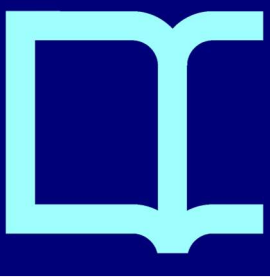

Determination of resveratrol and piceid in beer matrices by solid-phase extraction and liquid chromatography-tandem mass spectrometry

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ABSTRACT

Beer is one of the most commonly consumed undistilled alcoholic beverages in many countries. In recent studies, the stilbenes resveratrol and piceid have been found in some hop varieties which are used in the production of beer. Therefore, they could be transferred to beer. The aim of the present work was to validate a method to study the potential content of *trans*- and *cis*-resveratrol and piceid in 110 commercial beers from around the world.

The resveratrol and piceid content of 110 beers was analyzed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) after a solid-phase extraction (SPE) using optimized and validated procedures for the beer matrix. The beer matrix effect was also studied.

Stilbenes were found in quantifiable amounts in 92 beers, while concentrations below the limit of quantification (LOQ) were found in 18 beers. Resveratrol was found in the range of 1.34 - 77.0 μg/L in 79% of the beers analyzed, and piceid was found in the range of 1.80 - 27.3 μg/L in only 33% of them. The mean of total resveratrol in all the beers was 14.7 ± 20.5 μg/L. The content of resveratrol has been compared with other resveratrol containing foods. A serving of beer contains similar amounts of stilbenes as berries, less than chocolate and grape products but more than pistachios, peanuts or tomatoes. Overall, beer is one of the products with the lowest levels of total resveratrol (μg/L), and despite its high consumption should not be considered as a representative source of resveratrol.

KEYWORDS: Beer; resveratrol; piceid; wine; matrix effect; LC-MS/MS.

1 INTRODUCTION

Resveratrol (3,5,4'-trihydroxystilbene) is a phenolic *phytoalexin* with potentially preventive activity in several human diseases [1-6]. The described health effects depend on the ingested amount and bioavailability of these compounds. The presence of *trans*-resveratrol, *trans*-piceid (the resveratrol glucoside) and their respective *cis*- isomers in the human diet is limited. The major sources of resveratrol include grapes and grape products such as wines and grape juice [7]. Although it has been found in other foods such as peanuts, pistachios and some berries, their total resveratrol levels are from 10 to 100-times less than in grape products [7]. Recently, it has also been found at low levels in the skin of some kinds of tomatoes [8] and in chocolate products [9].

Beer is one of the most commonly consumed undistilled alcoholic beverages in many countries. It is a complex mixture of bioactive substances including carbohydrates, amino acids, minerals, vitamins and phenolic compounds [10]. The majority of phenolic compounds in beer are non-tannic and non-flavonoid compounds (98% of total phenolic compounds), such as phenolic acids [11,12]. Other minor phenols found in beer are flavonols, catechins, procyanidins, tannins and chalcones [13,14]. The content of polyphenols in beer is largely influenced by the genetic factors of its raw materials and therefore by the environmental conditions in which they grow, and also by technological brewing factors [13,15]. Hops are used in the brewing industry to add flavor and bitterness to beer [16]. Although it has been noticed that the nature of the harvest year can have a strong influence, [17-19] *trans*- and *cis*-piceid have been found in different hop cultivars and in hop pellets in concentrations ranging from 0.5 to 11.7 mg/kg, up to 2 mg/kg of *trans*-resveratrol [20], and *cis*-resveratrol has been found up to 0.3 mg/kg in hop pellets [21] and up to 1.2 mg/kg in hop cones [22]. Moreover, about 20-30% of beer polyphenols originate from hops, and 70-80% from malt [10,20],

