

# Multivariate spectroscopy for targeting phenolic choreography in wine with A-TEEM<sup>TM</sup> and NMR crosscheck non-targeted metabolomics

J.E. Herbert-Pucheta<sup>1</sup>, G. Padilla-Maya<sup>2</sup>, D. Milmo-Brittingham<sup>2</sup>, D. Lojero<sup>3</sup>, A.M. Gilmore<sup>4</sup>, L. Raventós-Llopart<sup>5</sup>, K.E. Hernández-Pulido<sup>5</sup>, and L.G. Zepeda-Vallejo<sup>6</sup>

<sup>1</sup> Consejo Nacional de Ciencia y Tecnología-Laboratorio Nacional de Investigación y Servicio Agroalimentario y Forestal. Universidad Autónoma Chapingo. Carretera México-Texcoco Km 38.5, C.P. 56230, Chapingo, Estado de México, Mexico

<sup>2</sup> Consejo Mexicano Vitivinícola A.C. Montecito No 38 Piso 15 Despacho 22 – WTC México, C.P. 03810, Ciudad de México, Mexico

<sup>3</sup> Intercovamex, Nueva China No. 400 Colonia Rincón del Valle, C.P. 62240, Cuernavaca, Morelos, Mexico

<sup>4</sup> Horiba Scientific, 3880 Park Avenue Edison, NJ 08820. United States of America

<sup>5</sup> Finca Sala-Vivé – Freixenet, Carretera San Juan del Río Cadereyta, Km 40.5, Colonia los Pérez Ezequiel Montes, Querétaro, Mexico

<sup>6</sup> Departamento de Química Orgánica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional. Prolongación de Carpio y Plan de Ayala s/n, Colonia Santo Tomás, 11340 Ciudad de México, Mexico

**Abstract.** Present work comprises the use of different multivariate spectroscopic methods for tracking novel metabolomics signatures related to red wine chemistry. It is presented for the first time the proton nuclear magnetic resonance metabolomics fingerprint of a monovarietal Mexican Merlot, obtained with acquisition improvements recently proposed to the OIV Methods of Analysis sub-commission. Effective multi-presaturation solvent schemes have revealed a rich (poly)-phenolics aromatic region, so far not exploited for wine-fingerprinting or – targeted profiling routines. It is presented as well for the first time the use of simultaneous absorbance-transmission and fluorescence excitation-emission matrix “push-one-bottom” method (A-TEEM<sup>TM</sup>) at specific chemical conditions for a rapid, effective and high-sensitivity characterization of phenolic choreography in wines, as novel observables to quantify oenological practices and aging.

## 1. Introduction

Economic and cultural importance of wine is chemically supported mostly by its phenolics content, as said primary and special metabolites’ family contribute to wines’ organoleptic properties such as colour, taint, mouth-feel and aromas [1]. Standard quantification of phenolics comprises colorimetric and/or chromatographic approaches, whereas despite their robustness, they present certain complexity in terms of sample preparation, chemical manipulations, being in turn time consuming, laborious, costly and require some level of analytical expertise. OIV cross-commissions shall promptly evaluate the ratio between wineries and oenological research institutes *in all member states* that could provide the manoeuvre of analytical experts certified to carry out said standard methods.

This work presents a “push-one-bottom”, rapid, user-friendly and non-invasive spectroscopic solution to track a robust phenolic profile in wines, with simultaneous absorbance-transmittance (A-T) and fluorescence excitation – emission matrix spectroscopy (EEM), branded as A-TEEM<sup>TM</sup> [2], wherein the simultaneous AT & EEM acquisition is carried out at each excitation increment. Immediate applications of the A-TEEM<sup>TM</sup> device comprise the characterization of human consumption water quality, in terms of quantification of Total dissolved

Organic Carbon (TOC) metabolites like low-and high-molecular aromatics, as well as protein compounds from humic/fluvic sources [3]. More recently, A-TEEM<sup>TM</sup> technology has been used to determine phenolic and anthocyanin profiles in fresh and oxidized Italian red wines, in the excitation-emission range between 250–800 nm [3,4]. Construction of reliable meta-data bases in terms of reproducible A-TEEM<sup>TM</sup> phenolic-anthocyanin libraries for quantitative analysis of mostly special metabolites in wine with a fast/high sensitivity/push-one-bottom solution needs an orthogonal crosscheck with robust OIV methods. For instance, high-resolution proton Nuclear Magnetic Resonance Spectroscopy (<sup>1</sup>H-NMR) [5–7] is used to obtain the first reported metabolomics fingerprint and profiling of a monovarietal Mexican Merlot (2018, Sala Vivé, Freixenet, Querétaro MX), targeting the most abundant primary metabolites, in agreement to an OIV resolution project under course [7]. <sup>1</sup>H-NMR meta-data analysis is presented, for fingerprinting some spectral regions associated to phenolics that orthogonally correlates with A-TEEM<sup>TM</sup> observables.

