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Evaluation of Liquid Chromatographic Behavior of Lumazinic Derivatives, from α-Dicarbonyl Compounds, in Different C18 Columns: Application to Wine Samples Using a Fused-Core Column and Fluorescence Detection

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ABSTRACT: Several C18 columns, packed with totally porous particles of different sizes and shell thicknesses, have been compared for simultaneous determination of α-dicarbonyl compounds, previous derivatization to lumazinic derivatives. Chromatographic conditions for the separation have been optimized for each column, and chromatographic parameters have been calculated and exhaustively compared. A core−shell C18 column provided the best results, and a HPLC method with fluorimetric detection has been proposed. The developed method has been validated in terms of linearity, precision, and sensitivity. Detection and quantification limits obtained were comprised between 0.02 and 0.30 and 0.07 and 1.0 ng mL^{-1}, respectively, while RSD values obtained were lower than 6% and 5% in intraday and interday repeatability studies, respectively. The method has been applied to analysis of the α-dicarbonyl compounds in different types of wines. The higher levels of the total α-dicarbonyl compounds were found in sweet wines and the lower levels in white wines.

KEYWORDS: *α-dicarbonyl compounds, lumazinic derivatives, C18 columns, fluorescence detection, wine samples*

1. INTRODUCTION

α-Dicarbonyl compounds are reactive intermediates formed in physiological systems by lipid peroxidation of polyunsaturated fatty acids[1,2] and biological systems through the Maillard reaction by degradation of sugars.[3,4] In processed foods, these compounds are formed from carbohydrates during thermal processing in the course of the Maillard reaction, and they are also important compounds present in food products obtained by fermentation processes, such as wine. In this matrix, α-dicarbonyl compounds are formed as a consequence of the malolactic fermentation, a process that can occur after or simultaneously with alcoholic fermentation. During malolactic fermentation decarboxylation of L-malic acid takes place, and in consequence, acidity levels are reduced and several compounds related with the aroma and flavors are formed.[5,6] Among the identified formed compounds, dicarbonyl compounds with a short chain such as diacetyl (DIA), glyoxal (Gly), methylglioxal (MGly), 2,3-pentanedione (2,3-Pen), and phenylglyoxal (PhGly) are included.[5−7] These compounds are present in all types of wines, with levels higher in red wines,[8] and these concentrations are increased during age.[9] They play an important role in wines because they have a great influence on flavor and sensory characteristics. Specifically, DIA and 2,3-Pen are the most important compounds in the aroma of wines, and DIA is responsible for the buttery flavor of certain wines.[10] In addition, α-dicarbonyl compounds present reactivity with other components, which contribute to the loss of nutritional quality and increment production of toxic compounds in the wine.[11]

Glucosones, such as glucosone (GS) and 3-deoxyglucosone (3-DG), are α-dicarbonyl compounds with a C-6 backbone, which can be also formed from hexoses through monosaccharide autoxidation.[12] To date, to our knowledge, no data about the quantities of these compounds in wines have been reported.

A number of methods have been developed for determination of α-dicarbonyl compounds in several matrices such as biological samples and processed and fermented foods, and the prelabeled HPLC method is the most common. The more frequent derivatization reaction is formation of quinoxaline derivatives, and different diaminobenzenes have been proposed as derivatizing reagents: 2,4-dinitrophenylhydrazine,[13] 1,2-diaminobenzene,[14] and 1,2-diamino-4,5-dimethoxybenzene.[15,16] Other reagents used have been 6-hydroxy-1,2,3-triaminopirimidine,[17,18] 2,3-diaminonaphthalene,[19] or 5,6-diamino-2,4-hydroxypyrimidine.[20]

For analysis of these compounds in wine samples, methods such as gas chromatography with a mass-selective detector (GC-MS)[21] or a thermoionic detector (GC-NPD)[22] have been proposed as alternative techniques. However, the methodology more frequently used is based on formation of quinoxaline derivatives and analysis by RP-LC with photometric detection.[22−24] All HPLC methods employ C18 columns with particles of 5 μm for chromatographic separation of the corresponding derivatives.[25]

In this point, development of packed columns with smaller particle diameter to work in reverse phase could improve analysis in terms of separation efficiency and also in terms of reduction of the analysis time, which is an important factor to

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take into account. In this way, columns packed with core−shell and totally porous sub-2 μm particles have been most frequently employed in qualitative and quantitative analysis.[26] The efficiency of a packed column is described by the Deemter's plate height model,[27] which explains the variation of this parameter with the linear velocity. The key concept of porous shell (or fused-core) particles is to increase both efficiency and separation velocity by reducing the mass transfer (C term in the van Deemter curve).[28] This is possible because it has a silica core that allows keeping their diameter large enough to avoid pressure limitations at high linear velocities. This type of column is constituted by a solid silica core of 1.7 μm in size with a porous outer layer 0.5 μm thick and a total particle size of 2.7 μm. On the other hand, packing of columns with porous sub-2 μm particles consist of derivatized, high-purity porous-silica microspheres having reproducible bonded monolayers. The rigidity and extremely narrow particle-size distribution of totally porous particles allow high-resolution and fastest analysis since operation at high flow rates is possible. However, this column has the disadvantage that usually an ultra-high-pressure liquid chromatography (UPLC) system is required to achieve the best results, while a fused core column allows fast separation on a conventional LC system, without significant loss in efficiency or resolution.[29] In the past decade, comparative studies about the separation of compounds in columns with different particle size, such as pharmaceutical compounds[30] or aflatoxins,[29] among others, have been realized by several authors, and in all cases, they concluded that columns with small particle size achieve major separation efficiency and improve the analytical method developed. On the other hand, core−shell columns have been compared with sub-2 μm porous particles columns in pharmaceutical compound analysis,[31] and a higher capacity of the core−shell columns to accomplish better separation efficiency under the same operation conditions was demonstrated.

The present paper is focused in two ways: first, to investigate the chromatographic behavior, in different packed columns, of the lumazinic derivatives formed from α-dicarbonyl compounds, in order to explore the advantages of columns with core−shell and minor particle size in analysis of these compounds; second, apply the obtained results in development of a simple, rapid, and competitive method to determine these compounds in different types of wines.

