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Production of monoclonal antibodies against hop-derived (*Humulus lupulus* L.) prenylflavonoids and the development of immunoassays

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ABSTRACT

Monoclonal antibodies against the hop-derived prenylated chalcone xanthohumol (X) and the prenylated flavonoids isoxanthohumol (IX) and 8-prenylnaringenin (8-PN) were developed. Carboxylic acid haptens of X, IX and 8-PN were synthesized by linking a spacer to their C4′-OH group followed by subsequent coupling to bovine serum albumin (BSA) to form conjugates that were employed as immunogens in BALB/c mice to raise antibodies. The monoclonal antibodies that were secreted from the established hybridoma cell lines proved, in cross-reactivity studies, to possess highly specific binding capacities in an optimized competitive indirect ELISA. The immunoassays make use of immunogen-coated microtiterplates and a peroxidase-labeled anti-mouse IgG_1 secondary antibody with ABTS as a chromogenic substrate. For X the IC_{50} value derived from the standard curve was 62.91 ng mL^{-1}, and for both IX and 8-PN 37.15 ng mL^{-1}. The assay was validated for the quantitative analysis of X, IX and 8-PN in urine and serum. A simple sample pretreatment procedure using a diethyl ether extraction was optimized and the recoveries and matrix effects were assessed. The validity of the established assay was tested and mean inter- and intra-assay variations in urine were 2.32% and 1.91%, respectively for X, 6.24% and 2.39%, respectively for IX and 7.18% and 0.74%, respectively for 8-PN. In serum, the mean inter- and intra-assay variations were 8.90% and 1.37%, respectively for X, 6.13% and 1.57%, respectively for IX and 6.13% and 2.43%, respectively for 8-PN. Furthermore, the method demonstrated excellent accuracy and significant correlation with measurements by an established and validated HPLC–MS method.

Abbreviations: X, xanthohumol; IX, isoxanthohumol; 8-PN, 8-prenylnaringenin; EQ, equol; DAID, daidzein; GEN, genistein; COUM, coumestrol; END, enterodiol; ENL, enterolactone; E2, 17β-estradiol; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; ABTS, 2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt; IC_{50}, concentration of competitor that gives 50% inhibition; (RP-HP)LC, (reversed-phase high-performance) liquid chromatography; MS, mass spectrometry; UV (–VIS), ultraviolet (–visible); GC, gas chromatography; PEG, polyethylene glycol; pNPP, *para*-nitrophenylphosphate; PBS, phosphate-buffered saline; MWCO, molecular weight cut off; (k)Da, (kilo)Dalton; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SDS, sodium dodecyl sulfate–polyacrylamide; PBS(-Tw), phosphate buffered saline (with 0.05% Tween-20); ^1H and ^{13}C NMR, proton and carbon nuclear magnetic resonance; MHz, megaHertz; TLC, thin-layer chromatography; MSD, multimode source detector; APPI/APCI, atmospheric pressure photo-ionization/atmospheric pressure chemical ionization; HMPA, hexamethylphopshoramide; DCC, dicyclohexylcarbodiimide; NHS, *N*-hydroxysuccinimide; DMF, dimethylformamide; DMEM, Dulbecco's modified eagle medium; FBS, fetal bovine serum; HAT, hypoxanthine aminopterine thymidine; DMSO, dimethylsulfoxide; FPLC, fast protein liquid chromatography; TMB, 3,3′,5,5′-tetramethylbenzidine; OPD, *o*-phenylenediamine dihydrochloride; NC, negative control; B_i, absorbance in wells in the presence of a competitor; B_0, absorbance in wells without presence of a competitor (=maximum binding of the antibody); DMX, desmethylxanthohumol; 6-PN, 6-prenylnaringenin; QC, quality control; *r*, Pearson's correlation coefficient; CV, coefficient of variation; %RE, percentage relative error.

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1. Introduction

Hop (*Humulus lupulus* L.) proves to be a very rich source of prenylated flavonoids which contribute to the potential health-beneficial properties of beer [1]. The most prominent prenylchalcone xanthohumol (X) (up to 1%, w/w), is readily converted to the isomeric prenylflavanone isoxanthohumol (IX) by thermal treatment and under alkaline conditions [2,3]. X has been characterized as a broad spectrum cancer chemopreventive agent in *in vitro* studies, acting on different stages of carcinogenesis [4–6]. Furthermore, it was shown to have antioxidant and anti-infective properties as well [7–9]. About one-third of the human population hosts a microbial community that can convert IX into the estrogenic 8-prenylnaringenin (8-PN) [10]. Consequently, the non-estrogenic IX possesses estrogenic potential through its conversion to 8-PN. While beer is the main dietary source of hop-derived prenylflavonoids, dietary supplements containing these compounds are available on the market, thereby substantially increasing an individual's exposure. Given the low bioavailability of X [11] and the great inter-individual variation in IX- and 8-PN levels in body fluids [12], analytical methods covering wide concentration ranges in different biological matrices are required. Most of the reported quantitative methods for the determination of X, IX and 8-PN are based on reversed-phase liquid chromatography (RP-HPLC) combined with UV detection or mass spectrometric detection (MS or tandem MS) [3,13,14]. Immunoassays offer a low-cost, high throughput alternative to labs that do not have access or skills to do LC–MS(/MS). To the best of our knowledge, only Schaefer et al. [15] have reported on the generation of polyclonal antibodies against 8-PN and the subsequent development of a radioimmunoassay for the quantitative determination of 8-PN in beer and urine. Additionally, over the last few years, various papers have reported on immunoassays for soy- or flax-derived phytoestrogens for quantitation in biological samples [16–20]. Therefore, we present here the first report on the successful generation of monoclonal antibodies raised against 4′-*O*-(carboxymethyl)ether-bovine serum albumin (BSA) conjugates of X, IX and 8-PN. Monoclonal antibodies are known to show a highly specific binding to their haptens, to exert negligible cross-reactivities compared to polyclonal antisera and ensure the continuous production of a well-characterized product. Furthermore, we established and validated a competitive enzyme-linked immunosorbent assay (ELISA) using immunogen-coated microtitration plates and a peroxidase-labeled anti-mouse IgG_1 secondary antibody to measure X, IX and 8-PN concentrations in human biological fluids.

