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Analytical methods for quantitation of prenylated flavonoids from hops

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Abstract

The female flowers of hops (*Humulus lupulus* L.) are used as a flavoring agent in the brewing industry. There is growing interest in possible health benefits of hops, particularly as estrogenic and chemopreventive agents. Among the possible active constituents, most of the attention has focused on prenylated flavonoids, which can chemically be classified as prenylated chalcones and prenylated flavanones. Among chalcones, xanthohumol (XN) and desmethyloxanthohumol (DMX) have been the most studied, while among flavanones, 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN) have received the most attention.

Because of the interest in medicinal properties of prenylated flavonoids, there is demand for accurate, reproducible and sensitive analytical methods to quantify these compounds in various matrices. Such methods are needed, for example, for quality control and standardization of hop extracts, measurement of the content of prenylated flavonoids in beer, and to determine pharmacokinetic properties of prenylated flavonoids in animals and humans. This review summarizes currently available analytical methods for quantitative analysis of the major prenylated flavonoids, with an emphasis on the LC-MS and LC-MS-MS methods and their recent applications to biomedical research on hops. This review covers all methods in which prenylated flavonoids have been measured, either as the primary analytes or as a part of a larger group of analytes. The review also discusses methodological issues relating to the quantitative analysis of these compounds regardless of the chosen analytical approach.

Keywords

hops; mass spectrometry; 8-prenylnaringenin; prenylated flavonoids; quantitation; xanthohumol

1. INTRODUCTION

The female flowers of hops (*Humulus lupulus* L.) are used in the brewing industry to add aroma and bitterness to beer. Recently, there has been interest in the possible health benefits of hops. Hops has been traditionally promoted as a mild sedative, but research on its clinical efficacy and possible active constituent(s) remains inconclusive [1]. In recent years, most of the attention has focused on potential estrogenic and cancer chemopreventive properties of hops. The research in this area has advanced to the point that both Phase I [2–4] and Phase II clinical trials have been completed with hop extracts or purified constituents [5, 6].

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Among the possible active constituents, prenylated flavonoids have received the most attention. Chemically, they can be divided into two groups: prenylated chalcones and prenylated flavanones (Figure 1). In hop cones, the most abundant prenylated chalcone is xanthohumol (XN) whose content can reach up 1% of dry weight [7, 8]. XN has been primarily studied for its cancer chemopreventive properties. It exhibits strong antiproliferative activity against breast, colon and ovarian cancer cell lines and is a potent inducer of quinone reductase (NQO1) [9–11]. In-depth reviews of the biological properties of XN have been published recently [7, 12, 13]. Desmethylxanthohumol (DMX), a demethyl analog of XN, is much less abundant in hops and occurs at levels 1/30 to 1/5 of those of XN [7, 8, 14, 15]. While DMX may have chemopreventive activities (reviewed in [15]), the interest in this compound primarily comes from its propensity to isomerize into flavanones 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN) (see below).

In contrast to chalcones, prenylated flavanones are minor constituents of hops occurring at concentrations 10–100 fold lower than XN [7, 8]. This class of compounds has been primarily investigated for estrogenic properties. Among prenylated flavanones, 8-PN has been identified as one of the most potent phytoestrogens [16] and its estrogenic properties have been confirmed in numerous *in vitro* and animal studies [17–19]. Isoxanthohumol (IX), the 5-*O*-methyl analog of 8-PN, has much weaker estrogenic activity [17]. However, several *in vitro* and *in vivo* studies have shown that IX can be metabolically converted into 8-PN either by the action of cytochrome P450 or by intestinal microflora [20–23]. Thus, IX can be considered a pro-estrogen, which provides an important rationale for inclusion of this compound in the standardization of various hop extracts. More extensive coverage of the phytochemistry and biological activities of hops is available in several excellent reviews [24–27].

Because of the increased interest in medicinal properties of prenylated flavonoids, there is a demand for accurate, reproducible and sensitive analytical methods to quantify these compounds in various matrices. Examples of research areas that demand such methods include quality control and standardization of hop extracts, measurement of the content of prenylated flavonoids in beer to estimate human exposure, and investigations of pharmacokinetic properties of prenylated flavonoids in animals and humans.

The purpose of this review is to summarize currently available methods for quantitative analysis of the major prenylated flavonoids of hops with emphasis on high performance liquid chromatography-mass spectrometry (LC-MS) and LC-tandem mass spectrometry (LC-MS-MS). The review will cover all methods in which prenylated flavonoids have been measured, either as the primary analytes or as part of a larger group of compounds. Methodological issues relevant to the quantitative analysis of these compounds will be discussed that are independent of the final analytical technique that is used.

2. SOURCES AND PURITY OF REFERENCE STANDARDS

XN and DMX are primarily obtained by isolation from spent hops, a byproduct from supercritical CO₂ extraction of the bitter acid flavor components from hop strobiles. Isolation procedures using both adsorbent-based chromatography and high-speed countercurrent chromatography have been reported [8, 28]. Synthetic methods for preparation of both XN and DMX have also been reported [29, 30]. Given the high abundance of XN in spent hops and the availability of this material, it is unlikely that total synthesis will replace isolation as the primary source of this compound. However, synthesis of DMX might be preferable, since this compound is present in much smaller quantities and is often difficult to obtain in good yields and purity from spent hops.

