RESEARCH ARTICLE

Disposition of hop prenylflavonoids in human breast tissue

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Hop-derived products may contain xanthohumol (XN), isoxanthohumol (IX), and the potent phytoestrogen 8-prenylnaringenin (8-PN). To evaluate the potential health effects of these prenylflavonoids on breast tissue, their concentration, nature of metabolites, and biodistribution were assessed and compared with 17β-estradiol (E_2) exposure. In this dietary intervention study, women were randomly allocated to hop ($n = 11$; 2.04 mg XN, 1.20 mg IX, and 0.1 mg 8-PN *per* supplement) or control ($n = 10$). After a run-in of ≥ 4 days, three supplements were taken daily for 5 days preceding an aesthetic breast reduction. Blood and breast biopsies were analyzed using HPLC-ESI-MS/MS. Upon hop administration, XN and IX concentrations ranged between 0.72 and 17.65 nmol/L and 3.30 and 31.50 nmol/L, and between 0.26 and 5.14 pmol/g and 1.16 and 83.67 pmol/g in hydrolyzed serum and breast tissue, respectively. 8-PN however, was only detected in samples of moderate and strong 8-PN producers (0.43–7.06 nmol/L and 0.78–4.83 pmol/g). Phase I metabolism appeared to be minor (~10%), whereas extensive glucuronidation was observed (> 90%). Total prenylflavonoids showed a breast adipose/glandular tissue distribution of 38/62 and their derived E_2-equivalents were negligible compared with E_2 in adipose (384.6 ± 118.8 fmol/g, $p = 0.009$) and glandular (241.6 ± 93.1 fmol/g, $p < 0.001$) tissue, respectively. Consequently, low doses of prenylflavonoids are unlikely to elicit estrogenic responses in breast tissue.

Keywords:
8-prenylnaringenin / 17β-estradiol / Bioavailability / Breast tissue / *Humulus lupulus*

1 Introduction

It is increasingly recognized that some non-nutrients possess biological activities that may be relevant to human health. Whether dietary exposure to such compounds may result in beneficial or adverse effects is, however, often unclear because different phytochemicals, and even mixtures thereof are involved that affect diverse targets and act through several molecular mechanisms [1]. In particular,

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Abbreviations: 8-PN, 8-prenylnaringenin; **E_2,** 17β-estradiol; **$E_2\alpha/\beta$,** E_2-equivalents towards estrogen receptor α/β; **ER,** estrogen receptor; **IX,** isoxanthohumol; **RT,** room temperature; **SRM,** selected reaction monitoring; **UDP,** uridine diphosphate; **XN,** xanthohumol

dietary exposure to phytoestrogens, a group of non-nutrients capable of interfering with the endogenous estrogen signaling and associated processes *in vitro* and/or *in vivo*, causes a lot of controversy and safety concerns [2–5]. As estrogens are implicated in the etiology of breast cancer, these hormonally active compounds are being evaluated as potential cancer chemopreventive or promoting agents [6, 7].

In contrast to the rigorously studied soy phytoestrogens genistein and daidzein, comparatively less is known about the prenylflavonoids xanthohumol (XN), isoxanthohumol (IX), and 8-prenylnaringenin (8-PN) found in hops (*Humulus lupulus* L.) and hop-derived products such as beers [8] and food supplements [9, 10] (Fig. 1). Whereas XN and IX possess no or weak estrogenic activity, 8-PN is one of the most potent dietary phytoestrogens [11] and unique with respect to its estrogen receptor (ER) specificity, as it binds preferentially to ERα [12]. In addition, IX, which usually predominates over 8-PN by over tenfold [8], functions as a precursor to 8-PN as it can be *O*-demethylated by cytochrome P450 enzymes [13, 14] and/or by intestinal microbiota [15, 16]. The extent of this bioactivation varies considerably among individuals, which can be classified into weak, moderate, and strong 8-PN producers [15]. Therefore, the estrogenic potency and potential health effects of hop-derived products do not only depend on the ingested amount of 8-PN, but also on the IX concentration and the 8-PN producer phenotype [15].

To properly evaluate the impact of prenylflavonoid exposure on breast carcinogenesis, more information on their bioavailability, especially their absorption, metabolism, and distribution in human breast tissue, is needed. As phase I and II reactions alter the pharmacological profiles of phytoestrogens [17, 18] and as cell-type specific responses to estrogen exposure have been reported [19], not only the concentration, but also the nature of the metabolites and the biodistribution (either adipose or glandular tissue) of orally administered prenylflavonoids in breast tissue were assessed and compared with the endogenous 17β-estradiol (E_2) exposure. To the best of our knowledge, this is the first controlled human study addressing these issues.

