Resveratrol analogues in hop, malt and adjuncts

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Descriptors:
Hops, HPLC, malt, polyphenol, sorghum

SUMMARY

Resveratrol, already identified in wine and other food matrices, seems to be linked to anti-carcinogenic, anti-viral, anti-oxidant, anti-inflammatory, and estrogenic activities. The aim of the present work was to investigate stilbenes in hop, malt, and adjuncts. Hop emerged as by far the most interesting raw material in terms of polyphenol concentration and profile. Up to 10 ppm *trans*-resveratrol, *trans*-piceid, and *cis*-piceid were found in some varieties. Furthermore, an analogue of pterostilbene was detected at concentrations up to 1 ppm. This compound is interesting in the perspective of increasing beer health potential. As stilbene synthase appears absent from hop, closely related chalcone synthases are most probably involved in stilbene synthesis. For the first time, traces of *trans*-resveratrol and *trans*-piceid were also detected in red sorghum. On the other hand, no stilbene was found in malt, barley, corn, or white sorghum.

INTRODUCTION

Although hop polyphenols have been widely studied in the last decade for their antioxidant activity in the boiling kettle (1), very little is yet known about their real impact on health. Resveratrol (trans-3,4',5-trihydroxystilbene), already identified in wine and other food matrices, seems to be linked to anti-carcinogenic, anti-viral, anti-oxidant, anti-inflammatory and estrogenic activities (2).

Free *trans*-resveratrol and its glucosides *trans*-piceid and *cis*-piceid have been recently found in hop cones, hop pellets, and spent hop from CO_2 extraction (3-5). Resveratrol was quantified by HPLC-APCI-MS/MS (3) and, as previously shown for total polyphenols and flavonoids (1), the low α-acid varieties such as Saaz, Cascade, and Willamette emerge as the most interesting, especially when stilbene concentrations are given for a same bitterness potential (6). However, as for other phytoalexins, a strong influence of harvest year and growing area has been observed (6).

In hop, concentrations of *trans*-resveratrol and *trans*-piceid range from 0.2 to 1 ppm and from 4 to 9 ppm, respectively (3, 4). Pelletisation induces extensive stilbene degradation in some cultivars (5). Similarly, one-year storage at 4 °C has been shown to cause a huge loss of *trans*-resveratrol and its glucoside, especially in the case of hop cones (5).

The aim of the present paper was to investigate the presence of stilbenes in various beer raw materials. In addition, in order to understand how stilbenes are synthesised in hop, we have sought to detect stilbene synthase activity.

ANALYTICAL METHODS

Materials

Hop samples from the harvest 2006 were a kind gift from Yakima Chief (Louvain-la-Neuve, Belgium) and from the Hop Growers Union of the Czech Republic (Zatec, Czech Republic). All samples were stored under vacuum at 4 °C until needed. Barley, malt, wheat, corn and sorghum were a kind gift of the Centre de Référence pour la Qualité des Malts et de la Bière (Louvain-la-Neuve, Belgium). All samples were stored at room temperature until needed.

Chemicals

Ethanol was obtained from Belgaco (Belgium). Acetonitrile, toluene, hexane and cyclohexane were supplied by Fisher Scientific (UK). Methanol was supplied by Romil (UK). *trans*-Resveratrol, *trans*-piceid, *trans*-piceatannol, *trans*-pterostilbene and formic acid were supplied by Sigma-Aldrich (Belgium). *trans*-Astringin and *trans*-pinosylvin were obtained from Sequoia Research Products (UK). Aqueous solutions were made with Milli-Q (Millipore, USA) water.

Extraction of stilbenes from hop and cereals

All extraction steps have been done in duplicate with protection against day light. The method was initially developed to analyze stilbenes in hop (3). All samples were crushed in a mortar. Hop ground samples (2.5 g) were delipidated, in successive 10 min steps at room temperature under gentle stirring, three times with 50 ml toluene and three times with 50 ml cyclohexane. For cereals, hydrophobic compounds were removed by successive washings with hexane. At the end of each step, the sample was centrifuged for 10 min at 3000 g. At the last step, the powder was dried under vacuum to get rid of residual solvent. Delipidated 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 a 50:50 (v/v) mixture of ethanol:water.

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, 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, 3V; capillary temperature, 175 °C; sheath gas, 40 psi; auxiliary gas, 7 psi; discharge current 5 µA. After the first monitoring on the m/z = M+1, collision-induced dissociation spectra were recorded at 37 % relative collision energy.

RESULTS AND DISCUSSION

Search for stilbenes in malt, barley, and different brewing adjuncts

The recent discovery of a stilbene synthase in cereals (7) led us to investigate stilbenes in barley, malt, wheat, corn, and sorghum. Hexane was used for delipidation. In standard addition experiments, recovery of *trans*-piceid was 92 % and recovery of *trans*-resveratrol was 75 % (ethanol:water, 80:20 v/v at 60 °C for stilbene extraction). For the first time, traces of stilbene (0.5-1 ppm) were detected by HPLC-MS/MS-APCI(+) in red sorghum (selection of M+1 = 229 for resveratrol and piceid, 245 for piceatannol, 257 for pterostilbene, 213 for pinosylvin, 421 for rhaponticin, 407 for astringin) (figure 1). No stilbenes were found in barley, malt, corn, or white sorghum.

