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Original Article

Stereospecific quantitation of 6-prenylnaringenin in commercially available *H. lupulus*-containing natural health products and dietary supplements

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Abstract

6-Prenylnaringenin (6PN) is a chiral prenylflavonoid found most prevalently in hops (*Humulus lupulus*) and present in hops and hop products. It is an isomer of the potent phytoestrogen, 8-prenylnaringenin. An enantiospecific method for quantitation 6PN by LC-ESI-MS has been developed. Baseline enantiomeric resolution of 6PN was attained on a Chiralpak® AD-RH column with an isocratic mobile phase consisting of acetonitrile and 10 mM ammonium formate (pH 8.5) (39:61, v/v) and a flow rate of 1.25 mL/min. Quantitative MS data were obtained by selected ion monitoring of the $[M-H]^-$-ion of both enantiomers of 6PN (*m/z* 339.10) and the internal standard, 4-acetamidobenzoic acid (*m/z* 178.05). The method was found to be accurate and precise for enantiospecific quantification of 6PN. The method was successfully applied to the content analysis of 39 commercially available natural health products and dietary supplements reported to contain *H. lupulus* plant material, extracts and label claims of 6PN. 6PN was present in 25 of 34 products containing plant material or extracts of *H. lupulus*. Of the five products with claimed amounts of 6PN, all were found to possess <50% of label claims. Results of the content analysis indicated a lack of uniformity in botanical nutraceuticals claiming 6PN content.

Keywords: 6-Prenylnaringenin; *Humulus lupulus* L.; Chiral; Enantioseparation; Analysis; Nutraceuticals

INTRODUCTION

6-Prenylnaringenin (6PN) (2,3-dihydro-5,7-dihydroxy-2-(4-hydroxyphenyl)-6 - (3-methyl-2-butenyl)-4H-1-benzopytan-4-one), $C_{20}H_{20}O_5$, MW 340.37 g/mol (Fig. 1), is a naturally occurring prenylflavonoid with a single chiral carbon center that is most commonly found in the female flowers (cones) of hops (*Humulus lupulus* L.) (1,2) but has also been described in *Wyethia invenusta* (3), *W. glabra* (4), *Glycyrrhiza glabra* (5), *Psoralea corylifolia* (6–9), *Lupinus luteus* (10), and *Sophora tomentosa* L. (11).

Currently, there are no studies in the literature investigating the enantiospecific bioactivity of 6PN despite the potential for individual enantiomers to exhibit different biological activities and FDA recommendation for assessment of each enantiomer's biological activities for new chiral drugs (12,13).

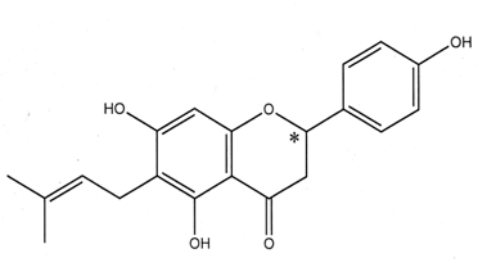

Fig.1. Chemical structure of 6-prenylnaringenin. * Denotes chiral carbon center.

One study investigating the enantiospecific estrogenic activities of the structural isomer, 8-prenylnaringenin, found that both 8-prenylnaringenin enantiomers displayed high affinity and selectivity for human estrogen receptor-α and –β but *S*-8-prenylnaringenin exhibited an overall higher affinity for both receptors than *R*-8-prenylnaringenin (14). Differences in 6PN bioactivity between enantiomers may exist but remain unexplored.

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Considerably less information on the bioactivity of 6PN is available in the literature compared to the well-studied related compounds of xanthohumol, isoxanthohumol and 8-prenylnaringenin. Previous studies have shown that racemic 6PN possesses substantially weaker estrogenic activity than 8-prenylnaringenin (presently the most potent phytoestrogen known) (15). 6PN has demonstrated antimicrobial and antifungal activity against several pathogens (1,16), potent anti-proliferative activities in prostate cancer cell lines (17), induction of quinone reductase (18), anti-osteoporoporotic properties (19) and inhibition of aldose reductase (7). Additionally, there is no pre-clinical or clinical pharmacokinetic data available in the literature for 6PN following the administration of pure 6PN.