although hops are added in 100-times lesser amounts than malt [23,24]. Therefore, a low content of stilbenes from the hops could be expected to be found in the final beer product [18]. Recently, low amounts of *trans*-resveratrol and *trans*-piceid (5 and 15 μg/L, respectively) have been found in four and five regular beers, respectively [25]. Other authors, after analyzing only two beers by High Performance Liquid Chromatography (HPLC-UV), with detection at 280 nm, found up to 200-times higher concentrations of resveratrol (ranging between 0.3 mg/L and 3 mg/L) [26], however neither the *trans*- or *cis*- forms nor the piceid content were specified. Therefore, very sensitive, selective and validated analytical methods are necessary to strengthen scientific evidence of the presence of *trans*- and *cis*- resveratrol and piceid in beers. Added to this, when performing MS analysis of food components, a large matrix effect can be observed, which leads to a diminution in the signal intensity of the analytes and the sensitivity of the method. The matrix effect during validation of analytical methods may be best examined by comparing the response of an analyte at any given concentration spiked into the target matrix, to the response of the same analyte present in the "neat" mobile phase [27-29].

The aim of this study was to validate an analytical method for beer matrix and to study the content of *trans*- and *cis*- resveratrol and piceid in 110 beers from around the world, including alcohol-free, lager, ale, weissbier, stout, and abbey beers, and compare them with other dietary sources of resveratrol, like red wine or grape products and other foods with stilbenes.

2 MATERIALS AND METHODS

2.1 Standards and Reagents. All samples and standards were handled avoiding exposure to light. Standards of *trans*-resveratrol (99% purity), *trans*-3,4',5-trihydroxystilbene-3-ß-D-glucopyranoside (*trans*-piceid) (97% purity) and ethyl gallate were purchased from Sigma-Aldrich-Fluka (St. Louis, MO), *cis*-resveratrol (97% purity) from Toronto Research Chemicals Inc (Toronto, ON, Canada), and taxifolin (>90% purity) from Extrasynthèse (Genay, France). Methanol, acetone, glacial acetic acid, ethyl acetate and acetonitrile of HPLC grade were purchased from Scharlab (Barcelona, Spain). Ultrapure water (Milli-Q) was obtained from Millipore system.

2.2 Samples. A total of 110 international commercial beers were analyzed (Table 1): 52 lagers, 20 ales, 15 abbey beers, 11 weissbiers, 7 stouts and 5 alcohol-free beers. The alcohol content ranged between less than 0.05 and 14% (v/v). All beers were purchased from local commercial markets. Some of the beers selected in this study are the most widely consumed in Spain (19%), while the others are a variety of beers consumed in Europe (66%) and worldwide (15%).

2.3 Sample Preparation. Prior to the analysis, all beers were sonicated for 4 minutes for degasification. All experiments were performed on ice, avoiding light exposure, and all reagents were maintained in an N_2 atmosphere to avoid the oxidation of phenolic compounds. All beers were analyzed immediately after being opened.

2.4 Quality parameters of the method. To obtain the maximum detectivity and sensitivity in the analysis of resveratrol in beer by Liquid Chromatography-Electrospray Ionization- Tandem Mass Spectrometry (LC-ESI-MS/MS), sample extraction was optimized, and the quality of the method and the matrix effect were evaluated. Optimization of sample extraction was carried out through the analysis of different parameters, including beer volume and pre-cleaning of samples as recommended by

Jerkovic *et al.* [25]. Different volumes of samples (5 and 1 mL) were considered for loading onto HLB® cartridges (30 mg; 30 µm particle size and 80 Å pore size) (Waters). The pre-cleaning of beers before the solid-phase extraction (SPE) consisted of cleaning beers with toluene (1:1, v/v) followed by a double extraction with cyclohexane (1:1, v/v) as described previously by Jerkovic et al. [25].

After the optimization of the sample extraction, the method was evaluated for selectivity, detectivity, sensitivity, linearity, recovery, accuracy and precision, according to the Food and Drug Administration (FDA) acceptance criteria [30]. Selectivity is the ability of an analytical method to differentiate and quantify the analyte in the presence of other components in the sample. This was assessed by analyzing blank beer samples. A blank beer sample was obtained after applying the SPE analysis procedure to 1 mL of blank beer (beer number 57, Table 1). The selected beer for the evaluation of the method was a lager beer because it is the most widely consumed kind of beer worldwide and more common both on the market and in this study. The detectivity of the method was evaluated by determining the limit of detection (LOD) and the limit of quantification (LOQ). The LOD was determined as the concentration of analytes with a signal-to-noise ratio of at least 3 and the LOQ was the lowest standard with a signal-to-noise ratio of at least 10. Sensitivity was expressed as the slope of the analytical curve. Linearity was evaluated by spiking blank beer matrix with known concentrations of analytes at 6 concentration levels (4-100 µg/L for *trans*-resveratrol and 2-50 µg/L for *cis*-resveratrol and *trans*-piceid). Recovery was calculated as the ratio of the mean peak area of the analytes spiked before extraction to the mean peak area of the analytes spiked post-extraction multiplied by 100 in a six-point calibration curve of beer matrix (n=3).