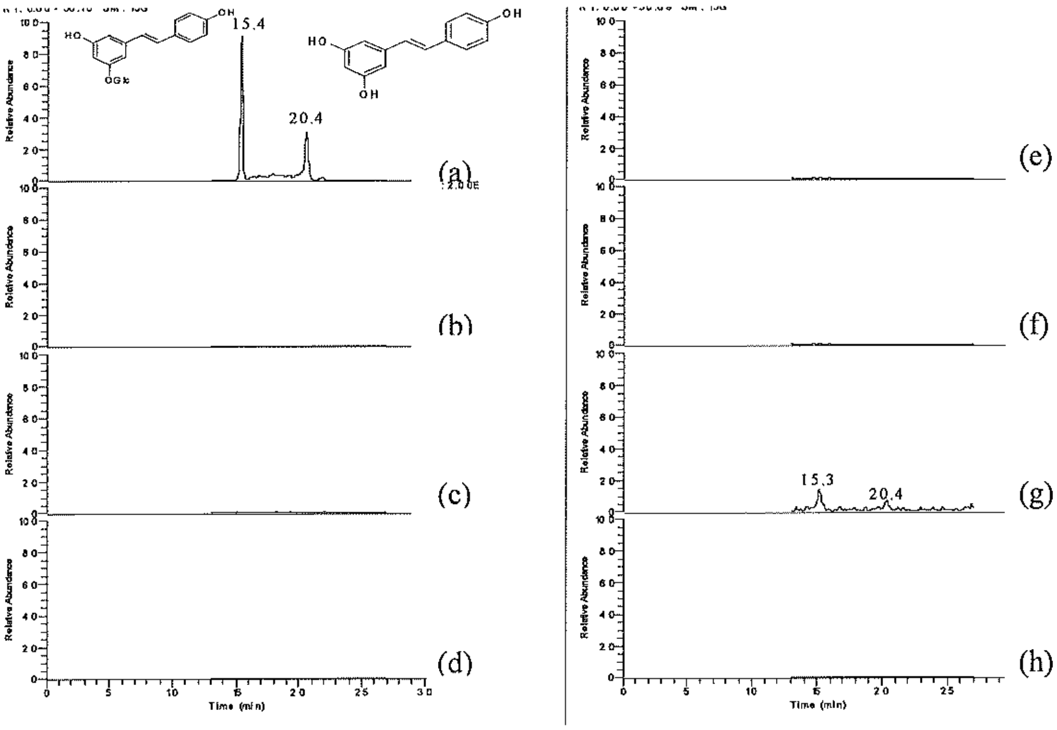


Figure 1: RP-HPLC-APCI(+)-MS/MS chromatogram of: a pale malt extract spiked (a) or not (b) with *trans*-piceid (tr = 15.4 min) and *trans*-resveratrol (tr = 20.4 min); (c) a dark malt extract; (d) a barley extract; (e) a corn extract; (f) a white sorghum extract; (g) a red sorghum extract; (h) a wheat extract.

Search for other stilbene analogues in hop samples

As expected (3), *trans*-piceid, *cis*-piceid, and *trans*-resveratrol were identified in the eight hop cone samples (American and European varieties) from harvest 2006 (figure 2). *trans*-Piceid ranged from 2.3 to 7.3 ppm and 0.7 to 2.2 ppm free resveratrol was detected. Worth stressing is the presence in all samples of another stilbene, closely related to *trans*-pterostilbene (same M+1 = 257, peak (d) in figure 2), at a concentration near 1 ppm in pterostilbene equivalents. Its retention time was 1 minute longer than that of *trans*-pterostilbene (31 min). By comparison to a stilbene library, this compound appears not to be *trans*-5-hydroxy-3,4'-dimethoxystilbene (t_R = 31.2 min). Complementary analyses will be needed to identify which isomer it might be.