Use of botanical natural health products and dietary supplements has increased in North America and globally. In the US, sales of botanical preparations increased 86% from 1995 to 2006 with 2012 sales reaching $5.5 billion USD (20). While in Canada, as of 2010, 3 in 4 Canadians use natural health products with 33% reporting daily use (21). Globally, there are over 1,000 companies producing 29,000 different preparations of botanical products which equates to a $60 billion USD industry (22,23). Natural health products and dietary supplements containing *H. lupulus* plant materials or extracts of purified 6PN may represent a significant dietary source of 6PN. Products containing *H. lupulus* plant materials and extracts are marketed for a variety of uses including; menopause support and hormone replacement therapy, anti-anxiety and relaxation aids, breast enhancement, pain relief and sleep aids (24). Unlike conventional pharmaceuticals which must undergo scrupulous evaluations for safety and efficacy and are subject to strict chemical standardization in both the US and Canada by the FDA and Health Canada, respectively, natural health products and dietary supplements are not required to pass through an equally scrupulous development, standardization or approval pathway in either country (20,25). The development of simple analytical techniques for analysis of bioactive constituents in botanical products may facilitate the monitoring of botanical product claims and standardization (26).

While there are several validated methods in the literature capable of quantitation of 6PN (27), they are not enantiospecific. To comprehensively evaluate a chiral compound, enantiospecific methods of analysis should be developed and employed in all aspects of research of chiral compounds. Presently, there is only one study in the literature describing chromatographic seperation of 6PN enantioemrs using a triacetyl cellulose column and 100% methanol. However, no supporting data or validation parameters were provided (28). The present study describes a novel, isocratic, LC-ESI-MS method for the stereospecific analysis and quantitation of 6PN and its application to content analysis of 6PN in commercially available *H. lupulus*-containing natural health products and dietary supplements.

MATERIALS AND METHODS

Chemicals and reagents

Racemic 6PN analytical standard was purchased from Toroma Organics Ltd. (Saarbruecken, Germany). 4-acetamidobenzoic acid (acedoben) was purchased from Sigma-Aldrich (St. Louis, MO). Ammonium formate was purchased from Acros Organics (Geel, Belgium). HPLC-grade acetonitrile was purchased from EMD Chemicals (Gibbstown, NJ). Ultra-pure, reagent-grade water from a Milli-Q water system was used (Millipore, Billerica, MA).

Analytical system and conditions

The LC-ESI-MS system used was a Shimadzu LCMS-2010 EV liquid chromatography mass spectrometer system (Kyoto, Japan) connected to the LC portion consisting of two LC-10AD pumps, a SIL-10AD VP auto injector, a SPD-10A VP UV detector and a SCL-10A VP system controller. Data analysis was accomplished using Shimadzu LCMS Solutions software (version 3). The mass spectrometer conditions consisted of a curved desolvation line (CDL) temperature of 250 °C and a block temperature of 200 °C. The CDL, interface, and detector voltages were -20.0 V, 4.5 kV, and 1.2 kV, respectively. Nitrogen was used as nebulizer

gas (1.5 L/min) from a Parker Balston NitroFlow 60 NA nitrogen generator (Parker Hannifin, Haverhill, MA).

The analytical column used was an amylose Tris (3,5-dimethylphenylcarbamate) coated silica-gel Chiralpak® AD-RH (5 μm, 150 × 4.6 mm) (Chiral Technologies, West Chester, PA). The mobile phase consisted of acetonitrile and 10 mM ammonium formate (pH 8.5) (39:61, v/v) that was filtered and degassed under reduced pressure prior to use. Separation was carried out isocratically at ambient temperature (23 ± 1 °C) with a flow rate of 1.25 ml/min. 4-Acetamidobenzoic acid was used as the internal standard (IS). Both enantiomers of 6PN were monitored in selected ion monitoring negative ion mode with single plot transition at *m/z* 339.10. IS was monitored at *m/z* 178.05. For LC monitoring, absorbance was measured at 291 nm.

