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Quantification of xanthohumol, isoxanthohumol, 8-prenylnaringenin, and 6-prenylnaringenin in hop extracts and derived capsules using secondary standards

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ABSTRACT

Hop is a well-known and already frequently used estrogenic phytotherapeutic, containing the interesting prenylflavonoids, xanthohumol (XN), isoxanthohumol (IXN), 8- and 6-prenylnaringenin (8-PN and 6-PN). Since the use of secondary standards can form a solution whenever the determination is required of certain components, not commercially available or too expensive, it was decided to develop an accessible HPLC-DAD method for the determination of these prenylflavonoids. The amounts were determined in hop extract and capsules, using quercetin and naringenin as secondary standards. After optimization of the sample preparation and HPLC conditions, the analysis was validated according to the ICH guidelines. The response function of XN, 8-PN, quercetin and naringenin showed a linear relationship. For the determination of XN, a calibration line of at least three concentrations of quercetin has to be constructed. The correction factors for XN (quercetin) and for 8-PN (naringenin) were validated and determined to be 0.583 for XN, and 1.296 for IXN, 8-PN and 6-PN. The intermediate precision was investigated and it could be concluded that the standard deviation of the method was equal considering time and concentration (RSD of 2.5–5%). By means of a recovery experiment, it was proven that the method is accurate (recoveries of 96.1–100.1%). Additionally, by analysing preparations containing hop extracts on the Belgian market, it was shown that the method is suitable for its use, namely the determination of XN, IXN, 8-PN and 6-PN in hop extract and capsules, using quercetin and naringenin as secondary standards.

1. Introduction

Hop (*Humulus lupulus* L.) is a dioecious perennial plant belonging to the Cannabaceae. Although it grows in the wild in Europe, Asia and North America, it is mainly cultivated for its female inflorescences which are used in the brewing industry to add bitterness and aroma to beer [1,2]. In pharmacotherapy products derived from the hop inflorescences (strobiles) are used to treat insomnia and nervousness [3].

Xanthohumol (XN) is a prenylated chalcone present in hop plants, which has shown interesting activities such as cancer chemoprevention, antiviral effects against HIV-1, and even antiplasmodial activity by inhibiting the replication of *Plasmodium falciparum* [4–6]. It also inhibits the oxidation of low-density lipids [7]. XN is the main prenylflavonoid in hops, but it is accompanied by other prenylflavonoids, that are typically present in much lower concentrations [8]. During boiling, XN is largely converted into isoxanthohumol (IXN) and, thus, IXN is the most prevalent prenylflavonoid in beers [9]. Interstingly, IXN has been shown to act as a precursor for 8-prenylnaringenin (8-PN), the main estrogenic principle derived from hops [10,11]. It was established that this phytoestrogen is more potent than the well-known soy-derived isoflavonoids such as genistein and daidzein, and the alfalfa-derived coumestrol [12]. Preparations standardized on 8-PN are being used to help relief menopausal complaints [13,14].

Many analytical methods have been described. A semi-quantitative TLC method has been established for the analysis of XN, humulones, and lupulones in hop strobiles [15]. This lead to a sensitive HPTLC method developed for the quantification of XN in hops and hop products [16]. Similar quantification was achieved by HPLC with UV or MS detection and by microemulsion electrokinetic chromatography [17–19]. Since LC coupled to MS-MS provides improved sensitivity and greater selectivity, it can be used for the analysis of minor components in complex matrices. This is

Abbreviations: XN, xanthohumol; IXN, isoxanthohumol; 8-PN, 8-prenylnaringenin; 6-PN, 6-prenylnaringenin; RSD, relative standard deviation.

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why Stevens et al. used this technique to quantify XN and other prenylflavonoids in hops and beer [18]. XN and IXN were also determined in different commercial hop products by HPLC/DAD and HPLC-ESI-MS/MS [1]. For the quantification of 8-PN an analytical method was developed using HPLC-MS with electrospray ionization [20].

Although many analytical methods already have been developed for the quantification of XN, IXN and 8-PN, there still is a lack of validated methods in reach for routine control. Several methods showing good separation were reported though not always validated questioning the suitability [21]. Even in the case of properly validated methods for the determination of several prenylflavonoids, primary reference material was used meaning that these compounds need to be isolated first in order to perform the analysis [22]. The use of secondary standards enables any laboratory, without access to expensive or simply unavailable reference materials, to determine the amounts of these interesting components. Therefore the aim of this research was to develop and validate a simple and readily available analytical method for the quantification of XN, IXN, 8-PN and 6-PN in the crude extract of hop and capsules containing this extract, using quercetin and naringenin as secondary standards. A previously reported HPLC method from Possemiers et al. was used as starting point for the development and optimization of this analysis [23].

2. Materials and methods

2.1. Reagents

Acetonitrile HPLC far UV, methanol for HPLC, and formic acid for analysis were provided by Acros Organics (Morris Plains, NJ, USA). Distilled water was prepared with a Millipore water purification system (Millipore, Bedford, MA, USA). The reference quercetin (8.2% water; 98.3% purity, determined by HPLC) that was used as a secondary standard for the determination of XN, was obtained from Sigma–Aldrich (Bornem, Belgium). The reference naringenin (<1% water; 99.3% purity, determined by HPLC) was used as secondary standard for the determinations of IXN, 8-PN and 6-PN, and was purchased from Sigma–Aldrich (Bornem, Belgium).

2.2. Plant material and capsules

The hop extract standardized on 8-PN content was derived from hydroalcoholic extraction of spent hops, the material remaining after extraction of hops with liquid or supercritical carbondioxide (Lifenol®, Naturex, Avignon, France). It is identical to that used in two clinical trials [13,14].

