Scientific paper

Comparison of Methods for Determination of Polyphenols in Wine by HPLC-UV/VIS, LC/MS/MS and Spectrophotometry

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Received: 20-10-2008

Abstract

Phenolic antioxidants are usually grouped into flavonoids and non-flavonoids, according to their structure. With regard to the tannic character, phenolic antioxidants are further subdivided to tannic phenols and non-tannic phenols. Collectively, these compounds contribute to the high antioxidant capacity of wine.

In this work, we compare determination of gallic acid, catechin, epicatechin, resveratrol, quercetin, dihydrobenzoic acid, sinapic acid, vanillic acid, caffeic acid, chlorogenic acid, ferullic acid, ellagic acid, *p*-coumaric acid and caftaric acid in 141 wine samples using two liquid chromatographic methods and detection systems, i.e. with UV detection and mass-spectrometric detection. In addition, we applied the conventional Folin-Ciocalteu spectrophotometric method for determination of the total phenolic content in wine samples and compared the results with those obtained using the chromatographic methods.

Despite satisfactory correlations statistically significant differences between HPLC-UV/VIS and LC/MS/MS were established, which could be related to coelution not detectable with UV/VIS detectors. The correlations between results of the spectrophotometric method and sum of LC/MS/MS determinations are not satisfactory and are different for white, red, and rosé wines.

Keywords: Food analysis; wine; antioxidants; chromatography

1. Introduction

The concentration of phenolic substances in wine depends on the winemaking practice, climate, viticultural practice, infections and pests.[1] Considering the accumulated knowledge on the effect of phenolic antioxidants on human health and the resulting market requirements it is highly important to have well developed, robust and established methods for their determination.[2–4]

For separation and determination of phenolic acids and flavonoids, HPLC is the established technique.[5–8] The chromatographic conditions include the use of, almost exclusively, a reversed phase C18 column; UV/VIS diode array detector, and a binary solvent system containing acidified water and a polar organic solvent.[9]

In addition to separation methods, methods for determination of the so-called total phenolic content are also routinely in use. The one described by Singleton and Rossi[10], where oxidation of phenolic compounds with the Folin-Ciocalteu reagent[11] (mixture of phoshotungstate and phosphomolibdate) results in production of coloured products, of which the absorption is measured at 765 nm, is among the most often used. While a number of articles are focussed on determination of phenolic compounds and antioxidant properties of wines, [12–21] a comprehensive comparison of the most often used methods is still lacking.

It is safe to assume that LC/MS/MS in the multiple reaction monitoring (MRM) mode is probably most free of interferences, and it could potentially be used as an appropriate reference method. On the other hand, matrix effects may lead to different results obtained using other methods. In order to obtain a better insight into the extent

of these effects, we examined 73 red, 54 white and 14 rosé wines using all three methods.

With the chromatographic methods, we examined the content of phenolics which are most abundant in wines: gallic acid, catechin, epicatechin, *cis*- and *trans*-resveratrol and quercetin. Additionally, we compared the results with the total content of phenolics as obtained with the spectrophotometric method.

2. Materials and Methods

2. 1. Standards

Gallic acid, ellagic acid, sinapic acid, caffeic acid, chlorogenic acid, *p*-coumaric acid, dihydro benzoic acid and *trans*-resveratrol were purchased from Sigma (St. Louis, USA), (+)catechin hydrate, (-)epicatechin, vanillic acid, ferrulic acid and quercetin dihydrate were purchased from Fluka (St. Gallen, Switzerland). *Cis*-resveratrol was obtained after *trans*-resveratrol isomerization at 360 nm for 24 h.[22] All reagents and standards were prepared using Milli Q deionized water (Millipore, Bedford, USA).