Since prenylated flavanones are minor constituents of spent hops, isolation is not the most economical way to obtain sufficient amounts of pure reference standards. Consequently, various synthetic and semi-synthetic approaches for preparation of prenylated flavanones have been developed. IX is typically prepared by isomerization of XN in basic medium [14, 31]. In this procedure, XN is dissolved in 1% aqueous or 5% ethanolic sodium hydroxide. After the reaction is complete, the mixture is acidified and the resulting IX purified by preparative HPLC. Both 8-PN and 6-PN can be synthesized by a number of methods that rely on prenylation of naringenin. Older approaches typically resulted in nonselective prenylation, while newer methods are more selective for either 8-PN or 6-PN [32, 33]. Recently, viable procedures for preparation of 8-PN by demethylation of IX have been developed [34]. A welcome development in this field is that commercial suppliers are now offering reference standards for some of the prenylated flavonoids. Currently, the most pressing need in this area is stable isotope labeled analogs that can be used as internal standards for quantitative analysis using LC-MS-MS. Given the interest in prenylated flavonoids, it is likely that some of the published synthetic procedures will soon be optimized for the preparation of these analogs.

The purity of the reference standards (when stated) is typically reported as better than 90%, but the methods used to determine purity are not always stated. Some methods reported purity as low as 90% [35, 36], which is substandard for good analytical work. When stated, the purity was typically determined by HPLC-UV or LC-MS. The impurities that accompany the main component are rarely reported. This is unfortunate since complete separation of related chalcones and flavanones is often difficult during isolation. For example, 6-PN and 8-PN can often be found as impurities in isolated XN [26]. If these issues are not addressed, errors can be introduced into the final analytical results. Furthermore, only a few reports mention water content [31], and we could find no report concerning the content of residual solvents remaining from purification procedures. A recently developed approach of quantitative NMR (qNMR) [37, 38] may be a better alternative to the chromatographic methods in that it provides both the quantitative and qualitative information about impurities [17].

3. SOLUTION STABILITY OF PRENYLATED FLAVONOIDS

8-PN and 6-PN are generally stable compounds, and no major issues regarding their stability have been reported. Wyns *et al.* noted only a slight degradation of low levels of IX and 8-PN in urine after three freeze-thaw cycles [39]. In contrast, isomerization of prenylchalcones into the corresponding flavanones is an important issue that needs to be carefully addressed during analytical method development. This isomerization is brought about intramolecular nucleophilic addition of the 2-hydroxyl group onto the Michael acceptor of the chalcone skeleton. XN isomerizes into IX while DMX isomerizes to form a mixture of 8-PN and 6-PN due to the possibility of rotation around the carbon bond of the B ring (see Figure 1). The ratio of 8-PN to 6-PN varies with the experimental conditions, but 6-PN is typically the major isomer. Isomerization of prenylchalcones into flavanones is not only of analytical significance, but also of great biological significance. The reaction sequence by which XN first cyclizes into IX and then becomes demethylated to form 8-PN is a possible *in vivo* pathway for conversion of abundant, but not estrogenic XN into the potent estrogen 8-PN. Similarly, through cyclization, non-estrogenic DMX can be converted into estrogenic 8-PN. Therefore, if artifactual isomerization of chalcones during sample preparation and analysis is not properly controlled, erroneous conclusions about biological conversion of chalcones into flavanones will be made.

Of the two prenylchalcones, XN is more resistant to cyclization. Stevens *et al.* [14] noted that addition of 1% formic acid improved stability of XN, particularly in aqueous solutions.

Methanolic stock solutions in 99:1 methanol:formic acid (v/v) were reported stable for up to 4 months. The recommendation to use acid does not appear to be widely followed, with most groups using pure methanol or DMSO for stock solution preparation.

In contrast, DMX is very unstable particularly in aqueous solutions. Low stability of DMX in solution likely originates from the intramolecular general acid catalysis by the 6-OH group as demonstrated for other chalcones [40]. Rapid isomerization of DMX into 8-PN and 6-PN has been cited as the main reason why this compound has not been found in beer [14]. Interestingly, Chen *et al.* found that degassing dramatically decreased stability of the methanolic solution of DMX [38]. Chen *et al.* [38] and Stevens *et al.* [14] also noted that isomerization could not account for all of the loss of DMX and that some degradation of the prenyl group likely occurred, although the structure of degradation products were not reported. Because of these considerations, it has been recommended that stock solutions of DMX be prepared weekly [14]. The low solution stability of DMX is a significant problem during quantitative analysis of this chalcone and is likely the reason why far fewer methods have been developed for this prenylchalcone compared to XN. Even when values for DMX content in hop extracts were reported, no detailed investigation of the stability of DMX under analytical conditions was reported [14, 41]. The stability of DMX in biological matrices has not been investigated to date. A careful investigation of this issue is needed to answer the important research question of whether DMX can serve as a precursor of the potent estrogen 8-PN *in vivo*.

4. SAMPLE PREPARATION METHODS

Various sample preparation methods have been employed depending on the type of matrix and demands of the analytical method, i.e., whether a single flavonoid or a mixture of compounds was analyzed.

4.1. Beer analysis

Most methods for beer analysis have used simple degassing to prevent frothing during pipetting, followed by sample filtration prior to injection. Despite a relatively simple sample preparation, very different recoveries of the target flavonoids from “flavonoid-free” beer have been reported. Stevens *et al.* [14] and Intelmann *et al.* [42] reported good recovery of prenylated flavanones ranging from 88–110%, which was in contrast to 68.8% reported for 8-PN by Maragou *et al.* [43]. Stevens *et al.* [14] reported recovery of DMX and XN of 78–88% (depending of the target level), which was in contrast to 94% recovery reported by Intelmann *et al.* [42]. Low recovery of some flavonoids has been attributed to the complexation with macromolecules present in beer such as complex carbohydrates and proteins [14, 44]. In contrast, Maragou *et al.* attributed low recovery of 8-PN to strong binding to the PTFE filter used to filter the sample [43]. Whatever the reason(s) for low recovery, it appears that methods that use simple degassing and filtration tend to underestimate concentrations of prenylated flavonoids in beer.

Several methods have used an extraction technique such as solid phase extraction (SPE) [44], cloud point extraction (CPE) [45] or magnetic mixed hemi-micelles SPE (MMHSPE) to enrich the target analytes prior to analysis [46]. SPE resulted in poor recovery of 8-PN (63.4–72.8%), while CPE and MMHSPE showed excellent recovery of XN from beer (90–103%). The latter method achieved a concentration factor of 60 enabling very low quantitation limits.

