Comparison of Procedures for Resveratrol Analysis in Beer: Assessment of Stilbenoids Stability through Wort Fermentation and Beer Aging

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ABSTRACT

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Three resveratrol extraction procedures were compared in beer. Two-step pre-cleaning with toluene and cyclohexane allows 76% *trans*-resveratrol recovery by solid-phase extraction (SPE) before RP-HPLC-MS/MS analysis. This procedure proved much more efficient than liquid/liquid extraction, which requires solvents that are too hydrophobic for stilbenoids. SPME-GC-MS, where the main limiting factor is non-reproducibility from fibre to fibre, is an interesting alternative for resveratrol quantification in complex mixtures where pre-cleaning is too difficult. With the optimized SPE procedure, 5 $\mu g.L^{-1}$ *trans*-resveratrol was detected in four commercial beers. However, concentrations of stilbenoids in beer should be higher taking into account the presence of *cis*-stilbenoids. During fermentation, *trans*-resveratrol was partially regenerated from its glucoside, more stable through beer aging. Adding a stilbenoids-enriched ethanolic hop extract after fermentation significantly increases the beer stilbenoids potential.

Key words: Aging, beer, fermentation, piceid, resveratrol, RP-HPLC-MS/MS, SPME-GC-MS, stilbenoids, wort.

INTRODUCTION

About 20-30% of the polyphenols in beer are issued from hop. Flavonoids, the major constituent of hop polyphenols (1-5%), also derive from malt and adjuncts[4,5]. On the other hand, hop is the only source of prenylchalcones and stilbenoids[3,25,26]. Total *trans*-stilbenoids concentrations range from 0.5 to 12 $mg.kg^{-1}$ in hop cones[9,12] and up to 9 $mg.kg^{-1}$ in hop pellets[10-12].

Numerous methods have been published for determining *trans*-resveratrol in liquid matrices such as grape juice, wine, urine, plasma, or blood[1,2,14,15,18,28,30-32,34]. Most of them are direct analyses[1,2,15,18]. In the case of liquid extraction, ethyl acetate is often used[14,32]. The extract is further analyzed by HPLC hyphenated to UV absorption[8,19,21], fluorescence[17], or mass spectrometry[3,32]. GC/MS has also been used to determine *trans*-resveratrol, after derivatization with bis(trimethylsilyl)trifluoroacetamide (BSTFA)[16,18,22-24,29]. Luan *et al.*[16] developed a method using silylation of a solid-phase microextraction (SPME) fibre.

The aim of the present work was to compare three different procedures for the analysis of stilbenoids in beer. After optimization, the methods giving the best results were applied to four commercial lager beers, four commercial top-fermented beers, and four laboratory-scale enriched beers. The stability of piceid and resveratrol through fermentation and beer aging was also assessed.

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EXPERIMENTAL

Chemicals

Trans-Resveratrol (99%), *trans*-piceid (97%), bis(trimethylsilyl)trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMS), formic acid (pa), and hexane (97%) were supplied by Sigma-Aldrich (Belgium). Toluene (97%), ethyl acetate (97%), cyclohexane (99.96%), diethyl ether, and acetonitrile (99.99%) were supplied by Fisher Scientific (UK). Isooctane (99.8%) was obtained from Merck (Germany). Sodium chloride (97%) was supplied by Fluka (UK). Ethanol (97%) was obtained from Belgaco (Belgium). Methanol (99.9%) was supplied by Romil (UK). Aqueous solutions were made with Milli-Q (Millipore, USA) water.

Pre-cleaning procedures before SPE or liquid-liquid extraction

In each case, 100 mL (or 300 mL) beer was mixed three times for 10 min with 100 mL (or 300 mL) solvent (isooctane, diethyl ether, toluene, cyclohexane, or a combination of toluene/cyclohexane) at room temperature. At the end of each step, the sample was centrifuged for 10 min at 3000 g. At the last step, the beer was evaporated under vacuum to get rid of residual solvent.