2.2. HPLC-UV/VIS Analysis

The HPLC system Waters 600E was composed of the isocratic pump W600, the autosampler Waters 717+ and the Waters 996 photodiode array detector. Experimental conditions were the following: mobile phase A: 0.1% ortophosphoric acid; mobile phase B: methanol; mixed in a linear gradient as follows: 0 min: 90% A, 10% B; 15 min: 78% A, 22% B; 25 min: 50% A, 50% B; 34 min: 34% A, 66% B; 35 min: 90% A, 10% B; flow-rate: 1.0 mL/min; detection at 210 nm, 253 nm, 278 nm, 303 nm and 335 nm; injection volume: 50 μL; HPLC column: Synergi Hydro RP 150 × 4.6 mm, 4 μm (Phenomenex, Torrance, California, USA), column temperature: 35 °C. Retention times: gallic acid 4.2 min, catechin 13.5 min, epicatechin 20.6 min, *trans*-resveratrol 28.5 min, *cis*-resveratrol 29.9 min, quercetin 32.1 min. Optimum wavelengths are 210 nm for gallic acid, epicatechin and *cis*-resveratrol, 278 nm for catechin, 303 nm for *trans*-resveratrol and 253 nm for quercetin. All solvents were HPLC-grade and were degassed before use.

2. 3. HPLC/MS/MS Analysis

The HPLC system Perkin Elmer PE200 was composed of a binary pump, a column thermostat and an autosampler. The mass spectrometer used was 3200 QTRAP MS/MS with ESI ionisation (Applied Biosystems/MDS Sciex, Foster City, USA).

The experimental conditions were: mobile phase A: 50% acetonitrile, 50% acetic acid (0.5%); mobile phase B: 2% acetic acid; gradient elution: 0 min 30% A, 70% B; 10 min 30% A, 70% B; 30 min 100% A, 0% B; 35 min 100% A, 0% B; 40 min 30% A, 70% B for reconditioning of the system; flow rate: 0.7 mL/min; injection volume: 10 ìL; ionisation: ESI negative; dwell time 50 ms; MRM transitions: gallic acid 169/125, dihydro benzoic acid 153/109, sinapic acid 223/164, catechin and epicatechin 289/245, vanillic acid 167/123, caffeic acid 179/135, quercetin 301/151, chlorogenic acid 353/191, ferullic acid 193/134, resveratrol 227/185, ellagic acid 301/145, *p*-coumaric acid 163/119, caftaric acid 311/179. All solvents were HPLC-grade and were filtered and degassed before their use.

2. 4. Determination of Total Phenols

The determination of total phenols (TP) was performed according to the Folin-Ciocalteu procedure.[9,21] Briefly, 25 μL of a red wine sample or 250 μL of a white wine sample, 15 mL of distilled water, 1.25 mL of the diluted (1:2) Folin–Ciocalteu reagent, 3.75 mL of a sodium carbonate solution (20%) are mixed and distilled water is added to make up the total volume of 25 mL. The solution is agitated and left to stand for 120 min for the reaction to take place. The absorbance at 765 nm is determined in a cuvette of 1 cm. The absorbance measurements were performed using a Varian Cary 1E spectrophotometer.

The calibration curve was prepared with gallic acid solutions in concentration from 0 to 1000 mgL^{-1}. The results were expressed as millimols of gallic acid equivalent (GAE) per litre. The results for standards were highly reproducible (calibration curve squared regression coefficient >0.9993). All determinations were performed in triplicate.

The Folin–Ciocalteu reagent was purchased from Merck (Darmstadt, Germany). It contains sodium tungstate, sodium molybdate, ortophosphoric acid, hydrochloric acid, lithium sulphate, bromine, hydrogen peroxide.[11]

2. 5. Calibration and Quantification

Stock solutions of standards were diluted in the mobile phase to obtain working standard solutions. Concentrations of the analytes were calculated from chromatogram peak areas on the basis of calibration curves. In HPLC-UV/VIS, identification of the different compounds was achieved by comparison of both the retention times and the absorption spectra with those obtained for the standards.