2 Materials and methods

2.1 Chemicals

The isolation of XN, IX, and 8-PN from spent hops was performed as described by Stevens *et al.* [20]. Standards of 8-(4″-hydroxyisopentenyl)-5-methoxy-7,4′-dihydroxyflavanone (IX alcohol) and 8-(4″-hydroxyisopentenyl)-naringenin (8-PN alcohol) were synthesized as described by Nikolic *et al.* [21]. Glucuronides of XN, IX, and 8-PN were prepared by incubation of aglycones with pooled human liver microsomes from 50 donors (0.374 nmol cytochrome P450/mg total protein; In Vitro Technologies, Baltimore, MD, USA) in the presence of alamethicine and uridine diphosphate (UDP) glucuronic acid [14, 21]. Aliquots of 40 and 4 nmol/L solutions of a synthetic 8-PN analogue (8-isopentylnaringenin synthetized according to Roelens *et al.* [22]) in sodium acetate buffer (0.1 mol/L, pH = 5) were used as internal standards in the analyses of urine, serum, and breast tissue, respectively.

For the hydrolysis of conjugated prenylflavonoids, a 33 g/L solution of Type H-1 *Helix pomatia* extract (min. 300 U β-glucuronidase/mg and 15.3 U sulfatase/mg; Sigma-Aldrich, Bornem, Belgium) in sodium acetate buffer (0.1 mol/L, pH = 5) was prepared.

2.2 Hop-derived food supplements

One batch of commercially available hop-derived food supplements (MenoHop®, Metagenics Europe, Ostend, Belgium containing the Lifenol® extract (Naturex, Avignon, France)) was kindly provided by the manufacturer. The composition and manufacturing information have been described previously [10]. The concentrations of prenylflavonoids were measured in triplicate at study onset and closure according to Bolca *et al.* [15]; each capsule contained 2.04 ± 0.06 mg XN, 1.20 ± 0.04 mg IX, and 0.10 ± 0.01 mg 8-PN.

2.3 Subjects

A total of 21 generally healthy Belgian or Dutch women, scheduled for an aesthetic breast reduction, were recruited for this study. The exclusion criteria were breast cancer and antibiotic treatment within the previous month. Ethical approval was granted by the Ethics Committee of the Ghent University Hospital (EC UZG 2005/022; initial recruitment date: February 20th, 2007). The volunteers were fully informed on the aims of the study and gave their written consent.

2.4 Study design

This study was designed as a randomized dietary intervention trial with a run-in phase of at least 4 days and a supplementation phase of 5 days preceding the breast reduction. Following eligibility assessment, volunteers were randomly allocated to hop supplement ($n = 11$: H01-11) or control ($n = 10$: C01-10). All participants were counseled not to change their habitual, Western-type dietary patterns, but were asked to abstain from hop-based products during the whole experimental period. A detailed list of prenylflavonoid-containing foods (all beers, some herbal teas claiming sedative properties, and young hop shoots), dietary supplements, and homeopathic treatments, including examples and brand names, was distributed in order to guide the volunteers in this respect. Additionally, subjects were instructed to report every case of doubt or fortuitous consumption, and to provide detailed information on that eating occasion, including type and portion size.

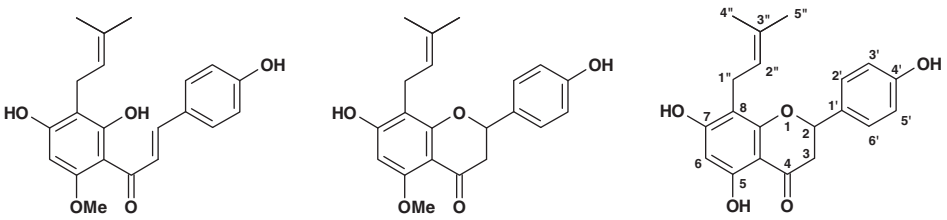

Figure 1. Structures of the most important prenylflavonoids found in hop-derived products: XN, IX, and 8-PN.

During the supplementation phase, hop-derived supplements were taken daily with meals at breakfast, lunch, and dinner (three capsules/day). The control group did not receive any supplementation before surgery. Compliance was evaluated by subject inquiry and urinary prenylflavonoid excretion.

After the run-in phase and before anesthesia, subjects delivered spot urine samples. During surgery, blood and breast biopsies were collected. Serum was obtained by centrifugation (10 min at 600 *g*, room temperature (RT)) after coagulation. Aliquots of both urine and serum samples were stored at −20°C until analysis. The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. In addition, a general questionnaire was used to collect information on the subjects' history of antibiotic treatments, hormonal therapies, use of any other medication, dietary habits and food supplement intakes, and anthropometric measures.

2.5 Sample preparation and analytical methods

2.5.1 Urine

After spiking with 100 μL internal standard, 500 μL urine were mixed with sodium acetate buffer (0.1 mol/L, pH = 5) (50:50, v/v), incubated with or without 15 μL β-glucuronidase/sulfatase for 18 h at 37°C, and extracted twice with 5 mL diethyl ether. Pooled upper organic layers were evaporated to dryness at RT under a gentle stream of N_2, reconstituted in 250 μL methanol/water (40:60, v/v), and stored at −20°C prior to HPLC-MS/MS analysis.

