



# Influence of ripeness and maceration of the grapes on levels of furan and carbonyl compounds in wine – Simultaneous quantitative determination and assessment of the exposure risk to these compounds



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## ABSTRACT

The validated method based on the use of headspace solid phase microextraction (HS-SPME) coupled with the comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometric detection (GC × GC/TOFMS) proved to be appropriate for this first simultaneous quantitative determination of six toxic compounds (formaldehyde, acetaldehyde, ethyl carbamate, furan, furfural and acrolein) found in wines. Acetaldehyde and acrolein coeluted with other wine compounds, which indicated that difficulties could arise if only one-dimensional gas chromatography was used for the determination of these compounds. The advancement of the ripeness degree and increasing the grape maceration time seems to result in higher concentrations of toxic compounds. The exposure to furan, acrolein and ethyl carbamate through wine consumption may pose risks to consumer health, since calculated MOE values were lower than 10,000.

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## 1. Introduction

The ripeness degree and grape maceration time are important parameters influencing the chemical profile of wine. The ripeness degree defines the harvest time that must be based on technological and phenolic maturity of grapes. The technological maturity includes soluble solids content of around 20°Brix and titratable acidity of 6–8 g L<sup>-1</sup> (Ribéreau-Gayon, Dubourdieu, Donèche, & Lonvaud, 2006). Phenolic maturity is related to the quantity and extraction capacity of anthocyanins and tannins obtained from

grapes during the maceration. In this stage, the quantity of extractable tannins from grape seed decreases due to the polymerization of these compounds, resulting in lower wine astringency. Furthermore, an increase occurs in the concentration of anthocyanins, which are responsible for the color of grape skins, since the degradation of the cellular walls of skins facilitates the extraction of these compounds during the maceration (Cadot, Caillé, Samson, Barbeau, & Cheynier, 2012).

The enological quality may be satisfactory when grapes with technological and phenolic maturity are harvested, resulting in a wine with the adequate alcoholic degree, “harmonious”, pleasant to palate, with balance of astringency and bitterness, and other positive characteristics (Meléndez, Ortiz, Sarabia, Iñiguez, &

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Puras, 2013). Levels considered ideal of soluble solids and acidity in grapes can be achieved in a shorter time with a combination of high temperature and incidence of intense solar radiation on the vine. However, the phenolic maturity may not be achieved under these climatic conditions (Ferrer-Gallego, Hernández-Hierro, Rivas-Gonzalo, & Escribano-Bailón, 2012). In this way, the prolongation of maceration time may resolve problems arising from insufficient phenolic maturity of the grapes at harvest, since the extraction of tannins increases during maceration. The proportion of tannins and anthocyanins affects the stability and color of red wine and the ideal ratio between the concentration of these two groups of phenolic compounds must be equal or higher than 10:1 (tannins: anthocyanins) (Peynaud, 1997).

The effect of ripeness degree and/or maceration time of grapes have been studied in relation to phenolic composition (Ferrer-Gallego et al., 2012), sensory quality (Cadot et al., 2012), physico-chemical properties (Meléndez et al., 2013), and volatile profile of wines (Yilmaztekin, Kocabey, & Hayaloglu, 2015). To the best of the authors' knowledge, there is no report about the influence of these parameters on the levels of toxic compounds. Furan and carbonyl compounds represented by acetaldehyde, formaldehyde, ethyl carbamate (EC), furfural and acrolein are toxic compounds that may be formed during winemaking and their quantification in wines has been rarely reported in literature (Jeong et al., 2015; Kächele, Monakhova, Kuballa, & Lachenmeier, 2014; Nóbrega et al., 2015; Perestrelo, Silva, & Câmara, 2015). Acetaldehyde and formaldehyde were found in red wines from South Korea in average levels of 17232.7 and 40.9  $\mu\text{g L}^{-1}$ , respectively (Jeong et al., 2015). The average concentration of EC reported by Nóbrega et al. (2015) in Brazilian wines was 14.6  $\mu\text{g L}^{-1}$ . Fortified wines from Portugal presented furfural in average levels of 1843.1  $\mu\text{g L}^{-1}$  (Perestrelo et al., 2015). Acrolein was found in German wines at 0.7  $\mu\text{g L}^{-1}$  (Kächele et al., 2014) and no study has reported quantitative data on furan in wine. Regarding legislation for these compounds, only EC has limits established for wine. Canada (30  $\mu\text{g L}^{-1}$ ), Czech Republic (30  $\mu\text{g L}^{-1}$ ) and USA (15  $\mu\text{g L}^{-1}$ ) are the countries that set maximum levels for this ester in wine. There is no legislation about formaldehyde, however the International Programme on Chemical Safety of World Health Organization (WHO) has established a tolerable concentration of 2600  $\mu\text{g L}^{-1}$  based on the no-observed-effect level (NOEL) of 260  $\text{mg L}^{-1}$  for the histopathological effects in the oral and gastric mucosa of rats (IPCS, 2012).

Furan and carbonyl compounds may pose risk to consumer health since they have been related to various diseases. Acetaldehyde exposure may increase the risk of cancer of the upper aerodigestive tract (oral cavity, pharynx, larynx and esophagus), liver, large intestine and breast (Seitz & Stickel, 2010). Formaldehyde may be related to leukemia and nasopharyngeal cancer (Bachand, Mundt, Mundt, & Montgomery, 2010). EC has shown to contribute to the occurrence of tumors in liver, mammary gland (Cui, Wang, Qiu, & Wu, 2016). Furan and furfural may be associated to liver neoplasms (hepatocellular adenomas or carcinomas) (Arts et al., 2004; Dong et al., 2016) and furan has been related to leukemia (Bakhiya & Appel, 2010). Acrolein may play a role in multiple sclerosis, Alzheimer's disease, cardiovascular disease, hepato and nephro-toxicity (Mogue et al., 2015). On the cellular level, the occurrence of the toxic effects of these compounds may be related to DNA and protein adduction, oxidative stress, mitochondrial disruption, membrane damage, endoplasmic reticulum stress, and immune dysfunction (Arts et al., 2004; Bachand et al., 2010; Cui et al., 2016; Dong et al., 2016; Seitz & Stickel, 2010). The International Agency for Research on Cancer (IARC) classifies the acetaldehyde and formaldehyde ingested specifically through alcoholic beverages as carcinogenic to humans (group 1), EC and furan as causing probable (group 2A) and possible (group 2B) carcinogenic

effects to humans, respectively, acrolein and furfural are in group 3, in which the IARC needs further study to classify this compound regarding carcinogenic effects (IARC, 2016).