2. 6. Linearity and Repeatability

The method linearity was assessed by means of linear regression of the mass of analyte injected vs. its peak area. The repeatability was expressed as standard deviation (SD) of three separate determinations.

2. 7. Sample Preparation

141 commercially available wine samples (73 red, 54 white and 14 rosé wines) were purchased and directly

analysed. All samples ware filtered through 0.45 µm Chromafil polyamide/nylon syringe filters (Macherey–Nagel, Düren, Germany) before injection.

For all chromatographic analyses, the wine samples were diluted ten times with the respective mobile phases described above.

3. Results and Discussion

3. 1. Spectrophotometric Determination

Spectrophotometric determination of total phenolic content (TP) is a simple method for estimating the total content of reducing phenolic compounds in wine. Although it is an empirical method, it is still in routine use as it is robust, fast and simple. The TPs determined for red wines ranged from 4.11 to 14.57 GAE mmolL^{-1}, rosé wines 1.94 to 4.11 GAE mmolL^{-1}, and white wines 0.94 to 2.35 GAE mmolL^{-1}. It is of interest, how the TP results correlate with the sum of phenolic compounds determined using LC/MS/MS (Figure 1).

Unsatisfactory correlations between the methods are evident (Figure 1). We not only obtained three different calibration curves for the different types of wine, the high data scatter is additionally discouraging.

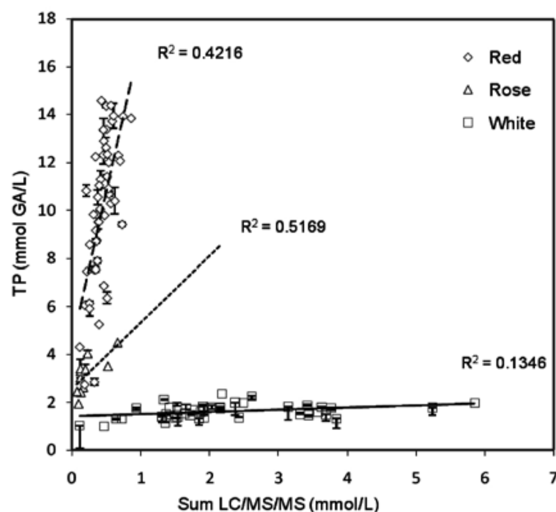

Fig. 1: Comparisons between determinations of TP and sum of gallic acid, catechin, epicatechin, resveratrol, quercetin, dihydrobenzoic acid, sinapic acid, vanillic acid, caffeic acid, chlorogenic acid, ferullic acid, ellagic acid, p-coumaric acid and caftaric acid, determined using LC/MS/MS in white wines, rosé wines and red wines.

A comparison of the typical standard deviations for both methods is given in Table 1, again showing that the SDs for the spectrophotometric method are mostly higher. The conclusion is that the spectrophotometric method is not suitable for quantitative determination of the total phenolic content of wine; it may only serve for rough estimations, and even then only for red wines.

The exhibited differences may also be the result of synergistic and/or antagonistic effects of different wine compounds contributing to the chemistry leading to colour formation according to the Folin-Ciocalteu method. These are not assessed using separation methods.

It is interesting to observe, however, that there is a relatively good correlation between the content of gallic acid (GA) determined using LC/MS/MS and the total phenolic content determined using the spectrophotometric method (Figure 2). This leads to the conclusion that determinations of TP may be regarded as a good measure of GA concentration.

