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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

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Nowadays, hop is used almost exclusively by brewers for bitterness and flavor. Although hop polyphenols have been widely studied in the past decade for their antioxidant activity in the boiling kettle, very little is known about their real impact on health. The discovery of resveratrol in hop pellets highlights the potential health-promoting effect of moderate beer consumption. Here, we have optimized a quantitative extraction procedure for resveratrol in hop pellets. Preliminary removal of hydrophobic bitter compounds with toluene and cyclohexane at room temperature allows 99% *trans*-resveratrol recovery by ethanol:water (75:25, v/v) solid/liquid extraction at 60 °C. Reverse phase liquid chromatography proves an excellent means of separating isomers. In addition, we have compared two mass spectrometry ionization methods—atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI)—in both the positive and the negative modes. On the basis of standard additions applied with the optimized extraction procedure and reverse phase high-performance liquid cromatography−APCI(+)−tandem mass spectrometry, it appears that Tomahawk hop pellets (T90, harvest 2002) contain 0.5 ppm *trans*-resveratrol, 2 ppm *trans*-piceid, no *cis*-resveratrol, and 0.9 ppm *cis*-piceid.

KEYWORDS: Hop; extraction; resveratrol; polyphenols; health; HPLC-MS/MS; APCI; ESI

INTRODUCTION

Hop (*Humulus lupulus* L.) is a dioecious plant of the Cannabacea family, cultivated in most temperate zones of the world for its female inflorescences. For centuries, hop was used in traditional medicine. Nowadays, the plant is used almost exclusively by brewers for aroma, bitterness, foam stability, and antimicrobial protection (*1*). Hop varieties can be differentiated on the basis of secondary metabolites (resins, oils, and polyphenols) essentially located in the lupulin glands (*2*, *3*). In wort and beer, up to 30% of the polyphenols present are derived from hop, despite the fact that malt is added in much larger amounts (nearly 100 times more). Depending on the hop variety, its geographic origin and freshness, the harvesting procedure, and the manner in which the dried hop cone is packaged, total polyphenols account for 3−6% (w/w) (*4*). They consist mainly of phenolic acids, oligomeric flavanoids, and prenylflavonoids. Among them, xanthohumol and hopein have been extensively investigated by De Keukeleire et al. (*4*, *5*) for their exceptional estrogenic activities. Stevens et al. (*6*) and Lermusieau et al. (*7*), furthemore, have emphasized the huge antioxidant activity of hop proanthocyanidins.

Surprisingly, stilbenes appear never to have been investigated in this polyphenol-rich matrix before our work in 2003 (*8*). The phytoalexin *trans*-resveratrol is usually synthesized by plants in response to injury or fungal attack (*9*, *10*). It was first reported in grapevines (*Vitis vinefera*) in 1976 (*10*) but later discovered in cranberries (*11*), blueberries (*12*), bilberries (*12*), peanuts (*13*, *14*), and *Polygonum cuspidatum* (*15*, *16*), a traditional Chinese medicinal herb. The decrease in coronary heart disease observed among wine drinkers, known as the famous "French paradox" (*17*), has led many groups to investigate this compound in red wines but also in rosé and white wines (*18*−*20*). *trans*-Resveratrol has also been linked to various health benefits, such as anticarcinogenic, antioxidant, antiinflammatory, and estrogenic activities (*21*).

Various solid−liquid extraction methods have been applied to extract *trans*-resveratrol from a solid matrix, usually with methanol (*9*−*12*, *15*, *22*, *23*), ethanol (*13*, *16*, *24*), ethyl acetate (*9*−*12*, *15*, *25*), diethyl ether (*22*), acetone (*25*), acetonitrile (*14*), or even a supercritical fluid (*26*). Quantification has been

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achieved either with gas chromatography [if the analyte is prederivatized (*9*, *10*)], centrifugal partition chromatography (*15*, *25*), capillary electrophoresis (*16*), or reverse phase high-pressure liquid chromatography (RP-HPLC). The latter is usually hyphenated to UV absorbance (*13*, *14*, *24*, *26*), fluorescence (*22*), or mass spectrometry detection techniques such as atmospheric pressure chemical ionization (APCI) (*11*) and electrospray ionization (ESI) (*12*, *23*).