Gas chromatography with mass spectrometric detection has usually been the technique of choice for the determination of these compounds in wine (Kächele et al., 2014; Nóbrega et al., 2015; Paiano et al., 2014). Furthermore, these compounds have been analyzed individually or at best, furan and furfural are analyzed in the same analytical procedure (Perestrelo et al., 2015). The determination of these compounds can be challenging, since they are present in the concentration range of  $\text{ng L}^{-1}$  to  $\text{mg L}^{-1}$  and wine is a complex matrix, in which compounds of different chemical classes are present. Comprehensive two dimensional gas chromatography ( $\text{GC} \times \text{GC}$ ) offers superior separation capabilities due to high peak capacity, selectivity, sensitivity, structural chromatographic peak organization, when compared to 1D-GC and has already been applied to the investigation of other wine compounds in our previous study (Welke, Manfroi, Zanús, Lazarotto, & Zini, 2012). This is the first report that aims to: (i) develop and validate a method for simultaneous quantification of six toxic compounds (formaldehyde, acetaldehyde, acrolein, furan, EC and furfural) formed during the vinification, using headspace solid phase microextraction (HS-SPME) coupled with  $\text{GC} \times \text{GC}$  with time-of-flight mass spectrometric detector (TOFMS); (ii) evaluate the influence of ripeness degree and maceration time of grapes used in winemaking in relation to the levels of these toxic compounds; and (iii) assess the risk of the exposure to these compounds through wine consumption.

## 2. Material and methods

### 2.1. Samples, analytical reagents, and supplies

Syrah wines elaborated by Brazilian Agricultural Research Corporation (Embrapa, from portuguese: Empresa Brasileira de Pesquisa Agropecuária) located in Petrolina, Pernambuco state, Brazil, were evaluated in order to verify the influence of ripeness degree and maceration time of grapes on levels of toxic compounds, in addition to the assessment of the exposure risk to these compounds. Other 11 commercial Syrah wines obtained from different vintages (2010–2013) were also analyzed in relation to the exposure risk. These samples were provided by wineries located in Brazil and Chile (Details on the year and local of production of each sample are in Table S1 of Supplementary Material).

2,2,2-Trifluoroethyl hydrazine (TFEH, Aldrich, Steinheim, Germany) was used as derivatizing agent as suggested by Kim and Shin (2011) to determine aldehydes, including formaldehyde and acetaldehyde, and ketones in water. An aqueous solution of 62,000  $\text{mg L}^{-1}$  of TFEH was prepared and 100  $\mu\text{L}$  were added to each wine sample before extraction. Sodium chloride (0.3 g NaCl, analytical grade, Nuclear, São Paulo, Brazil) was also added to samples to increase the ionic strength of the solution and consequently improve the extraction of analytes. NaCl was previously dried at 100 °C for 1 h and stored in a desiccator until use.

Standard compounds: formaldehyde, acetaldehyde, EC, acrolein, furan and furfural (analytical purity higher than 98%) were purchased from Fluka (Ronkonkoma, USA) and individual stock solutions (1000  $\text{mg L}^{-1}$ ) of each component were prepared in double distilled ethanol. Octanal, 2-furfurylthiol and methyl nonanoate were used as internal standards (IS) and purchased from Sigma (St. Louis, USA). A solution of each IS (1000  $\text{mg L}^{-1}$ ) was prepared in double distilled ethanol. A solution (10  $\text{mg L}^{-1}$ ) containing the three IS was prepared in double distilled ethanol and 10  $\mu\text{L}$  of this mix was added to each wine sample before HS-SPME. This IS mix (10  $\mu\text{L}$ ) was also used in standard solutions intended to elaborate calibration curves. Internal standards were chosen having in

mind that their chemical structure should be similar to the analytes (Table S2 of Supplementary Material). In addition, former tests were performed to verify their absence in wine samples (results not shown).

A model wine solution was prepared with (+)-tartaric acid ( $6 \text{ g L}^{-1}$ , Synth São Paulo, Brazil) and 10% of ethanol (Nuclear São Paulo, Brazil) in MilliQ deionised water (Millipore purification system, Bedford, MA, EUA). The pH of this solution was adjusted to 3.5 with sodium hydroxide (Nuclear, São Paulo, Brazil). The calibration curves were prepared by diluting the standard compounds in the model wine solution. This model solution was used because the extraction efficiency is influenced by pH and especially alcohol content, as discussed in a former study (Welke, Zanús, Lazarotto, Schmitt, & Zini, 2012). Ethanol is the major volatile compound of the wine samples and consequently two phenomena may be found: (i) massive sorption of ethanol by the fiber that may impart a change in the coating nature and this may decrease the amount of compounds sorbed by the coating and/or (ii) ethanol may compete with other components of the sample for the active sites of the fiber and may also cause displacement of other compounds during the adsorption step. Calibration curves were used to quantify the toxic compounds found in wine samples as described in Section 2.4.

The SPME fiber 50/30 divinylbenzene-carboxen-polydimethylsiloxane (DVB/CAR/PDMS) StableFlex of 2 cm was purchased from Supelco (Bellefonte, USA) and was conditioned according to the manufacturer's recommendations ( $270^\circ\text{C}$  during 1 h) prior to its first use. Twenty microliter headspace vials with magnetic screw caps sealed with silicone septa have also been bought from Supelco.

## 2.2. Wine elaboration

The wines were made from Syrah grapes grown in a vineyard (latitude:  $9^\circ 16'\text{S}$ , longitude  $40^\circ 52'\text{O}$ ; altitude: 413.5 m) located in Sub-middle region of São Francisco Valley, City of Casa Nova, Bahia state, Brazil. This grape variety is the most widely cultivated in this region, which is characterized by high temperature and incidence of intense solar radiation on the vine.

Grapes harvested in 2013 at three different stages of maturation [T1: before technological or industrial maturity ( $19^\circ\text{Brix}$ ), T2: ideal ripeness degree ( $21^\circ\text{Brix}$ ), T3: overripening ( $23^\circ\text{Brix}$ )] and macerated during three different periods (C1: 10 days, C2: 20 days and C3: 30 days) were used to produce wines. The vinifications were conducted in triplicate and eight different combinations of ripeness degree and maceration times resulted in the following experiments: T1\_C1, T1\_C2, T2\_C1, T2\_C2, T2\_C3, T3\_C1, T3\_C2 and T3\_C3. The T1\_C3 was not evaluated because there were oenological problems related to the excessive astringency of this wine. There are less polymerized tannins in grapes harvested before technological maturity (T1) and the extension of maceration for more than 20 days results in unacceptable astringency in wine, which makes this combination of ripeness degree and maceration time an unfeasible industrial practice. Forty-eight plants were assigned to each of the eight experiments. The choice of ripeness degree was based on the expected alcohol content of produced wine since the Brazilian legislation establishes that the alcohol content of wine should be between 8.6 and 14% (Brasil, 2004). The alcohol content of wines was shown in Table S3 of Supplementary Material.

