Journal of Microbiology, Biotechnology and Food Sciences

Bajčan et al. 2013: 2 (Special issue 1) 1699-1708

REGULAR ARTICLE

OPTIMIZING CONDITIONS FOR SPECTROPHOTOMETRIC DETERMINATION OF TOTAL POLYPHENOLS IN WINES USING FOLIN-CIOCALTEU REAGENT

Daniel Bajčan [1], Ľuboš Harangozo[1], Diana Hrabovská [1], Dominika Bončíková [1]*

Address: RNDr. Daniel Bajčan PhD.

[1]Department of Chemistry, Faculty of Biotechnology and Food Sciences, Slovak University of Agriculture, Tr. A. Hlinku 2, 949 76 Nitra, Slovak republic, Phone no.: +421376414369.

*Corresponding author: bajcan@gmail.com

ABSTRACT

Wine is a complex beverage that obtains its properties mainly due to synergistic effect of alcohol, organic acids, carbohydrates, as well as the phenolic and aromatic substances. At present days, we can observe an increased interest in the study of polyphenols in wines that have antioxidant, antimicrobial, anti-inflammatory, anti-cancer and many other beneficial effects. Moderate and regular consumption of the red wine especially, with a high content of phenolic compounds, has a beneficial effect on human health. The aim of this work was to optimize conditions for spectrophotometric determination of total polyphenols in wine using Folin-Ciocaulteu reagent. Based on several studies, in order to minimize chemical use and optimize analysis time, we have proposed a method for the determination of total polyphenols using 0.25 ml Folin-Ciocaulteu reagent, 3 ml of 20% Na_2CO_3 solution and time of coloring complex 1.5 hour. We found out that our optimized method and standard method provides the same results, so our optimized method can be futher used for the determination of total polyphenol content in wines. In doing so, the cost of chemicals for one analysis will decrease more than 10 times.

Keywords: determination, Folin-Ciocalteu reagent, method, polyphenols, spectrophotometry, wine

INTRODUCTION

Phenolic compounds are important components of wine, especially red wine, and they significantly contribute to the antioxidant and sensory properties of wine. Polyphenols are extensive and complex group of compounds. The most represented group of phenolic compounds in wine are phenolic acids, flavonoids (anthocyanins, flavonols and catechins, tannins), stilbenes and lignans **(Jackson, 2008; Lea *et al*., 1979)**. **Waterhouse (2002)** devides wine phenolics into 2 groups: non-flavonoids: hydroxycinnamates, hydroxybenzoates and the stilbenes; plus the flavonoids: flavan-3-ols, the flavonols, and the anthocyanins. The amount of polyphenols in wine is influenced by many factors, including climatic and pedological conditions of vineyard, temperature and length of maceration, variety of grapes, yeast spiece, winemaking technology, SO_2 and alcohol content, pH, etc. **(Villano *et al*., 2006; Lachman and Šulc, 2006)**.

Different types of methods are used to analyze the content of phenolic compounds in wines, such as chromatography, electroseparation and especially optical methods. One of the most used and also one of the easiest methods for determination of total polyphenols is the spectrophotometric method using Folin-Ciocalteu (FC) reagent which was suggested by **Folin and Denis (1912)** and later was modified by **Folin and Ciocalteu (1927)**. Its principle is based on the oxidation of phenolic compounds in alkaline medium with molybdenum and tungsten phosphate to form a blue-colored complex. The intensity of blue-colored tungsten-molybdenum complexes with polyphenols is measured spectrophotometrically at the wavelength of 750 nm. This method was later modified many times and the method proposed by **Singleton and Rossi (1965)** is the most widely used for analysis of total polyphenol (TP) content in wines. Many other authors **(Alonzo *et al*., 2002; Lachman *et al*., 2003; Šulc *et al*., 2003; Vichitphan *el al*., 2007; Majo *et al*., 2008; Satora *et al*., 2008; Geldenhuys, 2009; Weingerl *et al*., 2009; Amendola *et al*., 2010; Ivanova *et al*., 2011;** etc.**)** reported and proposed modification of this method.