## 2. Materials and methods

### 2.1. Wine samples

A set of eleven Mexican monovarietal Merlot wines from Queretaro, México (Finca Sala Vivé, Freixenet México),

from two different years of vintage (2017 and 2018), aged at different conditions were analysed, hereafter numbered as follows: (1): Merlot 2017 aged within a 2017-Tonnellerie d'Aquitaine French barrel, (2): Merlot 2017 Gran Reserva taken from a 24-months bottled aging, (3–4): Merlot 2018 aged within a 2018-Tonnellerie d'Aquitaine French barrel with a duplicate sampling, (5): Merlot 2018 aged within a 2016-Tonnellerie d'Aquitaine French barrel, (6): Merlot 2018 directly taken from the fermentation tank, (7–9): Merlot 2018 aged within a 2016-Boutes French barrel with a triplicate sampling, (10–11): Merlot 2018 aged within a 2018-Demptos American barrel, with a duplicate sampling.

Sample preparation for A-TEEM<sup>TM</sup> spectroscopy was carried out by dissolving 30  $\mu$ L of wine samples (3), (6) and (11) with a 200 $\times$  dilution factor, using a 12% ethanol v/v solution as solvent at three different pH levels: 1, 3, 7. A final volume of 3 mL per sampling was versed in each case within a (1  $\times$  1) cm path length Cuvette Quartz. A-TEEM<sup>TM</sup> lectures were done with a temperature of 25  $^{\circ}$ C.

Sample preparation for NMR studies comprised the addition of 100  $\mu$ L of a mixture of D<sub>2</sub>O and chemical-shift reference sodium 3-(trimethylsilyl)-propionate-2, 2, 3, 3-d<sub>4</sub> (TSP), phosphonate buffer KH<sub>2</sub>PO<sub>4</sub> 0.1% and 2% NaN<sub>3</sub> to 900  $\mu$ L of wine sample, whereas pH was finally adjusted to a value of 3.9 for all samples. Samples were finally versed in standard 5 mm NMR tubes.

## 2.2. UV-VIS absorbance-transmittance coupled with excitation emission matrix fluorescence

Simultaneous UV-VIS absorbance-transmission and fluorescence excitation-emission matrix spectra were carried out on an A-TEEM<sup>TM</sup> Aqualog system (Horiba Jobin Yvon, Inc.) with simultaneous absorption – excitation wavelength spans from 240–800 nm (5 nm interval) and emission wavelengths spanning from 248–826 nm with an average increment of 4.66 nm. Analysis of the fully corrected A-TEEM<sup>TM</sup> data was carried out with the Aqualog DataStream package based on the multivariate routine known as parallel factor analysis (PARAFAC, Solo + MIA package from Eigenvector Research Inc.) [10]. Best fit of the data was achieved with a five component model.

## 2.3. Nuclear Magnetic Resonance (NMR) spectroscopy

All spectra were recorded on a Bruker 600 AVANCE III HD equipped with a 5 mm <sup>1</sup>H/ D TXI probehead with z-gradient. 1D-<sup>1</sup>H experiments with water-to-ethanol solvent presaturation were carried out as elsewhere reported [5].