2. Materials and methods

2.1. Chemicals and materials

X, IX, 8-PN, desmethylxanthohumol (DMX), and 6-prenylnaringenin (6-PN) were available in our laboratory [10]. Equol (EQ) was purchased from Extrasynthèse (Genay, France). Daidzein (DAID), genistein (GEN) and naringenin were obtained from Acros Organics (Morris Plains, NJ, USA). The other competitors, coumestrol (COUM), enterodiol (END), enterolactone (ENL) and 17β-estradiol (E2) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA), polyethylene glycol (PEG 1500), Tween-20, alkaline phosphatase-labeled anti-mouse IgG antibody, a Mouse Monoclonal Antibody Isotyping kit and SIGMA *FAST para*-nitrophenyl phosphate (*p*NPP) tablets were purchased from Sigma–Aldrich. All other chemicals and solvents for the synthetic work and supplements for the biological work were obtained from Acros Organics and were all of analytical reagent grade. The solvents for the chromatography were all of LC–MS quality and purchased from Biosolve (Valkenswaard, The Netherlands). Phosphate-buffered saline (PBS) solution was prepared as 0.137 M NaCl and 2.7 mM KCl in 0.01 M phosphate buffer, pH 7.4. Complete serum-free Dulbecco's modified eagle medium (DMEM) (Invitrogen, Carlsbad, CA, USA) was supplemented with L-glutamine (0.05%), penicillin (100 units mL^{-1}), and streptomycin (100 $\mu g\,mL^{-1}$). The murine NS0 cell line, a non-secreting plasmacytoma cell line (myeloma) from BALB/c mice was kindly provided by the Laboratory for Cellular Immunology (P. De Baetselier, Vrije Universiteit Brussel, Belgium). For the cultivation of the NS0 cell line complete DMEM was supplemented with heat-inactivated FBS (10%, Invitrogen) and thioguanine (10 μM). HAT medium was used to select and culture hybridomas and consisted of complete DMEM with heat-inactivated FBS (20%), hypoxanthine (120 μM), thymidine (19 μM) and aminopterin (0.6 μM). Dialysis of the antigen conjugates and cell media was performed with Slide-A-Lyzer cassettes (0.5–3.0 mL; MWCO 7000 Da and 12.0–30.0 mL; MWCO 10 kDa) from Pierce (Rockford, IL, USA). For the production of monoclonal antibodies on large scale Integra Biosciences CELLine bioreactors (type CL350) were used and were purchased from Integra Biosciences (Chur, Switzerland). For the purification of the monoclonal antibodies, HiTrap protein G columns from GE Healthcare (Piscataway, NJ, USA) were used. The ultracentrifugation was performed with Amicon Ultra-15 centrifugal filter units (Millipore, Billerica, MA, USA). For the protein gel electrophoresis TEMED, sodium dodecyl sulfate–polyacrylamide (SDS) and β-mercaptoethanol were purchased from Bio-Rad Laboratories (Richmond, CA, USA) and all other used products were from Acros. The acetate buffer (0.1 M; pH 5.0) was made from sodium acetate hydrate obtained from UCB (Leuven, Belgium). The enzymatic deconjugation solution (33 mg mL^{-1}) was prepared in acetate buffer with sulfatase from *Helix pomatia* (type H-1 with sulfatase activity: 15.3 units mg^{-1} and β-glucuronidase activity: 300 units mg^{-1}) from Sigma–Aldrich. Peroxidase-labeled anti-mouse IgG_1 antibody, unlabeled mouse IgG antibody and ABTS stop solution were obtained from SouthernBiotech (Birmingham, Alabama, USA). ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt) tablets and peroxidase substrate buffer were purchased from Pierce. The ELISA technique was carried out on microtiter plates (Nunc Maxisorp, Nunc, Roskilde, Denmark) with 96 wells.