A capsule of 229.1 mg contains 178.6 mg of hop extract, 48.5 mg dicalcium phosphate, 1.0 mg of silicium dioxide and 1.0 mg of magnesium stearate.

2.3. Primary standards

Primary reference material of XN (4.2% water; 99.5% purity, determined by HPLC) was obtained by semi-preparative HPLC of Xanthoflav™, a commercially available hop extract enriched in XN (Hopsteiner, Mainburg, Germany), on a Varian Omnisphere C-18 column (250 mm × 21.4 mm, 10 μm, Varian, Sint-Katelijne-Waver, Belgium) using a Giilson 322 pump with a Gilson UV-VIS 156 detector and a Gilson 206 fraction collector (Gilson, Middleton, United States of America). 8-PN (4.7% water; 99.9% purity, determined by HPLC), 6-PN and IXN were obtained following procedure. IXN was prepared from XN by isomerisation under reflux in a 5% ethanolic KOH solution. IXN was purified from the reaction mixture by semi-preparative HPLC. 8-PN was prepared by prenylation of naringenin with 2-methylbut-3-en-2-ol in dioxane in the presence of boron trifluoride [24]. All chemicals were obtained from Sigma–Aldrich (Bornem, Belgium). Using semi-preparative HPLC, 8-PN was purified from a fraction resulting from flash chromatography containing both 8-PN and 6-PN and a B ring prenylated naringenin. The identities of XN, IXN, and 8-PN were confirmed by comparison of ^{1}H NMR and ^{13}C NMR data (Varian 300 MHz) with literature values [25].

IXN and 6-PN were only used for the identification in the HPLC chromatogram.

2.4. Sample preparation

For the reference solutions, about 25 mg of quercetin was accurately weighed and dissolved in 50.0 ml of methanol. 3.0 ml, 4.0 ml, and 5.0 ml of this solution was diluted to 25.0 ml with methanol. 15 mg of naringenin was accurately weighed and dissolved in 100.0 ml of methanol. 1.0 ml of this solution was diluted to 100.0 ml with methanol 50%.

As test solution, about 100 mg of the extract was weighed accurately in a 100.0 ml volumetric flask and dissolved with methanol. For the capsules, 60 mg of the mixed content of 20 capsules was accurately weighed and dissolved in 50.0 ml methanol. The solutions were placed in the ultrasonic bath for 30 min before filtering it through a 25 mm syringe filter (0.45 μm Nylon). For the determination of XN, 20 μl was injected, whereas for 8-PN, IXN and 6-PN, 50 μl was used.

2.5. Analytical method

The instrument used was a Beckman (System Gold 168) HPLC with diode array detection (DAD) and an automatic injector (Analis, Gent, Belgium). The gradient for the analysis was set at 0 min, 80:20 (A:B); 3 min, 80:20; 33 min, 25:75, using 0.25% formic acid in water as solvent A and 0.25% formic acid in acetonitrile as solvent B at a flow rate of 1.0 ml/min. A Lichrospher HPLC column, RP–18e (5 μm), 244 × 4 mm, was used and the column thermostat was set at 30 °C. The chromatogram was recorded at 370 nm for the determination of XN, and at 290 nm for the determinations of IXN, 8-PN and 6-PN.

3. Results and discussion

3.1. Method development

In this project, the HPLC-DAD method described in [23] was further optimized and validated for the determination of XN, IXN, 8-PN and 6-PN in hop extract and capsules containing the extract. Although previous reports stated that this technique could not offer sufficient sensitivity and selectivity for the determination of prenylflavonoids in complex matrices [1,20], this research revealed that by comparing UV spectra of the peaks, it was possible to identify the components.

3.1.1. Chromatographic conditions

Starting from the HPLC method used in previous work [23], the analytical method was developed and optimized. Originally methanol was used, but a change to acetonitrile and addition of 0.25% formic acid resulted in an improvement of the peak shape and higher sensitivity. This led to a small change in the gradient with (A) 0.25% formic acid in water and (B) 0.25% formic acid in acetonitrile: 0 min, 80:20 (A:B); 3 min, 80:20; 33 min, 25:75; 35 min, 80:20. Except for the HPLC column of Merck (Darmstadt, Germany), LiChrospher RP18 EC, 5 μm, 244 × 4 mm, also one with a high carbon load of Grace Davison (Lokeren, Belgium), Alltima HP C18 HL, 3 μm, 150 × 3 mm, was used. However, using the first column a better peak purity was obtained and therefore the LiChrospher column was maintained for the analysis. The temperature of the column

