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Rapid quantification of 4-ethylphenol in wine using high-performance liquid chromatography with a fluorimetric detector

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Summary

A rapid method was established for quantifying 4-ethylphenol in wine using HPLC with a detector usually present in wine laboratories. It does not require sample preparation and carries out chromatographic separation in less than 5 min, making control of wine production processes easier. The method is linear up to 2000 µg·l^{-1} with RSD < 3 % over 20 µg·L^{-1} and gives a detection limit of 4.0 µg·L^{-1}. It was validated in comparison with the HPLC-coulometric array detector, giving comparable results. Its application to the analysis of 720 DOC and table Italian red wines revealed that the 45 % of them had contents of 4-ethylphenol potentially affecting sensory perception of the aroma.

K e y w o r d s : wine, volatile phenols, ethylphenols, process control.

Introduction

Volatile phenols, *i.e.* 4-ethylphenol (4-EP), 4-ethylguaiacol (4-EG), 4-vinylphenol (4-VP) and 4-vinylguaiacol (4-VG) are one of the most significant problems in modern wine-making, as they can give the wine "off-flavours", described as phenolic, medicinal, pharmaceutical, smoky, spicy and clove-like flavours (Montedoro and Bertuccioli 1986, Rapp and Versini 1996).

White wines can contain vinylphenols in varied amounts, up to several hundred µg·L^{-1} but usually lack ethylphenols, while the contrary is true for red wines, where ethylphenols can reach amounts of a few mg·l^{-1} (Chatonnet *et al.* 1992, 1993; Chatonnet 1993). This compositional framework is due to the genesis of the quoted compounds. Vinylphenols, contributing to "band-aid", gouache, genista-like and spicy scents (Dubois 1983, Versini 1985, Versini *et al.* 1992, van Wyk and Rogers 2000), are mainly formed during alcoholic fermentation by strains of *S. cerevisiae* called POF+ (Phenolic Off Flavour positive) capable of stereospecific enzymatic decarboxylation of the *trans* forms of *p*-coumaric and ferulic acids (Albagnac 1975, Chatonnet *et al.* 1993, Grando *et al.* 1993). Oligomer proanthocyanidins inhibit cinnamate decarboxylase of *S. cerevisiae* (Chatonnet *et al.* 1990), justifying the very low amounts of vinylphenols in red wines.

Ethylphenols come from the enzymatic activities of decarboxylation of the cited cinnamic acids and subsequent reduction of vinylphenols by the *Brettanomyces*/*Dekkera* genus' yeast, apart from small amounts produced, in peculiar growth conditions, by some yeasts and lactic acid bacteria (Chatonnet *et al.* 1995; Barata *et al.* 2006; Couto *et al.* 2006). In contrast to *S. cerevisiae*, *Brettanomyces* has a vinylphenol reductase, and its decarboxylase is not inhibited by proanthocyanidins (Chatonnet *et al.* 1993). 4-EP was found in wine at the end of the 1960s (Webb 1967, Dubois and Brulè 1970) and confers odours defined as stable-, horse sweat-, leather-like (Etievant 1991). In blends with 4-EG, which is also described as sweet in beer (Meilgaard 1975), it gives stable- and animal-like odours in red wine (Chatonnet *et al.* 1992). The 4-EP/4-EG ratio usually ranges from 3.5 to 16 (Chatonnet *et al.* 1992; Pollnitz *et al.* 2000; Alessandria *et al.* 2005; Nicolini *et al.* 2006). Because of the type of aroma, its relatively low limit preference threshold (Chatonnet *et al.* 1992) and rather frequent appearance in wine, 4-EP is the most critical volatile phenol for red wine production.

