Jerkovic, V. and Collin, S.

The *cis*-resveratrol Concentration is Proposed as a New Indicator of the Hop Freshness

***trans*-Piceid, *cis*-piceid and *trans*-resveratrol contents of hop cones and hop pellets from six American varieties (harvest 2004) were monitored by RP-HPLC-APCI(+)-MS/MS over 12 months of storage. *trans*-Resveratrol, *cis*-piceid and *trans*-piceid were found in all samples. After 8 months of storage, the overall stilbene content was decreased in the same range whatever the conditioning. Absent in fresh hop cones or pellets, *cis*-resveratrol was released from *cis*-piceid in all stored samples. *cis*-Resveratrol concentration revealed very interesting for assessing hop freshness.**

Descriptors: resveratrol, stilbene, polyphenols, hop conditioning, hop storage, resveratrol stability

1 Introduction

In the brewing industry, female inflorescences of hop plant (*Humulus lupulus*, L.) are processed in several different ways, yielding cones (leaf hop), pellets, isomerised pellets, organic solvent extracts, supercritical, isomerised and reduced extracts [1]. According to the conditioning, hop can impart bitterness, flavor, antioxidant activity, foam stability and bacteriostatic activity to beer [2, 3]. After harvest in the autumn, hop is dried (from 75–80 % moisture to below 10 % [4]) at a temperature close to 60 °C [1, 3, 5]. Even compressed in bales, hops cannot be stored for a long time without protected atmosphere because of flavor oxidation and a decrease in α-acid content [1, 4–9]. It is therefore very usual to decrease the volume by pelletizing dried milled hop [10]. In order to prevent degradation, hop pellets are then stored under vacuum (after nitrogen flushing) between –2 and 4 °C [6, 7, 11–14]. Under these conditions, the α-acid content remains relatively constant during one-year storage [14], whilst the essential oil content is inevitably affected after a few months (e.g. : farnesene in aromatic varieties) [15]. Polyphenol oxidation [7] may also take place, depending on the variety of hop. Cold storage doesn't prevent deterioration but slows down oxidation [14].

The hop storage index (HSI), defined as the ratio of absorbance at 275 nm to the absorbance at 325 nm of an alkaline methanolic solution of a non-polar extract of hop, is usually used to indicate hop freshness [16]. The absorption values of α- and β-acids extracts are maximal at 325 nm and minimal at 275 nm. Oxidised α- and β-acids extracts have a maximum absorption at about 250–280 nm. Therefore, hop oxidation induces an HSI increase. However, the variation between samples is sometimes very scarce and the specificity of this assay is insufficient.

In 2005, *Callemien et al.* [17] mentioned for the first time the presence of three cardioprotective stilbenes [18] in hop: *trans*-resveratrol, *trans*-piceid and *cis*-piceid (total concentration close to 3.5 ppm). In hop pellets, *Jerkovic et al.* [19] measured stilbene concentrations from 5 to 16 ppm mg/kg, *trans*-piceid being in all cases the major constituent (4 to 8.8 mg/kg). Among the hop cultivars investigated, it appeared that the lower the pellets α-acid content, the higher the resveratrol potential, except in highly oxygen-sensitive varieties [19]. Recently, *Jerkovic* and *Collin* [20] evidenced the huge impact of the harvest year. No stilbenes were detected in supercritical hop extracts. Spent hop thus emerged as a very cheap and delipidated raw material for the production of resveratrol-enriched hop extracts [21].

The aim of the present work was to evidence a new indicator of the hop freshness, taking advantage of the instability of hop stilbenes. American varieties were analyzed through storage in two differently conditioned forms, all under protected atmosphere: leaf hop and derived pellets.

2 Experimental

2.1 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 and T90 pellets were industrially produced and vertically sampled from the same batches. Pelletisation took place within a week after the harvest. All samples were stored at 4 °C under inert atmosphere until needed.

