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HPLC determination of phenolics adsorbed on yeasts

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Abstract

An analytical HPLC method was developed to determine monomeric anthocyans as catechin (CA), flavonols as rutin (RU) and phenol acids as gallic acid (GA) adsorbed on the cell wall of 23 *Saccharomyces cerevisiae* strains grown on two media containing high levels of phenolic compounds, from grape seeds and grape skins, and on one control medium. Microbial biomass purification by liquid–liquid extraction of the phenolics is followed by reversed-phase chromatographic separation and CA, RU and GA detection by ultraviolet detector. The method was linear over the studied range of concentrations: GA at 0.12–0.96 μg/ml, CA at 0.25–20.00 μg/ml and RU at 0.02–0.20 μg/ml. The correlation coefficient for each analyte was greater than 0.9983. The recovery was greater than 85% for both GA and RU, and greater than 94% for CA. The detection limits for GA, CA and RU were determined to be 0.015, 0.025 and 0.029 μg/mg of biomass, respectively. The proposed method is highly responsive for the determination of different phenolics, and seems to be useful to evaluate their adsorption profile on yeasts.

Keywords: HPLC; Monomeric anthocyans; Phenolics; Yeasts

1. Introduction

Considerable epidemiological research supports that the consumption of foods and beverages rich in phenolic compounds is associated with a low incidence of cardiovascular diseases in humans and, moreover, due to their antioxidant activity, it could protect against cancer [1–4].

More than 4000 phenolic compounds, such as phenolic acids, flavonoids and proanthocyanins, have been found in the vegetable kingdom. Phenolics give fruits, juices and fermented beverages many properties, such as colour, browning, bitterness and astringency [5,6].

During food processing these compounds can lead, based on their characteristics, to various products formed by enzymatic or chemical reactions [7].

Among various red wines constituents, anthocyanins, flavonols, catechins and other flavonoids play a major role in wine quality and determine peculiar characteristics, such as colour and astringency [8].

In spite of some authors have determined phenolic compounds in wines by direct injection in high performance liquid chromatography (HPLC), coupled with mass spectrometry [9], the solid-phase extraction (SPE) is the common technique used for concentration and purification prior to HPLC separation of these compounds in wines [10–12].

Separation of phenolics in grapes and/or wines was performed commonly by reverse-liquid chromatography followed by ultraviolet [13], photodiode array [14], fluorimetric [15], electrochemical [16] or mass spectrometric detection [17].

Phenolics content in wines vary notably, according to several parameters [18,19], such as grape variety [20], maceration temperature [21], length of grape pomace contact [22] and other vinification conditions [23,24].

Wine yeasts are among the causes that decrease the phenolic content of wines [25]. Some author suggested that a mechanism could be exclusively physical, involving the establishment of weak and reversible interactions mainly between anthocyanins and yeast walls by adsorption [26].

Different yeast strains have shown different amount of anthocyanins adsorbed and, moreover, the adsorption mechanism involves hydrophobic interaction seeing that anthocyanins with a greater degree of methoxylation are more retained than those

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most hydroxylated besides different polarity and porosity of cell walls [27].

Other studies, instead, have confirmed that the highest anthocyanin removal was for compounds with higher polarity and have demonstrated the formation during fermentation of anthocyanin-derived pigments, which have a role in maintaining wine colour [28].

An interesting correlation between the yeast strain used for winemaking and the phenolic composition of wine have recently demonstrated, elucidating that strain behaviour can somewhat modify chromatic properties, phenolic profile and antioxidant power of wine [29].

Recently, to determine the interaction of wine polyphenols and the external components of yeast lees, polyphenolic compounds remaining in solution and those adsorbed on yeast lees at different times were analysed during a simulation of wine aging [30].

A simple screening method was proposed to differentiate yeasts with low, medium and high aptitude to adsorb phenolics, considering the colour modifications of the microbial biomass, i.e. white, grey and hazel [31].

At present, the available analytical methods able to detect chromatographically phenolics adsorbed on microbial cell walls are limited and complex. So, we decided to develop a new rapid analytical methodology of extraction and chromatographic determination, using HPLC associated to an ultraviolet detector (UV), to survey phenolics adsorbed on yeasts. Moreover, in this method the yeasts have been grown in different vegetal matrices as selective biological extractors.

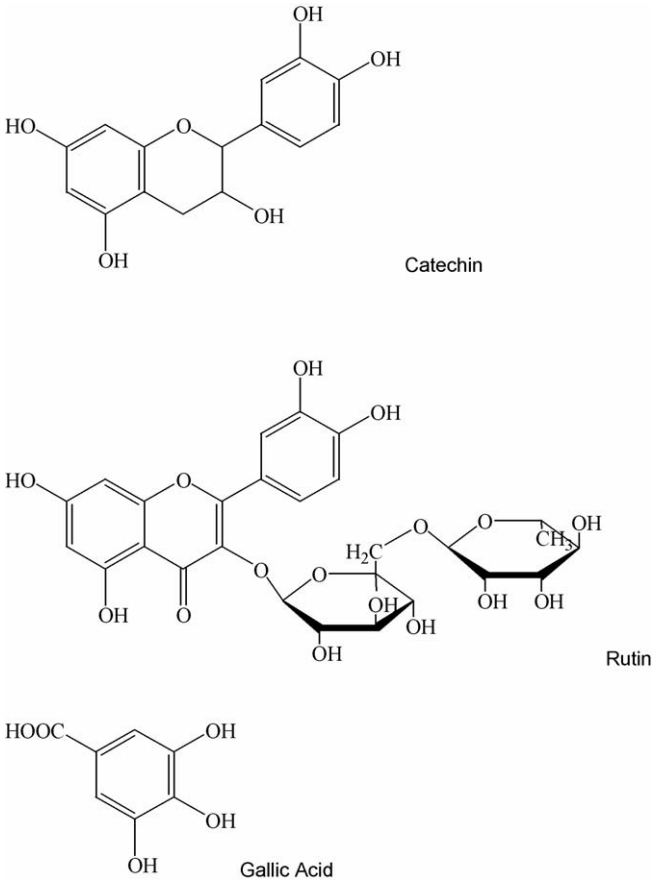

Fig. 1. Structures of phenolics.