Wines were elaborated following the traditional method of red winemaking (Peynaud, 1997). Musts were placed in 20 L glass bottles fitted with Muller valves. Dry yeast ( $0.35 \text{ g L}^{-1}$ , *Saccharomyces cerevisiae* – Maurivim PDM®, Amazon Group, Monte Belo do Sul, RS, Brazil), fermentation activator ( $0.20 \text{ g L}^{-1}$ , Gesferm plus®, Amazon Group) and potassium metabisulfite preservative ( $0.1 \text{ g L}^{-1}$ ,

Synth, São Paulo, SP, Brazil) were used in winemaking. The alcoholic fermentation occurred at  $25 \pm 1^\circ\text{C}$  and its evolution was monitored daily by the measurement of density using an electronic hydrostatic balance (Super Alcomat, Gibertini Elettronica SRL, Milano, Italy). The fermentation was considered complete when the density became constant and lower than 0.997, which according to Ribéreau-Gayon et al. (2006) corresponds to a residual sugar content lower than the maximum concentration of  $5 \text{ g L}^{-1}$  of sugar for dry wine established by Brazilian legislation (Brasil, 2004). Malolactic fermentation was performed at  $18 \pm 2^\circ\text{C}$  until all malic acid was converted into lactic acid, which was verified by paper chromatography. The stabilization of wines occurred in cold storage ( $0^\circ\text{C}$ ) for 30 days. Before bottling, the free sulfur dioxide content was adjusted to  $30 \text{ mg L}^{-1}$ . The wines have been bottled (750 mL) and stored in cellar ( $18^\circ\text{C}$ ) for 30 days. The results of physicochemical analysis of the wines (density, alcohol content, pH, total acidity, volatile acidity, dry extract, free  $\text{SO}_2$ , total  $\text{SO}_2$  and phenolic content) are shown in Table S3 in the Supplementary Material.

## 2.3. Headspace solid phase extraction (HS-SPME) of toxic compounds

Headspace extraction of wine samples was performed with a CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) with a conditioning station for SPME fiber. HS-SPME was carried out according to previous work: 1 mL of wine in 20 mL glass headspace vials, 30% of NaCl (m/v), without sample agitation, extraction time of 45 min and extraction temperature of  $55^\circ\text{C}$  (Welke et al., 2012).

The derivatization step using TFEH was added to the previously developed method in order to improve detection of three toxic compounds, which are highly volatile: acetaldehyde (boiling point, BP:  $20.2^\circ\text{C}$ ), formaldehyde (BP:  $-19.3^\circ\text{C}$ ) and acrolein (BP:  $53^\circ\text{C}$ ). Boiling points and other characteristics of analytes evaluated in this study, as well as the derivatization reaction are shown in Supplementary Material (Table S4 and Fig. S1, respectively). After HS-SPME, compounds were thermally desorbed in the injector of GC  $\times$  GC/TOFMS at  $250^\circ\text{C}$  for 5 min.

## 2.4. Determination of the toxic compounds using GC $\times$ GC/TOFMS

The GC  $\times$  GC system consisted of an Agilent 6890 N (Agilent Technologies, Palo Alto, CA, USA) equipped with a Pegasus IV time-of-flight mass spectrometer (Leco Corporation, St. Joseph, MI, USA). A polar column (DB-Wax, 100% polyethylene glycol;  $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ , J&W Scientific Inc., Folsom, CA, USA) was used as first-dimension ( $^1\text{D}$ ) column, and a DB-17 ms (DB17 ms 50% phenyl-methylpolysiloxane;  $1.70 \text{ m} \times 0.18 \text{ mm} \times 0.18 \mu\text{m}$ , J&W Scientific Inc., Folsom, CA, USA) was employed as a second-dimension ( $^2\text{D}$ ) column. This column set showed the best results related to the separation among the compounds of wine, better occupation of the bidimensional separation space and consequently lower number of coeluted compounds than those obtained when other two combinations of chromatographic columns were used in  $^1\text{D}$  and  $^2\text{D}$ : (i) DB-WAX ( $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ )  $\times$  DB1 ms (100% dimethylpolysiloxane;  $1.70 \text{ m} \times 0.10 \text{ mm} \times 0.10 \mu\text{m}$ ) and (ii) DB-WAX ( $30 \text{ m} \times 0.25 \text{ mm} \times 0.25 \mu\text{m}$ )  $\times$  DB17 ms (50% phenyl 50% dimethyl arylene siloxane;  $1.70 \text{ m} \times 0.18 \text{ mm} \times 0.18 \mu\text{m}$ ) (Welke et al., 2012).

The GC system was equipped with a secondary column oven and a dual stage thermal modulator. During modulation, cold pulses were generated using dry nitrogen gas cooled by liquid nitrogen (Linde, Canoas, RS, Brazil), whereas heated dry air was used for hot pulses. The injector, transfer line and ion source temperature were at  $250^\circ\text{C}$ . Programme conditions of oven temperature were as follows: initial temperature of  $35^\circ\text{C}$  for 5 min,

programmed at 3 °C min<sup>-1</sup> to 250 °C, where it remained for 5 min. The secondary oven was kept 10 °C above the primary oven throughout the chromatographic run. The modulator was offset by +25 °C in relation to primary oven. Helium (99.9999% purity, White Martins, Porto Alegre, RS, Brazil) was used as carrier gas at a constant flow of 1 mL min<sup>-1</sup>. The MS parameters included electron ionization at 70 eV, detector voltage of -1750 V, mass range of 45–450 *m/z*, and acquisition rate of 100 spectra s<sup>-1</sup>. All samples were analyzed in triplicate. These conditions were used in a previous study (Welke et al., 2012).

Analytes were positively identified comparing the retention times and mass spectra of unknown compounds with those of standard compounds (mentioned in Section 2.1) through co-injections. In addition, the mass spectra of the analytes were compared with the spectra available on the NIST mass spectral library (2005).

The quantitation of the analytes was performed using the external standard method. Work solutions of the standard compounds were prepared with the wine model solution (described in Section 2.1) and the range of concentrations of each one of the compounds is listed in Table 1. The analytical curves with at least six concentration levels for each standard compound have been obtained by linear regression using the ratio between the area of quantifier ion of each toxic compound and the area of the quantifier ion of an IS. The quantifier ions are listed in Table S2 of the Supplementary Material.

The method used to quantify toxic compounds was evaluated according to the following parameters: linearity, recovery, precision, repeatability, limit of quantification (LOQ) and detection (LOD) (ICH, 1996). Recovery, repeatability and precision were performed by spiking three different concentrations of each toxic compound in model wine solutions. The lowest, intermediate and highest concentration of the analytical curve of each toxic compound were used to determine these parameters. Repeatability was obtained by the coefficient of variation (CV) of six independent

assays performed under the same analytical conditions on the same day and the precision was calculated by the CV of four independent assays performed under the same analytical conditions in four different days.

## 2.5. Calculation of the estimated daily intake of toxic compounds

The theoretical estimated exposure of the toxic compounds found in wine was calculated as follows: estimated daily intake of toxic compounds (μg kg<sup>-1</sup> body weight per day) = [wine consumption expressed in mL per day × concentration of toxic compound in wine expressed in μg mL<sup>-1</sup>] ÷ body weight of 60 kg as recommended by the World Health Organization (WHO, 2013).