To allow standardization of diuresis, urinary excretion of creatinine was measured in triplicate according to the conventional kinetic Jaffé method [23] as described by Bolca *et al.* [24]. Based on a creatinine clearance rate of 0.163 mmol/(d*kg) [25], daily urinary prenylflavonoid excretions were calculated.

2.5.2 Serum

Serum samples (200 μL), spiked with 200 μL internal standard, were mixed with sodium acetate buffer (0.1 mol/L, pH = 5) (50:50, v/v), incubated with or without 100 μL β-glucuronidase/sulfatase for 18 h at 37°C, and extracted twice with 5 mL diethyl ether. Pooled upper organic layers were evaporated to dryness at RT under a gentle stream of N_2, reconstituted in 100 μL methanol/water (40:60, v/v), and stored at −20°C prior to HPLC-MS/MS analysis.

2.5.3 Breast tissue

Breast tissue samples were dissected into fractions containing almost exclusively either pure fat or glandular tissue, based on gross inspection. Areas of adipose tissue intimately intermixed with fibroglandular tissue were avoided and connective tissue was removed.

For prenylflavonoid quantification, approximately 1.2 g adipose or glandular breast tissue, spiked with 200 μL internal standard, were homogenized in 1 mL ice-cold 200 mmol/L hydrochloric acid in methanol and 1 mL hexane with a System POLYTRON® PT2100 (Kinematica AG, Luzern, Switzerland). After centrifugation (2 × 10 min at 12 000 *g*, 4°C), the hydroalcoholic phases were evaporated to dryness at RT under a gentle stream of N_2 and reconstituted in 1.6 mL sodium acetate buffer (0.1 mol/L, pH = 5). Upon incubation with or without 200 μL β-glucuronidase/sulfatase during 18 h at 37°C, samples were extracted twice with 6 mL diethyl ether. Pooled upper organic layers were evaporated to dryness at RT under a gentle stream of N_2, reconstituted in 100 μL methanol/water (40:60, v/v), and stored at −20°C prior to HPLC-MS/MS analysis.

Endogenous estrogens were extracted as described by Chetrite *et al.* [26]: approximately 200 mg adipose or glandular breast tissue were homogenized in 5 mL ethanol/water (70:30, v/v) using a T10 ULTRA-TURRAX® (Ika, Werk Staufen, Germany), and, after precipitation (2 × 24 h at −20°C), extracted with 5 mL ethyl acetate/hexane (60:40, v/v). The upper organic layer was evaporated to dryness at 37°C under a gentle stream of N_2 and reconstituted in 500 μL steroid-free serum (Std0-DRG, DRG Instruments, Marburg, Germany). Samples were analyzed for E_2 in triplicate using a commercial quantitative ELISA (EIA-4399, DRG Instruments) [27]. According to the manufacturer, this kit had a sensitivity of <5.13 pmol/L serum, an intra- and interassay CV of 6.4 and 7.6%, respectively, and a cross-reactivity of 0.2% with estrone, 0.05% with estriol, and

<0.05% with 17α-estradiol. A cross-reactivity of 0.5% was measured for prenylflavonoids.

2.5.4. Quantitative HPLC-MS/MS analysis of prenylflavonoid aglycones

Quantification of total, *i.e.* free and deconjugated, prenylflavonoids in hydrolyzed urine, serum, and tissue samples (duplicate extractions, single HPLC-MS/MS measurements) was performed by HPLC-MS/MS using a Waters 2695 Alliance separations module (Waters, Milford, MA, USA) and a triple quadrupole MS operated in negative ESI mode (TSQ Quantum, Thermo Scientific, San Jose, CA, USA). Separations were carried out on an Atlantis® T3 C18 reversed-phase column (5 μm; 2.1 × 100 mm; Waters) at 35°C and using a gradient of solvent A (*i.e.* 8.7 mmol/L aqueous acetic acid) and solvent B (*i.e.* methanol) with the following elution profile: 0–8 min, from 83% to 100% B in A; 8.1–13 min, 100% B; 13.1–20 min, from 100% to 53% B in A, and a flow rate of 250 μL/min. The injection volume was 10 μL. Ionization and detection parameters were optimized during infusion experiments with standards of XN, IX, 8-PN, IX alcohol, 8-PN alcohol, and internal standard. MS/MS data were collected in selected reaction monitoring (SRM) mode with specific transitions of parent and product ions for each analyte: XN and IX (*m/z* 353/233/119), 8-PN (*m/z* 339/219/119), IX alcohol (*m/z* 369/249/119), 8-PN alcohol (*m/z* 355/235/119), and internal standard (*m/z* 341/119). For XN, IX, and 8-PN linear ($r^2 > 0.99$) calibration curves were obtained over a range of 0.5–500 nmol/L in urine, 0.05–50 nmol/L in serum, and 0.1–100 pmol/g in tissue, with LOD ($S/N = 3$) and LOQ ($S/N = 10$) of 0.003 nmol/L and 0.010 nmol/L in urine and serum, and 0.026 pmol/g and 0.088 pmol/g in tissue, respectively.

