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► To cite this version:

Monica Rosa Loizzo, Stefania Nigro, Damiano de Luca, Francesco Menichini. Detection of ochratoxin A and cis- and trans-resveratrol in red wines and their must from Calabria (Italy). *Food Additives and Contaminants*, 2011, 99999 (1), pp.1. 10.1080/19440049.2011.590454 . hal-00715433

HAL Id: hal-00715433

<https://hal.science/hal-00715433>

Submitted on 7 Jul 2012

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Journal:	<i>Food Additives and Contaminants</i>
Manuscript ID:	TFAC-2011-077.R1
Manuscript Type:	Original Research Paper
Date Submitted by the Author:	09-May-2011
Complete List of Authors:	Loizzo, Monica; University of Calabria, Pharmaceutical sciences Nigro, Stefania; CALAB De Luca, Damiano; CALAB Menichini, Francesco; CALAB
Methods/Techniques:	Chromatography - HPLC, Extraction
Additives/Contaminants:	Ochratoxin A
Food Types:	Wine
Abstract:	The natural occurrence of ochratoxin A (OTA) in red wines has been widely reported by several authors, as well as cis- and trans-resveratrols. The aim of this work was to evaluate the ochratoxin A (OTA) and both cis- and trans-resveratrol content from must to wine during the red wine making process in a pilot scale of vinification in Calabria (Italy). Eleven samples were collected at different stages of the vinification process. Analysis was done by HPLC. Wine from manufacturer 3 present the highest amount of trans-resveratrol (3.41 mg l ⁻¹). This wine was characterized by both Aglianico-Magliocco grape variety. Interestingly, data regarding OTA revealed that the value of this contaminant is low in all analyzed samples and in each case below the legal limit (2.0 mg l ⁻¹ (ppb)). Overall, the obtained results demonstrated the high quality of wines produced in Calabria.

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Detection of ochratoxin A and *cis*- and *trans*-resveratrol in red wines and their musts from Calabria (Italy)

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The natural occurrence of ochratoxin A (OTA) in red wines has been widely reported by several authors, as well as *cis*- and *trans*-resveratrols. The aim of this work was to evaluate the ochratoxin A (OTA) and both *cis*- and *trans*-resveratrol content from must to wine during the red wine-making process in a pilot scale of vinification in Calabria (Italy). Eleven samples were collected at different stages of the vinification process. Analysis was carried out by HPLC. Wine from manufacturer 3 contained the highest amount of *trans*-resveratrol (3.41 mg l⁻¹). This wine was characterized by both Aglianico-Magliocco grape variety. Interestingly, data regarding OTA showed that the value of this contaminant is low in all analyzed samples and in each case below the legal limit (2.0 mg l⁻¹ (ppb)). Overall, the results demonstrated the high quality of wines produced in Calabria.

Keywords: Ochratoxin A; *cis*- and *trans*-resveratrol; wine; vinification process

Introduction

Calabria has been well known for the quality of its local grapes and wines since immemorial time so much as that in Ancient Greece they were awarded as prizes to the Olympic winners. This is the region that was first called Enotria, or 'Land of Wine', the name that ultimately defined Italy for the Ancient Greeks. We are talking about Calabria, the area whose viticulture and winemaking traditions are interwoven with the region's own history, which have been this way for thousands of years. To this day, viticulture represents an important part of the local economy. In fact, Calabria has long been an important contributor of the Italian winemaking sector. The area dedicated to vitiviniculture in Calabria dropped from 50,000 hectares in the 1950s, to the current 18,000 hectares. This is partly due to the voluntary abandonment of some vintners, and in part to incentives proposed by the European Community. Yearly production is about 100,000,000 cases of wine, of which 80% are red wines and 20% are white. There are 12 regional DOC and 13 IGT wines, that account for about 890,000 cases per year. The local wines strongly express the unique local climate, characterized by constant sun exposure and balanced heat and humidity levels during the maturation period. Today, the regional winemaking industry is still strongly linked to the local traditions, which in practice mark well-defined zones.