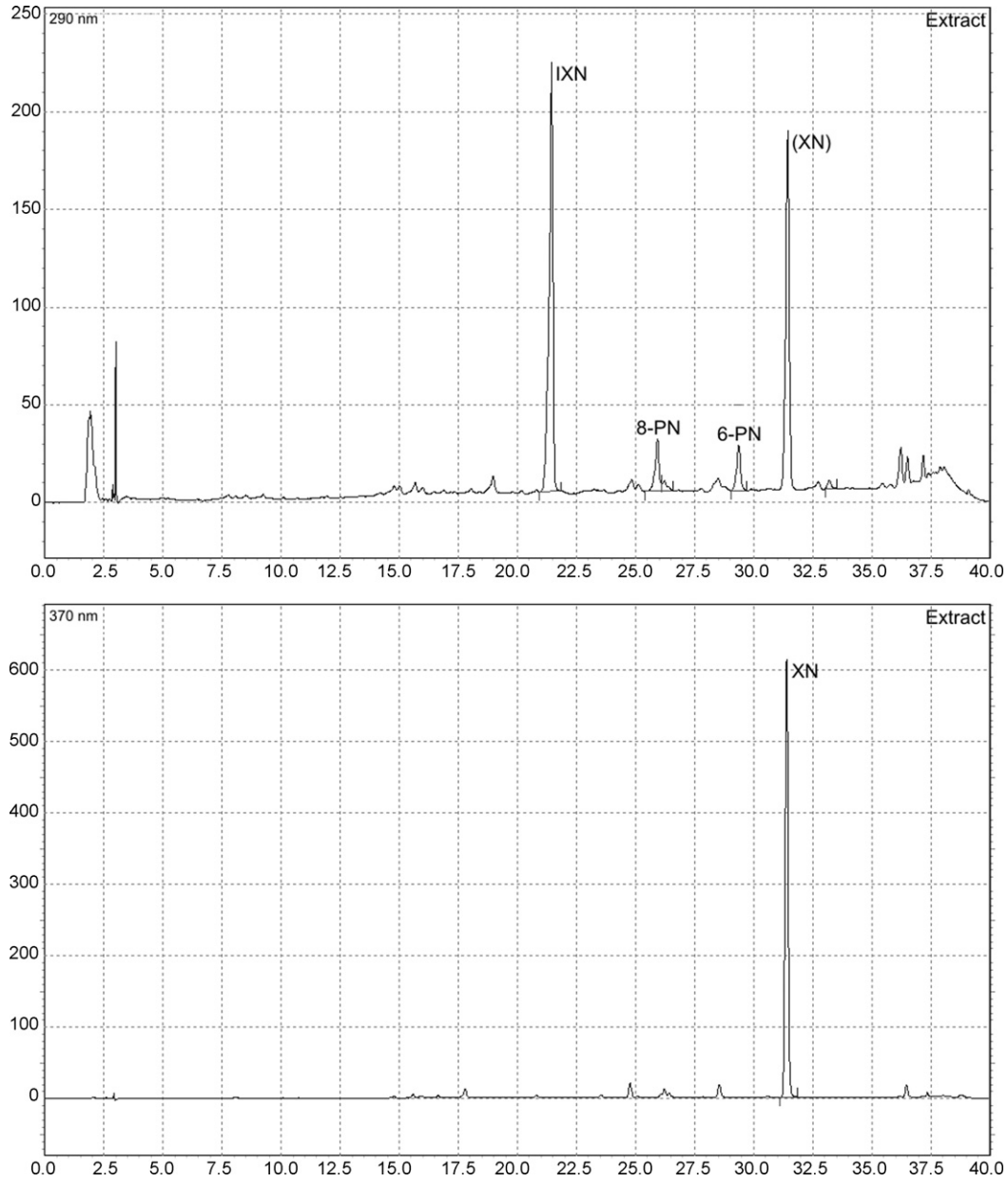

Fig. 1. HPLC chromatogram of the extract using the developed method at 290 nm (upper) and 370 nm (lower) with isoxanthohumol (IXN), 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), and xanthohumol (XN).

compartment was set at 30 °C in order to prevent chromatographical changes due to differences in room temperature. By comparing the UV spectra of the peaks and the retention times to those of the standard material, they could be identified as XN, IXN, 8-PN, and 6-PN. The chromatograms of the extract and the capsules are shown in Figs. 1 and 2, respectively. The UV spectra of the relevant peaks in the extract, needed for identification whenever no standard material is available, are shown in Fig. 3.

3.1.2. Sample preparation

The sample preparation was thoroughly adjusted compared to the sample preparation mentioned in [23]. First the amount of the aliquot of extract was determined by dissolving different weights of the extract (25, 50, 100 mg) in 50.0 ml of pure methanol. The solution with a concentration of 1 mg/ml gave the best results (finally 100 mg in 100.0 ml was used). Different solvents were investigated. By dissolving the extract in methanol:water (1:1) containing formic acid, which would be preferred in order to resemble the starting conditions of the HPLC, only very small peaks were observed. When the acid was omitted and a mixture of methanol and water was used, lower amounts of XN were detected and the different solvent did not improve the peak shape of 8-PN and 6-PN. A mixture of methanol and DMSO was tested as well, but since no improvement was observed, 100% methanol was kept for dissolving the samples. The duration of sonication was set at 30 min after testing the effect of 10, 20, 30, and 60 min in the ultrasonic bath. Because of the big difference in quantity between XN and the related prenylflavonoids, it was investigated which injection volume should be used in order to determine all selected components at the same time. However, this was not feasible. A higher injection volume resulted in a tailing peak for XN and the amounts of the other flavonoids was too low to use smaller injection volumes. Thus, it was concluded to inject 20 μl for the determination of XN, and 50 μl for the determinations of IXN, 8-PN and 6-PN. It was necessary to remove particles from the samples before injection, and therefore the difference between centrifuging and filtrating