2.5.5 Qualitative HPLC-MS/MS analysis of prenylflavonoid metabolites

Qualitative analyses of non-hydrolyzed urine, serum, and tissue samples (single extractions, single HPLC-MS/MS measurements) were performed with the equipment and conditions described above, except for the following changes. The LC mobile phase gradient was adapted: 0–30 min, from 42 to 82% B in A; 30.1–40 min, 42% B in A, with a flow rate of 220 μL/min and an injection volume of 20 μL. Ionization and detection parameters were optimized during infusion experiments with both aglycones and glucuronides of XN, IX, and 8-PN, and internal standard. MS/MS data were collected in SRM mode with specific transitions of parent and product ions for each analyte: XN and IX (*m/z* 353/233), 8-PN (*m/z* 339/219), IX alcohol (*m/z* 369/119), 8-PN alcohol (*m/z* 355/235), XN glucuronide and IX glucuronide (*m/z* 529/353/233), 8-PN glucuronide (*m/z* 515/339/219), and internal standard (*m/z* 341/119).

2.6 Statistical analyses

SPSS for Windows version 15.0 (SPSS, Chicago, IL, USA) was used for all statistical analyses. Results were considered statistically significant at an α two-tailed level of 0.05. Means and SEM of urine, serum, and tissue concentrations were calculated. Tests for normality and equality of the variances were performed using the Kolmogorov–Smirnov and Levene's test, respectively. Intrasubject comparisons were evaluated with the paired Student's *t*-test or Wilcoxon's matched-pairs signed-rank test, whereas the Student's *t*-test was used to compare means between groups. Using the TwoStep cluster analysis protocol, subjects were phenotyped as weak, moderate, or strong 8-PN producers based on the urinary excretion of 8-PN/(8-PN+IX) [15].

3 Results

3.1 Study population

A total of 21 generally healthy women undergoing an aesthetic breast reduction participated in this study. Their age and BMI, based on self-reported weight and height measurements, ranged between 18 and 60 years and 19 and 36 kg/m^2, respectively, and did not differ significantly ($p = 0.758$ and $p = 0.088$) between groups. Six women (32%; H01-03 and C01-03) were in the follicular phase of their menstrual cycle and 2 (11%; H04 and C04) in the luteal phase, whereas 11 (46%; H05-10 and C05-09) were menopausal. Four women followed a therapy with exogenous estrogens: *i.e.* oral (5%; C03) or intra-uterine (10%; H11 and C10) contraceptives, or estrogen therapy (5%; H10); while one was treated with anti-estrogens (5%; C09). Based on their urinary excretion profiles, three participants (27%; H06, H09, and H10) were phenotyped as moderate 8-PN producers and 3 (27%; H02, H03, and H07) as strong 8-PN producers.

3.2 Total exposure

The exposure to XN, IX, and 8-PN upon hop supplementation was assessed as the sum of unconjugated aglycones and deconjugated (sulfo)glucuronides and sulfates as measured in hydrolyzed urine, serum, and breast adipose and glandular tissue (Table 1). None of the control samples contained detectable amounts of hop-derived phytoestrogens. The estimated daily urinary prenylflavonoid excretion profiles varied considerably between individuals and were in the nmol/day range (4.45–52.64 nmol XN/day, 84.47–850.09 nmol IX/day, and 13.18–104.14 nmol 8-PN/day). XN and IX were found in all hydrolyzed serum samples and ranged between 0.72 and 17.65 nmol/L and 3.30 and 31.50 nmol/L, respectively. However, only good 8-PN producers (three strong and two moderate) had circulating 8-PN concentrations

Table 1. Urinary excretion, and serum and breast tissue concentrations of XN, IX, and 8-PN aglycones equivalents upon prenylflavonoid supplementation (*i.e.* 6.12 mg XN, 3.6 mg IX, and 0.3 mg 8-PN *per* day, for 5 days)

	Urine (nmol/day)		Serum (nmol/L)		Breast tissue (pmol/g)			
					Adipose		Glandular	
	n	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM	*n*	Mean ± SEM
XN	11	22.52 ± 4.93	11	4.99 ± 1.79	9	0.69 ± 0.17	10	2.58 ± 0.45
IX	11	526.16 ± 70.83	11	14.86 ± 2.71	11	3.68 ± 1.27	11	12.02 ± 7.20
8-PN	11	44.28 ± 7.82	5	2.20 ± 1.26	6	1.44 ± 0.34	7	2.50 ± 0.46

