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Title:

Determination of Trans-resveratrol in Wines, Spirits, and Grape Juices Using Solid-Phase Micro Extraction Coupled to Liquid Chromatography with UV Diode-Array Detection

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Abstract

Solid phase microextraction (polyacrylate fiber), coupled to liquid chromatography with UV–diode array detection, has been optimized for the determination of trans-resveratrol in in wines, spirits, and grape juices. The main aspects influencing fiber adsorption (fiber coating, extraction time, ethanol content, salt addition) and desorption (desorption and injection time, desorption solvent mixture composition, carryover) of the analyte have been investigated. The method permitted a fast and simple determination of free trans-resveratrol in commercial wines and spirits. It was found in all the analysed samples at concentration levels ranging from 0.007 to 4.486 μg mL^{-1}. Total trans-resveratrol concentrations were also evaluated after enzymatic deconjugation of piceid.

Keywords: Trans-resveratrol . SPME . LC/UV-DAD .Wines . Spirits . Grape juices

Introduction

Antioxidants are important in preventing and repairing damages caused by oxidative stress, playing a major role in the fight against chronic and degenerative human illnesses (Khurana et al. 2013). Research on the effects of polyphenols on human health has developed considerably in the past decade (Hanhineva et al. 2010; Khurana et al. 2013). It strongly supports a role for polyphenols in the prevention of degenerative diseases, particularly cardiovascular diseases and cancers, due to their antioxidant properties.

Resveratrol is a polyphenol belonging to the stilbenes subclass (Orallo 2008; Fernández-Mar et al. 2012), produced by more than 70 plant species, possessing a well documented bioactivity (Fernández-Mar et al. 2012; Shindikar et al. 2016). In fact, not only resveratrol is a good antioxidant, but also exhibits anti-inflammatory, anti-ageing, anti-tumour and anti-mutagenic properties. Recently, preclinical studies have also shown its involvement in regulation of several epigenetic mechanisms affecting gene expression. In fact, resveratrol is an inhibitor of different histone deacetylase enzymes (HDACs), an inducer of the metastasis-associated protein 1 (MTA1), and of specific microRNAs (miRNAs) in human cancer (Venturelli et al. 2013; Dhar et al. 2015; Kumar et al. 2015). Further, studies on its structure, function and mechanism of action have shown that it exists in two isomers, trans- and cis-resveratrol, and their glucosides (trans- and cis-piceid) in Vitis vinifera L. (*Vitaceae*) (Vitrac et al. 2005; Fernández-Mar et al. 2012). However, among the four forms, the trans isomer is the one with greater therapeutic potential and recently it has been proposed as an anti-ageing and anticancer drug (Orallo 2006; Kumar et al. 2015; Varoni et al. 2016)

The main commercial source of resveratrol is the Fallopia japonica (*Polygonum cuspidatum*), a large perennial herb native to East Asia. The main resveratrol dietary sources are peanuts, pistachios, dark chocolate and grapes, in particular red grape skin (Stervbo et al. 2007; Fernández-Mar et al. 2012). Therefore, considerable amounts are expected in red wines.

Generally, it is difficult to predict resveratrol wine concentration since many factors affect its biosynthesis in grapes: grape varieties, geographic region, climatic factors, plant stress conditions, oenological practices, alcohol content and ageing of beverage (Gambelli and Santaroni 2004; Downey et al. 2006; Stervbo et al. 2007). Accordingly, concentrations ranging from undetectable to 14.3 mg/L have been described in wines (Minussi et al. 2003; Gambelli and Santaroni 2004; Gobbi et al. 2004; Vitrac et al. 2005; Stervbo et al. 2007). Instead, its levels have never been assessed in hard liquor or spirits probably because the excessive alcohol consumption is always advised to avoid, being responsible of serious physical and social damage (De Benedetto et al. 2009). Nonetheless, it is widely proven that a regular and moderate intake of alcohol drinks has beneficial effects on human health (German 2000).