The aim of this study was to propose and optimize the conditions for the spectrophotometric determination of TP content in wines using FC reagent. We have optimized the following parameters: volume of FC reagent, volume of 20% Na_2CO_3 solution and time of colouring molybdenum-tungsten complex with polyphenols.

MATERIAL AND METHODS

We have been using the method proposed by **Šulc *et al.* (2003)** for the analysis of TP content in wines in our laboratory, which is based on method published by **Singleton and Rossi (1965)**. Analysis procedure was as follows: 1 ml of wine sample was pipetted into 50 ml volumetric flask an then 5 ml of destilled water was added. 2.5 ml of FC reagent was added into volumetric flask and also 7.5 ml of 20% Na_2CO_3 solution after 3 minutes. The mixture was filled with distilled water up to volume 50 ml and it was left at the laboratory temperature for 2 hours to coloring the complex. By the same procedure the blank and calibration solutions of gallic acid were prepared. Finally the absorbance of samples and calibration solutions was measured against blank solution at the wavelength 765 nm. Total polyphenol content in wines was calculated as amount of gallic acid equivalent (GAE) in mg per 1 litre of wine.

Method of **Šulc *et al.* (2003)** we have chosen as a standard (default) method. When we optimized the method, we examined the possibilities of addition smaller amount of FC reagent (0.1; 0.25 and 0.5 ml) against standard volume (2.5 ml) of FC reagent. Simultaneously we examined the possibilities of addition smaller amount of 20% Na_2CO_3 solution (2; 3 and 4 ml) against standard volume (7.5 ml) of 20% Na_2CO_3 solution. As a final factor, we examined the time required for coloring molybdenum-tungsten complexes with polyphenols. We tested the possibility of using various times (0.5; 1 and 1.5 hour) compared with the standard time (2 hours). For optimization of the parameters, we used 3 kinds of quality, dry wines which characteristics are listed in Table 1.

Table 1 Wine samples used in optimization of the method and their characteristics

Sample	Variety	Producer	Vineyard area	Colour	Vintage	Alcohol content
MT	Müller Thurgau	Vindevie, s.r.o. Rimavská Sobota	South Slovak	white	2010	10.5%
CS	Cabernet Sauvignon	Vinárske závody Topoľčianky, s.r.o.	Nitra	red	2010	11%
FM	Blaufränkisch	Vinárske závody Topoľčianky, s.r.o.	Nitra	rosé	2009	11%

All analyses were performed as four parallels. Statistical analysis were performed using the software Statistica 6.0 (StatSoft) and the results were evaluated by analysis of variance ANOVA at the significance level $\alpha = 0.05$.

RESULTS AND DISCUSSION

Optimization of the amount of FC reagent used in determination of TP content

The first step in the optimization of the method was to compare the results of determination of TP contents in real wine sample (MT) using tested (0.1 – 0.5 ml) and standard volume of FC reagent (2.5 ml). Using the above mentioned quantities of FC reagent we chose such a concentration range of standard solutions containing gallic acid (0.4 - 2.0 $mg.l^{-1}$), in which all three calibration curves were linear (Fig. 1).