The aim of the present work was to quantify accurately for the first time *trans*-resveratrol and analogues in hop pellets. Procedures for hop resin removal and resveratrol extraction were optimized. The sensitivity was improved by comparing different atmospheric pressure ionization techniques for mass spectroscopy.

MATERIALS AND METHODS

Chemicals. Ethanol (97%) was obtained from Belgaco (Gent, Belgium). Acetonitrile (99.99%), toluene (99.97%), and cyclohexane (99.96%) were purchased from Fischer Scientific (United Kingdom). Acetone (99.9%), *n*-hexane (99%), and formic acid (pa) were purchased from Aldrich (Germany). Methanol (99.9%) and dichloromethane (99.9%) were purchased from Romil (Cambridge, United Kingdom). Acetic acid (99.8%) came from Acros (Geel, Belgium). Petroleum ether (100−140 °C) was obtained from Vel (Darmstadt, Germany). Aqueous solutions were made with Milli-Q (Millipore, Bedford, MA) water. *trans*-Resveratrol (99%), *trans*-piceid (97%), catechin (98%), epicatechin (90%), rutin (95%), myricetin (85%), quercetin (98%), and kaempferol (90%) were purchased from Sigma-Aldrich (Bornem, Belgium). *cis*-Piceid and *cis*-resveratrol were obtained by irradiating for 10 min the respective *trans* forms dissolved in methanol at a wavelength of 254 nm.

Stability of *trans*-Resveratrol. A stock solution of 100 ppm *trans*-resveratrol was prepared in methanol. The stability of the phytoalexin was tested under various conditions: (i) 20 °C with exposure to white light, (ii) 20 °C with exposure to white light under nitrogen, (iii) 20 °C with exposure to red light, (iv) 20 °C with exposure to red light under nitrogen, (v) 4 °C (dark refrigerator), and (vi) −20 °C (dark freezer). Aliquots of each sample were taken at 0, 1, 3, and 9 days.

Optimization of *trans*-Resveratrol Extraction from Hop Pellets. For these experiments, recovery factors were calculated by standard addition from normal phase HPLC-UV ($\lambda = 306$ nm) data. Extractions were carried out on Tomahawk hop pellets (T90, 2002 harvest) under red light.

Removal of Hydrophobic Compounds. Hop pellets (2.5 g) were reduced to powder and introduced into a centrifugal vial. In successive 10 min steps, resins and lipids were removed at room temperature under gentle stirring first with 50 mL of toluene (three times) and then with 50 mL of cyclohexane (three times). At the end of each step, the sample was centrifuged for 10 min at 3000*g*. At the last step, hop powder was also dried under vacuum to get rid of residual solvent. For the optimization, other procedures were tested as follows: no delipidation or delipidation with petroleum ether (three times) and *n*-hexane (three times) or with petroleum ether alone (six times).

trans-Resveratrol Extraction. Delipidated hop powder was extracted three times with 40 mL of ethanol:water (75:25, v/v), each time for 10 min under gentle stirring at 60 °C. This procedure was compared with extraction at room temperature with either acetone, ethanol, or methanol mixed with water (75:25, v/v). 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.

Standard Addition Method. Hop was spiked with increasing amounts of either *trans*-resveratrol (0, 0.5, 1, and 5 ppm), *trans*-piceid (0, 5, 10, and 20 ppm), catechin, (0, 75, and 150 ppm), epicatechin (0, 100, and 250 ppm), quercetin (0, 75, 150, and 225 ppm), myricetin (0, 0.5, 1, and 5 ppm), rutin (0, 500, and 1000 ppm), or kaempferol (0, 10, 20, and 30 ppm).

