Determination of the Hop-Derived Phytoestrogen, 8-Prenylnaringenin, in Beer by Gas Chromatography/Mass Spectrometry

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A method was developed to determine 8-prenylnaringenin, a novel hop-derived phytoestrogen, in beer. Matrix purification involved solid-phase extraction on octadecyl silica followed by liquid/liquid extraction on a ChemElut 1010 column connected to a Florisil adsorption/desorption cartridge. 8-Prenylnaringenin was eluted from the tandem columns using a 1:1 mixture of diethyl ether and ethyl acetate and subsequently determined as tris(trimethylsilyl) ether by GC/MS-SIM. The recovery of 8-prenylnaringenin in beer samples was between 61.1 ± 6.6 and 82.2 ± 8.8% for levels of 37 and 92.5 μg L^{-1}, respectively, and the detection limit was ~5 μg L^{-1}. Although most beers do not contain 8-prenylnaringenin in detectable quantities, the highest concentration found was 19.8 μg L^{-1}. The concentration of 8-prenylnaringenin in beers and, possibly, its absence depend on the selection of particular hop varieties, the hopping rate, or the type of hop product used in brewing. The efficiency of transfer of 8-prenylnaringenin from hops to beer is between 10 and 20%.

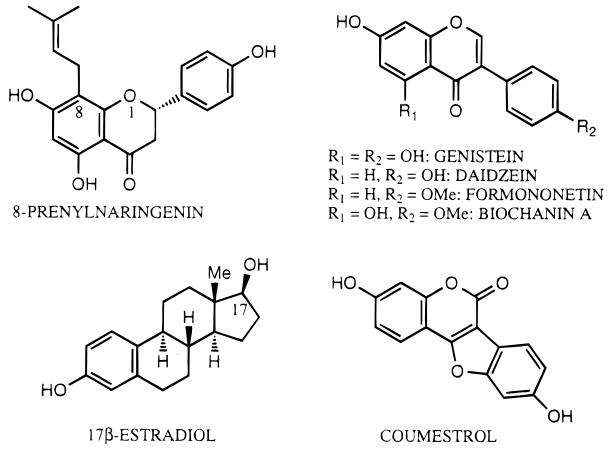
Keywords: *Phytoestrogens; 8-prenylnaringenin; beer; hops (Humulus lupulus L.); gas chromatography/mass spectrometry*

INTRODUCTION

Many plant secondary metabolites inhibit tumor growth, prevent oxidative damage, or affect the metabolism of steroid hormones (Caragay, 1992). Isoflavones, particularly genistein and daidzein (Chart 1), have been implicated in cancer prevention (Messina and Barnes, 1991; Messina et al., 1994; Adlercreutz, 1995), whereas populations exposed to a high isoflavone intake through the diet have particularly low incidences of breast (Adlercreutz et al., 1991) and prostate (Adlercreutz et al., 1993) cancer. Soy is the prevalent source of such isoflavones, and a variety of soy products have become well-known for their health-protective action.

The presence of genistein and daidzein in beer was first reported by Rosenblum et al. (1992) by GC/MS, and these isoflavones, together with their respective precursors formononetin and biochanin A (Chart 1), were recently determined in beer by radioimmunoassays in combination with reversed phase HPLC (Lapcik et al., 1998). Isoflavones were isolated from beer by continuous extraction with chloroform followed by fractionation using Sephadex LH-20 in the solvent system isooctane/chloroform/methanol (2:1:1, v/v) (Rosenblum et al., 1992), as well as by extraction using diethyl ether prior to silica TLC and octadecyl HPLC chromatography

Chart 1



(Lapcik et al., 1998). The source of isoflavones remains unclear, although hops should be a prime candidate. A number of polyphenols have been identified in hops, and the quantitatively most important classes are proanthocyanidins and flavonols, including their glycosides. A less common class of polyphenols, prenylflavonoids, is currently arousing high interest, in particular because of their pronounced and varied biological activities (Stevens et al., 1998). The occurrence of this series is restricted to only a few plant families, including the Moraceae and the Cannabaceae, to which hops (*Humulus lupulus* L.) belong. The content of prenylflavonoids in hops ranges from 0.2 to 0.6%. Our recent studies in this area have led to the identification of 8-prenylnaringenin (8-PN) (Chart 1) as the estrogenic principle in

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hops (Milligan et al., 1999). This finding resolves conflicting literature reports regarding the estrogenicity of hops, which has been known in traditional medicine for a very long time (De Keukeleire et al., 1997a). Comparison of the in vitro estrogenic activity of 8-PN with that of 17β-estradiol (Chart 1) and reputed phytoestrogens such as coumestrol (Chart 1) and genistein revealed that it is more potent than any other known phytoestrogen (Milligan et al., 1999).