2. MATERIALS AND METHODS

2.1. Chemicals, Standards, and Samples. DIA, 2,3-Pen, and PhGly, all of 97% purity, and aqueous solutions of Gly and MGly, 40% purity, were purchased from Sigma-Aldrich (Madrid, Spain). GS (98%) and 3-DG (95%) were obtained from Santa Cruz Biotechnology (California, USA). Stock standard solutions of 3-DG (100 μg mL^{-1}), GS (400 μg mL^{-1}), and PhGly (135 μg mL^{-1}) were prepared by dissolving in ultrapure water adequate amounts of the powder presentation of each compound. Stock solutions of DIA (600 μg mL^{-1}), 2,3-Pen (150 μg mL^{-1}), Gly (50 μg mL^{-1}), and MGly (70 μg mL^{-1}) were prepared by weighting and dilution of the adequate aliquots in ultrapure water. Stock analyte solutions were prepared separately and stored at 4 °C. Working standard mixture solutions were daily prepared by suitable dilution of stock analyte solutions with ultrapure water. 5,6-Diamino-2,4-hydroxypyrimidine sulfate (DDP) (95%) was purchased from Sigma-Aldrich, and a 8.6 mM stock solution was daily prepared by dissolving adequate amounts in ultrapure water containing 200 mM β-mercaptoethanol (β-ME), also provided from Sigma-Aldrich. Ammonia/ammonium chloride buffer was prepared by dissolving ammonium chloride from Panreac (Barcelona, Spain) in ultrapure water and fixing the pH at 10.0 with ammonia (Panreac). Phosphoric acid (85%) was provided by Scharlau (Barcelona, Spain), acetic acid (95.5%) by Romil Chemical LTD (Cambridge, England), and formic acid (98%) by Fluka (Seelze, Germany). Different solutions of pH between 2.9 and 4.0 were prepared by dilution of an adequate volume of concentrated acid in ultrapure water. Methanol (MeOH), HPLC grade, was purchased from Panreac. Ultrapure water was obtained from a Milli-Q water system (Millipore S.A.S., Molsheim, France). Wines analyzed were acquired from local markets and kept at 4 °C, avoiding exposure to direct light. Specifically, red wines (Monasterio de Tentudía, Ribera del Duero, and Merlot), white wines (Rioja Comportillo, Viña Canchal, and Manzanilla), red sweet wines (Vin Santo and Port Tawny), and white sweet wines (Málaga Dulce and Oremus Takaji) were purchased. Most of them are from Spain, with the exception of red sweet wines (from Italy and Portugal) and Oremus Tokaji wine (from Hungary).

2.2. Instrumentation and Software. Chromatographic studies were performed on an Agilent model 1100 LC instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with an online degasser, quaternary pump, manual six-way injection valve, UV−vis diode-array detector, rapid scan fluorescence spectrophotometer detector, and the Chemstation software package to control the instrument, data acquisition, and data analysis. Chromatographic studies and analytical separation were carried out in columns purchased from Agilent. The column temperature was controlled by a coil with recirculating water, in which the temperature was selected through a thermostatic bath. The injection volume was set at 20 μL for the Zorbax-Eclipse XDB C18 column and at 10 μL for columns with minor particle size. The flow rate was 1 mL min^{-1} for Eclipse columns and 0.5 mL min^{-1} for the Poroshell column. Detection was performed with a fluorimetric detector at 450 nm, exciting at 270, 330, and 350 nm.

A Crison MicropH 501 m (Barcelona, Spain), equipped with a combined glass/saturated calomel electrode, was used for pH measurements.

Calibration curves and analytical figures of merit were performed by means of the ACOC program,[32] in MATLAB code.

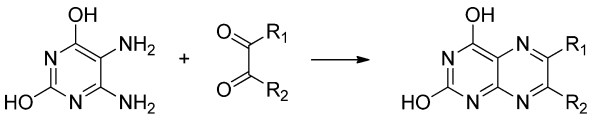
2.3. General Procedure: Calibration Curves. To build the calibration curves, aliquots of each α-dicarbonyl compound in variable concentration were placed in 25 mL volumetric flasks, and 0.125 mL of ammonia/ammonium chloride buffer (0.5 M, pH = 10) and 1.5 mL of 8.6 mM DDP solution containing 200 mM β-ME were added. After 30 min at 60 °C, solutions were cooled in ice water and diluted with 0.4 mM phosphoric acid solution (pH 3.2) up to the mark. Resulting solutions were filtered through a 0.22 μm nylon filter, and aliquots of 10 μL were injected in the chromatographic system. Separation was performed with a Poroshell 120 column, employing a solution of 0.4 mM phosphoric acid solution (pH 3.2)/MeOH (95:5, v/v) (eluent A) and MeOH (eluent B) as mobile phase, with the following gradient mode: 0−5 min, 0% B; 5−13 min, 30% B; 13−13.5 min, 40% B. These conditions were maintained until 25 min, and finally, the eluent B content was decreased to the initial conditions (0% B) and the column was re-equilibrated for 5 min. The eluate was fluorimetrically monitored at 450 nm (exciting at 270, 330, and 350 nm), and peak areas were used as analytical signal. Three replicas of each standard were used. Temperature was fixed at 25 °C, and a flow rate of 0.5 mL min^{-1} was employed.

2.4. Analysis of α-Dicarbonyl Compounds in Wine Samples. Analysis of the α-dicarbonyl compounds in red, white, and sweet wines was carried out by the standard addition method. For each wine sample, adequate volumes were added in a 25 mL volumetric flask. Increasing volumes of a standard mixture of the dicarbonyl compounds were added, and the general procedure was followed for the derivatization step. Separation of derivatives was carried out in a Poroshell 120 column thermostated at 25 °C. A mobile phase composed of 0.4 mM phosphoric acid solution (pH 3.2)/MeOH (95:5, v/v) (eluent A) and MeOH (eluent B) was employed by following the same gradient mode described in section below. Fluorescence excitation/emission wavelengths of 330/450 nm were employed.

3. RESULTS AND DISCUSSION

The first aim of this work is to realize a comparative study of the chromatographic behavior of the lumazinic derivatives from α-dicarbonyl compounds using three different C18 columns. In any case, the focus was to obtain baseline separation of all compounds in the shortest time and with the lowest solvent consumption as possible in order to achieve a simple, a fast, and an environmental friendly method, susceptible of being applied to analysis of these compounds in wine samples.

3.1. Formation of Lumazinic Derivatives. The most extensively applied methods in wine analysis for control of α-dicarbonyl compounds levels are based on reaction with 2,3-diaminobenzene to form quinoxaline derivatives that can be determined by HPLC with UV detection at 313 nm,[22] fluorimetric detection exciting at 350 nm,[33] or GC with a MS or NPD detector.[34] These derivatization reactions need high temperatures and reaction times close to 3 h. In this paper, DDP is proposed as derivatizing reagent in order to simplify the derivatization reaction. Optimization of the conditions for formation of the lumazínic derivatives for Gly and MGly was described in previous work.[20] In this work, use of DDP as derivatizing reagent was expanded to the seven α-dicarbonyl compounds that can be present in wine samples. The scheme of the derivatization reaction is shown in Figure 1.



Gly: $R_1 = R_2 = H$

MGly: $R_1 = H$, $R_2 = CH_3$

DIA: $R_1 = R_2 = CH_3$

2,3-Pen: $R_1 = CH_3$, $R_2 = CH_2CH_3$

PhGly: $R_1 = H$, $R_2 = Ph$

3-DG: $R_1 = H$, $R_2 = -CH_2-(CHOH)_2-CH_2OH$

GS: $R_1 = H$, $R_2 = -(CHOH)_3-CH_2OH$

Figure 1. Derivatization reaction of α-dicarbonyl compounds with 5,6-diamino-2,4-dihydropyrimidine (DDP) to yield the corresponding lumazinic derivatives.