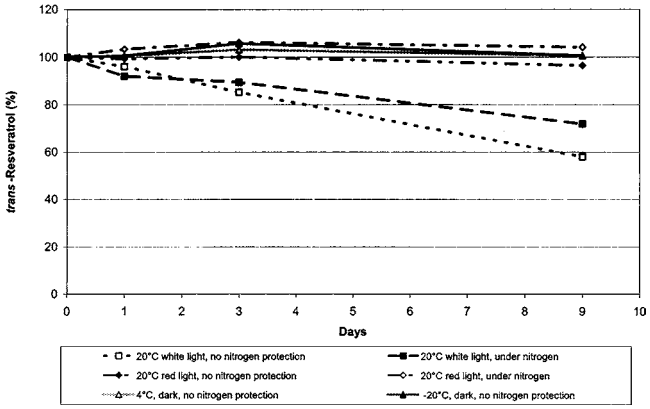

Figure 1. Stability of *trans*-resveratrol under various conditions. Quantification by HPLC-UV at 306 nm.

HPLC-UV Analysis. For the extraction procedure optimization, separations were carried on a Luna silica column (250 mm × 4.6 mm, 5 μm) (Phenomenex, Holland) eluted with a linear gradient from dichloromethane to methanol and a constant 4% level of acetic acid: water mixture (50:50, v/v). The gradient elution was as follows: from 82 to 68% dichloromethane in 30 min, 68 to 46% in 30 min, 46 to 10% in 5 min, and finally isocratic for 5 min at a flow rate of 1 mL/min. Twenty microliters of sample was injected into the column kept at 25 °C. *trans*-Resveratrol was monitored at 306 nm with a UV6000LP diode array detector.

RP-HPLC-MS/MS Analysis. Quantifications were performed on a C18 Prevail column (150 mm × 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. The 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 of 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 or ESI source (Finnigan Mat). APCI inlet conditions were applied as follows: vaporization temperature, 470 °C; capillary voltage, 3 V; capillary temperature, 175 °C; sheath gas, 40 psi; auxiliary gas, 7 psi; and discharge current, 5 μA. For the ESI source, conditions were applied as follows: source voltage, 4.5 kV; capillary voltage, 36 V; capillary temperature, 225 °C; sheath gas, 70 psi; and auxiliary gas, 20 psi. In both methods, collision-induced dissociation spectra were recorded at 37% relative collision energy.

RESULTS AND DISCUSSION

Assessment of *trans*-Resveratrol Stability. As depicted in **Figure 1**, *trans*-resveratrol can be stored for 1 week at 20, 4, or −20 °C without significant deterioration if protected from white light. In the presence of white light, however, *trans*-resveratrol is very quickly degraded at room temperature, especially if oxygen is also present (40% degradation after 9 days vs 25% if protected with a nitrogen headspace). Accordingly, all subsequent extraction procedures were applied under red light at room temperature. The extracts obtained were then stored at −20 °C in the dark until used.

Optimization of *trans*-Resveratrol Extraction. According to the literature (*6*), preliminary removal of hop lipids and resins is most likely necessary in order to recover high amounts of polyphenols. Ether is often chosen for preliminary cleaning because of its ability to remove hard and soft hop resins efficiently (*27*). Unfortunately, as indicated by its short retention time on a polar HPLC column (*8*), *trans*-resveratrol is much more hydrophobic than other hop polyphenols such as catechin, epicatechin, or procyanidin oligomers. Therefore, it exhibits significant solubility in diethyl ether (0.11%, w/w or 1.1 mg/