Wine consumption was estimated using two approaches based on the: (i) recommended daily intake of a cup of wine (150 mL), as wine presents beneficial properties derived from its moderate consumption due to the presence of flavonoids and stilbenes (Boban et al., 2016); and (ii) amount of wine (242 mL) consumed by the Brazilian population according to data of Analysis of Personal Food Consumption done in Family Budget Research, abbreviated as POF (from portuguese: Pesquisa de Orçamentos Familiares). This is the most recent evaluation performed by Brazilian Institute of Geography and Statistic, which included 34,003 respondents, aged from 10 years old and upward. The consumption of 1121 food items was mentioned in POF, including wine. (IBGE, 2011). The consumption of 242 mL of wine is reported in POF considering only people who declared consuming this beverage. The exposure to the toxic compounds found in wine was estimated considering the concentration of each compound quantified in wines.

## 2.6. Risk characterization

The Joint FAO/WHO Experts Committee on Food Additives (JECFA) does not provide parameters for safe ingestion of formaldehyde, acetaldehyde, acrolein, furan and EC, and establishes that the

**Table 1**

Figures of merit of the analytical method based on the use of HS-SPME-GC × GC/TOFMS for the determination of formaldehyde, acetaldehyde, acrolein, furan, ethyl carbamate (EC) and furfural found in Syrah wine. Experimental conditions are described in Sections 2.3 and 2.4.

Compound	Linearity (μg L <sup>-1</sup> )	Regression equation	r <sup>2a</sup>	LOD <sup>b</sup> (μg L <sup>-1</sup> )	LOQ <sup>c</sup> (μg L <sup>-1</sup> )	Conc. (μg L <sup>-1</sup> ) <sup>d</sup>	Rec. (%) <sup>e</sup>	Repe. (%) <sup>f</sup>	Prec. (%) <sup>g</sup>
Formaldehyde	11–39	y = 368.78x + 1.562	0.9920	0.5	1.5	11	94	7	11
						22	95	8	10
						39	100	8	10
Acetaldehyde	11–39	y = 3135.7x – 23.054	0.9898	3.0	9.0	11	103	6	12
						22	94	6	8
						39	96	8	8
Acrolein	10–35	y = 18.443x – 0.025	0.9939	1.0	3.0	10	90	11	12
						20	98	7	11
						35	99	8	9
Furan	45–270	y = 14.731x – 0.431	0.9732	5.2	15	45	95	13	10
						180	100	11	7
						270	96	8	9
EC	51–303	y = 0.1053x + 0.004	0.9978	0.3	0.9	51	97	9	12
						150	97	7	9
						303	99	11	8
Furfural	50–503	y = 594.44–22.758	0.9970	1.1	3.3	50	106	11	10
						201	98	9	10
						503	92	11	12

<sup>a</sup> Determination coefficient.

<sup>b</sup> Limit of detection.

<sup>c</sup> Limit of quantification.

<sup>d</sup> Concentration corresponding to the lowest, intermediate and highest concentration of the analytical curve of each toxic compound that was used to determine the percentage of recovery, repeatability and precision.

<sup>e</sup> Percent of recovery.

<sup>f</sup> Repeatability: coefficient of variation of six independent assays performed under the same analytical conditions on the same day.

<sup>g</sup> Precision: coefficient of variation of four independent assays performed under the same analytical conditions in four different days.



margin of exposure (MOE) must be used in the risk characterization, as these compounds are genotoxic (Bachand et al., 2010; Bakhiya & Appel, 2010; Cui et al., 2016; Dong et al., 2016; Mogue et al., 2015; Seitz & Stickel, 2010). The MOE is calculated by the ratio of the dose that produces a specific toxic effect in studies using animals (benchmark dose, BMD) and the estimated daily intake calculated as mentioned in Section 2.5. The lower limit of the 95% confidence interval of the dose required to give a 10% increase in the occurrence of a toxic effect compared to the control (BMDL10) are presented in Table S5 of the Supplementary Material. MOE value lower than 10,000 has been proposed as an indication of concern from the perspective of public health (WHO, 2013).

Since furfural is a non-genotoxic compound and may even be used in food as flavouring agent, there is an acceptable daily intake (ADI) of 0.5 mg per kg of body weight established by JECFA (JECFA, 2016). In this case, the comparison of estimated intake of furfural (calculated as mentioned in Section 2.5) with its ADI was used to assess the potential risks to human health. Risk may exist if the estimated intake exceeds the ADI.

### 2.7. Statistical analysis

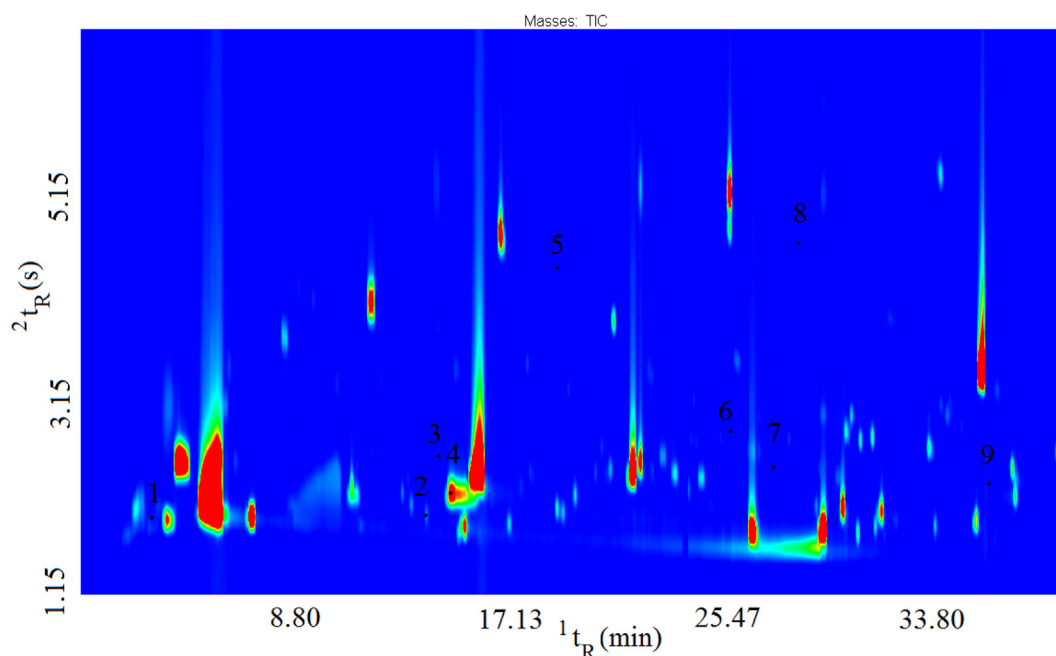
Analysis of variance (ANOVA) followed by Tukey test ( $P < 0.05$ ) using Microsoft Excel was applied to the physicochemical analyses and the data related to levels of toxic compounds found in experimental wines elaborated using grapes of three different stages of maturation and macerated for three different periods.