The physicochemical variables that influence the yield and reaction rate, such as pH, temperature, reaction time, and DDP excess, were studied chromatographically by monitoring the derivatization reaction. In accordance with previous studies, the yield of the reaction of α-dicarbonyl compounds with DDP was optimal at pH 10, and this value was fixed in all solutions with 0.125 mL of ammonia/ammonium chloride buffer (0.5 M). Temperature and reaction time are two very important parameters that influence in the DDP−dicarbonyl compound reaction rates. For this reason, a series of standard mixture solutions containing 1.0 μg mL^{-1} GS, 400.0 ng mL^{-1} 3-DG, 100.0 ng mL^{-1} Gly, 100.0 ng mL^{-1} MGly, 100.0 ng mL^{-1} DIA, 120.0 ng mL^{-1} 2,3-Pen, 500.0 ng mL^{-1} PhGly, and DDP solution (7.5×10^{-2} mM) (concentrations enough so that all obtained derivatives provide a good signal, easily measurable, and whose variations are detectable, when the conditions of the reaction are changed) were placed in 25 mL volumetric flasks at pH 10 and incubated at 45, 60, and 80 °C during several heating times in the range 2−60 min. After completing the reaction times, the flasks were cooled in ice water and made to volume with 0.4 mM phosphoric acid (pH 3.2), and aliquots of 10 μL of these solutions were injected in the chromatographic system. For all α-dicarbonyl compounds, except for DIA and PhGly, the peak area of the derivatives increased with temperature and is maintained constant between 60 and 80 °C. The peak area of DIA and PhGly derivatives increased up to 60 °C, and for higher temperatures, the signal decreases. Finally, we selected 60 °C as the optimum heating temperature. With respect to the heating time, the peak area of the seven lumazinic derivatives increased up to 15 min. However, the reaction rate for formation of GS and 3-DG lumazinic derivatives is more dependent on the heating time, and after 60 min, these reactions had not been completed. With the object of not extending the total time of the derivatization reactions, a compromise value of 30 min was selected as optimum.

In the optimized conditions of temperature and reaction time, the DDP/total aldehyde ratio was studied in a range between 10:1 and 70:1. For all compounds a 10:1 ratio was enough to obtain the maxima signal; however, for GS and DIA a 30:1 ratio was necessary, and this relation was chosen as optimum.

Studies carried out showed that in the derivatization conditions DDP is degraded into several products, with the object of minimizing the appearance of degradation products that can interfere in analysis of some of the lumazinic derivatives, and in accordance with previous research[14,18] β-ME is added to the DDP solution. The influence of this reagent in the stabilization of DDP has been studied by preparing DDP solutions (0.8 mM) in the presence of different concentrations of β-ME (200, 400, and 600 mM). Aliquots of each solution were placed in a 25 mL volumetric flask, heated at 60 °C for 30 min at pH 10, diluted with 0.4 mM phosphoric acid (pH = 3.2) until the mark, and injected into the chromatographic system. It can be observed that degradation of DDP was avoided in the same extension, independent of the concentration of β-ME employed. Therefore, a solution of DDP was prepared in the presence in 200 mM of this compound. Figure 2A shows the chromatograms of a DDP solution in the presence and in the absence of 200 mM β-ME, and it can be observed that, in the presence of β-ME, degradation of the reagent is practically avoided and signal decreases.

In Figure 2B, a representative chromatogram from lumazinic derivatives obtained in physicochemical-optimized conditions is shown. As can be seen, all α-dicarbonyl compounds become a single lumazinic derivative, except 2,3-Pen and PhGly, which form two derivatives. For unequivocal identification of the derivatives, a comparison of the retention time of Gly derivative with a reference standard of lumazine was done. A standard solution containing 50 ng mL^{-1} of lumazine was injected in the chromatographic system, in the conditions previously described. As expected, retention time and excitation spectra of both peaks are coincident, and this allows confirmation of formation of the lumazinic derivatives.

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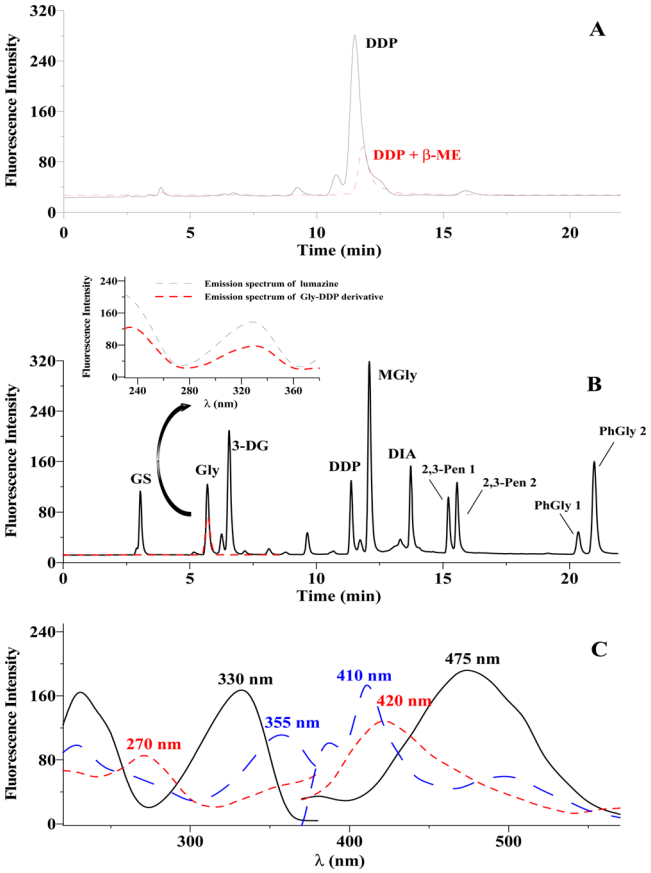

Figure 2. (A) Influence of the presence of β-ME in the degradation process of DDP in the derivatization reaction conditions. Chromatograms correspond to a DDP solution (7.5×10^{-2} mM) in the absence of β-ME (continuous line) and in the presence of 200 mM of β-ME (dashed line). $\lambda_{exc}/\lambda_{em}$ = 330/450 nm. (B) HPLC/FLD chromatograms from a stock standard solution of the α-dicarbonyl compounds in the presence of DDP solution (7.5×10^{-2} mM) at pH 10, derivatized in optimized conditions (heating for 30 min at 60 °C) (continuous line), and of a standard solution of lumazine, 50 ng mL^{-1} (dashed line). $\lambda_{exc}/\lambda_{em}$ = 330/450 nm. [GS] = 1.0 μg mL^{-1}, [3-DG] = 400.0 ng mL^{-1}, [Gly] = 100.0 ng mL^{-1}, [MGly] = 100.0 ng mL^{-1}, [DIA] = 100.0 ng mL^{-1}, [2,3-Pen] = 120.0 ng mL^{-1}, and [PhGly] = 500.0 ng mL^{-1}. (C) Excitation and emission spectra of lumazinic derivatives GS, 3-DG, Gly, MGly, DIA, and 2,3-Pen (—), PhGly 1 (---), and PhGly 2 (———).

