GC-MS (*14*), and solid-phase microextraction (SPME) coupled with GC-MS (*15*). Concentration levels of 4EP were found in 61 bottled Australian red wines ranging from 2 to 2660 μg/L with a mean concentration of 795 μg/L, while levels of 4EG ranged from 1 to 437 μg/L with a mean concentration of 99 μg/L (*8*). Recently, Larcher et al. reported an HPLC method to measure volatile phenols in wine using a coulometric detector (*16*). LODs for this procedure were between 1 and 3 μg/L, with a good linearity from 50 to 2000 μg/L.

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10.1021/jf071156m CCC: $37.00
Published on Web 08/04/2007

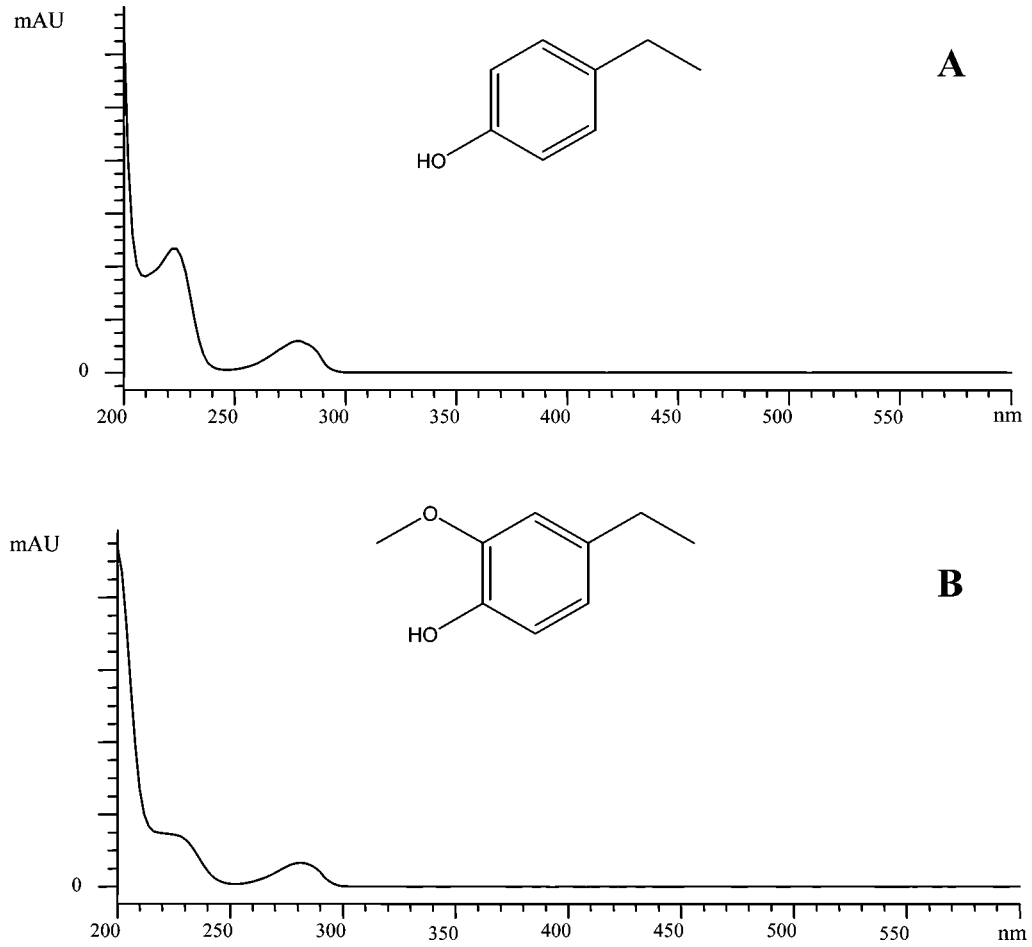

Figure 1. UV spectra of 4EP (**A**) and 4EG (**B**).