## 3. Results and discussion

Wines elaborated with grapes from eight different combinations of ripeness degree and maceration times showed no statistical difference according to Tukey test ( $P < 0.05$ ) for density, pH, total acidity, volatile acidity, dry extract, free and total  $\text{SO}_2$ .

Table S3 of Supplementary Material presents the results of the physicochemical analyses. The phenolic content and the total polyphenol index (TPI) were the parameters that presented significant difference in wines. Phenolic levels ranged from 1375 to 2210  $\text{mg L}^{-1}$ . Wines elaborated with grapes harvested before technological or industrial maturity (T1) showed the lowest levels of phenolic and the increase of maceration time (from 10 to 20 days, experiment T1\_C1 and T1\_C2, respectively) has not showed significant difference in this parameter. However, the advancement of the ripeness degree (comparing T1, T2 and T3) increased the levels of these compounds in wines. Overripe grapes (T3 experiment) resulted in wines with the highest concentration of phenolics, however the prolongation of the maceration time (10 to 20 or 30 days) of these grapes seems to negatively influence the concentration of these compounds, since their levels decrease. A deep discussion about the influence of ripeness degree and maceration time on the profile of phenolic compound and other variables will be undertaken in a further study. TPI is around 40 for wines of treatment T1 and higher than 60 to the treatment T2 and T3. This index is a qualitative indicator for the evaluation of the potential for wine aging. Only wines with IPT greater than 60 have potential for aging. Wines with IPT between 45 and 55, are better as young wines and those with IPT below 40 are considered low quality wines (Harbertson & Spayd, 2006). As T1 wines do not have good aging potential, it is not industrially feasible to prolong their maceration for more than 20 days. In contrast, wines obtained from grapes of the treatments named T2 and T3 present potential for aging, since the IPT is close to 60, justifying the use of prolonged maceration until 30 days.

The separation of the toxic compounds (formaldehyde, acetaldehyde, EC, furan, furfural and acrolein) and IS (octanal, 2-furfurylthiol, methyl nonanoate) from the other compounds of the sample in the two-dimensional space is shown in Fig. 1. This chromatogram refers to the wine elaborated with grapes harvested at overripe stage (23°Brix) and macerated during 30 days (T3\_C2

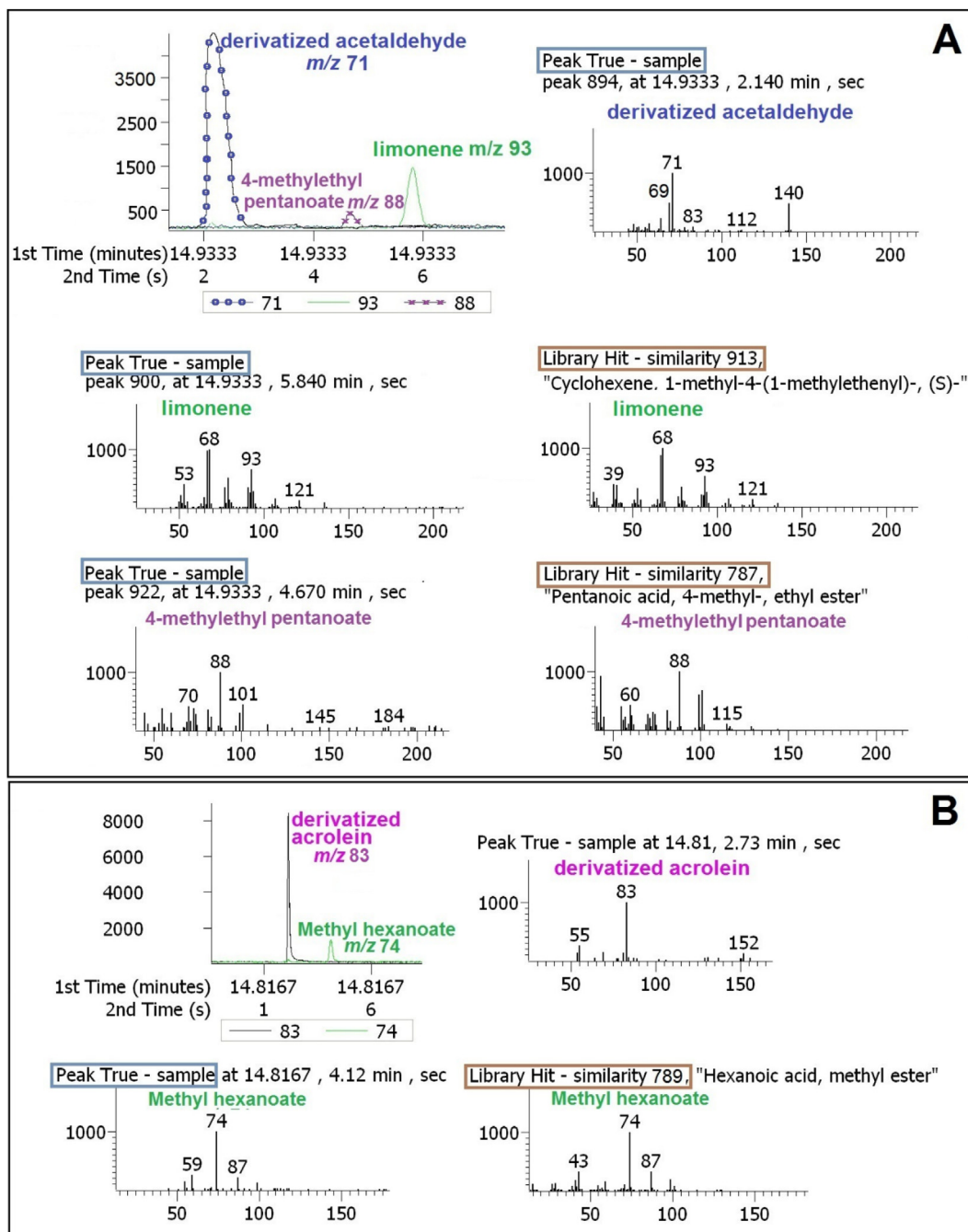


**Fig. 1.** Colour plot of GC  $\times$  GC/TOFMS total ion current chromatogram (TICC) of compounds extracted from the headspace of a Syrah wine demonstrating the separation of toxic compounds, internal standards (IS) and other volatiles according to the retention time in first ( $1t_R$ ) and second ( $2t_R$ ) chromatographic dimensions: [1] formaldehyde, [2] acrolein, [3] furan, [4] acetaldehyde, [5] octanal (internal standard, IS), [6] 2-furfurylthiol (IS) [7] furfural, [8] methyl nonanoate (IS) and [9] ethyl carbamate. Black dots indicate the apexes of the chromatographic peaks of the compounds of interest. This chromatogram refers to the wine that presented the highest levels of toxic compounds (T3\_C2 experiment; grapes harvested at overripe stage (23°Brix) and macerated during 30 days. The chromatograms of other wines were shown in Fig. S2. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