Stock and working standard solutions

Methanolic stock solutions of racemic 6PN (100 μg/ml) and the IS were prepared. These solutions were protected from light and stored at -20 °C between uses for no longer than 3 months. Calibration standards were prepared from the stock solutions of racemic 6PN by sequential dilution, yielding a series of concentrations; 0.05, 0.1, 0.5, 1.0, 5.0, 10, 50 and 100 μg/ml for both *R*- and *S*-6PN. Quality control (QC) samples were prepared from stock solutions of racemic 6PN by dilution to yield target concentrations of 0.075, 7.5 and 75 μg/ml. Calibration curves were constructed over the range of 0.05-100 μg/ml (147 nM–294 μM).

Circular dichroism

A Jasco J810 circular dichroism spectropolimeter-flurometer (Easton, MD) was used to verify the identities of the 6PN enantiomers. Fractions of each 6PN enantiomer from a racemic stock solution were collected after chromatographic separation using the described analytical method. Fractions were dried to completion using a stream of compressed nitrogen. Samples were suspended in methanol and deposited in a 0.5 mm path length water-jacked cylindrical quartz cell. Molar ellipticity was monitored from 420 to 190 nm at ambient temperatures (23 ± 1 °C). Enantiomer identities were confirmed using published circular dichroism spectra for 8-prenylnaringenin, a positional isomer of 6PN (24,29).

Natural health products and dietary supplements

Six herbal teas and 33 preparations reported to contain hops and hops extracts readily available in the United State and Canada were selected for investigation. The products chosen also represented the spectrum of heath claims for hops-containing products available on the market. Five of the preparations possessed label claims for 6PN. Table 1 contains the products analyzed and label claims. All products were purchased on the open market through retail stores, online retailers and direct sales or multi-level marketing companies in both Canada and the United States. No products were accepted directly from manufacturers. All products were from lots that were not past noted expiry dates.

Sample extraction and preparation

The 6 tea products underwent two types of extraction; hot water and methanolic. For the hot water extractions, individual infusion instructions (volume of water and steeping time) were followed for each tea product. Boiling (100 °C) ultra-pure water was used for the hot water extractions. The tea bags were undisturbed during the hot water extractions. Following the steeping times, the extractions were collected in 100 μl aliquots. For the methanolic extractions, the contents of each tea bag were emptied into a 15 ml tube. Four ml of methanol were added to each tube and then the tubes were placed on a rocking platform shaker and incubated at room temperature (22 ± 1 °C) for 3 h. Tubes were centrifuged for 10 min at 4,400 rpm. The supernatant was collected in 100 μl aliquots.

Of the 33 preparations, 14 were in capsule form and 12 were tablets. The remainder of the products were in softgel, tincture, gum or topical dosage forms. For the extraction and preparation of the products, one recommended dose, as described by the accompanying product information, was used.

Table 1. Natural health product and dietary supplement label claims per preparation. † Indicates a product claiming to contain *H. lupulus*. • Indicates a product claiming an amount of 6-prenylnaringenin.