2.2 Chemicals

Ethanol (97 %) was obtained from Belgaco (Gent, Belgium). Acetonitrile (99,99 %), toluene (97 %) and cyclohexane (99,96 %)

Authors:

Vesna Jerkovic, Sonia Collin, Unité de Brasserie et des Industries Alimentaires, Faculté d'Ingénierie biologique, agronomique et environnementale, Université catholique de Louvain, Louvain-la-Neuve, Belgium, sonia.collin@uclouvain.be

Tables and figures see Appendix

were supplied by Fisher Scientific (UK). Formic acid (pa) was obtained from Aldrich (Germany). Methanol (99.9 %) and diethyl ether (99 %) was supplied by Romil (Cambridge, UK). Aqueous solutions were made with Milli-Q (Millipore, Bedford, MA, USA) water. *trans*-Resveratrol (99 %) and *trans*-piceid (97 %) were supplied by Sigma-Aldrich (Bornem, Belgium). *cis*-Resveratrol was synthesized from *trans*-form under UV light. δ-Viniferin and ε-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).

2.3 Extraction of stilbenes from hop [17]

All extraction steps have been done 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, three times with 50 ml toluene and three times with 50 ml cyclohexane, in order to remove hydrophobic compounds. At the end of 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 three 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 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.

2.4 RP-HPLC-MS/MS analysis of stilbenes

Quantifications were performed on a C18 Prevail column (150 x 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 % water to 55 % in 23 min, 55 % to 0 % in 7 min, and isocratic for 10 min at a flow rate of 200 μl/min. Ten microliters 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 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 for resveratrol and piceid and on the m/z = 455 for viniferins, collision-induced dissociation spectra were recorded at 37 % relative collision energy.

2.5 Determination of α-acids in hop

The content of α-acids in hop cones and pellets was determined by HPLC-UV according to the Analytica EBC (2005).

2.6 Determination of the hop storage index

The hop storage index was determined according to the ASBC H-6,12 method (1992).

3 Results and discussion

3.1 Stability of stilbenes in hop cones and pellets.

The *trans*-piceid, *cis*-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 [17]. As depicted in Figure 1, concentrations ranging from 4.5 to 11 mg/kg *trans*-piceid, 2.1 to 6.1 mg/kg *cis*-piceid (in *trans*-piceid equivalents) and up to 1 mg/kg *trans*-resveratrol were measured in the fresh cones (0 month of storage). Four months of storage at 4 °C proved sufficient to strongly affect the stilbene content of leaf hop (30–80 % degradation), despite the protective atmosphere in each package. In some samples (e.g. Nugget), *trans*-piceid seemed more affected than its aglycon, probably because *trans*-resveratrol is partially regenerated from the glycoside [21].

Relative stilbene concentrations in fresh pellets (0 month of storage) derived from the six cones samples described above are depicted in Figure 2. Concentrations ranging from 1.7 to 5.8 mg/kg *trans*-piceid, 0.9 to 5.0 mg/kg *cis*-piceid (in *trans*-piceid equivalents) and less than 1 mg/kg *trans*-resveratrol were found before storage. Pelletization induced strong stilbene degradation in some cultivars (> 50 % in Willamette or Tomahawk), whereas other varieties (Warrior or Nugget) proved much more resistant. Yet pellets emerged as more stable than leaf hop during storage (0–50 % degradation after the first four months). After 8–12 months of storage, the residual piceid content was in the same range whatever the conditioning.

3.2 Identification of degradation products.

Strong stilbene degradation, especially in cones, was observed during storage. The presence of a new peak identified as *cis*-resveratrol in all chromatograms of 8 and 12 months-stored samples (number 4 in Fig. 3b; mass spectrum similar to that of *trans*-resveratrol) indicates a possible release from *cis*-piceid (conversion from *trans*-resveratrol only light-induced) [21]. Expressed in *trans*-resveratrol equivalents, up to 1.2 mg/kg *cis*-resveratrol was formed in Willamette hop cones over eight months at 4 °C (Fig. 4a). Over the same period, 4.3 mg/kg of cis-piceid and 6.9 mg/kg of *trans*-stilbenes disappeared, indicating that other degradation products must exist (Fig. 1). *trans*-Resveratrol is also known to dimerize oxidatively in plant cells to ε-viniferin or δ-viniferin through the action of peroxidases or phenoloxidases [22]. We therefore looked for these viniferins in 4-, 8-, and 12-months-aged Willamette cones. ε-Viniferin [tR = 24.1 min, HPLC-MS-MS-APCI (+) fragments of m/z = 455 at m/z = 437, 361, and 349 [23]] and δ-viniferin [tR = 26.1 min, HPLC-MS-MS-APCI (+) fragments of m/z = 455 at m/z = 437 and 361] proved to be absent. In hop pellets, *cis*-resveratrol was also generated from *cis*-piceid through storage but, logically, in lower amounts (Fig. 4b).