Analytically, GC-FID and GC-MS, both coupled with several possible extraction methods, are the most frequent approach to measuring volatile phenols (Versini 1985, Chatonnet and Boidron 1988, Chatonnet *et al.* 1993, Ferreira *et al.* 1996, Aznar *et al.* 2001, Dominguez *et al.* 2002, Monje *et al.* 2002, Boido *et al.* 2003, Diez *et al.* 2004; Rocha *et al.* 2004). A recently proposed method by HPLC-CoulArray which does not include any sample preparation simultaneously measures 4-ethylphenol, 4-ethylguaiacol, 4-vinylphenol, and 4-vinylguaiacol in wine (Larcher *et al.* 2006). Other HPLC methods use fluorimetric detection (FLD) to quantify volatile phenols (Larroque *et al.* 1987, Madigan *et al.* 1994, Bettin *et al.* 2002, Meyer *et al.* 2003), but with sample extraction.

In this paper we present a very rapid HPLC-FLD analytical approach usable for systematic process control in wine-making, measuring 4-EP without any sample preparation, with the exception of filtration. A survey of the 4-EP content in commercially available Italian red wines is also shown.

Material and Methods

C h e m i c a l s a n d r e a g e n t s : The chemicals used for the preparation of HPLC mobile phases and the standards used to calibrate and to evaluate the selectivity of the method are given in Tab. 1.

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Table 1

Chemicals and reagents

Materials	Producer
acetonitrile (ACN); HPLC grade; methanol (MeOH); HPLC grade; phosphoric acid (H_3PO_4); 85 %; gallic acid;	VWR-International, Darmstadt, Germany
sodium monobasic phosphate (NaH_2PO_4); 98-102 %; salicylic acid; p-hydroxybenzoic acid; sinapaldehyde;	Carlo Erba Reagents, Rodano, Milan, Italy
water; HPLC grade;	Milli-Q system, Millipore, Bedford, MA
4-vinylphenol (4-VP);	Aldrich Chemical Co., Milwaukee, MI
4-vinylguaiacol (4-VG); 4-ethylphenol (4-EP); 4-ethylguaiacol (4-EG);	Lancaster Eastgate, White Lund, Morecambe
caffeic acid; vanillic acid; ferulic adid;	Roth, Germany
p-coumaric acid; protocatechic adid; tryptophol; tyrosol; eugenol;	Fluka Chemical, Buchs,, Swiss
sinapic acid; gentisic acid; 4-methyl guaiacol; syringaldehyde; syringol; stearic acid; vanillin; umbelliferone; epicatechin;	Sigma-Aldrich, Steinheim, Germany
syringic acid;	Soc. D.ri Mascia-Brunelli Reagents, Milan, Italy
guaiacol;	Lamberto Gallo Reagents, Milan, Italy
malvidin-3-monoglucoside;	In home purification

HPLC-FLD method: The wine sample was filtered on 25 mm x 0.45 µm PTFE syringe cartridge (Alltech, Deerfield, IL) and directly transferred into a 2 ml glass screw-top vial. Analysis was carried out with HPLC Agilent 1100 (Agilent Technologies, Inc., Santa Clara, CA) equipped with a fluorimetric detector (excitation at 225 nm; emission at 320 nm) and Chemstation. Isocratic separation (50 mM NaH_2PO_4 buffer adjusted to pH 3.40 with H_3PO_4: ACN:MeOH, 65:30:5, by vol) was performed on a Zorbax Eclipse Plus C18 column (4.6 mm x 50 mm, 1.8 µm particle size; Agilent). The temperature of the column was 25 °C, the flow rate 1.5 ml·min^{-1}. The injection volume was 10 µl. The analysis time was 5 min.

Validation of the method: Precision (as a RSD %) was studied between 1 and 2000 µg·L^{-1}, covering the range of the most frequent concentrations of 4-EP in wines. 13 concentration levels (1, 2, 5, 10, 20, 50, 100, 200, 500, 750, 1000, 1500, and 2000 µg·L^{-1}) with 10 repetitions per level were studied. Linearity was checked between 50 and 2000 µg·L^{-1}. Critical (L_C) and detection (L_D) limits were calculated according to Hubaux and Vos (1970) and Currie (1997). To this aim, the 4-EP signal was measured in samples spiked at very low concentration levels with appropriate additions of standard to a zero-level wine. The zero-level sample was obtained from a sound and sensorially off-flavour free red wine treated with charcoal (3 g·L^{-1}) to remove any detectable 4-EP amount. The accuracy of the method was checked in comparison with the HPLC-coulometric array method (HPLC-ED) proposed by Larcher *et al.* (2006), analysing 52 commercial Italian red wines.