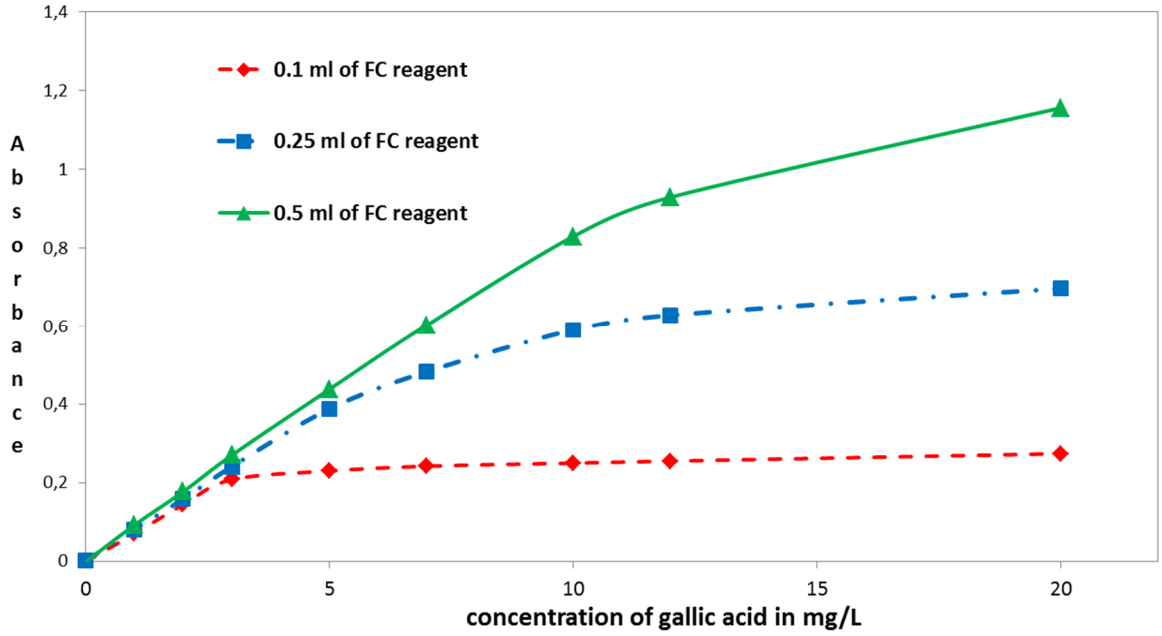

Figure 1 Testing of linearity of the calibration curves using various volumes of FC reagent

When testing the conformity of the results, we found that already amount of 0.25 ml FC reagent is sufficient for analysis of TP content in wines (Tab. 2).

Using this amount of FC reagent (0.25 ml), 10-fold increase in saving will be achieved. Using this amount of FC reagent, the cost of chemicals for one analysis will decrease from 25 eurocents to 2.5 eurocents. Using of 0.1 ml of FC reagent, the results of conformity testing were statistically different and this quantity (0.1 ml) of FC reagent is not sufficient for the TP content analysis.

Table 2 Comparison of total polyphenol contents (in $mg.l^{-1}$) in wine sample MT using various amounts of FC reagent

Volume of FC reagent in ml	2.5 standard	0.1	0.25	0.5
Equation of calibration curve	y=0.108x + 0.0108	y=0.07x + 0.0042	y=0.0768x + 0.0095	y=0.0927x + 0,0075
Correlation coeficient R^2	0.997	0.9994	0.9983	0.9968
1. measurement	282.4	271.4	288.1	272.9
2. measurement	273.1	271.4	281.6	286.9
3. measurement	291.7	275.0	281.6	283.7
4. measurement	275.5	271.4	281.6	279.1
5. measurement	289.4	267.9	288.1	275.9
Arit. Average	282.4	271.4	284.2	279.7
Variation range	18.6	7.1	6.5	14.0
Conformity[A]	-	**no**	**yes**	**yes**

[A] - conformity of measurement with the measurement of default method

Optimization of the amount of 20% Na_2CO_3 solution used in determination of TP content

Disodium carbonate is a very strong alkali and its effects on the skin are significantly negative (caustic) when working with the solution with high content (20%) of Na_2CO_3.

Table 3 Comparison of total polyphenol contents (in $mg.l^{-1}$) in wine sample MT using various amounts of 20% Na_2CO_3 solution

Volume of 20% Na_2CO_3 in ml	7.5 standard	2.0	3.0	4.0
Equation of calibration curve	y=0.0765x + 0.0056	y=0.0828x + 0.0037	y=0,0818x + 0.0053	y=0.0775x + 0.006
Correlation coeficient R^2	0.9991	0.9998	0.9992	0.999
1. measurement	281.1	294.0	286.7	288.0
2. measurement	277.8	291.0	283.7	284.7
3. measurement	287.8	285.0	289.8	288.0
4. measurement	274.4	285.0	289.8	288.0
5. measurement	287.8	297.1	286.7	297.7
Arit. average	281.8	290.4	287.3	289.3
Variation range	13.4	12.1	6.1	13.0
Conformity[A]	-	**no**	**yes**	**yes**

[A] - conformity of measurement with the measurement of default method

Similarly, high levels of Na_2CO_3 can sometimes cause precipitation of some substances in wine. Therefore, it was necessary to optimize (minimize) the amount of 20% Na_2CO_3 solution used in determination of total polyphenols in wine.