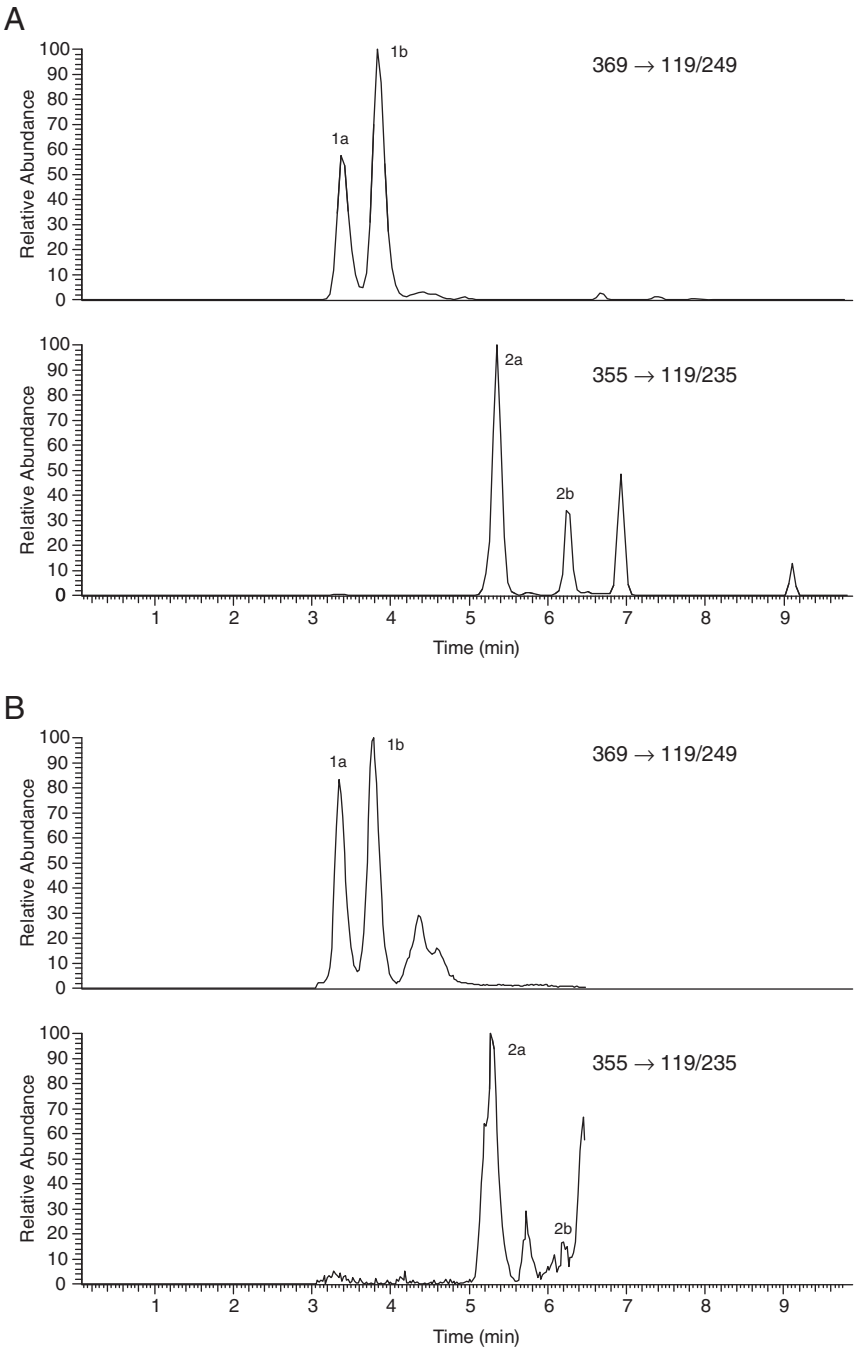

Figure 2. SRM trace chromatograms of *cis* and *trans* alcohols of IX (1a,b; *m/z* 369/119/249) and 8-PN (2a,b; *m/z* 355/119/235) (A) standards in control urine and (B) in hydrolyzed urine after prenylflavonoid supplementation.

(0.43–7.06 nmol/L) exceeding the LOQ. In breast adipose and glandular tissue, exposure levels of 0.26–5.14 pmol XN/g, 1.16–83.67 pmol IX/g, and 0.78–4.83 pmol 8-PN/g were measured. Unlike XN, IX could be quantified in all tissue samples, whereas 8-PN was detected in both adipose and glandular breast tissue of the moderate and strong 8-PN producers, but also in glandular tissue of one poor 8-PN producer (H11). No statistically significant correlations were observed between the serum and tissue concentrations of XN, IX, and 8-PN.

3.3 Phase I and II metabolism

In hydrolyzed urine, both *cis* and *trans* alcohols of IX were observed in varying ratios (0.45–0.89) and were estimated to compose $9.3 \pm 1.0\%$ of the total IX equivalents (Fig. 2). Conversely, only one isomer of the monooxidation products of 8-PN, accounting for $12.3 \pm 1.4\%$ of the total 8-PN equivalents, was found. No phase I-metabolites were detected in serum or tissue samples.

Upon enzymatic hydrolysis, urinary aglycone concentrations raised 10–20 times (IX: 13.3 ± 7.3; 8-PN: 11.6 ± 2.2), whereas the corresponding glucuronides disappeared (Fig. 3). Only traces of aglycones were observed in non-hydrolyzed serum and tissue samples suggesting an extensive glucuronidation (> 90%).