Ochratoxin A (OTA) is a toxic fungal secondary metabolite synthesized by *Penicillium verrucosum*, *P. nordicum* and some species of the genera *Aspergillus*, *Petromyces* and *Neopetromyces* (Frisvad and Samson 2000; Frisvad and Thrane 2000; Larsen et al. 2001; Castella et al. 2002). OTA is a potent nephrotoxic mycotoxin which is carcinogenic and possesses teratogenic, immunotoxic and possibly neurotoxic properties (Scientific Committee on Food 1998). The International Agency for Research on Cancer (IARC) has classified OTA in Group 2B as possibly carcinogenic to humans based on sufficient evidence for carcinogenicity in experimental animals and inadequate evidence in humans (IARC 1993). The Joint Expert Committee on Food Additives (JECFA) of the World Health Organization (WHO) and the Food and Agriculture Organization (FAO) established at its 44th meeting (FAO/WHO JECFA 1995) a provisional tolerable weekly intake (PTWI) rounded to 100 ng kg⁻¹ body weight; this PTWI was retained at the 56th meeting, the last time the JECFA evaluated OTA (FAO/WHO JECFA 2001). Recently, the Scientific Panel on Contaminants in the Food Chain of the European Food Safety

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3 Authority (EFSA) has derived a tolerable weekly intake (TWI) of 120 ng kg⁻¹ body
4 weight for OTA, which equivalent to a daily intake of about 17 ng kg⁻¹ body weight
5 (Scientific Panel on Contaminants in the Food Chain 2006). Wine was identified as
6 the second most important source of OTA after cereals in the European diet,
7 accounting for as much as 13% of the total contribution, according to the conclusions
8 of the Report of the 2nd Scientific Cooperation Task promoted by the European
9 Commission in 1999, concerning the assessment of dietary intake of OTA in the
10 population of the European Union Member States (Miraglia and Brera 2002).

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12 The epidemiological research undertaken to date ensures, with a certain degree
13 of certainty, that the moderate consumption of wine, is beneficial for one's health, the
14 so-called French paradox (Renaud and de Lorgeril, 1992). The presence of the
15 polyphenolic compounds, such as resveratrol, that are present in high concentrations
16 in grape skin, seeds, and wine, mainly in red wine, act positively in the human body
17 (Soleas et al. 1997; Sun et al. 2002). Resveratrol occurs in two isomeric forms (*trans*
18 and *cis*) (Fig. 1): the *trans*-resveratrol or 3,5,4'-trihydroxystilbene is the most
19 abundant form and is mainly located in grape skins (Monagas et al. 2005). Several
20 studies have demonstrated that resveratrol has a biological effect that provides health
21 benefits. Besides its antioxidant activity, this has proved to have an anticancer effect
22 in the process of the initiation, promotion and progression of cancer
23 (Chanvitayapongs et al. 1997; Jang et al. 1997; Belguendouz et al. 1998; Rotondo et
24 al. 1998; Jannin et al. 2001). In fact, while inhibiting the formation of free radicals, it
25 could act as an antimutagenic and, while stopping the oxygenase cycle (Belguendouz
26 et al. 1998; Rotondo et al. 1998), it could reduce the presence of substances deriving
27 from arachidonic acid which stimulate the growth of cancer. Jannin et al. (2001)
28 underlined that *trans*-resveratrol produces a strong inhibition in the proliferation of
29 hepatoblastomas' cellular lines both in human subjects and in mice; furthermore, the
30 presence of alcohol as a solvent enhances its effect. Recently, it was found
31 responsible of the reduction of the effects of some neurological diseases, as
32 Alzheimer or Parkinson, (Bastianetto et al. 2000; Youdim and Joseph, 2001; Alarco'n
33 de la Lastra and Villegas, 2005) among others.

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35 The aim of this study was to detect OTA and resveratrol in samples collected in
36 Calabria (Italy) at different stages of the vinification process. Data on the distribution
37 of resveratrol-related compounds and OTA in Italian red wine were gathered.
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Materials and methods

Chemicals

OTA and *trans*-resveratrol standards were purchased from Sigma – Aldrich s.r.l. (Milan, Italy). *cis*-Resveratrol was obtained by UV irradiating a methanolic solution of *trans*-isomer (100 mg l⁻¹) at 366 nm for 30 min. The resulting solution has two peaks, the first one corresponding to *trans*-Resveratrol and the second one corresponding to *cis*-Resveratrol. The obtained concentration of *cis*-resveratrol is estimated to be 94%. All chromatographic solvents were high-performance liquid chromatography (HPLC) ultra gradient grade solvents and were purchased from Sigma-Aldrich s.r.l. (Milan, Italy). HPLC grade acetonitrile is obtained from Carlo Erba (Mila, Italy). Analytical reagent grade phosphoric acid and methanol were also supplied by Carlo Erba. Ultrapure water was produced by a Milli-Q system (Millipore, Bedford, MA).