Spectral characteristics of all derivatives were studied in order to achieve higher sensitivity in their analysis, and excitation and emission spectra were obtained for each peak (Figure 2C). All of them, except the PhGly derivatives, presented excitation and emission wavelengths characteristics of the lumazines at 330 and 475 nm, respectively. Also, the two derivatives of 2,3-Pen have identical spectral characteristics, with excitation and emission wavelengths at 330 and 475 nm, respectively, and both are formed in similar extension, as can be seen in the chromatogram shown in Figure 2B, where the peak areas of the two derivatives are similar. In the case of the PhGly derivatives, its spectral characteristics are different. Thus, the lumazinic derivative with minor elution time (PhGly 1) presents excitation and emission wavelengths at 270 and 420 nm, respectively, while the other derivative (PhGly 2) shows excitation at 355 nm and emission at 410 nm. On the other hand, in this case formation of one of the derivatives (PhGly 2) is more favorable that the other, probably due to steric impediments.

As a consequence of the different excitation and emission wavelengths of the lumazinic derivatives, a fluorescence detector was programed in multiexcitation mode and each chromatogram was recorded simultaneously exciting at 270, 330, and 350 nm. A wavelength of 450 nm was selected as the emission wavelength as a compromise in order to analyze all compounds in only one run.

3.2. Optimization of Separation and Comparison of Columns Efficiency. With the aim of selecting the more appropriate column for separation of the seven α-dicarbonyl-DDP derivatives, we realized a systematic comparison of three columns assayed. This study was performed in order to develop a simple, reliable, and robust method for efficient separation and quantification of these compounds. In this study, reversed-phase C18 columns with different dimensions and different size and surface particles were tested. The columns used were Zorbax-Eclipse XDB C18 (5.6 μm), Poroshell 120 (2.7 μm), and Zorbax-Eclipse XDB-C18 RRHT (1.8 μm). The most important parameters that influence chromatographic separation, such as mobile phase composition, flow rate, and column temperature, were assayed with each column.

The composition of the mobile phase has a large influence in complete resolution of the derivatives. Due to the analytes differing widely in polarity, isocratic elution is unviable and it was necessary to use a binary gradient. The two solvents employed in the gradient elution were mixtures of acid:MeOH, as solvent A, and MeOH 100% as solvent B. The presence of acid in the mobile phase is essential to complete resolution of the lumazinic derivatives, so the influence of the nature of the acid used in solvent A and the pH of the mobile phase was studied. Acetic, phosphoric, and formic acids were assayed, and the pH was varied between 2.9 and 4.0 for each acid. The behavior of the α-dicarbonyl–DDP derivatives was identical with the three columns assayed, independent of the acid used. In general, the retention times of DIA, 2,3-Pen, and PhGly lumazine derivatives are not affected, and the retention times of the less retained derivatives decreased as the pH increased. Finally, 0.4 mM phosphoric acid (pH 3.2) was selected as the aqueous component of solvent A for the three columns.

Below, the composition of solvent A was optimized for each column by modifying the phosphoric acid (0.4 mM)/MeOH ratio between 99/1 and 99/5, v/v. Also, different gradients with methanol as solvent B were assayed to achieve baseline separation of all derivatives in a time as short as possible. Optimized conditions for each column are summarized in Table 1.

Also, the influence of the temperature in the separation has been studied with the three columns. In general, the capacity factors decreased with increasing temperature for all compounds. It is noticeable that this effect is greater for the less retained compounds and when the mobile phase is 100% aqueous, as can be seen in Figure 3. In gradient mode, as the percentage of methanol increased and the mobile phase viscosity was varying, the effect of temperature in the capacity factors decrease, and the variation in the last four analytes eluted is lower.

Representative chromatograms obtained in the optimized conditions with the three columns assayed, XDB-C18, Poroshell, and XDB-C18 RRHT, are shown in Figure 4. As can be seen, use of a conventional monomeric C18 column results in a lack of complete separation between the peak of the

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Table 1. Column Characteristics and Optimum Chromatographic Parameters for Each Column

column	mobile phase	gradient mode	other parameters
Agilent Zorbax Eclipse XDB-C18 (150 mm × 4.6 mm, 5.6 μm)	40 mM H_3PO_4/MeOH (98/2, v/v) (eluent A) MeOH (eluent B)	0–8 min, 0% B; 8–13 min, 20% B; 13–14 min, 35% B; 35% B until 25 min flow rate: 1 mL min^{-1}	T_{column}: 30 °C $V_{injection}$: 20 μL t_0: 1.4 min P_0: 145 bar
Agilent Poroshell 120 EC-C18 (150 mm × 3 mm, 2.7 μm)	40 mM H_3PO_4/MeOH (95/5, v/v) (eluent A) MeOH (eluent B)	0–5 min, 0% B; 5–13 min, 30% B; 13–13.5 min, 40% B; 40% B until 25 min flow rate: 0.5 mL min^{-1}	T_{column}: 25 °C $V_{injection}$: 10 μL t_0: 1.8 min P_0: 245 bar
Agilent Zorbax Eclipse XDB-C18 RRHT (50 mm × 4.6 mm, 1.8 μm)	40 mM H_3PO_4/MeOH (98/2, v/v) (eluent A) MeOH (eluent B)	0–5 min, 0% B; 5–13 min, 35% B; 35% B until 18 min flow rate: 1 mL min^{-1}	T_{column}: 20 °C $V_{injection}$: 10 μL t_0: 0.5 min P_0: 180 bar