These finding suggested the need to develop a specific, rapid, and repeatable analytical method to examine levels of the phenolic off-flavor 4EP and 4EG in red and white wines to help winemakers in monitoring and control *Brettanomyces* contamination. Here, we report the development of a method for the determination of 4EG and 4EP in red and white wines based on the use of liquid chromatography with tandem mass spectrometric detection (LC-MS-MS) and HPLC-DAD-fluorescence.

MATERIALS AND METHODS

Chemicals. Orthophosphoric acid was from Carlo Erba (Milan, Italy), and standards of 4EP and 4EG were from Sigma-Aldrich (Milan, Italy). Water was distilled and filtered through a Milli-Q apparatus (Millipore, Milan, Italy). Methanol and acetonitrile were the HPLC solvents (Carlo Erba, Milan, Italy). Stock standard solutions of the active ingredient were prepared in methanol. Working standard solutions for HPLC and LC-MS-MS analyses were prepared daily by diluting the stock solutions with water and methanol respectively. PTFE syringe filters of 0.45 μm were from PALL Life Sciences (Ann Arbor, MI).

Apparatus and Chromatography. *LC-MS-MS Analysis.* A Varian tandem mass spectrometer (Palo Alto, CA) consisting of a ProStar 410 autosampler, two ProStar 210 pumps, and a 1200 L triple quadrupole mass spectrometer equipped with an electrospray ionization source (ESI) was used. Varian MS workstation version 6.7 software was used for data acquisition and processing. The chromatographic separation was performed on a Phenomenex Synergy MAX-RP column (4.6 mm $\times$ 150 mm i.d., 4 μm particle size, Bologna, Italy). The gradient was as follows: initial methanol and water (70:30 v/v) to methanol and water (82:18 v/v) in 12 min and maintained for 3 min. The mobile phase was pumped at a flow rate of 0.3 mL/min. The injection volume was 10 μL, and the analysis time was 15 min per sample.

The ESI-MS was operated in the negative ion mode. The electrospray capillary potential was set to -54 V. Air was used as a drying gas for solvent evaporation. The atmospheric pressure ionization (API) housing and drying gas temperatures were kept at 50 and 300 °C. Deprotonated analyte molecules were subjected to collision induced dissociation using argon at 2.60 mTorr as the collision gas using multiple reaction monitoring (MRM) data acquisition for the transitions of precursor ion mass charge (*m/z*) 121 $\rightarrow$ 106 for 4EP and 151 $\rightarrow$ 136 for 4EG. The collision energy was 14 eV for 4EP and 12 eV for 4EG. The scan time was 1 s, and the detector multiplier voltage was set to 1400 V.

HPLC Analysis. An Agilent Technologies (Waldbronn, Germany) model 1100 liquid chromatograph fitted with a diode array detector (DAD) and a fluorescence detector (Hitachi L-7485, Tokyo, Japan) was used. The separation of phenols was achieved with a Waters Spherisorb ODS2 column (250 mm $\times$ 4.6 mm, 5 μm particle size) thermostated at 20 °C and using a solvent gradient as follows: initial mobile phase acetonitrile−aqueous orthophosphoric acid 0.1% (10:90 v/v), reaching acetonitrile−aqueous orthophosphoric acid 0.1% (90:10 v/v) in 25 min, and reconditioned for 10 min with the initial concentration of the mobile phase. The injection volume was 100 μL, and the flow rate was set at 1 mL/min. The UV-DAD analysis was performed at a wavelength of 280 nm according to the 4EP and 4EG maxima reported in the UV spectrum (**Figure 1**). A good linearity was obtained in the range from 10 to 5000 μg/L with correlation coefficients between 0.9996 and 0.9999.

The fluorescence analysis was performed at 4EP maximum excitation and emission wavelengths of 260 and 305 nm. A good linearity was obtained in the range from 1 to 10000 μg/L for 4EP and from 10 to 10000 μg/L for 4EG with correlation coefficients between 0.9996 and 0.9999.