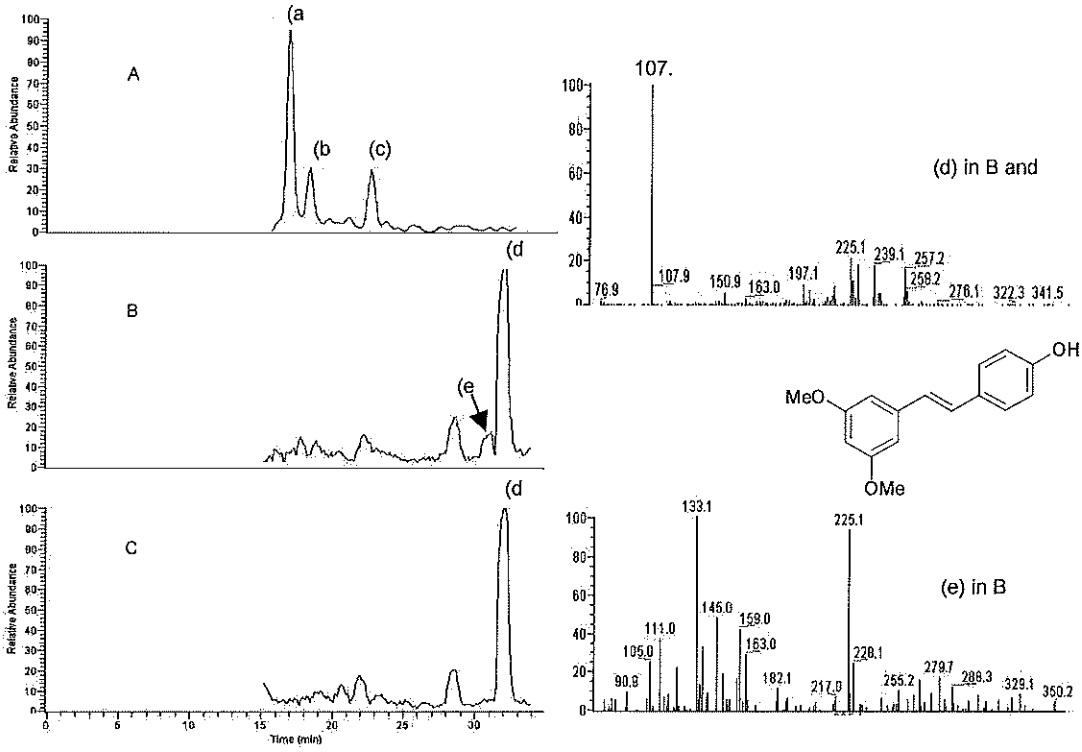


Figure 2: RP-HPLC-APCI(+)-MS/MS chromatogram and experimental mass spectra of a Tomahawk 2006 hop extract (A) selection at m/z = 229 (B) selection at m/z = 257 after a standard addition before extraction of 1 ppm of *trans*-pterostilbene (C) selection at m/z = 257 (pterosilbene + 1) (a) *trans*-piceid, (b) *cis*-piceid, (c) *trans*-resveratrol (d) = the unknown (e) *trans*-pterostilbene.

Search for a stilbene synthase in hop

With selected primers (table 1) we were able to amplify by PCR a sequence in hop DNA. As stilbene synthase is well characterised in grapevine, we used grape DNA as a control (figure 3).

Table 1: Degenerate primers used for PCR amplification of a Stilbene synthase (STS) in hop. Between brackets are given degenerate nucleotides.

STS forward	5'-AA(AG) GA(GA) TGG GG(TCA) CA(AG) CC(ATC) A(AT)(AG) TC-3'
STS reverse	5'-AC(TGA)CC CCA (TGA)TC (AC)A(GA) TCC (ACT)TC-3'

