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CONTENT OF ENDOGENOUS SULFUR DIOXIDE IN WINES

Vlastimil Kubáň, Vlastimil Fic, Petr Marcinčák, Stanislav Kráčmar, Jozef Golian

ABSTRACT

Content of free and total endogenous sulfur dioxide were evaluated by classical iodometric titration in must, during winemaking processes and in bio-wine. No exogenous sulfur dioxide was added in any technological operations to simplify the evaluations. In addition, the results were corrected on the content of reductons (total content of reducing substances). The results confirmed formation of endogenous sulfur dioxide from sulfur containing substances (sulfur containing amino acids etc.) in both experiments. Microbial sulfur dioxide is preferably bound to carbonyl substances. Only minor part is present in the free (active) form of the sulfur dioxide. In addition, total content of polyphenols (TPC) and total antioxidant capacity (TAC) were determined by spectrophotometry at the same time. A procedure OIV-MA-AS323-O4B: R 2009 was used. Contents of "free" and "total" sulfur dioxide (with/without correction on contents of reductons) and total content of reductones were determined after complexing the sulfur dioxide with formaldehyde. A standard spectrophotometric method using Folin-Ciocalteu reagent was applied for determination of total content of polyphenolics (TPC) at 765 nm after 60 min incubation. The results were expressed as tannin equivalents (in $mg.L^{-1)}$. A standard DPPH (2,2´-difenyl-1-picrylhydrazyl dissolved in methanol) spectrophotometric method was applied for determination of total antioxidant capacity (TAC) at 515 nm. Depletion of the color intensity was measured after 60 min incubation against blank (methanol) and absorbance decrease $\Delta(A) = (A_0 - A_1)/A_0$ was calculated and used for construction of calibration curve. The TAC values were expressed as ascorbic acid concentrations (in $mg.L^{-1}$).

Keywords: Endogenous sulfur dioxide; wine; reductons; iodemetric titration; spectrophotometry

INTRODUCTION

Bio-wine is a wine that strictly produced by fermentation of wine musts obtained by pressing of wine grapes grown according the rules of ecological agriculture. In Europe, most of the bio-wines are produced in small wineries. Plantation and subsequent complete technological process, from collection of grapes up to bottling of final wine, forms a fully closed cycle with respect to the abovementioned rules and in accordance with the environmental protection. The special attention must be focused on elimination or substantial reductons of losses of content of biologically active substances and thus to prevent reductons of total antioxidant activity/capacity (TAA/TAC). The appropriate high levels of TAA/TAC thus allow reductons of usage of synthetic additives. Typical and individual natural aroma characteristics of bio-wine could be preserved.

Sulfur dioxide can be present as endogenous (formed during fermentation processes) and especially also as exogenous (added in individual technological operations) in wines, and generally in all fermented drinks (e.g. beers, ciders etc.). Endogenous sulfur dioxide is produced (in addition to the other metabolites) in sulfite non-supplied grape must, but mostly during fermentation by enzymatic transformation of sulfur containing substances (e.g. thioamino acids, i.e. cysteine, cystine, methionine, glutathione, thio-compounds, sulfates, elemental sulfur etc.) by *Saccharomyces cerevisiae* action. Production of endogenous sulfur dioxide depends on many factors (type of microorganism, matrix composition **(Rankine, 1968**; **Eschenbruch, 1974; Romano and Suzzi, 1993; Špakovská et al., 2012; Bajčan et al., 2016)**. Contents of endogenous sulfur dioxide can reach from several $mg.L^{-1}$ up to 30 $mg.L^{-1}$ and its concentrations can be as high as 100 $mg.L^{-1}$ in some extreme conditions (**Rankine and Pocock 1969; Eschenbruch, 1974; Dott et al., 1976; Suzzi et al., 1985)**. Higher concentrations of exogenous SO_2 together with endogenous SO_2 could be critical for malolactic fermentation **(Henick-Kling and Park, 1994)**. Endogenous sulfur dioxide is mostly present in bound forms, but minor concentrations of free (active) form could be also present **(Wells and Osborne, 2011)**. Presence of both forms of the endogenous SO_2 must be known before

addition of the exogenous (technological) sulfur dioxide in any step of technological processes.