## 3. Results and discussion

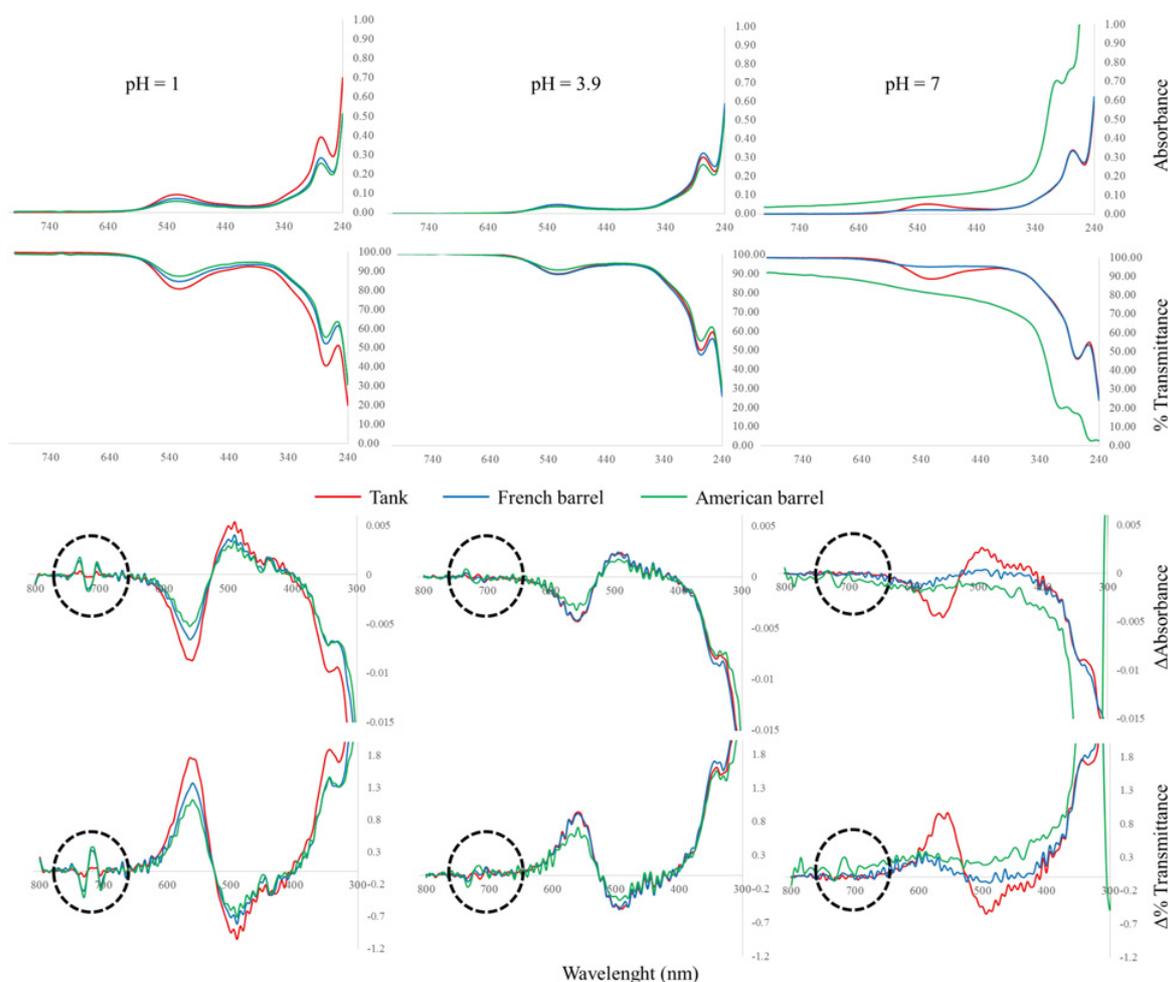
Figure 1 presents the raw and 1<sup>st</sup> derivative absorbance and % transmittance spectra of a set of Mexican Merlot 2018 red wines with no barrel aging (Tank, red) and three months aged, even within a 2018-Tonnellerie d'Aquitaine French barrel (blue) and with a 2018-Demptos American barrel (green), at three different acidic conditions (pH = 1, 3, 7). Peaks on raw spectra, attenuated in the derivative

graphs, accentuate the following bands: a major extinction peak at 275 nm, a second minor peak at 520 nm and a third residual peak at around 715 nm, well observed at pH=1, in a lesser extent at pH=3, but only for aged samples in barrels. No residual 715 nm peak is observed, neither for Tank samples at any pH value and at a pH = 7 for the rest of the aged Merlot wines. Whereas the 275 nm absorbance – transmittance lines are commonly associated with simple phenolic compounds and the 520 nm peak region has been associated to stable anthocyanin compounds [12], the 715 nm absorbance peak could strongly be associated to flavylum cations of most common anthocyanidines in red wines that serve as dyes [13]. Deep inspection of first derivative absorbance and % transmittance spectra of Figs. 1 and 2, reveals a noticeable increase of the 520 nm band for not aged or poorly aged species, whilst absorption band at 715 nm is present in samples with presumably better aging processes, an effect that is better appreciated at acidic conditions. The last correlates with previous EEM studies [12] that claim the possibility to distinguish monomeric and polymeric anthocyanin species. Present results opens the venue to distinguish monomeric (at 520 nm) and polymeric (715 nm) anthocyanin species with first derivative absorption spectra, whereas at acidic conditions, said spectroscopic signature is more pronounced.

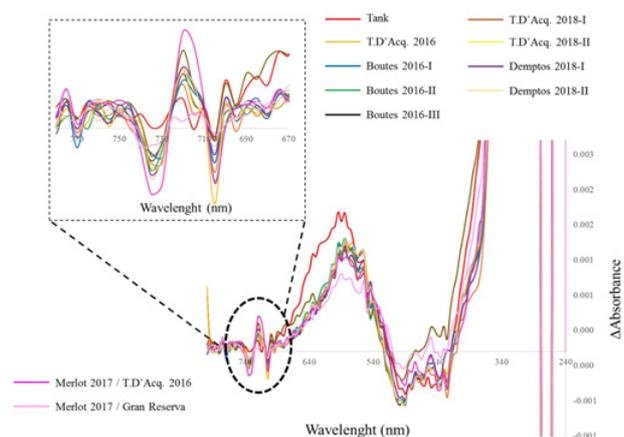
Proton nuclear magnetic resonance (<sup>1</sup>H-NMR) metabolomics fingerprint of Mexican monovarietal Merlot red wines, with the use of key methodological improvements [5,7] that noticeably increases spectral signal- to-noise ratio, is presented in Fig. 3.