Preparation of Samples for Analysis. Italian bottled red and white wines were purchased at a local market (Cagliari, Italy). Wine samples were filtered, fortified at the desired level of 4EG and 4EP, and directly

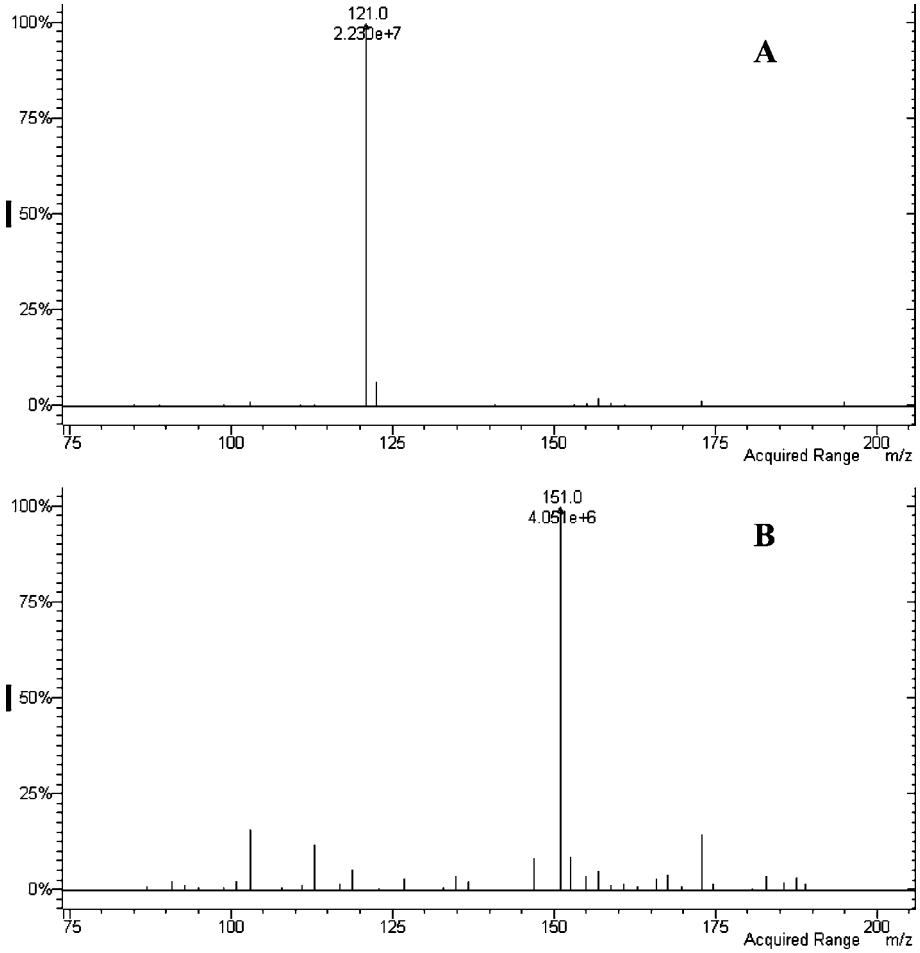

Figure 2. Negative ion mass spectra of 4EP (**A**) and 4EG (**B**).

Table 1. HPLC and LC-MS (ESI) Characteristics of the Two Alkylphenols

compound	formula	log *p*[a]	HPLC t_R (min)	LC-MS t_R (min)	mol wt	LC-MS (ESI) *m/z* (amu) (% relative abundance)
4-ethylphenol	$C_8H_{10}O$	2.35	14.51	10.81	122	121 $[M-H]^-$ 100, 122 $[M]^-$ 7
4-ethylguaiacol	$C_9H_{12}O_2$	2.48	14.89	10.92	152	151 $[M-H]^-$ 100, 103 $[M-CH_5O_2]^-$ 14, 112 [unknown] 9

[a] Log *p* values were calculated with the CS ChemDraw Pro Cambridge Software (Cambridge, MA).

injected for analysis after dilution (1:10 v/v) with methanol for LC-MS-MS and bidistillated water for HPLC-DAD-fluorescence analysis.