experiment). The chromatograms of other wines were shown in Fig. S2 of Supplementary Material. The comparison of the mass spectra of the standard compounds and the compounds identified in the samples is shown in Figs. S3 and S4 of Supplementary material for toxic compounds and internal standards, respectively. The derivatized form of the acetaldehyde, formaldehyde and acrolein were detected with TOFMS and the ion  $m/z$  69 corresponding to the  $^{-}CF_3$  (present in the derivatizing agent) appears on the mass spectra of these compounds. The molecular ions of acetaldehyde, formaldehyde and acrolein are 71, 57 and 83, respectively. They correspond to the rest of the molecule structures of the derivatized

compounds, as shown in Fig. S1 of Supplementary material. The derivatized compounds are more stable to be analyzed using gas chromatography. Other analytes (furan, furfural and EC) have not required derivatization since these compounds have suitable stability/volatility for GC analysis (Table S4 of Supplementary Material). These compounds did not react with TFEH and consequently the derivatizing ion ( $^{-}CF_3$ ,  $m/z$  69) was not found in mass spectra of furan, furfural and EC showed in Fig. S3 of Supplementary Material.

The use of GC  $\times$  GC/TOFMS allowed correct identification and quantification of two of the toxic compounds (acetaldehyde and



**Fig. 2.** Mass spectra and part of chromatogram of modulated peaks related to the separation in the second dimension ( $^2D$ ) of two toxic compounds (acetaldehyde and acrolein) that coeluted in the first chromatographic dimension ( $^1D$ ) with other compounds present in Syrah wines analyzed using HS-SPME-GC  $\times$  GC/TOFMS. (A) acetaldehyde that was separated in  $^2D$  from 4-methylethyl pentanoate and limonene; (B) acrolein that was separated in  $^2D$  from methyl hexanoate. Experimental conditions are reported in Sections 2.3 and 2.4.

acrolein) that coeluted in the first chromatographic dimension (<sup>1</sup>D) with other compounds present in Syrah wines. Acetaldehyde (<sup>1</sup>t<sub>R</sub>:14.93 min and <sup>2</sup>t<sub>R</sub>: 2.14 s) was separated from ethyl 4-methyl pentanoate (<sup>1</sup>t<sub>R</sub>:14.93 min and <sup>2</sup>t<sub>R</sub>: 4.8 s) and limonene (<sup>1</sup>t<sub>R</sub>:14.93 min and <sup>2</sup>t<sub>R</sub>: 5.95 s) only in the second chromatographic dimension (<sup>2</sup>D). Acrolein (<sup>1</sup>t<sub>R</sub>:14.82 min and <sup>2</sup>t<sub>R</sub>: 2.7 s) was also separated from methyl hexanoate (<sup>1</sup>t<sub>R</sub>:14.82 min and <sup>2</sup>t<sub>R</sub>: 4.1 s) in <sup>2</sup>D (Fig. 2). The compounds that coeluted with acetaldehyde and acrolein have no toxic potential and their presence in wines may have a positive effect on the wine aroma. Esters (ethyl 4-methyl pentanoate and methyl hexanoate) may impart fruity odor and the terpene (limonene) may be related to floral notes (Welke et al., 2012).

The performance of the method used to quantify six toxic compounds found in wines is shown in Table 1. The calibration curves showed good linearity, as determination coefficients (*r*<sup>2</sup>) were in the range of 0.9898 to 0.9978. The LOD and LOQ values show that the method is sufficiently sensitive to quantify toxic compounds in wines. The lowest and highest LOD value was 0.3 µg L<sup>-1</sup> and 5.2 µg L<sup>-1</sup> for EC and furan, respectively. The lower LOQ was found for EC (0.9 µg L<sup>-1</sup>) and furan showed the highest LOQ (15.5 µg L<sup>-1</sup>). Furthermore, recovery was found to be higher than 90% and coefficients of variation were lower than 13.1% (obtained in assays of repeatability and precision), which demonstrate the efficiency of the method.

The lowest levels of toxic compounds were observed in wines produced from grapes harvested before technological or industrial maturity (T1\_C1 and T1\_C2), except for furan and EC that reached

lower levels in T1\_C1 followed by T2\_C1 (Table 2). In contrast, wines done with overripe grapes (T3\_C1, T3\_C2 and T3\_C3) showed the highest levels for all toxic compounds, especially high amount of acetaldehyde, whose concentration was statistically different from the other samples according to Tukey test (*P* < 0.05). Results of ANOVA are shown in Table S6 of Supplementary Material. The concentration of formaldehyde and EC showed no statistical difference between the samples. Acrolein, furan and furfural were found in lower concentrations in wines of experiments T1 (T1\_C1 and T1\_C2) and these levels were statistically different from the other samples (experiment T2 and T3).

In general, the advancement of the ripeness degree and increasing of maceration time of grapes appears to result in higher levels of toxic compounds in the wines, although wines resulting from T3\_C3 did not follow this trend (Table 2). The increase of the concentration of aminoacids (especially arginine, alanine and citrulline) (Lamikanra & Kassa, 1999), glucose, phenolic compounds, and others (Bindon, Varela, Kennedy, Holt, & Herderich, 2013), which are precursors of toxic compounds, may be related to the progress of ripening degree of the grapes (Bindon et al., 2013; Lamikanra & Kassa, 1999). In the same way, during the maceration step occurs the extraction of compounds present in the grape (peel, pulp and seeds) that provide precursors of toxic compounds to the must, such as aminoacids, pentoses, glycerol and pectin hydrolysis products (Cadot et al., 2012; Gil et al., 2012; Lamikanra & Kassa, 1999).

Among these precursors of toxic compounds, whose concentration increases during ripening and maceration of grapes, glucose is

**Table 2**  
Concentration of toxic compounds [formaldehyde, acetaldehyde, acrolein, furan, ethyl carbamate (EC) and furfural] of wines elaborated with Syrah grapes harvested at three different stages of maturation [T1: before technological or industrial maturity (19°Brix), T2: ideal ripeness degree (21°Brix), T3: overripening (23°Brix)] and macerated during three different periods (C1: 10 days, C2: 20 days and C3: 30 days).