Evaluation of the role of reducing substances (reductones) on content of "total" and "free" sulfur dioxide in technological processes of winemaking of bio-wine (from the treatment of wine grape musts up to bio-wine bottling) was the main aim of the study. No exogenous sulfur dioxide was added during the technological process in any form.

Scientific hypothesis

Microorganisms (*Saccharomyces cerevisiae*) produce so called endogenous sulfur dioxide during alcoholic fermentation in the range measurable concentrations (from several $mg.L^{-1}$ up to 50 $mg.L^{-1}$ and under extreme conditions up to 100 $mg.L^{-1}$) directly in the sulfite-supported grape musts. The values presented in literature are controversial, since they probably represent not only the sulfur dioxide, but also so called reductones.

MATERIAL AND METHODOLOGY

A pH meter Level 1 equipped with a combined pH electrode (WTW GmbH, Weilheim, Germany) that was regularly calibrated with a set of buffers of pH 4.01, 7.00 and 9.23. All spectrophotometric measurements were performed using UV-VIS spectrophotometer Helios Delta (Spectronic Unicam, Cambridge, UK) in fused silica rectangular cuvettes with 10 mm optical lengths.

NaOH (c_m = 0.1, 1.0 and 2.0 $mol.L^{-1}$), oxalic acid dihydrate, buffer solution (20 ml acetic acid conc. and 80 ml 27% sodium acetate filled up to 250 ml with distilled water), reagent solution (5 g NH_4VO_3 dissolved in 75 ml of 1 $mol.L^{-1}$ NaOH and after addition of 100 ml 270 $g.L^{-1}$ sodium acetate solution filled up to 250 ml with distilled water), tartaric acid (c_m = 10 $g.L^{-1}$), charcoal, I_2 solution with $c(I_2)$ = 0.01 $mol.L^{-1}$, 16% (v/v) H_2SO_4, 1% (v/v) formaldehyde (CH_2O), EDTA (c_m = 1 $g.L^{-1}$), starch solution, arsonium oxide (all from Pliva-Lachema, Brno, Czech Republic) were used.

Wine grape must and intermediate products in individual steps of the technological processes of winemaking of white bio-wines Veltlinské zelené (VZ) and Ryzlink rýnský (RR) and red bio-wine Rulandské modré (RM) production of moderate climatic region from Marcinčák Winery - Bio-wine Ltd., (Mikulov, Czech Republic) winery sub-region Moravia were analyzed. Contents of sulfur dioxide ("free" and "total") and content of reductones (sum of all substances oxidized by iodine in strongly acidic condition of sulfuric acid, except of sulfur dioxide), in addition to total contents of polyphenols (TPC) and total antioxidant capacity (TAC) were determined in all wines. Samples of the individual wines were collected from storage balloons in repetitive two-week intervals and immediately analyzed.

Determination of SO_2 in wine

A procedure OIV-MA-AS323-O4B: R 2009 that is used for determination of free and total content of sulfur dioxide in grape wines, fruit wines, malt and Tokay wines was used. Contents of "free" and "total" sulfur dioxide (with/without correction on contents of reductons) and total content of reductones (organic substances with hydroxyl groups on double bonds, i.e. 2,3-dihydroxypropenal, L-ascorbic acid, gallic acid, etc. and some inorganic substances having strong reductions properties) were determined.

Determination of the "free SO_2"

A wine sample (50 mL) was pipetted with a pipette touching the bottom into an Erlenmeyer bottle (500 mL) and 16% H_2SO_4 (10 mL), EDTA solution (1 mL, c = 10 $g.L^{-1}$), starch solution (5 mL) were immediately added and mixed. The mixture was titrated quickly with standard I_2 solution ($c(I_2)$ = 0.02 $mol.L^{-1}$) until blue color persisting 30 s was observed against the white background. Based on the standard I_2 solution (V_1) consumption, the concentration (in $mg.L^{-1}$) of "free sulfur dioxide" in wine was calculated. The sum of the "free sulfur dioxide" and total content of reductones was detected.