A simple procedure (Lu et al., 1995; Tekel' et al., 1999) for the isolation of genistein and daidzein from aqueous samples (human urine) is based on liquid/liquid extraction using ChemElut columns containing diatomaceous material that adsorbs and retains water from the matrix. The high surface area of the cartridge filling allows efficient emulsion-free interaction between the sample and an organic extraction solvent. This procedure was adapted to the isolation of the hop-derived phytoestrogen, 8-PN, from beer. Subsequently, quantitative analysis was carried out by GC/MS in the SIM mode on the tris(trimethylsilyl) ether of 8-PN. We presently detail this method in view of its potential to detect health-beneficial compounds in beer and its feasibility as a routine operation in breweries.

EXPERIMENTAL PROCEDURES

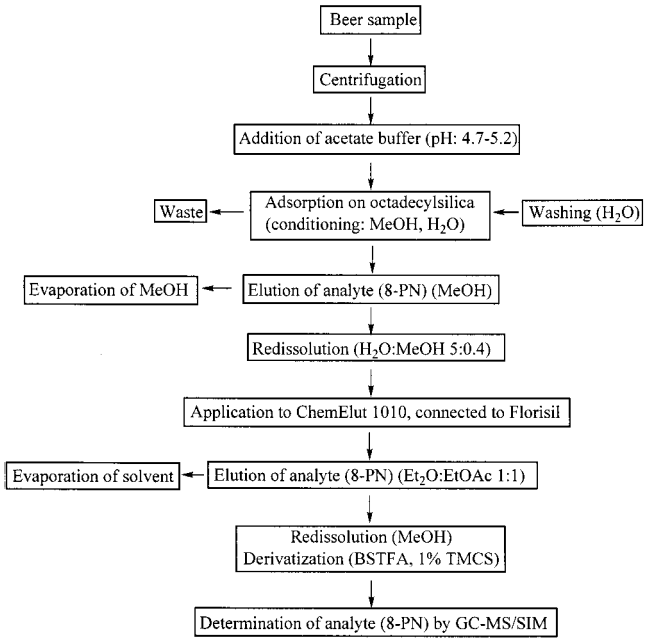
Apparatus. GC/MS analyses were carried out on a GCQ Finnigan mass spectrometer (Finnigan, San Jose, CA), hyphenated to an HP 5890 Series II gas chromatograph (Hewlett-Packard, Palo Alto, CA), which was equipped with an HP-5MS (cross-linked 5% phenyl methyl siloxane) fused silica capillary column (30 m × 0.25 mm i. d., film thickness = 0.25 μm). Injections were carried out in the splitless mode. The carrier gas was high-purity helium (Air Liquide, Liège, Belgium) at a flow rate of 1 mL min^{-1}. The injector and interface temperatures were 280 and 275 °C, respectively. The oven temperature was programmed from 70 °C (held for 1 min) to 200 °C at 40 °C min^{-1} and then to 280 °C at 10 °C min^{-1}, the final temperature being held for 20 min. The temperature of the ion source was 200 °C, and the electron voltage was 70 eV.

Reagents. Methanol and water (HPLC grade) were obtained from BDH Laboratory Supplies (Poole, Dorset, U.K.), diethyl ether and ethyl acetate were from Acros Organics (Geel, Belgium), and toluene was from Merck (Darmstadt, Germany). (*S*)-8-Prenylnaringenin [8-(3-methylbut-2-enyl)-5,7,4′-trihydroxyflavanone; 8-PN] was purified from a chloroform hop extract by preparative HPLC (purity ≥ 99% as established by analytical HPLC and ^{1}H NMR) (De Keukeleire et al., 1997a). Kaempferol (3,5,7,4′-tetrahydroxyflavone; robigenin) was obtained (purity ≥ 96%) from Fluka Chemie (Buchs, Switzerland). [*N*,*O*-Bis(trimethylsilyl)trifluoroacetamide] (BSTFA) containing 1% trimethylchlorosilane (TMCS) was from Alltech Associates (Deerfield, IL). The derivatization vials were silanized with a solution of 5% dimethyldichlorosilane (Merck) in toluene before use.