Improvements of water-to-ethanol multi-presaturation schemes for having a full set of proton resonances of both primary and specialized wine metabolites towards fast acquisition NMR fingerprinting & targeting, has been recently presented at OIV SCMA experts' group [7]. Advantages of the use of said methodological <sup>1</sup>H-NMR aspects, is exemplified in Fig. 3 and should be read as follows: <sup>1</sup>H-NMR-OIV resolution project comprises the quantitation of solely six primary metabolites for wine analysis [7], most probably due to poor signal-to-noise ratio with the use of standard <sup>1</sup>H-NMR solvent-suppression schemes, that severely penalizes the limits of detection – quantification of lower concentration metabolites. Accurate water-to-ethanol multipresaturation schemes allowed the fingerprinting and profiling of at least 15 novel metabolites, having excellent agreements with respect prestigious plant metabolomics meta-data Repositories [14,15]. It is worth noting to highlight that with selected acquisition conditions [5], an important number of non-assigned resonances at the phenolics region (5.58–8.0 ppm) present an accurate signal-to-noise ratio for increasing known NMR fingerprinting & targeting of wines.

With the use of accurate multipresaturation schemes for water and ethanol intense signals, a rich (poly)-phenolics aromatic region is exposed within the full NMR-fingerprint of studied Querétaro Merlot wines, with reasonable acquisition times per sample (i.e. 4 minutes per experiment, with 64 transients). Despite a full NMR pre-processing treatment (signal bucketing, integration and quantification) of present data will be elsewhere discussed in detail, mostly from rich



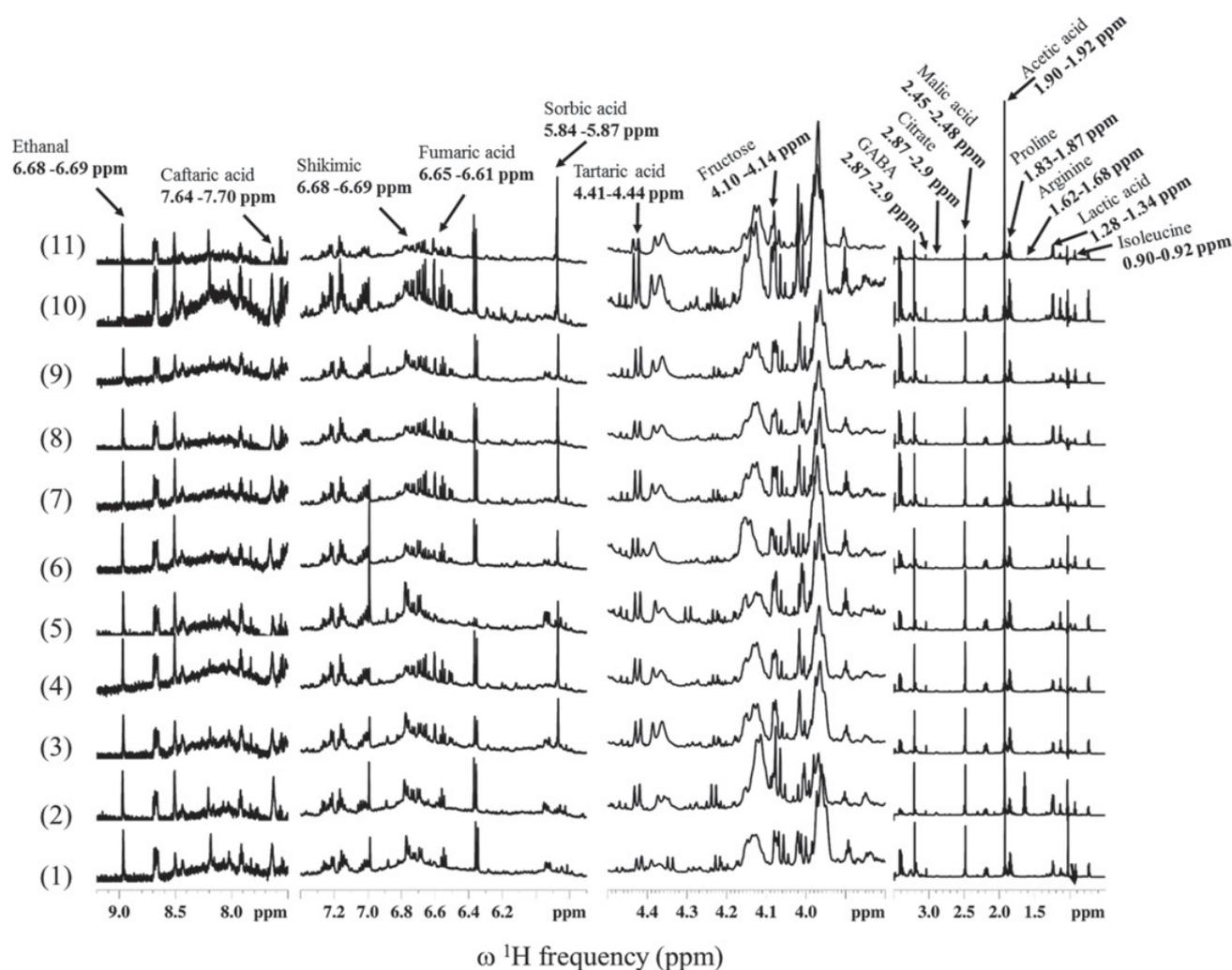
**Figure 1.** Raw and first derivative ( $\Delta$ ) UV-VIS absorbance – % transmittance spectra of Mexican monovarietal Merlot wines (samples 3 (red spectra), 6 (blue spectra) and 10 green spectra); see Materials and Methods) at three different pH values (pH = 1, extreme Left; pH = 3.9, Middle; pH = 7, extreme Right). Relevant absorption-%Transmittance peaks at 715 nm is highlighted with a dotted-line circle.