Product	Country of manufacture	Recommended dose	Label claim
Teas			
Preferred Nutrition SleepTea™	Canada	1 bag	†
Celestial Seasoning® Tension Tamer® Tea	United States	1 bag	†
Virage Santé Lalma Nightly	Canada	1 bag	†
Traditional Medicinals® Nighty Night®	United States	1 bag	†
Algonquin Tea Company Peace Tea	Canada	1 bag	†
Celebration Herbals Hops Flowers with	United States	1 bag	†
Sleep aids			
Nature's Harmony® Herbal Insomnia	Canada	1 tablet	†
Swiss Natural® Herbal Sleepwell	Canada	3 tablets	†
Hyland's® Calms Forté®	United States	1 tablet	†
Jamieson™ Relax & Sleep™	Canada	2 capsules	†
Iovate Health Sciences International Inc. Sleep	Canada	1 caplet	†
Swiss Natural® Solutions® Sleep	Canada	2 capsules	†
SISU Easy Rest	Canada	1 capsule	†
Relaxants/Anti-anxiety			
Futurebiotics Chill Pill®	United States	3 tablets	†
Nature's Harmony® Herbal Nerve	Canada	2 tablets	†
Metagenics® MyoCalm P.M.®	United States	4 tablets	†
Renew Life® Smokers' Cleanse™ 3	United States	1 capsule	†
Land Art Pure FEEL NO1	Canada	5mL glycerol tincture	†
Anti-inflammatories			
Source Naturals® Minor Pain Comfort™	United States	2 tablets	†
New Chapter® Zyflamend® Nighttime	United States	2 softgels	†
Menopause support/Hormone replacement			
Vitanica® Woman's Passage™	United States	1 capsule	†
Nature's Own™ MenoSleep	Australia	2 tablets	•
AOR™ Advanced Series Estro Detox™	Canada	2 capsules	•
Swanson® Estro8PN™	United States	1 capsules	†
Natural Factors® MenoPause Formula	Canada	1 capsule	†
Life Extension® Natural Female Support	United States	1 capsule	•
Enzymatic Therapy® AM/PM PeriMenopause	United States	1 tablet	†
BioCeuticals® MenoPlus 8-PN™	Australia	1 tablet	•
EuroPharma® (Terry Naturally®)	United States	2 capsules	†
MenoComplete™	United States	2 capsules	†
Solaray® Estro-3™	United States	1 capsule	†
Garden of Life® Oceans 3™ Healthy Hormones®	United States	3 softgels	•
Breast enhancement			
Breast Xcell		3 capsules	†
Eyefive Breast Success	United States	3 capsules	†
Eyefive Breast Success	United States	1tablet	†
ZOFT® Fulfill	United States	2 pieces chewing gum	†
Miscellaneous			
worldProducts™		Apply ointment as necessary	†
WiseWays Herbals™ Crystal Visions Dream Balm	United States	Apply ointment as necessary	†
Vitanica® LactationBlend™	United States	1 capsule	†
Nature's Way® Hops Flowers	United States	2 capsules	†

For products without a recommended dosage, 1 g of the product was taken. Capsules were emptied of contents and tablets were ground to a fine powder. The gum product was frozen with liquid nitrogen and ground to a fine powder. Softgels were perforated to provide easy escape of internal contents. Products placed in 15-ml tubes were extracted with 4 ml of methanol by being placed on a rocking platform shaker and incubated at room temperature (22 ± 1 °C) for 3 h. Tubes were centrifuged for 10 min at 4,400 rpm. The supernatant was collected in 100 µl aliquots. It should be noted that tincture did not undergo the extraction procedure given that it was already in extract-form.

To the 100 µl aliquots, 30 µl of IS (4-acetamidobenzoic acid) was added. Samples were dried to completion using a Thermo Scientific Savant™ SPD1010 SpeedVac® Concentrator (Waltham, MA, USA) with a low heat setting (45 °C). The residues were reconstituted in 200 µl of mobile phase and 10 µl were injected into the LC/MS system.

Data analysis

The quantification of concentrations was based on calibration curves constructed using the peak area ratio (PAR) of the enantiomers of 6PN to IS, against the concentrations of 6PN enantiomers using unweighted least squares linear regression.

RESULTS

Enantiospecific LC-ESI-MS analysis of 6-prenylnaringenin

Numerous compositions of mobile phases and chiral columns were evaluated to achieve the best separation and resolution of the two 6PN enantiomers. To our knowledge, there are no previously published enantiospecific methods of detection for 6PN in the literature. Optimal separation was achieved with the 39:61 (v/v) acetonitrile and 10 mM ammonium formate (pH 8.5) with a flow rate of 1.25 ml/min using a Chiralpak® AD-RH column. A basic mobile phase was required for two reasons; stability and ionization. In acidic medium, cyclization of the prenyl side chain results in the transformation of 6PN into a four ring structure and decreased signal intensity over time during analysis (30,31). Ionization of 6PN was improved with the use of basic conditions as opposed to neutral conditions as indicated by increased signal.

