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Determination of Stilbenes in Hop Pellets from Different Cultivars

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About 30% of the polyphenols in wort and beer derive from hop, but little is yet known about their nutritional impact. The recent discovery of *trans*-resveratrol and piceid isomers in hop opens new doors to understanding beer health benefits. In the present work, resveratrol was quantified by HPLC−APCI−MS/MS in pellets from 9 different cultivars. Concentrations ranging from 4 to 9 mg/kg *trans*-piceid, from 2 to 6 mg/kg *cis*-piceid, and up to 1 mg/kg *trans*-resveratrol were detected. As previously shown for total polyphenols and flavonoids, the lower the α-acid content, the higher the total stilbene content.

KEYWORDS: **Resveratrol; stilbene; polyphenols; hop cultivars; beer**

INTRODUCTION

Female inflorescences of hop plant (*Humulus lupulus* L.) are used in the brewing industry for bitterness and aroma (*1*, *2*) in beer. About 30% of the polyphenols in wort and beer derive from hop, but little is yet known about their nutritional impact. Most studies have focused on their contribution to wort reducing power (*3*), beer color, and haze (*4*).

In the past decade, hop xanthohumol and flavonoids (*5*) have received a little more attention. The recent discovery in hop of 0.5 mg/kg *trans*-resveratrol (*6*) (**Figure 1 A**), famous in wine for its potential role in the "French paradox" (*7*), opens new doors to understanding hop health benefits. Noteworthy is also the presence of both piceid isomers (parts **C** and **D** of **Figure 1**) in the investigated Tomahawk (harvest 2002) hop cultivar [2 and 0.9 mg/kg *trans*- and *cis*-piceid, respectively (*6*)]. Already known as a potent antioxidant, *trans*-resveratrol has also been investigated for some interesting physiological effects, including antiplatelet, anti-inflammatory, estrogenic, cardioprotective, antitumor, and antiviral properties (*7*). Although less potent than its aglycon, *trans*-piceid would appear to limit the elevation of lipid concentration and to inhibit eicosanoid synthesis (*8*). According to Hollman (*9*), piceid absorption from food may be enhanced because of the presence of sugar. Yeast (*10*) or bacterial (*9*) β-glucosidase activity may lead to piceid hydrolysis yielding free aglycon.

trans-Resveratrol levels in wine are profoundly influenced by factors such as growth region (*11*), grape variety (*11*), interactions with pathogens such as *B. cinerea* and *P. viticola* (*12*, *13*), or winemaking conditions (*14*). Free resveratrol has been found in red wine in the 1.33−5.13 mg/kg range (*15*, *16*), while its glycoside *trans*-piceid has been reported at concentrations ranging from 0.22 to 7.5 mg/kg (*16*, *17*) to 20 mg/kg (*18*). In white wine, *trans*-resveratrol ranges only from 0.002 to 0.452 mg/kg and *trans*-piceid ranges from 0.033 to 0.438 mg/kg (*19*, *20*). Few foods are known to contain *trans*-resveratrol. Among them are especially cranberries, cranberry juice (*21*), peanuts, and peanut products (*22*).

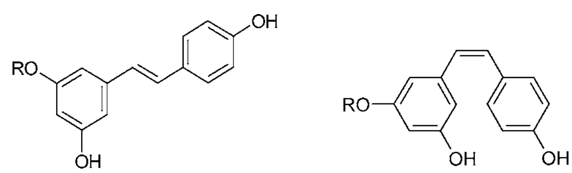

Figure 1. *trans*- (A and C) and *cis*-resveratrol (B and D) and their glucosides. A and B: R = H, resveratrol. C and D: R = glucose, piceid.

