

## Fate of Resveratrol and Piceid through Different Hop Processings and Storage Times

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*trans*-Piceid and *trans*-resveratrol contents of hop cones, hop pellets, CO<sub>2</sub> extracts, and spent hop from American varieties (harvest 2004) were determined by reverse-phase high-performance liquid chromatography–atmospheric pressure chemical ionization–tandem mass spectrometry [RP-HPLC–APCI(+)-MS/MS]. Pelletization induced strong stilbene degradation in some cultivars. Similarly, 1 year of storage at 4 °C led to a huge loss of *trans*-piceid, especially in the case of hop cones (much faster than in model media, although well protected from light and oxygen). Therefore, after 8 months of storage, the overall stilbene content was in the same range whatever the conditioned form. Absent in fresh hop cones or pellets, *cis*-resveratrol was released from *cis*-piceid in all stored samples. On the other hand, no  $\delta$ -viniferin was detected despite it is present in light-protected model media spiked with *trans*-piceid. Because supercritical carbon dioxide proved inefficient for recovering resveratrol and piceid from pellets, spent hop emerged as the most interesting material for subsequent specific stilbene extraction.

**KEYWORDS:** Resveratrol; stilbene; polyphenols; hop conditioning; hop storage; beer; resveratrol stability

### INTRODUCTION

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 (1). Although less potent than its aglycon, *trans*-piceid would appear to limit the elevation of the lipid concentration and inhibit eicosanoid synthesis (2).

Hop flavanoids have been widely studied in the past decade for their exceptional antioxidant activity in the boiling kettle (3). In 2005, Callemien et al. (4) mentioned for the first time the presence of *trans*-resveratrol, *trans*-piceid, and *cis*-piceid in hop pellets (total concentration close to 3.5 ppm). Jerkovic et al. (5) compared various hop pellets from harvest 2003. In this case, the total stilbene concentration ranged from 5 to 16 ppm, with *trans*-piceid being in all cases the major constituent. Except for very high oxygen-sensitive varieties, it appeared that the lower the  $\alpha$ -acid content, the higher the resveratrol potential. Recently, Jerkovic and Collin (6) evidenced, however, the huge impact of the harvest year.

Only female inflorescences of hop plant (*Humulus lupulus* L.) are used in the brewing industry (7, 8). After harvest in the autumn, hop is dried (from 75 to 80% moisture to below 10% (9) at a temperature close to 60 °C before storage for up to 1 year (9–11). Even compressed in bales, hops cannot be stored for a long time without a protected atmosphere because of flavor oxidation and a decrease in  $\alpha$ -acid content (9–15).

It is therefore very usual to decrease the volume by pelletizing dried milled hop (16). In this process, shear forces raise the temperature of the hop material up to 50 °C (9, 11, 16). To prevent degradation, hop pellets are then stored under vacuum (after nitrogen flushing) between –2 and 4 °C (12, 13, 17–20). Under these conditions, the  $\alpha$ -acid content remains relatively constant during 1 year of storage (20), while the essential oil content is inevitably affected after a few months (e.g., farnesene in aromatic varieties) (21). Polyphenol oxidation (13) may also take place, depending upon the variety of hop. Cold storage does not prevent deterioration but slows down oxidation (20).

Hop extraction is another way to prevent hop resin from decomposing. Liquid and supercritical carbon dioxide are the most efficient apolar solvents for extracting  $\alpha$ -humulones without other chemicals, such as pesticides, hard resins, chlorophylls, and polyphenols (11). In the case of hop extract, the  $\alpha$ - and  $\beta$ -acid concentrations do not change as the extracts age (14).

The aim of the present work was to assess the impact of conditioning and storage on hop stilbene concentrations. American varieties from harvest 2004 were analyzed through storage in three differently conditioned forms, all under protected atmosphere: leaf hop, derived pellets, and CO<sub>2</sub> extracts. In addition, model media were monitored over 1 year. Spent hop from the supercritical extraction of two samples was also investigated.