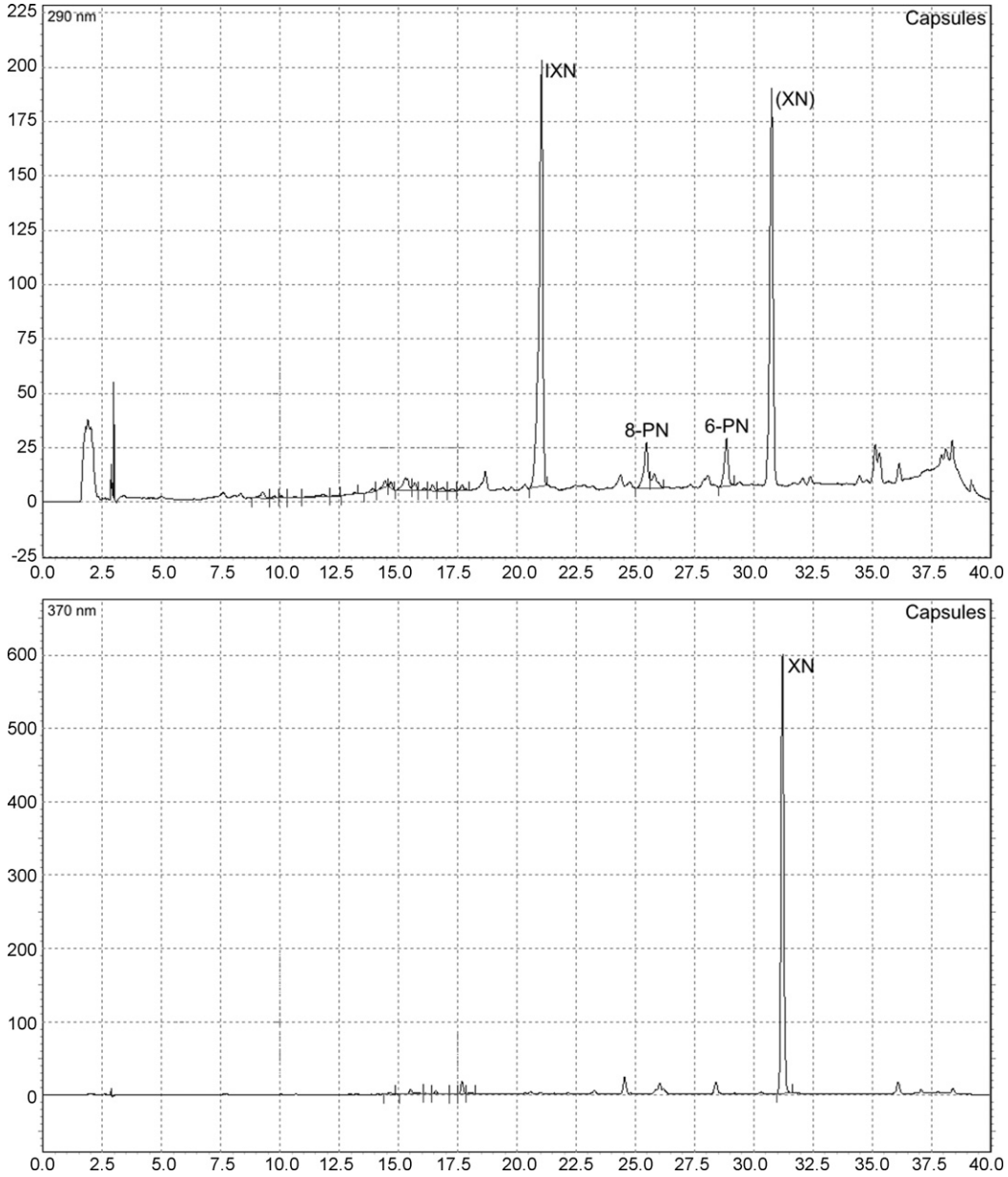

Fig. 2. HPLC chromatogram of the capsule content using the developed method at 290 nm (upper) and 370 nm (lower) with isoxanthohumol (IXN), 8-prenylnaringenin (8-PN), 6-prenylnaringenin (6-PN), and xanthohumol (XN).

was investigated. However, the same results were obtained and filtration was preferred since this is faster.

3.1.3. Secondary standards

Firstly, rutin was used as the secondary standard for the determination of XN. However, during the investigation of the precision, no repeatable results could be obtained. This was caused by the different absorption maximum between rutin and XN. An important feature for the choice of secondary standards in cases like this, is the maximum absorption wavelength, which should be similar to those of the components under investigation. Thus, quercetin was selected as the secondary standard for the determination of XN in the hop extract and capsules, and naringenin was used for the determinations of IXN, 8-PN and 6-PN. The UV spectra of the standards are shown in Fig. 4. Since IXN and 6-PN are less important components to determine and their structures resemble very well the structure of 8-PN, the correction factor of 8-PN was used for the determination of these compounds. Since naringenin dissolved in pure methanol resulted in a broadened peak, it was further diluted using methanol:water (1:1) instead of pure methanol, improving the peak shape.

3.2. Method validation

3.2.1. Response function – calibration model

The calibration models of XN, 8-PN, quercetin, and naringenin were investigated by injecting at least 5 different concentration levels of the standards and constructing the calibration lines. The linearity was investigated by means of the correlation coefficient, the 95% confidence interval of the intercept, the significance of the slope using the Student's *t*-test, and an analysis of variance (ANOVA). Also graphical inspection was performed on the calibration line and the residuals. The results are summarized in Table 1.

All calibration models showed a linear relationship in the range of 18.50–73.99 μg/ml XN, 19.32–154.56 μg/ml quercetin,