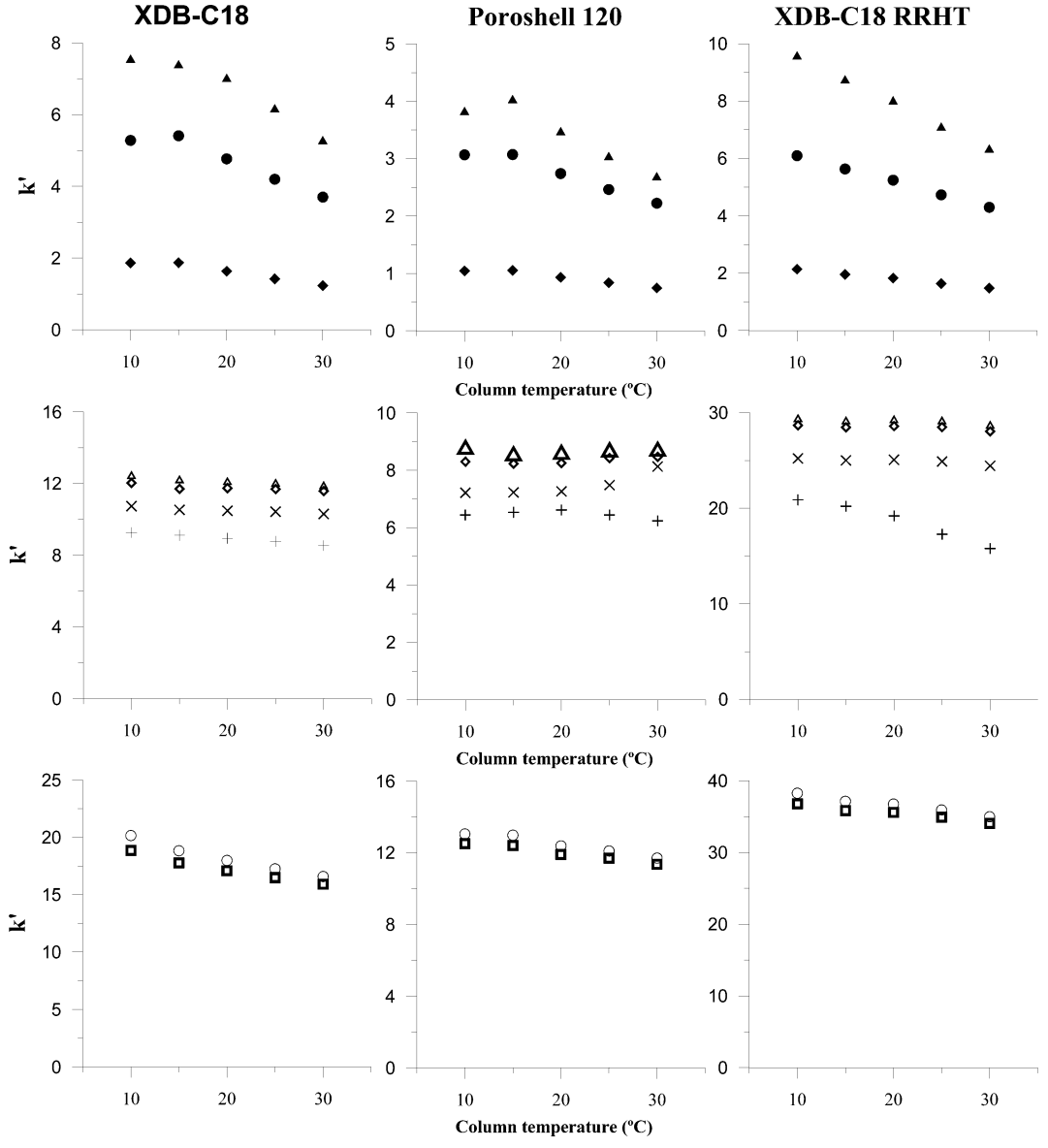

Figure 3. Variation of capacity factor (k') with column temperature for the three columns studied: (◆) GS, (●) Gly, (▲) 3-DG, (+) MGly, (×) DIA, (◇) 2,3-Pen (1), (△) 2,3-Pen (2), (□) PhGly (1), and (○) PhGly (2).

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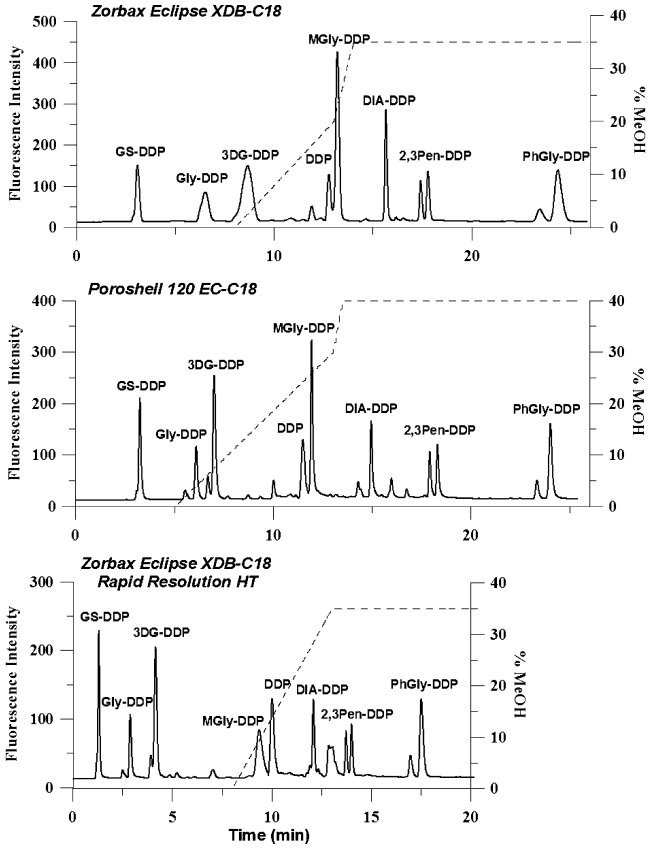

Figure 4. Optimum separation of lumazinic derivatives in the three columns assayed. Dashed lines indicated the gradient program employed. $\lambda_{exc}/\lambda_{em}$ = 330/450 nm.

MGly−DDP derivative and the peak of the DDP reagent. In addition, more polar analytes presented peak tailing, and in general, peak shape was wider than in the other columns. On the other hand, lower analysis time is obtained with the XDB-C18 RRHT column. It is important to remark that elution order of MGly derivative and DDP is inverted in this column. The Poroshell column achieves complete separation of the compounds in 25 min with narrow and symmetric peaks.

Once elution conditions were established, chromatographic parameters (column resolution (R), capacity factor (k'), and theoretical plate number (N)) were calculated to obtain information about the efficiency of each column in the chromatographic separation of the lumazinic derivatives. These parameters have been calculated following eqs 1−3, and the obtained values are summarized in Table 2.

$$R = \frac{1.17(t_{R_2} - t_{R_1})}{(w_{1/2})_1 + (w_{1/2})_2} \quad (1)$$

$$k' = \frac{t_R - t_0}{t_0} \quad (2)$$

$$N = 5.54x(t_R/w_{1/2})^2 \quad (3)$$

t_R is the retention time, t_0 is the dead time, and $w_{1/2}$ is the width of the peak at half height.

Retention time is the most important parameter to know the length of analysis. For all compounds, using the optimized elution conditions, retention times were shorter with the XDB-C1 8 RRHT column than with conventional C18 and Poroshell columns, getting a total scan time of 18 min, against the 25 min for the other two columns. Thus, the column length reduction decreases analysis time, as expected. Retention times of the lumazinic derivatives in the three columns assayed can be seen in Table 2.

Peak resolution describes the degree of separation between two compounds. Usually an R value of 1 is accepted to consider that a separation is satisfactory, but R must be equal to or higher than 1.5 to achieve a baseline resolution. As can be seen in Table 2, better resolution for all compounds is achieved when the separation was carried out with a Poroshell column. It is important to highlight that R has been calculated with respect to the previous peak eluted; thus, in the case of columns XDB-C18 and Poroshell, MGly resolution is referred to its separation from the DDP. However, as the order of elution between DDP and MGly derivative changes in XDB-C18 RRHT, the resolution of MGly is calculated with regard to the 3-DG derivative, with a resolution value of DDP-MGly of 1.4 ± 0.1. In relation to k' values, in the case of the Poroshell column, they are comprised between 0.84 and 12.7, which are near the ideal values (1 and 10),[35] while for XDB-C18 RRHT, k' values are too high (approximately 35 for the last derivative). This is in agreement with the fact that superficially porous particles typically have about one-half to three-quarters the surface area of totally porous particles, resulting in a smaller capacity factor value for columns of core−shell particles in size comprised between 2.5 and 2.7 μm.[36] On the other hand, N values, calculated based on the width of peak at half height, were greater for the Poroshell column in all cases, which indicates more efficient chromatographic separation.