Determination of the "total SO_2"

A wine sample (50 mL) was pipetted with a pipette touching the bottom into an Erlenmeyer bottle (500 mL) containing NaOH solution (8 mL, c = 4 $mol.L^{-1}$). Erlenmeyer bottle was sealed, solutions were mixed and allowed to stand in dark place. After 5 min, 16% H_2SO_4 (15 mL), EDTA solution (1 mL, c = 10 $g.L^{-1}$), starch solution (5 mL) were added and immediately mixed. The mixture was titrated quickly with standard I_2 solution ($c(I_2)$ = 0.02 $mol.L^{-1}$) until blue color persisting 30 s was observed against the white background. Based on the standard I_2 solution (V_2) consumption, the concentration (in $mg.L^{-1}$) of "total sulfur dioxide" in wine was calculated. The sum of the "total sulfur dioxide" and total content of reductones was detected.

Correction on reductones

A wine sample (50 mL) was pipetted with a pipette touching the bottom into an Erlenmeyer bottle (500 mL) containing formaldehyde solution (1 mL, c = 10 $g.L^{-1}$). Erlenmeyer bottle was sealed, solutions were mixed and allowed to stand in dark place. After 30 min, 16% H_2SO_4 (10 mL), EDTA solution (1 mL, c 10 $g.L^{-1}$) and starch solution (5 mL) were added and immediately mixed. The mixture was titrated quickly with standard I_2 solution ($c(I_2)$ = 0.02 $mol.L^{-1}$) until blue color persisting 30 s was observed against the white background. Based on the standard I_2 solution (V_4) consumption, the concentration (in $mg.L^{-1}$) of "total reductones" in wine was calculated and "total reductones" concentration was subtracted from the "free sulfur dioxide" or "total sulfur dioxide" concentrations in wines. Content of reductones was expressed ascorbic acid and/or sulfur dioxide concentration (in $mg.L^{-1}$).

Determination of the total polyphenols content (TPC)

A standard spectrophotometric method using Folin-Ciocalteu reagent was applied for determination of total content of polyphenolics (TPC) at 765 nm after 60 min incubation. The results (TPC) were expressed as tannin equivalents (in $mg.L^{-1}$). Repeatability was verified by 10 repetitive determination of gallic acid (c_m = 0.4 $mg.L^{-1}$) and tannin (0.5 $mg.L^{-1}$). A six points calibration curve A =

$f(c_m)$ was constructed using standard solutions. Samples were collected regularly in two-week intervals **(Figures. 1 – 5)** and immediately analyzed.

Determination of the total antioxidant capacity/activity (TAC/TAA)

A standard DPPH spectrophotometric method was applied for determination of total antioxidant capacity (TAC) at 515 nm. A precisely weighed sample of 2,2´-difenyl^{-1}-picrylhydrazyl (DPPH, 24 mg) was dissolved in methanol (100 mL) and the standard solution was stored for max. 2 months in refrigerator. Working solution was prepared by dilution of the DPPH standard solution (10 mL) with methanol (45 mL). Absorbance (A_0) of the working solution has to be in the range A_0 = 0.8 – 1.0, at wavelength 515 nm. A six points calibration curve $A = f(c_m)$ was constructed using standard solutions of ascorbic acid (40, 60, 80 a 100 mg.L^{-1}). Samples were collected regularly in two-week intervals **(Figures. 1 – 5)** and immediately analyzed.

The DPPH working solution (8.55 mL) was added to 450 µl of the calibration solution or sample solution and mixture was mixed. The mixture was allowed to stand in cold and dark place. Depletion of the color intensity was measured after 60 min incubation against blank (methanol) and absorbance decrease $\Delta(A) = (A_0 - A_1)/A_0$ was calculated and used for construction of calibration curve. The TAC values were expressed as ascorbic acid concentrations (in mg.L^{-1}).