Liquid-liquid extraction

Cleaned beer (300 mL) was extracted three times with 300 mL ethyl acetate and 5 g sodium chloride. Combined supernatants were concentrated by rotary evaporation

Table I. Stilbenoids recovery factors (%) for various pre-cleaning procedures applied on 100 mL beer. In all cases, stilbenoids were further extracted by Oasis solid phase extraction with 24 mL ethanol. n.d.: not detected.

Solvent for hydrophobic compounds removal	*trans*-Resveratrol	*trans*-Piceid
No pre-cleaning	2 +/- 2	9 +/- 2
Diethyl ether (3*100 mL)	n.d.	19 +/- 2
Isooctane (3*100 mL)	17 +/- 10	38 +/- 8
Cyclohexane (3*100 mL)	33 +/- 7	25 +/- 5
Toluene (3*100 mL)	34 +/- 13	43 +/- 4
Toluene (1*100 mL) and cyclohexane (2*100 mL)	76 +/- 8	37 +/- 4

Table II. Stilbenoids recovery factors (%) for various eluents applied on the Oasis solid phase extraction cartridge (500 mg, 6 cc) loaded with 100 mL beer. Comparison with liquid/liquid extraction with ethyl acetate. Hydrophobic compounds were removed in all cases with toluene (1*100 mL) and cyclohexane (2*100 mL).

Stilbenoids extraction	*trans*-Resveratrol	*trans*-Piceid
Liquid-liquid extraction		
3*300 mL ethyl acetate	53 +/- 6	7 +/- 0.01
Oasis solid phase extraction cartridge		
24 mL ethanol:water (80:20), v/v	38 +/- 3	13 +/- 3
24 mL methanol:water (80:20), v/v	48 +/- 16	25 +/- 3
24 mL methanol (1.5 mol/L formic acid)	51 +/- 3	13 +/- 2
24 mL ethanol (1.5 mol/L formic acid)	71 +/- 8	11 +/- 6
24 mL ethanol	76 +/- 11	37 +/- 1

Table III. Comparison between RP-HPLC-MS/MS and SPME-GC-MS procedures for stilbenoids analysis. n.d.: not detected.

	trans-Resveratrol		*trans*-Piceid		
	Limit of Detection	Limit of Quantification	Detection	Quantification	Time for analysis
RP-HPLC-MS/MS	5 $\mu g.L^{-1}$	15 $\mu g.L^{-1}$	5 $\mu g.L^{-1}$	15 $\mu g.L^{-1}$	8 hours
SPME-GC-MS	5 $\mu g.L^{-1}$	15 $\mu g.L^{-1}$	n.d.	n.d.	1.5 hours

(35°C) to dryness. The residue was solubilized in 2 mL ethyl acetate for RP-HPLC-APCI(+)-MS/MS analysis.

Solid-phase extraction (SPE)

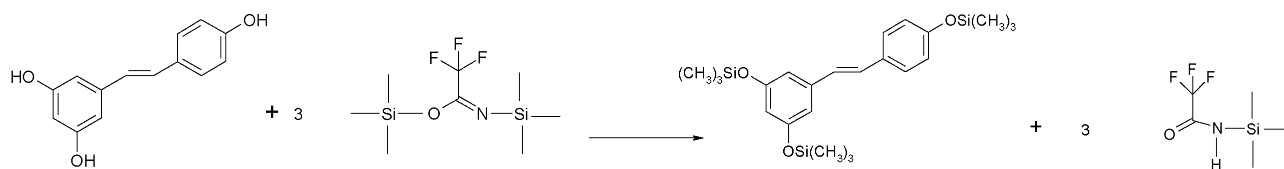
This procedure was inspired from the method of Urpí-Sardà *et al.*[30] applied to stilbenoids in human LDL. Cleaned beer (100 mL) was loaded onto an Oasis HLB (500 mg, 6 cc) active cartridge from Waters (USA). The cartridge was then washed with 16 mL of 1.5 M formic acid and 16 mL of an aqueous solution containing 5% methanol and 2% formic acid. Stilbenoids elution was performed with different kinds of eluents (usually 24 mL). Best results were obtained with 24 mL ethanol. After filtration to remove residual particles, the supernatant was concentrated to dryness by rotary evaporation (35°C) and solubilized in ethanol:water (50:50, v/v) before concentration under nitrogen to 0.2 mL and RP-HPLC-APCI(+)-MS/MS analysis.