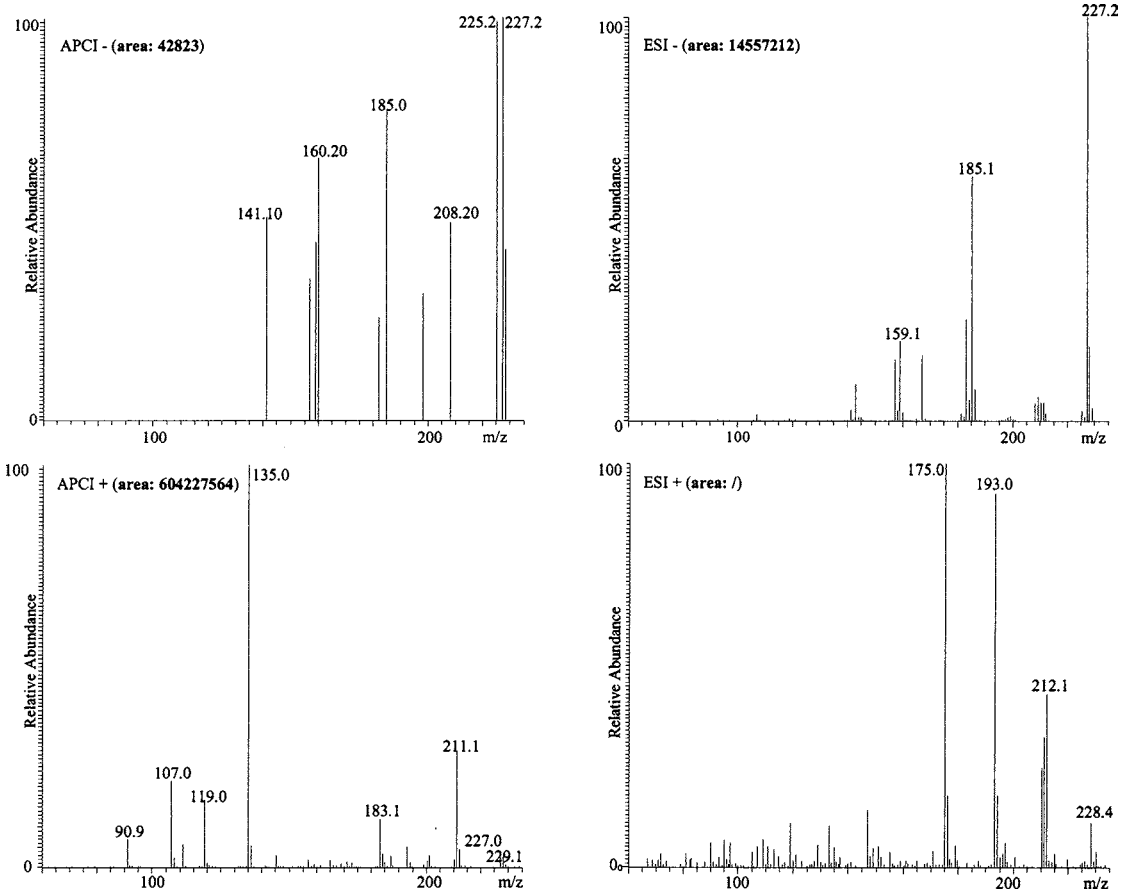

Figure 2. Comparison of ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) in MS/MS analyses. Injection in triplicates of 5 μL *trans*-resveratrol (10 ppm in methanol) into a 200 μL/min RP-HPLC flow.

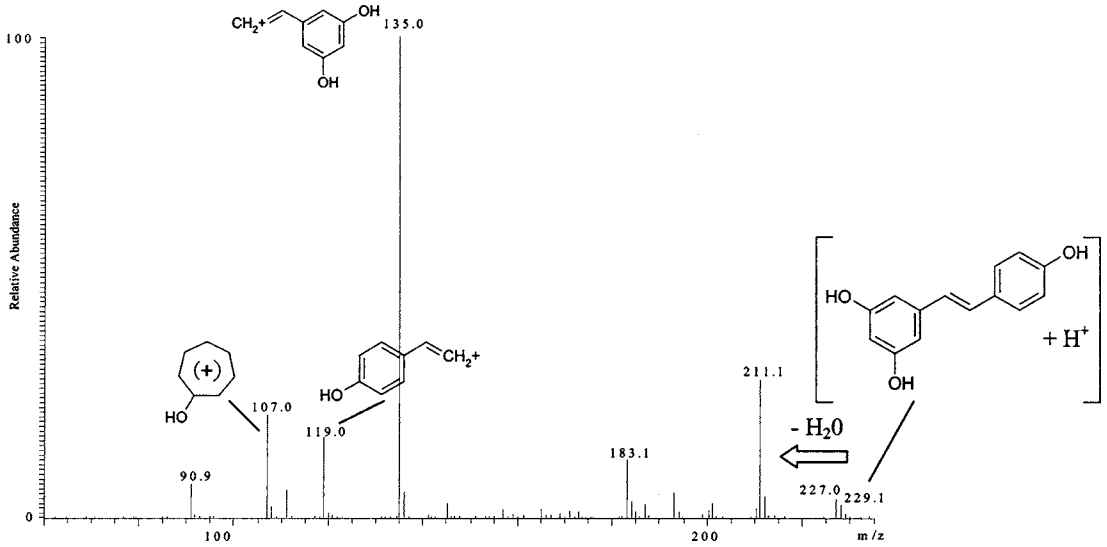

Figure 3. *trans*-Resveratrol MS/MS mass spectrum in positive mode APCI.