2.2. Instruments

Semi-preparative chromatography was performed with a Varian Omnisphere C_{18} column (250 mm × 21.4 mm, 10 μm) from Varian (Palo Alto, CA, USA) using a Gilson 322 pump (Gilson, Middleton, WI, USA) with a Gilson UV–VIS 156 detector and a Gilson 206 fraction collector. The identity of the haptens was confirmed by interpretation of proton magnetic resonance spectra (^{1}H NMR, 300 MHz) and carbon magnetic resonance spectra (^{13}C NMR, 75 MHz) data obtained with a Varian Mercury-VX 300 MHz spectrometer. Thin-layer chromatography (TLC) was performed using silica-coated aluminium plates with fluorescent indicator (Alugram SIL G/UV 254 plates, 0.2 mm; Macherey-Nagel & Co., Düren, Germany) and TLC separations were examined under UV light at 254 nm and 350 nm and revealed by a sulfuric acid–anisaldehyde spray. The hapten/carrier protein ratio of the antigen conjugates was estimated by UV-spectrometry at characteristic wavelengths using a Shimadzu UV-1600PC spectrophotometer (Shimadzu, Kyoto, Japan) in scan mode in a 1 cm quartz cuvette and using UV Probe 2.20 software. Purification of the produced monoclonal antibodies was performed on an AKTA-FPLC apparatus (GE Healthcare). Microtiter plates from the primary screening assays were read with a Molecular Devices (Wokingham, Berkshire, UK) microplate reader and the absorbances of the com-

petitive immunoassays were determined with a Beckman Coulter Paradigm Detection Platform (Fullertan, CA, USA). The HPLC–MS system was an Agilent 1200 (Agilent Technologies, Waldbronn, Germany) equipped with vacuum degasser (G1379B), binary pump (G1312A), autosampler (G1329A), column thermostat (G1316A) and diode array detector (G1315B). The system was coupled online to the mass selective MSD detector (G1956B), SL version, controlled by Chemstation software (Rev. B 02.01) and the MSD was tuned with Agilent APPI/APCI tuning mix. Statistical analyses during validation were performed using SPSS version 17.0 for Windows (SPSS Inc., Chicago, IL, USA).

2.3. Synthesis of 4′-O-carboxymethyl-xanthohumol, 4′-O-carboxymethyl-isoxanthohumol and 4′-O-carboxymethyl-8-prenylnaringenin

4′-*O*-carboxymethyl-xanthohumol was prepared by selective alkylation of the phenolic 4′-hydroxy group using dry potassium-*tert*-butoxide and ethyl bromoacetate in dry HMPA according to Al-Maharik et al. with minor modifications [21]. After the alkylation the ethylester was hydrolyzed under acidic conditions to the desired acid in good yield. The 4′-*O*-carboxymethyl-derivative of isoxanthohumol was obtained by cyclization of the xanthohumol-derivative under alkaline conditions. For the preparation of the 4′-*O*-carboxymethyl-derivative of 8-prenylnaringenin synthesis was started from naringenin. After protection of the 7-OH-group and subsequent linking with the spacer, naringenin was prenylated at the 5-OH-group [22]. Through a Claisen rearrangement the 8-prenylnaringenin-derivative was obtained.

2.4. Immunogen synthesis

For activation of the haptens the dicyclohexylcarbodiimide (DCC)/*N*-hydroxysuccinimide (NHS) coupling method of Hosoda et al. [23] was adapated. Briefly, a solution of the haptens was put for 2–5 h at room temperature with DCC and NHS in dry DMF. The mixtures were then centrifuged to deposit the crystals of dicyclohexylurea. The supernatants containing the respective NHS-esters were conjugated with BSA in a reversed micellar system [24] at a molar ratio (between the activated compound and BSA) of approximately 70:1 which is sufficient for a high degree of haptenisation, since approximately 60 amino groups can be used for modification. As there is a positive correlation between the number of haptens incorporated and the buffer/octane ratio, a large amount of buffer was used (6/1 buffer/octane). Upon clearance of the mixture, the activated haptens in DMF solution were added immediately under vigorous shaking. After a slight transient turbidity was observed the reaction mixture was allowed to react 24–36 h at room temperature. Total disappearance of the activated hapten was checked by TLC (4/1 trichloromethane/methanol). Hapten-BSA conjugates (Fig. 1) were isolated from the mixture by precipitation with three volumes cold acetone at 0 °C followed by centrifugation. The supernatant was removed, the sediment was washed against PBS^{D-} and dialyzed overnight at 4 °C under continuous stirring against PBS^{D-} using Slide-a-Lyzer cassettes. The remaining solution was filtered through a 0.2 μm filter and lyophilized. The hapten/carrier protein ratio of the conjugates was determined by spectrophotometric analysis. Aliquots of the conjugates were stored at −20 °C until use.

2.5. Immunization

Three to four BALB/c female mice (12–16 weeks old) (Charles River Laboratories, Wilmington, DE, USA) were immunized subcutaneously (100 μL) with the respective hapten-BSA conjugate (100 μg). Two and four weeks after the initial injection,