In Figure 3 we show the comparison between sum of phenolic compounds determined using HPLC, and TP. For

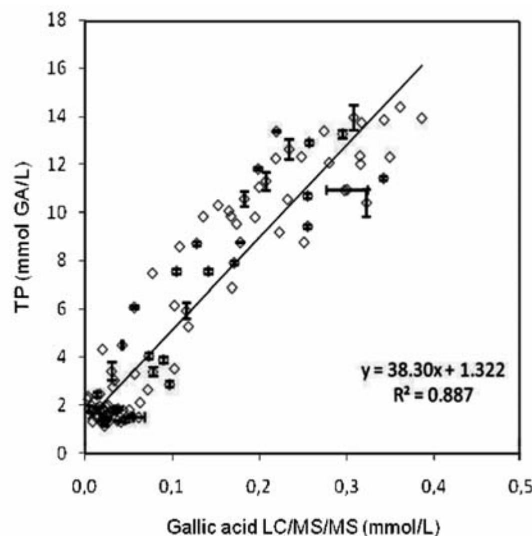

Fig. 2: Comparison between the concentrations of gallic acid determined using LC/MS/MS and TP. All wine samples included.

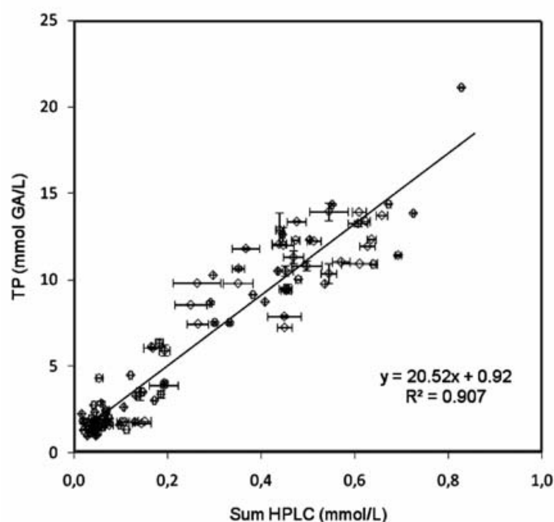

Fig. 3: Comparison between the sum of gallic acid, catechin, epicatechin, trans-resveratrol, cis-resveratrol and quercetin, determined using HPLC and TP. All wine samples included.

Table 1: Typical standard deviations for determinations of total phenolic content (TP) and for determinations of the sum of phenolic compounds determined using LC/MS/MS.

Wine sample	SD (TP) (mmol/L)	SD (Sum LC/MS/MS) (mmol/L)
Red	0.10	0.012
Rose	0.09	0.024
White	0.02	0.070

catechin, epicatechin, trans-resveratrol, cis-resveratrol and quercetin we obtained similar correlations to those in Fig. 2 and Fig. 3. On the other hand, dihydrobenzoic acid, sinapic acid, caffeic acid, chlorogenic acid, ferullic acid, *p*-coumaric acid and caftaric acid, determined using LC/MS/MS exhibit different correlations with total phenolic content determined in white, rosé and red wines (similar to those in Fig. 1). It is important to highlight that concentrations of dihydrobenzoic acid, sinapic acid, caffeic acid, chlorogenic acid, ferullic acid, *p*-coumaric acid and caftaric acid are much higher in white wines than in red wines, but in contrary, TP of white wines is much lower than TP of red wines, which is the reason for the three different correlation lines for the three types of wine in Figure 1.

3. 2. Comparison of Chromatographic Methods

In this section, we compare the determination of several wine phenolic compounds that can easily be determined using HPLC-UV/VIS: gallic acid, quercetin, catechin, epicatechin and *trans*-resveratrol. We used all red wine samples and some white wines in the comparison.

From Figure 4, it is evident that there is considerable data scatter and that the quality of correlations varies considerably. With the exception of quercetin and *trans*-resveratrol, determinations using HPLC-UV/VIS are systematically higher by 15–20% than those obtained with HPLC/MS/MS, since the slopes of correlation lines are >1. Determinations of quercetin using the two methods correlate well, while the determination of *trans*-resveratrol is in average 30% lower if the HPLC-UV/VIS method is used. However, data scatter is also the highest for this particular analyte ($R^2 = 0.894$).

For all analytes, the standard deviation of determinations is higher in the case of HPLC-UV/VIS than LC/MS/MS. There seems to be no systematic bias, as the intersections are small in comparison with the determinations. We assume that the systematic error in HPLC-UV/VIS determinations is due to matrix effects, which is the subject our further research.