4.2. Hop extracts

Hop cones and supplements containing hop extracts are typically extracted by sonication in methanol or ethanol, both of which are excellent solvents for prenylated flavonoids [36, 47]. Recoveries in excess of 90% have been observed for all prenylflavonoids.

4.3. Biological fluids

For analysis of prenylated flavonoids in serum/plasma or urine, liquid-liquid extraction (LLE) and SPE have been the usual sample preparation methods, except for two reports that used protein precipitation [17, 48]. In biological specimens, prenylated flavonoids are predominantly found in the form of conjugates (glucuronides and/or sulfates) and prior enzymatic deconjugation with glucuronidase/sulfatase mixture is necessary to release free aglycones. Methyl-*tert* butyl ether (MTBE), diethyl ether and ethyl acetate have been the most commonly used solvents for LLE. A typical procedure uses 2–3 extractions with 3–10 volumes of organic solvent. Combined organic phases are then evaporated to dryness and reconstituted in an appropriate solvent.

At this stage, care needs to be taken to ensure that all residue has dissolved. We have observed that for serum samples, a high percentage of methanol (>70%) is needed to fully redissolve the extract. Lower methanol content resulted in a cloudy solution not suitable for injection onto HPLC columns. This is likely due to presence of hydrophobic serum constituents (lipids) which are not soluble in a polar medium but were extracted by a non-polar solvent such as MTBE. Defatting the serum with hexane prior to extraction as carried out by Bolca *et al.* may help alleviate this problem [49]. Excellent recoveries (> 90%) from serum, urine and fecal cultures were observed for all prenylflavonoids [22, 23, 50, 51], except in the report by Wyns *et al.* [39], who reported recoveries of 78.7% and 74.1% for 8-PN and XN, respectively.

SPE was also used for extraction of IX and 8-PN from urine [21] and serum [39]. While good recovery of IX and 8-PN from urine was observed [21], Wyns *et al.* noted poor recovery of IX and XN (40.6–46.8% and 58.8–69.1% for IX and XN, respectively) from serum [39]. In our laboratory, we also observed lower recovery of XN using SPE compared to LLE particularly for concentrations above 100 ng/ml. A possible reason for poor recovery of XN is its strong binding to serum proteins which is not disrupted during SPE [52]. Based on these observations, LLE appears to be a method of choice for efficient extraction of prenylflavonoids from biological fluids.

4.4. Tissue analysis

Overk *et al.* reported analysis of 8-PN and IX in rat liver and mammary gland [17]. Liver samples were first homogenized in phosphate buffer (pH 7.4) followed by LLE with ethyl acetate. Mammary glands were homogenized in phosphate buffer followed by protein precipitation using methanol/acetonitrile. After centrifugation, the supernatant was evaporated to dryness and redissolved in 70% methanol prior to analysis using LC-MS-MS.

For the analysis of prenylflavonoids in breast tissue, sample preparation has involved homogenization of tissue biopsies in a mixture of cold 0.2 M hydrochloric acid in methanol and hexane (1:1, v/v) [53]. After centrifugation, the hydroalcoholic phase was removed, evaporated to dryness and reconstituted in sodium acetate buffer. After enzymatic deconjugation, prenylflavonoid aglycones were extracted with diethyl ether.

5. LC-MS AND LC-MS-MS METHODS

Because of its speed, sensitivity and selectivity, mass spectrometry has become the dominant technique for quantitative analysis of natural products, particularly in complex matrices such as biological samples. A summary of published LC-MS and LC-MS-MS methods for analysis of prenylflavonoids is presented in Table 1.

Because molecular masses and tandem mass spectra are nearly identical for the isomeric pairs XN/IX and DMX/6-, 8-PN (see below), good chromatographic separation is essential for accurate quantitation. Fortunately, separation is easily accomplished using common C₈ or C₁₈ reversed phase stationary phases. Due to different polarities of the target flavonoids, gradient elution during reversed phase chromatography has been invariably used. Most methods have carried out separations under acidic conditions using either formic or acetic acid to control the pH, except the method of Maragou *et al.*, which used water as the weak solvent [43]. Separation under acidic conditions is preferable because low pH suppresses ionization of phenolic groups providing better chromatographic peak shape. In terms of column geometry, there has been a general trend toward using shorter columns (100 mm or shorter) packed with smaller particles (3.5 μ m or smaller), which ultimately results in faster separations.

The latest development in this area is ultrahigh-pressure liquid chromatography (UHPLC). UHPLC separations use columns packed with ultra high efficiency sub 2 μ m particles. Because these particles provide such high efficiency, good resolution can be obtained even on very short columns. Another key to the success of these columns is the fact that their efficiency is not reduced under high flow rate conditions. Thus, separations can be carried out using short columns and high flow rate, which results in very short analysis times. Since a combination of high flow rate and fine particles results in very high column backpressures, specialized equipment is necessary to carry out these separations. Recently, our group has begun applying UHPLC technology for fast analysis of prenylated flavonoids in biological fluids and tissues [57, 58]. An example of fast separation of four major prenylflavonoids using UHPLC-MS-MS is shown in Figure 2. Baseline separation of all analytes can be achieved in 2.5 min. This method has been used in support of an ongoing Phase I clinical trial of hops [3].