In order to consider the yeast biodiversity and selectivity of adsorption toward phenolics with different chemical structure, we choose to determine a polyphenol non-flavonoid, *gallic acid* (GA), a flavanol, *catechin* (CA) and a flavonol, *rutin* (RU).

It is well known that grape seeds are richer in catechins and proanthocyanidins that grape skins, in both red and white cultivars [32]. On the contrary, flavonols, such as RU, are particularly contained in grape skins [33].

Chemical structures of target compound are shown in Fig. 1.

In details, the purpose of this study was to determine CA, RU and GA adsorbed by 23 strains of *Saccharomyces cerevisiae*, grown on two media containing high levels of phenolic compounds, from grape seeds and grape skins, and on one control medium. The new assay was validated determining linearity, precision, accuracy, recovery and stability.

2. Experimental

2.1. Microorganisms

Twenty-three strains of yeast of the species *S. cerevisiae*, previously selected for oenology, were inoculated in Petri dishes containing a control medium and two media, one rich in tannins (grape seeds) and the other in anthocyans (grape skins). These media had the following composition:

Control medium (Sabouraud Agar): glucose 40 g/l, peptone from casein 10 g/l, agar 15 g/l.
Grape Seed Agar: homogenised grape-seed 50 g/l, peptone from casein 7.5 g/l, yeast extract 4.5 g/l, agar 20 g/l.
Grape Skin Agar, homogenised grape-skin 50 g/l, peptone from casein 7.5 g/l, yeast extract 4.5 g/l, agar 20 g/l.

Media were sterilised at 121 °C for 15 min, poured into Petri dishes, inoculated with a small quantity of yeast biomass and incubated at 25 °C for 7 days.

2.2. Chemicals, solutions, disposables and preparation of standards

Sabouraud Agar, peptone from casein, yeast extract and agar from Biokar Diagnostics (Beauvais, France), acetonitrile (HPLC grade), methanol (HPLC grade) and acetic acid (analytical grade) from Carlo Erba Reagenti (Milano, Italy), and standards of GA 97%, CA 98% and RU 95% from Aldrich (Milwaukee, USA) were used. Distilled and deionised water was prepared with Milli-Q water purification system. A standard solution of each studied compound was prepared in methanol at a concentration of 1 mg/ml. Stock solutions containing GA, CA and RU were prepared by diluting the standard solutions with methanol to yield concentrations of 0.1, 1 and 10 μg/ml, respectively. Working standard solutions were freshly prepared for spiking control medium inoculated in four replicates and at appropriate dilutions with the strain TT51, chosen because its

Table 1
Biomass colour of the 23 yeast strains grown for 7 days at 25 °C on Grape Seed Agar (A), Grape Skin Agar (B) and on a control medium (C)

A		B		C	
Biomass colour in seeds	Strain	Biomass colour in skins	Strain	Biomass colour in control medium	Strain
White	Sc 1303	White	Sc 226	White	Sc 226
White	Sc 1304	White	Sc 708	White	Sc 708
White	Sc 1864	White	Sc 1661	White	Sc 1661
White	Sc 2659	White	Sc 1826	White	Sc 1826
White	Sc 2717	White	Sc 2659	White	Sc 2659
Grey	1042	White	Sc 2717	White	Sc 2717
Grey	MCR 4	Grey	1042	White	1042
Grey	MCR 5	Grey	Sc 560	White	Sc 560
Grey	Sc 1596	Grey	Sc 1303	White	Sc 1303
Grey	Sc 1661	Grey	Sc 1596	White	Sc 1596
Grey	Sc 1826	Grey	Sc 1864	White	Sc 1864
Grey	Sc 2489	Grey	Sc 2489	White	Sc 2489
Grey	TT 51	Grey	TT 51	White	TT 51
Grey	TT 77	Grey	TT 241	White	TT 241
Grey	TT 241	Grey	TT 244	White	TT 244
Grey	TT 244	Grey	TT 254	White	TT 254
Grey	TT 254	Hazel	MCR 4	White	MCR 4
Hazel	Sc 226	Hazel	MCR 5	White	MCR 5
Hazel	Sc 560	Hazel	Sc 1304	White	Sc 1304
Hazel	Sc 708	Hazel	Sc 1483	White	Sc 1483
Hazel	Sc 1483	Hazel	Sc 2621	White	Sc 2621
Hazel	Sc 2621	Hazel	TT 77	White	TT 77
Hazel	TT 173	Hazel	TT 173	White	TT 173

biomass colour on Grape Seed Agar and Grape Skin Agar was intermediate, as reported in Table 1. Working solution were used to yield the following concentrations: for GA 0.015–0.96 μg/mg of biomass, for CA 0.25–20.00 μg/mg of biomass and for RU 0.02–0.2 μg/mg of biomass.