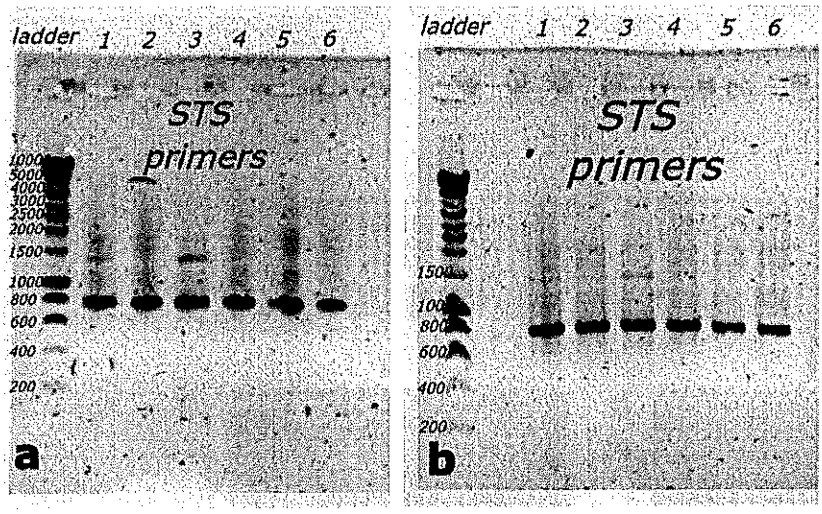
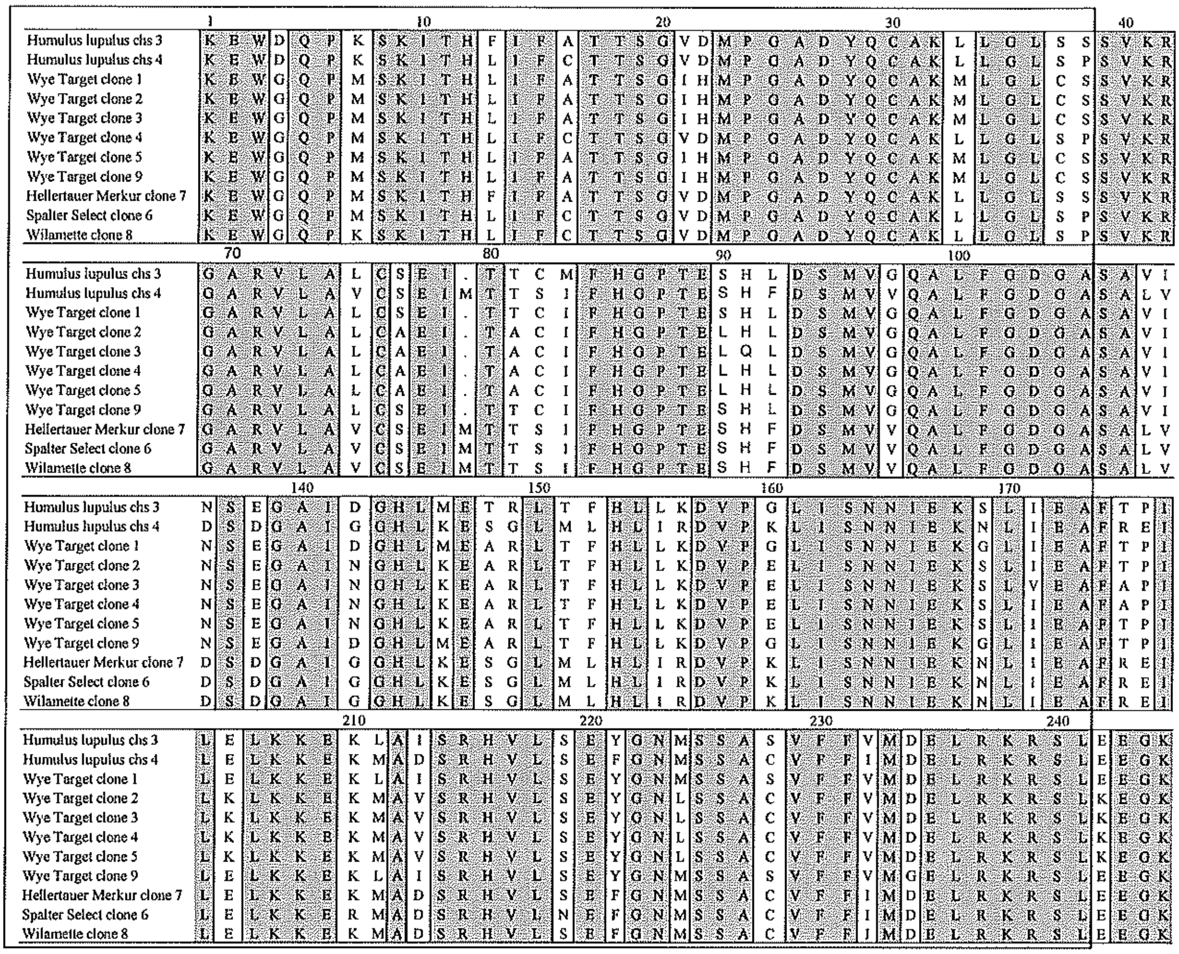


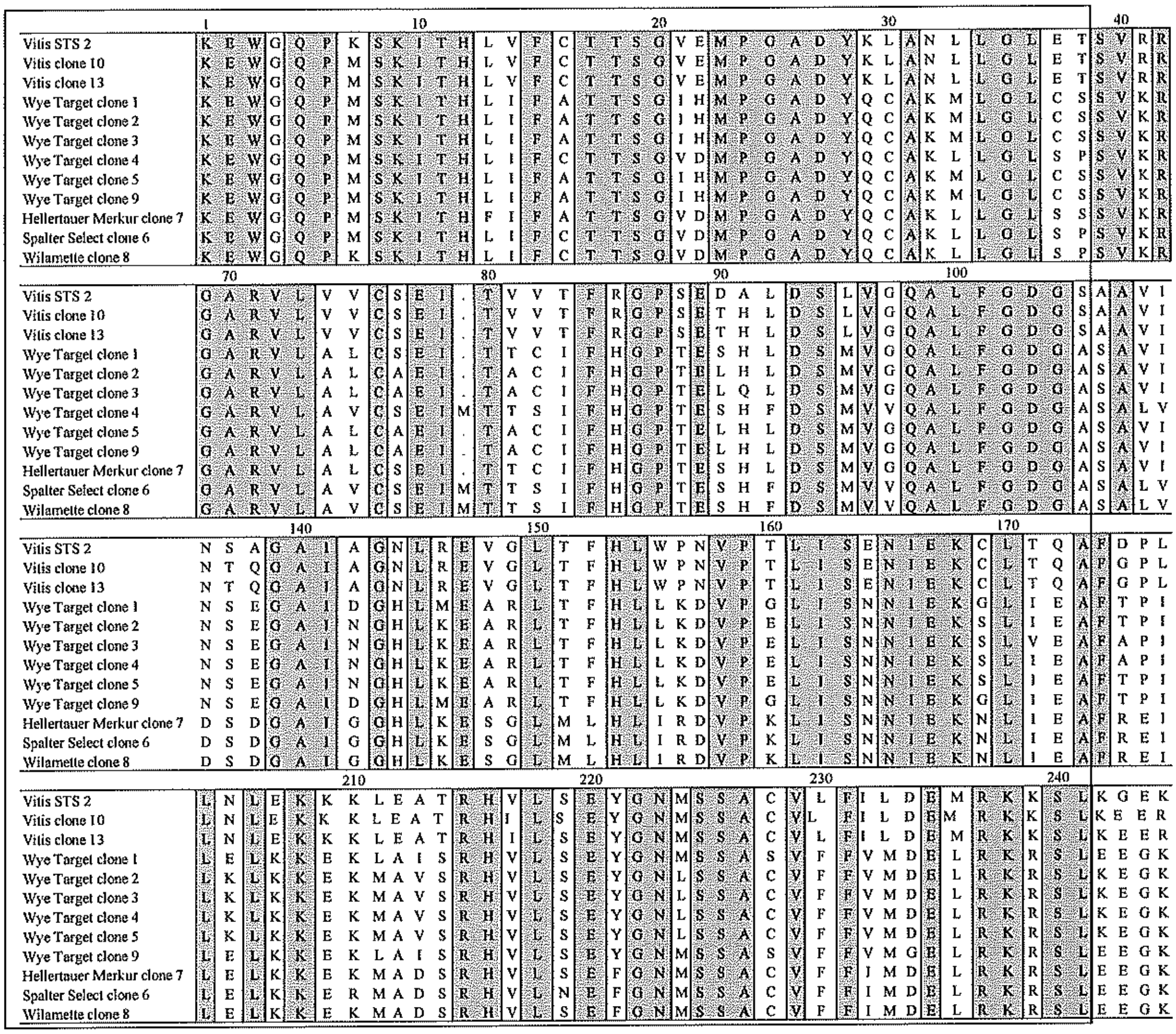
Figure 3: Product of two independent PCR amplifications. Numbers correspond to hop samples: (1) Spalter select, (2) Hallertau Merkur, (3) Willamette, (4) Wye target, (5) and (6) grapes samples.