Statistical methods

The obtained data were subjected to analysis of variance using the Minitab 17 statistical software program (Minitab, Coventry, United Kingdom). Where statistical differences were noted, differences among data were determined, using the Tukey's test. Significance was defined at p <0.05.)

RESULTS AND DISCUSSION

Samples for the determination of acidity (pH), the total antioxidant capacity (TAC) and the total content of polyphenols (TPC) and the "total" and "free" sulfur dioxide were collected in regular two weeks intervals during grapes ripening, production of the grape musts by pressing, in principal steps of the winemaking up to bottling of the final bio-wine and during storage of the bio-wine in bottles. Collection of the samples for the total content of reductones was done immediately after pressing and in accordance with the collections for the other analyses.

pH values **(Figure 1)** of the samples of ripped grapes varied in the interval 2.5 – 2.7 and then pH values rapidly increased up to pH values close to 3. The changes of pH values could be explained by some biochemical a physico-chemical processes (i.e. sugar destruction, production of ethanol by yeast, biotransformation of acids, production of CO_2, temperature changes of fermentation media etc.) taking part in alcoholic fermentation. Acidity of fermentation medium was in the pH intervals 3.0 – 3.4, 3.0 - 3.4 and 3.0 – 3.4 for VZ, RR and RM in the beginning of fermentation. Small decrease of the pH values in individual wines was observed in must samples before its first decantation. More evident decrease of the pH values was observed in samples of RM (from pH 3.41 to 3.23). Increase of the pH was observed in samples of VZ probably due to the more intense contacts with atmospheric oxygen and subsequent oxidation of some wine components. Very small decrease of pH values was observed in samples of RR during the technological process. Less evident changes of pH values were observed during winemaking processes of all wine samples. Thus the processes were „standardized".

As can be seen from **Figsure 2** and **Figure 3**, the values of the total antioxidant capacity (TAC) and the total contents of polyphenols (TCP) were relatively high and their values oscillated around 800 – 1000 gallic acid equivalent (GAE). The values of both parameters close to pressing (ripped grapes) rapidly decreased and oscillated around 500 GAE a 50 ascorbic acid equivalents (AAE) for TPC and for TAC. Both values insignificantly increased after pressing the grape must and were practically constant (in the range of experimental errors) during the whole vinification processes. It means that both values were stabilized.

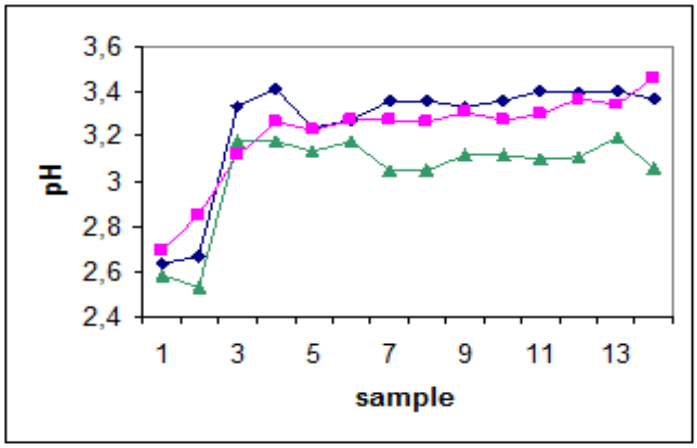

Figure 1 Changes of pH values during technological processes of bio-wine production.
Note: Rulandské modré ♦, Veltlínské zelené ■, Ryzlink rýnský ▲, sample: 1 - must after pressing, 2 - must after first decantation, 3 - 14 samples during fermentation (two-week intervals).