Wine and must samples

Eleven Calabrian (Italy) wines were selected based on their geographic origin, representing either Denomination of Controlled Origin (DOC) or Identification of Geographical Tipicity (IGT) Italian wines. All wine samples used in this study were obtained from grapes cultivated in different areas of the province of Cosenza during the 2007 vintage year. Further information on the geographical origin, grape varieties, vintage year are reported in Table I. All wines were analyzed for OTA and *cis*-, *trans*-resveratrol. Analysis were performed in triplicate.

Analysis of cis- and trans-resveratrol

Analysis was carried out by solid-phase extraction (SPE) method and HPLC. The SPE method provided the use of SPE ISOLUTE C18 (1g, 6 ml) cartridge (Stepbio, Bologna, Italy). Samples are dissolved in distilled water. The sample solution is transferred into an SPE cartridge preconditioned with methanol and distilled water. This is then washed with water and eluted with methanol. The solutions are evaporated to dryness under a stream of nitrogen. The samples are re-dissolved by adding methanol. For identification of *cis*- and *trans*-resveratrol a HPLC system HP 1100, equipped with a pump, UV-vis detector, column oven, injector, and a C18 RP column (Phenomenex Luna 5 µm C18, 250 x 4.60 mm) was employed. The elution

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3 was performed under binary gradient conditions (Abrin et al. 2005). Pump A: glacial
4 acetic 12 in water (pH 2.4) and Pump B: phase A/acetonitrile (20:80). The gradient
5 profile was 0 min 18% B; 11 min, 18% B; 18 min, 23% B; 31 min, 100% B. The
6 flow-rate was 1.0 ml min⁻¹. The eluate was monitored at two different wavelengths,
7 285 and 306 nm, where *cis*- and *trans*-isomers have absorbance maxima, respectively.
8 Identification of *trans*-resveratrol was performed by comparison of both retention
9 time and UV spectrum with those obtained with the standard. A calibration curve was
10 obtained for *trans*-resveratrol at 306 nm. *cis*-Resveratrol was identified by comparing
11 retention time and UV spectrum with the new peak appearing after UV irradiation of
12 *trans*-resveratrol standard solution. As *cis*-resveratrol is not commercially available,
13 its calibration curve was obtained at 285 nm using the same solutions as for the *trans*-
14 isomer, after exposure of the solution to UV light at 366 nm for 30 min. This time of
15 exposure was necessary to determine at least 90% of the *trans*- to *cis*-isomer
16 conversion that was ascertained on the basis of the decrease in the *trans*-isomer (Trela
17 & Waterhouse 1996). The concentrations of *cis*-resveratrol standard solutions were
18 obtained from *trans*-resveratrol concentrations corrected by the relevant conversion
19 factor (Goldeberg, 1995). Calibration curves were linear, with a correlation
20 coefficient of 0.9999 over the ranges of 0.07-53.50 mg l⁻¹ for *trans*-resveratrol and
21 1.25-9.50 mg l⁻¹ for *cis*-resveratrol. LODs were 0.02 mg l⁻¹ for *trans*-resveratrol and
22 0.03 mg l⁻¹ for *cis*-resveratrol.
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40 *Ochratoxin A Analysis*

41 OTA analyses were performed according to the AOAC Official Method 2001.01
42 (Visconti et al. 2001). Wine samples (20 ml) were diluted with 20 ml of a solution
43 containing 1% polyethylene glycol (PEG 8000) and 5% sodium bicarbonate and
44 filtered through glass microfiber filters. Filtered extract (10 ml) was cleaned up
45 through an OchraTest immunoaffinity column (VICAM, Watertown, MA) at a flow
46 rate of about 1 drop per second. The column was washed with 5 ml of solution
47 containing sodium chloride (2.5%) and sodium bicarbonate (0.5%) followed by 5 ml
48 of distilled water at a 1-2 drops per second flow rate. OTA was eluted from the
49 column with 2 ml of methanol, and the eluted extract was evaporated under air stream
50 at ca. 50 °C and reconstituted with 250 µl of the HPLC mobile phase. Re-constituted
51 extract (100 µl) was injected into the same chromatographic apparatus used for
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3 resveratrol analysis by a full loop injection system. The toxin was determined by
4 HPLC/fluorescence detector (Agilent 1100 Series, Agilent Technology) set at an
5 excitation wavelength of 333 nm and an emission wavelength of 470 nm. The
6 analytical column was a Luna C18 (250 x 4.6 mm, 5 μm ,) preceded by a 0.45 μm
7 Rheodyne guard filter. The mobile phase consisted of a mixture of phosphoric acid
8 0.083M/acetonitrile/methanol (1:1:1v/v/v) eluted at a flow rate of 0.8 ml min⁻¹.
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14 15 16 *Statistical analysis*