Efficiency. *Standard Curves and Linearity*. For LC-MS-MS analysis, a six-point standard curve for each phenol was prepared. Standard solutions were prepared in triplicate containing 4EP and 4EG at 10, 50, 100, 500, 1000, and 5000 μg/L. Calibration curves were created by plotting the concentration of each phenol against the standard peak area of the monitored transition. Simple linear regression analysis was performed to calculate the slope and intercept. The correlation coefficient (*r*) for each phenol was also determined.

For the HPLC-DAD-fluorescence method, an aliquot of red or white wine was fortified with different amounts of 4EP and 4EG to reach concentrations of 50, 100, 500, 1000, and 5000 μg/L. Calibration curves were created by plotting the concentration of each phenol against the standard peak area following the standard addition method.

Repeatability. To evaluate precision, the repeatability of both the instrument and the analytical procedure proposed was determined. Intermediate precision was calculated by performing six injections of the same standards for six consecutive days.

The proposed LC-MS-MS and HPLC-DAD analytical methods for the determination of 4EP and 4EG in wines has been demonstrated to be adequate, fast, precise, and accurate. Good linearity and repeatability were achieved for the two volatile phenols.

RESULTS AND DISCUSSION

LC-MS-MS Analysis. ESI and atmospheric pressure chemical ionization (APCI) were tested in positive and negative modes, respectively. ESI in the positive mode and APCI in the negative mode did not give any signal when infusion at the rate of 0.6 mL/h of standard solutions of the two phenols at 1000 μg/L were recorded. Moreover, ESI responses in the negative mode were superior to APCI in the positive mode. Thus, ESI in the negative mode was chosen for the identification, quantification, and confirmation of 4EP and 4EG in wines for its most intense response. For the ESI method operated in the negative mode, air gave the most intense response if compared with nitrogen.

Full scan spectra of phenols were acquired with a scan range of 80−200 amu, scan time of 0.75 amu, scan width of 0.70 amu, and detector at 1100 V. The main ions were observed at *m/z* 121 $[M-H]^-$ and 122 $[M]^-$ for 4EP and *m/z* 151 $[M-H]^-$ and 103 $[M-CH_5O_2]^-$ for 4EG (**Figure 2** and **Table 1**).

We then used product ion scans in a MS-MS breakdown experiment to look for the most abundant product ions. The collision energy was optimized to achieve the highest sensitivity. Product ion spectra for 4EP consisted of a fragment ion at *m/z*