The precision and accuracy of the method were evaluated using three different concentrations [low (near the LOQ), medium and high] within the linear range of the calibration curve of the analytes in the beer matrix (n=8). Accuracy and precision were calculated as the percentage and relative standard deviation, respectively, of the ratio of the mean calculated concentration and the true value of the known added concentration in blank beer samples (n=8) for each concentration.

The matrix effect of the beers was also evaluated by calculating the matrix factor (MF or suppression coefficient), process efficiency (PE) [28] and the variations in the sensitivity of the method [29]. The MF was expressed as the ratio of the mean peak area of the analytes spiked after the SPE procedure in blank beer matrix to the mean peak area of the same analyte standards in aqueous matrix without SPE procedure multiplied by 100. PE was calculated as the ratio of the mean peak area of the analytes spiked before the SPE procedure to the mean peak area of the same analytes standards in aqueous matrix without SPE procedure multiplied by 100. Differences in the sensitivity of the method within the two matrices (beer and water) were expressed as the ratio of the slope of the analytical curve in beer matrix (spiked after the SPE procedure) to the slope of the analytical curve in aqueous matrix without SPE procedure multiplied by 100.

Short and long term stability of standards was previously evaluated by our group [31].

2.5 Determination of Resveratrol and Piceid in Beer by LC-MS/MS. Resveratrol and piceid analyses were carried out by LC-ESI-MS/MS after an SPE, based on the Urpi-Sarda *et al.* [31,32] method and optimized for beer samples.

Extraction procedure. Samples (1 mL of each presonicated beer, diluted with ultrapure water to reduce the alcohol percentage to below 5%) with the internal standard (ethyl gallate) all maintained on ice, were loaded onto a Waters Oasis® HLB 96-well plate that

had been preconditioned with 1 mL of methanol and equilibrated with 1 mL of 2 mol/L acetic acid in water. Samples were washed with 1 mL of 2 mol/L acetic acid in water and 1 mL of 2 mol/L acetic acid in water/methanol (85/15 v/v). Elution was achieved with 0.5 mL of 1 mol/L acetic acid in methanol and 2 x 0.75 mL of 1 mol/L acetic acid in ethyl acetate. The eluate was evaporated to dryness under a gentle stream of N_2. The residue was reconstituted with 100 µL of initial mobile phase, with 1.64 µmol/L of taxifolin as an additional external standard. Ethyl gallate was used as the internal standard (mean recovery: 99%, CV=10%) and taxifolin was used as an additional external standard to assess the performance of the mass spectrometer. Both compounds are absent in beers.

LC-MS/MS analyses. LC analyses were performed using an Agilent 1100 system equipped with a quaternary pump and a refrigerated plate autosampler (Waldbronn, Germany). An Applied Biosystems API 3000 triple quadrupole mass spectrometer, equipped with a turbo ion spray source ionizing in the negative mode, was used to obtain the mass spectrometry data.

A Phenomenex Luna C_{18} column, 50 x 2.0 mm i.d., 5 µm (Torrance, CA) maintained at 40 °C was used for chromatographic separation. The injection volume was 15 µL, and the flow rate was 500 µL/min. Gradient elution was carried out with 0.5 mL/L acetic acid as mobile phase A and 700 mL/L acetone, 300 mL/L acetonitrile with 0.4 mL/L acetic acid as mobile phase B. A non-linear gradient profile was applied as follows: 0-0.5 min, 10-15%B; 0.5-5 min, 15-100%B and 5-5.6 min, 100%B. The column was re-equilibrated for 6 min, to return to 10%B.