Baseline separation of 6PN enantiomers was successfully achieved under reverse-phase conditions using the Chiralpak® AD-RH column. The resolution factor for the 6PN enantiomers was 1.4. There were no interfering peaks co-eluted with the peaks of interest (Fig. 2). The retention times for *R*- and *S*-6PN were approximately 13.8 and 17.6 min, respectively. The IS had a retention time of 2.375.

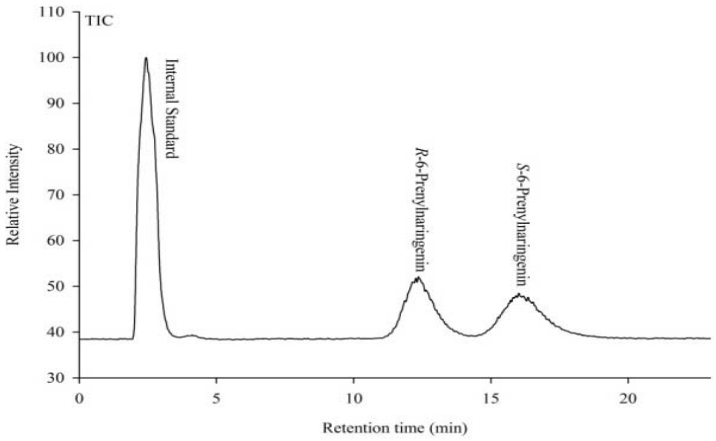

Fig. 2. Representative LC-ESI-MS chromatogram of IS and 6-prenylnaringenin at a concentration of 10 µg/ml shown as total ion concentration (TIC).

Table 2. Accuracy and precision of the enantiospecific LC-ESI-MS quantitative analysis of 6PN (*n*=3).

Analyte	Nominal value (µg/ml)	Measured value (µg/ml)	CV (%)	Bias (%)
R-6PN				
Low	0.075	0.083	3.84	11.0
Medium	7.5	7.04	5.55	-6.19
High	75	80.4	3.88	7.19
S-6PN				
Low	0.075	0.078	10.0	4.38
Medium	7.5	6.79	7.67	-9.47
High	75	78.8	8.73	5.10

The calibration curves for 6PN were linear in the range of 0.05-100 μg/ml (Fig. 3). The calibration curves for each enantiomer showed good coefficients of determination (R^2 >0.99). The LC-ESI-MS assay showed excellent accuracy with bias of less than 15% for the QC samples evaluated at low (0.075 μg/ml), medium (7.5 μg/mL) and high concentrations (75 μg/ml) (Table 2). The limit of quantitation for the assay is 0.05 μg/ml and the limit of detection is 0.01 μg/ml as determined by signal to noise ratio of 1:3.

Circular dichroism for enantiomer identification of 6-prenylnaringenin

Circular dichroism measurements were carried out from collected fractions and the resulting CD spectra (Fig. 4) were compared to CD spectra in the literature for 8-prenylnaringenin (24,29) for enantiomer identification as no published spectra were available for 6PN. CD (*R*-6PN, in methanol): $[\theta]_{330}$ - 1,079; $[\theta]_{291}$ + 16,158; $[\theta]_{217}$ - 11,202; $[\theta]_{201}$ + 3,019. (*S*-6PN, in methanol): $[\theta]_{330}$ + 7,194; $[\theta]_{293}$ - 15,385; $[\theta]_{215}$ + 20,484; $[\theta]_{204}$ + 3,519.

Enantiospecific quantification of 6-prenylnaringenin in natural health products and dietary supplements