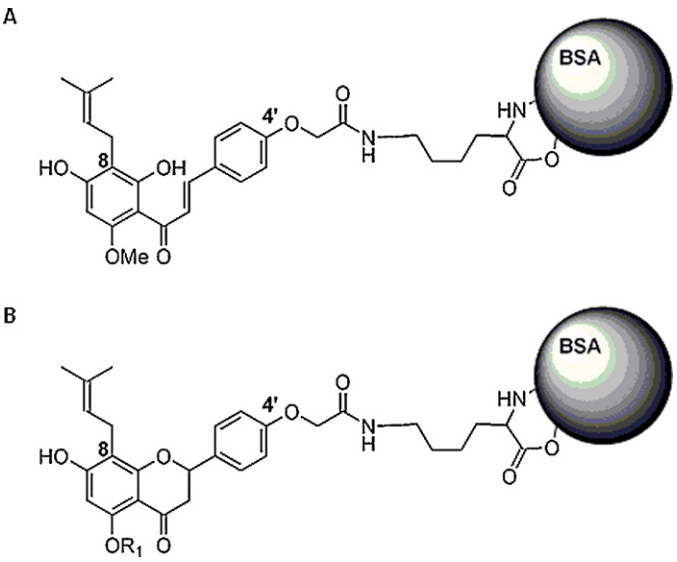

Fig. 1. Structures of the three immunogens: (A) 4′-*O*-carboxymethyl-xanthohumol and (B) isoxanthohumol-BSA (R_1 = Me) and 8-prenylnaringenin-BSA (R_1 = H).

booster injections were given intraperitoneally with an equal amount of immunogen. Three days after the last booster injection the mice were sacrificed for lymphocyte/myeloma cell fusion. Afterwards their spleens were isolated and transferred to 10 mL DMEM.

2.6. Cell fusion

The procedure described by Catty et al. [25] was used as basis for the cell fusion. Murine NS0 myeloma cells were cultured in complete DMEM supplemented with 10% FBS. Mice spleens were perfused with 20 mL serum-free DMEM, and the resulting lymphocyte suspension was mixed with the myeloma cells at a ratio of 10:1 and centrifuged. A few drops of alkaline DMEM were added to the cell pellet and after rolling of the tube 700 μL of a 50% PEG solution (w/v, in 1 mM NaOH) at 37 °C was added. Next, the procedure was completed with alternating of rolling steps and the addition of (in total) 15 mL serum-free DMEM. Finally, after completion of the fusion procedure, the cells were spun down and resuspended in 20 mL of HAT selection medium. The resulting cell suspension was distributed (100 μL $well^{-1}$) in 96-well tissue culture plates and incubated in a 10% CO_2 incubator at 37 °C. The next day one drop of HAT medium was added to the wells and every two days culture medium was replaced.

2.7. Hybridoma selection and cloning

Two weeks after the cell fusion, culture supernatants from all fusion wells were screened for the presence of anti-hapten antibodies. Initial screening and selection was performed using two replicate non-competitive enzyme-linked immunosorbent assays (ELISA) with X/IX/8-PN-BSA as the coating antigens. Next, antibodies from the selected hybridomas were tested in a competitive indirect ELISA to determine whether they recognize pure X/IX/8-PN and the coating antigen on a competitive basis. The monoclonality of the produced antibodies was ensured via limiting dilution (two rounds of subcloning) of the selected hybridomas [25]. Several aliquots of the first hybridomas and their clones were cryopreserved in liquid nitrogen (in 10% FBS in DMSO) at several stages during the development.

2.8. Production and characterization

The clones of interest were cultured for a period of 3 up to 5 months *in vitro* in Integra CELLine bio-reactors. After dialysis against PBS using Pierce Slide-A-Lyzer cassettes, monoclonal antibodies (mAbs) were purified from the collected culture media with fast protein liquid chromatography (FPLC) by their affinity for HiTrap Protein G columns and, subsequently, ultrafiltered against PBS. The protein concentration of the final antibody solutions was determined using a Bio-Rad protein concentration determination assay. Then, using the Sigma mouse monoclonal antibody isotyping kit, the isotype class was determined. Finally, the purity of the antibody solutions was confirmed by 12% SDS gel electrophoresis, followed by Coomassie blue staining [26]. The mAb stocks were stored at a 1 mg mL^{-1} concentration in PBS at −20 °C until further use.

2.9. Indirect non-competitive screening ELISA

The selection of the hybridoma's was carried out using an antigen-coated ELISA, with supernatants from the hybridoma cultures. Briefly, microtiterplates were coated overnight at 4 °C with 100 μL of a 10 μg mL^{-1} solution of X-BSA, IX-BSA and 8-PN-BSA. After washing four times with PBS containing 0.05% (v/v) Tween-20 (PBS-Tw), the wells were blocked for 1 h at 37 °C with 1% BSA in PBS. Next, after washing, 100 μL of supernatant was added and incubated during 2 h at 37 °C. After washing, 100 μL of the alkaline phosphatase-labeled rabbit anti-mouse IgG antibody at a 1:3000 dilution was added and incubated for 1 h at 37 °C. Finally, after a final washing step, 100 μL of the *p*NPP substrate solution was added at room temperature and after 30 min the absorbance was measured at 405 nm.

2.10. Indirect competitive ELISA

After two screenings with the non-competitive ELISA, the specificity of the antibodies from the selected hybridomas for X, IX or 8-PN was tested in an indirect, but competitive ELISA. Briefly, the same procedure as in Section 2.9 was applied, except for the extra addition of pure X, IX or 8-PN (10 μL of a 1 mg mL^{-1} solution in methanol) together with the culture supernatants to the wells. Whenever the binding of the antibodies to the coating antigen was inhibited by their affinity for X, IX or 8-PN, thereby decreasing the absorbance, the respective hybridomas were included for further experiments. Next, the different steps and parameters of this competitive ELISA were thoroughly optimized, using the purified monoclonal antibodies. In summary, checkerboard titrations were completed to establish the optimal working dilutions of the coating antigen, blocking buffer, primary and secondary antibody, and the type of secondary antibody and enzymatic substrate, incubation temperatures and time periods were optimized. Furthermore, the influence of organic solvent (i.e., methanol) for dissolvation of the prenylated flavonoid standards, on assay performance was examined.