Following the high interest generated by resveratrol in a lot of scientific areas, such as medicine, biology, chemistry, agriculture and food science, many analytical methods, principally based on chromatographic technique, have been developed for its quantification in biological or food matrices (Villamor et al. 2013; Andrei et al. 2014). Of course, since wine is a complex alcoholic beverage containing volatile and nonvolatile compounds, sample pre-treatment procedures were always necessary to simplify the matrix and make the sample suitable for instrumental analysis. Conventional techniques such as liquid-liquid extraction (LLE) or solid-phase extraction (SPE) have been often used for the purpose (Villamor et al. 2013). However, these techniques require multiple steps, a lot of work and the use of large amounts of solvents.

Solid-phase micro extraction (SPME) represents an effective alternative to conventional techniques, because it integrates sampling, extraction, concentration, and sample introduction into a single solvent-free step, increasing the method performances and reducing the cost and time of each analysis (Aresta et al. 2016). SPME coupled to liquid chromatography with fluorescence detection (Vînas et al. 2008) was employed for the quantification of resveratrol in wine, even if the carbowax-templated resin fiber (CW-TPR) used in the work is no longer commercially available. SPME coupled to gas chromatography-mass spectrometry has been also used, even if an on-fiber derivatization procedure was always necessary before the instrumental analysis (Luan et al. 2000; Caia et al. 2009; Vînas et al. 2009).

In the present paper, SPME of trans-resveratrol was optimized and interfaced with liquid chromatography with UV–diode array detection, testing different fibers, polydimethylsiloxane (PDMS), polydimethylsiloxane/ divinylbenzene (PDMS/DVB) and polyacrylate (PA). Short extraction times were necessary to reach the equilibrium and very short desorption times were employed. The developed procedure was then successfully applied to the extraction of trans-resveratrol from red wine and spirit samples. The whole method permitted a fast and simple determination of free and total (after enzymatic deconjugation) trans-resveratrol in the selected samples.

Material and methods

Materials

trans-Resveratrol was supplied by Sigma-Aldrich (Milano, Italy). Stock solutions were prepared in ethanol and stored in the dark at -20°C. All organic solvents and water used in this study were HPLC grade and were purchased from Sigma-Aldrich. The mobile phase was filtered through a 0.20 μm nylon membrane (Lab Service Analytica, Bologna, Italy).

SPME fibers for LC, i.e. polyacrylate (PA) 85 μm, polydimethylsiloxane–divinylbenzene (PDMS–DVB) 65 μm and polydimethylsiloxane (PDMS) 7 or 100 μm, respectively, were supplied by Supelco (Sigma-Aldrich).

Apparatus

The LC system (ThermoQuest, San Jose, CA) consisted of a Spectra System Pump, model P2000, an SPME interface (Supelco), with a standard six-port Rheodyne valve with a special fiber desorption chamber (total volume: 60μL) installed in place of the sample loop. The detector was a Spectra System model UV6000LP photodiode array (ThermoFinnigan,San Jose, CA) controlled by ChromQuest software running on a personal computer. The column used was a Phenomenex (Torrance, CA, USA) Kinetex C18, 4.6 mm x 100 mm (2.6 μm).

Chromatographic and detection conditions

The mobile phase was an acetonitrile/methanol/water (10:30:60, v/v/v). mixture with 0.05 % formic acid. The flow rate was 0.7 mL min^{-1} and detection took place at room temperature. The detection wavelength was 310 nm (5 nm band-width). Spectra were acquired in the 220–400 nm range at the apex and on the ascending or descending part of each peak. Peak purity was checked by the technique of spectra overlaying, after normalization.