RP-HPLC-APCI (+)-MS/MS analysis

Quantifications were performed on a C18 Prevail column (150 × 2.1 mm, 2 µm) (Alltech, Deerfield, IL, USA) eluted with a linear gradient from water (containing 1% acetonitrile and 0.1% formic acid) to acetonitrile. Gradient elution was as follows: from 95% to 55% water in 23 min, from 55% to 0% water in 7 min, and isocratic elution for 10 min at a flow rate of 200 µl/min. A ten-microliter sample was injected into the column kept at 30°C. A SpectraSystem equipped with an AS3000 autosampler and a P4000 quaternary pump was used. The system was controlled with Xcalibur software version 1.2 (Finnigan Mat). Mass spectra were acquired using an LCQ mass spectrometer equipped with an APCI source (Finnigan Mat). The following APCI inlet conditions in positive mode were applied: vaporization temperature, 470°C; capillary voltage, 3V; capillary temperature, 175°C; sheath gas, 40 psi; auxiliary gas, 7 psi; discharge current 5 µA. After the first monitoring at m/z = 229, collision-induced dissociation spectra were recorded at 37% relative collision energy.

Direct SPME-GC-MS analysis

The method used was inspired from the method of Luan *et al.*[16], initially applied to resveratrol in wine. All extraction steps were carried out in duplicate, with protection against daylight. The fibres were pre-conditioned for 2 h at 300°C in the GC injection port. Sampling was performed by immersing a polyacrylate SPME fibre (85 µm thickness, Supelco Inc., USA) for 30 min in a 6 mL liquid sample kept at room temperature under magnetic stirring. After extraction, the fibre was placed for 20 min in the headspace of a 2-mL vial containing 100 µL BSTFA (with 1% TMS). The GC program started as soon as the fibre was inserted into the GC injector. The set desorption time was 7 min at 280°C. A TRACE GC gas chromatograph equipped with a 50 m × 32 mm, i.d. 1.2 µm CP-Sil 5 CB column, a TRACE MS mass spectrometer (Intersciences-Thermo Finnigan) and the MS chemstation with Xcalibur software version 1.2 (Finnigan Mat) were used for analysis. Helium was maintained at a flow rate of 1 mL/min.