3.4 Breast tissue disposition

Compared with adipose breast tissue, higher mean XN, IX, and 8-PN concentrations were measured in glandular tissue ($p = 0.011$, $p = 0.059$, and $p = 0.105$, respectively), with an adipose/glandular tissue distribution of 22/78 (46/54–8/92), 34/66 (77/23–2/98), and 36/64 (49/51–14/86), respectively (Fig. 4).

The exposure to 8-PN was translated into E_2-equivalents towards ERα and ERβ (E_2α- and E_2β-equivalents) assuming relative estrogenic potencies towards ERα of 1/10 and towards ERβ of 1/100 [11] and 98% attenuation due to glucuronidation [28]. Upon prenylflavonoid supplementation, breast tissue was exposed to phytoestrogen-derived E_2α-equivalents (adipose fractions: 2.9 ± 0.7 fmol/g; glandular fractions: 5.0 ± 0.9 fmol/g), which were significantly lower than the measured unconjugated E_2 tissue concentrations (adipose fractions: 384.6 ± 118.8 fmol/g, $p = 0.009$; glandular fractions: 241.6 ± 93.1 fmol/g, $p < 0.001$), and even lower (tenfold) E_2β-equivalents.

4 Discussion

For the first time, the absorption, metabolism, and distribution of the prenylflavonoids XN, IX, and 8-PN were assessed in normal human breast tissue after hop-derived supplement intake. These hop-derived prenylflavonoids are known to possess pleiotropic bioactivities *in vitro* and *in vivo*. XN was shown to prevent carcinogen-induced preneoplastic lesions in mouse mammary gland organ cultures at nmol/L concentrations (IC_{50}: 20 nmol/L) [29]. As a full agonist of both ERα and ERβ [22], 8-PN can mimic or modulate estrogenic effects, depending on the endogenous estrogen levels and the target tissue. Longer-term (3 m) oral administration of high doses of pure 8-PN (equipotent human dose ~650 mg/day) had similar although milder effects than E_2 (equipotent human dose ~7 mg/day) on the uterus, vagina, and mammary gland of ovariectomized rats [5, 30]. Even though a tenfold lower 8-PN dosing did not elicit estrogenic responses and other, ER-independent, mechanisms of action such as inhibition of aromatase activity (*in vitro* IC_{50}~75 nmol/L, [31, 32]) and angiogenesis (*in vivo* IC_{50}~125 nmol/L, [33]), have been proposed, safety questions arise concerning unrestricted long-term use of freely accessible hop-derived food supplements [5, 30, 34]. However, to test and evaluate the suggested mechanisms of action on breast tissue, information on the levels of orally ingested prenylflavonoids that actually reach their target site in a bioactive conformation is needed. Our results indicate that, >12 h after last hop supplement intake, breast adipocytes and mammary gland epithelial cells were exposed to 0.05–0.50 pmol/g total prenylflavonoid aglycones and 2.5–20 pmol/g total prenylflavonoid glucuronides.

Although differences in time of sampling, dosing regimen, and formulation [35] often hamper sound comparisons between intervention trials, the urine and serum concentrations observed in this study were in line with previous reports on human exposure to prenylflavonoids [15, 16, 28, 36]. The prenylflavonoid tissue levels were lower compared with the corresponding serum concentrations. Moreover, the lack of correlation between serum and tissue levels suggests that serum concentrations do not predict tissue disposition, as described for isoflavones [37, 38].

In this study, *cis* and *trans* alcohols of IX and 8-PN, the most abundant human liver microsomal metabolites *in vitro* [14, 21], were only detected in hydrolyzed urine samples. Similarly, Overk *et al.* found no 8-PN alcohol aglycones, but detected the monoglucuronides of these metabolites in non-hydrolyzed serum samples of rats [34]. Overall, phase I metabolism appeared to play a minor role (~10%) probably due to rapid conjugation to glucuronic acid and/or sulfate moieties in the intestinal epithelium and liver, as observed in *in vitro*-studies [39–41]. In accordance with previous reports on human exposure to 8-PN [28, 36] and isoflavones [42–47], prenylflavonoid monoglucuronides were the predominant circulating metabolites. However, diglucuronides, sulfoglucuronides, mono- and disulfates were not monitored, but expected to be minor. Like serum, breast tissue contained mostly glucuronidated prenylflavonoids. Besides the studies of Bolca *et al.* [42] and Guy *et al.* [44], we are not aware of other reports on the disposition of phase II metabolites of phytoestrogens in human tissue.