17 All experiments were carried out in triplicate. Data were expressed as means \pm S.D.
18 Regression analyses of OTA versus stilbenes levels were performed on OTA positive
19 wine samples ($n = 10$) in order to calculate correlation coefficients (r).
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25 **Results and discussion**

26 OTA and both *cis*- and *trans*-resveratrol content from must to wine during the
27 red wine-making process in a pilot scale of vinification in the area of Cosenza
28 (Calabria, Italy) were reported. Eleven samples selected based on their geographic
29 origin, representing either Denomination of Controlled Origin (DOC) or Identification
30 of Geographical Tipicity (IGT) Italian wines (Table I) were investigated. Among the
31 analyzed samples, the sample from manufacturer 3 characterized by both Aglianico
32 and Magliocco grape varieties exhibited the highest amount of total resveratrol (2.23,
33 6.22 and 5.61 mg l⁻¹ for must, wine stopped after FML and commercial wine,
34 respectively) (Figure 1, Table II). Samples from manufacturer 7 characterized only by
35 the Magliocco grape variety showed the lowest content of resveratrol. In particular in
36 commercial wine the *cis*- and *trans*-resveratrol content were 0.62 and 0.72 mg l⁻¹,
37 respectively. This data is of interest because it is possible that the Aglianico grape
38 variety can contribute significantly to the production of these polyphenolic
39 compounds since samples from manufacturer 1 containing only Aglianico grape
40 variety exhibited a *trans*-resveratrol content of 1.41 mg l⁻¹. Commercial wine samples
41 from manufacturer 5 containing Greco, Guarnaccia and Malvasia grape variety
42 exhibited a *cis*- and *trans*- resveratrol content of 1.81 and 2.28 mg l⁻¹, respectively. A
43 *cis*- and *trans*-resveratrol content of 1.40 and 1.00 mg l⁻¹, respectively was found in
44 commercial wine containing Merlot and Cabernet Sauvignon from manufacturer 10.
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60 A lower content of total resveratrol was found in samples from manufacturer 11