The MS and MS/MS parameters were as previously described [31]. Briefly, the following parameters were used: capillary voltage –3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), collision gas (N_2) 6 (arbitrary

units), focusing potential -200 V, entrance potential -10 V, declustering potential -50 V, drying gas (N_2) heated to 400 ºC and introduced at a flow rate of 6000 cm^3/min. The collision energy was -25 V for resveratrol, piceid and taxifolin, and -30 V for ethyl gallate.

Quantification of analytes. For quantification of *trans*- and *cis*-resveratrol and *trans*- and *cis*-piceid in beer samples, the multiple reaction monitoring (MRM) mode was used with a dwell time of 300 ms, monitoring four transitions for each analysis: *trans*- and *cis*-resveratrol (227/185), *trans*- and *cis*-piceid (389/227), ethyl gallate (197/169) and taxifolin (303/285). t*rans*-Resveratrol was quantified using a six-point calibration curve determined by weighted ($1/x^2$) linear regression between 4 and 100 µg/L in the beer matrix and *cis*-resveratrol and *trans*-piceid between 2 and 50 µg/L in the beer matrix (n=3 for each calibration curve). Ethyl gallate was used for quantification purposes. *cis*-Piceid was expressed as *trans*-piceid equivalents as no commercial standard was available. To identify *cis*-piceid, *trans*-piceid isomerated by light exposure for 10 minutes on ice was used [33].

3 RESULTS AND DISCUSSION

3.1 Quality parameters of the method. Optimization of the sample extraction procedure and evaluation of the methodology were performed.

3.1.1 Sample Extraction Optimization. The main objective of the sample extraction optimization was to reach the highest detectivity and sensitivity while minimizing the matrix effect in LC-MS/MS. In the first step, we compared the recovery of resveratrol and piceid from the spiked beer matrix (1 µg/mL of final concentration) using different sample volumes (5 and 1 mL) with or without the pre-cleaning procedure (n=4). The recovery values of *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid from the pre-

cleaned samples increased by 86%, 157% and 112% respectively, compared to the non-pre-cleaned ones when 5 mL were used, and this is in accordance with Jerkovic et al. [25]. However, when we compared 1 mL of a sample with and without the pre-cleaning procedure, no differences were observed for the recovery of the compounds. These results suggested a higher matrix effect when a higher volume was considered, affecting the analyte ionization and obtaining best signal-to-noise ratio for the analytes extracted with lesser volumes. When we compared different volumes of pre-cleaned samples (1 and 5 mL), the recovery values for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid after loading 1 mL were 2, 2.5 and 1.3-fold higher, respectively, than when 5 mL were considered. Again, the volume of the sample influenced the matrix effect and the resveratrol ionization. Therefore, as no differences were observed loading 1 mL of sample with and without pre-cleaning, and the recovery was higher than after loading 5 mL of pre-cleaned sample, 1 mL of beer without the pre-cleaning procedure was the selected volume used for the total resveratrol determination in the commercial beers and for the standard calibration curves.

3.1.2 Evaluation of the method. The method met the criteria of selectivity because no endogenous peaks were observed at the same retention time as the analytes in the blank beer samples. The LOD and LOQ of analytes are shown in table 2, and sensitivity was $3.62 \cdot 10^{-5}$, $2.60 \cdot 10^{-4}$ and $9.47 \cdot 10^{-5}$ cpm·L/μg for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively. The 6-point calibration concentrations (n=3) in blank beer matrix determined by weighted ($1/x^2$) least-square regression analysis showed correlation coefficients for all analytes > 0.99. The calibration curves were linear over the concentration range studied. Recovery was evaluated comparing a 6-point calibration curve of analytes with and without the SPE procedure in the beer matrix (n=3). *cis*-Resveratrol, trans-resveratrol and *trans*-piceid showed recovery values of

99%, 90% and 102%, respectively, after SPE in the beer matrix. The recovery for the internal standard ethyl gallate was also evaluated at the concentration used in the analysis (50 µg/L) (n=8). Ethyl gallate showed a recovery of 99%. The precision and accuracy of the analytes in the beer matrix after SPE met the acceptance criteria of the FDA [30] and are shown in table 2.