Octadecyl (C_{18}) disposable extraction columns (500 mg) and the solid-phase extraction system Baker-10 were from Baker (Phillipsburg, NJ). ChemElut CE-1010 columns for liquid/liquid extraction were obtained from Varian Sample Preparation Products (Harbor City, CA) and Florisil cartridges Sep-Pak from Waters Corp. (Milford, MA).

Procedure. A simple cleanup procedure of the aqueous beer matrix and isolation of 8-PN involved sequential solid-phase extraction (SPE; C_{18}), liquid−liquid extraction (ChemElut 1010), and adsorption−desorption (Florisil). This original protocol was recently developed for the determination of free isoflavones in human urine (Tekel' et al., 1999) and adapted to the analysis of 8-PN in beer, thereby using methanol for elution from the SPE column and diethyl ether and ethyl acetate (1:1, v/v) for desorption from the Florisil cartridge.

After centrifugation of a beer aliquot (20 mL) for 10 min at 3600 rpm, 3 mL of acetate buffer (3 M, 9.9 g of acetic acid and 18.4 g of sodium acetate trihydrate in 100 mL of water) was added to the supernatant. The content of the glass tube was premixed, and the pH (between 4.7 and 5.2) was controlled using an indicator strip and adjusted, if necessary, by addition of acetic acid. The beer sample was preconcentrated on a C_{18} column after conditioning with methanol (2 × 5 mL) and water (2 × 5 mL). Next, the SPE column was washed with water (2 × 5 mL), and the fraction of interest was eluted with methanol (2 × 3 mL). The eluate was dried in a water bath at 40 °C using a gentle stream of nitrogen. The residue was dissolved in methanol (0.4 mL) and water (5 mL) was added, followed by vortexing. This sample was applied on a ChemElut 1010 column, which was connected with a Teflon stopcock to a Florisil cartridge. After 10 min, 8-PN was eluted using diethyl ether and ethyl acetate (1:1, v/v; 50 mL). The eluate was collected, and the solvent was evaporated under vacuum at 40 °C to dryness. The residue was redissolved in methanol (1 mL) and transferred to a silanized derivatization vial. Methanol was evaporated to dryness at 60 °C in a gentle stream of nitrogen. After addition of the internal standard, BSTFA (100 μL) containing 1% TMCS was added to the vial under vortex mixing. Subsequently, the solution was heated for 60 min at 60 °C. After cooling, 2 μL of this solution was injected in the GC/MS instrument. 8-PN was analyzed in the single ion monitoring mode (GC/MS-SIM). The calibration curve for 8-PN was constructed using a blank beer sample. The blank beer was brewed without hop addition, but using a commercially available isohumulone extract (Wigan Products, Eardiston, Near Tenbury Wells, Worcestershire, U.K.; 10% aqueous solution) at the pilot brewery of the KaHo, Educational Unit of Biochemistry, Gent, Belgium). For the determination of 8-PN in the beer samples, kaempferol was added as internal standard.



RESULTS AND DISCUSSION

The electron impact (EI) mass spectrum of the tris-(trimethylsilyl) ether of 8-PN is shown in Figure 1.

The retention times of the internal standard (kaempferol) and 8-PN were 19.5 and 20.2 min, respectively, whereas the selected mass fragment ions were *m*/*z* 559, 487, 458, and 415 for kaempferol and *m*/*z* 556, 541, 513, and 485 for 8-PN. A recovery study was performed on beer samples spiked with 37 and 92.5 μg L^{-1} (n = 5). The internal standard was added prior to derivatization. The ratios of the area of the peak (sum of four selected ions) of the compound of interest to the area of the peak