4. Conclusions

In this work, we compared three routinely used methods of determination of the following wine phenolic antioxidants: gallic acid, catechin, epicatechin, resveratrol, quercetin, dihydrobenzoic acid, sinapic acid, vanillic acid, caffeic acid, chlorogenic acid, ferullic acid, ellagic acid, *p*-coumaric acid and caftaric acid. We used liquid chromatographic methods with UV detection and mass-spectrometric detection and the conventional Folin-Ciocalteu spectrophotometric method for determination of the total phenolic content. The comparisons led us to the following conclusions:

- While satisfactory correlations between HPLC-UV/VIS and LC/MS/MS determinations were established, there is a significant data scatter and the results of LC/MS/MS may be overestimated by 15–20% in average, relative to LC/MS/MS determinations, except in the case of *trans*-resveratrol, where the results may be underestimated by 30%
- The uncertainties of results using HPLC-UV/VIS are higher than those obtained with LC/MS/MS. The uncertainties of results using the spectrophotometric method are still higher.
- Different correlations between determinations using the spectrophotometric method and LC/MS/MS determinations were obtained for white, red, and rosé wines.
- Determinations of the total phenol content correlate with the individual contents of gallic acid, catechin, epicatechin, trans-resveratrol, cis-resveratrol and quercetin as obtained using LC/MSMS in all wine samples.
- The contents of dihydrobenzoic acid, sinapic acid, caffeic acid, chlorogenic acid, ferullic acid, *p*-coumaric acid and caftaric acid, determined using LC/MS/MS exhibit different correlations with total phenolic content determined in white, rosé and red wines. The reason is probably that concentrations of these compounds are considerably higher in white wines than in rosé and red wines, but the total phenolic content of white wines is up to 15 times lower than those of red wines.

In routine work, it is important to understand that HPLC-UV/VIS determinations may be biased to up to 30% relative to LC/MS/MS determinations. The Folin-Ciocalteu method may only be used for comparative purposes, and even then only for red, rosé and white wines separately. The exhibited differences may also be the result of synergistic and/or antagonistic effects of difference wine compounds contributing to the chemistry leading to colour formation according to the Folin-Ciocalteu method. These are not assessed using separation methods.

5. Acknowledgement

The financial support of the Slovenia Research Agency, Programme no. P1-0153 is gratefully acknowledged.