mL). Four more hydrophobic solvents were tested as follows: *n*-hexane, petroleum ether, toluene, and cyclohexane (unfortunately, these solvents removed few hop hard resins). As shown in **Table 1**, no delipidation led, as expected, to poor recovery (50%, standard addition method). A gain of about 20% was recorded when we applied two-step solid/liquid extraction with petroleum ether and hexane or one-step solid/liquid extraction with petroleum ether alone (still 4% better). Two-step solid/liquid extraction with toluene and cyclohexane proved even more efficient (30%), probably thanks to the ability of toluene to remove part of the hard resins.

Three organic solvent:water mixtures (75:25, v/v), usually used for stilbene extraction, were then compared as regards their ability to extract resveratrol at room temperature. As shown in **Table 2**, ethanol emerged as the most efficient, just above methanol (about 10% more). Although very often used for procyanidin recovery (*27*), the acetone:water mixture proved inappropriate for extracting *trans*-resveratrol. Gentle heating at 60 °C, as advised by Romero-Pérez (*24*), improved the recovery still further (by 8%). When two-step solid/liquid extraction with toluene and cyclohexane was combined with this last procedure, 99% recovery was achieved. As depicted in **Table 3**, this method

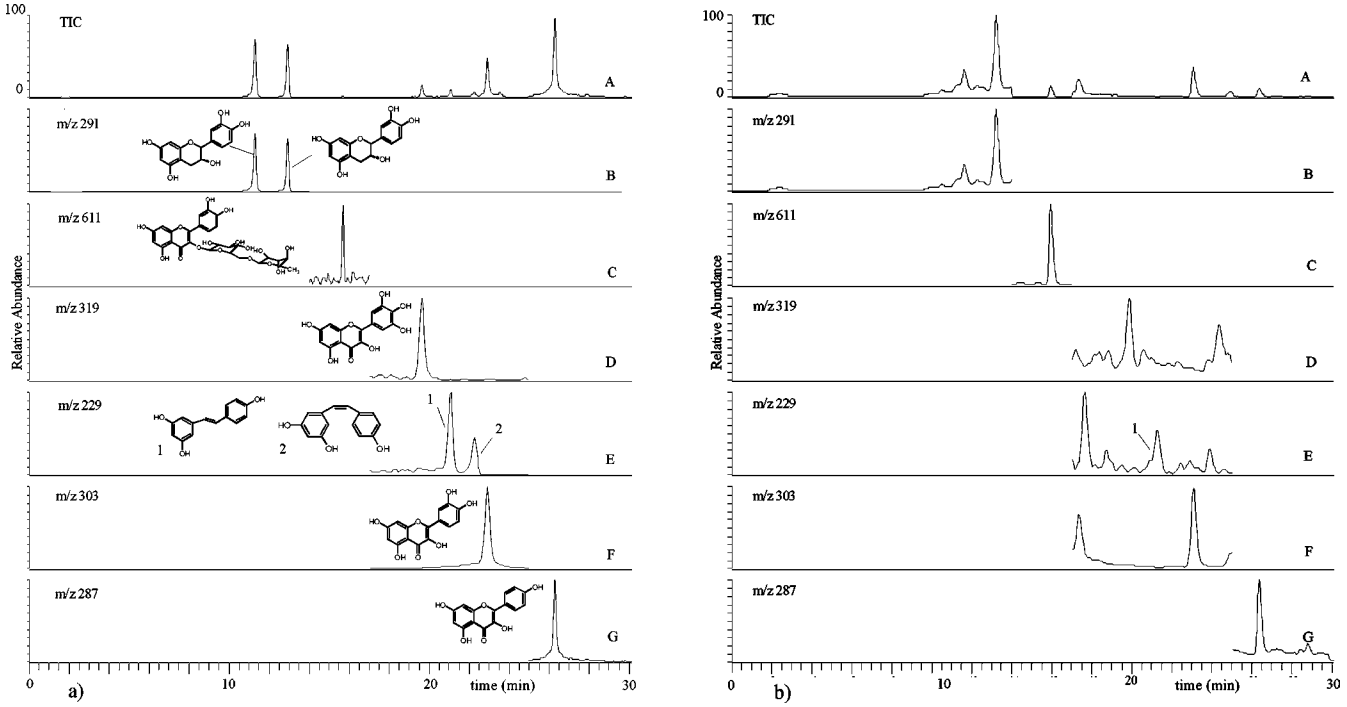

Figure 4. (**a**) Separation of eight polyphenolic standards (5 ppm in methanol) by RP-HPLC-APCI(+)/MS-MS. (**b**) Analyses of Tomahawk hop extract (T90, harvest 2002). (A) TIC (total ion chromatogram), (B) catechin and epicatechin, (C) rutin, (D) myricetin, (E) *trans*-resveratrol-1 and *cis*-resveratrol-2, (F) quercetin, and (G) kaempferol.

Table 1. Recovery Factors of *trans*-Resveratrol for Various Hydrophobic Compounds Removal Procedures[a]

solvent for hydrophobic compounds removal	recovery factor (%)
no delipidation	50
petroleum ether and *n*-hexane	68
petroleum ether	72
toluene and cyclohexane	82

[a] Polyphenols were extracted in all cases with methanol:water (75:25, v/v); UV (λ = 306 nm) detection.

Table 2. Recovery Factors of *trans*-Resveratrol According to the Solvent Used for Polyphenol Extraction[a]

solvent for polyphenol extraction	recovery factor (%)
acetone:water (75:25, v/v)	34
methanol:water (75:25, v/v)	72
ethanol:water (75:25, v/v)	82
ethanol:water (75:25, v/v)[b]	90