The aim of the present work was to investigate the stilbene content of hop pellets from 9 different cultivars (harvest 2003, T90 pellets) and to compare the concentration data with amounts of total polyphenols, flavanoids, and isohumulones in each cultivar. A new method developed by Callemien et al. (*6*) enabled us to recover 99% *trans*-resveratrol and 95% *trans*-piceid from hop. After removal of resins by means of several cleaning steps with toluene and cyclohexane, stilbenes were extracted with ethanol/water (80:20) at 60 °C and analyzed by high-performance liquid chromatography (RP-HPLC) coupled with APCI(+) tandem mass spectrometry (MS/MS).

EXPERIMENTAL PROCEDURES

Materials. Sterling, Wilhamette, Cascade, Nugget, Vanguard, Simcoe, Warrior, Tomahawk, and Amarillo varieties, all pellets T90 from the harvest 2003, were a kind gift from Yakima Chief (Strombeek-Bever, Belgium). All samples were stored at −80 °C until needed.

Chemicals. Ethanol (97%) was obtained from Belgaco (Gent, Belgium). Acetonitrile (99.99%), toluene (97%), and cyclohexane (99.96%) were supplied by Fisher Scientific (U.K.). Formic acid (pa) was obtained from Aldrich (Germany). Methanol (99.9%) and diethyl ether (99%) was supplied by Romil (Cambridge, U.K.). Aqueous

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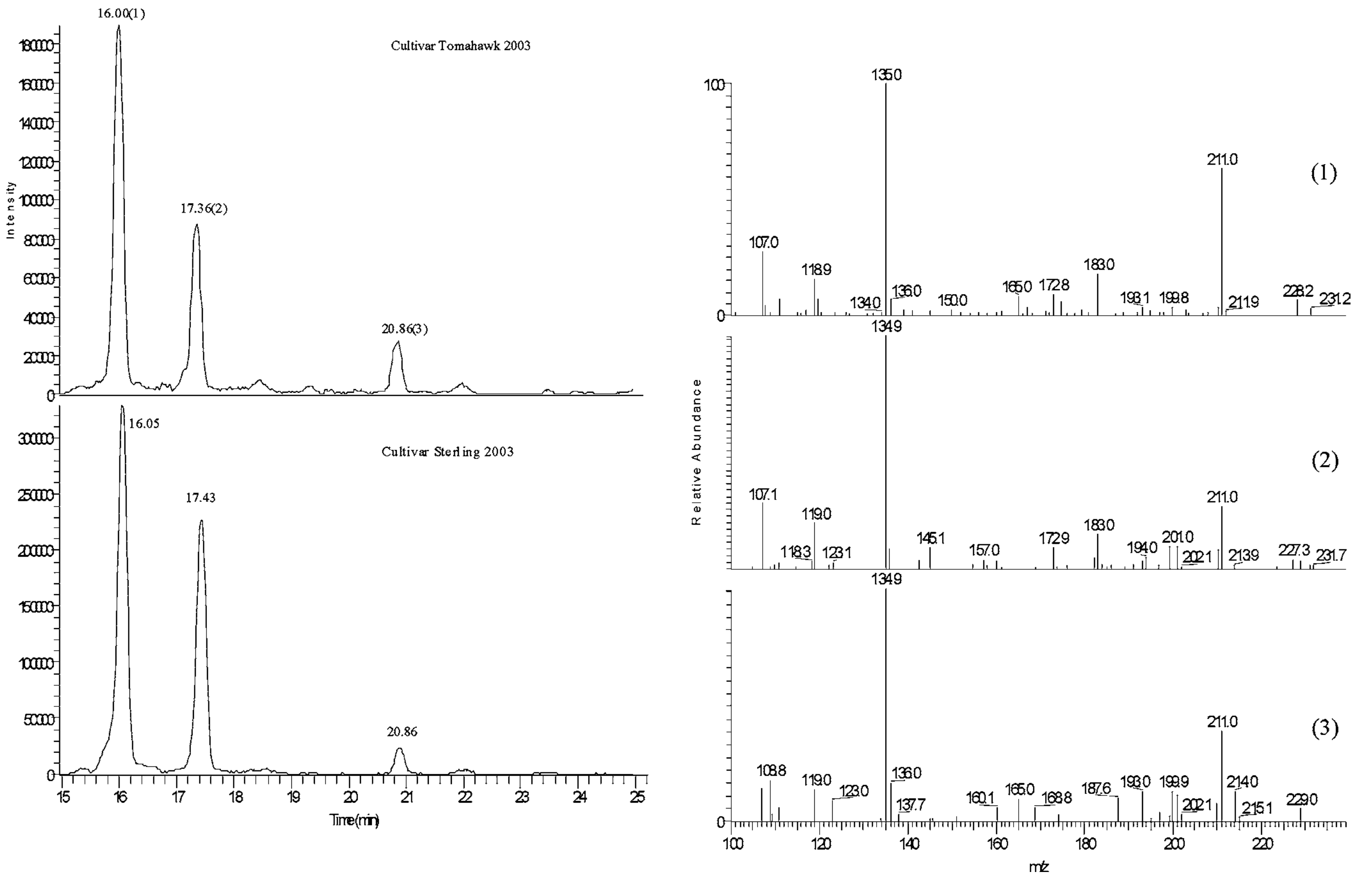