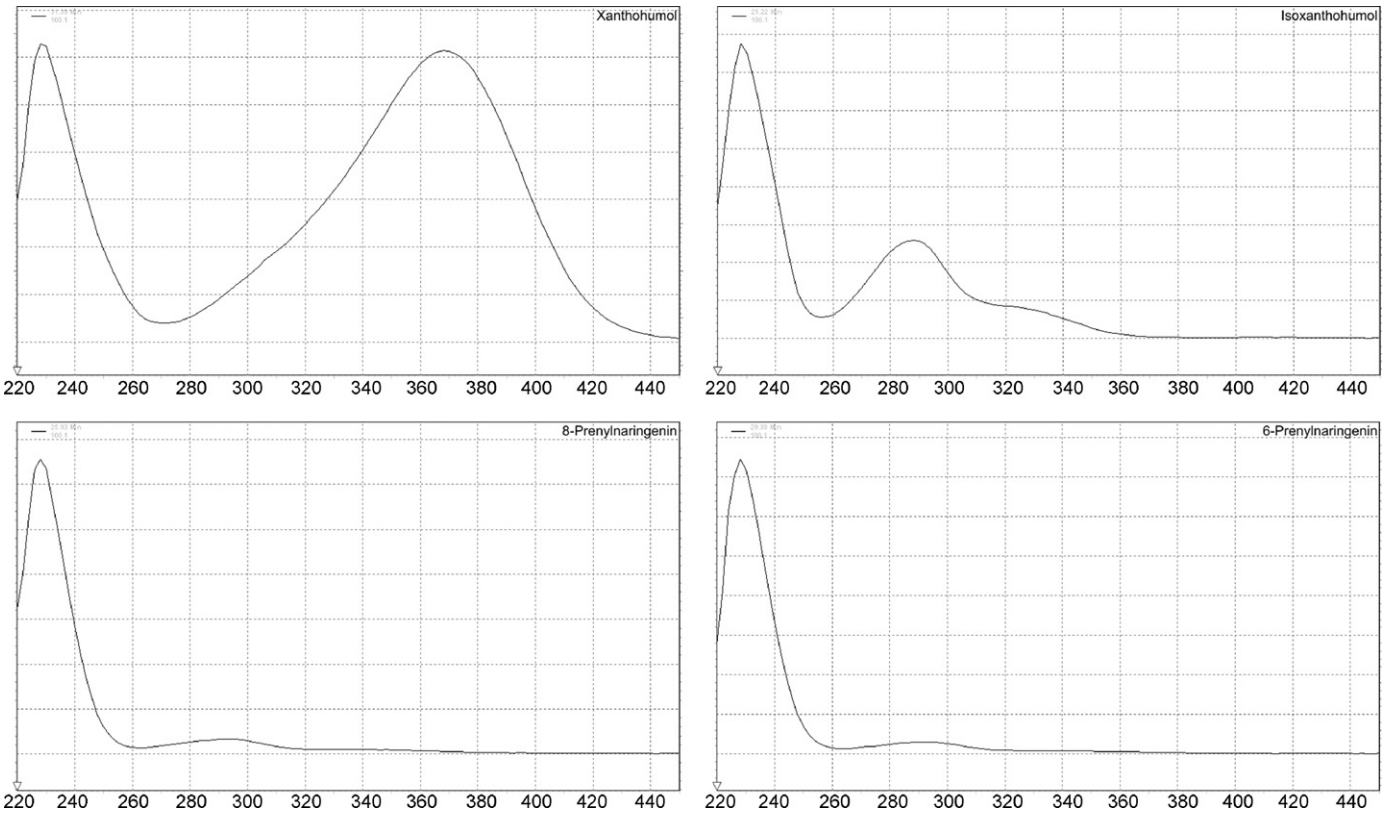

Fig. 3. The UV spectra of xanthohumol, isoxanthohumol, 8-prenylnaringenin, and 6-prenylnaringenin in the hop extract.

0.86–42.77 μg/ml 8-PN, and 0.38–26.82 μg/ml naringenin. For quercetin, the intercept was significantly different from the point (0,0), meaning that for each analysis a calibration line with at least three standard levels has to be created. In the ANOVA for 8-PN, the obtained *F*-value was higher than the critical *F*. However, the quality coefficient was calculated to be lower than 2.5% meaning that these results are still in agreement with the ICH guidelines [26–28].

3.2.2. Correction factor for response

The repeatability of the correction factor was investigated for XN using quercetin, and for 8-PN using naringenin. The correction factor for response was calculated for different concentration levels ($C_p/A_p \times A_s/C_s$) using the concentration (C) and area (A) of the primary standards (p), XN and 8-PN, and secondary standards (s), quercetin and naringenin. Different parameters were calculated: the average, the standard deviation and the relative standard deviation. Both the within-level and the between-level precision were examined. The Cochran's test was used in order to determine whether the standard deviation of the correction factor could be considered equal for the different concentration levels, and then an ANOVA (single factor, $\alpha = 0.05$) was performed. The results are shown in Table 2.

Since the Cochran's test was positive and the ANOVA showed that, from a statistical point of view, the results obtained on the three different concentration levels were not significantly different from each other, it could be concluded that both the correction factors for XN and for 8-PN can be considered to be precise within the respective XN and 8-PN concentration range from 50% to 200%. In future analyses, a correction factor of 0.583 should be used for XN, and 1.296 for IXN, 8-PN and 6-PN. The concentrations determined against the respective secondary standards must be multiplied by the respective correction factor after which the percentage (w/w) is calculated against the weighed sample amount.

3.2.3. Precision

The analysis of the extract and of the capsules was performed six times on the same day in order to investigate the repeatability. For the intermediate precision the analyses were performed (six times) on three different days with freshly prepared standard

Table 1
Summary of the results concerning the linearity of the calibration models of XN, quercetin, 8-PN and naringenin.

	XN	Quercetin	8-PN	Naringenin
N	6	6	6	7
Range (μg/ml)	18.50–73.99	19.32–154.56	0.86–42.77	0.38–26.82
Corr. coefficient	0.9993	0.9995	0.9999	0.9999
Intercept	25,470	−306,116	−1039	−2310
CI 95%	−142,766 to 129,007	−467,363 to −144,869	−15,387 to 13,308	−28,853 to 24,233
Slope	132.6	74,804.4	147.6	196.4
t-test (t_{crit})	82.28 (2.23)	104.41 (2.23)	441.17 (2.23)	207.90 (2.18)
F_{calc} (F_{crit})	1.30 (4.53)	1.50 (4.53)	4.90 (4.53)	0.20 (3.97)
Quality coefficient			0.85%	
Residuals	Randomly scattered	Randomly scattered	Randomly scattered	Randomly scattered