DNA sequencing revealed, however, that the fragment amplified from hop is more closely related to the chalcone synthase of hop than to grapevine stilbene synthase (98% homology with the *Humulus lupulus* chs3 gene coding for chalcone synthase) (figure 4).



	1 10 20 30 40	70 80 90 100	140 150 160 170	210 220 230 240
Humulus lupulus chs 3	KEWDQPKSKITHFIFATTSGVDMPGADYQCAKLLGLSSSVKR	GARVLALCSEI.TTCMFHGPTESHLDSMVGQALFGDGASAVI	NSEGAIDGHLMETRLTFHLLKDVPGLISNNIEKSLIEAFTPI	LELKKEKLAISRHVLSEYGNMSSASVFFVMDELRKRSLEEGK
Humulus lupulus chs 4	KEWDQPKSKITHLIFCTTSGVDMPGADYQCAKLLGLSPSVKR	GARVLAVCSEIMTTSIFHGPTESHFDSMVVQALFGDGASALV	DSDGAIGGHLKESGLMLHLIRDVPKLISNNIEKNLIEAFREI	LELKKEKMADSRHVLSEFGNMSSACVFFIMDELRKRSLEEGK
Wye Target clone 1	KEWGQPMSKITHLIFATTSGIHMPGADYQCAKMLGLCSSVKR	GARVLALCSEI.TTCIFHGPTESHLDSMVGQALFGDGASAVI	NSEGAIDGHLMEARLTFHLLKDVPGLISNNIEKGLIEAFTPI	LELKKEKLAISRHVLSEYGNMSSASVFFVMDELRKRSLEEGK
Wye Target clone 2	KEWGQPMSKITHLIFATTSGIHMPGADYQCAKMLGLCSSVKR	GARVLALCAEI.TACIFHGPTELHLDSMVGQALFGDGASAVI	NSEGAINGHLKEARLTFHLLKDVPELISNNIEKSLIEAFTPI	LKLKKEKMAVSRHVLSEYGNLSSACVFFVMDELRKRSLKEGK
Wye Target clone 3	KEWGQPMSKITHLIFATTSGIHMPGADYQCAKMLGLCSSVKR	GARVLALCAEI.TACIFHGPTELQLDSMVGQALFGDGASAVI	NSEGAINGHLKEARLTFHLLKDVPELISNNIEKSLVEAFAPI	LKLKKEKMAVSRHVLSEYGNLSSACVFFVMDELRKRSLKEGK
Wye Target clone 4	KEWGQPMSKITHLIFCTTSGVDMPGADYQCAKLLGLSPSVKR	GARVLALCAEI.TACIFHGPTELHLDSMVGQALFGDGASAVI	NSEGAINGHLKEARLTFHLLKDVPELISNNIEKSLIEAFAPI	LKLKKEKMAVSRHVLSEYGNLSSACVFFVMDELRKRSLKEGK
Wye Target clone 5	KEWGQPMSKITHLIFATTSGIHMPGADYQCAKMLGLCSSVKR	GARVLALCAEI.TACIFHGPTELHLDSMVGQALFGDGASAVI	NSEGAINGHLKEARLTFHLLKDVPELISNNIEKSLIEAFTPI	LKLKKEKMAVSRHVLSEYGNLSSACVFFVMDELRKRSLKEGK
Wye Target clone 9	KEWGQPMSKITHLIFATTSGIHMPGADYQCAKMLGLCSSVKR	GARVLALCSEI.TTCIFHGPTESHLDSMVGQALFGDGASAVI	NSEGAIDGHLMEARLTFHLLKDVPGLISNNIEKGLIEAFTPI	LELKKEKLAISRHVLSEYGNMSSASVFFVMGELRKRSLEEGK
Hellertauer Merkur clone 7	KEWGQPMSKITHFIFATTSGVDMPGADYQCAKLLGLSSSVKR	GARVLAVCSEIMTTSIFHGPTESHFDSMVVQALFGDGASALV	DSDGAIGGHLKESGLMLHLIRDVPKLISNNIEKNLIEAFREI	LELKKEKMADSRHVLSEFGNMSSACVFFIMDELRKRSLEEGK
Spalter Select clone 6	KEWGQPMSKITHLIFCTTSGVDMPGADYQCAKLLGLSPSVKR	GARVLAVCSEIMTTSIFHGPTESHFDSMVVQALFGDGASALV	DSDGAIGGHLKESGLMLHLIRDVPKLISNNIEKNLIEAFREI	LELKKERMADSRHVLNEFGNMSSACVFFIMDELRKRSLEEGK
Wilamette clone 8	KEWGQPKSKITHLIFCTTSGVDMPGADYQCAKLLGLSPSVKR	GARVLAVCSEIMTTSIFHGPTESHFDSMVVQALFGDGASALV	DSDGAIGGHLKESGLMLHLIRDVPKLISNNIEKNLIEAFREI	LELKKEKMADSRHVLSEFGNMSSACVFFIMDELRKRSLEEGK