3.3 *cis*-resveratrol as indicator of the hop freshness.

Table 1 gives the HSI values of the six here-investigated hop cultivars after 8 months of storage. HSI values from 0.28 to 0.33 usually indicate that hop has been well handled. In both conditionings, only our Willamette sample (well known for its sensitivity

against oxidation [19]) clearly emerged in this HSI assay as more degraded, the five other samples exhibiting very close values.

After 8 months, *cis*-resveratrol was detected in all samples, even in pellets. Its amount still increased between 8 and 12 months (Fig. 4b). Aged-Willamette emerged as the richest in *cis*-resveratrol in both conditionings (2.1 and 0.3 mg/kg after 12 months in leaf hop and pellets, respectively). Therefore, *cis*-resveratrol could be used as another interesting indicator of hop freshness.

4 Conclusions

trans-Resveratrol, *cis*-piceid and *trans*-piceid were found in all fresh leaf hops and pellets samples. After 8 months of storage, the overall stilbene content was in the same range whatever the conditioning. No viniferin was detected in aged hop samples. On the other hand, *cis*-resveratrol revealed to be released from *cis*-piceid. The *cis*-resveratrol concentration can be proposed as a new indicator of hop freshness. Significant higher values are measured in leaf hop samples.

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Appendix

Table 1 **Hop Storage Index (HSI) in hop cones and pellets after 8 months of storage (determination by ASBC H-6,12 method (1992)).**

Variety	Cones	Pellets
Willamette	0.39	0.38
Tomahawk	0.33	0.33
Cascade	0.33	0.31
Nugget	0.31	0.31
Simcoe	0.31	0.31
Warrior	0.28	0.33