The optimized procedure for the competitive ELISA was as follows: wells were coated overnight (at least 12 h) at 4 °C with 200 μL of a 0.5 μg mL^{-1} solution of the respective antigen and after washing with PBS-Tw blocked with 250 μL of 3% BSA in PBS during 1 h at 37 °C. Then, per well 100 μL of serial dilutions of the competitor, i.e. X in 50% methanol in PBS or IX or 8-PN (in 10% methanol in PBS) and an equal volume of the specific antibodies (5 ng mL^{-1} in PBS with 0.1% BSA) were added and incubated during 2 h at 37 °C under slow agitation. In negative control (NC) wells, the primary antibody was replaced by an antibody of irrelevant specificity (unlabeled mouse IgG; 1:5000 dilution in 0.1% BSA in PBS). The plate was washed with PBS-Tw and 200 μL of peroxidase-labeled anti-mouse IgG_1 antibody was added to each well. The plate was left at 37 °C for 1 h. After washing the plate four times with PBS-Tw, 200 μL ABTS substrate solution in peroxide substrate buffer was added and reaction took place in the dark at room temperature and was then stopped after 30 min by the addition of stop solution. Absorbance was measured at 405 nm and calibration curves were constructed by plotting $[(B_i - NC)/(B_0 - NC)]$ (B_i = absorbance of wells with standard/biological sample; B_0 = absorbance from maximum binding of the antibody to the antigen without competition) against log (competitor concentration) and fitted to a four-parameter logistic equation using Sigmaplot version 11.0 (Systat Software, Chicago, IL, USA). For each point of the calibration curve a minimum of five replicates was used and absorbances were the mean value of duplicate readings. From the equation of the calibration curves, IC_{50} values (concentration at which the binding of the antibody to the coating antigen is inhibited with 50%) were determined and subsequently used to express the sensitivity and the detection range of the ELISA.

2.11. Determination of cross-reactivity

A panel of other phytoestrogens (daidzein, genistein, enterodiol, enterolactone, equol, coumestrol), 17β-estradiol and structurally related compounds (desmethylxanthohumol, 6-prenylnaringenin) were tested for cross-reactivity by determining their respective IC_{50} values in the competitive assay as described above. Cross-reactivity values were calculated as the ratio of the IC_{50} of the X, IX or 8-PN standard to the IC_{50} of the test compounds and expressed as a percentage.

2.12. Preparation of samples for validation and extraction of urine and serum

For the validation of the competitive ELISA, spiked urine and serum samples, as well as some urine samples from a dietary intervention study [12], were prepared. Briefly, for the hydrolysis of glucuronide and sulfate conjugates, one volume of (spiked) urine or serum was mixed with one volume of sodium acetate buffer and incubated with a β-glucuronidase/sulfatase solution overnight at 37 °C. Next, the samples were extracted using five volumes of diethyl ether under vigorous vortex mixing during 30 s. This extraction was repeated three times to ensure complete recovery of the compounds. Finally, the collected solvent layers were evaporated to dryness under nitrogen and the residue was dissolved in assay buffer (50% methanol in PBS for X and 10% methanol in PBS for IX/8-PN).

3. Results and discussion

3.1. Immunoassay optimization

The concentrations of X, IX and 8-PN in biological samples, upon ingestion of a hop-based dietary supplement, most often only reach to lower ppb-levels. Therefore, the analytical sensitivity of the competitive ELISA, which is represented by its IC_{50} value, has to be as low as possible. Consequently, the amount of primary antibody and conjugate, as well as the concentration and type of blocking buffer (1–3% BSA in PBS or 0.5% milk powder in PBS), the amount of secondary antibody (1:2000–1:8000), type of substrate (ABTS, TMB or OPD) and the duration (0.5–2 h) and incubation temperature (room temperature or 37 °C) of each step were optimized in order to enhance the analytical sensitivity. The standard curve characteristics were optimal at conjugate and antibody concentrations of 0.5 μg mL^{-1} and 5 ng mL^{-1}, respectively, for X, IX and 8-PN and the other parameters were defined as described in Section 2.10. Furthermore, the effects of different concentrations of methanol on

Table 1
Influence of methanol (expressed as percentage in the well) on the binding capacity of the primary antibodies (0% methanol as reference).