In the mass spectrometer, prenylated flavonoids ionize well in either positive ion or negative ion mode using standard atmospheric pressure ionization methods such as electrospray and atmospheric pressure chemical ionization (APCI). As shown in Table 1, electrospray appears to be a more popular choice, with the preference towards negative ion mode. The choice of ionization method and polarity is based on a combination of factors including the design of the particular ion source, compatibility with the chromatographic mobile phase, required detection sensitivity, and the presence of possible chromatographic interferences or matrix effects. Maragou *et al.* reported a detailed investigation of the effect of mobile phase composition on the instrument response and found that methanol-containing mobile phase and negative ion electrospray mode provided the best signal-to-noise [43]. It has also been our experience that negative ion mode provides slightly better sensitivity even when acidified mobile phase is used. In addition, negative ion mass spectrometry provides a lower background compared to the positive ion mode, which ultimately results in better signal-to-noise. Negative ion mode is also preferred if glucuronide or sulfate metabolites are measured along with the parent compound, because these metabolites tend to ionize more efficiently in the negative ion mode. However, the ultimate decision of which mode to use depends on the performance of the particular instrument, and no specific recommendation based on the literature review can be made.

In contrast to electrospray which is compatible with solvent flow rates in the nl/min to lower $\mu\text{l/min}$ range, APCI requires mobile phase flow rates $\sim 100 \mu\text{l/min}$ and can accommodate larger diameter column (3.0 or 4.6 mm) than can electrospray without flow splitting. Therefore, higher mobile phase flow rate requirements make APCI ideally suited for coupling with UHPLC columns, which are typically used under high flow rate conditions. Most published methods describing LC-MS and LC-MS-MS of analyses of prenylated flavonoids have used APCI in positive ion mode with acidified mobile phases for chromatographic separation. Compared to electrospray, APCI is considered less prone to matrix effects and provides wider dynamic range. However, no specific study addressing these issues for analysis of prenylated flavonoids has been published.

Among analyzers used for mass spectrometric detection, quadrupoles occupy the dominant place. The minimal vacuum requirements, sensitivity, wide dynamic range, ease of operation, and relatively low cost have made quadrupole mass spectrometers ideal choices for quantitative analysis of small molecules, and prenylflavonoids are no exception. Data acquisition on single quadrupole mass spectrometers is carried out in selected ion monitoring mode (SIM), where the voltages on the quadrupole are adjusted to transmit only the ions of interest. The major disadvantage of single quadrupoles is possible interference from isobaric compounds, i.e., compounds that have the same molecular mass as the target analyte. To minimize this problem, careful chromatography and sample clean-up are necessary, which usually translates into long analysis times and low throughput. Even with these precautions, the risk of isobaric interference is high during analysis of prenylflavonoids in complex matrices such as biological fluids or tissues. Despite these disadvantages, methods based on LC-MS with SIM detection can perform adequately for prenylflavonoid analysis as shown by Wynn *et al.* [39].

In contrast to single quadrupoles, triple quadrupole mass spectrometers and hybrid quadrupole-linear ion trap instruments provide an additional level of selectivity by monitoring the transitions from specific precursor ions to specific fragment ions in a process called selected reaction monitoring (SRM). Due to the extremely high selectivity of MS-MS detection, these methods are preferred for analysis of prenylflavonoids. Method development using SRM typically begins by acquisition of product ion spectra of the target analyte(s). Product ion spectra of prenylated flavonoids and their metabolites have been investigated in detail in several publications [8, 20, 59–61]. In negative ion mode, retro-Diels-Alder (RDA) cleavage is the dominant pathway with little to no fragmentation of the prenyl group (see Figure 3 and Scheme 1A). The $^{1,3}\text{A}^-$ ion at m/z 219/233 (for 6-PN, 8-PN/DMX and IX/XN, respectively) and $^{1,3}\text{B}^-$ at m/z 119 are typically used for quantitative analysis. In positive ion mode (Table 2), the two dominant fragmentation channels are RDA cleavage and loss of 2-methylpropene (C_4H_8) from the prenyl group (Scheme 1B). Transitions from precursor ion to either the $^{1,3}\text{A}^+$ or $^{1,3}\text{A}^+-\text{C}_4\text{H}_8$ ion are typically used for quantitation in positive ion mode. It is important to note the similarity of the product ion spectra for isomeric pairs of chalcone/flavanones and isomeric flavanones 8-PN and 6-PN. Since there is no specific fragment ion that is unique for a particular isomer, SRM detection cannot distinguish between 8-PN and 6-PN or XN and IX. This example is a good reminder that occasionally even the most selective MS-MS detection is not sufficient without resolving chromatography. Thus, chromatography is essential for quantitation of isomeric prenylflavonoids, and analysis time is usually that needed for baseline separation.

Ideally, a SRM method should employ two transitions. One transition serves for quantitation (quantifier) while the other serves to ensure that the measured peak is indeed the target analyte (qualifier). If the measured peak is free from interferences, then the ratio between quantifier and qualifier should be the same as in the reference standard. Any deviation from the expected ratio is an indication that a co-eluting impurity is interfering with measurement

of the analyte. Among published reports, only the methods of Bolca *et al.* [53] and Yuan *et al.* [57] used both the qualifier and the quantifier transition.

In addition to the quadrupole mass spectrometers, ion trap-based methods have also been reported [54, 62]. Ion trap mass spectrometers have been traditionally considered qualitative analyzers, although recent work suggests that can be effectively used for quantitation. The major drawback of ion trap instruments is their limited dynamic range due to space charge effects. The advantage of ion traps compared with single quadrupole mass spectrometers is that they can provide qualitative information about the analytes through MS/MS measurements, which results in additional assurance that the desired compound is being measured.

Since mass spectrometric response can vary over time, use of an internal standard is highly recommended. When sample preparation includes extraction, use of an internal standard is critically important. Stable-isotope labeled analyte, known as a surrogate standard, is the ideal internal standard for a mass spectrometry-based method. At the time of writing, no stable isotope-labeled analogs of prenylated flavonoids were available. Instead, closely related compounds have been used as internal standards (see structures in Figure 4). 4,2 - Dihydroxychalcone (4,2 -DHC), 8-isopentylnaringenin and naringenin have been the most common choices [17, 43, 53]. In contrast, Wyns *et al.* used the unrelated compound 4-hydroxybenzophenone as an internal standard [39]. A disadvantage of using a single internal standard for quantitation of both prenylated flavanones and chalcones is the fact that these classes of compounds have different chemical properties. For example, a flavanone internal standard cannot account for possible isomerization of the chalcones during sample preparation. This issue was investigated in more detail by Maragou *et al.* [43] who noted that 4,2 -DHC is not the best internal standard for quantitation of 8-PN in beer. 4,2 -DHC tends to cyclize over time which reduces its concentration, and this distorts the measured area ratios versus 8-PN. An ideal method should perhaps include two surrogate standards, one for flavanones and one for chalcones.