3.1.2 Evaluation of the Matrix effect. To assess the strength of the matrix effect, 6-point calibration curves of analytes (n=3) in the beer matrix and in an aqueous matrix (pure solvent) without the SPE procedure were compared. Briefly, blank beer was prepared with the full extraction procedure and standards were added *a posteriori* to this matrix to compare the differences of peak signal intensity of the analytes due to the matrix effect to calculate the MF, and *a priori* to calculate the PE. The MF for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid were 5.2%, 5.8% and 1.1% respectively. These matrix factors highlight a great suppression of the ionization of the analytes due to the matrix effect [28,29]. The PE of the method considers the MF and the recovery. The PE was 12.6%, 28.2% and 3.25% for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively. Taking into account that the recovery is >90% for all the analytes, the low PE value is attributable to the high MF. As shown in figure 1, standard curves in the beer matrix showed a decrease of the sensitivity of 95%, 94% and 99% for *trans*-resveratrol, *cis*-resveratrol and *trans*-piceid, respectively, when compared to the aqueous matrix. This great loss in the peak intensity signal highlights that calibration curves in an adequate matrix, in this case the beer matrix, are needed to avoid an underestimation of the analyte concentration in beer samples. This enormous matrix effect may also explain the differences in the enhancement of the peak intensity when loading 1 or 5 mL of pre-cleaned beer samples compared to non-pre-cleaned samples.

To our knowledge, this is the first time that matrix effect of beer in the resveratrol analysis by LC-MS/MS has been highlighted and calculated.

FIGURE 1 HERE

The matrix effect (MF) for the internal standard ethyl gallate was also evaluated at the concentration used in the analysis (50 µg/L) (n=8). Ethyl gallate in the beer matrix spiked after the SPE procedure showed a decrease of the peak intensity of 85% compared with the aqueous matrix without SPE procedure.

3.2 Determination of Resveratrol and Piceid in Beers. The 110 commercial beers were quantified using MRM transitions of 227/185 for *trans*- and *cis*- resveratrol and 389/227 for *trans*- and *cis*- piceid in LC-MS/MS. In this study, *trans*- and *cis*-piceid showed a retention time of 3.15 and 3.62 minutes, respectively, and *trans*- and *cis*-resveratrol showed a retention time of 3.85 and 4.18 minutes, respectively (Figure 2A). The confirmation of resveratrol and piceid in beer samples was based on their retention time and ion fragmentation in the MS/MS mode as compared with those of commercially available standards. Finally, to verify the identity of the peaks, beer samples (Figure 2B) and spiked beer samples (Figure 2C) were injected and compared, confirming the presence of *trans*- and *cis*- resveratrol and *trans*- and *cis*- piceid in beers.

FIGURE 2 HERE

Of the 110 analyzed beers, 79% of them contained free resveratrol (mainly in the *trans*-form), while only 33% of the beers analyzed contained piceid in quantifiable amounts. Table 1 shows that 59 beers contained *trans*-resveratrol between 3.68 and 66.74 µg/L, 69 beers contained *cis*-resveratrol in a range between 1.34 and 22.65 µg/L, 6 beers contained *trans*-piceid between 1.8 and 9.31 µg/L, and 38 beers contained *cis*-piceid between 1.80 and 24.24 µg/L. The beer with the greatest amount of stilbenes (beer

number 104) contained 66.74 µg/L, 10.31 µg/L and 4.17 µg/L of *trans*- and *cis*-resveratrol and *cis*-piceid, respectively.

TABLE 1 HERE

TABLE 2 HERE

Jerkovic *et al.* [17,18,21,22] found *trans*-resveratrol (up to 1 mg/kg) and *cis*-resveratrol (up to 1.2 mg/kg) in significantly lower quantities than *cis*- and *trans*-piceid in hops (2-6 mg/kg and 4-9 mg/kg, respectively). In our analyses, resveratrol (mainly in its *trans*-form) has been found to be the most abundant stilbene in beer. This can be attributable to the fact that resveratrol (in the *trans*- and *cis*- form) can be partially regenerated by its glucoside, although piceid in beer remains more stable during the brewing process than resveratrol [26]. It could also be possible that hydrolysis of glycosides by yeast or bacterial β-glucosidase activity through beer fermentation may lead to piceid hydrolysis yielding free resveratrol [17,18]. In addition, an isomerase activity on phenols by the yeast during fermentation has been described previously by Jeandet *et al.* [34] and other authors [35]. As well as these factors, the amount of resveratrol and piceid extraction from hops to beer may depend on the commercial form of the hops [21,36], as well as hop freshness [22]. These factors can explain the differences in the *trans*- and *cis*-amounts of resveratrol and piceid in hops and beers.