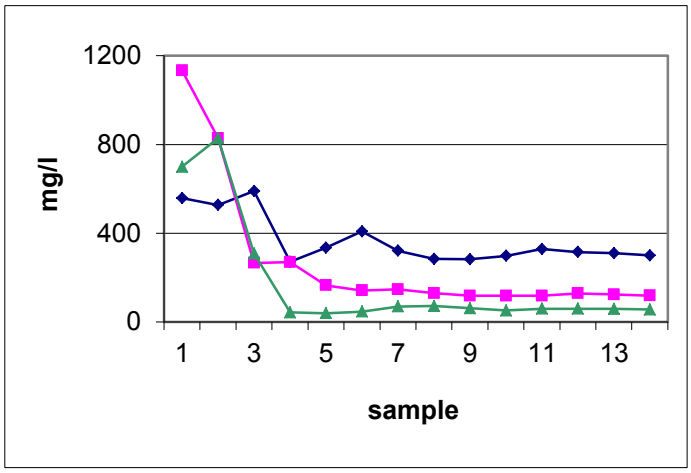

Figure 2 Changes of the total antioxidant capacity TAC in mg.L^{-1} ascorbic acid. Note: Rulandské modré ♦, Veltlínské zelené ■, Ryzlink rýnský ▲, sample: 1 - hard grapes, 2 - soft grapes, 3 – ripped gapes, 4 – must after pressing, 5 - 14 samples during fermentation (two-week intervals).

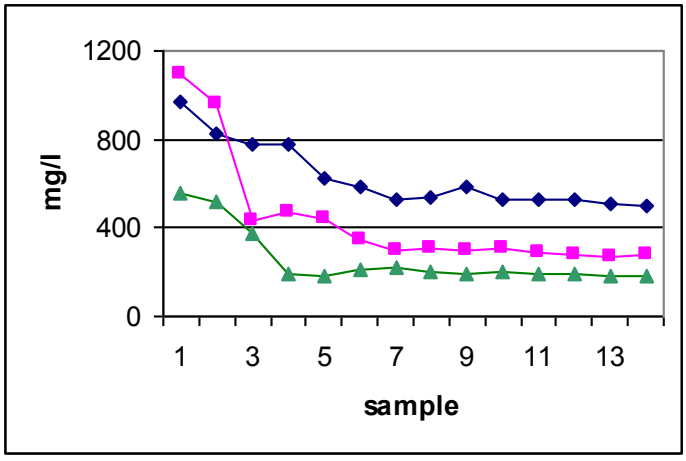

Figure 3 Changes of the total contents of polyphenols during technological processes of bio-wine production. Note: Rulandské modré ♦, Veltlínské zelené ■, Ryzlink rýnský ▲, samples: see **Figure 2.**

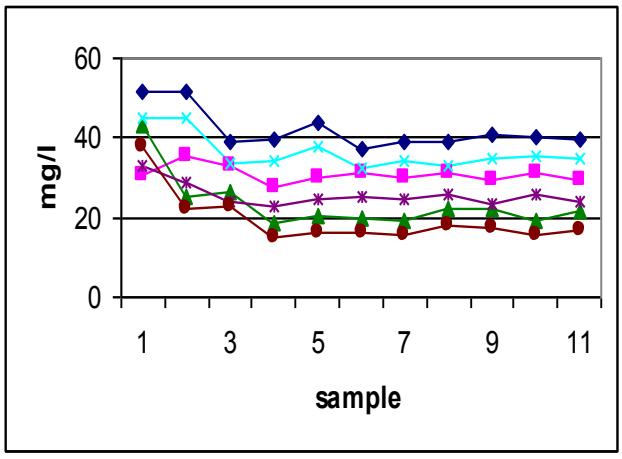

Figure 4 Changes of the contents of endogenous total SO_2 during technological processes of bio-wine without and with the corrections on reductones contents. Note: Rulandské modré ♦, Veltlínské zelené ■, Ryzlink rýnský ▲, samples: see Figure 2.