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3 characterized only by Gaglioppo grape variety (1.06 mg l^{-1}). In disagreement with
4 data reported in literature we found no correlation between grapes grown in high
5 altitudes and high resveratrol content (Bavaresco et al. 2007).
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9 The quantity of resveratrol in the wine is determined by the amount of time that
10 the grape skins and pulp ferment. Moreover its concentration varies considerably
11 (from 0.1 to 20 mg l^{-1}) depending on multiple factors such as grape variety, fungal
12 infection, winemaking process, and climatic conditions. In disagreement with a
13 previous study that analyzed wines obtained with Aglianico, Piediroso and Nerello
14 Mascalse grapes, we have found an influence of grape variety and *trans*-resveratrol
15 content (Gambutì et al. 2004). Goldeberg et al. (1995) analyzed 300 wine samples
16 from around the world for their *trans*-resveratrol content. Among them, 67 Italian
17 wines from Veneto, Tuscany and Piedmont were analyzed and a *trans*-resveratrol
18 content ranged from 1.51 to 1.76 mg l^{-1} was found. La Torre et al. (2002) reported the
19 *trans*-resveratrol content in 10 Calabrian wine produced in the Crotona area (Calabria,
20 Italy) with different grape varieties including Greco bianco, Gaglioppo,
21 Montepulciano, Nebbiolo, Nero d'Avola, Malvasia, Colorino and Cirò. All of the
22 results, except Cirò wine which exhibited the highest *trans*-resveratrol content, (6.76
23 mg ml^{-1}) are in line with our values. The high content in *trans*-resveratrol founded in
24 analyzed samples is of certain interest since this stilbene have demonstrated health
25 benefits such as protection against coronary heart diseases (Hertog et al. 1993), cancer
26 (Jang et al. 1997), and the reduction of the effects of some neurological diseases, such
27 as Alzheimer or Parkinson, (Alarcón de la Lastra et al. 2005).
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31 The presence of OTA in wines of different types, origins and designations has
32 been reported worldwide by many authors (Festas et al. 2000; Otteneder and Majerus
33 2000; Tateo et al. 2000; Pietri et al. 2001; Lòpez de Cerain et al. 2002; Hocking et al.
34 2003; Shephard et al. 2003; Soufleros et al. 2003; Rosa et al. 2004; Ng et al. 2004;
35 Sugita-Konishi et al. 2006). The presence of OTA in wines seems to be due
36 exclusively to the colonization of grapes by OTA-producing moulds. At least in
37 Europe, *Aspergillus carbonarius* seems to be by far the species with the highest OTA
38 production potential in grapes, followed by other species of the section Nigri, such as
39 *A. niger* or *A. tubingensis* (Abarca et al. 2001; Battilani and Pietri 2002; Battilani et
40 al. 2003; Perrone et al. 2006). In most of the occurrence studies the levels of OTA
41 found in red wines were higher than those in rose wines, followed by white wines
42 (Belli et al. 2002). OTA content in all analyzed samples (Table III) revealed that the
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3 value of this contaminant ranging from 0.002 to 0.100 $\mu\text{g l}^{-1}$ for commercial wines
4 obtained from manufacturer 10 and 5, respectively. Interestingly, this content is very
5 low and in each case below the legal limit that is fixed at 2.0 $\mu\text{g l}^{-1}$ or ppb. Previously,
6 Perrone et al. (2007) reported how OTA contamination is prevalent in wines from
7 Southern Italy (mean OTA content of 1.36 $\mu\text{g l}^{-1}$) with an increasing trend “north to
8 south”. In particular the highest values of OTA were found in two Puglia wines,
9 Negroamaro and Primitivo that showing also the highest content of stilbenes. Our data
10 evidenced how the warm climatic conditions, that characterized Calabria region, not
11 always correlate with OTA contamination since a mean OTA content in analyzed
12 Calabrian wines is 0.026 $\mu\text{g l}^{-1}$ with a lowest OTA value of 0.070 $\mu\text{g l}^{-1}$ in wine
13 sample from manufacturer 3.
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18 Statistical analysis performed on Calabrian wine samples ($n = 11$) resulted in a
19 positive correlation between OTA content and resveratrol related compounds. In
20 particular we have found correlation coefficients (r) for *trans*-resveratrol versus OTA
21 and *cis*-resveratrol versus OTA of 0.50 and 0.54, respectively. Previously Perrone et
22 al. (2007) and Bavaresco et al. (2003) reported a positive correlation between OTA
23 contamination and *trans*-resveratrol content with $r = 0.52$ and $r = 0.20$, respectively.
24 Collectively these evidences suggested that resveratrol synthesis is to a certain extent
25 related to the infection of fungus. However, Perrone et al. (2007) reported that the
26 effect of fungal infection on resveratrol response is relevant only when high levels of
27 OTA are formed, that is not true in our samples since the OTA content is very low.
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32 Overall, the obtained results demonstrated the high quality of wines produced in
33 Calabria (Italy).
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Acknowledgments

The authors are grateful to the enologist Dr. G. Biondi and Dr. A. Di Lorenzo for technical support.

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Table I. General characteristics

Manufactures	Geographical origin	Altitude	Grape variety	Wine classification
<i>1</i>	Donnici Inferiore	480 – 500	Aglianico	DOC – IGT
<i>2</i>	Amendolara	25	Aglianico - Magliocco	IGT
<i>3</i>	Saracena	350 - 500	Aglianico - Magliocco	IGT
<i>4</i>	Lattarico	350	Magliocco	-
<i>5</i>	Verbicaro	450	Greco – Guarnaccia - Malvasia	DOC
<i>6</i>	San Vincenzo La Costa	450	Magliocco	-
<i>7</i>	Bisignano	550 - 650	Magliocco	IGT
<i>8</i>	Rogliano	550 - 600	Gaglioppo - Greco Nero - Magliocco	DOC
<i>9</i>	Scigliano	650	Gaglioppo - Greco Nero - Magliocco	DOC
<i>10</i>	Saracena	650	Merlot - Cabernet Sauvignon	-
<i>11</i>	Bisignano	400	Gaglioppo	-

Table II. *cis*- and *trans*-Resveratrol content (ppm or mg l⁻¹) in commercial wine, wine must and must after malolactic fermentation during vintage year 2007