The data were statistically evaluated using Origin® 7.0 RS0 v 7.0220 software (OriginLab Corporation, Northampton, MA, USA)

HPLC-ED method: A HPLC 2695 Alliance system, controlled by an Empower Pro 2002 data station (Waters Corporation, Milford, MA) was used. The electrochemical detector 5600A (ESA, Bedford, MA), piloted by the CoulArray ESA data processor, was equipped with 8 electrodes set at 280, 340, 380, 430, 490, 550, 650 and 800 mV *versus* Pd/H_2 reference electrode, being the dominant channel at 550 mV for 4-EP. Samples were filtered with a 0.45 µm PTFE syringe filter and collected in 2 ml glass screw-top vials for instrumental analysis.

Red wine samples: 493 DOC wines (Designation of controlled origin) and 227 table wines without geographic specification were analysed. Wine samples which had passed through the chemical laboratory of IASMA Research Centre (Trento) in 2006 for different commercial purposes, *e.g.* authorisation to export or use the DOC mark, were mainly used. For this reason, the bulk of the DOC wines (181) came from the Trentino South-Tyrol region. Many other samples were from the neighbouring regions of Lombardy (61) and Veneto (38), and from Tuscany (79), a region where quality red wines have a significant role. The remaining wines were from 11 other regions.

Results and Discussion

HPLC-FLD method performance: The chromatogram in Fig. 1 shows the specific HPLC-FLD peak patterns of volatile phenols measured in a natural red wine fortified with 4-VG, 4-VP, 4-EP and 4-EG. These compounds elute in a short time and in a zone of the chromatogram apparently not affected by the presence of the compounds shown in Tab. 2 chosen among the normal components of wine liable to possible interference. Nevertheless, poor correlations between the 2 methods for 4-VG, 4-VP and 4-EG were observed (data not shown). In particular, co-elution problems for several wines were highlighted by the CoulArray detector, which takes advantage

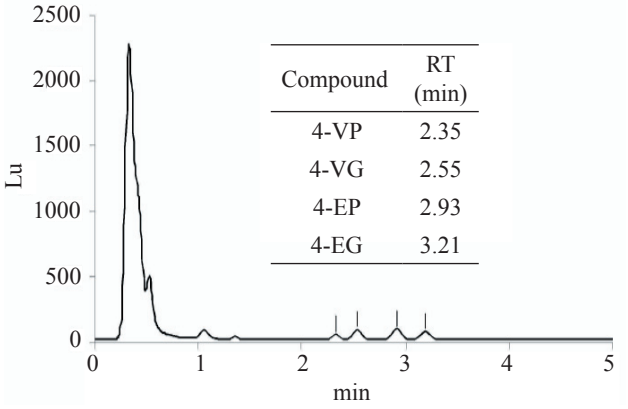


Fig. 1: Chromatogram of the volatile phenols measured with HPLC-FLD in a natural red wine fortified with 4-vinylphenol (4-VP), 4-vinylguaiacol (4-VG), 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG).

Table 2

Retention times (RT) of volatile phenols and possible interfering phenolic compounds

Compound	RT (min)
malvidin	0.37
gallic acid	0.38
protocatechic acid	0.44
epicatechin	0.46
tyrosol	0.52
caffeic acid	0.52
syringic acid	0.53
vanillic acid	0.53
p-hydroxybenzoic acid	0.54
p-coumaric acid	0.67
salicylic acid	0.68
ferulic acid	0.70
gentisic acid	0.71
sinapic acid	0.71
syringaldehyde	0.73
vanillin	0.77
sinapaldehyde	0.81
tryptophol	1.18
4-methyl-guaiacol	1.96
4-vinylphenol	**2.35**
4-vinylguaiacol	**2.55**
4-ethylphenol	**2.93**
4-ethylguaiacol	**3.21**
eugenol	4.13

of its higher discriminative capabilities due to the voltammetric peak pattern based on 8 electrodes. On the contrary, no co-elution interference was ever observed for 4-EP.