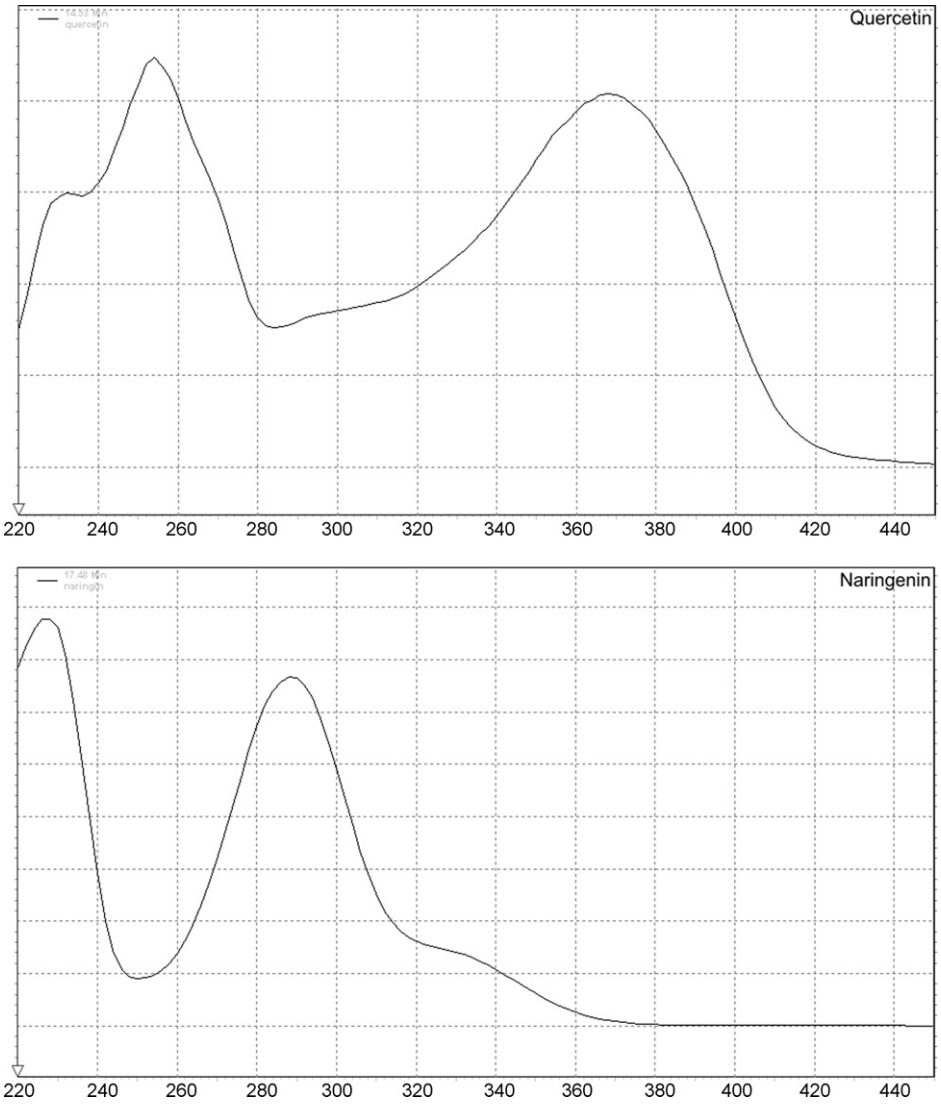

Fig. 4. The UV spectra of the standards quercetin and naringenin.

solutions. The repeatability on different concentration levels was investigated by analysing 50% and 200% of the previously described sample amounts, i.e. 50 and 200 mg for the crude extract, and 30 and 120 mg for the capsules.

The amounts of XN, IXN, 8-PN and 6-PN were calculated by means of the correction factors. Also the total amount of prenylflavonoids was determined by adding up the partial results of each individual compound. The averages, standard deviations

Table 2
Repeatability results on the correction factor.

	Correction factor for XN			Correction factor for 8-PN		
	50%	100%	200%	50%	100%	200%
Results	0.565	0.593	0.571	1.260	1.301	1.317
	0.588	0.589	0.597	1.258	1.298	1.353
	0.564	0.587	0.592	1.256	1.299	1.309
	0.573	0.581	0.586	1.279	1.313	1.284
	0.585	0.599	0.594	1.264	1.339	1.294
	0.579	0.573	0.585	1.288	1.325	1.286
Mean	0.576	0.587	0.588	1.267	1.313	1.307
RSD	1.74	1.56	1.58	1.02	1.28	1.97
Precision						
Mean		0.583			1.296	
RSD_{within}		1.63			1.49	
$RSD_{between}$		1.88			2.33	
C (C_{crit})		0.31 (0.71)			0.60 (0.71)	
F (F_{crit})		3.04 (3.68)			9.77 (3.68)	

Table 3
Overview of the determined amounts of XN, IXN, 8-PN and 6-PN in the crude extract and in the capsules, obtained on the different days and at different concentration levels.