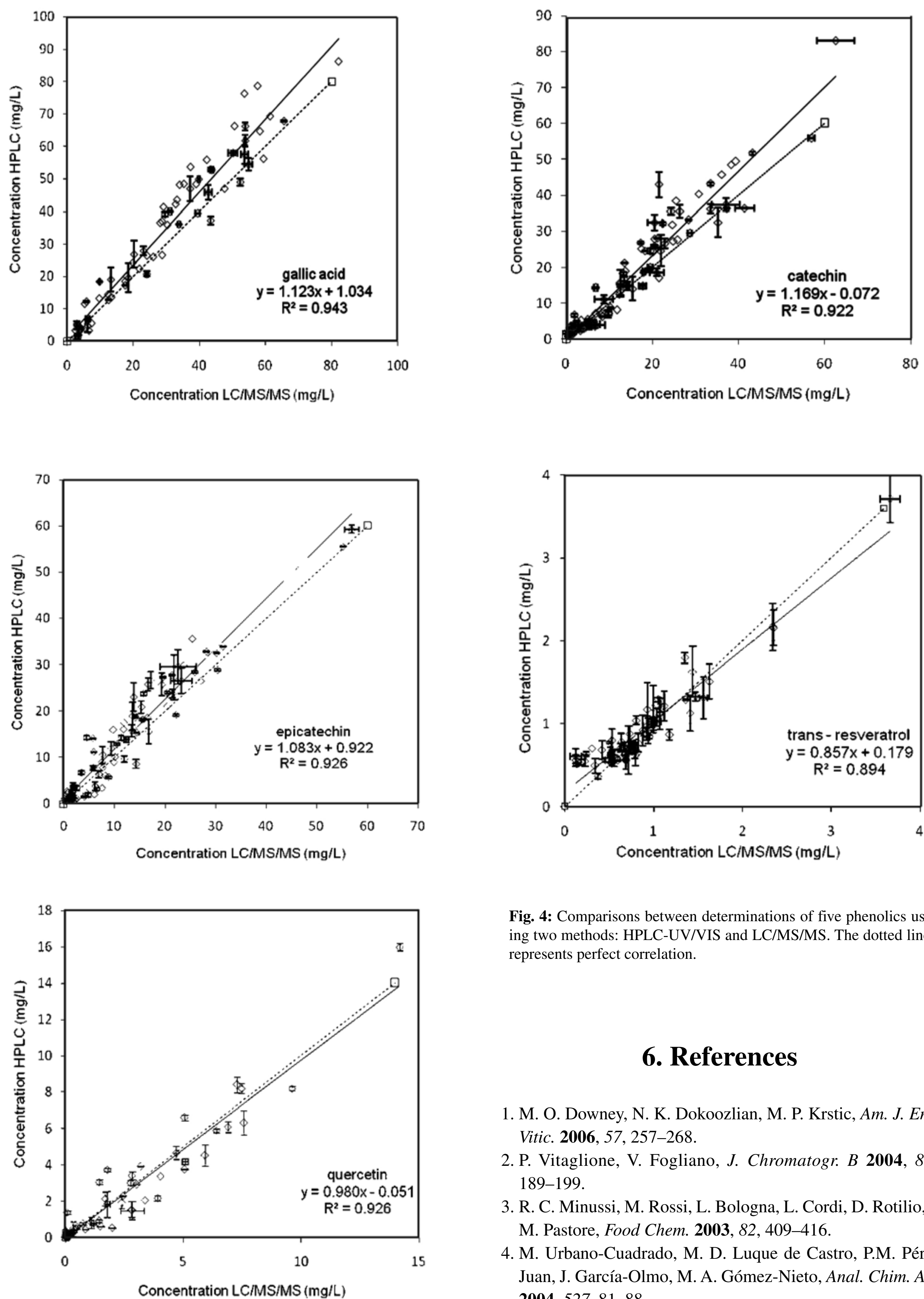

Fig. 4: Comparisons between determinations of five phenolics using two methods: HPLC-UV/VIS and LC/MS/MS. The dotted line represents perfect correlation.

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Povzetek

Glede na kemijsko sestavo delimo fenolne antioksidante na flavonoide in neflavonoide. Glede na taninski značaj jih delimo na taninske in netaninske fenole. Omenjene fenolne komponente so odgovorne za visok antioksidativni potencial vina. Primerjali smo določanje galne kisline, katehina, epikatehina, resveratrola, kvercetina dihidrobenzojske kisline, sinapinske kisline, vanilinske kisline, kavne kisline, klorogenske kisline, ferulne kisline, elaginske kisline, p-kumarne kisline in kaftarjeve kisline v številnih vzorcih vin z uporabo dveh metod tekočinske kromatografije z UV/VIS detekcijo in z masnospektrometrično detekcijo (LC/MS/MS).
Uporabili smo konvencionalno spektrofotometrično metodo s Folin-Ciocalteu-jevim reagentom za določanje vsebnosti skupnih fenolov in rezultate primerjali z rezultati kromatografskih metod.
Kljub zadovoljivim korelacijam smo ugotovili statistično signifikantne razlike med HPLC-UV/VIS in LC/MS/MS, kar je lahko posledica koelucije, ki je z UV/VIS detektorjem ne zaznamo. Primerjava med rezultati, dobljenimi s spektrofotometrično metodo in vsoto fenolnih komponent, določanih z LC/MS/MS je pokazala različne korelacijske premice za rdeča, rosé in bela vina.