Methanol (%)	Relative binding capacity (%)		
	Anti-X	Anti-IX	Anti-8-PN
0	100	100	100
0.25	111	92	98
0.5	106	93	107
2.5	123	92	106
5	139	97	106
12.5	155	90	94
25	155	85	81
50	75	81	77

the performance of the ELISA were studied. Given the relatively apolar nature of X, IX and 8-PN, a certain amount of organic solvent is necessary to ensure full solubilization in PBS. Although DMSO is often used for this purpose, we opted for methanol, as it is generally the best tolerated solvent in ELISA. Methanol dissolves X, IX and 8-PN very well and we did not notice any difference in solubility with the former. Consequently, a compromise had to be found between solubility and influence of methanol on assay performance. For that, the maximum signal or the maximum binding of the antibody was observed with increasing amounts of methanol added to the assay buffer (Table 1; amount of methanol expressed as percentage of the well). For the anti-IX and anti-8-PN antibodies the binding was attenuated starting from 12.5% methanol in assay buffer. The binding of anti-X, on the other hand, benefits from increasing amounts of methanol, and only starts to decrease with 50% methanol in the well. The solubility of X, IX and 8-PN in PBS with added amounts of methanol in the assay buffer, respectively 50% for X and 10% for IX and 8-PN, was examined by triplicate analysis with HPLC-UV (quantification at 370 nm for X and 295 nm for IX and 8-PN) [27]. The signal intensity of X, IX and 8-PN (1.25 $\mu g\,mL^{-1}$) in increasing methanol concentrations versus the same concentration in 100% methanol (or 100% dissolvation) was investigated. Analysis results showed that the solubility of X increased from 76.10% in 1% methanol in PBS to 98.70% in 50% methanol in PBS. For IX and 8-PN (both at a concentration of 1.25 $\mu g\,mL^{-1}$), the solubility increased from 84.40% and 83.30% in 1% methanol in PBS to 97.03% and 97.97% in 10% methanol, respectively. Additionally, calibration standards were prepared just before the beginning of each assay, to secure full solubilization and to minimize degradation of the analytes in PBS.

Standard curves (mean ± SD) for X, IX and 8-PN from 15 assays, measured on three different days under optimized conditions, are shown in Fig. 2(A–C). The IC_{50} values were 62.91 $ng\,mL^{-1}$ for anti-X, 37.15 $ng\,mL^{-1}$ for anti-IX and for anti-8-PN and their respective limits of detection (10% inhibition) were 14.45 $ng\,mL^{-1}$, 5.62 $ng\,mL^{-1}$ and 4.37 $ng\,mL^{-1}$. Values for the LLOQ (limit of quantitation) were calculated as 10 × SD of the blank and were 41.01 $ng\,mL^{-1}$, 13.90 $ng\,mL^{-1}$ and 17.10 $ng\,mL^{-1}$ for X, IX and 8-PN, respectively.

3.2. Specificity of the monoclonal antibodies

The cross-reactivities of the three monoclonal antibodies against a panel of other phytoestrogens, chemically related compounds and 17β-estradiol (E2) are shown in Table 2. Generally, all three antibodies revealed excellent specificity for their antigens. For anti-8-PN the negligible cross-reactivity (<0.03%) presented by DMX and 6-PN is probably due to the impurity of the respective standards or in situ isomerization (for DMX). On the other hand, anti-X showed some, negligible cross-reactivity (<0.05%) with IX and 8-PN.

Table 2
Cross-reactivity of three monoclonal antibodies against structurally related compounds.

Competitor	Cross-reactivity (%)		
	Anti-X	Anti-IX	Anti-8-PN
GEN	<0.01	<0.01	<0.01
DAID	<0.01	<0.01	<0.01
END	<0.01	<0.01	<0.01
ENL	<0.01	<0.01	<0.01
EQ	<0.01	<0.01	<0.01
COUM	<0.01	<0.01	<0.01
DMX	<0.01	<0.01	<0.03
6-PN	<0.01	<0.01	<0.03
E2	<0.01	<0.01	<0.01
X	100	<0.01	<0.01
IX	<0.05	100	<0.01
8-PN	<0.05	<0.01	100

Table 3
Recovery of X, IX and 8-PN in spiked urine and serum samples after solvent extraction at three QC levels.

Recovery (CV) (%)	X	IX	8-PN
Urine			
Low	110.38 (0.76)	89.68 (1.59)	89.05 (3.65)
Medium	103.87 (0.22)	96.44 (3.28)	95.61 (0.95)
High	96.26 (3.67)	96.88 (0.25)	97.13 (3.52)
Serum			
Low	95.34 (8.43)	97.65 (2.45)	85.71 (7.07)
Medium	84.98 (4.44)	96.99 (4.00)	90.92 (1.07)
High	88.05 (2.33)	100.58 (3.76)	95.28 (1.24)

3.3. Analytical recovery

The recovery after solvent extraction with diethyl ether was assessed by spiking a known amount of X, IX or 8-PN in blank urine and serum before extraction and after extraction. By comparing the measured concentrations (from triplicate analyses) at low (=QC_1; 50 $ng\,mL^{-1}$), medium (=QC_2; 250 $ng\,mL^{-1}$) and high (=QC_3; 750 $ng\,mL^{-1}$) QC level from the competitive ELISA, the extraction recovery and the possible influence of matrix components are assessed. In Table 3 the mean recoveries (with coefficients of variation) are presented for X, IX and 8-PN, for three concentration levels in urine and serum. In urine the recoveries for X ranged from 96.3% to 110.4%, for IX from 89.7% to 96.9% and for 8-PN from 89.1% to 97.1% and in serum the recoveries ranged from 85.0% to 95.3% for X, 97.0% to 100.6% for IX and 85.7% to 95.3% for 8-PN. It can be concluded, from these recovery experiments, that the sug-

Table 4
Influence of urine and serum on the maximum binding of the antibodies (without preliminary extraction).