Other aspects like solvent consumption were taken into account. In this way, although the analysis time for the XDB-C18 RRHT column is lower, solvent consumption is higher than with Poroshell (12.5 against 18 mL per run) due to the flow rate employed with this column being one-half.

In order to evaluate the precision of analysis with the three columns, repeatability was assessed by injection of 10 standard solutions, in the optimized conditions, for each column. The RSD from the signal of the peak area was between 1.6% and 7.4% for XDB-C18, 2.4% and 6.3% for XDB-C18 RRHT, and 2.4% and 6.1% for Poroshell. Therefore, significant differences were not found.

In summary, although the XDB-C18 RRHT column has the fastest elution time, resolution was enhanced with the core−shell column. Also, the solvent reduction achieved with this column is a good advantage with the aim of developing a method of analysis less polluting as possible; thus, the Poroshell 120 column was chosen for the following experiments.

3.3. Method Validation: Analytical Parameters. For validation of the method, a calibration curve of each compound was established employing the peak area as analytical signal and using the optimized conditions for the selected Poroshell column (Table 1). The linearity of the method was assessed by preparing calibration standards with concentrations ranging from 10.0 to 600 ng mL^{-1} of GS, from 4.0 to 120.0 ng mL^{-1} of Gly and 3-DG, from 4.0 to 40.0 ng mL^{-1} of MGly and DIA, from 4.0 to 150.0 ng mL^{-1} of PhGly, and from 4.0 to 60.0 ng mL^{-1} of 2,3-Pen. The pH 10 of the derivatization reaction was maintained with ammonia/ammonium chloride buffer, and the concentration of DDP was 0.86 mM, as previously explained in section 2.3. Standard solutions containing all α-dicarbonyl compounds were prepared in triplicate for each concentration level, and the analytical figures of merit were calculated

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Table 2. Chromatographic Parameters Calculated for Each α-Dicarbonyl Compound in the Three Columns Assayed

column	α-dicarbonyl compound	$t_R \pm SD^a$ (min)	$R \pm SD^a$	$k' \pm SD^a$	$N \pm SD^a$
Zorbax Eclipse XDB-C18	GS	2.89 ± 0.05	6.59 ± 0.06	1.06 ± 0.03	1480 ± 77
	Gly	5.90 ± 0.10	7.41 ± 0.18	3.24 ± 0.09	1984 ± 148
	3-DG	7.80 ± 0.20	3.03 ± 0.04	4.57 ± 0.16	2052 ± 145
	MGly	12.79 ± 0.07	1.17 ± 0.07	8.12 ± 0.03	27 283 ± 1593
	DIA	15.34 ± 0.03	8.57 ± 0.10	9.93 ± 0.02	47 071 ± 2150
	2,3-Pen (1)	17.20 ± 0.02	7.07 ± 0.06	11.27 ± 0.02	81 245 ± 1595
	2,3-Pen (2)	17.55 ± 0.02	1.34 ± 0.01	11.51 ± 0.02	72 407 ± 1703
	PhGly (1)	22.70 ± 0.06	12.67 ± 0.22	15.18 ± 0.04	27 549 ± 1303
	PhGly (2)	23.51 ± 0.07	1.40 ± 0.02	15.76 ± 0.04	24 488 ± 754
Poroshell 120 EC-C18	GS	3.30 ± 0.02	9.79 ± 0.08	0.84 ± 0.01	4348 ± 117
	Gly	6.25 ± 0.05	13.04 ± 0.11	2.49 ± 0.01	10 018 ± 177
	3-DG	7.31 ± 0.06	4.06 ± 0.03	3.07 ± 0.01	12 178 ± 149
	MGly	12.05 ± 0.04	1.58 ± 0.03	5.72 ± 0.04	34 227 ± 632
	DIA	15.04 ± 0.05	11.68 ± 0.11	7.39 ± 0.05	57 992 ± 688
	2,3-Pen (1)	18.03 ± 0.04	12.06 ± 0.12	9.05 ± 0.06	88 847 ± 2298
	2,3-Pen (2)	18.45 ± 0.04	1.68 ± 0.02	9.29 ± 0.06	82 818 ± 2207
	PhGly (1)	23.70 ± 0.10	17.63 ± 0.45	12.28 ± 0.10	74 283 ± 5729
	PhGly (2)	24.60 ± 0.10	2.08 ± 0.09	12.70 ± 0.11	76 963 ± 9321
Zorbax Eclipse XDB-C18 RRHT	GS	1.30 ± 0.02	6.34 ± 0.17	1.65 ± 0.05	931 ± 52
	Gly	2.88 ± 0.03	7.96 ± 0.16	4.87 ± 0.13	2605 ± 122
	3-DG	4.13 ± 0.04	5.10 ± 0.14	7.42 ± 0.21	4023 ± 188
	MGly	9.37 ± 0.09	12.13 ± 0.23	18.10 ± 0.44	3927 ± 186
	DIA	12.08 ± 0.03	8.54 ± 0.16	23.63 ± 0.55	69 012 ± 7705
	2,3-Pen (1)	13.73 ± 0.03	8.81 ± 0.20	27.00 ± 0.63	86 418 ± 4532
	2,3-Pen (2)	14.00 ± 0.03	1.45 ± 0.03	27.55 ± 0.64	80 582 ± 7418
	PhGly (1)	16.95 ± 0.06	10.83 ± 0.44	33.54 ± 0.75	39 543 ± 3950
	PhGly (2)	17.20 ± 0.10	1.57 ± 0.06	34.63 ± 0.77	43 576 ± 3225

[a]SD: standard deviation.

Table 3. Calibration Data and Validation Parameters

α-dicarbonyl compound	linear range (ng mL^{-1})	intercept ± SD[a]	slope ± SD[a] (ng mL^{-1})	R^2	LOD[b] (ng mL^{-1})	LOQ[c] (ng mL^{-1})	precision[d] intraday (n = 10)	precision[d] interday (n = 6)
GS	10–600	(−29) ± 17	2.10 ± 0.02	0.9989	0.31	1.03	2.9	5.0
Gly	4–120	464 ± 33	11.80 ± 0.20	0.9975	0.05	0.18	4.5	4.9
3-DG	4–125	70 ± 34	7.74 ± 0.08	0.9986	0.08	0.28	3.6	4.3
MGly	4–40	26 ± 26	32.50 ± 0.40	0.9978	0.02	0.07	4.5	2.1
DIA	4–30	188 ± 22	24.00 ± 0.50	0.9963	0.03	0.10	6.0	2.3
2,3-Pen (1)	4–150	(−72) ± 32	7.80 ± 0.10	0.9974	0.09	0.30	3.9	3.4
2,3-Pen (2)		(−12) ± 18	8.75 ± 0.06	0.9994	0.08	0.27	2.3	3.2
PhGly (1)	4–60	(−16) ± 13	8.00 ± 0.10	0.9978	0.16	0.52	4.7	5.0
PhGly (2)		32 ± 15	14.30 ± 0.10	0.9991	0.07	0.25	5.7	3.3