Quality control samples used for the study of intra- and inter-day accuracy and precision, extraction recovery and stability were prepared in the same way as the calibration samples. Specific quality control samples representing low, middle and high concentration were 0.025, 0.5 and 1 μg/mg for GA;

Table 2
Biomass colour and related biomass content in phenolics, gallic acid and catechin of the 23 yeast strains grown for 7 days at 25 °C on Grape Seed Agar

Strain	Biomass colour	Folin–Ciocalteu index	Gallic acid (μg/mg of biomass)	Catechin (μg/mg of biomass)
1042	Grey	[a]	[a]	9.036
MCR 4	Grey	0.663	0.289	7.737
MCR 5	Grey	0.628	0.025	1.274
Sc 226	Hazel	0.989	0.135	20.504
Sc 560	Hazel	1.124	0.070	4.025
Sc 708	Hazel	0.723	0.025	5.774
Sc 1303	White	0.653	0.166	8.467
Sc 1304	White	0.653	0.290	11.735
Sc 1483	Hazel	0.891	0.117	2.289
Sc 1596	Grey	0.631	0.073	1.374
Sc 1661	Grey	0.768	0.038	2.173
Sc 1826	Grey	0.737	0.045	2.433
Sc 1864	White	0.680	0.232	13.166
Sc 2489	Grey	0.850	[a]	1.692
Sc 2621	Hazel	0.820	0.104	1.881
Sc 2659	White	0.509	0.185	4.419
Sc 2717	White	0.440	0.198	3.418
TT 51	Grey	0.669	0.328	4.389
TT 77	Grey	0.641	0.015	1.308
TT 173	Hazel	0.670	0.714	4.064
TT 241	Grey	0.770	0.028	1.662
TT 244	Grey	0.728	0.048	1.395
TT 254	Grey	0.772	0.034	1.154

[a] Not determined.

0.5, 4.00 and 16.00 μg/mg for CA; 0.03, 0.1 and 0.2 μg/mg for RU.

All samples were filtered through a 0.22 μm Millipore Filters (Bedford, USA) before analysis.

2.3. Total polyphenol determination

The Folin–Ciocalteu (FC) index was determined on the biomass of the 23 yeast strains grown for 7 days at 25 °C on Grape Seed Agar and Grape Skin Agar. The official method [34] was modified as follows:

Ten micrograms of yeast biomass was taken using a calibrated loop.
The biomass was suspended in a 10 ml-volumetric flask with 0.5 ml of distilled water.
0.5 ml of FC reagent was added.
Two milliliters of sodium carbonate anhydrous solution 20% (w/v) was added.
The flask was filled with distilled water and, after vortex mixing, the suspension was poured off into a centrifuge test-tube.
After 30 min the suspension was centrifuged at 4000 rpm for 5 min (centrifuge 4235 A, ALC Laboratory Instruments, Cologno Monzese, Italy);
The absorbance was read at 750 nm, subtracting the value of a control solution prepared using distilled water instead of the biomass (spectrophotometer Anadeo 1, Bibby Sterilin Ltd., Staffordshire, England).
The FC index was calculated multiplying the absorbance by 200.

2.4. Chromatography

A Jasco PU 980 pump and LG 980-02 ternary unit (Tokyo, Japan) with a 20 μl loop injection valve was used. The chromatographic system was associated to an ultraviolet detector Jasco UV-975 (Tokyo, Japan). The separation was performed on a Tracer HYPERSIL ODS (25 cm × 0.46 cm, 5 μm i.d.) reversed-phase column (Tecnokroma, Barcelona, Spain), with a ODS guard (4.5 cm × 0.46 cm). A block heater Gastorr GF 103 (Jones Chromatography, Colorado, U.S.A.) was utilised to maintain the analytical column at 25 °C. A mixture of acetic acid, methanol and water was used as mobile phase. Phase A with a solvent ratio of 5:20:75 (v/v/v) was used to detect GA while phase B with a modified ratio of 5:45:50 (v/v/v) was chosen to separate CA and RU. The column was flushed with a flow rate of 0.5 ml/min. Data were processed using Borwin chromatography software (Version 1.21) from Jasco (Tokyo, Japan).

2.5. Extraction procedure

The extraction of the phenolic fraction was performed directly on the microbial biomass. Around 60 mg, exactly weighted, of yeast biomass were withdrawn with calibrated loops and mixed with methanol in a fixed ratio, weight over volume, of 20:1. Samples were shaken by vortex for 5 min and then centrifuged at 6000 × *g* for 10 min. The supernatant was withdrawn, divided in three aliquots in Eppendorf test tubes and then centrifuged at 5000 × *g* for 5 min. The extracts obtained were filtered through membranes filters 0.22 μm and aliquots of 20 μl were used for chromatographic analysis. In conclusion,

Table 3
Biomass colour and related biomass content in phenolics, gallic acid, catechin and rutin of the 23 yeast strains grown for 7 days at 25 °C on Grape Skin Agar

Strain	Biomass colour	Folin–Ciocalteu index	Gallic acid (μg/mg of biomass)	Catechin (μg/mg of biomass)	Rutin (μg/mg of biomass)
1042	Grey	[a]	[a]	0.255	0.036
MCR 4	Hazel	0.243	0.059	0.575	[a]
MCR 5	Hazel	0.275	0.110	0.584	[a]
Sc 226	White	0.220	0.161	1.402	0.112
Sc 560	Grey	0.310	0.118	0.767	[a]
Sc 708	White	0.306	0.070	0.945	[a]
Sc 1303	Grey	0.275	0.109	1.502	[a]
Sc 1304	Hazel	0.255	0.088	0.637	[a]
Sc 1483	Hazel	0.322	0.030	0.275	0.159
Sc 1596	Grey	0.227	0.020	0.404	0.046
Sc 1661	White	0.205	0.088	1.125	[a]
Sc 1826	White	0.205	0.061	0.767	[a]
Sc 1864	Grey	0.262	0.300	2.774	[a]
Sc 2489	Grey	0.520	0.061	1.423	0.065
Sc 2621	Hazel	0.355	0.012	1.118	0.149
Sc 2659	White	0.381	0.070	0.539	0.042
Sc 2717	White	0.160	0.020	0.482	0.054
TT 51	Grey	0.268	0.179	1.231	0.072
TT 77	Hazel	0.323	0.039	0.372	0.029
TT 173	Hazel	0.290	0.122	0.964	[a]
TT 241	Grey	0.349	0.147	0.969	[a]
TT 244	Grey	0.292	0.172	1.136	[a]
TT 254	Grey	0.337	0.104	1.247	0.050