### EXPERIMENTAL PROCEDURES

**Materials.** Willamette, Cascade, Nugget, Simcoe, Warrior, and Tomahawk hop varieties from the harvest 2004 were a kind gift from Yakima Chief (Louvain-la-Neuve, Belgium). Cones, T90 pellets, CO<sub>2</sub>

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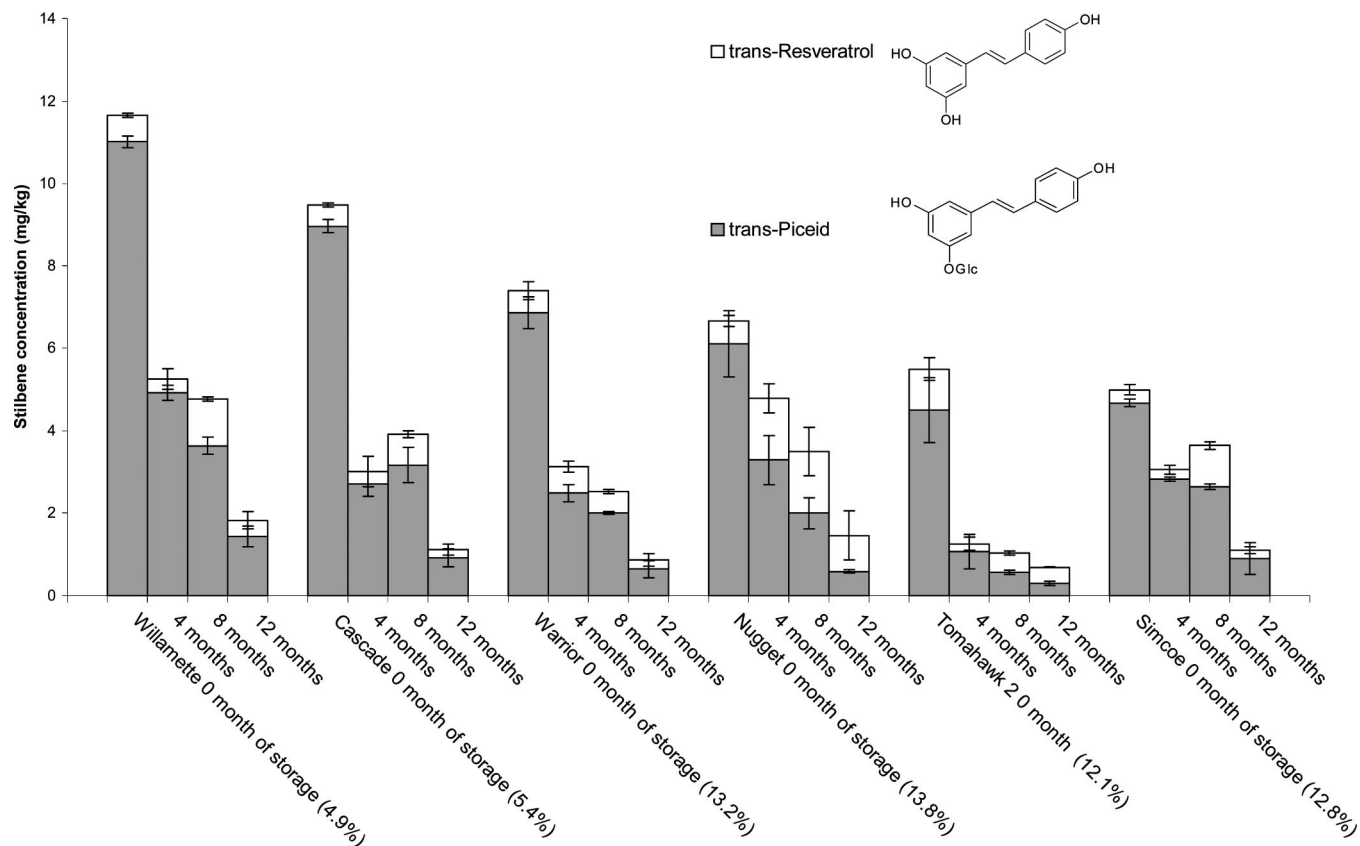


Figure 1. Concentrations and standard deviations of stilbenes in hop cones through storage.  $\alpha$ -Acid content (%) given between brackets. Assay in duplicate.