Solid-Phase Micro Extraction

A manual SPME device (Supelco) was used to hold the fiber. Extractions were performed for 30 min at 20°C, in 1.5 mL amber vials (Supelco) with PTFE hole caps (Supelco), by direct immersion of the fiber in the sample extracts under magnetic stirring, in the presence of 6.6% of ethanol and 10% of sodium chloride. Analyte desorption was performed in static desorption mode by soaking the fiber in the mobile phase into the desorption chamber of the interface for 15 min. Then the valve was changed to the inject position for 10 s. After each run, the fiber was soaked for 5 min in ethanol and subsequently rinsed with water, in order to ensure the removal of residual adsorbed compounds. All the experiments were performed in triplicate.

Samples collection and pre-treatment

Two red wines (samples 1 and 2), one rosé wine (3), one white wine (4) and two spirits (one artisan Grappa, 5 and one aged Grappa, 6) were purchased from a local supermarket. All samples but

sample 6 were produced in Italy between 2012 and 2014. The ABV of selected wines was in the range 9-13%, the ABV of spirits was 33 and 40% for sample 5 and 6, respectively.

0.5 mL of each sample were dried (60 min) in a vacuum concentrator. Then, variable amounts (4.5 mL for red wine, 3 mL for rosé and white wines, 1.5 mL for spirits) of a 0.1 M acetic acid solution with 10% NaCl and 6.6 % ethanol were added, and 1.5 mL of the resulting mixtures subjected to SPME.

Solutions for glycosides hydrolysis were prepared by dissolving 10 mg of β-glucuronidase from Helix pomatia (300.000 units g^{-1} solid, Sigma-Aldrich) in 5 mL acetate buffer (0.1 M, pH 5.0) and then stored in 0.5 mL aliquots at -20 ° C. For the enzymatic deconjugation samples dry residues were mixed with 0.5 mL of the β-glucuronidase solution, incubated for 17 h at 37 °C, diluted with 1.0 mL of 0.1 M acetic acid with 15% NaCl and 9.9 % (v/v) ethanol, and subjected to SPME. All the experiments were performed in triplicate. Quantitation was performed with the standard addition method.

Results and discussion

Preliminary experiments were performed in order to compare the extraction efficiency obtained using polar 85μm PA, semi-polar 65 μm PDMS-DVB and non-polar 7 μm PDMS coated fibers, respectively. The relevant extraction time profiles obtained at 20°C by plotting the area counts versus the extraction time are reported in Figure 1. As apparent, equilibrium was reached after 60 minutes and the PA fiber was capable of the most efficient extraction and was then selected for further experiments. Furthermore, since it is possible to obtain good extraction yields and reliable analysis also in non-equilibrium conditions, an extraction time of 30 minutes was chosen for further experiments. The extraction profile was also established at higher temperatures; however, a response decrease was observed in this case (data not shown).

Generally speaking, salt addition improves the recovery, especially in the case of polar (hydrophilic) compounds that are difficult to extract. Thus, experiments were performed by increasing progressively the ionic strength of the extraction solutions. A signal enhancement was obtained by the addition of 10% sodium chloride, that was chosen as working concentration, since higher salt levels did not produce an additional signal increase.

The effects of ethanol content on trans-resveratrol extraction efficiency were also studied. Figure 2 compares the results obtained without ethanol and by adding increasing amounts of ethanol (6.6 or 13.2%) to the extraction solution in the presence of 10% sodium chloride. As can be seen, a significant response increase was obtained in the presence 6.6% of ethanol, amount that was used for further experiments.

The dynamic mode was first employed to desorb the analyte from the fiber in the SPME-LC interface; this approach produced quantitative recoveries but very broad chromatographic peaks. Thus, the static desorption technique was used for further experiments. The fiber was soaked in mobile phase for a variable period of time before injection into the LC column. The best conditions were found using 15 minutes of static desorption followed by 10 seconds of fiber exposition to the mobile phase stream. Under these conditions, the analyte peak (10% of its height) showed a small width (0.2) and good symmetry (1.09, B/A), even if a carryover of 13.23± 4.34 % was estimated. Thus, to ensure a complete cleaning of the fiber after each run, the fiber was subjected to the cleaning procedure described in the experimental section.