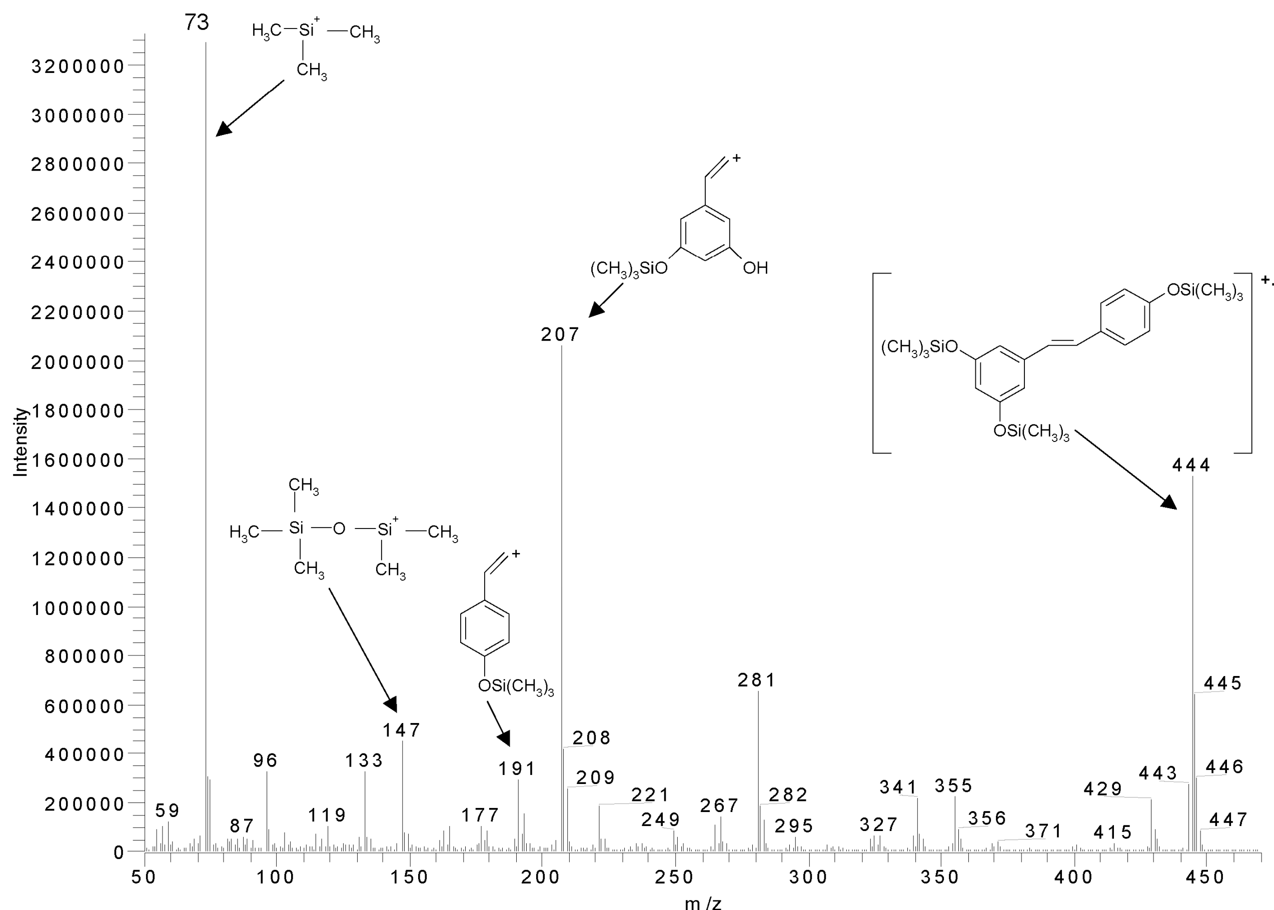


Fig.1. Experimental mass spectrum of derivatized *trans*-resveratrol from beer.

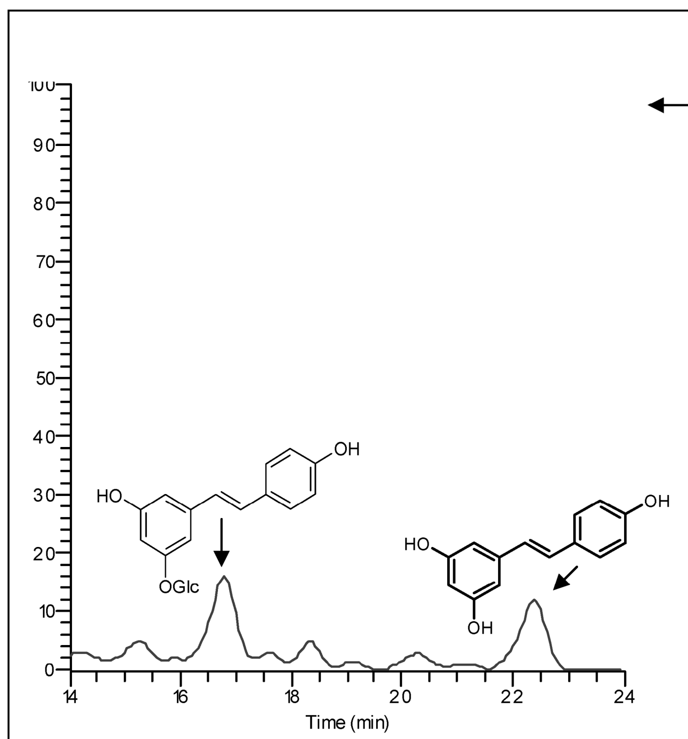
The temperature program was set as follows: from 100 to 250°C at 10°C/min, constant at 250°C for 30 min. The GC injector port was used in splitless mode and maintained at 280°C during the run. The MS operated in electron impact ionization mode (70 eV), and the mass-to-charge ratio scan ranged from 50 to 550 amu. The MS ion source was kept at 230°C. To increase sensitivity, MS was also used in the selected ion monitoring mode (SIM, m/z = 444 for *trans*-resveratrol).