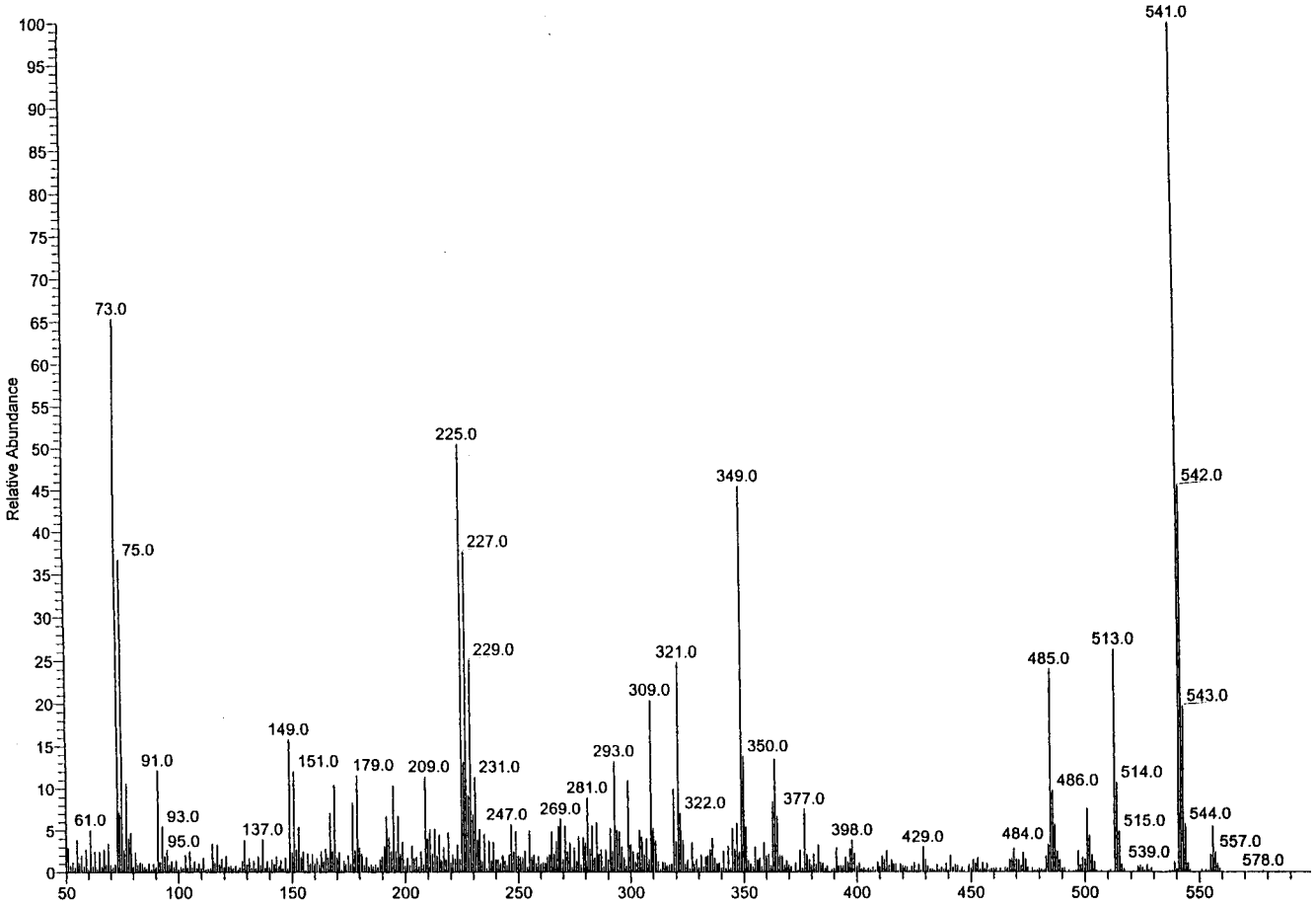


Figure 1. Mass spectrum (EI) of the tris(trimethylsilyl) ether of 8-PN (for experimental conditions, see Experimental Procedures).

(sum of four selected ions) of the internal standard were calculated. These values were compared to the ratios obtained for purified beer extracts, which were spiked with 37 or 92.5 μg L^{-1}. The recoveries of 8-PN in beer are 61.1 ± 6.6 and 82.2 ± 8.6%, respectively.

The detection limit was determined by analyzing samples of increasing concentrations of 8-PN (0, 9.25, 18.5, 37, and 74 μg L^{-1}, respectively) and a constant amount of internal standard. Ratios of the area of the peak of 8-PN to the area of the internal standard were determined. A linear calibration curve ($r = 0.997$) was established, and the detection limit was calculated as $3 \times S_b/m$, where S_b is the standard deviation of the intercept on the y-axis and m is the slope of the calibration curve (Verwaal et al., 1996). The detection limit of ~5 μg L^{-1} permits semiquantitative (incomplete recoveries, see above) monitoring of 8-PN at low levels in beer samples.