The response of the developed SPME-LC procedure was linear in the range 0.1 and 500 ng mL^{-1}, with correlation coefficients better than 0.999 and an intercept not significantly different from zero at 95% confidence level. The estimated LOD and LOQ obtained on standard solutions were 0.4 and

188 1.3 ng mL^{-1}, respectively, calculated according to IUPAC as three and ten-fold the standard
189 deviation of the intercept of the calibration curve.
190 The within-day precision of the method was investigated on standard solutions at a concentration
191 level of 2, 20 and 200 ng mL^{-1} by performing daily three replicates. The same solutions were
192 analyzed three times each day for a period of seven days for the day-to-day precision evaluation.
193 The within day RSD% were 12.6, 6.7 and 6.5 % at 2, 20 and 200 ng mL^{-1}, respectively. The day-to-
194 day RSD% were 18.5, 7.5 and 7.0, respectively, at 2, 20 and 200 ng mL^{-1}.
195
196 The developed procedure was eventually applied to the analysis of several commercial alcoholic
197 drinks. Since the ethanol content has a deep influence on the extraction process, its removal during
198 the drying step was very important. Furthermore, literature data show that wine concentration of
199 resveratrol is variable, therefore samples dry residues were suspended in different volumes of the
200 0.1 M acetic acid solution with 10% NaCl and 6.6 % ethanol, as described in the experimental
201 section.
202 Figure 3, A and B, reports, for instance, two typical SPME–LC chromatograms relevant to the
203 analyses of A) red wine (1) and B) Grappa (6) samples, respectively. As apparent, the
204 chromatographic profiles clearly show the absence of interfering peaks at the retention time for the
205 analyte (13.14 ± 0.16), and the same results were obtained in the case of all the analyzed samples.
206 Both free and total trans-resveratrol concentration in the selected samples were determined.
207 Therefore, enzymatic hydrolysis of the samples was conducted to remove aglycone portions from
208 piceid. Table 1 lists the estimated concentration levels of trans-resveratrol in the selected samples,
209 before and after enzymatic deconjugation, together with the obtained percentage recoveries. As
210 apparent, variable concentrations of the analyte were found in all the considered samples. The
211 measured levels of free trans-resveratrol in wine samples were between 0.019 (5) and 3.486 (1) μg
212 mL^{-1}, which were in good agreement with literature data (Gambelli and Santaroni, 2004, Minussi et
213 al. 2003). The increase of trans-resveratrol levels after enzymatic hydrolysis of the samples ranged
214 from 0 (Grappa samples) to 18% (red wine 1). This experimental evidence was quite surprising,
215 since the concentration of the piceid in wines was supposed to be higher than that of the aglycone as
216 already reported (Romero-Pérez et al. 1999). However, it is also known that winemaking techniques
217 such as grapes maceration with skins or the use of clarifying agents and filters can widely modify
218 trans-resveratrol and piceid levels in wine. As also reported in Table 2, the recovery of the analyte
219 from spiked samples varied from 92.5 to 99.6% with an average recovery ± SD of 96.5± 2.5,
220 demonstrating the accuracy of the method and the efficiency of the SPME procedure.
221

Conclusions

A sensitive, reproducible, and low-cost analytical method for the determination of free and total trans-resveratrol in selected alcoholic beverages was successfully developed, using solid phase microextraction (85 μm PA fiber) coupled to liquid chromatography with UV–diode array detection, i.e. standard equipment easily available in most laboratories. The method could be easily transferable in the production facility and useful for many purposes.

Acknowledgements

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Conflict of Interest

Antonella Aresta declare that she has no conflict of interest. Pietro Cotugno declare that he has no conflict of interest. Federica Massari declare that she has no conflict of interest. Carlo Zambonin declare that he has no conflict of interest. This article does not contain any studies with human or animal subjects.

Ethical Approval

In this study, humans are not involved.

Informed Consent

Not applicable

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Table 1. Estimates of trans-resveratrol in wines and spirits, before and after β-glucuronidase hydrolysis, and recoveries.