Beers

Eight commercial Belgian beers were purchased from local supermarkets: four lager beers (LG1-LG4) and four top-fermented beers (TFW-white, TFA-amber, TFP-with pine, and TFL-late hopping). In addition, four stilbenoids-enriched beers (EX1-EX4) were prepared. EX1 and EX2 were prepared respectively by adding 10 mL or 20 mL stilbenoids-enriched hop extract (89 mg total stilbenoids.L^{-1}, obtained from spent Tomahawk 2006[29] by an ethanol:water 80:20, v/v extraction) to LG1 (final volume 100 mL). For EX3, 10 mg.L^{-1} *trans*-piceid and *trans*-resveratrol commercial standards were added to LG1. For aging investigations, EX3 was kept at 4°C or 20°C and protected against light for one year. EX4 was fermented in the laboratory from industrial wort (a kind gift of Inbev, Leuven, Belgium). The wort was diluted to 12° Plato and 10 mg.L^{-1} *trans*-piceid and *trans*-resveratrol commercial standards were added. Fermentations and maturations were conducted in 3L EBC tubes with lager yeast ($15*10^6$ cells/mL at pitching) as follows: at 12°C for 5 days, 13°C for 1 day, 15°C for 3 days, 7°C for 3 days, and 0°C for 24 h.

RESULTS AND DISCUSSION

Three different procedures for resveratrol determination in beer were compared. Two involved the use of RP-HPLC-APCI(+)-MS/MS after beer pre-cleaning. The third involved determination of derivatized resveratrol by SPME-GC-MS.



Beers	*trans*-Resveratrol (µg.L^{-1})	*trans*-Piceid (µg.L^{-1})
LG1	>5*	>5*
LG2	>5*	>5*
LG3	<5	>5*
LG4	<5	<5
TFW	>5*	>5*
TFA	>5*	>5*
TFP	<5	<5
TFL	<5	<5
EX1	327 (19)**	n.d. (83)**
EX2	997 (67)**	n.d. (147)**
EX3	10000	10000
EX4	8300	6000

Fig. 2. RP-HPLC-APCI(+)-MS/MS (m/z = 229) chromatogram for LG1. Concentrations of *trans*-resveratrol and *trans*-piceid in eight commercial and four experimentally enriched beers. * Under the quantification limit (15 µg.L^{-1} of the SPE procedure). ** True values obtained by SPME (SPE data given in brackets for information).

Comparison of pre-cleaning procedures

According to the literature[6], preliminary removal of hydrophobic beer constituents is necessary to improve polyphenol recovery. In the absence of pre-cleaning, the *trans*-resveratrol recovery factor reached only 2% (Table I, standard addition method). Because of the S1-type solubility of resveratrol[3,6,20], solvents more hydrophobic than diethyl ether were required for pre-cleaning. One-step liquid/liquid pre-cleaning with isooctane, cyclohexane, or toluene still gave poor results. On the other hand, a two-step liquid/liquid procedure using toluene and cyclohexane (followed by the optimized extraction procedure described below) made it possible to improve *trans*-resveratrol recovery by 50%. For *trans*-piceid, no significant difference was observed between toluene alone or a mixture of toluene and cyclohexane.