Figure 2. RP-HPLC–APCI(+)–MS/MS data for a Sterling and Tomahawk hop extract (T90, harvest 2003). MS/MS chromatogram (*m*/*z* 229) and experimental mass spectra for *trans*-piceid (1), *cis*-piceid (2), and *trans*-resveratrol (3).

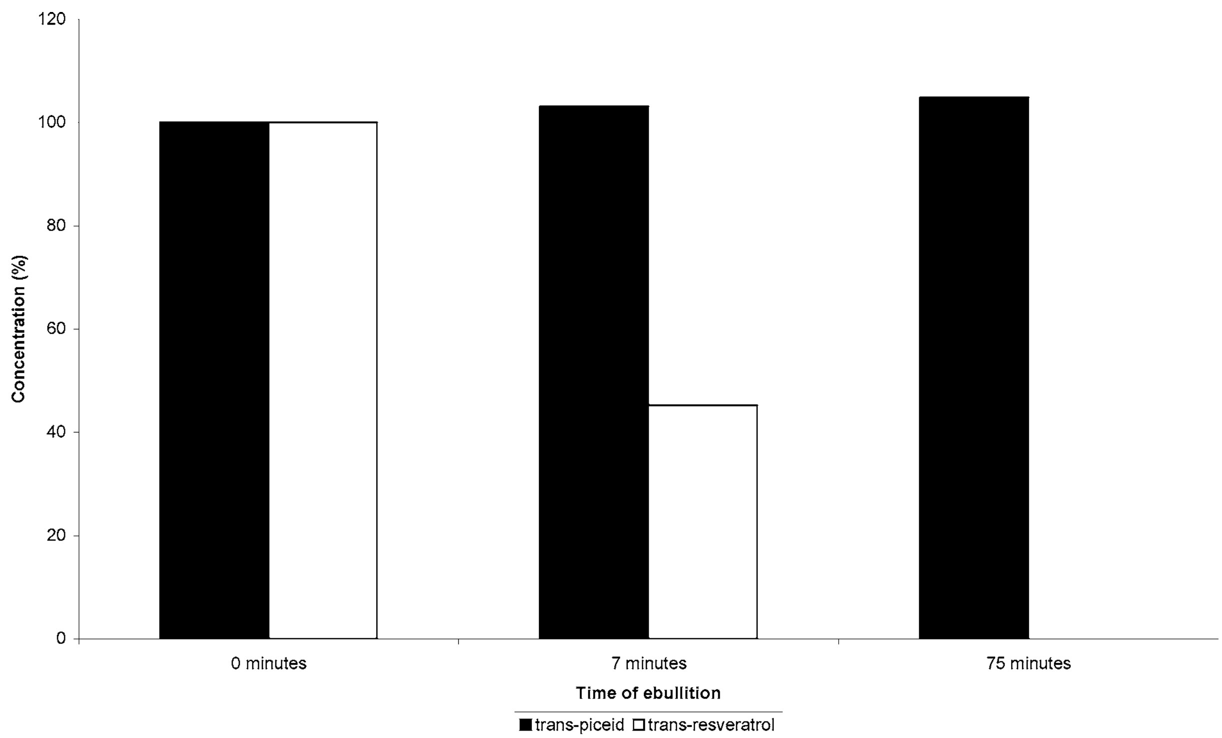

Figure 3. Follow-up of the degradation of *trans*-resveratrol and *trans*-piceid in an aqueous model medium previously flushed by nitrogen. The temperature inside the flask was settled at 100 °C. Concentrations were measured by HPLC–MS/MS.

solutions were made with Milli-Q (Millipore, Bedford, MA) water. *trans*-Resveratrol (99%) and *trans*-piceid (97%) were supplied by Sigma−Aldrich (Bornem, Belgium).

Extraction of Stilbenes from Hop. This method has been developed in our laboratory (*6*) to analyze stilbenes in hop pellets. All extraction steps have been done with protection against day light, in duplicate. Hop pellets were crushed in a mortar. Ground samples (2.5 g) were extracted, in successive 10 min steps at room temperature under gentle stirring, 3 times with 50 mL toluene and 3 times with 50 mL cyclohexane, to remove hydrophobic compounds. At the end of