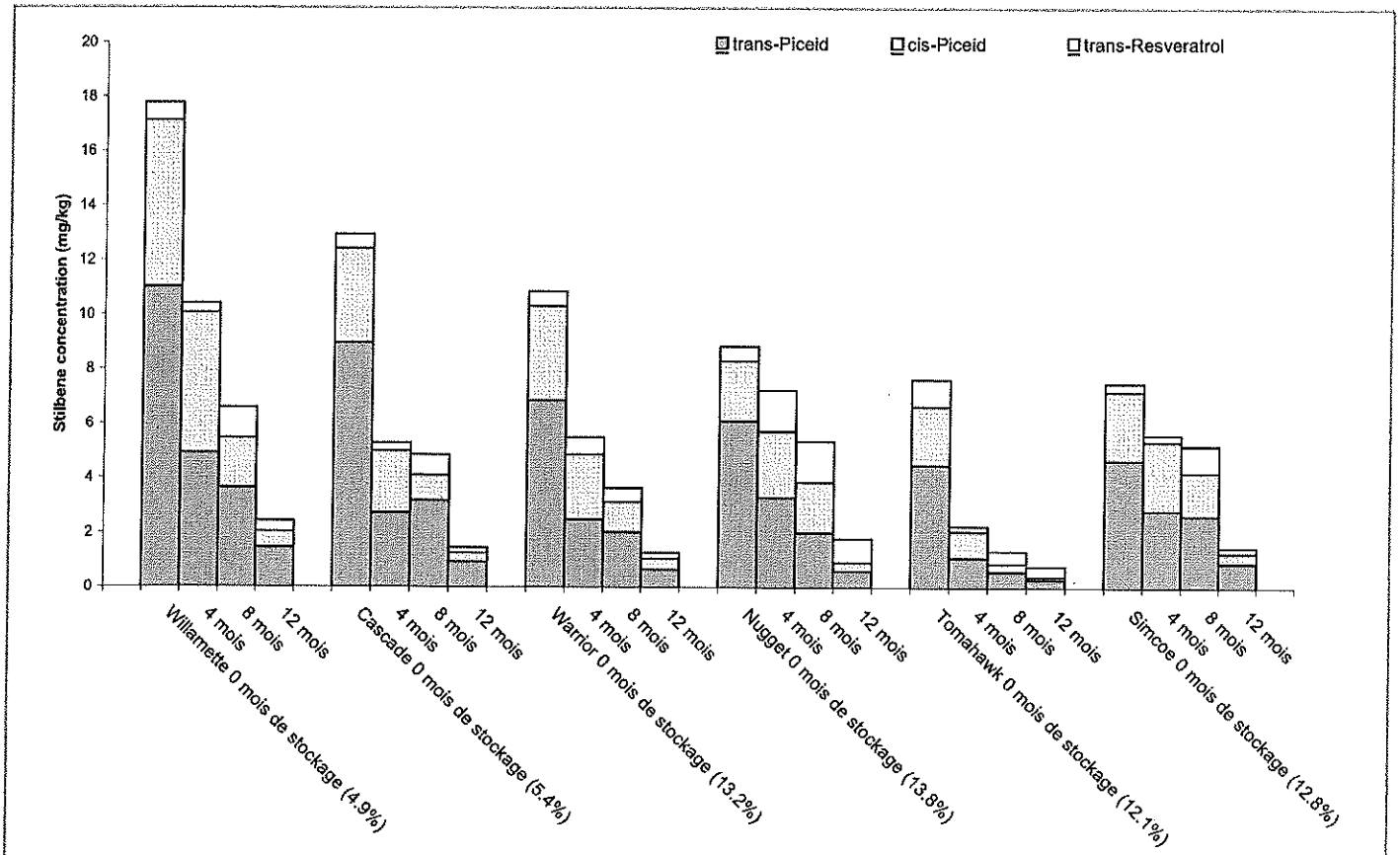


Fig. 1 **Concentrations of stilbenes in hop cones through storage. α-Acid content (%) given between brackets. Assay in duplicate. Standard deviation < 7 %**

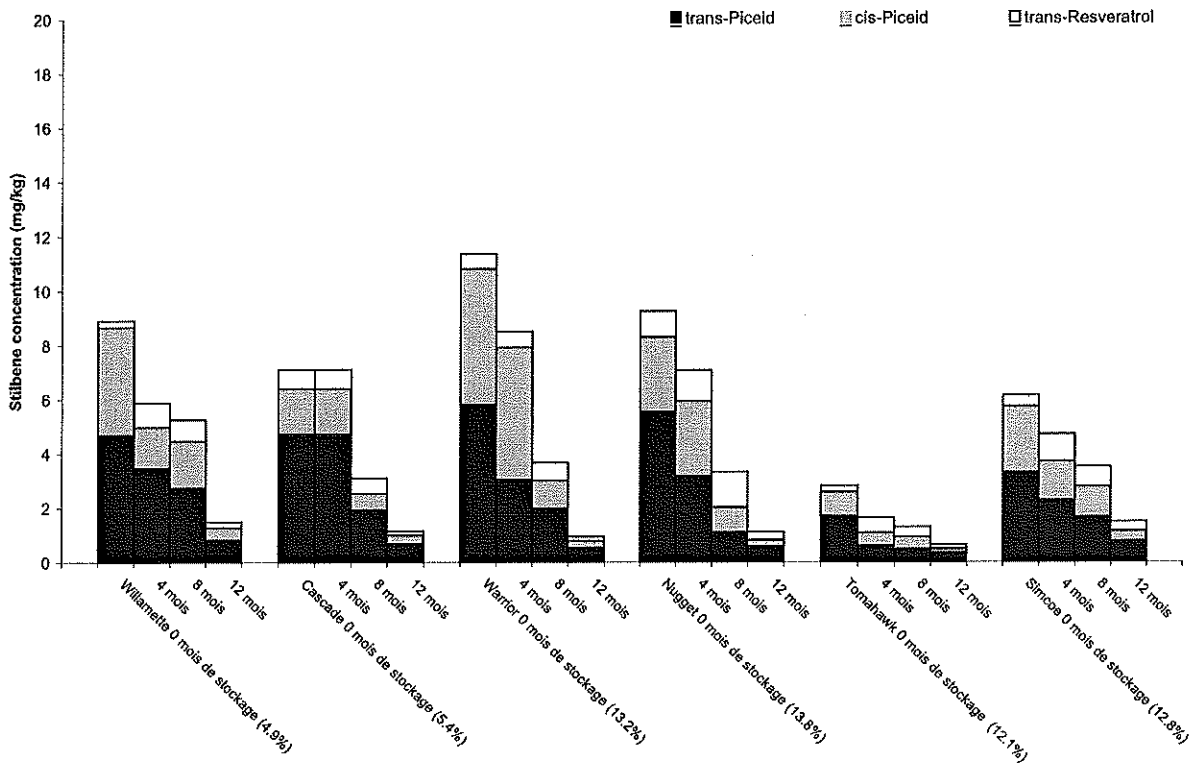


Fig. 2 **Concentrations of stilbenes in hop pellets through storage. α-Acid content (%) given between brackets. Assay in duplicate. Standard deviation < 7 %**