Resveratrol recovery when either oasis HLB SPE or liquid-liquid extraction was used before RP-HPLC-MS/MS analysis

As depicted in Table II, although often used for stilbenoids extraction from wine[14,32], ethyl acetate proved too hydrophobic to extract resveratrol directly from beer. Very efficient for hop extraction[3], ethanol:water (80:20, v/v) recovered only 38% of resveratrol from the cartridge. Less hydrophilic solvents, such as pure ethanol with or without a trace of formic acid, emerged as the best compromise. When 24 mL ethanol was used for elution, 5 µg.L^{-1} resveratrol was detectable in beer (limit of quantification = 15 µg.L^{-1}) (Table III). Although not fully optimized for the glucoside, the procedure also allowed detection of 5 µg.L^{-1} piceid in beer (the poor recovery factor = 37% being balanced by a higher response at the MS detector).

Comparison with the direct SPME-GC-MS procedure

Compared to RP-HPLC-MS/MS analysis, SPME-GC-MS avoids pre-cleaning and long extraction steps. Yet the low volatility of *trans*-resveratrol required derivatization on the fibre by silylation (BSTFA gaseous phase) (Fig. 1). This procedure also allowed detection of 5 µg.L^{-1} resveratrol in beer (Table III). The greatest limitation of the technique was non-reproducibility between successive fibres. It was thus necessary to carry out the entire standard addition process very often. Moreover, *trans*-piceid was undetectable. As shown below, however, this method proved to be a very good alternative when pre-cleaning became the limiting factor.

Stilbenoids concentrations in commercial beers

The optimized HPLC-MS/MS-APCI(+) procedure was applied to the Oasis eluates of eight commercial beers.

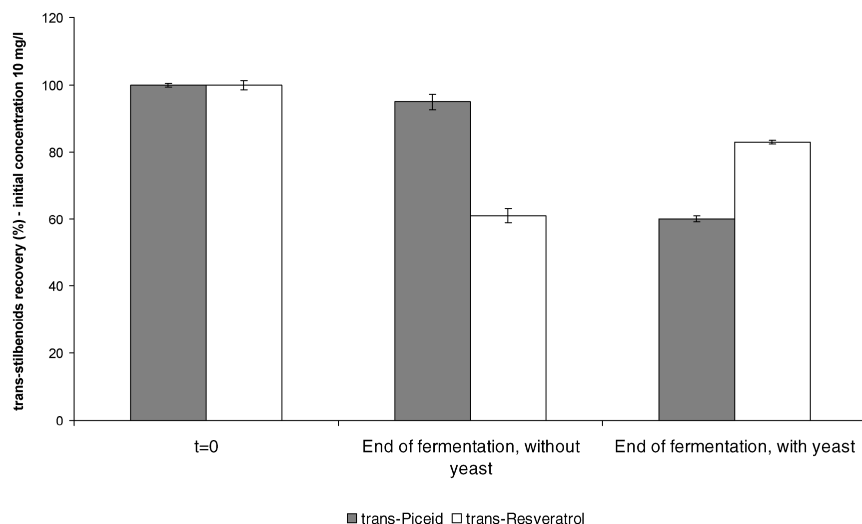


Fig. 3. Evolution of *trans*-resveratrol and *trans*-piceid concentrations in EX4 (with standard deviation) through wort fermentation.

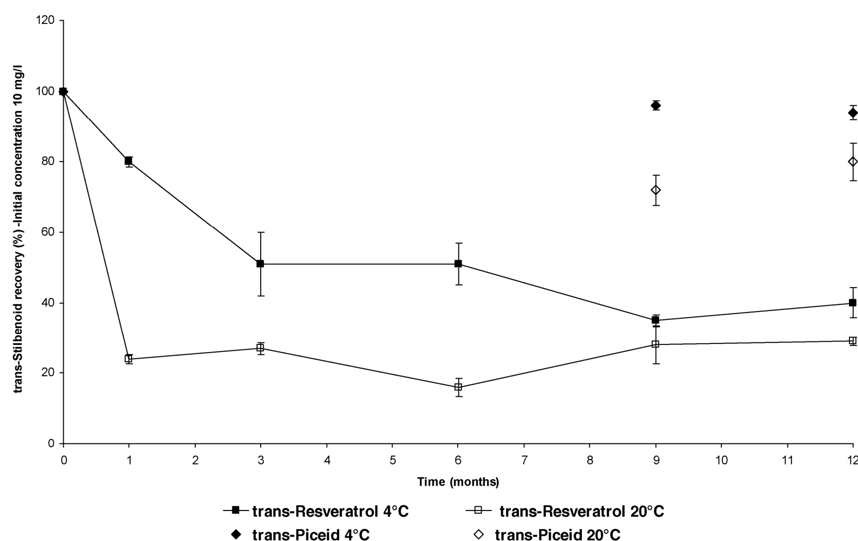


Fig. 4. Evolution of *trans*-resveratrol and *trans*-piceid concentrations in EX3 (with standard deviation) through beer aging.