We have examined 17 Belgian beers, 12 beers from other countries, and 3 experimental beers. The results are presented in Table 1. Figure 2 represents, as an example, the GC/MS analysis of 8-PN in beer sample 4, in which mass fragment ions 541, 513, and 485 are positively detected at a retention time of ~20.4 min (detection of the molecular ion is hampered by its low intensity). A striking observation pertains to the absence of 8-PN in 14 beers (10 Belgian beers, 2 beers from abroad, and 2 experimental beers), whereas 6 beers contained 8-PN barely at the detection level (5.0 μg L^{-1}). Only 12 of 32 beers showed the presence of 8-PN in concentrations varying between 5.5 and 19.8 μg L^{-1}. Some of the beers were brewed using advanced hop products, which are manufactured by further manipulation of hops extracted using liquid or supercritical carbon dioxide (Benitez et al., 1997). Most polyphenols are too polar to be extracted in these conditions and, consequently, they are retained in the spent hops. However, prenylated flavonoids, by virtue of the presence of one or more prenyl side chains, may be dissolved to some extent in the carbon dioxide phase depending on the extraction conditions.

A potential relationship between the contents of isohumulones (the bitter-tasting compounds in beer) and 8-PN could provide valuable information. Brewers, indeed, aim at a certain isohumulone concentration in

Table 1. Concentration of 8-PN in Various Beers (for Experimental Conditions, See Experimental Procedures)

beer sample[a]	concn (μg L^{-1})	beer sample[a]	concn (μg L^{-1})
1	5.0	17	5.0
2	5.0	18	11.6
3	nd[b]	19	8.0
4	nd	20	6.7
5	nd	21	5.5
6	nd	22	6.3
7	nd	23	17.9
8	19.8	24	nd
9	16.1	25	10.1
10	nd	26	5.0
11	5.0	27	14.9
12	nd	28	6.5
13	nd	29	nd
14	5.0	30	nd
15	nd	31	nd
16	nd	32	7.9

[a] Samples 1−17, Belgian beers; samples 18−29, beers from other countries; samples 30−32, experimental beers. [b] nd, not detectable.