Using various amounts of 20% Na_2CO_3, we found out that already amount of 3 ml of 20% Na_2CO_3 is sufficient for the analysis of TP content in wines (Tab. 3).

Optimization of the time of coloring complex in the determination of total polyphenols

Two hours is the standard time for colouring of blue-colored tungsten-molybdenum complex with polyphenols in standard method. We tested the possibility of using various times (0.5; 1 and 1.5 hours) compared with the standard time. We found out that already time – 1.5 hour is sufficient for the analysis of TP content in wines (Tab. 4).
Using times – 0.5 and 1 hour for colouring complex, the results of conformity testing were statistically different and these times are not sufficient for the TP content analysis.

Table 4 Comparison of total polyphenol contents (in $mg.l^{-1}$) in wine sample MT using various times for colouring tungsten-molybdenum complex with polyphenols

Time of coloring the complex in hours	**2.0 standard**	**0.5**	**1.0**	**1.5**
Equation of calibration curve	y=0.0825x + 0.0072	y=0.0795 + 0.0076	y=0.0808x + 0.0081	y=0.082x + 0.0082
Correlation coeficient R^2	0.9996	0.9983	0,9991	0,9996
1. measurement	279.4	291.6	286.0	281.2
2. measurement	282.2	288.9	288.3	283.9
3. measurement	282.2	290.7	288.9	281.2
4. measurement	285.2	289.8	288.9	283.9
5. measurement	285.2	289.8	288.9	278.2
Arit. average	282.8	290.2	288.2	281.7
Variation range	5.8	2.7	2.9	5.7
Conformity[A]	-	**no**	**no**	**yes**

[A] - conformity of measurement with the measurement of default method

Verification of optimized method for the analysis of TP content using real wine samples

At the end of our work, we compared the optimized method and the standard method for the determination of total polyphenols in wine using 3 real samples of wines (white, rosé and red) with different TP contents.

The results of determination of total polyphenols in all 3 real wine samples showed statistical conformity (at the significance level $\alpha = 0.05$) of measurements obtained by optimized and standard method (Tab. 5). It follows that our optimized method can be futher used for the determination of total polyphenol content in wines. In doing so, the cost of chemicals for one analysis will decrease more than 10 times (from 25 to 2.5 eurocents), if we consider only the price of the most expensive chemical - FC reagent, as the price of other chemicals used in analysis of TP content in wines is negligible compared to price of FC reagent.

Table 5 Comparison of the results of determination of total polyphenols in various wine samples (in mg GAE.l^{-1}) obtained with standard and optimized methods

Sample	CS^S	CS^O	FM^S	FM^O	MT^S	MT^O
1. measurement	2885	3011	442.8	469.7	279.8	288.4
2. measurement	2885	2979	451.7	488.7	286.5	282.1
3. measurement	2952	2916	478.5	463.4	279.8	285.2
4. measurement	2907	2916	456.2	463.4	282.1	278.9
5. measurement	2952	2979	442.8	469.7	282.1	291.5
Arit. average	2916.2	2960.2	454.4	470.98	282.06	285.22
Variation range	67	95	29.7	25.3	6.7	12.6
Conformity^B	**yes**		**yes**		**yes**	

^{S} – standard method ^{O} – optimized method
^{B} - conformity between measurements obtained with optimized and standard method