The critical (L_C) and detection (L_D) limits calculated according to Hubaux and Vos (1970) and Currie (1997) for 4-EP were 2.0 and 4.0 µg·L^{-1}, respectively. These values are absolutely acceptable in the light of the sensory threshold of this compound and of its usual concentration in wine. It is known that the use of on-line electrochemical derivatization prior to HPLC- fluorescence detection gives significantly lower limits of detection (Meyer *et al.* 2003), but this approach does not seem to be essential in the case of technological control of 4-EP in wine.

The precision (RSD %) of the HPLC-FLD method in a wide range of concentrations is shown in Fig. 2. The RSD

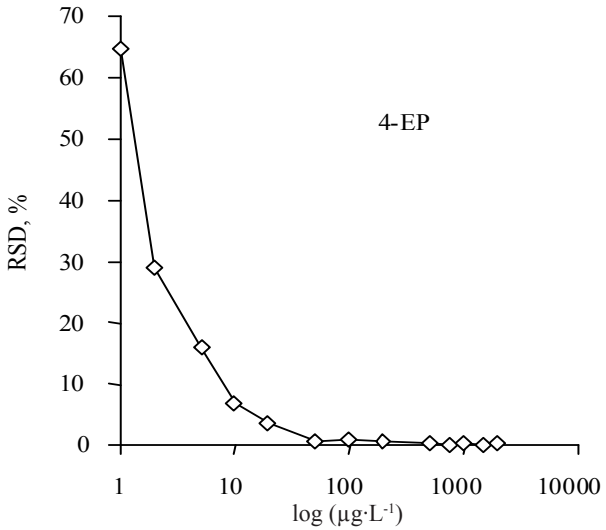


Fig. 2: Semi-logarithmic plot of precision (RSD %) *versus* concentration of 4-ethylphenol.

value is below 3 % for concentration levels higher than 20 µg·L^{-1}, and below 10 % for concentrations higher than 8 µg·L^{-1}. Therefore, the 8 µg·L^{-1} values can be assumed as the quantification limit for the proposed approach. These findings agree with the precision levels usually accepted for general and impurity methods respectively (Currie 1997, Green 1996, Vial and Jardy 1999, Larcher *et al.* 2006).

The linearity of the method is proved up to 2000 µg·L^{-1}, as shown by the parameters of the regression analyses given in Tab. 3.

A positive and statistically highly significant correlation was noted between the values achieved with the 2 methods (Fig. 3).

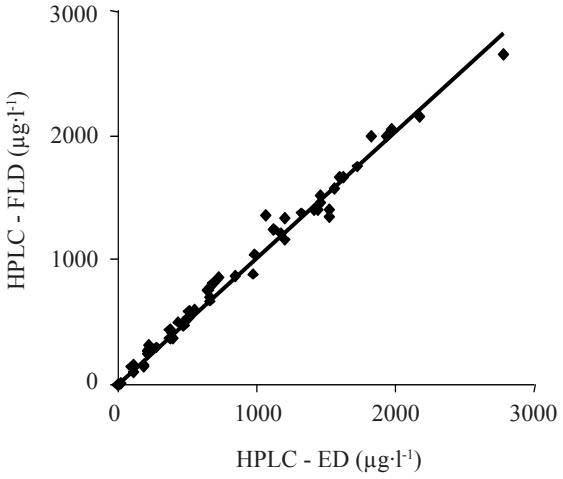
Application of the HPLC-FLD method: The values of classical parameters for statistical distribution (10°, 25°, median, 75° and 90° percentile) of the 4-EP content in the 720 wines analysed were respectively: 48, 140, 325, 860 and 1580 µg·L^{-1}, while the remaining 10 % of samples ranged up to 6.2 mg·L^{-1}. On the basis of the limit preference threshold of 426 µg·L^{-1} (4-EP:4-EG, 10:1) found by Chatonnet *et al.* (1992) for wines distinctively affected by adverse phenolic characteristics, and assuming that the same ratio between ethylphenols is also right for our samples, we estimated that the aroma of 45 % of the wines in the present sample could be negatively affected by ethylphenols.