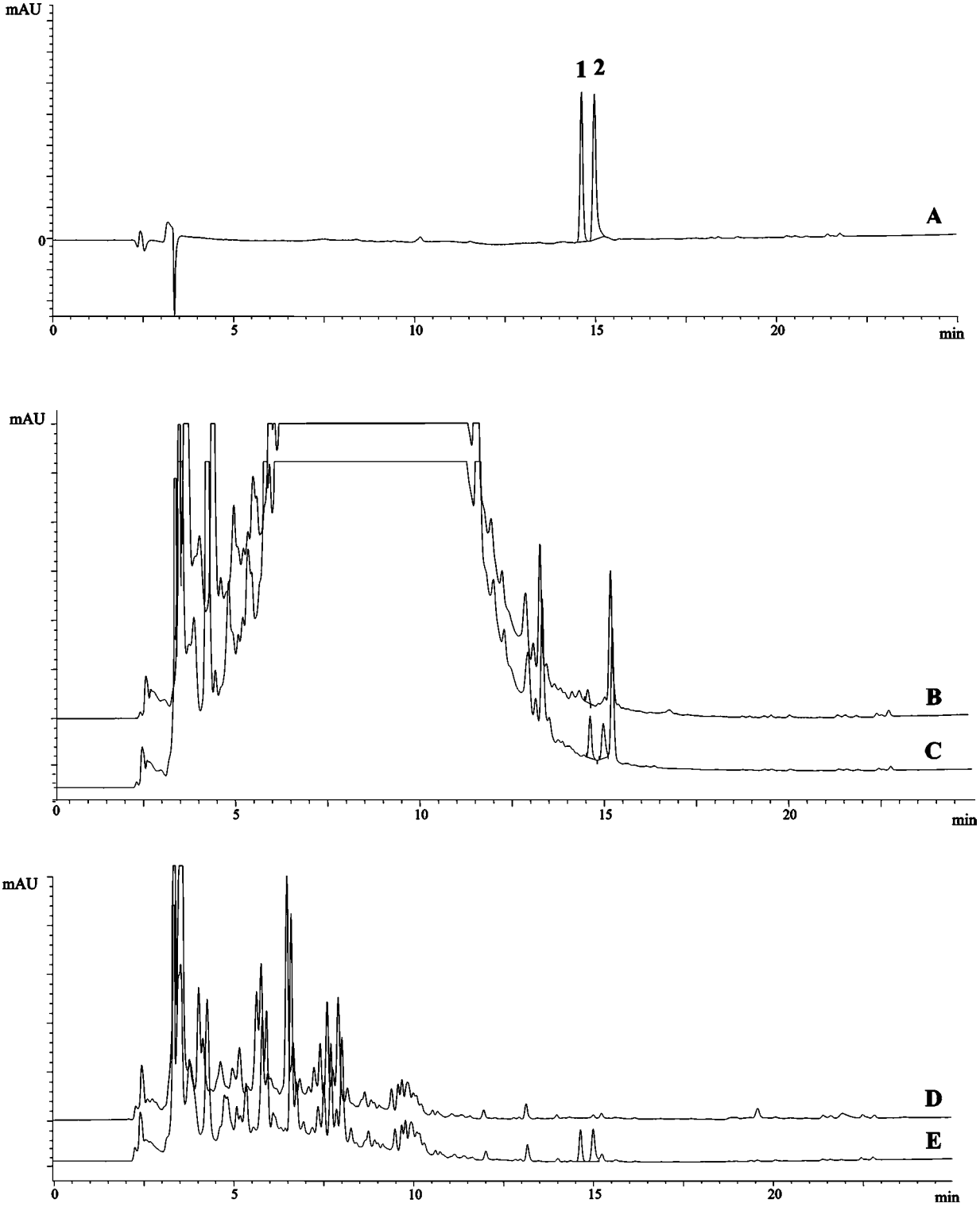

Figure 3. HPLC-DAD chromatogram at 280 nm for the analysis of phenols: (1) 4EP and (2) 4EG. (**A**) Standard of phenols at 500 μg/L; (**B**) red wine non-fortified; (**C**) red wine fortified at 500 μg/L; (**D**) white wine non-fortified; and (**E**) white wine fortified at 500 μg/L.

106 when CE was 14 V. 4EG gave the most intense ion at *m*/*z* 136 at a collision energy of 12 V with two less intense ions at *m*/*z* 121 and 93 at 22 and 32 V, respectively.

Selected reaction monitoring of the precursor−product ion transitions *m*/*z* 121 → 106 for 4EP and 151 → 136 for 4EG, corresponding to a loss of a methyl group, were chosen for the quantitative determination of the two volatile phenols. For the LC-MS-MS quantitation, an external standard method was used. Peak areas obtained from the MRM of phenol standards were used for the quantitative determination of 4EP and 4EG in wines. Sample solutions of wines were filtered and directly injected after a 10-fold dilution with methanol and detected under the optimum conditions mentioned earlier. Retention times of 4EP and 4EG were 10.81 and 10.92 min, respectively.