**Figure 2.** First derivative ( $\Delta$ ) UV-VIS absorbance spectra of Mexican monovarietal Merlot wines with different aging schemes (samples 1 to 11, refer colour code to figure legends and Materials and Methods) acquired at pH = 3.9. As in Fig. 1, absorbance region at 715 nm is expanded.

(poly)-phenolics aromatic region, some remarks can be done. Figure 4 present expansions of Fig. 3 per data set (see Materials and Methods), whereas Querétaro Merlot's NMR-signatures are stacked in terms of aging

reservoirs and year of vintages: (1–2) Merlot 2017; (3–4): Merlot 2018/2018-Tonnellerie d'Aquitaine; (10–11): Merlot 2018/2018-Demptos; (7–8): Merlot 2018/2016-Boutes; (5) Merlot 2018/2016-Tonnellerie d'Aquitaine; (6): Merlot 2018/tank. This specific classification in Fig. 4, was done by means of the UV-VIS  $\Delta$ Absorbance 715 nm peak intensity that each set of samples present (full UV-VIS  $\Delta$ Absorbance depicted in Fig. 2). As observed and expected, aged 2017-Merlot in a 2017-Tonnellerie d'Aquitaine barrel, present a maximum 715 nm  $\Delta$ Absorbance peak intensity. In counterpart, said sample present a minimum signal intensity of  $\Delta$ Absorbance at 520 nm wavelength (Fig. 2), strongly suggesting the presence of a major amount of poly-anthocyanin with a minor amounts of mono-anthocyanin moieties. In extreme contrast, Merlot 2018/tank samples present no  $\Delta$ Absorbance signal at 715 nm and a maximum at 520 nm, confirming the UV-VIS  $\Delta$  Absorbance antagonist mono/poly-anthocyanin profile. Last observations could be cross-checked with the use of the NMR-Merlot profiling. Despite the lack of spectral resolution at the novel revealed (poly)-phenolics region around 5.58–8.0 ppm, signal integration could shed light on phenolics content in agreement with UV-VIS observations.



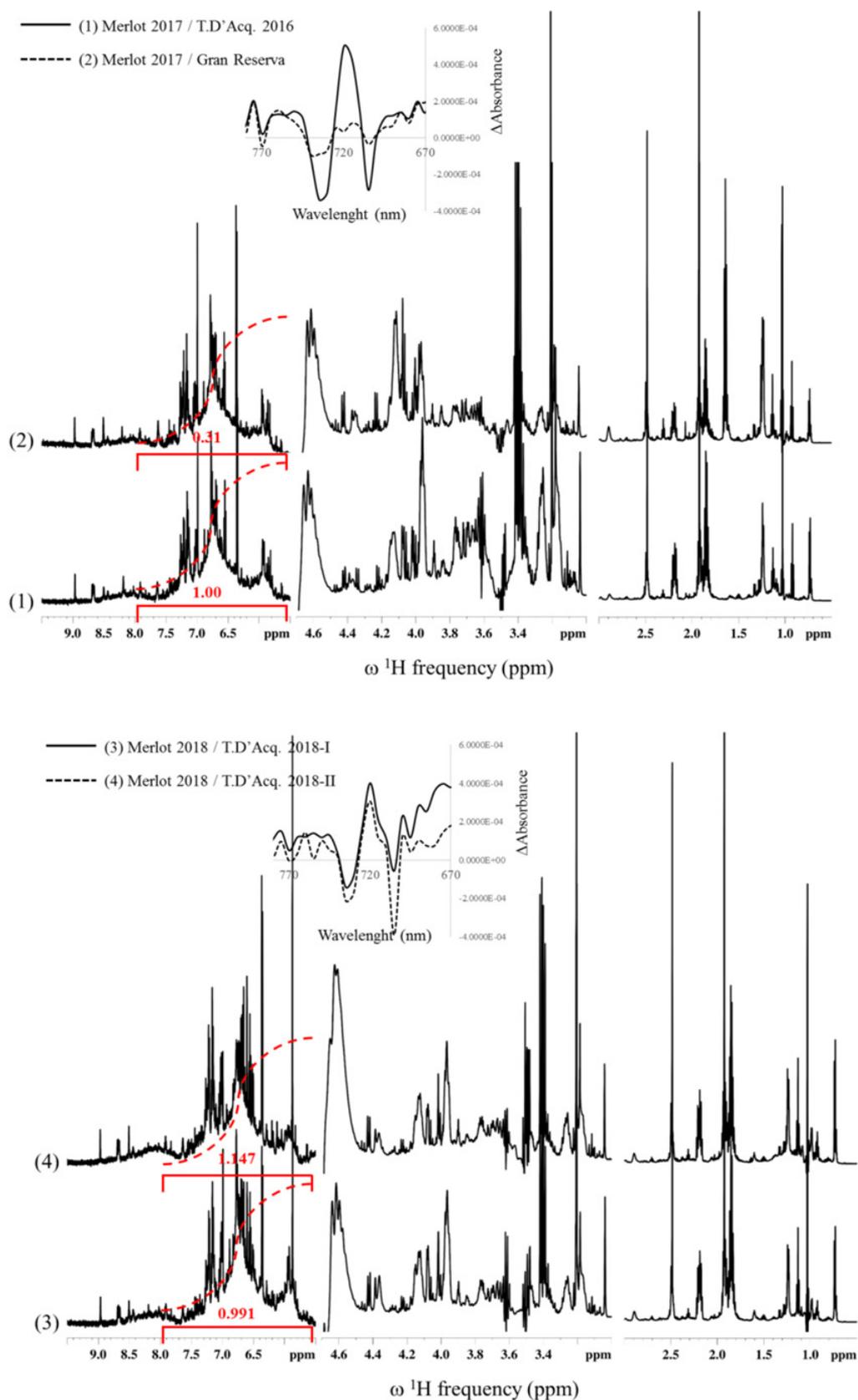
**Figure 3.** Stacked proton one-dimensional Nuclear Magnetic Resonance spectra ( $^1\text{H-NMR}$ ) with improved water-to-ethanol multipresat. Scheme [5] of Mexican monovarietal Merlot wines, with different aging schemes (samples 1 to 11, refer to Materials and Methods). NMR signature of Mexican Merlot from Queretaro Region (Finca Sala Vivé / Freixenet) present signal assignment of relevant metabolites, with excellent agreements with respect foreign Merlot noir genotypes' NMR fingerprints in plant metabolomics databases [15].