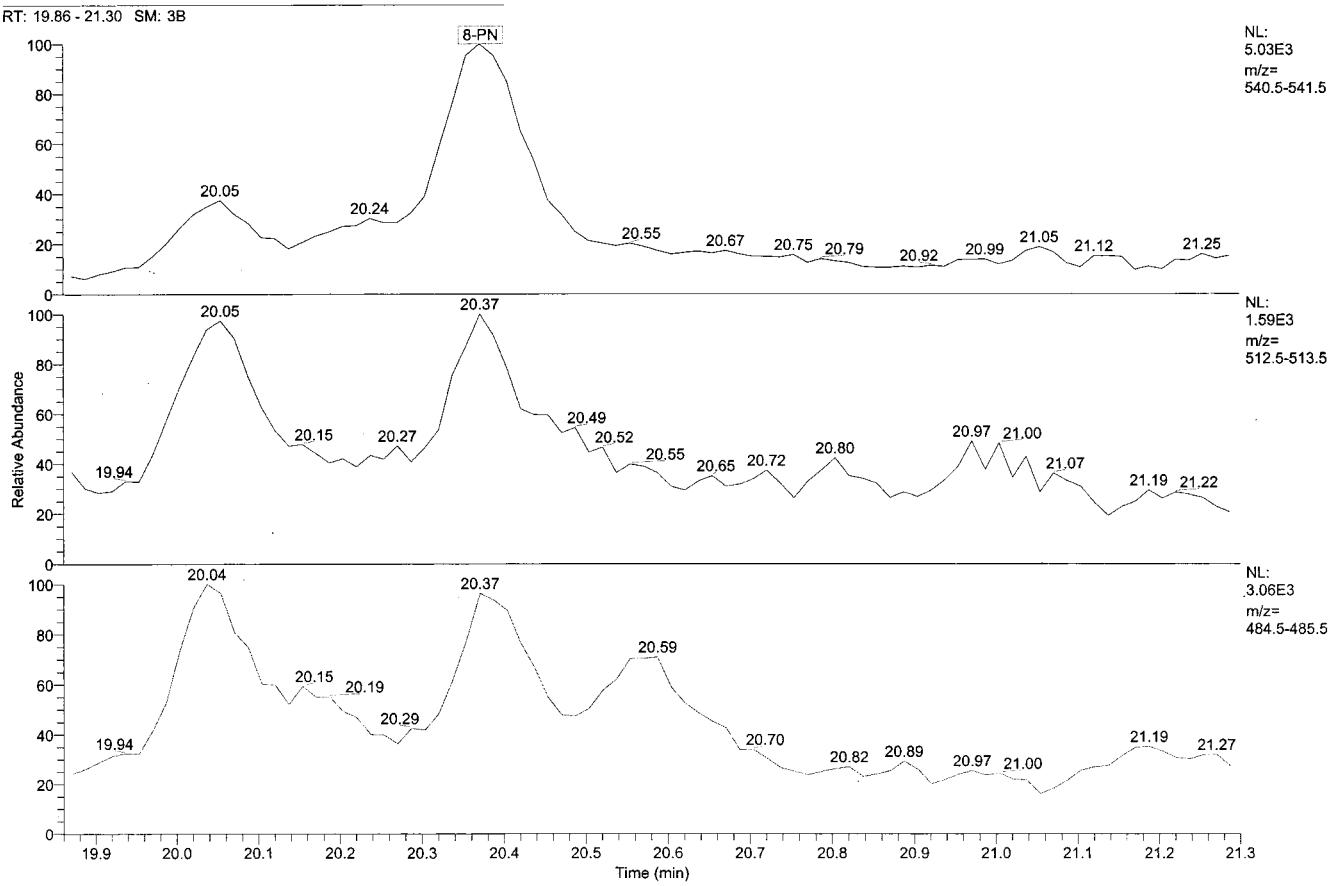


Figure 2. GC/MS-SIM detection of 8-PN in beer sample 4 (for experimental conditions, see Experimental Procedures).

the final beer and, accordingly, they decide on the level of hop addition. However, application of a hop product consisting of pure isohumulones may lead to the desired levels of isohumulones, yet the beers wil be devoid of 8-PN. Thus, there is no correlation between the concentrations of the isohumulones and of 8-PN. The 8-PN content in beer depends above all on the variety and the quantities of hops used. These parameters are unknown as the hopping regimen in each brewery is considered to be proprietary knowledge.

We determined the concentration of 8-PN in pellets prepared from the hop variety Hallertau Magnum following the method outlined above, and the average semiquantitative value was around 90 mg kg^{-1} (w/w). The concentration of 8-PN in the beer brewed using these pellets was 7.9 μg L^{-1}. Taking into account that ~0.5 g of hops was used per liter of beer, the transfer efficiency of 8-PN was, in this case, ~17%. We have previously shown that Hallertau Magnum exhibits one of the highest estrogenic activities within a series of hop varieties investigated (De Keukeleire et al., 1997b). In view of our recent findings regarding the hop estrogen, Hallertau Magnum should have a high concentration of 8-PN. When hop varieties are applied in the brewery that have much lower 8-PN levels, the estrogen could escape detection, which, in fact, has been observed for most beers. Moreover, 8-PN is expected to be absent in those beers that were brewed using advanced hop products. There is, furthermore, no correlation with beer types as we found, for example, in lager beers, both very high and undetectable levels of 8-PN. This would appear to limit a major influence of brewing technology on 8-PN concentrations in beer, because we, additionally, measured significant levels of 8-PN in a bottom-fermented and a top-fermented beer from the same brewery.

Our observations compare with the analyses of prenylated chalcones and related prenylflavonoids in hops and beer performed by Stevens et al. (1999) using sophisticated HPLC/tandem mass spectrometry. Detection of prenylflavonoids, following HPLC separation, by positive ion multiple-reaction monitoring showed levels of 8-PN varying between 13 and 17 μg L^{-1} in U.S. pilsner beers, whereas the degree of quantitative variation of 8-PN in imported beers and microbrews was very large (from 1 to 240 μg L^{-1}). It was not our concern to analyze isoflavones, but it appeared to us that they may be present in hops and also in beer, albeit in very low quantities. Isoflavones have been reported to occur in beers in concentrations of up to 29 nM (Lapcik et al., 1998), but it is questionable whether hops are the source of the isoflavones.

Both their results and our observations highlight the diversity of hop applications in the brewing practice. Obviously, the most important differentiating factor refers to the use of whole hops, on the one hand, or hop extracts and advanced hop products, on the other hand. A high hopping rate, however, does not necessarily correlate with a high 8-PN content as a large spread of 8-PN levels in various hop varieties is noted in accordance with a more or less pronounced estrogenic activity (De Keukeleire et al., 1997a). Prenylflavonoids, including 8-PN, are transferred from hops to beer to extents varying between 10 and 30% (Stevens et al., 1998), and the results observed here confirm the literature data. It is not clear whether beers brewed using advanced hop products are completely free of 8-PN as

traces may still be present in such materials. At present, it is not possible to speculate on possible health-related effects of consumption of 8-PN-containing beers as long as bioavailability data are unknown.

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