[a] Not determined.

phenolics were extracted by methanol addition to the biomass in ratio of 1 ml for 20 mg of biomass.

2.6. Stability

The stability of the three analytes was determined in two assays. GA (0.1 and 1 μg/ml), CA (2.5 and 20 μg/ml) and RU (0.05 and 0.2 μg/ml) were determined in methanol stored at −20 °C, over a period of 1 month. The stability of the same concentrations of the three analytes, after extraction from control medium and dissolving in methanol, was controlled for up to 48 h at room temperature.

2.7. Validation of the method

Validation of the HPLC method was performed by determining the intra-, inter-day accuracy and precision, and percentage of recovery of the three analytes under the extraction and analytical condition. The chromatographic identification of the compounds CA, RU and GA was obtained by their retention times. Moreover, in order to verify the specificity of the analytical method, the presence of interfering peaks and changes in retention times was assessed in chromatograms obtained from biomass analysis.

The quality control samples were analysed in a set of five on a single assay day to determine intra-day precision and accuracy, and analysed in duplicate on each of seven separate days to determine inter-day precision and accuracy.

The extraction recovery was determined in sets of five by measuring the amount of each compound recovered after extraction. The quality control samples in triplicate at low, middle and high concentration were used.

2.7.1. Calibration curves

The control medium inoculated with the strain TT51 and incubated at 25 °C for 7 days was used as blank sample. The standard calibration samples of GA, CA and RU were added and then extracted as described in Section 2.4. Calibration curves were constructed by plotting the peak-area of each analyte versus analyte concentration in control medium sample. In order to avoid undue bias to the low concentration of the standard curve by the high concentrations, the calibration curve of each compound was split into two ranges: 0.015–0.12 and 0.12–0.96 μg/mg for GA, 0.25–2.5 and 2.5–20.00 μg/mg for CA and 0.02–0.05 and 0.05–0.2 μg/mg for RU.

2.7.2. Limits of detection

Blank control medium was spiked with decreasing concentration of the three studied compounds and the samples were analysed as described above. The limit of detection was defined as the lowest concentration of the drug resulting in a signal-to-noise ratio of 3:1.

3. Results and discussion

The method proposed in this study, validated regarding detection limits, quantification, sensitivity, linearity, precision, accuracy and extraction efficiency allows identifying and quantifying phenolic compounds adsorbed on *S. cerevisiae* cell walls.

Table 1 reports the behaviour towards phenolic compounds of each strain according to the substrate used. Results show