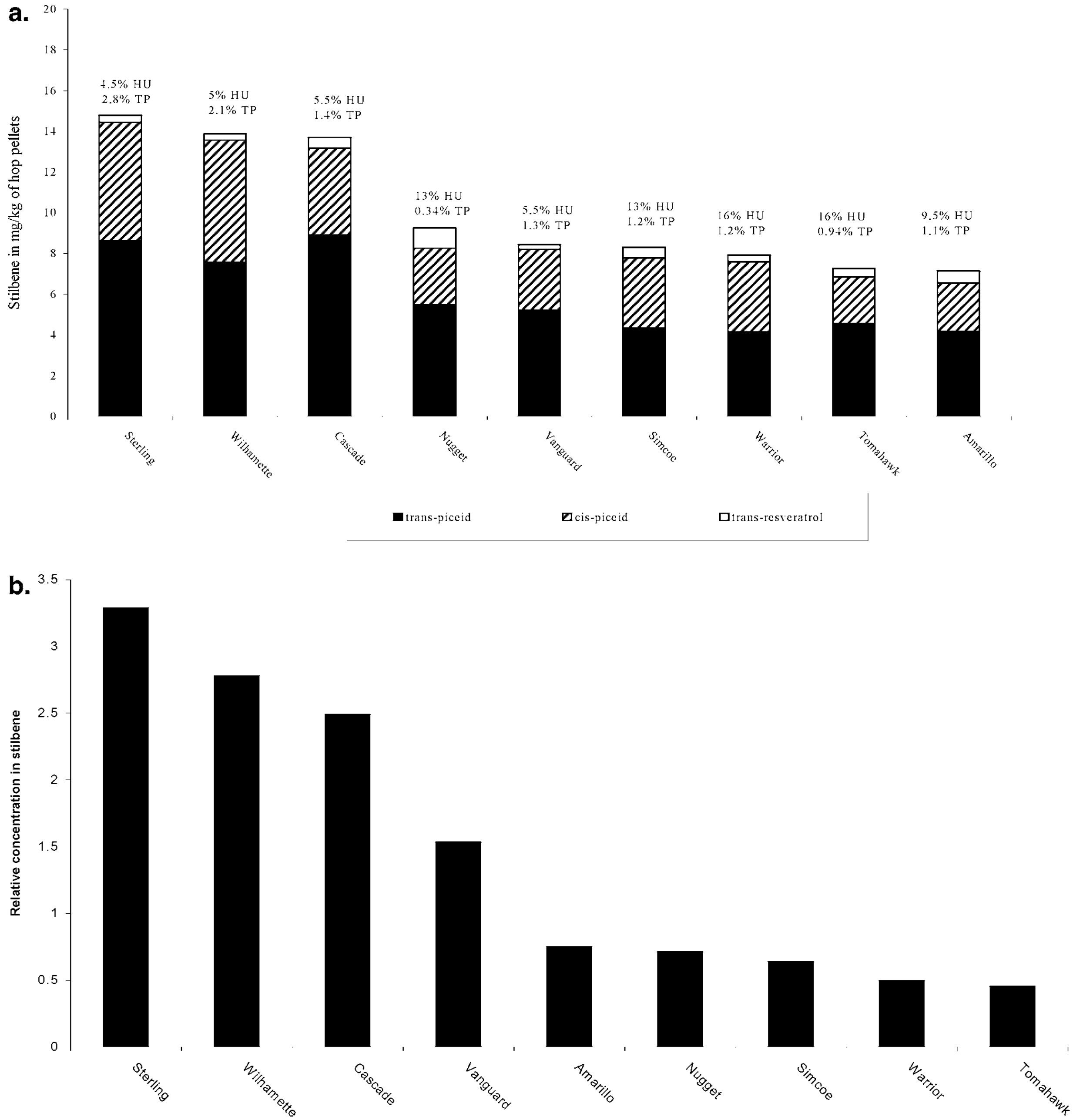

Figure 4. (a) Concentration (mg/kg) of *trans*-piceid, *cis*-piceid, and *trans*-resveratrol. % HU = humulone concentration. % TP = total polyphenols concentration. The Sterling variety is issued from Saaz × Cascade. (b) Relative concentrations of stilbenes for the same α content.

Table 1. Characteristics and Stilbene Content of Nine Hop Varieties

hop variety	α-acids value[a] (g/100 g)	total polyphenols (g/100 g)	flavanoids (%, catechin equivalent)	*trans*-piceid (mg/kg)[b]	*cis*-piceid (mg/kg)[b]	*trans*-resveratrol (mg/kg)[b]	total stilbene content (mg/kg)[b]
Sterling	4.5	2.8	0.29	8.63	5.81	0.35	14.79
Wilhamette	5	2.1	0.28	7.55	6.01	0.33	13.89
Cascade	5.5	1.4	0.23	8.89	4.28	0.53	13.70
Nugget	13	0.3	0.09	5.50	2.76	1.00	9.26
Vanguard	5.5	1.3	0.19	5.21	2.99	0.22	8.43
Simcoe	13	1.2	0.1	4.34	3.45	0.52	8.31
Warrior	16	1.2	0.13	4.15	3.44	0.33	7.92
Tomahawk	16	0.9	0.09	4.55	2.30	0.43	7.27
Amarillo	9.5	1.1	0.17	4.17	2.38	0.59	7.14

[a] According to the analytica EBC (1987). [b] Assay in duplicate; variation coefficient under 2%.

each step, the sample was centrifuged for 10 min at 3000*g*. At the last step, hop powder was dried under vacuum to get rid of residual solvent. Delipidated hop powder was extracted 3 times with 40 mL ethanol/water (80:20, v/v); each time for 10 min under gentle stirring at 60 °C. After each extraction, the sample was centrifuged for 10 min at 3000*g* and the supernatant was collected. After filtration to remove residual particles, the combined supernatants were concentrated by rotary evaporation (35 °C) to dryness. The residue was solubilized in 2 mL of 50:50 (v/v) mixture of ethanol/water.