An interesting approach to bypass the need for stable isotope labeled internal standards is the use of so-called ECHO technique [63]. This approach involves injecting unlabeled analyte standards shortly before and/or after the injection of samples to create an “echo” of the target analyte. Since retention times of the target analyte and the ECHO standard will be close, matrix effects and/or day-to-day variation in instrument response will be compensated. An application of this technique for IX and XN analysis in beer was demonstrated by Intelmann *et al.* [42]. A disadvantage of this method is that concentration of the ECHO standard should be carefully matched to the concentration of the analyte in the test samples. Because the two peaks elute close to each other, a much larger peak may partially overlap with the smaller one causing error in integration. It should also be noted that since the ECHO standard and the test samples are not mixed during sample preparation, the ECHO technique cannot account for sample losses during extraction. The utility of this method for analysis of prenylflavonoids in other matrices remains to be seen.

6. APPLICATIONS OF LC-MS AND LC-MS-MS METHODS TO BIOLOGICAL RESEARCH ON HOPS

Current interest in the medicinal properties of prenylflavonoids has focused most recent LC-MS and LC-MS-MS methods on understanding the ADME properties of these compounds. As a part of estrogenic evaluation of 8-PN *in vivo*, Overk *et al.* measured plasma and tissue levels of 8-PN and their major metabolites in rats after intraperitoneal injection of pure compound [17]. As part of the same study, IX was injected subcutaneously to monitor its conversion into estrogenic 8-PN. In all samples, 8-PN was primarily found in the form of

glucuronide conjugates. Monoglucuronides of Phase I metabolites of 8-PN were also detected. In plasma, unconjugated 8-PN was detected only at the highest administered dose of 40 mg/kg, while in the liver and mammary tissues it was also detected in rats dosed with lower doses (0.4 and 4.0 mg/kg). This finding suggests that local deconjugation by glucuronidases might be responsible for the release of aglycones in tissues. The levels of 8-PN in the liver were approximately 8-fold higher than those in the mammary gland. Another interesting observation of this study was lack of conversion of IX into 8-PN which is in contrast to the results from several studies in humans (see below).

Bolca *et al.* [49] studied *in vivo* conversion of IX into 8-PN as a part of a large dietary intervention that included soy-, flax- and hop-based food supplements. The hop supplement used in the study was a commercially available product containing 2.4 mg XN, 1.2 mg IX and 0.1 mg of 8-PN per capsule (MenoHop®). The main objective of the study was to examine microbial bioactivation of dietary phytoestrogens and to determine whether co-supplementation may alter bioactivation profile. The study found significant inter-individual differences in conversion of IX into 8-PN that were attributed to differences in microbial phenotype among study participants (classified as poor, moderate or strong producers). Combined with earlier results from the same group [23, 64], this study provided strong support to the hypothesis that microbial conversion is one of the main mechanisms for the production of 8-PN from IX *in vivo*. The analytical challenge in this study was accurate quantitation of 8-PN and IX in the presence of many other phytoestrogens that were administered during the co-supplementation phase [49], and this problem was addressed successfully using LC-MS to measure multiple phytoestrogens during the same analytical run.

In the follow-up study by the same group, disposition of XN, IX and 8-PN in breast tissue was measured after consumption of the same MenoHop® product as described above. A total of 21 healthy subjects scheduled for aesthetic breast surgery were recruited for the study. The subjects consumed three capsules per day (6.12 mg XN, 3.6 mg IX and 0.3 mg 8-PN) for 5 days after which blood, urine and breast tissue biopsy samples were taken for analysis. Because this study demanded a much more selective and sensitive analytical method, the authors used a LC-MS-MS method instead of LC-MS used in the previous study. IX was the most abundant flavonoid in all samples tested indicating significant *in vivo* conversion of XN into IX. Conversion of IX into 8-PN was also observed in strong and moderate 8-PN producers. Phase I metabolites of IX and 8-PN were also detected, and this was the first study that conclusively demonstrated presence of these metabolites *in vivo*. All flavonoids were found in the form of monoglucuronides. A particularly noteworthy result of this study was that the investigators were able to measure levels of prenylflavonoids in breast tissue biopsies. At this point, this is the only study demonstrating that these compounds can reach breast tissue after oral supplementation. Furthermore, the levels of estrogenic 8-PN measured in breast tissue (0.78–4.83 pmol/g) were judged to be below levels necessary to induce any estrogen-receptor mediated responses relevant to breast carcinogenesis.

Sowell *et al.* [2] reported on the pharmacokinetics in humans of orally administered XN. During this study, 3 volunteers took a single dose of 10 mg XN, and blood samples were taken over a period of 72 hr. In a separate experiment, urine samples were collected at 6 hr intervals for a total of 5 days. An example of a chromatogram corresponding to LC-MS-MS analysis of a plasma sample from this study is shown in Figure 5. An important result of this study was identification of 8-PN and 6-PN in plasma samples, which indicated that XN cyclized into IX which was subsequently demethylated to form 8-PN. Therefore, XN can act a pro-estrogen *in vivo*. This study also showed that XN is poorly absorbed after oral administration, has a very long elimination half-life (32 hrs) and forms glucuronides as the

most abundant circulating form. Recently, the same research group reported on the pharmacokinetics of XN in rats after intravenous and oral dosing [51]. The results of this rat study were similar to those observed in the human study, including poor absorption, long half-life and extensive glucuronidation.