NMR signal integration of rich (poly)-phenolics region around 5.58–8.0 ppm within the  $^1\text{H-NMR}$  spectra of wine samples exposed in Fig. 4, present a coherent agreement with respect the UV-VIS  $\Delta$ Absorbance signature at 715 nm. Aged wines, such as the Merlot 2017/2017-Tonnellerie d'Aquitaine (sample 1), or Merlot 2018/2018-Tonnellerie d'Aquitaine (samples 3–4) present a normalized signal integration of the rich (poly)-phenolics region of around the unity and in turn a 715 nm UV-VIS  $\Delta$ Absorbance clear fingerprint. For the antagonist Merlot 2018 / Tank (sample 6), it is observed a relative signal integration of the 5.58–8.0 ppm region of 8% (with respect the reference (sample 1)) and no 715 nm UV-VIS  $\Delta$ Absorbance fingerprint, in agreement with the expected lack of complex polyphenolics. Ensemble of results preliminary suggest an aging efficiency trend corroborated by both multivariate methods: Tonnellerie d'Aquitaine (2017, 2018) French barrels promotes wine aging with slight better results of polyphenolics content. American Demptos and French Boutes barrels promotes wine aging with equivalent spectroscopic results. Tested 2016-Tonnellerie d'Aquitaine produced wine samples with

poor signal integration ( $I = 0.038$ ) and weak 715 nm UV-VIS  $\Delta$  Absorbance signal.

Finally, coupled A-T & EEMs could be regarded as genuine fingerprints of optical active (macro)-molecular composition of wines. As above mentioned, Absorbance – %Transmission profiles present maximum extinction peaks around 275, 520 and 715 nm (Figs. 1 and 2). In turn, EEMs produces broad intense fluorescence emission spectra spanning from 250 to 320 nm for a 240–325 nm excitation wavelength (Fig. 5, Top).

PARAFAC model decomposes 3D-signals into a fixed number of statistical components (scores) that in turn describe the variability of acquired AT-EEM. In our study, a five component PARAFAC model (C1–C5) best explained the variability of fluorescence signatures of red wines as a function of pH. The dominant component C4 present a maximum emission signal of 375 nm (emission) and 320 nm (excitation), whereas said emission/excitation profiles present accurate agreements with fluorescence of flavonoid like moieties [11]. C2 and C3 scores present emission/excitation peaks at respectively (375/260), (355/270) nm that most likely are due to



**Figure 4.** Proton one-dimensional Nuclear Magnetic Resonance spectra (<sup>1</sup>H-NMR) with improved water-to-ethanol multipresat. Scheme [5] of Mexican monovarietal Merlot wines, with different aging schemes (samples 1 to 11, refer to Materials and Methods). Novel rich polyphenolics exposed region (5.58–8.0 ppm) with multipresat scheme has been integrated in all cases, referenced with respect sample 1 ( $I = 1.0$ ). First derivative ( $\Delta$ ) UV-VIS absorbance spectra of each sample, at the 715 nm region is as well exposed, per case.