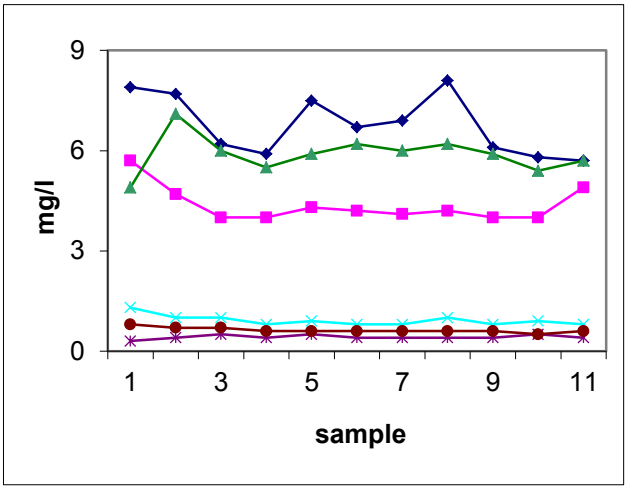

Figure 5 Changes of the contents of endogenous free SO_2 during technological processes of bio-wine without and with the corrections on reductones contents. Note: Rulandské modré ♦, Veltlínské zelené ■, Ryzlink rýnský ▲, samples: see **Figure 2**.

Contents of the „total sulfur dioxide“ and the „free sulfur dioxide“ were controlled **(Figure 4 and Figure 5)** from moment of pressing of ripped grapes. Contents of the „total sulfur dioxide“oscillated around 50 $mg.L^{-1}$ for RM and around 45 – 48 $mg.L^{-1}$ for RR and VZ. Contents of the „free sulfur dioxide“ were 4 – 7 $mg.L^{-1}$ much lower. The slightly higher values were found for RM while lower values were found for RR and VZ. All values decreased approximately of 10% during the following period and with the slight increase of the contents during the all time of the experiments to the levels between 33 – 41, 13 – 22 and 20 – 29 $mg.L^{-1}$ for RM, VZ and RR. Very similar trends were observed for the contents of „free sulfur dioxide“ in the intervals 5 – 7, 2.3 - 5.0 and 5 – 6 $mg.L^{-1}$ for RM, VZ and RR.

The obtained concentrations of the „total sulfur dioxide“ and „free sulfur dioxide“ included also total concentrations of reduced substances (reductones) with red-ox potentials lower than red-ox potential of the reaction of $I_2/2\ I^-$ and thus all substance easily oxidized by iodine under given experimental conditions (strongly acidic medium of H_2SO_4). Organic compounds with hydroxy-groups on the double bounds, having strong reductions properties (i.e. 2,3-dihydroxypropenal, L-ascorbic acid etc.) belong to the typical reductones. The substances interfere in the iodometric determinations of the „total”, “bound“ and „free” sulfur dioxide and they are source of false and seriously over-estimated results. After the corrections on the contents of reductones, the obtained contents of the „total sulfur dioxide“ (5 – 7, 3 – 4 and 4 – 6 $mg.L^{-1}$) and also the „free sulfur dioxide“ (0.1 – 1.3, 0.2 – 0.5 and 0.2 – 0.7 $mg.L^{-1}$) were one order of magnitude lower.

The results confirmed that microorganisms (*Saccharomyces cerevisiae*) produced so called endogenous sulfur dioxide during the alcohololic fermentation in the range measurable concentrations directly in the sulfite non-supplied grape musts **(Rankine, 1968; Eschenbruch, 1974; Romano and Suzzi, 1993; Bajčan et al., 2016)**. Concentrations of the endogenous sulfur dioxide can be from several $mg.L^{-1}$ up to 50 $mg.L^{-1}$ and under some extreme conditions up to 100 $mg.L^{-1}$ **(Rankine and Pocock, 1969; Eschenbruch, 1974; Dott et al., 1976; Suzzi et al., 1985)**. These values presented in the literature are controversial, since they probably represent not only the sulfur dioxide, but also reductones.