7. OTHER SEPARATION METHODS

Methods based on HPLC with UV detection were among the earliest used for quantitation of prenylflavonoids from hops [65]. The major disadvantages of UV-based methods compared to mass spectrometry are low sensitivity [55] and poor specificity, which necessitate extensive sample cleanup and long separation times. Compared with other hop prenylflavonoids, UV detection is more suitable for XN, which has strong absorption at 370 nm and can be quantified with good sensitivity with reported limits of quantitation ranging from 2.0–60 ng/ml for beer analysis [45, 46, 55, 66] and 35–100 ng/ml for biological specimens [67]. Prenylated flavanones are typically quantified at 290–295 nm. The reported LOQs have ranged from 17.7–60 ng/ml for IX and 100–1700 ng/ml for 8-PN depending on the type of matrix [67]. Most reported HPLC-UV methods have used diode array detection (DAD) to improve method selectivity, which is necessary when these methods are used for analysis of biological samples. In addition to standard HPLC, UHPLC-DAD has also been used for quantitation of XN and DMX extracted from hop cones along with other terpenophenolic [68].

Recently, Dhooghe *et al.* proposed a novel approach for quantitation of prenylflavonoids using HPLC-UV [31]. Instead of preparing calibration curves using primary reference standards, these authors used naringenin and quercetin as secondary calibration standards for quantitation of prenylflavanones and XN, respectively. For this approach to work, the maximum absorption wavelengths of the primary and secondary standards need to be very close. The key to the success of this method is correct determination of the response factor, which corrects for the difference in absorption coefficients between the primary and secondary analytes. Method validation demonstrated that the method has acceptable accuracy and precision. The overall utility of this method, however, remains questionable. As discussed above, primary standards of prenylated flavonoids are becoming more readily accessible, thus negating the main rationale for the development of this method. In addition, concentrations of 8-PN and 6-PN in hops extracts are rather low and therefore prone to interferences from closely eluting compounds that may be present in some extracts. The authors even noted that on several occasions, LC-MS was required because coeluting impurities prevented accurate integration of the 8-PN peak.

Despite low sensitivity and poor selectivity, there have been a surprisingly large number of publications describing applications of HPLC-UV methods to the quantitation of prenylflavonoids in biological samples [21, 23, 64, 67, 69]. Except for the report by Avula *et al.* [48] most of these studies did not show sample chromatograms, so it was not possible to determine the reliability of such data. We also note that only a few of these methods used an internal standard, despite dealing with a complex matrix and extensive sample preparation [69]. Overall, it is likely that in near future more sensitive and selective LC-MS-MS methods will replace HPLC-UV-based methods measurement of prenylflavonoids in biological samples.

In addition to UV, HPLC separation has recently been coupled to electrochemical detectors (ECD) for sensitive quantitation of XN in hop extracts. The LOQ for XN quantitation using HPLC-ECD was 0.32 ng/ml, which is comparable to those reported using LC-MS-MS and more than 10–20 fold better than HPLC-UV methods. In general, HPLC-ECD appears to be

a viable alternative to LC-MS-MS, and it will be interesting to see if this method can be extended to the quantitative analysis of other prenylflavanones in biological matrices.

GC-MS has also been used to measure 8-PN in beer [70]. The method requires long and elaborate sample preparation that includes derivatization prior to analysis. A detection limit of 5 ng/ml was reported.

Capillary electrophoresis (CE) has been applied in various forms to the analysis of prenylated flavonoids in hop extracts [71–73]. However, only the method of Kac *et al.* reported quantitative data [72]. The LOQ for XN was reported to be 0.15 µg/ml. CE methods generally suffer from poor reproducibility and require careful buffer selection for successful quantitative analysis.

High performance thin-layer chromatography was also used by Kac *et al.* to measure XN in hops and hop products [74]. TLC plates were developed using toluene-dioxane-acetic acid (77:20:3; v/v/v) as the mobile phase, and XN was detected by scanning the plate at 368 nm. The LOQ of HP-TLC quantitative analysis of XN was 0.5 µg/ml.

8. NON CHROMATOGRAPHIC METHODS

8.1. Immunoassays

Monoclonal antibodies against prenylated flavonoids have recently become available and have been used to develop immunoassays for quantitation of these compounds in biological fluids. Schaefer *et al.* reported a radioimmunoassay (RIA) that used tritiated 8-PN [22]. The monoclonal antibody against 8-PN employed in the final method showed very little cross-reactivity against other prenylated flavonoids. However, the authors were concerned about possible cross-reactivity against glucuronide metabolites. As expected for RIA, the method showed excellent sensitivity with the limit of quantitation of 0.1 ng/ml. The method was used in support of a Phase I clinical trial of 8-PN [4].

Recently, Wyns *et al.* [50] reported development of an ELISA using monoclonal antibodies against 8-PN, IX and XN. Each monoclonal antibody bound to one prenylflavonoid and showed little cross-reactivity against other prenylflavonoids or phytoestrogens. The method showed good correlation with a validated LC-MS method developed in the authors' laboratory for the measurement of 8-PN and IX in urine. However, the reported limits of quantitation for the ELISA method were almost 10 times worse than the corresponding values for the LC-MS method. The method was applied to the measurement of 8-PN and IX in urine samples from a dietary intervention trial [49]. However, the sensitivity of the method was insufficient to detect XN in the tested samples. Overall, these immunoassays provide an alternative way to measure prenylated flavonoids for laboratories lacking access to LC-MS-MS instrumentation. Wider applicability of the ELISA method will require additional improvements in the limit of quantitation.