Sample	Concentration (µg L <sup>-1</sup> ) ± CV <sup>b</sup> (%)					
	Formaldehyde	Acetaldehyde	Acrolein	Furan	EC	Furfural
T1_C1	33.1 ± 2.9 a	59 ± 4 b,c	42.5 ± 0.2 b	99.6 ± 0.3 b	23.6 ± 1.9 a	51.2 ± 1.0 b
T1_C2	41 ± 4 a	80 ± 4 b,c	78.7 ± 0.4 b	153 ± 1.3 b	86 ± 5 a	67 ± 4 b
T2_C1	79.6 ± 2.0 a	157 ± 9 b,c	134 ± 4 a	141 ± 6 b	66 ± 4 a	89 ± 7 a
T2_C2	83 ± 6 a	238 ± 8 b	144 ± 4 a	207 ± 1.5 a	76.3 ± 0.9 a	92.0 ± 2.9 a
T2_C3	112 ± 12 a	249 ± 6 b	125 ± 3 a	174 ± 2.1 b	89 ± 8 a	95.7 ± 0.7 a
T3_C1	1450 ± 9 a	282 ± 1.5 b	201 ± 10 a	231 ± 9 a	96 ± 7 a	161 ± 8 a
T3_C2	165 ± 9 a	962 ± 8 a	270 ± 12 a	330 ± 5 a	162 ± 10 a	224 ± 12 a
T3_C3	141 ± 8 a	313 ± 10 b	245 ± 9 a	218 ± 10 a	106 ± 8 a	103 ± 10 a

In columns, concentration of toxic compounds followed by same letter are not significantly different (*P* < 0.05) by Tukey test.

**Table 3**  
Concentration of toxic compounds [formaldehyde, acetaldehyde (ACT), acrolein, furan, ethyl carbamate (EC) and furfural] of commercially available Syrah wines.

N <sup>a</sup>	Concentration (µg L <sup>-1</sup> ) ± CV <sup>b</sup> (%)					
	Formaldehyde	ACT	Acrolein	Furan	EC	Furfural
1	206 ± 6	501 ± 13	149 ± 5	82 ± 12	17 ± 9	123 ± 8
2	119 ± 12	635 ± 6	410 ± 9	295 ± 9	200 ± 10	211 ± 6
3	40.3 ± 0.2	252 ± 6	32 ± 10	263 ± 7	144 ± 11	1715 ± 6
4	67 ± 10	226 ± 8	24 ± 12	145 ± 4	242 ± 12	208 ± 10
5	56 ± 12	67.4 ± 1.27	43 ± 5	256 ± 10	77 ± 8	316 ± 6
6	38 ± 3	112 ± 4	30 ± 12	243 ± 13	62 ± 15	366 ± 6
7	49 ± 2	131 ± 10	29 ± 6	199 ± 1.7	ND <sup>c</sup>	414 ± 0.7
8	23 ± 12	104 ± 5	23 ± 11	71 ± 9	69 ± 9	149 ± 11
9	23 ± 10	74 ± 7	8.4 ± 8.1	83 ± 6	ND <sup>c</sup>	204 ± 8
10	33 ± 6	51 ± 10	18.0 ± 1.0	64 ± 11	52 ± 6	243 ± 9
11	47 ± 10	192 ± 1.1	30.1 ± 1.0	56 ± 11	40 ± 7	84 ± 10
AM <sup>d</sup>	64	213	72	160	100	184
M <sup>e</sup>	47	131	30	145	69	211

<sup>a</sup> Sample number as specified in Table S2 of the Supplementary Material.

<sup>b</sup> Coefficient of variation.

<sup>c</sup> Not detected (concentration lower than LOQ <0.9 µg L<sup>-1</sup> for ethyl carbamate).

<sup>d</sup> Arithmetic mean.

<sup>e</sup> Median.

the primary substrate for acetaldehyde formation in wine. In addition, acetaldehyde may also be formed from alanine and by the oxidation of phenolic compounds and ethanol. Arginine, citrulline and their hydrolysis products may be precursors of EC (Araque et al., 2016). Acrolein is formed from the glycerol metabolism. Furan and furfural are formed from the degradation of sugars and formaldehyde is originated from pectin hydrolysis products (Jackson, 2014). The formation of these toxic compounds is catalysed by enzymes produced by microorganisms of the winemaking, especially during fermentation. As fermentation occurs during the maceration of the grapes, the reactions catalyzed by enzymes of the yeast (alcoholic fermentation) and lactic acid bacteria (malolactic fermentation) may have been potentiated with the advancement of the maceration period (Cadot et al., 2012).

Wines of T3\_C3 experiment did not follow the same trend of increasing the concentration of toxic compounds as ripening

advances and maceration period becomes longer. Degradation/oxidation reactions of toxic compounds may have occurred when the combination of overripe grapes and prolonged maceration time (30 days) was used in winemaking (Oliveira, Ferreira, De Freitas, & Silva, 2011). Carbonyls present in formaldehyde, acetaldehyde, acrolein, EC and furfural may react with sulfur dioxide (SO<sub>2</sub>) naturally present and/or added to wine and form  $\alpha$ -hydroxysulfonates that present low volatility and therefore, weak contribution to odor. The reaction among alcohols and carbonylic compounds produces acetals that result in green or pungent odor depending on the type of acetal compound and their concentration. Furthermore, the reaction of carbonyl compounds and sulfur aminoacids, such as cysteine, may result in the formation of thiols and thiazole compounds. These compounds may contribute to odor described as fruity, meaty, roasted, coffee, or cooked vegetable depending on the compound formed and its concentration in wine (Marchand,

**Table 4**

Values of the margin of exposure (MOE) calculated to the genotoxic compounds: formaldehyde, acetaldehyde, acrolein, furan and ethyl carbamate (EC) found in Syrah wines. The samples that represent risk to consumer health are highlighted in red (MOE value lower than 10,000). MOE was calculated using two wine consumption data: (i) based on a recommended daily intake of a cup of wine (150 mL), and (ii) based on the amount of wine (242 mL) consumed by the Brazilian population according to data of Analysis of Personal Food Consumption done in Family Budget Research.

BMDL10 <sup>a</sup>		Formaldehyde 28	Acetaldehyde 56	Acrolein 0.36	Furan 0.96	EC 0.25
Sample	Consumption (mL) <sup>b</sup>	Margin of exposure (MOE)				
Experimental wines <sup>c</sup>						
T1_C1	150	338,778	378,373	3389	3857	4241
	242	209,986	234,529	2100	2629	2391
T1_C2	150	273,304	280,486	1830	2501	1155
	242	169,403	173,855	1830	716	1551
T2_C1	150	140,615	142,372	1076	2718	1516
	242	87,158	88,247	1076	940	1685
T2_C2	150	135,511	93,928	1003	1858	1311
	242	83,995	58,220	1003	813	1152
T2_C3	150	100,188	89,802	1151	2205	1121
	242	62,100	55,662	1151	695	1367
T3_C1	150	74,896	79,368	718	1664	1037
	242	46,423	49,195	718	643	1031
T3_C2	150	67,866	23,285	534	1163	617
	242	42,066	14,433	534	382	721
T3_C3	150	79,433	71,642	588	1759	942
	242	49,235	44,406	588	584	1090
Commercially available wines <sup>d</sup>						
1	150	54,393	44,681	967	4682	5728
	242	33,715	27,695	600	3551	2867
2	150	94,193	35,279	350	1300	498
	242	58,384	21,867	217	309	796
3	150	277,948	88,800	4457	1459	695
	242	172,282	55,042	2763	431	893
4	150	166,613	98,883	6023	2644	412
	242	103,273	61,291	3734	256	1619
5	150	201,551	332,217	3359	1498	1295
	242	124,929	205,920	2083	803	917
6	150	293,994	200,603	4774	1581	1620
	242	182,228	124,341	2960	1004	968
7	150	229,615	170,616	4901	1933	>111,111 <sup>e</sup>
	242	142,324	105,754	3038	900	>68,870 <sup>e</sup>
8	150	478,582	215,583	6373	5401	1451
	242	296,642	133,626	3951	1200	900
9	150	475,797	303,274	17,087	4650	>111,111 <sup>e</sup>
	242	294,916	187,980	10,591	1550	>68,870 <sup>e</sup>
10	150	337,611	435,059	7989	5992	1935
	242	209,263	269,665	4952	2629	3669
11	150	238,275	116,476	4784	6875	2500
	242	147,692	72,196	2966	716	4210
Median	150	238,275	170,616	4784	2644	1620
	242	147,692	105,754	2965	1618	1004