Manufactures	<i>cis</i> – and <i>trans</i> -resveratrol					
	Must		FML		Commercial wine	
	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>	<i>cis</i>	<i>trans</i>
1	< 0.05	< 0.05	0.58 ± 0.04	1.11 ± 0.5	0.80 ± 0.03	1.41 ± 0.7
2	0.11 ± 0.02	0.08 ± 0.002	0.99 ± 0.09	2.14 ± 0.4	1.11 ± 0.5	2.04 ± 0.3
3	0.96 ± 0.03	1.27 ± 0.5	3.18 ± 0.8	3.04 ± 0.9	2.20 ± 0.6	3.41 ± 0.8
4	0.09 ± 0.004	< 0.05	1.05 ± 0.6	2.06 ± 0.04	3.42 ± 0.9	2.14 ± 0.6
5	0.20 ± 0.03	0.10 ± 0.008	2.12 ± 0.5	2.63 ± 0.07	1.81 ± 0.3	2.28 ± 0.5
6	0.11 ± 0.005	0.10 ± 0.006	1.25 ± 0.3	0.44 ± 0.02	1.51 ± 0.2	0.8 ± 0.07
7	0.48 ± 0.06	0.53 ± 0.07	0.67 ± 0.1	0.8 ± 0.005	0.62 ± 0.06	0.72 ± 0.04
8	0.17 ± 0.04	0.05 ± 0.001	1.81 ± 0.4	2.07 ± 0.8	1.83 ± 0.4	2.57 ± 0.8
9	0.18 ± 0.02	0.34 ± 0.06	1.72 ± 0.2	1.57 ± 0.3	2.27 ± 0.8	2.37 ± 0.4
10	0.70 ± 0.01	1.31 ± 0.8	0.78 ± 0.1	1.88 ± 0.5	1.40 ± 0.7	1.00 ± 0.6
11	0.35 ± 0.02	0.38 ± 0.05	0.45 ± 0.1	0.66 ± 0.07	0.42 ± 0.05	0.64 ± 0.02

Data are expressed as mean ± S.D. (*n* =3)

Table III. OTA content (ppb or $\mu\text{g l}^{-1}$) in commercial wine, wine must and must after malolactic fermentation during vintage year 2007

Manufactures	OTA content (ppb or $\mu\text{g l}^{-1}$)		
	Must	FML	Commercial wine
<i>1</i>	0.003 \pm 0.0001	0.003 \pm 0.0001	0.005 \pm 0.0001
<i>2</i>	0.035 \pm 0.007	ND	ND
<i>3</i>	0.050 \pm 0.007	0.080 \pm 0.021	0.070 \pm 0.023
<i>4</i>	0.010 \pm 0.0002	ND	0.050 \pm 0.020
<i>5</i>	0.250 \pm 0.042	0.130 \pm 0.042	0.100 \pm 0.042
<i>6</i>	0.080 \pm 0.020	0.020 \pm 0.009	0.020 \pm 0.009
<i>7</i>	0.005 \pm 0.0002	ND	0.005 \pm 0.0003
<i>8</i>	0.010 \pm 0.0004	0.020 \pm 0.007	0.010 \pm 0.0002
<i>9</i>	0.010 \pm 0.0004	0.010 \pm 0.0006	0.010 \pm 0.0003
<i>10</i>	0.010 \pm 0.0005	0.003 \pm 0.0002	0.002 \pm 0.0002
<i>11</i>	0.010 \pm 0.0004	0.01 \pm 0.0002	0.01 \pm 0.0004

Data are expressed as mean \pm S.D. ($n=3$); ND: Not detected.