[a]SD: standard deviation. [b]Limit of detection. [c]Limit of quantification. [d]Expressed as relative standard deviation (% RSD). Standard solution employed containing 300.0 ng mL^{-1} GS, 60.0 ng mL^{-1} Gly, 3-DG, 20.0 ng mL^{-1} MGly, DIA, 75.0 ng mL^{-1} 2,3-Pen, and 30.0 ng mL^{-1} PhGly.

employing the peak areas as analytical signal. The analytical figures of merit obtained for each α-dicarbonyl compound are shown in Table 3. Evaluation of the precision of the optimized method was done by analyzing standard solutions of the α-dicarbonyl compounds in the same day (intraday precision, n = 10) and in consecutive days (interday precision, n = 6). Intraday and interday repeatability values, expressed as relative standard deviation (RSD), are lower than 6.0% and 5.0%, respectively, so they may be considered as a guarantee of the goodness of the proposed method, and use of an internal standard is not required. The limits of detection (LOD) and quantification (LOQ) were calculated as concentrations corresponding to 3 and 10 times the standard deviation of the signal from baseline from chromatograms obtained in intraday precision study. Low values are obtained that are indicative of the high sensitivity of the developed method. Results obtained are summarized in Table 3.

3.4. Quantitative Measurement of α-Dicarbonyl Compounds in Wines. After evaluation and selection of the optimum column for separation of seven α-dicarbonyl compounds, the proposed method was applied to analysis of different red, white, and sweets wines. To carry out quantification of the α-dicarbonyl compounds in wine samples, the influence of the matrix effect over chromatographic

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Table 4. Concentrations of the Six α-Dicarbonyl Compounds Detected in Commercial Wine Samples

type of wine	[GS][a]	[Gly][a]	[3-DG][a]	[MGly][a]	[DIA][a]	[2,3-Pen][a]	total α-DC[a]
red wines[b]							
Monasterio Tentudía, 2008 (Extremadura, Spain)	4.50 ± 0.20	1.85 ± 0.08	13.50 ± 0.30	1.58 ± 0.05	3.10 ± 0.60	3.00 ± 0.04	27.53 ± 0.71
Ribera Duero, 2010 (Spain)	0.50 ± 0.20	1.35 ± 0.03	7.70 ± 0.40	0.68 ± 0.01	8.80 ± 0.20	1.45 ± 0.02	20.48 ± 0.49
Merlot, 2009 (Extremadura, Spain)	1.70 ± 0.20	7.10 ± 0.20	10.40 ± 0.50	0.42 ± 0.02	1.22 ± 0.05	5.60 ± 0.03	26.46 ± 0.58
white wines[c]							
Rioja Comportillo, 2011 (D.O. Rioja, Spain)	0.70 ± 0.10	1.53 ± 0.08	4.90 ± 0.20	0.73 ± 0.03	0.29 ± 0.01	2.22 ± 0.03	10.37 ± 0.24
Viña Canchal, 2011 (Extremadura, Spain)	0.40 ± 0.10	1.12 ± 0.05	7.90 ± 0.30	0.21 ± 0.01	0.63 ± 0.02	1.17 ± 0.03	11.43 ± 0.32
Manzanilla, dry wine (D.O. SanLúcar de Barrameda, Spain)	5.20 ± 0.20	1.10 ± 0.10	6.10 ± 0.20	0.28 ± 0.01	0.64 ± 0.02	0.90 ± 0.04	14.22 ± 0.30
red sweet wines[d]							
Vin Santo (Toscana, Italy)	138 ± 11	4.50 ± 0.30	133 ± 5	1.68 ± 0.08	1.02 ± 0.06	2.30 ± 0.10	280 ± 12
Port Tawny (Porto, Portugal)	89 ± 13	8.90 ± 0.40	39 ± 4	0.95 ± 0.05	1.50 ± 0.09	1.90 ± 0.10	141 ± 14
white sweet wines[d]							
Málaga Dulce (Málaga, Spain)	261 ± 7	8.40 ± 0.20	126 ± 5	0.90 ± 0.05	2.80 ± 0.10	2.61 ± 0.08	402 ± 9
Oremus Tokaji, 2008 (Hungary)	37 ± 1	5.50 ± 0.20	75 ± 2	3.21 ± 0.06	0.60 ± 0.07	4.60 ± 0.10	126 ± 2

[a]Concentration expressed as μg mL^{-1} ± SD (standard deviation) [b]Aliquot of wine samples: 500 μL for Gly, MGly, DIA and 2,3-Pen, and 120 μL for 3-DG and DIA wine/water (1/50, v/v) for GS and 3-DG. [c]Aliquot of wine samples: 1500 μL wine/water (1/50, v/v) for GS and 3-DG. [d]Aliquot of wine samples: 120 μL for Gly, MGly, DIA and 2,3-Pen, and 50 μL of a solution wine/water (1/50, v/v) for GS and 3-DG.