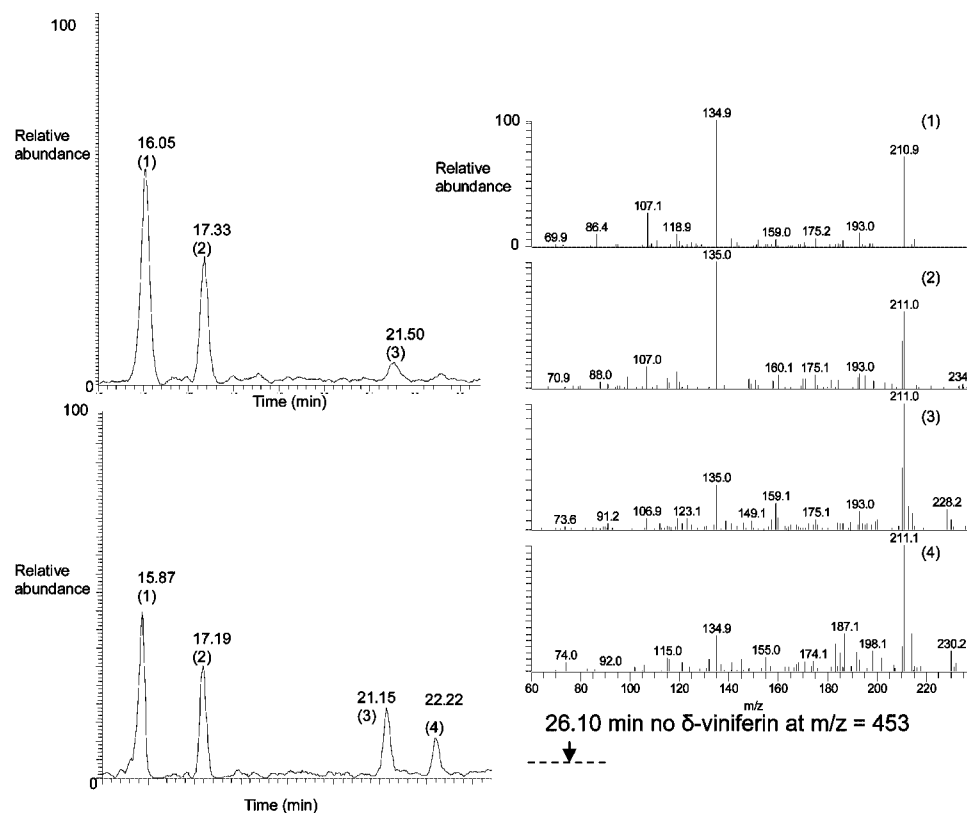
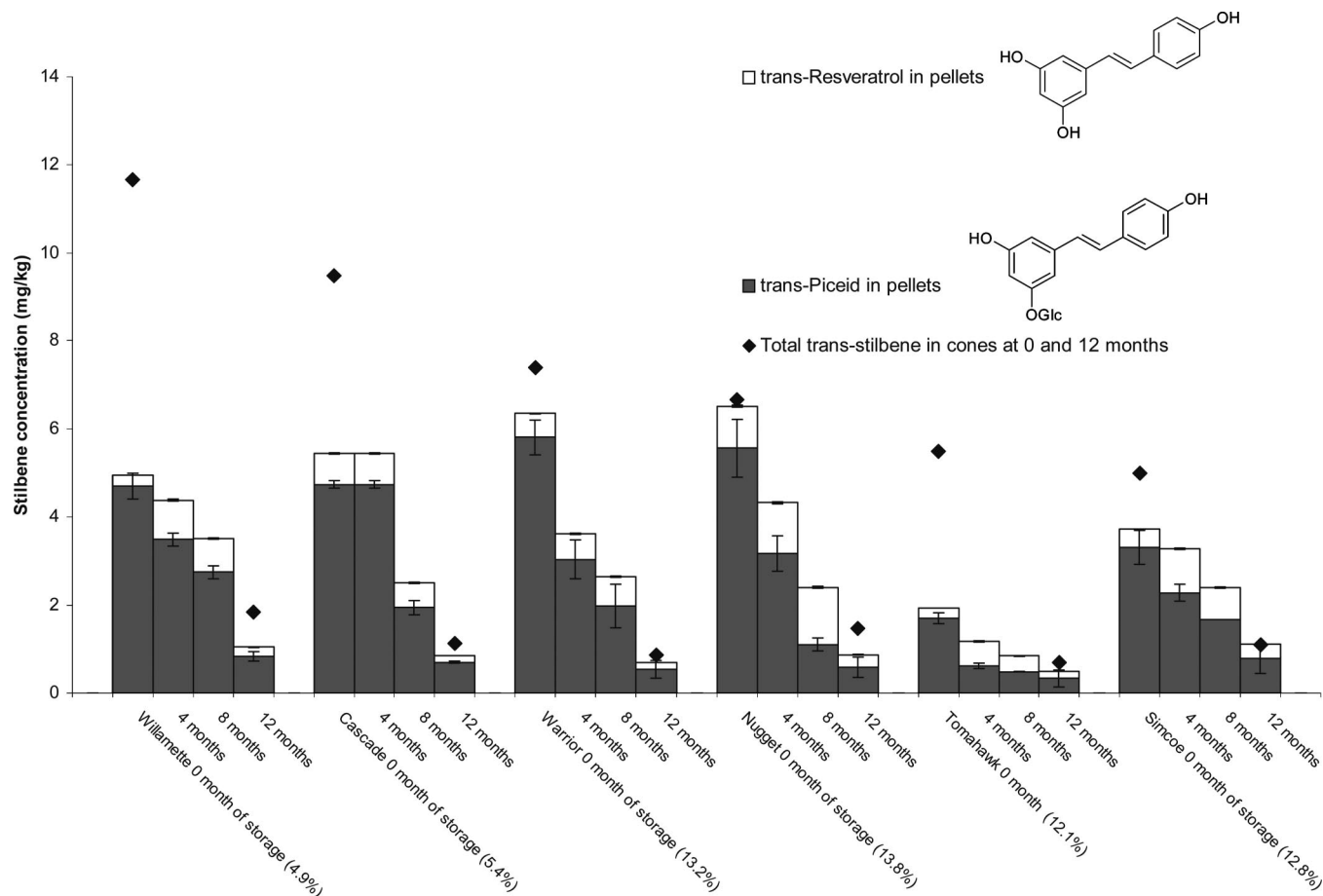