[a] Hydrophobic compounds were removed in all cases with petroleum ether; UV (λ = 306 nm) detection. [b] Extraction at 60 °C.

optimized specifically for resveratrol resulted in a low recovery of most hop polyphenols (except myricetin).

Optimization of the RP-HPLC-MS/MS Analysis. RP-HPLC provided higher chromatographic resolution (separation of *trans*-resveratrol, t_r = 20.85 min and *cis*-resveratrol, t_r = 22 min) than normal phase (only one peak at 5.6 min) (see also **Figure 4a**). To compare the ESI and APCI ionization methods, we injected 5 μL of *trans*-resveratrol solution (10 ppm in methanol) in triplicate into 200 μL/min RP-HPLC flow. Our data show that APCI in the positive mode and ESI in the negative mode gave the highest responses, the former giving a four-times higher response (**Figure 2**). APCI in the positive mode was thus retained for hop sample analysis (monitoring of the m/z = 229; see **Figure 3**).

Table 3. Recovery Factors Obtained for Nine Hop Polyphenols When the Optimized Resveratrol Extraction Procedure Is Applied [Toluene and Cyclohexane for Removing Hydrophobic Compounds and Ethanol:Water (60 °C) for Polyphenol Extraction; RP-HPLC-APCI(+)/MS-MS Detection][a]

compound	recovery factor (%)	concentration (ppm or mg/kg of hop pellets)
catechin	19	238
epicatechin	7	1483
rutin	16	1931
myricetin	80	1
trans-resveratrol	99	0.5
quercetin	21	132
kaempferol	22	16
trans-piceid	95	2.1
cis-piceid[b]	–	0.9

[a] Concentrations were found in Tomahawk hop pellets (T90, harvest 2002) by the standard addition method. [b] *trans*-Piceid equivalent.

Quantitative Analysis of Resveratrol in Hop. As depicted in **Figure 4b**, the RP-HPLC-APCI(+)MS/MS (m/z = 229) chromatogram obtained for Tomahawk hop pellets (T90, harvest 2002) revealed the presence of 0.5 ppm *trans*-resveratrol (standard addition method applied). Although no *cis*-resveratrol was found in the sample, two compounds showing higher polarity (t_r = 16.38 and 17.78 min) and the same mass spectrum were detected (**Figure 5**). MS/MS (m/z = 391) applied to standards and hop extract enable us to identify these compounds as *trans*- and *cis*-piceid. Standard addition applied with our aglycone-optimized method indicated concentrations of *trans*- and *cis*-piceid close to 2 and 0.9 ppm, respectively (in both cases, quantification was done in *trans*-piceid equivalents, for which a recovery factor of 95% was calculated).