The mean concentration of total resveratrol distributed in *trans*- and *cis*- resveratrol and piceid in different kinds of beers is shown in Figure 3. Abbey beers, ale, weissbier and stout beers contained significantly higher amounts of total resveratrol than lager and alcohol-free beers ($p<0.05$, Mann-Whitney test). These differences in stilbene concentrations could be due mainly to the different hop varieties used, as well as maceration, fermentation and the hopping rate in the boiling kettle during the brewing process [18].

FIGURE 3 HERE

The mean of piceid and resveratrol in their *trans*- and *cis*- forms, as well as the total resveratrol expressed per serving and per liter or kg in beers and in other resveratrol-containing foods is shown in Table 3. On an equal volume basis, beer had ~580-fold lower levels of total resveratrol than red wine [7], ~60-fold lower levels than grape juice and ~50-fold lower levels than white wine [7]. This means that ~260 L of beer contains the equivalent amount of total resveratrol found in one glass of wine (150 mL). Nevertheless, because of their alcoholic content, they should be consumed in moderation.

TABLE 3 HERE

In conclusion, a method to analyze resveratrol in beer matrix has been developed and evaluated and matrix effect of beer was determined. Total resveratrol was found in a range of 1.99 to 81.22 μg/L in 92 of the 110 commercial beers studied. *trans*-Resveratrol was the stilbene found in the highest levels and in the largest number of beers. Overall, beer contains only low levels of total resveratrol (μg/L), and despite its high consumption, it is not a representative source of dietary resveratrol.

ABBREVIATIONS USED

SPE, solid-phase extraction; LC-ESI-MS/MS, liquid chromatography-electrospray ionization- tandem mass spectrometry, MRM, multiple reaction monitoring; LOD, limit of detection; LOQ, limit of quantification; MF, matrix factor; PE, process efficiency.

SAFETY

We followed the general guidelines for working with organic solvents and acids. Universal precautions for the handling of chemicals were applied.

ACKNOWLEDGEMENTS

We are grateful to Drs. Isidre Casals and Olga Jauregui from the Scientific and Technical Services (University of Barcelona, Barcelona, Spain). This study was supported by the *INGENIO-CONSOLIDER Program, Fun-c-food CSD2007-063,* AGL2006-14228-C03-02 *from the Spanish Ministry of Science and Innovation and* the CIBER 30/06 Fisiopatología de la Obesidad y la Nutrición, Instituto de Salud Carlos III. Mireia Urpi-Sarda, Raul Zamora-Ros and Rafael Llorach would like to thank the *postdoctoral programs (Sara Borrell CD09/00134, F.I.S. CD06/00161 and CD09/00133, respectively) from the Ministry of Science and Innovation. Maria Rotches would like to thank the FI-DGR 2010 (AGAUR) fellowship from the Generalitat de Catalunya.*

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FIGURE CAPTIONS

Figure 1. Matrix effect of beer for: **A)** *trans*-resveratrol; **B)** *cis*-resveratrol; and **C)** *trans*-piceid.

Figure 2. Chemical structures of resveratrol and piceid in their *trans*- and *cis*- forms and Multiple Reaction Monitoring (MRM) chromatograms for: **A)** Standards of *trans*-resveratrol (1), *cis*-resveratrol (2), *trans*-piceid (3) and *cis*-piceid (4); **B)** Beer 62 and **C)** Beer 62 spiked with standards.

Figure 3. Mean concentration (μg/L) of *trans*- and *cis*- resveratrol and piceid content in different beer varieties. Bars with different letters are significantly different in total resveratrol content ($p<0.05$).