8.2. Other methods

The latest addition to the arsenal of quantitative methods for hop prenylflavonoids is square-wave adsorptive-stripping voltametry [35]. In this method, the analyte is accumulated at the hanging mercury drop electrode and then stripped off by applying a potential scan. The preconcentration of analyte (7-fold was reported) at the electrode allows for significant improvement in sensitivity without extensive sample preparation. In addition, because there is no chromatography involved, the method is much faster compared to classical HPLC-UV methods. The method was applied to the quantitation of XN in spent hops. The LOQ of 8.8 ng/mL and analysis time of 7 min make this method a viable option for rapid quality control of hops preparations.

9. MALDI IMAGING

Strictly speaking, imaging not a quantitative technique. Since it can be considered a semi-quantitative method, its applications will be briefly discussed. Imaging mass spectrometry using matrix-assisted laser desorption ionization (MALDI) mass spectrometry has become a popular method to study spatial distribution of target analytes in biological tissues [75]. In this method, thin tissues slices are mounted on microscope slides and then a suitable matrix is applied. The slices are then inserted into MALDI ion source, a pulsed laser is rastered across the slice and ion abundances are recorded to create an image of the ion current distribution across the slice.

Our group recently applied MALDI imaging to study spatial distribution of XN and its metabolites in the rat liver, intestine and colon after oral administration by gavage for five days at a dosage of 100 mg/kg. [76]. The rats were sacrificed and organs collected 24 hours after the last dose. The tissues were sliced to 12 μm thickness, mounted onto microscope slides and sprayed with 3-cyano-4-hydroxycinnamic acid (CHCA) as the matrix. The data were acquired on a hybrid quadrupole/time-of flight instrument equipped with a MALDI source. The high resolution MS-MS capabilities of this instrument facilitate highly selective detection of XN and its metabolites. XN was distributed diffusely in the liver and intestine (Figure 6). In colon slices, XN was found to be primarily concentrated in the colon folds. In the colon, distribution of XN glucuronides was found to be similar to that of free aglycones, while in the intestine glucuronides were predominantly found in the epithelium portion of the tissue. This study demonstrated significant accumulation of XN along the routes of absorption, which suggests that this compound is likely to be beneficial for prevention of cancer of the GI tract.

10. CONCLUSIONS AND FUTURE DIRECTIONS

Advances in modern analytical methodology, particularly LC-MS and LC-MS-MS have kept pace with the ever-increasing demands for fast, accurate and sensitive quantitation of prenylated flavonoids and their metabolites in complex matrices. A recent application that combined fast separation using UHPLC and a new fast-scanning triple quadrupole has pushed the speed of analysis to the point that sample preparation is the bottleneck in the overall throughput. Wide acceptance and adoption of this methodology is likely to continue [57].

Despite these advances, there are areas where further research is needed and new advances can be made. Analytical methods using stable isotope-labeled analytes are the gold standard in modern analytical chemistry, yet those standards are currently not available for prenylated flavonoids. These standards will be particularly valuable to properly account for spurious cyclization of prenylated chalcones during sample processing and analysis. Availability of these standards will be particularly significant for analytical methods that will support future clinical trials of hop-based supplements and purified flavonoids.

Recent development of monoclonal antibodies for prenylated hop compounds provides an opportunity to develop new sample preparation methods based on affinity purification. In the past, such methods have proven extremely effective in selectively extracting and enriching target analytes from complex matrices. Selective enrichment of target analytes can further improve achievable detection limits.

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Figure 1.

Isomerization of prenylated chalcones into flavanones. Xanthohumol (XN) isomerizes into isoxanthohumol (IX) while desmethylxanthohumol isomerizes to form a mixture of 8-prenylnaringenin (8-PN) and 6-prenylnaringenin (6-PN) due to rotation around B-ring. The rings are numbered according to the standard nomenclature.

Figure 2.

Fast separation of four major prenylated flavonoids and internal standard (8-isopentylnaringenin) using UHPLC-MS-MS.

Analytes: Standards of prenylated flavonoids at 5 ng/mL (10 μ L injection); column: Shimadzu Shim-pack XR-ODS III 2.0 $\times$ 50 mm, 1.6 μ m, C₁₈; mobile phase: 1.5 min gradient 45–70% acetonitrile/0.1% formic acid at 0.5 mL/min; temperature: 50°C; instrument: Shimadzu LCMS-8030 triple quadrupole; ionization: negative ion electrospray; detection: SRM, m/z 339 to 119 (6-,8-PN); m/z 353 to 119 (IX, XN), m/z 341 to 119 (IS).

Figure 3.

Negative ion electrospray CID product ion spectra of prenylated flavonoids. Note the similarity of product ion spectra for isomeric flavanones 8-PN and 6-PN as well as XN and IX. For structures of major fragment ions, see Scheme 1.

Instrument: Shimadzu LCMS-8030 triple quadrupole; collision energy 35 eV; collision gas: argon.

8-isopentylnaringenin

Naringenin

4,2'-dihydroxychalcone

4-hydroxybenzophenone

Figure 4.

Chemical structures of compounds used as internal standards for quantitation of prenylated flavonoids by LC-MS and LC-MS-MS.

Figure 5. LC-MS-MS chromatogram from a human plasma sample obtained 1 hour after ingestion of 10 mg of XN. Prior to extraction with MTBE, enzymatic hydrolysis was carried out to convert glucuronides and sulfate conjugates into aglycones. Detection of IX, 8-PN and 6-PN indicates that significant isomerization and metabolism of XN had occurred *in vivo* (Figure courtesy of Dr. Fred Stevens, Oregon State University).

Figure 6.

A) MALDI image of XN in rat liver. XN accumulated in the tissue is represented by light-colored area. Note the diffuse distribution of XN within the slice; B) Optical image of the same tissue slice.

Conditions: Slice thickness: 12 μm ; matrix: CHCA; instrument: Waters SYNAPT HDMS hybrid-quadrupole/time-of-flight mass spectrometer; detection: MS-MS of precursor ion of XN at m/z 355, positive ion mode. The image shows distribution of the fragment ion of m/z 299; data analysis: Pattern Creator Software (Waters Corporation). Data from reference [76], with permission.