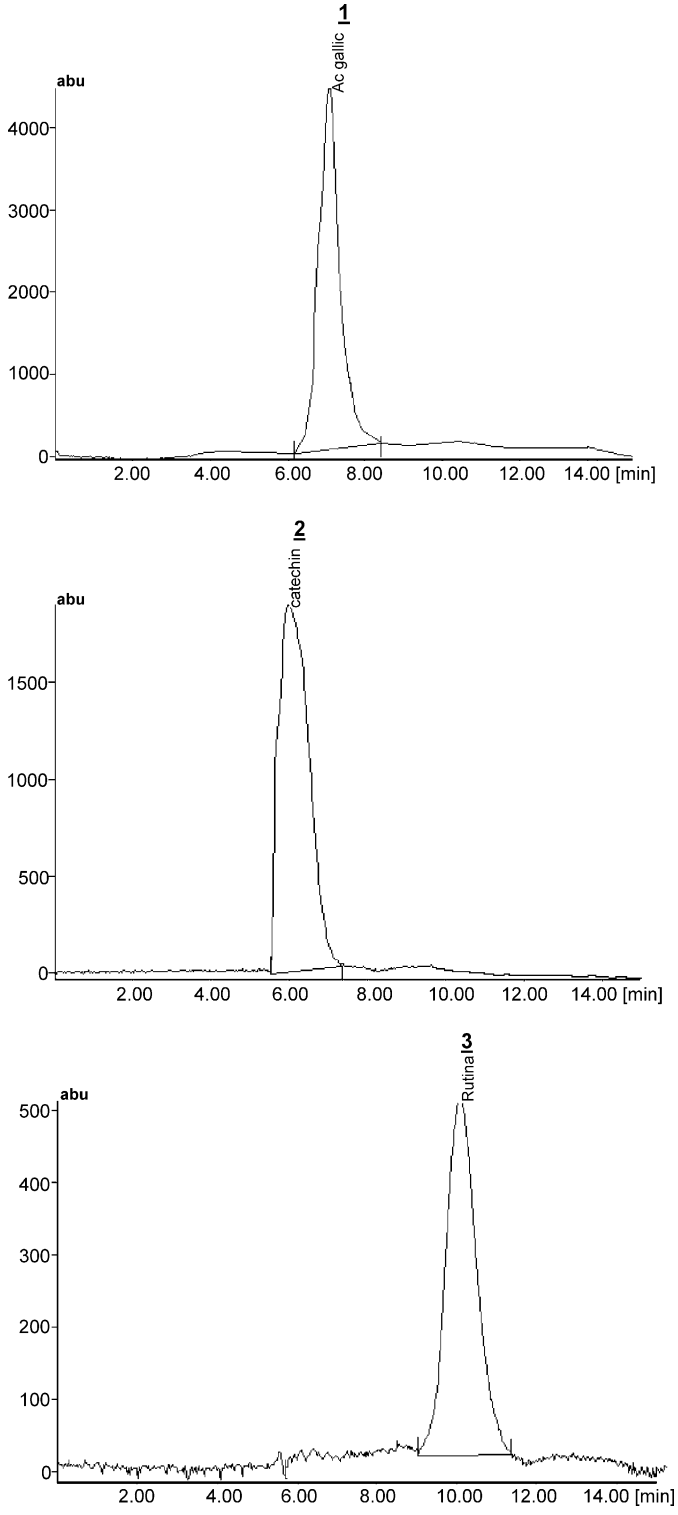

Fig. 2. Typical chromatograms of: gallic acid (1) in methanol (0.5 μg/ml), $t' = 7.2$, UV = 280 nm, mobile phase A; catechin (2) in methanol (0.6 μg/ml), $t' = 6.2$, UV = 280 nm, mobile phase B; rutin (3) in methanol (0.2 μg/ml), $t' = 10.1$, UV = 360 nm, mobile phase B.

differences between white and hazel strains due to the fact that the latter can adsorb more phenolics than the former [31].

Table 2 reports, for the yeast strains grown for 7 days at 25 °C on Grape Seed Agar, the biomass colour and the related content in total polyphenols, expressed as FC index, GA and CA. We have determined the FC index of the yeast biomass to compare the results obtained by HPLC with those found using a fast and simple spectrophotometric quantification.

Table 3 reports, for the yeast strains grown on Grape Skin Agar under the same growth conditions reported above, the biomass colour and the related content in total polyphenols, expressed as FC index, RU, GA and CA.

3.1. Total polyphenols determination

The FC index reported in Tables 2 and 3 shows that the total polyphenol content is always correlated with the biomass colour of the yeast strains but his value, especially for GA, RU and CA content, is not strictly correlated with the chromatographic data. This is probably due to the reaction of monomeric phenolics with other components of the agar plates to form complex structures, which could react with the FC reagent.

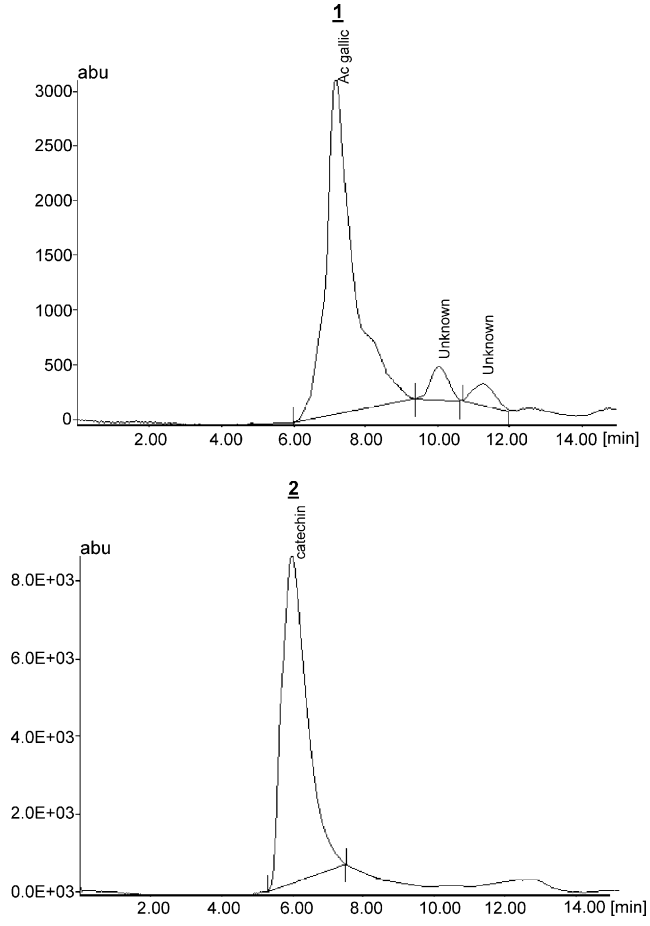

Fig. 3. Typical chromatograms of an extract obtained from grape seed matrix inoculated with the strain TT51. Gallic acid (1) $t' = 7.2$, UV = 280 nm, mobile phase A; catechin (2) $t' = 6.2$, UV = 280 nm, mobile phase B.

3.2. Chromatography

Chromatographic conditions were based on the isocratic separation on a reverse phase column. Preliminary studies