Figure 2. RP-HPLC-APCI(+)-MS/MS data of Willamette hop (harvest 2004). (a) Dried cones just after harvest. (b) Cones after 8 months of storage at 4 °C. MS/MS chromatogram ( $m/z$  229) and experimental mass spectra for *trans*-piceid (1), *cis*-piceid (2), *trans*-resveratrol (3), and *cis*-resveratrol (4).

extracts (supercritical conditions of 3100 psig and  $40 \pm 3$  °C) and spent were vertically sampled from the same batches. All samples were stored under vacuum (after nitrogen flushing) at 4 °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



**Figure 3.** Concentrations and standard deviations of stilbenes in hop pellets through storage.  $\alpha$ -Acid content (%) given between brackets. Assay in duplicate.

was obtained from Aldrich (Germany). Methanol (99.9%) and diethyl ether (99%) was supplied by Romil (Cambridge, U.K.). Aqueous solutions were made with Milli-Q (Millipore, Bedford, MA) water. *trans*-Resveratrol (99%) and *trans*-piceid (97%) were supplied by Sigma-Aldrich (Bornem, Belgium). *cis*-Resveratrol was synthesized from the *trans* form under UV light (2).  $\delta$ -Viniferin and  $\epsilon$ -viniferin standards were a kind gift of Dr. X. Vitrac and Prof. J.-M. Mérillon (Institut des Sciences de la Vigne et du Vin, Université Victor Segalen Bordeaux 2, France).