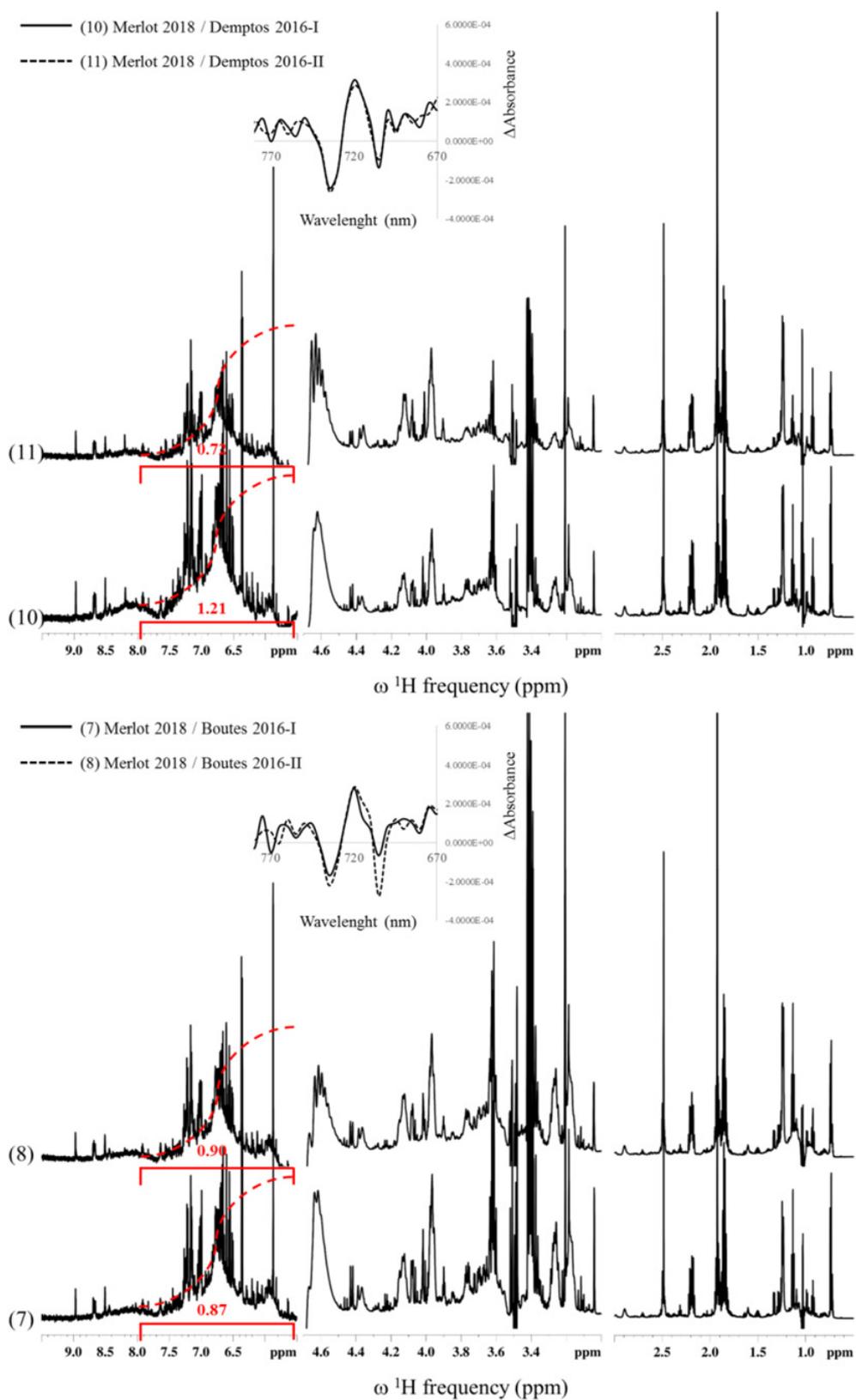


Figure 4. Continued.