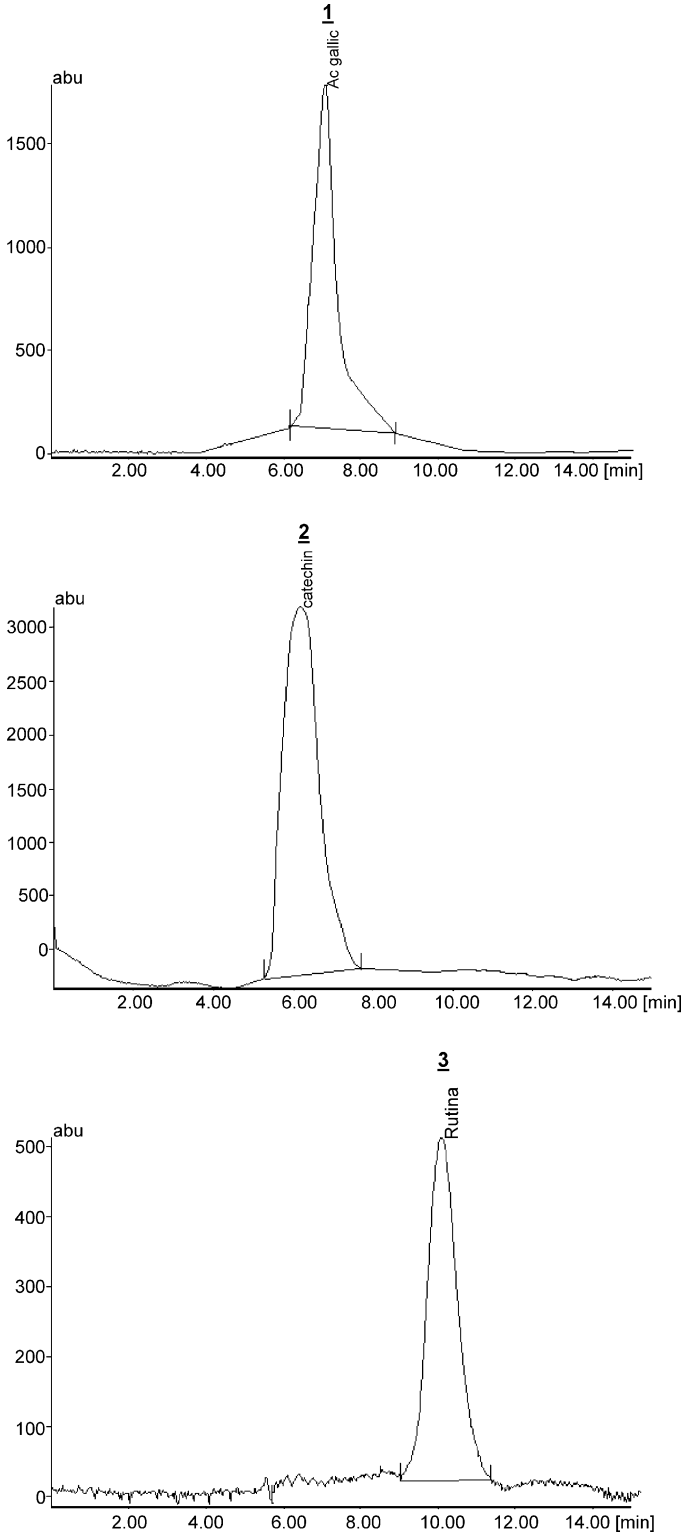

Fig. 4. Typical chromatograms of an extract obtained from grape skin matrix inoculated with the strain TT51. Gallic acid (1) $t' = 7.2$, UV = 280 nm, mobile phase A; catechin (2) $t' = 6.2$, UV = 280 nm, mobile phase B; rutin (3) $t' = 10.1$, UV = 360 nm, mobile phase B.

Table 4
Accuracy and precision for the determination of gallic acid (GA). catechin (CA) and rutin (RU)

Analyte	Nominal concentration (μg/mg)	Intra-day ($n = 5$)		Inter-day ($n = 14$)	
		Mean detected conc. (μg/mg)	R.S.D. (%)	Mean detected conc. (μg/mg)	R.S.D. (%)
GA	0.025	0.024	3.07	0.026	4.07
	0.500	0.495	2.98	0.501	3.56
	1.000	1.080	2.02	1.094	4.02
CA	0.500	0.540	1.51	0.470	3.14
	4.000	4.020	1.99	4.200	3.55
	16.000	16.100	1.55	16.01	2.39
RU	0.030	0.033	2.46	0.035	2.46
	0.100	0.101	1.22	1.020	3.09
	0.200	0.208	1.19	0.203	2.56

Measurements in the control medium Sabouraud Agar inoculated with the strain TT51. R.S.D., related standard deviation.

with different mobile phase combination of 20% acetonitrile in 25 mM phosphate buffer (pH 2.4) were considered. Moreover, a gradient elution of 20–80% methanol in acidified water with acetic acid (pH 1.75) was used. Some of the 46 chromatograms obtained reported also unknown-peaks. This was more evident for samples rich in GA.

Flavonols was detected by ultraviolet light showing a maximum at 360 nm. CA, GA and other phenolic acids were detected at 280 nm. Considering the concentration difference of the compounds in the matrices analysed, we determined each compound in a single chromatographic analysis. Therefore, a mobile phase used to determine GA (mobile phase A) was more hydrophilic than the mobile phase used to separate CA and RU (mobile phase B). Both mobile phases were formed by acetic acid, methanol and water with a ratio of 5:20:75 (v/v/v) and 5:45:50 (v/v/v) for mobile phases A and B, respectively.

Fig. 2 reported three typical chromatograms for methanolic solution of GA (1), CA (2) and RU (3).

3.3. Extraction

For all the 23 yeast strains grown in Sabouraud Agar, the biomass obtained did not contain any phenols and the chromatograms did not show any peaks. For each strain, the phenolic concentration was determined, considering the biomass produced on media containing grape seed and grape skin.

Large amount of CA was observed in both vegetal matrices analysed, especially in substrate inoculated with Sc 226 strain. Moreover, for this yeast strain, the detected CA concentration was higher in the medium containing grape seed, rich in tannins (C_{max} 20.504 μg/mg) than in the medium containing grape skin, rich in anthocyans (1.402 μg/mg). The maximum amount (C_{max} 2.774 μg/mg) has been detected in samples derived from the substrates inoculated with Sc 1864 strain. This strain, grown on the medium rich in anthocyans, was able to adsorb the highest concentration of GA (C_{max} 0.3 μg/mg), while yeast strain TT 173, grown on the medium rich in tannins, was able to adsorb the maximum concentration of GA (C_{max} 0.714 μg/mg).