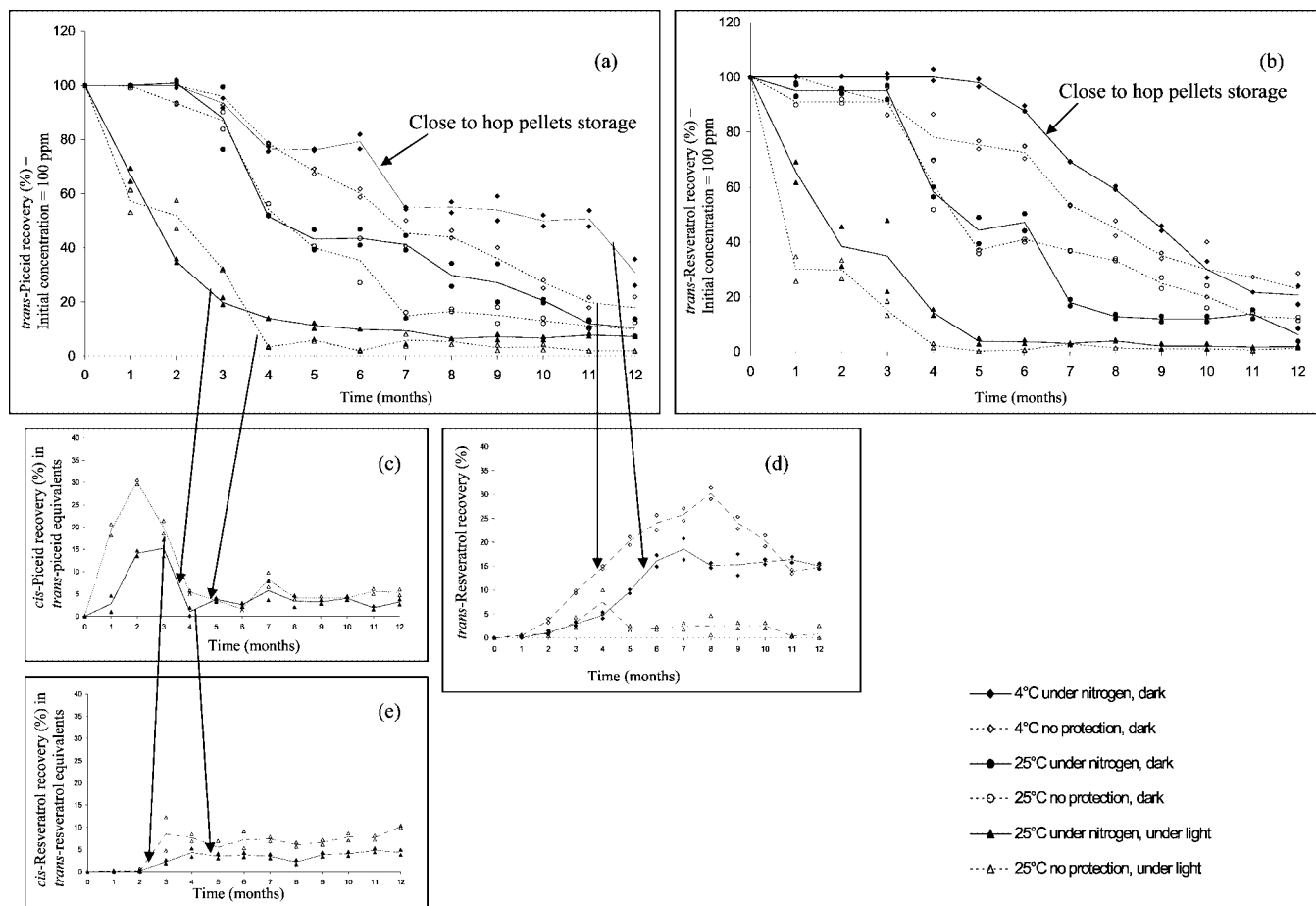
**Extraction of Stilbenes from Hop.** This method has been developed in our laboratory for *trans*-resveratrol and *trans*-piceid extraction (4). All extraction steps have been performed with protection against day light, in duplicate. Hop cones or pellets were crushed in a mortar. Ground samples (2.5 g) were extracted, in successive 10 min steps at room temperature under gentle stirring, 3 times with 50 mL of toluene and 3 times with 50 mL of cyclohexane, to remove hydrophobic compounds. At the end of each step, the sample was centrifuged for 10 min at 3000g. At the last step, hop powder was dried under vacuum to get rid of the residual solvent. Delipidated hop powder was extracted 3 times with 40 mL of 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 3000g 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 a 50:50 (v/v) mixture of ethanol/water. For spent hop, only the ethanol extraction procedure was applied. For viniferins, 10 g of ground samples were just cleaned 3 times with 50 mL of cyclohexane and then extracted 3 times with 40 mL of methanol/water (70:30, v/v), each time for 10 min under gentle stirring at 60 °C.

**Assessment of *trans*-Resveratrol and *trans*-Piceid Stability.** Two stock solutions of *trans*-resveratrol and *trans*-piceid (100 ppm) have been prepared in water/ethanol (96:4, v/v). The stability of the phytoalexins was tested under various conditions: (1) 25 °C with

exposure to white light, (2) 25 °C with exposure to white light under nitrogen, (3) 25 °C in the dark, (4) 25 °C in the dark under nitrogen, (5) 4 °C in the dark (refrigerator), and (6) 4 °C in the dark (refrigerator) under nitrogen. Aliquots of each sample were taken every months for 1 year.