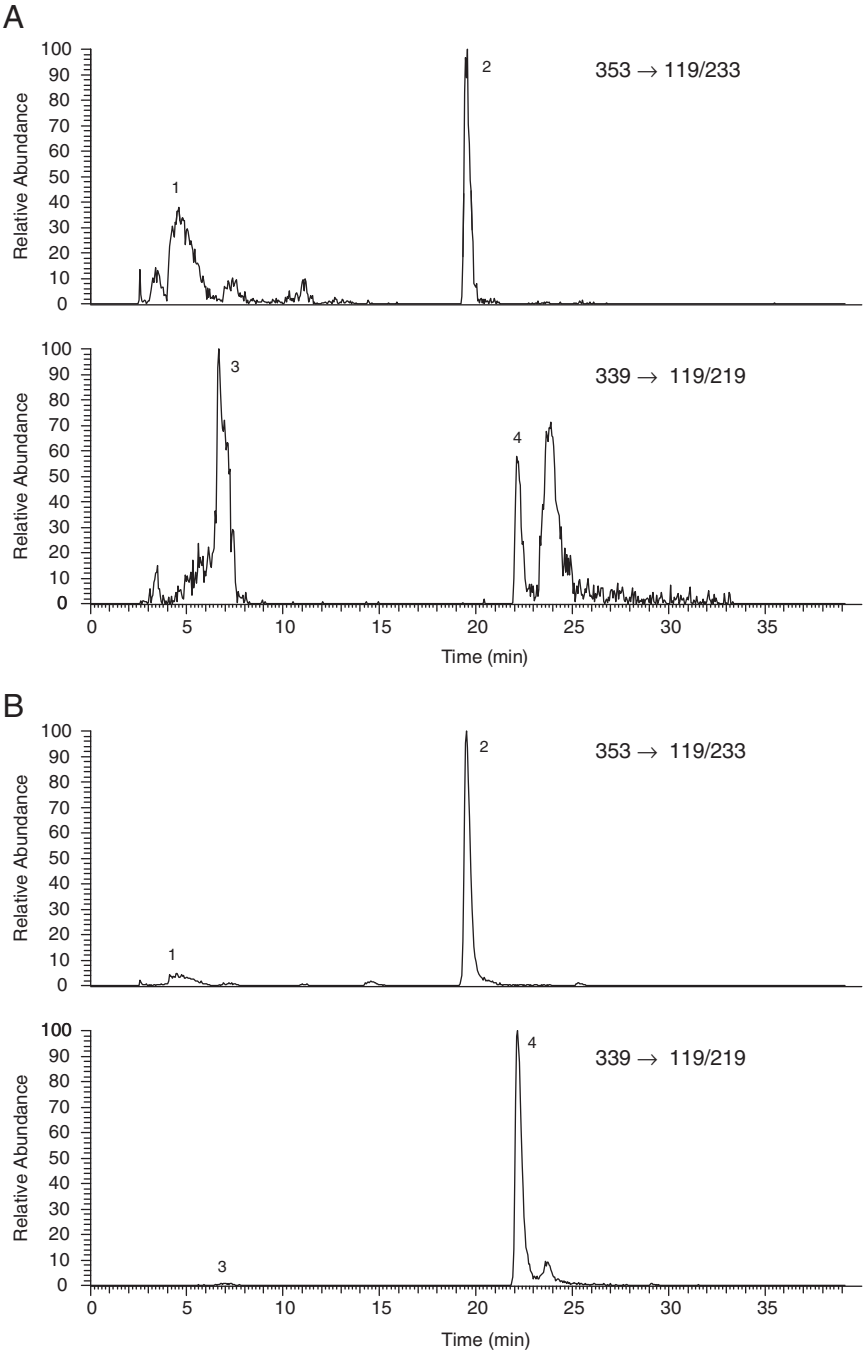

Figure 3. SRM trace chromatograms of monoglucuronides (1 and 3) and aglycones (2 and 4) of IX (*m/z* 353/119/233) and 8-PN (*m/z* 339/119/219) in (A) non-hydrolyzed and (B) hydrolyzed urine after prenylflavonoid supplementation.

The question is whether the exposure to XN, IX, and 8-PN, as observed in the present study, can result in any, protective or adverse, response related to breast carcinogenesis. Given the complexity of possible interactions, our information on *in situ* concentrations in addition to the current state of knowledge only allows for speculation on the potential activities of orally administered prenylflavonoids on breast tissue. The prevailing opinion is that treatments that trigger ER antagonistic effects, such as tamoxifen, and/or reduce E_2 and estrone levels, such as aromatase inhibitors, are protective against breast cancer and favorably affect the course of breast cancer once diagnosed [48]. Therefore, we discuss our findings in the context of the potential of hop-derived prenylflavonoids to interfere with ER-mediated signaling and steroidogenesis.

Both ER isoforms, *i.e.* ERα and ERβ, are expressed in breast tissue and estrogen-induced cell proliferation and breast carcinogenesis have mainly been linked to ERα signaling whereas ERβ can antagonize ERα-dependent transcription [49]. Consequently, the balance between ERα