The LC-ESI-MS method was successfully applied to the enantiospecific determination and quantification of 6PN in hops-containing natural health products and dietary supplements. To our knowledge, there are no previously published studies or information on the enantiospecific content of 6PN of natural health products and dietary supplements in the literature. The results of the analysis are reported in Table 3. To assess label claims of 6PN, appropriate labeling was defined as a product that contained at least 100% and no more than 120% of its claimed amount. If the label claimed to contain *H. lupulus*, appropriate labeling was attained if any amount of 6PN was quantifiable. None of the hot water extractions of the tea products contained detectable concentrations of 6PN. 6PN was quantified in all of the methanol extracts of the teas. Of the preparations claiming to contain *H. lupulus* but no claimed amount of 6PN, 19 of the 28 products contained 6PN. Of the 5 preparations claiming to contain specific concentrations of 6PN, all were found to possess the compound. However, all 5 products contained 6PN concentrations that fell far below the label claims. Percentages of detected amounts varied from 6.67% to 49% of label claims and none were within the range of 100% to 120%. The products with label claims for 6PN did not discriminate between enantiomers and are assumed to reflect the racemic concentrations. Several products which claimed to contain *H. lupulus* plant material or extract did not contain any detectable 6PN.

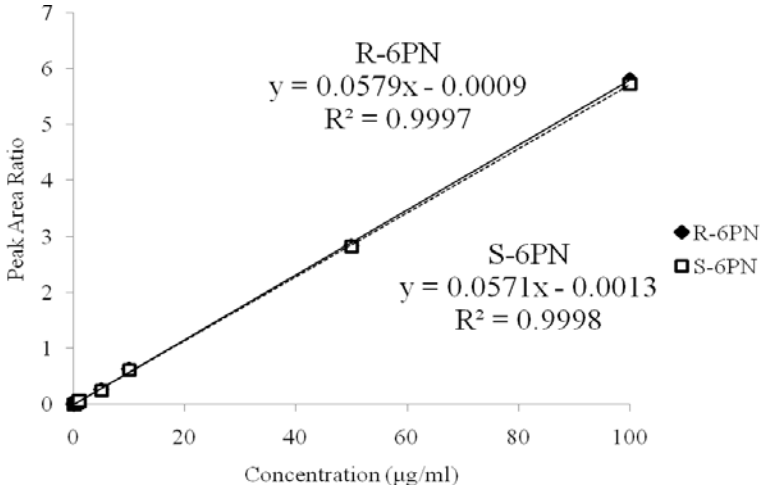

Fig. 3. Representative calibration curve for *R*- and *S*-6-prenylnaringenin (6PN), obtained using LC-ESI-MS.

Table 3. 6-Prenylnaringenin content in selected natural health products and supplements (mg per recommended dosage) following methanolic extraction. †; Indicates a product claiming to contain *H. lupulus*. If a specific racemic 6-prenylnaringenin concentration was claimed, the amount in mg is indicated below. ND; not detected, indicates that concentrations were below detectable limits.

Number	Claimed (mg)	*R*-6PN (mg)	*S*-6PN (mg)	Total 6PN (mg)
1	†	ND	ND	ND
2	†	ND	ND	ND
3	†	0.002	0.007	0.009
4	†	0.004	0.011	0.014
5	†	0.009	0.023	0.032
6	†	0.003	0.008	0.011
7	†	0.004	0.012	0.017
8	†	0.002	0.006	0.008
9	†	0.003	0.009	0.012
10	†	0.002	0.006	0.008
11	†	0.002	0.006	0.008
12	†	ND	ND	ND
13	†	0.005	0.014	0.019
14	†	0.002	0.007	0.010
15	†	0.002	0.008	0.010
16	†	0.002	0.006	0.008
17	†	ND	ND	ND
18	†	ND	ND	ND
19	†	ND	ND	ND
20	†	0.002	0.007	0.009
21	0.1	0.014	0.035	0.049
22	0.120	0.005	0.011	0.015
23	†	0.002	0.008	0.010
24	†	0.002	0.006	0.008
25	0.120	0.004	0.010	0.014
26	†	0.002	0.007	0.009
27	0.060	0.003	0.010	0.013
28	†	0.004	0.011	0.015
29	†	ND	ND	ND
30	†	0.002	0.006	0.008
31	†	ND	ND	ND
32	†	ND	ND	ND
33	0.120	0.002	0.006	0.008
34	†	0.006	0.017	0.023
35	†	0.008	0.024	0.032
36	†	0.002	0.007	0.009
37	†	0.006	0.018	0.024
38	†	0.009	0.025	0.034
39	†	0.032	0.077	0.108