**Reverse-Phase High-Performance Liquid Chromatography–Tandem Mass Spectrometry (RP-HPLC–MS/MS) Analysis of Stilbenes.** Quantifications were performed on a C18 Prevail column (150 × 2.1 mm, 2  $\mu$ m) (Alltech, Deerfield, IL) eluted with a linear gradient from water (containing 1% acetonitrile and 0.1% formic acid) to acetonitrile. The gradient elution was as follows: from 95% water to 55% in 23 min, from 55 to 0% in 7 min, and isocratic for 10 min, at a flow rate of 200  $\mu$ L/min. A total of 10  $\mu$ L of sample were 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 atmospheric pressure chemical ionization (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; and discharge current, 5  $\mu$ A. After the first monitoring on the  $m/z$  229 for resveratrol and piceid and on the  $m/z$  455 for viniferins, collision-induced dissociation spectra were recorded at 37% relative collision energy. For viniferins, an ESI source in negative mode was also tested: after the first monitoring on the  $m/z$  453, collision-induced dissociation spectra were recorded at 37% relative collision energy. The following conditions were applied: source voltage, 4.5 kV; capillary voltage, 36 V; capillary temperature, 225 °C; sheath gas, 70 psi; and auxiliary gas, 20 psi.

**Determination of  $\alpha$ -Acids in Hop.** The content of  $\alpha$ -acids in hop cones, pellets, extracts, and spents was determined by HPLC–UV according to the Analytica EBC.



**Figure 4.** Evolution of stilbenes in aqueous solutions under various conditions. (a) Model medium spiked with *trans*-piceid, (b) model medium spiked with *trans*-resveratrol, (c) *cis*-piceid, (d) *trans*-resveratrol, and (e) *cis*-resveratrol formation in the *trans*-piceid spiked media stored under light (c and e) or in the dark (d).

## RESULTS AND DISCUSSION

**Stability in Hop Cones.** The *trans*-piceid and *trans*-resveratrol concentrations of six leaf hop varieties (harvest 2004) were monitored over 12 months of storage by RP-HPLC-APCI(+)-MS/MS (4). As depicted in **Figure 1**, concentrations ranging from 4.5 to 11 mg/kg *trans*-piceid and up to 1 mg/kg *trans*-resveratrol were measured in the fresh cones (0 month of storage). A total of 4 months of storage at 4 °C proved sufficient to strongly affect the stilbene content of leaf hop (39–88% degradation), despite the vacuum (after nitrogen flushing) in each package. In some samples (e.g., Nugget), *trans*-piceid seemed more affected than its aglycon, perhaps because *trans*-resveratrol is partially regenerated from the glycoside (22).