Figure 4a.



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                            1                 10                  20                  30                  40
Vitis STS 2                 K E W G Q P K S K I T H L V F C T T S G V E M P G A D Y K L A N L L G L E T S V R R
Vitis clone 10              K E W G Q P M S K I T H L V F C T T S G V E M P G A D Y K L A N L L G L E T S V R R
Vitis clone 13              K E W G Q P M S K I T H L V F C T T S G V E M P G A D Y K L A N L L G L E T S V R R
Wye Target clone 1          K E W G Q P M S K I T H L I F A T T S G I H M P G A D Y Q C A K M L G L C S S V K R
Wye Target clone 2          K E W G Q P M S K I T H L I F A T T S G I H M P G A D Y Q C A K M L G L C S S V K R
Wye Target clone 3          K E W G Q P M S K I T H L I F A T T S G I H M P G A D Y Q C A K M L G L C S S V K R
Wye Target clone 4          K E W G Q P M S K I T H L I F C T T S G V D M P G A D Y Q C A K L L G L S P S V K R
Wye Target clone 5          K E W G Q P M S K I T H L I F A T T S G I H M P G A D Y Q C A K M L G L C S S V K R
Wye Target clone 9          K E W G Q P M S K I T H L I F A T T S G I H M P G A D Y Q C A K M L G L C S S V K R
Hellertauer Merkur clone 7  K E W G Q P M S K I T H F I F A T T S G V D M P G A D Y Q C A K L L G L S S S V K R
Spalter Select clone 6      K E W G Q P M S K I T H L I F C T T S G V D M P G A D Y Q C A K L L G L S P S V K R
Wilamette clone 8           K E W G Q P K S K I T H L I F C T T S G V D M P G A D Y Q C A K L L G L S P S V K R

                              70                80                  90                  100
Vitis STS 2                 G A R V L V V C S E I . T V V T F R G P S E D A L D S L V G Q A L F G D G S A A V I
Vitis clone 10              G A R V L V V C S E I . T V V T F R G P S E T H L D S L V G Q A L F G D G S A A V I
Vitis clone 13              G A R V L V V C S E I . T V V T F R G P S E T H L D S L V G Q A L F G D G S A A V I
Wye Target clone 1          G A R V L A L C S E I . T T C I F H G P T E S H L D S M V G Q A L F G D G A S A V I
Wye Target clone 2          G A R V L A L C A E I . T A C I F H G P T E L H L D S M V G Q A L F G D G A S A V I
Wye Target clone 3          G A R V L A L C A E I . T A C I F H G P T E L Q L D S M V G Q A L F G D G A S A V I
Wye Target clone 4          G A R V L A V C S E I M T T S I F H G P T E S H F D S M V V Q A L F G D G A S A L V
Wye Target clone 5          G A R V L A L C A E I . T A C I F H G P T E L H L D S M V G Q A L F G D G A S A V I
Wye Target clone 9          G A R V L A L C A E I . T A C I F H G P T E L H L D S M V G Q A L F G D G A S A V I
Hellertauer Merkur clone 7  G A R V L A L C S E I . T T C I F H G P T E S H L D S M V G Q A L F G D G A S A V I
Spalter Select clone 6      G A R V L A V C S E I M T T S I F H G P T E S H F D S M V V Q A L F G D G A S A L V
Wilamette clone 8           G A R V L A V C S E I M T T S I F H G P T E S H F D S M V V Q A L F G D G A S A L V