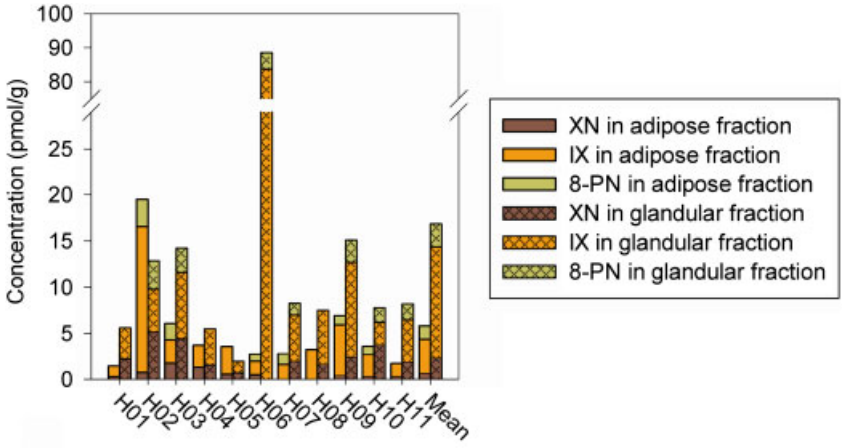

Figure 4. Disposition of XN, IX, and 8-PN in breast adipose and glandular tissue after prenylflavonoid supplementation. Mean values of duplicate extractions with single HPLC-MS/MS measurements.

and ERβ signaling has been hypothesized to determine the overall proliferative response to estrogens [49]. However, ligand-specific conformational changes in ER upon binding result in unique coregulator recruitment and transcriptional responses, as illustrated in animal studies *e.g.* [12]. The final outcome is, therefore, the result of the complex interplay and competition between all ligands, their metabolites, and even mixtures thereof, as far as their concentration and/or activity is physiologically relevant. Given the low exposure to XN, IX, and IX and 8-PN alcohol aglycones, all with weak or unknown estrogenic potencies [11, 17], their contribution was not considered in this evaluation. Although glucuronidation may increase the bioactivity of its substrate in some cases (*e.g.* morphine [50]), estrogenicity is typically attenuated upon glucuronidation (*e.g.* tamoxifen [51] and phytoestrogens [52–54]). Still, glucuronides may act as a source of tissue aglycones by means of *in situ* glucuronidase activity, but concomitantly intracellular UDP-glucuronosyltransferases can catalyze the opposite reaction. Considering all prior remarks, the overall E_2α- and E_2β-equivalents were calculated based on the 8-PN aglycone exposure levels only, thereby highlighting the importance of the 8-PN producer phenotype. Still, both hop-derived E_2α- and E_2β-equivalents were negligible compared with the E_2 tissue concentrations, which were in agreement with literature reports [26, 27, 55–58], suggesting that, in this case, hop consumption is unlikely to elicit important agonistic effects in human breast tissue. Yet, other bioactivities, directly or indirectly related to breast carcinogenesis, cannot be excluded based on these findings.

Breast tissue E_2 levels are maintained by active uptake of circulating estrogens and/or local synthesis (''intracrine organ'' concept) [59]. The putative attenuation of *in situ* steroidogenesis through aromatase inhibition by XN, IX or 8-PN [31, 32] is difficult to evaluate since these experiments were performed at supraphysiological concentrations (>10 nmol/L) and little or no information is currently available on the ability of phase I and II metabolites and mixtures of prenylflavonoids to modulate E_2 synthesis and metabolism. In the present study, no significant differences in unconjugated E_2 tissue levels were detected between the hop and control groups, but it was not designed to evaluate the effect of prenylflavonoid supplementation on the expression and/or activity of steroidogenic enzymes.

This work has some limitations. Firstly, sampling was done at a single time point, >12 h after last hop administration. Although steady-state levels are reached after 5 days of regular phytoestrogen intake throughout the day [60, 61], diurnal fluctuations are expected because of the discontinued dosing during night, as shown for isoflavone serum concentrations [35]. Secondly, tissue was obtained from a small, heterogeneous group of generally healthy women with mammary hypertrophy and it is difficult to predict to what extent our findings can be extrapolated to the general (female) population. Breast tumors, for instance, have a lowered ERβ expression [49], enhanced β-glucuronidase and decreased UDP-glucuronosyltransferase activities [62], and an overall altered estrogen metabolism resulting in higher E_2 levels [26, 57, 58, 63] and, therefore, a markedly different hormonal environment. Similarly, phytoestrogen disposition may be different *in utero*, during childhood, puberty or pregnancy and in men. Thirdly, the very short-term prenylflavonoid challenges applied in this study do not reflect dietary phytoestrogen exposure, which is often a combination of several phytochemicals with multiple and perhaps additive or interfering activities. Moreover, it is unclear whether and how other dietary constituents and doses of hop-derived phytoestrogens influence their disposition in breast tissue and, therefore, extrapolations should be done with caution. Finally, *in vitro* data were used to translate these prenylflavonoid levels to an overall exposure to E_2-equivalents. To strengthen these total estrogenicity estimates the use of ERα- and ERβ-driven reporter gene bioassays [64] is strongly recommended.

Taking these limitations and assumptions into consideration, we conclude that low doses of prenylflavonoids (~10 mg/day) are unlikely to elicit ER-mediated responses in breast tissue relevant to breast carcinogenesis.

In addition, this study provides data for a more comprehensive evaluation of the safety of hop-derived phytoestrogens, based on physiologically relevant exposure levels and metabolites.

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