Sample	[trans-Resveratrol] μg mL^{-1}		Recovery (%)
	before	after	
1	3.486±0.299	4.113±0.357	99.6
2	2.422±0.099	2.712±0.120	96.8
3	0.294±0.019	0.308±0.018	98.2
4	0.097±0.006	0.099±0.006	95.0
5	0.019±0.003	0.020±0.003	92.5
6	0.037± 0.004	0.038±0.004	97.0

Table 2. Linear range, equation and correlation coefficients calculated of each sample

Sample	Linearity range	Equation	R^2	Index (mM/mL)
1	2.5-5 μL	y=18.725x-4.462	0.9112	102
2	2.5-10 μL	y=7.650x+13.38	0.9415	42
3	7.5-30 μL	y=2.3825x+9,56	0.9933	13
4	20-80 μL	y=0.8451x+18.495	0.9777	5
5	150-500 μL	y=0.0475x+5.7611	0.7579	0.3
6	35-140 μL	y=0.3093x-4.435	1	2
standard	0.02-0.2 mM	y=183.11x+18.134	0.9986	-

342

343 **Figure captions**

344 **Figure 1.** Extraction time profiles obtained at room temperature with an 85 μm PA (□), a 65 μm
345 PDMS-DVB (■) and a 7 μm PDMS fiber (▲).

346 **Figure 2**. Effects of ethanol content on trans-resveratrol extraction efficiency in the presence of
347 10% sodium chloride using the 85 μm PA fiber.

348 **Figure 3**. SPME–LC-UV chromatograms relevant to the analysis of A) a red wine sample (1) and
349 B) a grappa (6) sample. t-R = trans-resveratrol.

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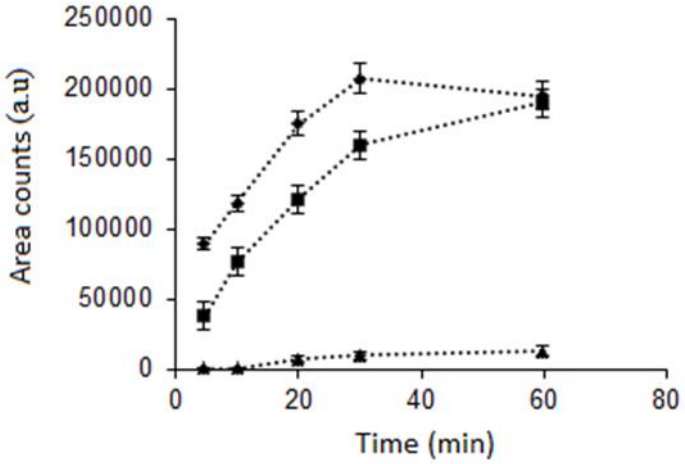
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Area counts (a.u)
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Figure 1

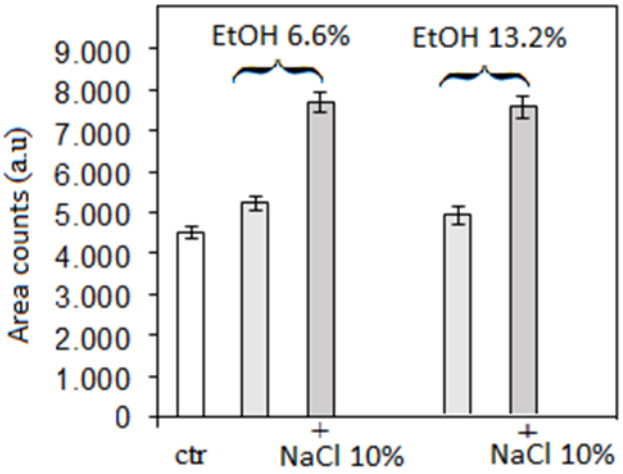
EtOH 6.6%
EtOH 13.2%
Area counts (a.u)
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Figure 2