% matrix (in PBS)	A_{max} (%)		
	Anti-X	Anti-IX	Anti-8-PN
Urine			
100	35.71	60.24	40.86
50	48.20	76.48	52.41
20	63.49	85.20	70.28
10	81.02	99.60	88.35
4	82.79	90.24	90.26
2	94.32	89.76	96.29
1	91.43	97.52	96.49
Serum			
100	41.63	56.32	60.69
50	46.39	57.33	62.96
20	46.75	65.94	70.25
10	54.99	74.55	71.43
4	62.21	77.27	77.34
2	70.81	84.33	81.97
1	78.13	89.60	94.68

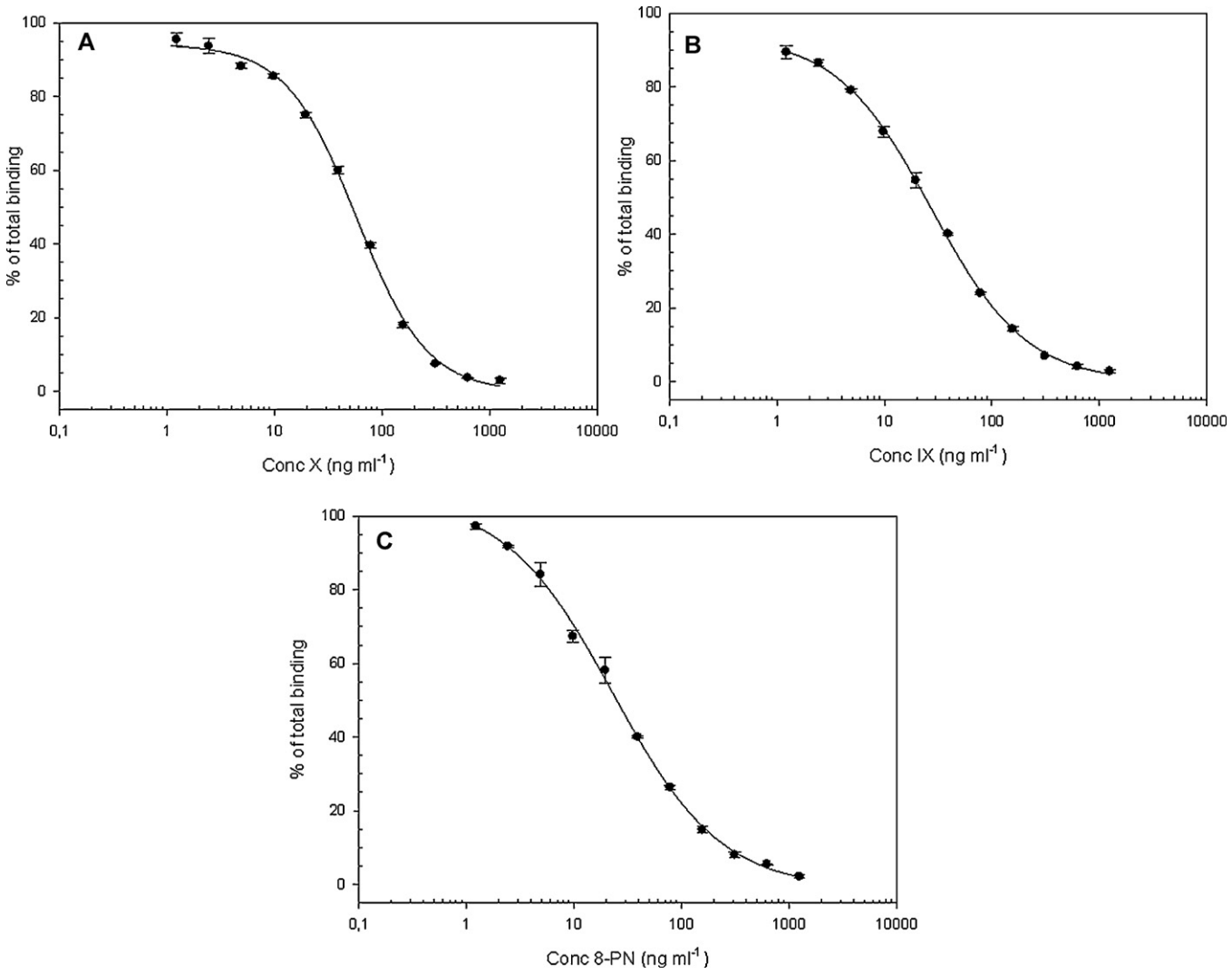

Fig. 2. (A) Standard curve for xanthohumol (X) obtained with the optimized ELISA, means based on 15 replicate calibration curves ($n = 15$) on three different days (± standard deviation). (B) Standard curve for isoxanthohumol (IX) obtained with the optimized ELISA, means based on 15 replicate calibration curves ($n = 15$) on three different days (± standard deviation). (C) Standard curve for 8-prenylnaringenin (8-PN) obtained with the optimized ELISA, means based on 15 replicate calibration curves ($n = 15$) on three different days (± standard deviation).

gested extraction method offers a simple sample preparation with satisfying recoveries of all three analytes at physiological relevant levels.