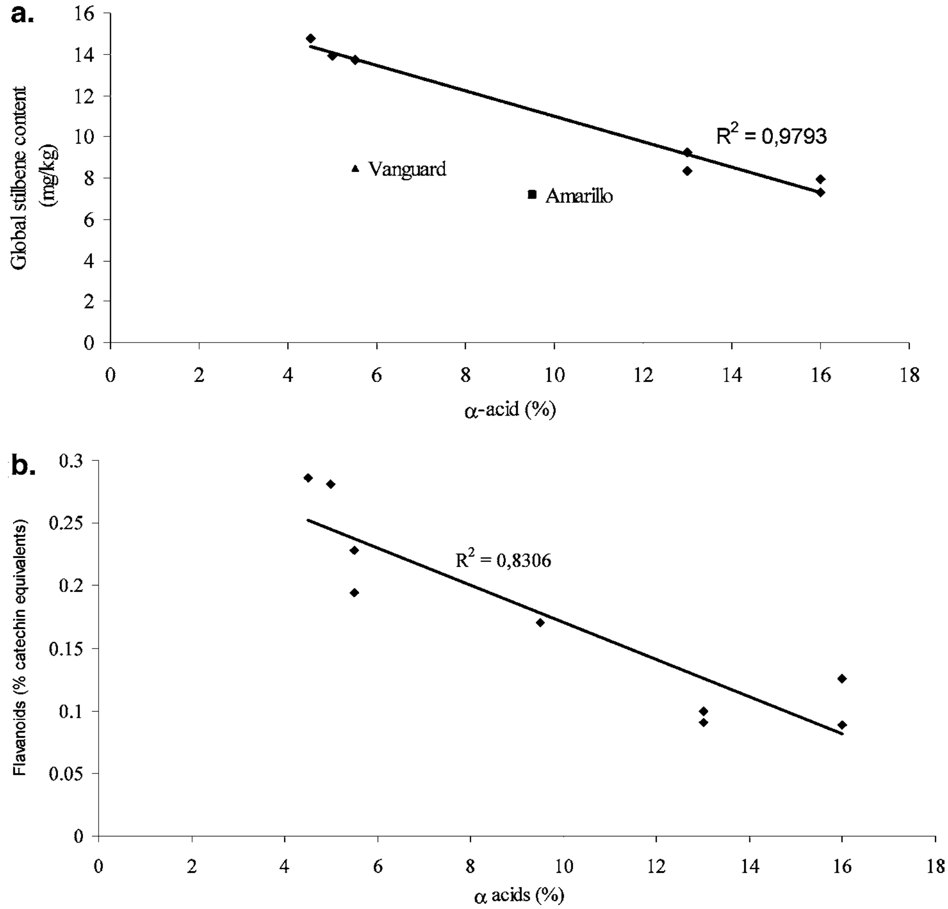

Figure 5. Relationship between the total stilbene content (a) or flavanoids (b) and the α-acid percentage for hop pellets varieties (α-acid value according to the Analytica EBC, 1987).

RP-HPLC−APCI(+)−MS/MS Analysis of Stilbenes. Quantifications were performed on a C18 Prevail column (150 × 2.1 mm, 2 μm) (Alltech, Deerfield, IL) 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% in 7 min, and isocratic for 10 min at a flow rate of 200 μL/min. A total of 10 μL of a 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 the Xcalibur software version 1.2 (Finnigan Mat). Mass spectra were acquired using a 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, 3 V; capillary temperature, 175 °C; sheath gas, 40 psi; auxiliary gas, 7 psi; discharge current, 5 μA. After the first monitoring on the *m*/*z* 229, collision-induced dissociation spectra were recorded at 37% relative collision energy.

Total Polyphenol and Flavanoid Quantification in Methanolic Hop Extracts. To remove humulones, 1 g of ground hop pellets was first extracted 3 times with 7 mL of diethyl ether by shaking for 15 min and sonicating for 5 min. After centrifugation (3500*g* for 10 min), the supernatant was removed. The residual hop was then extracted with methanol according to McMurrough and Hennigan (*23*). Residual diethyl ether was removed by rotary evaporation (35 °C) down to 15 mL. The volume was finally adjusted to 25 mL with methanol. Total polyphenols were determined according to Bishop (*24*). Flavanoids were determined by the method of Delcour and Janssens de Varebeke (*25*). Results are expressed in catechin equivalents (mg/kg of terminal flavanol able to react with *N*,*N*-dimethylaminocinnamaldehyde).