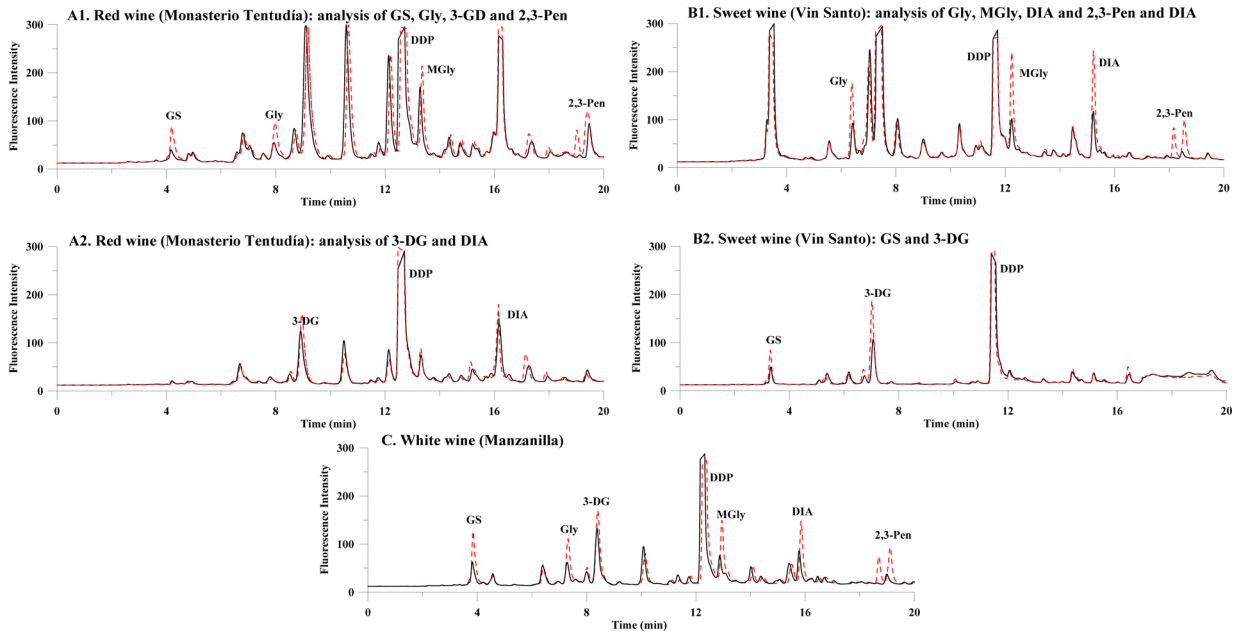

Figure 5. Chromatograms obtained from each type of wine analyzed (continuous line) and from wine samples spiked with 125.0 ng mL^{-1} GS, 60.0 ng mL^{-1} 3-DG, 40.0 ng mL^{-1} Gly, 6.0 ng mL^{-1} MGly, 6.0 ng mL^{-1} DIA, and 40.0 ng mL^{-1} 2,3-Pen (dashed line). (A1) Analysis of GS, Gly, MGly, and 2,3-Pen in red wine (Monasterio Tentudía, 0.50 mL of sample). (A2) Analysis of 3-DG and DIA in red wine (Monasterio Tentudía, 0.12 mL of sample. (B1) Analysis of Gly, MGly, DIA, and 2,3-Pen in sweet wine (Vin Santo, 0.12 mL of sample). (B2) Analysis of GS and 3-DG in sweet wine (Vin Santo, 0.50 mL of a solution wine/water (1/50 v/v)). (C) Analysis of six α-dicarbonyl compounds in white wine (Manzanilla, 1.50 mL of sample). $\lambda_{exc}/\lambda_{em}$ = 330/450 nm.

separation was first evaluated. For this standard addition calibration curves, from each of the α-dicarbonyl compounds in wine samples, were established, and calibration slopes were compared with the corresponding slopes of the external standard calibration plots of each analyte at the 95% confidence level. In all cases, considerable differences were obtained, and these results suggest that there is a matrix effect. As a consequence, calibration curves were constructed using the standard addition method for each of the wines analyzed and used to quantify each analyte in duplicate.

It is important to bear in mind that levels of α-dicarbonyl compounds vary with the type of wine, and for this reason, adequate sample dilutions were optimized for each type of wine analyzed. The volume chosen for each wine is shown in Table 4. The low volumes of samples needed for analysis, in comparison with other published methods, highlight the sensitivity of the proposed method.

Ten wines were analyzed following the methodology described in section 2.4: 3 red wines, 3 white wines, 2 sweet red wines, and 2 sweet white wines. Figure 5 shows an example of chromatograms obtained from each type of wine analyzed, and the results obtained for each α-dicarbonyl are shown in Table 4. Contrary to the literature,[22] PhGly was not detected in any of the different wines analyzed in this study. Also, in all

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samples, only one lumazinic derivative from 2,3-Pen was observed.

To date, the dicarbonyl compound more relevant in wines is DIA, since it has a pronounced butter odor and its presence in wines at high concentrations is undesirable.[8] Depending on the type of wine, the contents of DIA[37,38] ranged from 0.2 to 12 mg L^{-1}. As it can be seen in Table 4, in the samples analyzed in this paper the content of DIA varies between 0.29 and 8.8 mg L^{-1}. It can be observed that in red wines the content is higher than in white wines, in accordance with previous research, and no significant differences were found between not sweet and sweet wines, with the exception of Ribera Duero wine.

With respect to the other α-dicarbonyl compounds with a short chain, concentrations found for Gly and MGly were similar to those previously reported.[33] Values ranged from 1.1 to 1.85 mg L^{-1} for Gly and 0.28 to 1.68 mg L^{-1} for MGly, with the exception of Merlot wine, whose concentration of Gly found was 7.1 mg L^{-1}. Thus, the levels found in Merlot wine are close to the levels found for sweet wines, in which the quantities of Gly and MGly found were higher (between 4.5 and 8.9 mg L^{-1} and 0.9 and 3.2 mg L^{-1} for Gly and MGly, respectively). In all cases the relation Gly/MGly is greater than unity. With respect to the levels of 2,3-Pen, these are similar in all wines analyzed ranging between 0.9 and 5.60 mg L^{-1}, and no appreciable differences were found between sweet and not sweet wines.

On the other hand and with respect to the α-dicarbonyl sugars, 3-DG and GS have been detected in all wines analyzed. In red and white wines, the content of GS varies between 0.4 and 5.2 mg L^{-1} and the 3-DG content between 4.9 and 13.5 mg L^{-1}. In sweet wines, the concentration of both glucosones increases considerably, independent of whether they are red or white, and their concentrations vary between 37 and 261 mg L^{-1}.

Due to the high concentration of glucosones obtained in sweet wines, it was important to check if formation of the α-dicarbonyl compounds from sugars was not favored by the high temperature of the derivatization reaction conditions. Therefore, in sweet wines, the derivatization reaction was performed at room temperature for 2 h and 30 min and aliquots of these samples were analyzed. A comparison of the chromatograms, obtained at room temperature and higher temperature, does not show significant differences. This fact allows us to conclude that the elevated concentrations of glucosones found in sweet wines are related to its high levels of sugars.

Finally, if analyzing the total α-dicarbonyl concentration, it can be observed that no significant variations were found in each type of red or white wine analyzed. However, if we made a comparison between red and white wines, we can confirm that the total α-dicarbonyl concentration is significantly higher in red wines. On the other hand, in sweet wines, a great difference has been found between the wines analyzed, and values of total α-dicarbonyl concentration amounting to 402 mg L^{-1} have been found. In addition, Tokaji wine, which it is a sweet white wine produced with botrytized grapes, contains higher levels of MGly. In spite of this, the total α-dicarbonyl concentration found is lower compared to the other sweet wines analyzed.

In conclusion, all types of columns tested were able to separate the lumazinic derivatives with sufficient resolution and peak symmetry in the optimum chromatographic conditions, although they differed in analysis time and separation efficiency of the derivatives analyzed. The conventional C18 column had the lowest resolution and peaks wider than columns with minor particle size. By comparing the chromatographic behavior of lumazinic derivatives in small particle size columns, it can be observed that although the XDB-C18 RRHT column provides the lowest retention times the best separation efficiency and lowest solvent consumption is achieved with the Poroshell column. For this reason, the comparison study carried out showed that the Poroshell column was most appropriate for analysis of α-dicarbonyl compounds and previous derivatization to lumazines, and this column was selected for their analysis in wine samples. Derivatization reaction time, temperature, and sample volume employed in analysis have considerably decreased with respect to the previous published method. On the other hand, a very sensitive and reproducible method to analyze these compounds in different commercial wine samples has been developed, and no pretreatment of sample is required. The sensitivity of the method is presented as a useful alternative to other methods, even with MS detection, whose instrumentation is more costly and complicated and not available in many laboratories. A comparison between red and white wines shows that the total α-dicarbonyl concentration is significantly higher in red ones. In sweet wines, total amounts up to 400 mg mL^{-1} have been found.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS USED

GS, D-glucosone; 3-DG, 3-deoxyglucosone; Gly, glyoxal; MGly, methylglyoxal; DIA, diacetyl; 2,3-Pen, 2,3-pentanedione; PhGly, phenylglyoxal; DDP, 5,6-diamino-2,4-hydroxypyrimidine; β-ME, β-mercaptoethanol

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