RU, as expected, was detected only on the medium rich in anthocyans, where yeast strain Sc 1483 was able to adsorb the maximum concentration of RU (C_{max} 0.159 μg/mg). Yeast strain TT 77 have shown the lowest GA concentration in the medium rich in tannins (0.015 μg/mg) and the lowest RU concentration in the medium rich in anthocyans (0.029 μg/mg).

These data suggest a different yeast strain aptitude to adsorb phenolics. Therefore, it is possible to differentiate strains with high or low aptitude to adsorb phenolics. Moreover, these data may suggest yeast strains biodiversity referred to their aptitude to adsorb some phenolic derivatives more than the others. For example, yeast strain Sc 226 grown on the medium rich in tannins can adsorb very high concentration of CA (20.504 μg/mg) and low concentration of GA (0.135 μg/mg).

Fig. 3 reported typical chromatograms of extracts derived from grape seeds matrix inoculated with the strain TT 51. In the first chromatogram it is present the peak referred to GA (1); in the second one it is present the peak referred to CA (2). As reported in Table 2, the concentrations were 0.328 μg/mg for GA and 4.389 μg/mg for CA.

Fig. 4 reported three typical chromatograms of extracts derived from grape skin matrix inoculated with the strain TT 51. The peak 1 is referred to GA, the peak 2 is referred to CA and the peak 3 is referred to RU. As reported in Table 3, the

Table 5
Extraction recovery for gallic acid (GA), catechin (CA) and rutin (RU)

Analyte	Nominal concentration (μg/mg)	Mean recovery (%, $n = 5$)	R.S.D. (%)
GA	0.025	85.19	2.17
	0.500	91.35	1.49
	1.000	97.32	3.23
CA	0.500	93.94	2.98
	4.00	99.24	2.00
	16.00	101.11	1.56
RU	0.030	85.56	4.44
	0.100	94.92	1.20
	0.200	89.97	1.53

Measurements in the control medium Sabouraud Agar inoculated with the strain TT51. R.S.D., related standard deviation.

Table 6
Sensitivity and linearity of detection of gallic acid (GA), catechin (CA) and rutin (RU)

Analyte	Sensitivity (μg/mg)	Range studied (μg/mg)	Slope	Intercept	Correlation coefficient (r^2)
GA	0.015	0.015–0.12	0.65290	0.01060	0.97510
GA		0.12–0.96	0.54070	0.01800	0.98480
CA	0.025	0.25–2.5	0.49280	−0.04650	0.97280
CA		0.25–2.5	0.54070	0.06620	0.99180
RU	0.029	0.02–0.05	0.46390	0.10550	0.97430
RU		0.05–0.2	0.66020	0.02480	0.99170
Mean			0.55853	0.02977	0.98175
Standard deviation			0.08143	0.05179	0.00882

Measurements in the control medium Sabouraud Agar inoculated with the strain TT51.