<sup>a</sup> BMDL10 value expressed in mg kg<sup>-1</sup> of body weight as specified in Table S5 of the Supplementary Material.

<sup>b</sup> Consumption.

<sup>c</sup> Wines elaborated with Syrah grapes harvested at three different stages of maturation [T1: before technological or industrial maturity (19°Brix), T2: ideal ripeness degree (21°Brix), T3: overripening (23°Brix)] and macerated during 3 different periods (C1: 10 days, C2: 20 days and C3: 30 days).

<sup>d</sup> Commercial samples were provided by wineries located in Brazil (states of Rio Grande do Sul, Pernambuco, Bahia, Minas Gerais) and Chile (Details on the year and local of production of each sample are in Table S2 of Supplementary Material).

<sup>e</sup> ethyl carbamate wasn't found in these samples (concentration below the LOQ <0.9 µg L<sup>-1</sup>; this LOQ value was used to calculate MOE).



De Revel, & Bertrand, 2000). A detailed research on the volatile compounds related to the aroma of these wines will be reported as a separated further investigation.

The concentration of toxic compounds found in Syrah wine samples commercially available is shown in Table 3. Formaldehyde (ranging from 23.4 to 206  $\mu\text{g L}^{-1}$ ), acetaldehyde (51.5 to 635  $\mu\text{g L}^{-1}$ ), acrolein (8.4 to 410  $\mu\text{g L}^{-1}$ ), furan (55.8 to 295  $\mu\text{g L}^{-1}$ ) and furfural (83.9 to 1715  $\mu\text{g L}^{-1}$ ) were found in all samples under study. EC was not found (levels lower than the LOQ of the method, 0.9  $\mu\text{g L}^{-1}$ ) in only two commercially available wines. Some of these samples showed high concentration of furfural and acetaldehyde, which levels achieved 1715 and 635  $\mu\text{g L}^{-1}$ , respectively.

The comparison of the concentrations of toxic compounds quantified in wines with legislation limits is quite difficult, since only EC maximum levels in wine have been reported in some countries. Among the wines under study, 79% of samples showed EC levels higher than 30  $\mu\text{g L}^{-1}$  which is the limit established for wine in Canada (30  $\mu\text{g L}^{-1}$ ) and Czech Republic (30  $\mu\text{g L}^{-1}$ ) and 89% of the wines had EC concentration higher than 15  $\mu\text{g L}^{-1}$  (the maximum level set in USA). No sample showed formaldehyde level higher than the tolerable concentration (2600  $\mu\text{g L}^{-1}$ ) established by the International Programme on Chemical Safety of WHO (IPCS, 2012).

Toxic compounds quantified in experimental wines (elaborated using grapes harvested at three different stages of maturation and macerated during three different periods) (Table 2) and commercially available samples (Table 3) were used for calculation of estimated daily intake (Table S7 of Supplementary Material), which was employed to determine MOE (Table 4). Exposure to acrolein, furan and EC may pose risk on the consumer health, since MOE values were lower than 10,000 for both commercially available wines and experimental wines. The smaller the MOE value, the greater the risk associated with exposure to toxic compounds. The samples of experimental wines produced with overripe grapes (T3 experiment) showed the lowest MOE values.

According to data presented in Table 4, despite the high concentration of acetaldehyde and furfural found in some samples, these compounds did not represent risk to consumer health when ingested through wine. Acetaldehyde showed MOE values (170,616 and 105,754 related to daily consumption of 150 and 242 mL, respectively) higher than 10,000 and the estimated daily intake of furfural (Table S7 of Supplementary Material; median values of estimated intake of 0.5 and 0.8  $\mu\text{g kg}^{-1}$  of body weight for daily consumption of 150 and 242 mL, respectively) was lower than the ADI (500  $\mu\text{g kg}^{-1}$  of body weight) established by JECFA for this compound (JECFA, 2016). However, other foods/beverages may be a source of exposure to these toxic compounds. Acetaldehyde is naturally present in fruits, in addition to being formed in liver by metabolism of ethanol after ingestion of alcoholic beverages (Wang et al., 2016). Furfural is a characteristic flavour product of the Maillard reaction found in baked products, coffee, roast beef, and others (Jackson, 2014).

Data concerning quantification of toxic compounds formed during the preparation of wine are incipient. Moreover, the evaluation of exposure to these toxic compounds through wine consumption is reported in the literature only for acetaldehyde quantified in wine marketed in Italy (Paiano et al., 2014) and acetaldehyde, EC and formaldehyde using quantitative data previously published in other study (Lachenmeier, Przybylski, & Rehm, 2012). In both studies, the exposure to these toxic compounds seemed to represent risk (Lachenmeier et al., 2012; Paiano et al., 2014).

#### 4. Conclusion

The well-known high capacity of GC  $\times$  GC/TOFMS for wine analysis proved to be appropriate for this first quantitative determination of six toxic compounds found in Syrah wines. The coelution of

both acetaldehyde and acrolein with other wine compounds indicates the difficulties that could arise if only one-dimensional gas chromatography was used for the determination of such compounds that possibly would result in poor chromatographic separation and therefore, incorrect identification/quantification.

The advancement of the ripeness degree and longer maceration times of grapes are critical points related to the formation of toxic compounds. These steps may be related to an increasing of the concentration of precursors of toxic compounds. The use of over-ripe grapes and maceration of grapes greater than 20 days should be avoided since high levels of toxic compounds were formed during winemaking. The kinetics of formation of these compounds at all stages of winemaking will be evaluated in a future study. Special attention must be given to the formation of compounds that may cause risk to consumers' health, especially acrolein, EC and furan, whose MOE values indicated risk. The results of this study may contribute to define the harvest time and the duration of maceration to produce wines with the lowest possible levels of toxic compounds in order to get the most out of the beneficial functional properties of wine.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2017.03.090>.

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