                                  140                 150                 160                 170
Vitis STS 2                 N S A G A I A G N L R E V G L T F H L W P N V P T L I S E N I E K C L T Q A F D P L
Vitis clone 10              N T Q G A I A G N L R E V G L T F H L W P N V P T L I S E N I E K C L T Q A F G P L
Vitis clone 13              N T Q G A I A G N L R E V G L T F H L W P N V P T L I S E N I E K C L T Q A F G P L
Wye Target clone 1          N S E G A I D G H L M E A R L T F H L L K D V P G L I S N N I E K G L I E A F T P I
Wye Target clone 2          N S E G A I N G H L K E A R L T F H L L K D V P E L I S N N I E K S L I E A F T P I
Wye Target clone 3          N S E G A I N G H L K E A R L T F H L L K D V P E L I S N N I E K S L V E A F A P I
Wye Target clone 4          N S E G A I N G H L K E A R L T F H L L K D V P E L I S N N I E K S L I E A F A P I
Wye Target clone 5          N S E G A I N G H L K E A R L T F H L L K D V P E L I S N N I E K S L I E A F T P I
Wye Target clone 9          N S E G A I D G H L M E A R L T F H L L K D V P G L I S N N I E K G L I E A F T P I
Hellertauer Merkur clone 7  D S D G A I G G H L K E S G L M L H L I R D V P K L I S N N I E K N L I E A F R E I
Spalter Select clone 6      D S D G A I G G H L K E S G L M L H L I R D V P K L I S N N I E K N L I E A F R E I
Wilamette clone 8           D S D G A I G G H L K E S G L M L H L I R D V P K L I S N N I E K N L I E A F R E I

                                        210                 220                 230                 240
Vitis STS 2                 L N L E K K K L E A T R H V L S E Y G N M S S A C V L F I L D E M R K K S L K G E K
Vitis clone 10              L N L E K K K L E A T R H I L S E Y G N M S S A C V L F I L D E M R K K S L K E E R
Vitis clone 13              L N L E K K K L E A T R H I L S E Y G N M S S A C V L F I L D E M R K K S L K E E R
Wye Target clone 1          L E L K K E K L A I S R H V L S E Y G N M S S A S V F F V M D E L R K R S L E E G K
Wye Target clone 2          L K L K K E K M A V S R H V L S E Y G N L S S A C V F F V M D E L R K R S L K E G K
Wye Target clone 3          L K L K K E K M A V S R H V L S E Y G N L S S A C V F F V M D E L R K R S L K E G K
Wye Target clone 4          L K L K K E K M A V S R H V L S E Y G N L S S A C V F F V M D E L R K R S L K E G K
Wye Target clone 5          L K L K K E K M A V S R H V L S E Y G N L S S A C V F F V M D E L R K R S L K E G K
Wye Target clone 9          L E L K K E K L A I S R H V L S E Y G N M S S A S V F F V M G E L R K R S L E E G K
Hellertauer Merkur clone 7  L E L K K E K M A D S R H V L S E F G N M S S A C V F F I M D E L R K R S L E E G K
Spalter Select clone 6      L E L K K E R M A D S R H V L N E F G N M S S A C V F F I M D E L R K R S L E E G K
Wilamette clone 8           L E L K K E K M A D S R H V L S E F G N M S S A C V F F I M D E L R K R S L E E G K
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Figure 4b.

Figure 4: Sequence alignments: hop and *Vitis sp.* samples as compared to (a) *Humulus lupulus* chs 3 and chs 4 (b) *Vitis sp.* STS 2. Grey colour for similar sequences, white colour for different sequences.

Yet our grape fragment does correspond to a stilbene synthase, as observed in a phylogenetic tree (figure 5). Similar PCR results have been obtained by Schwekendiek *et al.* (8). Yamaguchi *et al.* (9) have close relationships between stilbene and chalcone synthases. When overexpressed in *Escherichia coli,* chalcone synthase has been shown to catalyse resveratrol synthesis (2-4 % in comparison to the major product)