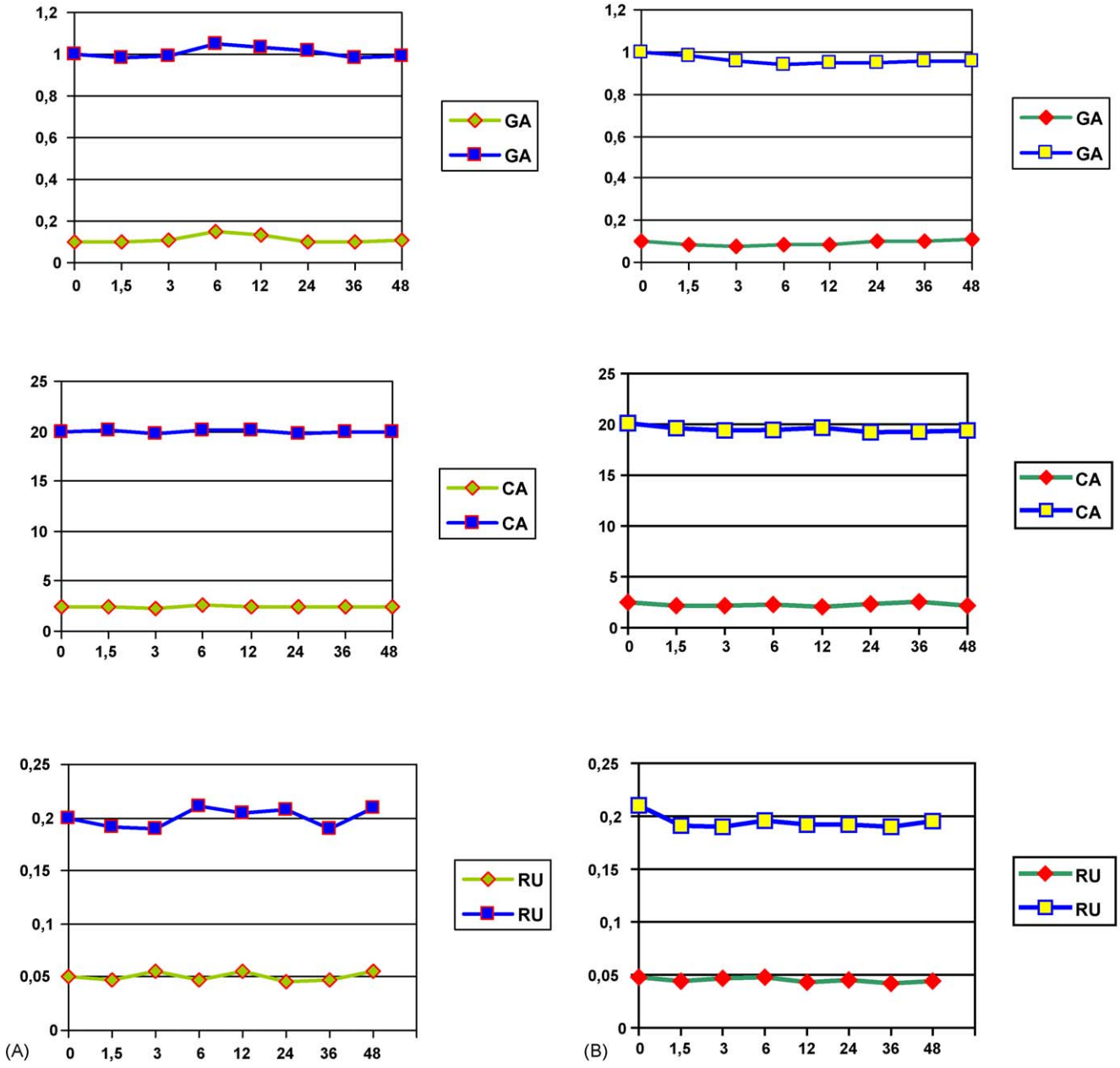

Fig. 5. Stability of gallic acid, catechin and rutin. (A) Stability of gallic acid (0.1 and 1 μg/ml); catechin (2.5 and 20 μg/ml); rutin (0.05 and 0.2 μg/ml) determined in methanol stored at −20 °C over a period of 1 month. *Abscissa*: Time of storing (days). *Ordinate*: Detected concentration (μg/ml). (B) Stability of gallic acid (0.1 and 1 μg/ml); catechin (2.5 and 20 μg/ml); rutin (0.05 and 0.2 μg/ml) determined after extraction from control medium and dissolving in methanol for up to 48 h at room temperature. *Abscissa*: Time of standing (h). *Ordinate*: Detected concentration (μg/ml).

concentrations were 0.179 μg/mg for GA, 1.231 μg/mg for CA and 0.072 μg/mg for RU.

3.4. Validation

All the measurements were performed in the control medium Sabouraud Agar inoculated with the strain TT 51.

Accuracy and precision for the determination of GA, CA and RU have been determined. Low, medium and high concentration were consider for the three analytes and specifically, 0.025–0.5 and 1 μg/mg for GA; 0.5–4 and 16 μg/mg for CA; 0.03, 0.1 and 0.2 μg/mg for RU. Within- and between-day precision and accuracy were performed. The results are shown in Table 4 and are expressed as mean detected concentration and related deviation (R.S.D.%). For the all three studied compounds, the precision at low, medium and high concentrations was really satisfactory.

The intra-day R.S.D. ranged from 1.19, referred to RU at 0.2 μg/mg, to 3.07 referred to GA at 0.025 μg/mg. The inter-day R.S.D. ranged from 2.39, referred to CA at 16 μg/mg, to 4.07 referred to GA at 0.025 μg/mg.

The recovery of all compounds was evaluated to test the efficiency and reproducibility of the extraction procedure. The recovery was calculated by comparing the respective peaks area of the extracted samples related to those equivalent methanolic standards. CA, RU and GA were added to the control medium to achieve the same concentration used to determine the accuracy. As reported in Table 5, the recovery ranged from 85.19 to 97.32% for GA, from 93.94 to 101.11% for CA and from 85.56 to 94.92% for RU.

3.4.1. Linearity and limits of detection

Calibration curves of the tested compounds were linear over the low and high concentration. Two calibration curves of each compound were determined using standard calibration samples of 0.015–0.03–0.06–0.12 and 0.12–0.24–0.48–0.96 μg/mg for GA; 0.25–0.75–1.25–2.5 and 2.5–5–10–20.00 μg/mg for CA; 0.02–0.03–0.04–0.05 and 0.05–0.1–0.15–0.2 μg/mg for RU.

The mean correlation coefficient was 0.98175 ± 0.00882. The results are summarized in Table 6.

The detection limit for GA, CA and RU in vegetal matrices was determined to be 0.015, 0.025 and 0.029 μg/mg of biomass, respectively, with S/N = 3:1 (Table 6). Measurements were performed in control medium prepared using Sabouraud Agar.

3.4.2. Stability

Stability of GA (0.1 and 1 μg/ml), CA (2.5 and 20 μg/ml) and RU (0.05 and 0.2 μg/ml), determined in methanol stored at −20 °C over a period of 1 month, was satisfactory. Moreover, it was found that the three analytes, determined after extraction from control medium and dissolving in methanol for up to 48 h at room temperature, were also stable at the same concentration. The results are summarized in graphics reporting the detected concentration (μg/ml) over the time (Fig. 5).

4. Conclusion

The proposed new method allows the possibility to perform a survey on phenolics adsorbed by yeasts.

The liquid extraction, associated to a high performance liquid chromatography has been used as a screening method to elucidate how yeasts interact with specific phenolics present in grape materials.

The assay requires low costs of testing and gives us the possibility to simply and quickly characterize yeast strains regarding their interaction with phenolics.

Because the choice of yeasts with low ability to adsorb phenolics may give wines richer in phenolic compounds with an enhanced antioxidant activity, the present analytical study could give an interesting contribute to predict the wine composition regarding phenolic monomers.

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