Scheme 1.

Proposed fragmentation pathways for prenylated flavanones in A) positive ion mode and B) negative ion mode (according to [8, 59]). The nomenclature of Retro-Diels-Alder fragment ions is according to Ma *et al.* [77]. Due to cyclization in the gas phase, XN shows similar fragmentation to IX.

Table 1
Summary of the LC-MS and LC-MS-MS methods for analysis of prenylated flavonoids from hops.

Matrix	Analytes	Sample prep	Column	Mobile phase	Ionization	MS	IS	LOD/LOQ	Ref
Hop products, beer	8-PN, 6-PN, IX, XN, DMX, 6-GN	Hop products: extraction w/MeOH Beer: degassing	C ₁₈ 4 × 250, 5 µm	1% FA-MeCN	(+) APCI	QQQ, SRM	4,2 -DHC	LOQ: 10 ng/ml	[14]
Hop products, beer	8-PN	Hop products: extraction w/MeOH/water (3:1) Beer: SPE	4.6 × 250, 5 µm	0.5% FA-MeOH	(+) ESI	Q, SIM	None	LOD: 1 ng/ml	[44]
Dietary supplements	08-PN, 6-PN, IX, XN, 6,8-diPN, 14 other phytoestrogens	Extraction w/80%MeOH	2.1x 150, 5 µm	10mM ammonium acetate-MeCN	(-) APCI	Ion trap	None	NR	[54]
Hop extracts, beer	8-PN, IX, XN, bitter acids	Hop extracts: extraction w/90% MeCN Beer: Degassing	C ₈ 4.0 × 250, 5 µm	Water-MeCN; 0.3% FA	(+) APCI	Ion trap	None	LOQ: 20–30 ng/ml	[55]
Human urine, serum	8-PN, IX, XN, 10 other phytoestrogens	Urine: LLE Serum: SPE	C ₁₈ 3.0 × 150, 3.5 µm	Water-MeOH/MeCN (80:20), 0.025% FA	(+) APCI	Q, SIM	4-OHBF	LOD Urine: 0.2–0.6 ng/ml Serum: 1.4–1.5 ng/ml	[39]
Beer	8-PN, zeaxenols	Degassing	C ₁₈ 4.6 × 250, 5 µm	Water, MeOH/MeCN	(-) ESI	Q, SIM	4,2 -DHC	LOQ: 2.4 ng/ml	[43]
Beer	IX, XN, bitter acids	Degassing	C ₁₈ 4.6 × 250, 5 µm	Water-MeCN; 0.5% FA	(-) ESI	Q-linear ion trap, SRM	ECHO	NR	[42]
Rat plasma, tissues	8-PN, IX, metabolites	Plasma: PP Liver: Homog. in buffer, LLE Mammary gland: Homog. in buffer, PP	C ₁₈ 2.1×10 0, 3.5 µm	0.1%FA-MeOH	(-) ESI	QQQ, SRM	NAR	LOQ: 58.8 fmol on column	[17]
Human urine, serum, breast tissue	8-PN, IX, XN; metabolites	Urine: LLE Serum: LLE Tissue: Homog. in 0.2M HCl, LLE.	C ₁₈ 2.1×10 0, 5 µm	0.05% AA-MeOH	(-) ESI	QQQ, SRM, quantif. +quantif.	8-IPN	LOQ: Urine, serum: 0.01nM Tissue: 0.088 pmol/g	[53]
Human serum, urine	8-PN, 6-PN, XN, IX,	LLE	C ₁₈ 2.1 × 150 4 µm	Water-MeCN/MeOH (95:5), 0.01% FA	(-) ESI	Q-Linear ion trap, SRM	4,2 -DHC	LOQ: 0.1–0.3nM	[2]
Rat plasma	8-PN, 6-PN, XN, IX,	LLE	C ₈ 2.0 × 50 5 µm	Water-MeCN; 0.1% FA	(-) ESI	Q-Linear ion trap, SRM	4,2 -DHC	LOQ: 0.4–0.5nM	[56]
Human serum, urine	8-PN, 6-PN, IX, XN	LLE	C ₁₈ 2.0 × 50 1.6 µm	0.1% FA-MeCN	(-) ESI	QQQ, SRM, quantif. + quantif.	8-IPN	LOQ: Urine: 0.5 ng/ml Serum: 1 ng/ml	[57]

Abbreviations:

AA= acetic acid; APCI=atmospheric pressure chemical ionization; ESI=electrospray ionization; FA= formic acid; IS=Internal standard; LOD=Limit of detection; LOQ=Limit of quantitation; 4,2 -DHC = 4,2 -dihydroxychalcone; 8-IPN=8-isopentylnaringenin; NAR=naringenin; 4-OHBF= 4-hydroxybenzophenone; NR= Not reported; Q= Single quadrupole; QQQ= triple quadrupole; SIM= Selected ion monitoring; SRM= Selected reaction monitoring; PP=Protein precipitation

Table 2

Tandem mass spectra of prenylated flavonoids in positive ion mode.^a

Compound	[M+H] ⁺	[MH-C ₄ H ₈] ⁺	L ₃ A ⁺	[L ₃ A-C ₄ H ₈] ⁺	L ₄ B ⁺
XN	355	299 (100) ^b	235 (5)	179 (65)	147 (3)
IX	355	299 (59) ^c	235 (17)	179 (85)	147 (6)
DMX	341	285 (100)	221 (20)	165 (89)	147 (3)
8-PN	341	285 (39) ^c	221 (4)	165 (12)	147 (1)
6-PN	341	285 (100)	221 (2)	165 (34)	147 (1)

^a Adapted from Stevens *et al.* [8], with permission

^b *m/z* (relative abundance)

^c Normalized to the precursor ion abundance