In conclusion, this study has yielded an optimized quantitative extraction method for resveratrol analysis in Tomahawk hop

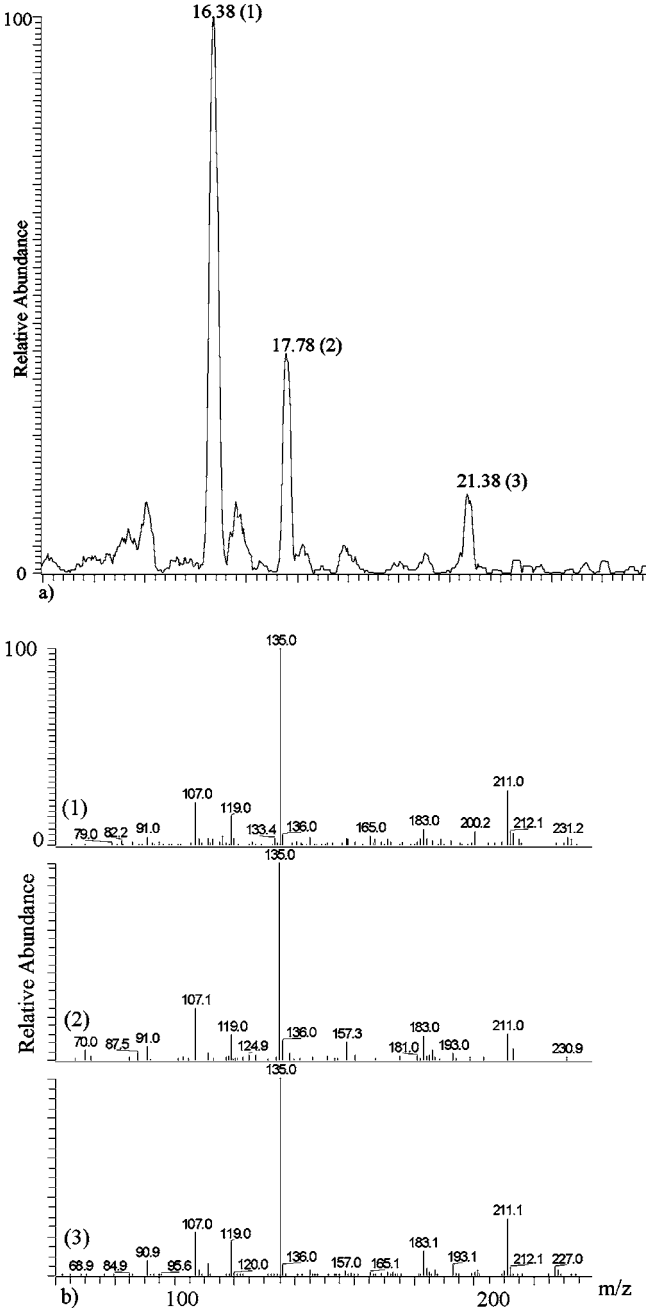

Figure 5. RP-HPLC-APCI(+)/MS-MS data for a Tomahawk hop extract (T90, harvest 2002): (**a**) MS/MS chromatogram (*m*/*z* = 229), (**b**) experimental mass spectra for *trans*-piceid (1), *cis*-piceid (2), and *trans*-resveratrol (3).

pellets. A recovery factor of 99% (95% for glycosides) was obtained when toluene and cyclohexane were used to remove hydrophobic compounds before polyphenol extraction with ethanol:water (75:25, v/v) at 60 °C. APCI in the positive mode emerged as the most efficient atmospheric pressure ionization technique. In our sample, we detected 0.5 ppm *trans*-resveratrol, no *cis*- resveratrol, and up to 2 and 0.9 ppm *trans*- and *cis*-piceid, respectively. Further investigations are now needed to quantify glycosides hydrolysis by yeast through beer fermentation. It would also be of prime interest to apply this optimized method to various hop cultivars and conditionings in order to select the most promising origins.

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