For efficiency experiments, standard solutions of phenols with concentrations ranging from 10 to 5000 μg/L were injected for the analysis in LC-MS-MS in a MRM experiment. For calibration curves, the correlation coefficients were 0.9992 for 4EP and 0.9990 for 4EG. For the precision experiment under conditions of repeatability, the highest and lowest variation

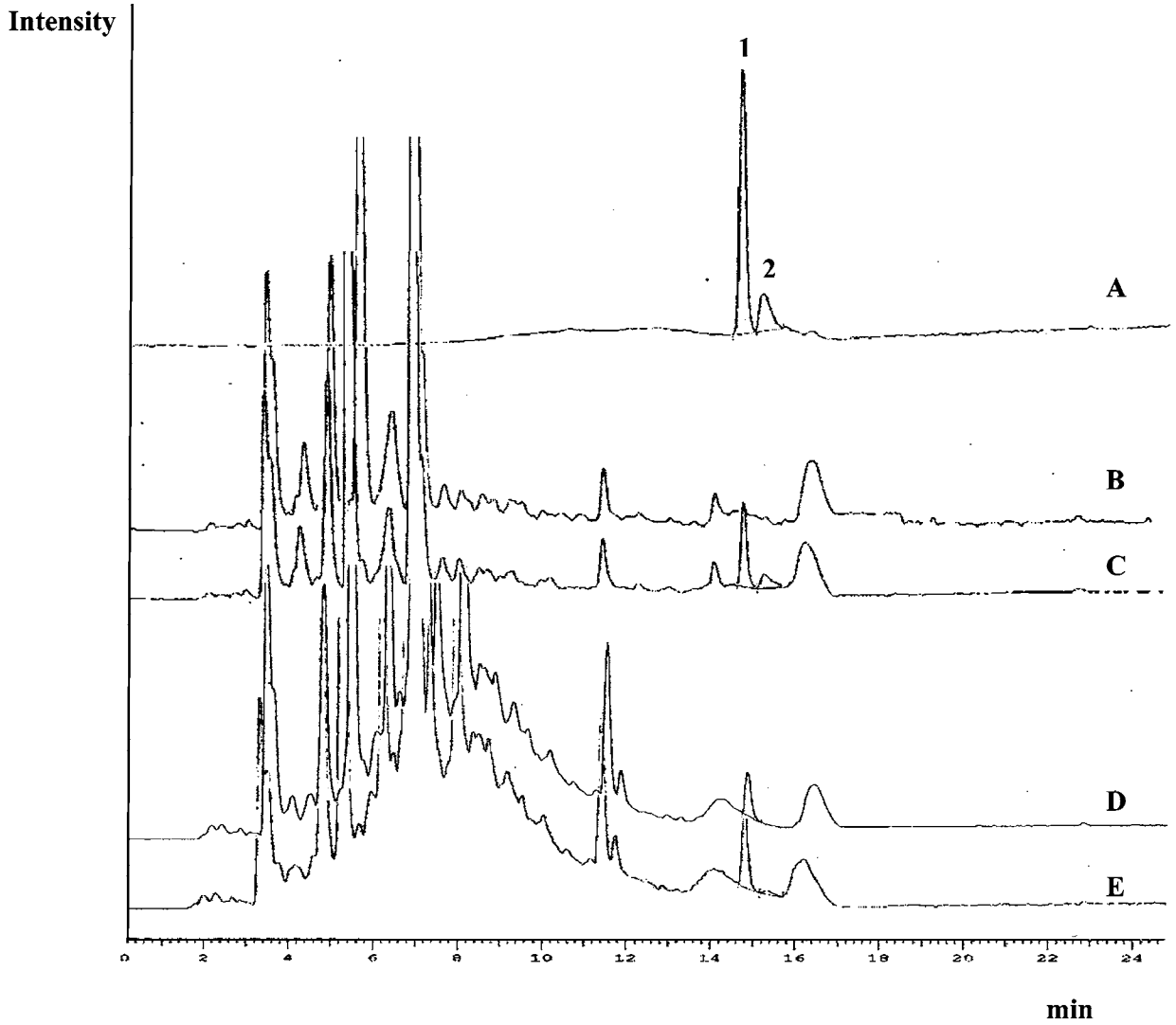

Figure 4. HPLC-fluorescence chromatogram at an excitation wavelength of 260 nm and emission of 305 nm for the analysis of phenols: (1) 4EP and (2) 4EG. (**A**) Standard of phenols at 50 μg/L; (**B**) white wine non-fortified; (**C**) white wine fortified at 50 μg/L, (**D**) red wine non-fortified; and (**E**) red wine fortified at 50 μg/L.

coefficients were 9.23 and 1.66%, while for the intraday experiment, the highest and lowest variation coefficients were 4.37 and 0.93%.

For the establishment of the limit of quantification and determination, 1000 μg/L standard solutions of phenols were gradually diluted with methanol. Each individual standard was injected 3 times. The LOD (S/N = 3) for 4EP and 4EG was 10 μg/L, while the LOQ (S/N = 10) was 50 μg/L for both phenols.

HPLC-DAD and Fluorescence Analysis. Since phenols have a strong UV absorption at 280 nm, a reversed-phase high-performance liquid chromatographic (HPLC) method was developed and validated using a photodiode array (DAD) for measuring levels of 4EP and 4EG in commercial wines. We also fitted downstream to the DAD detector a fluorescence detector to achieve a better sensitivity. **Figure 3** reports the HPLC-DAD chromatogram of 4EP and 4EG standards and a chromatogram of red and white wine fortified at 400 μg/L for the two phenols. HPLC retention times of 4EP and 4EG were 14.58 and 14.95 min, respectively. No interfering peaks were detected at the retention times of all analytes. Analysis of volatile phenols in wines was performed