RESULTS AND DISCUSSION

The stilbene contents of pellets from nine varieties (T90, harvest 2003) were determined by the method recently proposed by Callemien et al. (*6*). As shown in **Figure 2**, the RP-HPLC−APCI(+)−MS/MS chromatogram obtained enabled us to separate and quantify (by selecting *m*/*z* 135) *trans*-piceid, *cis*-piceid, and *trans*-resveratrol in all varieties. On the other hand, *cis*-resveratrol (tr = 22.02) was absent from all nine hop samples, as previously reported for *Vitis vinifera* grapevines (*12*, *16*, *17*). Processed juice products usually contain higher proportions of *cis*-resveratrol (*12*, *16*, *17*, *21*), probably owing to exposure to light, for instance, during the winemaking process or storage. Likewise, both isomers could be expected in final beer. However, stronger degradation could also occur in the boiling kettle, leading in that case to undetectable amounts in beer. As depicted in **Figure 3**, late hopping significantly improves the recovery (7 min at 100 °C allows us to recover 40% resveratrol and 100% piceid).

As depicted in **Figure 4 a**, concentrations ranging from 4 to 9 mg/kg *trans*-piceid, from 2 to 6 mg/kg *cis*-piceid, and up to 1 mg/kg *trans*-resveratrol were measured in hop pellets. As in grape juices and wines (*8*, *19*), *trans*-piceid emerged as the major form (**Table 1**). Cultivars with the highest amounts of free *trans*-resveratrol (e.g., Nugget with 1 mg/kg) did not turn out to be the most interesting sources of total stilbenes (e.g., Sterling with only 0.35 mg/kg *trans*-resveratrol but 14.79 mg/kg total stilbenes). Complementary studies are needed to identify the form most able to be solubilized in wort, to survive the brewing process, and of course, to induce *in vivo* health benefits.

Because the hopping rate in the boiling kettle is calculated according to the α-acid content of hop, total stilbene contents have been compared for a same bitterness potential (**Figure 4b**). The low-bitterness cultivars clearly emerge as the most interesting.

Figure 5 a shows for seven cultivars that the lower the α-acid concentration, the higher the total stilbene content ($R^2 = 0.9793$). A similar relationship was previously described by Lermusieau et al. (*3*) between humulones and total polyphenols or flavanoids (depicted with flavanoids for our 9 samples in **Figure 5b**; see also **Table 1**). Further investigations are needed to know how the α-acid level influences the stilbene content (higher intrinsic content or better resistance to oxidation). Indeed, we can suspect that resveratrol is less protected against oxidation in bitter varieties (lower antioxidant activity). Worth stressing, however, are the results of Biendl et al. (*26*) who measured the highest xanthohumol levels in bitter cultivars. Samples of Vanguard and Amarillo strongly deviated from the α-acid/stilbene relationship (**Figure 5a**). In both cultivars, well-known to hop producers for their high sensitivity to oxidation, considerable stilbene degradation probably occurred before analysis, leading to lower-than-expected piceid levels.

In conclusion, although hop polyphenols have been widely studied in the past decade for their antioxidant activity in the boiling kettle (*3*), very little is yet known about their real impact on health. The recent discovery of resveratrol in pellets (*6*) highlights what may be the key role of hop in producing the health benefits of moderate beer consumption. Total stilbene concentrations range from 5 to 16 mg/kg, with *trans*-piceid being in all cases the major constituent. The lower the α-acid content, the higher the resveratrol potential, except for very highly oxygen-sensitive varieties. Because resveratrol is a phytoalexin, the impact of the harvest year should now be considered. The most promising hop cultivars will be further investigated for the production of resveratrol-enriched hop extracts.

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