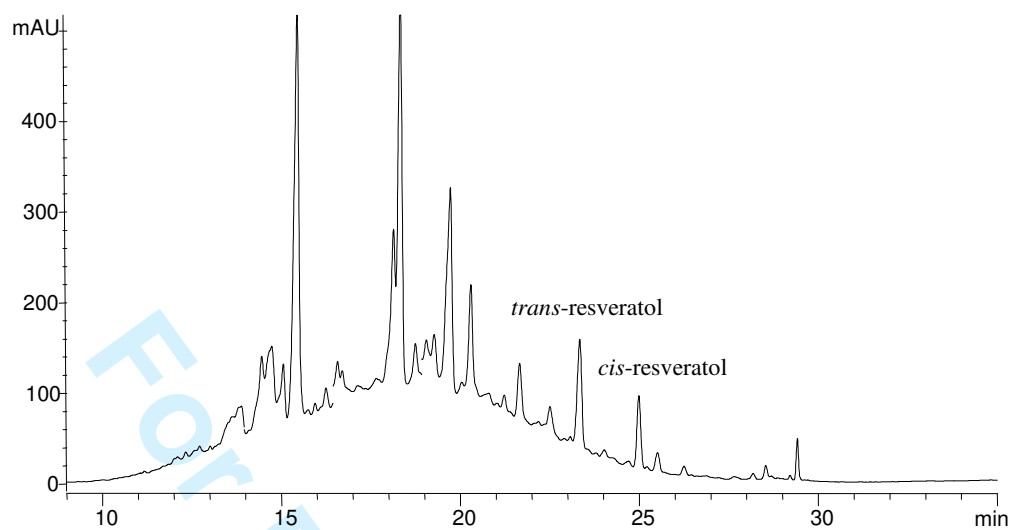
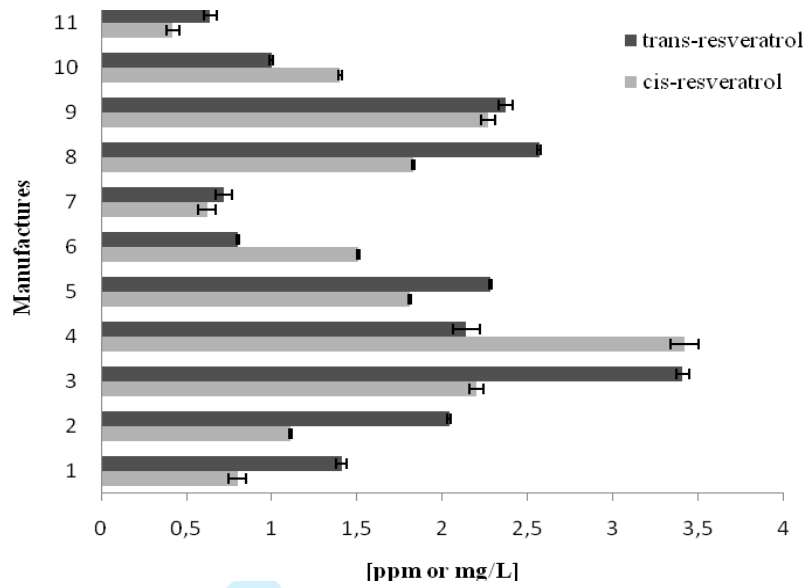
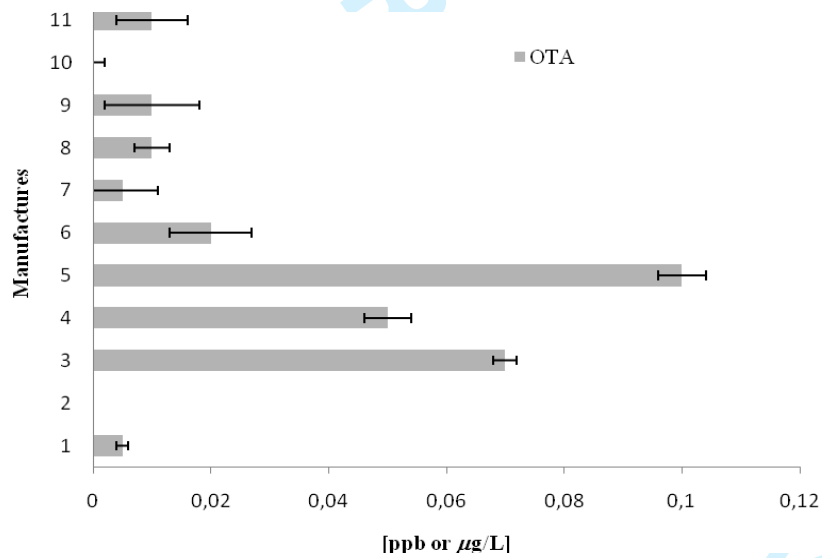


Figure 1. HPLC chromatogram of *cis*- and *trans*-resveratrol in commercial wine from manufacture

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(a)



(b)

Figure 2 Distribution of *cis*- and *trans*-Resveratrol content (a) and OTA level (b) in analyzed commercial wine. Data are means \pm SD ($n=3$).