Fig. 4 shows the distribution of 4-EP content. In the box plots, outlier and extreme values are defined accord-

Table 3

Linearity parameters for 4-ethylphenol measured by HPLC-FLD

n	Intercept (Lu)	SD Intercept (Lu)	Slope (Lu l·µg^{-1})	SD Slope (Lu l·µg^{-1})	SD Regression (Lu)	R^2
8	0.62	1.23	0.934	0.001	2.26	1.000



Intercept (µg·L^{-1})	SD intercept (µg·L^{-1})	Slope	SD slope	SD regression (µg·L^{-1})	R^2
45.2	17.61	0.987	0.016	75.58	0.9868

Fig. 3: Regression line and relative parameters of the contents of 4-ethylphenol in 52 wines measured using HPLC-ED and HPLC-FLD.

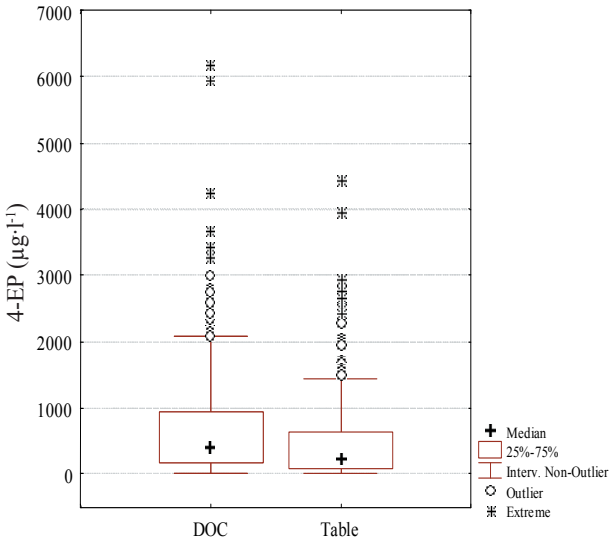


Fig. 4: Box plots of the distribution of the contents of 4-ethylphenol (4-EP) in DOC (Designation of controlled origin; n = 493) and table (n = 227) red Italian wines.

ing to the classical standard of STATISTICA® (StatSoft Italia 6.1 2003) where, if H is the difference obtained by subtracting the first from the third quartile, outliers and extremes are the values exceeding the upper limit of the third quartile of 1.5H and 3H, respectively.

Compared to DOC wines, table wines seem to have lower 4-EP content (Fig. 4), probably as a consequence of their shorter or complete lack of maturing in wood during the winemaking process and their usually shorter period of ageing, both factors limiting the effects of eventual "Brett" pollution.

Because of the kind of sampling and the lack of additional information about the winemaking procedures applied, any further discussion, e.g. "by region" or "by winemaking technique", is not justified.

Conclusion

The HPLC-FLD method proposed for the analysis of 4-ethylphenol - the most problematic and widely present volatile phenol in red wines - proved to be precise, accurate and sensitive. Furthermore, it has some important characteristics making it suitable for becoming a routine method for monitoring the risk from *Brettanomyces*, making the winemaking process control easier. It is indeed very fast and simple, as it does not require sample preparation, apart from preventive 0.45 µm filtration, and uses a relatively cheap and well-established detector in oenological laboratories as it is already routinely applied to the analysis of polyphenols, mycotoxins, and, after derivatization, amino acids and biogenic amines.

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