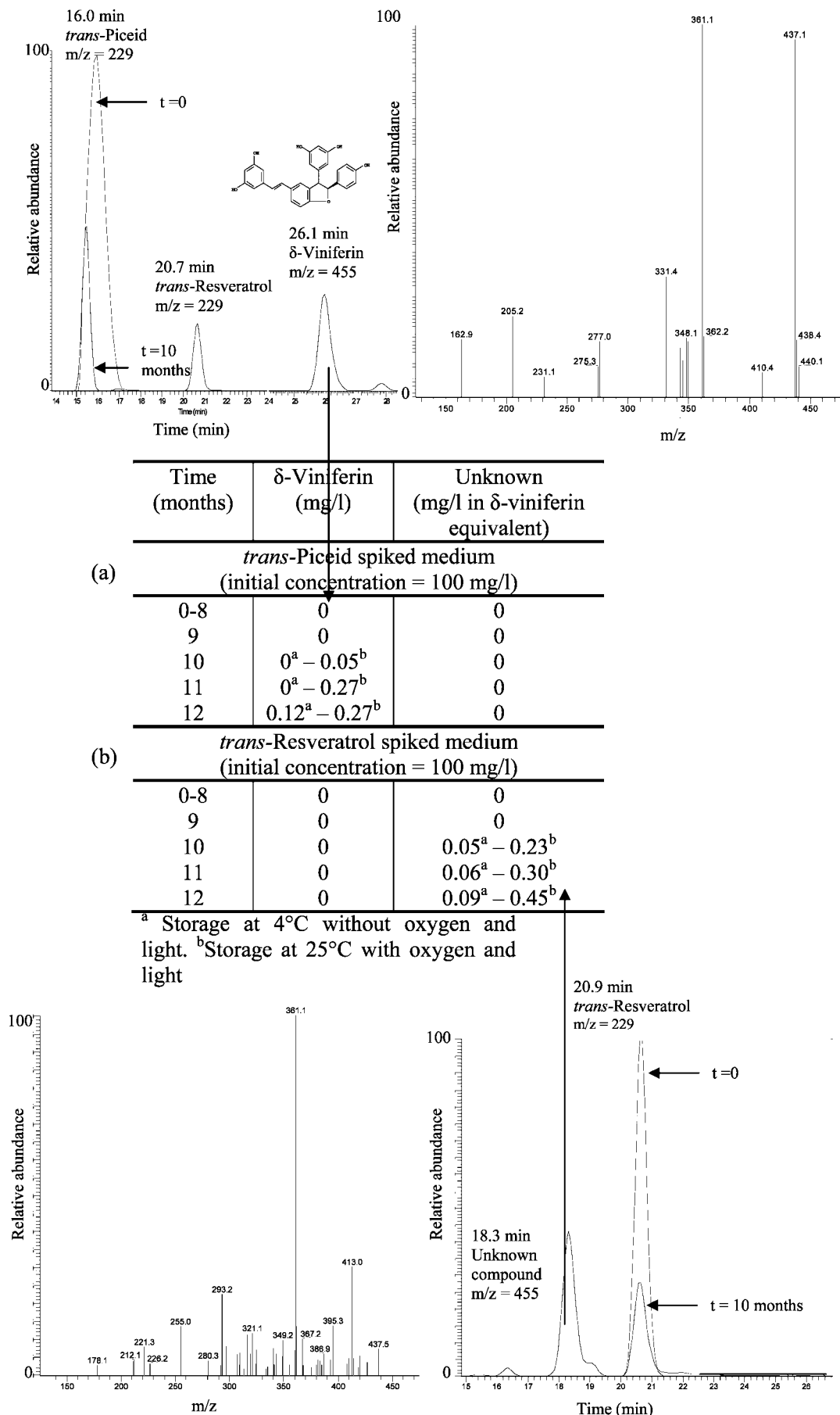
The presence of a new peak identified as *cis*-resveratrol in all chromatograms of >4 months-stored samples (number 4 in **Figure 2**; mass spectrum similar to that of *trans*-resveratrol) also indicates a release from *cis*-piceid (conversion from *trans*-resveratrol only light-induced). Likewise, Cantos et al. (23) have observed increased *trans*- and *cis*-resveratrol contents in grapes stored at 0 °C for 10 days.

Expressed in *trans*-resveratrol equivalents, up to 1.2 mg/kg *cis*-resveratrol was formed in Willamette hop cones over 8 months at 4 °C. Over the same period, 6.5 mg/kg of *trans*-stilbenes disappeared, indicating that other degradation products must exist. *trans*-Resveratrol is known to dimerize oxidatively in plant cells to  $\epsilon$ -viniferin (24, 25) or  $\delta$ -viniferin (26) through the action of peroxidases or phenoloxidases. Although viniferins are easily biosynthesized in model media (27–31), Prokop et al. (32) found no resveratrol dehydromer or viniferins in

whole-grape extract stored for 2 years. Logically, we therefore looked for these viniferins in 4-, 8-, and 12-month-aged Willamette cones.  $\epsilon$ -Viniferin [ $t_R = 24.1$  min, HPLC-MS/MS-APCI(+) fragments of  $m/z$  455 at  $m/z$  437, 361, and 349 (33)] and  $\delta$ -viniferin [ $t_R = 26.1$  min, HPLC-MS/MS-APCI(+) fragments of  $m/z$  455 at  $m/z$  437 and 361 (33)] proved to be absent, even when an adapted procedure [a cleaning step with only cyclohexane and extraction with 70:30 methanol/water (v/v) at 60 °C] was applied for these less polar dimers (recovery factor calculated by standard addition = 72% versus only 5% with the unmodified hop stilbene extraction procedure). The use of HPLC-MS/MS in ESI(-) mode (more suitable for compounds, such as viniferins, having a high molecular weight, fragments of  $m/z$  453 at  $m/z$  435, 411, 369, and 333 for  $\epsilon$ -viniferin and at  $m/z$  359, 347, and 435 for  $\delta$ -viniferin (33)) confirmed these results. Investigations on other oligostilbenes should be performed in the future (34, 35).