trans-Piceid and/or *trans*-resveratrol were detected in five of them (Fig. 2). The concentrations were, however, just below the quantification limits in most samples. Taking into account a concentration of 1-10 $mg.kg^{-1}$ stilbenoids in hop and hopping close to 200 $g.hL^{-1}$ in wort, a maximum of 2-20 $\mu g.L^{-1}$ stilbenoids could be expected in beer. Moreover, massive degradation of *trans*-resveratrol is known to occur in the boiling kettle (60% degradation after 7 min of boiling[10]). *trans*-Piceid is much more stable during heat treatment but, as depicted in Fig. 3, it can be converted to free resveratrol through wort fermentation (only 60% recovered from a beer prepared by spiking an industrial wort at 10 $mg.L^{-1}$ before fermentation = EX4). When the wort was not pitched, *trans*-piceid remained stable, suggesting that yeast enzymes catalyze this hydrolysis to free resveratrol, as previously described for wine[7,27,33]. On the other hand, the *trans*-resveratrol concentration decreased even in the absence of yeast (40% degradation), most likely because of reactions with wort components (apparent degradation of 17% in the presence of yeast, due to the equilibrium with piceid).

Stilbenoids concentrations reached in lab-scale productions

In order to better assess the reactivity and solubility of resveratrol- and piceid-enriched hop extracts in beer, beer LG1 was spiked with 10 or 20 mL hop extract containing 89 $mg.L^{-1}$ stilbenoids. These yielded beers EX1 and EX2, respectively (final volume: 100 mL). Similar spiking (10 $mg.L^{-1}$) was done with commercial standards (EX3). Our SPE procedure confirmed the presence of 10 $mg.L^{-1}$ in EX3 (Fig. 2), but although the expected total stilbenoids concentration in EX2 was 18 $mg.L^{-1}$ (including 1 $mg.L^{-1}$ free resveratrol), the concentrations determined by SPE extraction and HPLC-MS/MS analysis (taking into account the recovery factor usually obtained for beer—see

Table II) were much lower: only 67 µg.L^{-1} *trans*-resveratrol, 147 µg.L^{-1} *trans*-piceid, and 89 µg.L^{-1} *cis*-piceid. On the other hand, SPME-GC-MS analysis did allow detection of the expected concentration of *trans*-resveratrol (1.0 mg.L^{-1}, Fig. 2). In the SPE procedure supramolecular structures involving hop constituents most probably form, leading to excessive loss of stilbenoids during the pre-cleaning step (confirmed by pre-cleaning directly applied to our hop extract: 58% lost). No unpleasant descriptors characterized this 18 mg.L^{-1} enriched beer (according to 5 well-trained assessors taken in the laboratory). Of course, complementary analyses are needed to assess the bioavailability of stilbenoids in beers spiked in this way.

The enriched beer EX3 was also monitored over 12 months of storage at 4°C and 20°C. As depicted in Fig. 4, *trans*-piceid remained undegraded in beer for a year, although 80-90% degradation is known to occur in less antioxidant model media kept under the same conditions[13]. Conversely, although *trans*-resveratrol is reported not to be degraded after 3 months in model media kept at 4°C[13], here its recovery from aged beer was poor (50% recovery after 3 months at 4°C, 27% after 3 months at 25°C). Interactions with beer components are thus suspected. After 3 months, equilibrium with co-constituents was probably reached, as suggested by the constant level of 4 mg.L^{-1} maintained for up to one year.

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