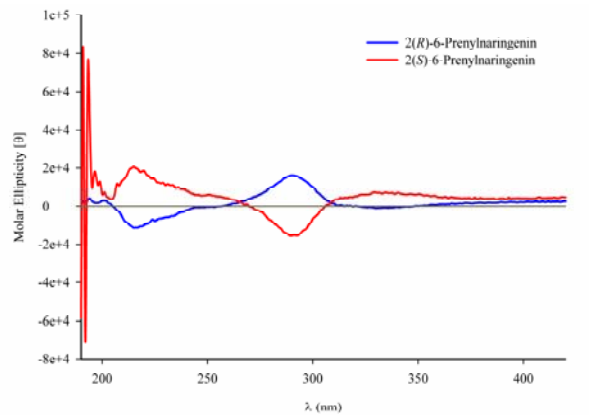

Fig. 4. Circular dichroism spectra for 6-prenylnaringenin.

DISCUSSION

The random sampling of products in the present study may indicate a lack of uniformity in botanical natural health products and dietary supplements such as polyphenol nutraceuticals. This is expected given the nature of plant variability in flavonoid production as well as the lack of standards for testing the uniformity of botanical natural health products and dietary supplements. The lack of manufacturer analysis may be due to the limited number of validated methods of analysis such as HPLC and LC-MS for measuring the polyphenol contents of products. Not only was the developed LC-ESI-MS method successfully able to assess claims of set amounts of 6PN, but it was also able to assess concentrations of 6PN in *H. lupulus* plant materials and extracts incorporated into the preparations. The applied method indicates a lack in uniformity of these *H. lupulus*-containing products, making further assessment of polyphenolic content uniformity and standardization of natural health products and dietary supplements and claims warranted.

The developed method has the potential to assess the stereospecific content of 6PN of a wide range of nutraceuticals incorporating the flavonoid or *H. lupulus* plant material or extract. The method can also be used to evaluate label claims of 6PN as well as plant extract claims. Additionally, the developed method of detection has the potential for evaluating product stability and spontaneous conversion to 8-prenylnaringenin via the chalcone, desmethylxanthohumol, as reported in the literature (20,32). Measuring flavonoid content may provide an appropriate means to monitor botanical preparation claims and content uniformity.

The results from this study as well as analyses of other nutraceuticals indicate that variability exists between products and manufacturers, even among products with similar indications for use (25,26,33). Variability in the quantity and recommended dose of a bioactive ingredient between manufacturers and formulations could prevent accurate and consistent replication of therapeutic results obtained from clinical trials or anecdotal reports. This variability may result in large part from a deficit of detection methods that can allow for reliable analysis and quantification. Studies such as this one may allow for development of new methods of analysis that can be easily applied to the content assessment of botanical natural health products and dietary supplements. Given the pharmacological effects, possible health-benefits and potential safety and toxicity concerns of these products, it is recommended that natural health products and dietary supplements be correctly and accurately labeled and regulatory procedures be adhered to for good manufacturing practices relating to standardization and content uniformity.

CONCLUSION

The developed LC-ESI-MS method for enantiospecific analysis of 6PN is novel, accurate and precise. It has been successfully applied to the content analysis of a wide variety of natural health products and dietary supplements with either 6PN and/or *H. lupulus* plant material label claims. Analysis of 6PN content indicated the expected presence of 6PN in 19 of 28 preparations and 6 of 6 tea products (only when extracted with methanol). While 6PN was found in all of the five preparations with 6PN label claims, none of the products passed strict evaluation criteria of content with 100-120% of label claims.

A complete analysis of flavonoid methods of analysis, pre-clinical and clinical pharmacokinetics, pharmacodynamics, safety and toxicology facilitates a better understanding of reported health-benefits and optimization for clinical utility (34). A further understanding of flavonoid enantiomeric forms would also provide comprehensive scientific regulation of products containing them. Studies are ongoing in our laboratory to characterize the enantiospecific pharmacokinetics, pharmacodynamics and safety of 6PN as well as related *H. lupulus* chiral prenylflavonoids.

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