### Stability during Pelletization and through Pellet Storage.

Relative stilbene concentrations in fresh pellets (0 month of storage) derived from the six cone samples described above are depicted in **Figure 3**. Pelletization induced strong stilbene degradation in some cultivars (>50% in Willamette or Tomahawk), whereas other varieties (Warrior or Nugget) proved much more resistant. However, pellets emerged as more stable than leaf hop during the first 4 months of storage (0–56% degradation). Again, the glycoside seemed to be more abundantly lost. After 8 months of storage, the overall stilbene content was in the same range whatever the conditioning (values in hop cones given by  $\blacklozenge$ ).



**Figure 5.** Concentrations of  $\delta$ -viniferin and the unknown compound at  $t_R = 18.3$  min in model media spiked with (a) *trans*-piceid and (b) *trans*-resveratrol. Corresponding RP-HPLC-APCI(+)-MS/MS chromatograms and mass spectra for spiked media kept 10 months at 25 °C under light and without a protected atmosphere.



**Table 1.** Stilbene Content in Fresh CO<sub>2</sub> Extract and Spent Samples<sup>a</sup>

| hop variety | <i>trans</i> -piceid (mg/kg) |       | <i>trans</i> -resveratrol (mg/kg) |       |
|-------------|------------------------------|-------|-----------------------------------|-------|
|             | CO <sub>2</sub> extract      | spent | CO <sub>2</sub> extract           | spent |
| Warrior     | <0.05                        | 7.7   | <0.05                             | 0.7   |
| Tomahawk    | <0.05                        | 6.9   | <0.05                             | 0.7   |

<sup>a</sup> Assay in duplicate, variation coefficient under 5%.

**Stability in Aqueous Model Media.** The stability of *trans*-piceid and its aglycon was monitored in water for 1 year under six different model conditions: 25 °C with exposure to white light, 25 °C in the dark, and at 4 °C in the dark, all with or without a nitrogen atmosphere (the highest protected medium is supposed to mimic hop storage conditions).

As depicted in **Figure 4a**, *trans*-piceid proved stable for 2 months at 4 or 25 °C if protected from white light and oxygen. The absence of light is the most important requirement, but oxygen and a temperature above 4 °C must be also avoided (35). In the highest protected conditions, 76 and 31% were recovered after 4 and 12 months, respectively. The degradation rate found in hop cones was much faster (close to the model media kept at 25 °C, ~50% recovery after 4 months), while pellets followed the 4 °C kinetics. Some hop components (pro-oxidants, etc.), partially degraded during the pelletization process, most likely influence the stability of stilbenes. In highly protected conditions (4 °C, dark, under nitrogen), *trans*-resveratrol (**Figure 4b**) emerged as a bit more stable than the glucoside (undegraded for 5 months). On the other hand, unprotected led to a strongest instability for the aglycone (70% degradation versus 43% for piceid after 1 month at 25 °C, without nitrogen, under light).

Under light, *cis*-piceid was recovered after 1 month in the *trans*-piceid spiked medium (**Figure 4c**) and, as in the case of *trans*-piceid (**Figure 4d**), release of *trans*-resveratrol in *trans*-piceid spiked medium), the free form *cis*-resveratrol was detected 2 months later (**Figure 4e**). In media containing both *cis*-piceid and *trans*-resveratrol (the case of hop samples), *cis*-resveratrol could be formed under light both by the release from its glucoside and by light conversion from *trans*-resveratrol.

Although not found in hop,  $\delta$ -viniferin was detected after a long storage (10 months with light and no protection at 25 °C and 12 months without light and under nitrogen at 4 °C) in the model medium spiked with *trans*-piceid [see **Figure 5a**,  $t_R$  = 26.1 min, fragments of  $m/z$  455 at  $m/z$  437 and 361 in APCI(+)]. On the other hand, it revealed absent in all of the *trans*-resveratrol spiked media, while another compound, probably also a dimer, was detected [**Figure 5b**,  $t_R$  = 18.3 min, fragments of  $m/z$  455 at  $m/z$  361 and 413 in APCI (+)].

**Stilbenes in Supercritical Hop Extracts and Spents.** Because of the high hydrophobicity of carbon dioxide, no stilbenes were detected in supercritical hop extracts (these are even more apolar than hop flavonoids, also recovered in a low amount in such extracts (21); see **Table 1**). On the other hand, very interesting were the levels detected in the two investigated spent samples: *trans*-piceid and *trans*-resveratrol reached, respectively, 7.7 and 0.7 mg/kg, in the Warrior sample. Spent hop thus emerges as a very promising, cheap, delipidated raw material for the production of resveratrol-enriched hop extracts.

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