The very similar trend was observed in sulfite supplied grape must and wines **(Jančářová et al., 2011)**. In that case, concentrations of „free sulfur dioxide“ were determined for four varieties of white wines (Müller-Thurgau, Rulandské bílé, Sauvignon, Muškát Ottonel) and two varieties of red wines (Dornfelder and Frankovka). Concentrations of reductones were as high as tens of percents of the concentrations of „sulfur dioxide“. In addition to the endogenous sulfur dioxide, microorganisms produce **(Wells and Osborn, 2011)** also a lot of other substances (acetaldehyde, acetoine, carbonyl compounds etc.) that together with the other substances present in wine matrix (sugars, aldehydes, colors etc.) rapidly bound produced endogenous sulfur dioxide.

From the above mentioned facts it can be concluded that classical iodometric titrations produce false and seriously overestimated (in units up to tens percents) results due to the interferences of the reductones with the red-ox potential lower than red-ox potential of the reaction between iodide and iodine ($I_2/2\ I^-$) and thus easily oxidized under strongly acidic conditions (H_2SO_4). Standard iodometric methods for the determination of “free”, “bound” and “total” SO_2 needs revision and it will be necessary to develop a more specific method for determination of different forms of SO_2, free of interference of reductones. The separation techniques (ion chromatography or capillary electrophoresis), gas-diffusion flow injection analysis **(Kubáň et al., 1989)** and spectroscopic methods **(Čmelík et al., 2005)** can be given as an examples.

The very similar problem can be probably expected in the methods for determination of total antioxidant capacities and total contents of polyphenols. These methods produce probably also false and overestimated results due to interferences of presented exogenous and endogenous sulfur dioxide. Both forms are strong antioxidants. Some interactions of reagents for determination of polyphenolics (Folin-Ciocalteu) and antioxidants (FRAP, DPPH, ABTS etc.) with SO_2 and their interferences in results were

reported by earlier authors and also present applicants of the methods (**Abramovic et al., 2015**).

CONCLUSIONS

The values of the total antioxidant capacity (TAC) and the total contents of polyphenols (TCP) were relatively high and their values oscillated around 800 – 1000 gallic acid equivalent (GAE). The values of both parameters close to pressing (ripped grapes) rapidly decreased and oscillated around 500 GAE a 50 ascorbic acid equivalents (AAE) for TPC and for TAC. Both values insignificantly increased after pressing the grape must and were practically constant.

It can be concluded that classical iodometric titrations produce false and seriously overestimated (in units up to tens percents) results due to the interferences of the reductones with the red-ox potential lower than red-ox potential of the reaction between iodide and iodine (I2/2 I-) and thus easily oxidized under strongly acidic conditions (H2SO4). Standard iodometric methods for the determination of "free", "bound" and "total" SO2 needs revision and it will be necessary to develop a more specific method for determination of different forms of SO2, free of interference of reductones. The separation techniques (ion chromatography or capillary electrophoresis), gas-diffusion flow injection analysis and spectroscopic methods can be given as an examples.

The very similar problem can be probably expected in the methods for determination of total antioxidant capacities and total contents of polyphenols. These methods produce probably also false and overestimated results due to interferences of presented exogenous and endogenous sulfur dioxide.

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Contact address:

Vlastimil Kubáň, Tomas Bata University in Zlín, Foculty of Technology, Department of Food Technology, Vavrečkova 275, 762 62 Zlín, Czech republic, E-mail: kuban@ft.utb.cz

Vlastimil Fic, Tomas Bata University in Zlín, Foculty of Technology, Department of Analysis and Food Chemistry, Vavrečkova 275, 762 62 Zlín, Czech republic, E-mail: fic@ft.utb.cz

Petr Marcinčák, Winery - Bio-wine Mikulov, Ltd., Vinařská 1561/6, 692 01 Mikulov, Czech republic, E-mail: marcincak@seznam.cz

Stanislav Kráčmar, College of Business and Hotel Management, Department of Gastronomy, Bosonožská 9, 625 00 Brno, Czech Republic, E-mail: kracmar@hotskolabrno.cz

Jozef Golian, Slovak University of Agriculture, Faculty of Biotechnology and Food Sciences, Department of Hygiene and Food Safety, Tr. A. Hlinku 2, 949 76 Nitra Slovakia, E-mail: jozef.golian@uniag.sk