	Crude extract					Capsules				
	XN	IXN	8-PN	6-PN	Total	XN	IXN	8-PN	6-PN	Total
Day 1										
Mean	4.01	1.60	0.19	0.17	5.97	3.08	1.16	0.14	0.13	4.50
Stdev	0.058	0.038	0.005	0.002	0.058	0.076	0.038	0.004	0.003	0.109
RSD%	1.44	2.38	2.59	1.22	0.98	2.45	3.26	2.68	2.66	2.42
Day 2										
Mean	3.93	1.48	0.18	0.16	5.75	2.96	1.15	0.14	0.12	4.37
Stdev	0.062	0.046	0.007	0.003	0.067	0.034	0.023	0.004	0.002	0.048
RSD%	1.58	3.12	3.64	1.99	1.16	1.16	1.97	2.79	1.57	1.11
Day 3										
Mean	3.88	1.55	0.20	0.17	5.79	2.96	1.10	0.13	0.12	4.31
Stdev	0.156	0.051	0.009	0.001	0.130	0.050	0.037	0.006	0.004	0.071
RSD%	4.04	3.26	4.68	0.88	2.24	1.69	3.38	4.37	3.50	1.66
Day 4										
Mean	3.85									
Stdev	0.137									
RSD%	3.55									
50%										
Mean	3.91	1.45	0.18	0.16	5.70	2.85	1.17	0.14	0.13	4.29
Stdev	0.202	0.048	0.009	0.001	0.239	0.054	0.033	0.005	0.004	0.080
RSD%	5.17	3.30	5.11	0.81	4.20	1.89	2.82	3.87	3.39	1.87
200%										
Mean	3.70	1.49	0.19	0.17	5.54	2.83	1.18	0.14	0.12	4.27
Stdev	0.066	0.016	0.006	0.001	0.063	0.030	0.041	0.004	0.002	0.049
RSD%	1.77	1.07	3.23	0.77	1.14	1.05	3.47	2.68	1.37	1.16

and relative standard deviations were calculated for each group of analyses and are shown in Table 3. The relative standard deviation within and between the different days and concentrations were determined, and the Cochran's test and ANOVA (single factor, $\alpha = 0.05$) were performed in order to investigate the results obtained on the different days and different concentration levels. These results are summarized in Table 4.

Since the Cochran's test was negative for the results of XN in the extract (the calculated C was higher than the C_{crit}), it was not allowed to perform an ANOVA. Instead, a fourth day was included and statistics were performed again. This time the Cochran's test was positive and an ANOVA could be performed. In all other cases the Cochran's test was positive, meaning that it was permitted to compare the different groups. When the ANOVA results in an F-value lower than F_{crit}, it can be concluded that from a statistical point of view, the results obtained on the different days or at the different concentration levels are not significantly different from each other. However, the F-value was higher in almost every case. By comparing the RSD_{within} and the $RSD_{between}$ for the different days, it was shown that there is no difference in variance between the different days. Since the $RSD_{between}$ for the different concentrations was similar to the $RSD_{between}$ for the different days, it could be concluded that the variance is not influenced by the concentration. In addition, the relative standard deviations between the days are in the order of magnitude as the limit set by Horwitz [29], and the between-level relative standard deviations are smaller than 5%. Therefore, the standard deviations of the method can be considered equal for the different days and the different concentration levels. In addition, graphical inspection of the results of each group showed there was no dependency on the days or on the different concentration levels.

Finally, the determined amounts in the hop extract were 3.88% XN, 1.51% IXN, 0.19% 8-PN, and 0.17% 6-PN, adding up to a total of 5.75% prenylflavonoids. The capsules contained 2.94% XN, 1.15% IXN, 0.14% 8-PN and 0.12% 6-PN, or 4.35% prenylflavonoids.

3.2.4. Accuracy

The accuracy was investigated by performing a recovery experiment. To 50% of the hop extract, known concentrations of XN and 8-PN were added in three concentration levels. To

Table 4
Summary of the results of the intermediate precision.

	Crude extract					Capsules				
	XN	IXN	8-PN	6-PN	Total	XN	IXN	8-PN	6-PN	Total
Time										
Mean	3.92	1.54	0.19	0.17	5.84	3.00	1.14	0.13	0.12	4.39
RSD_{within}	2.87	2.93	3.74	1.47	1.55	1.87	2.93	3.33	2.67	1.82
$RSD_{between}$	3.16	4.84	5.00	3.71	2.50	2.91	3.92	4.21	3.16	2.82
C (C_{crit})	0.49 (0.59)	0.42 (0.71)	0.55 (0.71)	0.60 (0.71)	0.68 (0.71)	0.61 (0.71)	0.43 (0.71)	0.54 (0.71)	0.58 (0.71)	0.61 (0.71)
F (F_{crit})	2.29 (3.10)	11.34 (3.68)	5.76 (3.68)	33.44 (3.68)	10.51 (3.68)	9.63 (3.68)	5.76 (3.68)	4.60 (3.68)	3.42 (3.68)	9.39 (3.68)
Horwitz	3.26	3.75	5.13	5.24	3.07	3.39	3.92	5.42	5.49	3.20
Concentration										
Mean	3.88	1.51	0.19	0.17	5.75	2.94	1.15	0.14	0.12	4.35
RSD_{within}	3.25	2.75	3.95	1.24	2.28	1.75	3.03	3.34	2.63	1.72
$RSD_{between}$	3.99	4.76	5.03	3.31	3.43	3.82	3.81	4.23	3.30	2.71
C (C_{crit})	0.43 (0.45)	0.30 (0.51)	0.32 (0.51)	0.52 (0.51)	0.67 (0.51)	0.44 (0.51)	0.28 (0.51)	0.31 (0.51)	0.35 (0.51)	0.42 (0.51)
F (F_{crit})	4.00 (2.53)	12.93 (2.76)	4.74 (2.76)	38.05 (2.76)	8.55 (2.76)	23.63 (2.76)	4.49 (2.76)	4.60 (2.76)	4.43 (2.76)	9.87 (2.76)

Table 5
Overview of the obtained recoveries of XN and 8-PN in the crude extract and of XN, IXN, 8-PN and 6-PN in the capsules.