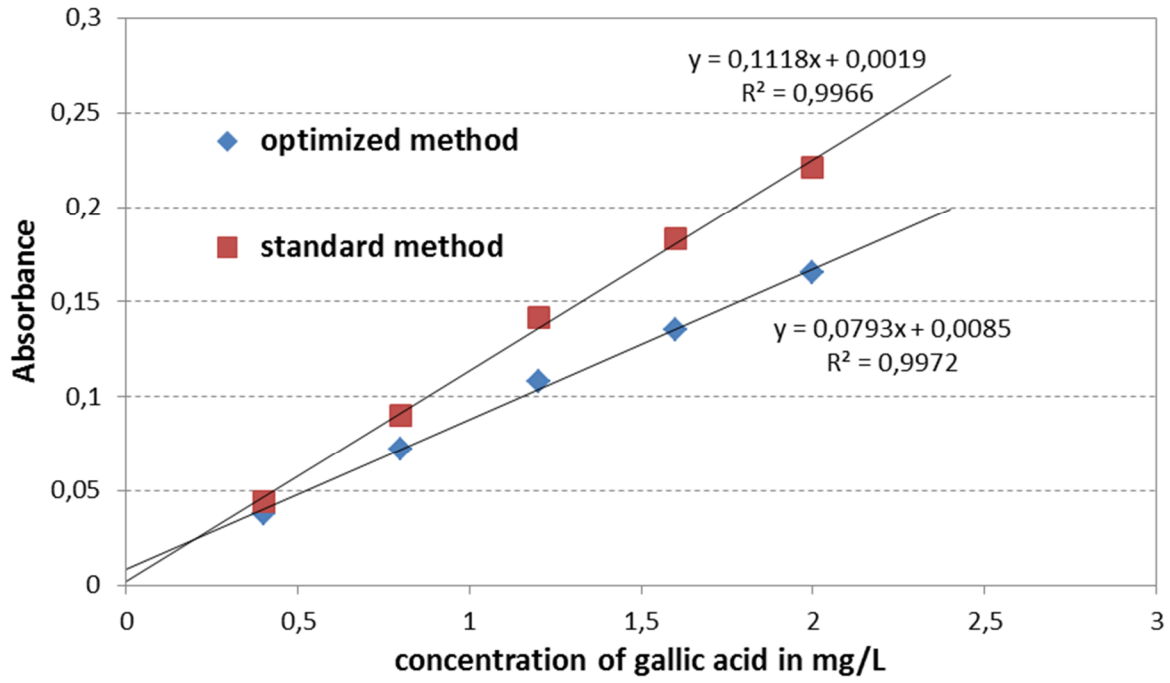

Figure 2 Calibration curves using standard and our optimized method

Although the sensitivity of the optimized method for detemination of total polyphenols in wines is approximately 1.4-times lower than the sensitivity of standard method (Fig. 2), but the factor of sensitivity is not very important due to high content of phenolic compounds in wines (200 - 4000 mg GAE per 1 liter).

CONCLUSION

We have optimized and verified the method for determination of total polyphenols in wines. The price of one analysis of the optimized method is 10-times lower than price of one analysis of the standard method, developed by **Šulc *et al.* (2003)**. We have optimized 3 main parameters of the determination of polyphenolic compounds in wines: the amount of FC reagent to 0.25ml, the amount of 20% Na_2CO_3 solution to 3 ml and time necessary for colouring blue-colored tungsten-molybdenum complex with polyphenols to 1.5 hour.

Acknowledgments: This work was funded by Grant Agency of the Slovak Republic VEGA no. 1/0456/12.

REFERENCES

ALONZO, M. – DOMNGUEZ, C. – GUILLN, D. A. et al. 2002. Determination of Antioxidant Power of Red and White Wines by a New Electrochemical Method and Its Correlation with Polyphenolic Content. In *Journal of Agriculture and Food Chemistry*, vol. 50, 2002, p. 3112-3115.

AMENDOLA, D. - DE FAVERI, D. M. - SPIGNO, G. 2010. Grape marc phenolics: extraction kinetics, quality and stability of extracts. In *Journal of Food Engineering*, vol. 97, 2010, p. 384-392.