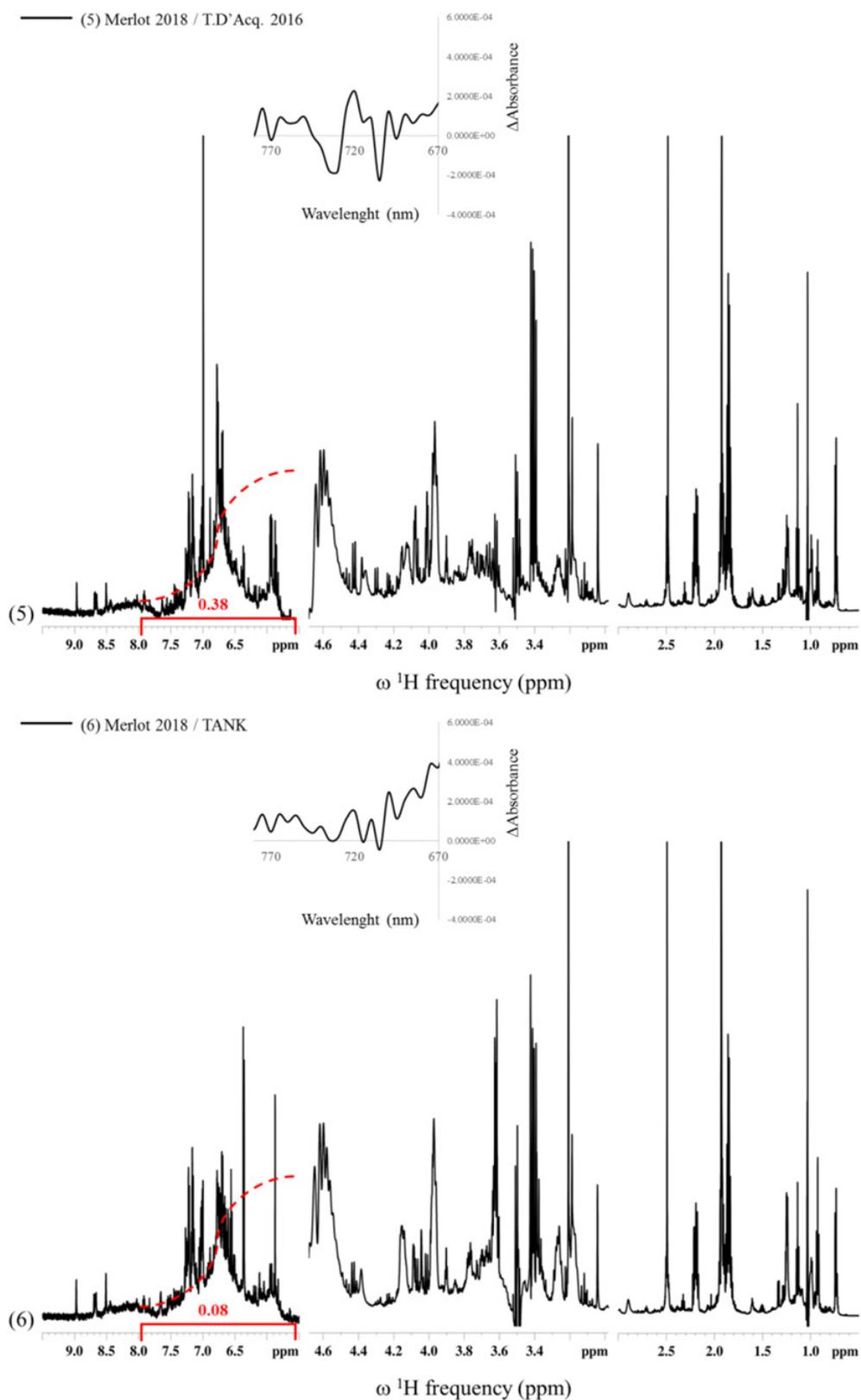
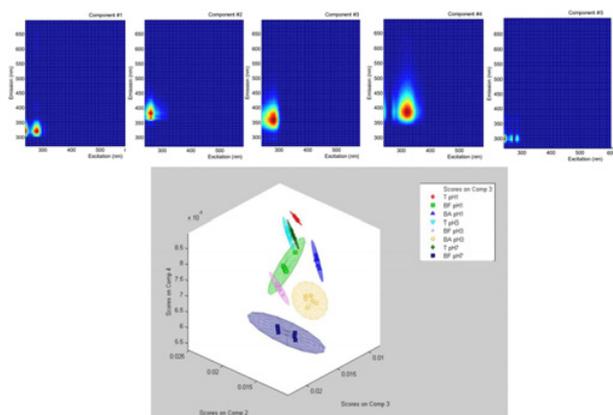


Figure 4. Continued.



**Figure 5.** Five component (C5) PARAFAC model constructed from the fluorescence EEMs comprising the evaluation of  $3 \times 3$  class groups as a function of storage vessel (tank, French barrel and American barrel) and solution pH (1, 3.9, 7) and 6 repetitions per class group (total of 54 files).

specific polyphenol content, whereas as appreciated by the absorbance – transmittance profile, it possess a pH dependency that can be traced within the PARAFAC cluster C4-C2-C3, depicted in Fig. 5.

#### 4. Conclusions

This work presents for the first time a NMR/ A-TEEM traceable molecular (polyphenolics) fingerprints, linked to the chemistry involved in aging processes, using a set of mono-varietal Querétaro Merlot samples as model system. First the use of raw and first derivative -pH dependent – UV-VIS ( $\Delta$ )Absorbance spectroscopy is proposed to elucidate a simple-to-complex phenolics' profile within wine samples, in terms of ( $\Delta$ ) Absorbance lines at 275, 520 and 715 nm. In parallel, methodological improvements allowed to obtain a proton NMR fingerprint of studied samples that in turn revealed a novel exploitable aromatics region, whereas signal integration of key regions, present excellent correlation with UV-VIS data, for cross-checking the novel method of analysis. Two-dimensional Absorbance (Transmittance) – Excitation

Emission Fluorescence Matrix and processing with a five component PARAFAC cluster accurately describe the variability of polyphenols in wines as a function of pH and different aging processes.

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*In memoriam* of Ernesto Ladron-de-Guevara.

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