by diluting 100 μL of filtered red or white wines with 900 μL of bidistilled water. To overcome matrix interferences, quantification of 4EP and 4EG was performed by the standard additions analysis. HPLC-DAD limits of quantification calculated as S/N = 10 were 50 μg/L for 4EP and 4EG, while limits of detection calculated as S/N = 3 were 10 μg/L for both phenols. For repeatability analysis, the highest and lowest coefficients of variation were 5.4 and 0.3%, while for intermediate precision, they were 1.62 and 0.30%, respectively.

Figure 4 shows a fluorescence chromatogram of a standard solution of the two phenols at 50 μg/L and chromatograms of white and red wines as well as wines fortified at the same concentration. HPLC-fluorescence retention times for 4EP and 4EG were 14.85 and 15.46 min, respectively. Limits of quantification calculated as S/N = 10 were 5 μg/L for 4EP and 50 μg/L for 4EG, while limits of detection calculated as stated previously were 1 and 10 μg/L for 4EP and 4EG, respectively. For repeatability analysis, the highest and lowest coefficients of variation were 6.88 and 0.74%, while for intermediate precision, they were 2.03 and 1.57%, respectively. Precision under conditions of repeatability were determined by performing in the same day three injections at 50, 100, and 500 μg/L for LC-MS-MS and HPLC-DAD analysis and three injections at 10, 50, and 100 μg/L for HPLC-fluorescence determination.

The proposed HPLC-DAD-fluorescence and LC-MS-MS methods, without the need of liquid−liquid extraction or

chemical derivatization steps, can be considered to be fast and easily applicable to monitoring wine quality.

ACKNOWLEDGMENT

The authors thank Davide Pera and Francesca Argiolas for helpful suggestions.

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Received for review April 20, 2007. Revised manuscript received June 26, 2007. Accepted June 27, 2007.

JF071156M