FOLIN, O. – CIOCALTEU, V. 1927. Tyrosine and tryptophan determinations in proteins. In *The Journal of Biological Chemistry*, vol. 73, 1927, p. 627-650.

FOLIN, O. – DENIS, W. 1912. On phosphotungstic-phosphomolybdic compounds as colour reagents. In *The Journal of Biological Chemistry*, vol. 12, 1912, p. 239-243.

GELDENHUYS, L. 2009. *Influence of oxygen addition on the phenolic composition of red wine*: dissertation work. Stellenbosch: Stellenbosch University, 2009, 99p.

IVANOVA, V. - DÖRNYEI, A. – MÁRK, L. – VOJNOSKI, B. – STAFILOV, T. – STEFOVA, M. – KILÁR, F. 2011. Polyphenolic content of Vranec wines produced by different vinification conditions. In *Food Chemistry*, vol. 124, 2011, p. 316-325.

JACKSON, R. S. 2008. *Wine Science: Principles and Applications*, *3rd ed.* New York: Academic Press, 2008, 751 p. ISBN 0123736463.

LACHMAN, J. – ŠULC, M. 2006. Phenolics and antioxidant activity of wines during the winemaking process. In *Control Applications in Post – Harvest and Processing Technology (CAPPT 2006)*. Potsdam: Leibniz-Institut für Agrartechnik Potsdam-Bornim, 2006, 9 p.

LEA, A.G.H. – BRIDLE, P. – TIMBERLAKE, C.F. SINGLETON, V.L. 1979. The procyanidins of white grapes and wines. In *American Journal of Enology the Viticulture,* vol. 30, 1979, p. 289–300.

MAJO, D. D. – GUARDIA, M. L. – GIAMMANCO, S. 2008. The antioxidant capacity of red wine in relationship with its polyphenolic constituents. In *Food Chemistry*, vol. 111, 2008, p. 45-49.

LACHMAN, J. – HEJTMÁNKOVÁ, K. – DUDJAK, E. 2003. Content of polyphenolic antioxidants and phenolcarboxylic in selected parts of yacon. In *Vitamins 2003 – Natural antioxidants and free radicals*. Pardubice: Univerzita Pardubice, p. 89 – 97, ISBN 80-7194-549-8.

SATORA, P. – SROKA, P. – DUDA-CHODAK, A. 2008. The profile of volatile compounds and polyphenols in wines produced from dessert varieties of apples. In *Food Chemistry*, vol. 111, 2008, p. 513-519.

SINGLETON, V. L. – ROSSI, J. A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. In *American Journal of Enology Viticulture,* vol. 16, 1965, p. 144-158.

ŠULC, M. – FAITOVÁ, K. – LACHMAN, J. - HEJTMÁNKOVÁ, A. - PIVEC, V. – DUDJAK, J. 2003. Obsah celkových polyfenolických látek v hroznech vybraných odrůd révy vinné. In *Vitamins 2003 – Přírodní antioxidanty a volné radikály*, Pardubice: Univerzita Pardubice, p. 181 – 187, ISBN 80-7194-549-8.

VICHITPHAN, S. – VICHITPHAN, K. – SIRIKHANSAENG, P. 2007. Flavonoid content and antioxidant activity of krachai-dum (*Kaempferia parviflora*) wine. In *KMITL Science and Technology Journal*, vol. 7, 2007, p. 97-105.

VILLANO, D. – Fernández-Pachón, M. S. – Troncoso, A. M. – García-Parrilla, M. C. 2006. Influence of enological practices on the antioxidant capacity and total polyphenols. In *Food Chem*istry, vol. 95, 2006, no. 3, p. 394-404.

WATERHOUSE, A.L. 2002. Wine phenolics. In Annals of New York Academy of Science, vo. 957, 2002, p. 21-36.

WEINGERL, V. – STRLIČ, M. – KOČAR, D. 2009. Comparison of methods for determination of polyphenols on wine by HPLC-UV/VIS, LC/MS/MS and Spectrophotometry. In *Acta Chimica Slovenica*, vol. 56, 2009, p. 698-703.