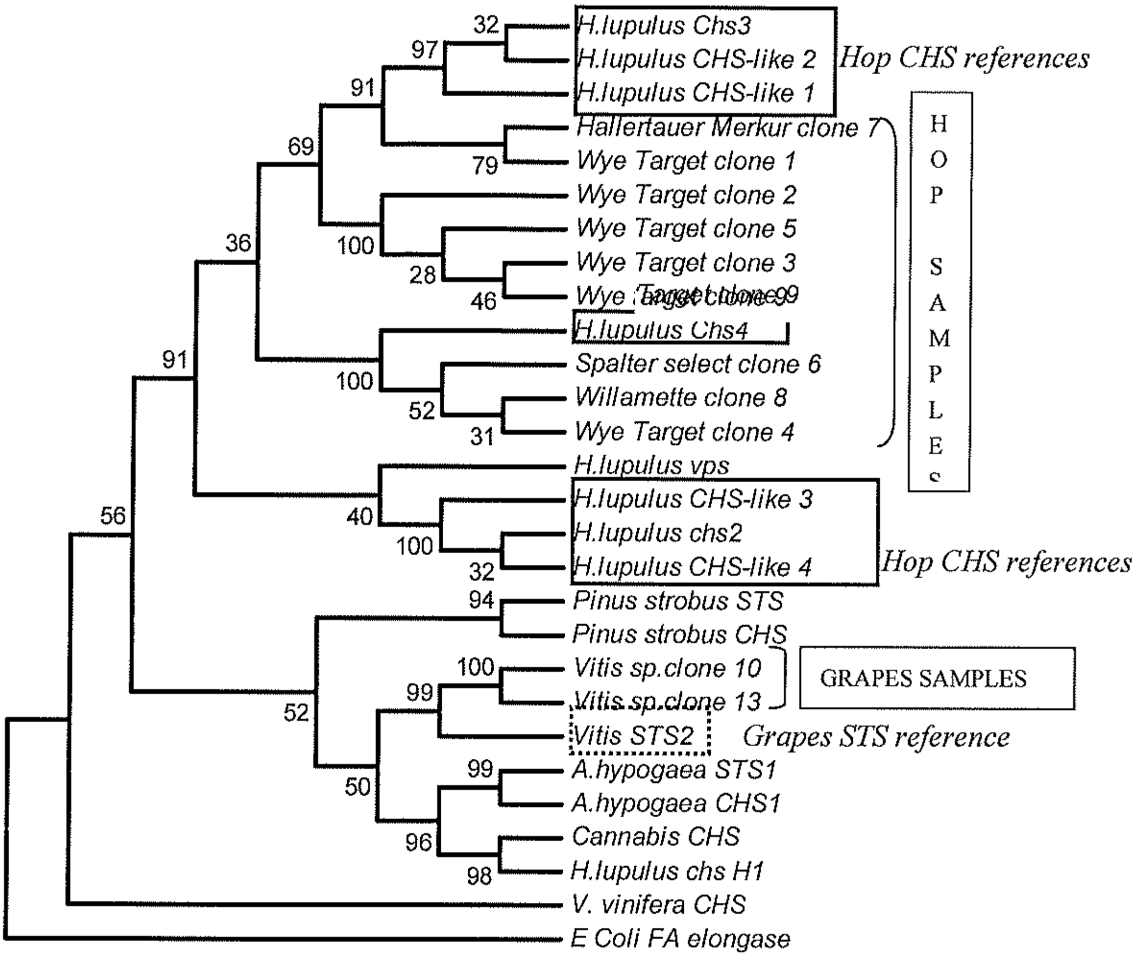

Figure 5: Phylogenetic tree for amplified DNA from 6 hop varieties and 2 grape varieties and literature-referenced primers for genes encoding stilbene synthase (STS) and chalcone synthase (CHS) in various species. The numbers given are homology percentages.

CONCLUSIONS AND PERSPECTIVES

Investigation of stilbenes in barley, malt, wheat, corn, sorghum and hop cones has led to the following conclusions:

1. hop and red sorghum are interesting sources of stilbenes for brewers;
2. in addition to *trans*-resveratrol, *trans*- piceid, and *cis*-piceid, hop varieties contain another stilbene analogue of pterostilbene that remains to be identified;
3. although no stilbene synthase was found in hop, chalcone synthase might be involved in stilbene biosynthesis.

ACKNOWLEDGEMENTS

Vesna Jerkovic is grateful to the InBev-Baillet Latour Foundation (Leuven, Belgium) for financial support. The authors thank Prof. François Chaumont of the Unité de Biochimie physiologique - Université catholique de Louvain, Stéphane Meulemans of

Yakima Chief, and Zdenek Rosa of the Hop Growers Union of the Czech Republic for their kind collaboration.

LITTERATURE

(1) Lermusieau, G., Liégeois, C. & Collin, S., Reducing power of hop cultivars and beer ageing, Food Chem., 2001, 72, 413-418.
(2) Frémont, L., Biological effects of resveratrol, Life Sci., 2000, 66, 663-673.
(3) Callemien, D., Jerkovic, V., Rozenberg, R. & Collin, S., Hop as an interesting source of resveratrol for brewers: Optimization of the extraction and quantitative study by liquid chromatography/atmospheric pressure chemical ionization tandem mass spectrometry, J. Agric. Food Chem., 2005, 53, 424-429.
(4) Jerkovic, V., Callemien, D. & Collin, S., Determination of stilbenes in hop pellets from different cultivars, J. Agric. Food Chem., 2005, 53, 4202-4206.
(5) Jerkovic, V. & Collin, S., Fate of hop stilbenes through different processing and storage time, submitted.
(6) Jerkovic, V. & Collin, S., Occurrence of stilbenes in American and European hop cones from three successive harvests, submitted.
(7) Yu, C., Springob, K., Schmidt, J., Nicholson, R., Chu, I., Yip, W. & Lo, C., A stilbene synthase gene (SbSTS1) is involved in host and nonhost defense responses in sorghum, Plant Physiology, 2005, 138, 393-401.
(8) Schwekendiek, A., Pfeffer, G. & Kindl, H., Hop *(Humulus lupulus L.)* transformation with stilbene synthase for increasing resistance against fungal pathogens, Acta Horticulturae, 2005, 668, 101-107.
(9) Yamaguchi, T., Kurosaki, F., Suh, D.Y., Sankawa, U., Nishioka, M., Akiyama, T., Shibuya, M. & Ebizuka, Y., Cross-reaction of chalcone synthase and stilbene synthase overexpressed in Escherichia coli, Febs Letter, 1999, 460, 457-461.