	Crude extract		Capsules			
	XN	8-PN	XN	IXN	8-PN	6-PN
Level	104.85	96.54	105.77	93.50	89.99	102.26
1	97.82	91.24	98.57	106.43	106.74	91.85
	99.77	101.31	97.19	103.78	109.98	92.35
Level	98.14	105.77	88.29	92.12	88.36	92.95
2	92.33	98.06	99.64	96.60	96.33	95.93
	99.95	104.84	106.92	95.22	88.23	97.92
Level	99.72	102.29	93.28	91.38	98.03	99.34
3	94.81	101.48	94.08	91.36	95.54	103.18
	97.90	99.38	89.75	94.75	101.80	96.12
Summary						
Mean	98.37	100.1	97.06	96.13	97.22	96.88
RSD%	3.56	4.45	6.67	5.65	8.05	4.29
t (t_{table})	1.40 (2.31)	0.07 (2.31)	1.36 (2.31)	2.14 (2.31)	1.07 (2.31)	2.25 (2.31)

Table 6
Overview of the analyzed hop-containing preparations.

Sample	Single extract or mixture	Form	8-PN per unit	Advised dose (per day)	Regulations
A	Single	Capsules	148.2 μg	1 capsule	OK*
B	Single	Capsules	13.8 μg	3 capsules	OK*
C	Single	Drops	14.9 μg/ml	Max 120 drops (=6.0 ml)	OK
D	Mixture	Capsules	–	2 capsules	OK
E	Mixture	Tablets	–	3 tablets	OK
F	Mixture	Tablets	18.8 μg	6 tablets	OK
G	Mixture	Tablets	32.9 μg	2 tablets	OK

* Although preparations were conform to the regulations, LC-MS analysis was needed to determine the exact amount of 8-PN, because of peak impurity that was observed.

50% of the capsule content three concentration levels of hop extract were added. For each concentration, three samples were prepared and analysed according to the previously described method. For each analysed sample the recovery was calculated ($[X_{after} - X_{before}] \div X_{added} \times 100$). A Student's t-test ($\alpha = 0.05$; $n-1$) was performed in order to check whether the obtained recovery was significantly different from 100%. The results of this experiment are shown in Table 5.

Since the Student's t-test was positive for all calculated recoveries, it could be concluded that they are not significantly different from 100% from a statistical point of view, and thus, the method yields accurate results. The mean recoveries were 98.37% and 100.1% for XN and 8-PN, respectively, in the extract, and for the capsules 97.06%, 96.13%, 97.22% and 96.88%, for XN, IXN, 8-PN and 6-PN, respectively.

3.2.5. Specificity

The peaks in the chromatogram were identified to be XN, IXN, 8-PN and 6-PN by comparing their UV spectrum (ranging from 220 to 450 nm) and their retention times to those of the reference material. The excipients from the capsules (dicalcium phosphate, silicium dioxide and magnesium stearate) do not interfere with the determination.

3.3. Analysis of samples on the Belgian market

In Belgium, food supplements are regulated by the Royal Decree of August, 29th, 1997 [30]. This Decree describes the notification procedure that has to be completed before a market authorization can be obtained next to other criteria that have to be fulfilled such as labelling and advertisements. The third annex of this document is a list of plants allowed to be commercialized as food supplements. For some of these plants maximal daily doses were established by the Advisory Committee for Plant Preparations. For *H. lupulus* the daily intake is limited to 400 μg of 8-PN. Each batch of hop-containing preparations should be checked for its compliance to this guideline. The list also proposes a suitable method for this analysis, which, for the determination of 8-PN, is described in reference [23].

However, when MS detection is not available, the validated HPLC method described in this paper can also be used for a rapid check-up of the hop preparations for their 8-PN content. This was done for several preparations on the Belgian market containing *H. lupulus* extract. It concerned single preparations, containing only hop extract, as well as more complex preparations containing a mixture of several plant extracts. In total seven different samples were analyzed and the results are shown in Table 6.

In each case it could be concluded that the preparations were conform, i.e. not exceeding the daily dose of 400 μg of 8-PN. However, when it is necessary to determine the exact amount of 8-PN in preparations, such as for sample A and B, it was noticed that there was a peak impurity and LC-MS analysis should be performed. Since, even when the impurity is included, the amount of 8-PN did not exceed the limit, it could nevertheless be concluded that these preparations are still in agreement with the regulations. In samples D and E no 8-PN was found.

4. Conclusion

Although many analytical methods were already developed, there was a need for the determination of XN, IXN, 8-PN and 6-PN in hop extract and capsules using secondary standards. This method will allow other laboratories to quantify these components without the necessity to acquire primary reference material first. The secondary standards used in this research, quercetin and naringenin, are readily available. Also the technique used, HPLC-DAD, makes this determination accessible and within the reach of other, smaller laboratories.

After optimization of the sample preparation and the chromatographic conditions, the developed method was validated according

to the ICH guidelines. The calibration models of XN, quercetin, 8-PN, and naringenin showed a linear relationship between the response and the concentration. It is necessary to prepare a calibration line for quercetin for each analysis, since (0,0) is not included and thus single-point calibration is not allowed. The repeatability of the correction factors for XN and for 8-PN were investigated and they were determined to be 0.583 and 1.296, respectively. Investigation of the intermediate precision showed no dependency on time or concentration of the results, and the between-days and between-levels relative standard deviations were lower than 5%. Therefore, the method can be considered to be precise. A recovery experiment was performed and it could be concluded that the method is accurate. In conclusion, the hereby reported method for the quantification of XN, IXN, 8-PN and 6-PN in the hop extract and in capsules, using the secondary standards quercetin and naringenin, is found suitable for its use.

This validated method was then used as a control to see whether hop-containing preparations were in agreement with the Belgian regulations, meaning that they did not exceed the maximum daily dose of 400 μg of 8-PN. Seven preparations were analyzed and all were found to be complying. Although the HPLC method was applicable for this kind of investigation, LC-MS analysis was necessary in two cases to determine the exact amount of 8-PN.

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