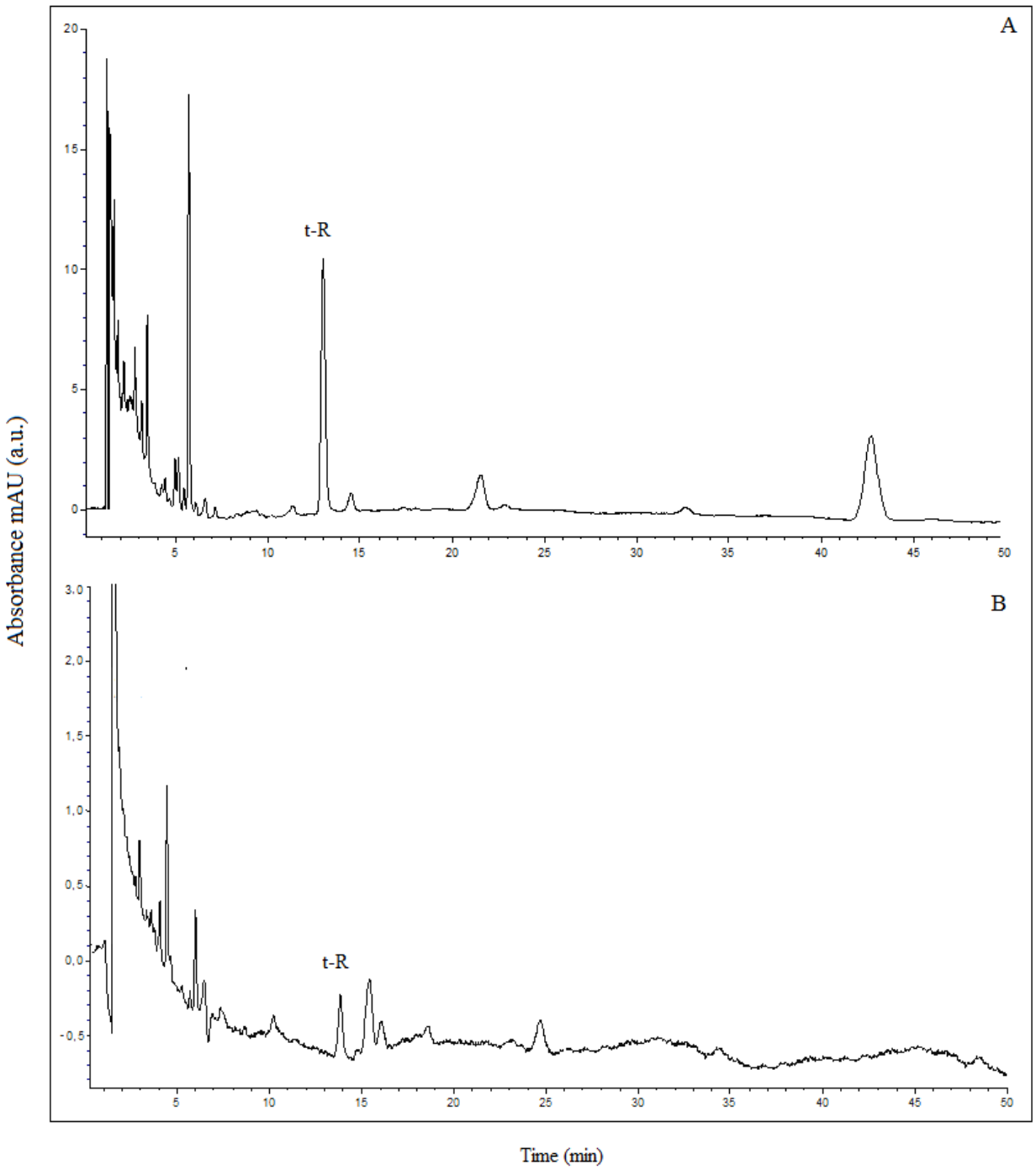

Figure 3