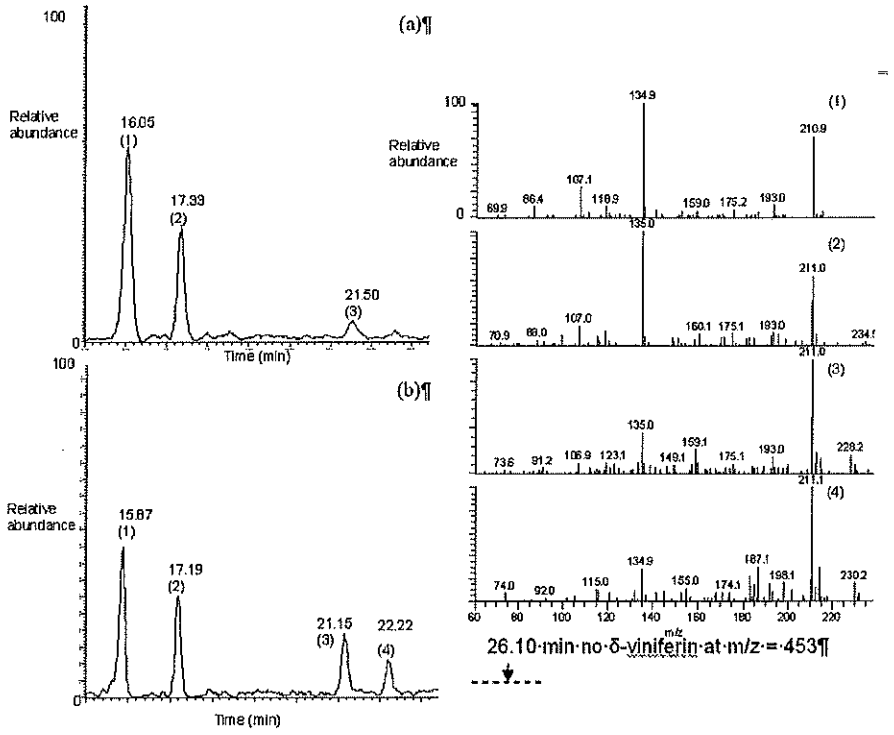
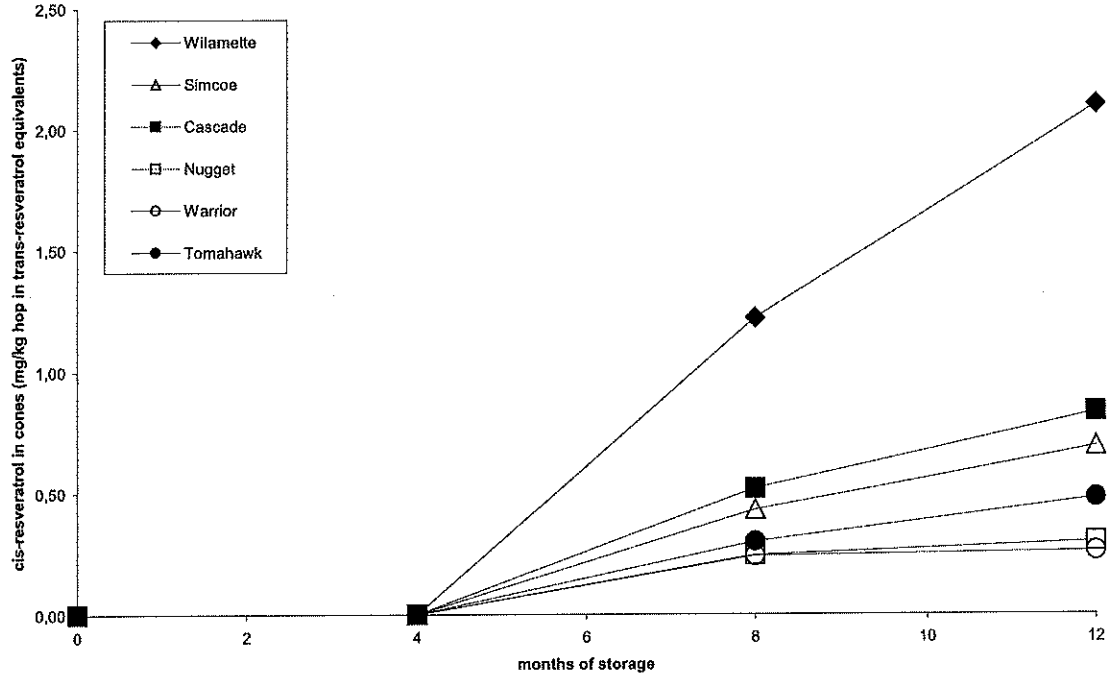
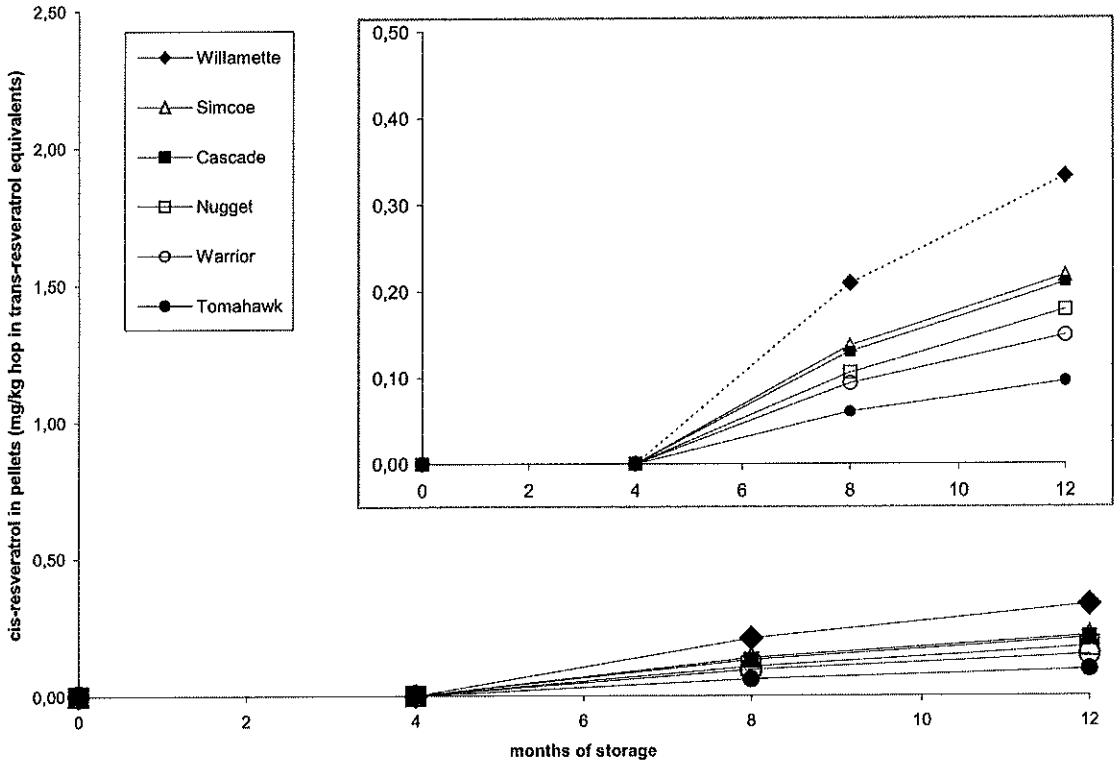


Fig. 3 **RP-HPLC-APCI (+)/MS-MS data of Willamette hop (harvest 2004) (a) dried cones just after harvest. (b) cones after 8 months of storage at 4°C. MS/MS chromatogram (m/z = 229) and experimental mass spectra for *trans*-piceid (1), *cis*-piceid (2), *trans*-resveratrol (3), and *cis*-resveratrol (4)**



(a)



(b)

Fig. 4 **Concentrations of *cis*-resveratrol (*trans*-resveratrol equivalents) in (a) hop cones and (b) hop pellets through storage. Standard deviation < 7%**