3.4. Matrix effects

The influence of certain matrix components in urine and serum was assessed, because they may interfere with the affinity of the antibody for its antigen. Our main goal was to develop a short and easy ELISA and therefore the possibility of measurement of unprocessed urine or serum samples was investigated. Blank urine and serum were serially diluted in PBS and a constant amount of antibody was added. Then, the changes in maximal absorbance were monitored (Table 4). For anti-X, in urine a minimum dilution factor of 10 was required to obtain at least 80% of the maximum signal and in serum at least a 100-fold dilution is required. For IX and 8-PN in serum, the effect of matrix components on the maximum absorbance was so large that at least 50-fold dilutions are recommended. In urine, the influence of the matrix on the binding capacity of anti-IX and anti-8-PN was smaller. As a result, we concluded that the influence of matrix components on the performance of our ELISA was too large and variable. Therefore, for the rest of the validation an extraction of urine and serum samples prior to our ELISA was recommended. The matrix effect was then also assessed at three QC levels, by comparison of spiked samples after extraction (100% recovery) and the spikes in assay buffer. As shown in Table 5, the extraction step with diethyl ether substantially improves the assay performance. In urine, matrix effects gave an average underestimation of the final concentrations of 9.6–13.2% for X, whereas for IX and for 8-PN matrix effects resulted in an underestimation of 0.8% to an overestimation of 9.6%. As expected, in serum, the underestimation of the final concentra-

Table 5
Matrix effects of extracted urine and serum samples on assay performance at three QC levels.

ME (%)	X	IX	8-PN
Urine			
Low	86.91	86.91	99.23
Medium	86.82	86.82	104.73
High	90.45	90.45	109.61
Serum			
Low	93.92	78.10	86.58
Medium	84.49	84.05	81.43
High	81.84	82.95	80.24

Table 6
Inter- and intra-assay variation (%CV) in urine and serum at three QC levels ($n = 3$).

	X	IX	8-PN
Urine			
Inter-assay			
Low	1.8	5.2	9.5
Medium	3.0	8.8	7.0
High	2.2	4.7	5.0
Intra-assay			
Low	1.7	3.0	0.7
Medium	2.1	1.3	0.4
High	1.9	2.9	1.1
Serum			
Inter-assay			
Low	14.7	4.7	12.3
Medium	7.6	7.0	2.7
High	4.4	6.7	3.4
Intra-assay			
Low	1.9	1.9	2.9
Medium	0.5	1.3	1.9
High	1.7	1.5	2.5

tions was slightly higher than in urine, and ranged for X from 6.1% to 18.2%, for IX from 16.0% to 21.9% and for 8-PN from 13.4% to 19.8%.

3.5. Precision and accuracy

Three quality control samples of X, IX and 8-PN in urine and serum were analyzed twice in three replicates in the same competitive assay on the same day, as well as in identical assays that were performed on three consecutive days and the inter- and intra-assay variations were calculated as %CV (Table 6). For urine and serum, the inter-assay variation was overall lower than 10%, except at the low level of X and 8-PN in serum, where the variation was 14.7% and 12.3%, respectively. The coefficients of variation were below 5% for the intra-assay variation, both in urine and serum and for all three QC levels. Certain factors, like evaporation of the solvents at the edge of the wells, non-homogeneity of the temperature or slight changes in the preparation of buffers and solutions, can cause these variations. Therefore, the use of daily calibration curves is highly recommended. Furthermore, accuracy was assessed as the percentage relative error and never exceeded 15%, both in urine and in serum (Table 7). In conclusion, the obtained results for the accuracy and precision assessment met the acceptance criteria in analytical validation guidelines [27], with emphasis on important issues described by Findlay and co-workers [28].

Table 7
Accuracy (%RE) in urine and serum at three QC levels ($n = 3$).

	Urine			Serum		
	X	IX	8-PN	X	IX	8-PN
Low	9.2	14.1	11.9	4.9	2.3	10.8
Medium	7.8	0.1	0.1	11.6	2.9	2.8
High	1.7	1.7	3.2	7.5	6.3	3.2

3.6. Correlation with LC–MS method

We determined the correlation between the competitive ELISA and an established and validated LC–MS method [29] by applying both methods on urine samples from a dietary intervention trial described in detail by Bolca et al. [12] and with Ethical Approval from the Ethics Committee of the Ghent University Hospital (EC UZG 2007/199; Belgian registration number: B67020072203; initial recruitment date: October 15, 2007). We selected 18 urine samples of subjects (both male and female, generally healthy individuals) who ingested with meals a hop-based supplement (MenoHop, Metagenics Europe): 1.20 ± 0.04 mg IX, 0.10 ± 0.01 mg 8-PN, 0.09 ± 0.01 mg 6-PN, and 2.04 ± 0.06 mg X per capsule) three times a day during 5 days, after a run-in phase of at least 4 days. On the last day of the intervention spot urine samples were collected. The urine samples were extracted and hydrolyzed as described in section 2.12 and IX and 8-PN concentrations were measured with our competitive ELISA and compared with the results from LC–MS analyses. For the ELISA, values were based on four measurements with the final immunoassay and for LC–MS duplicate determinations were used. The correlations for both IX and 8-PN urinary concentrations were found to be highly significant: correlation coefficients were $r = 0.945$ ($P < 0.01$; $n = 18$) and $r = 0.860$ ($P < 0.01$; $n = 18$) for IX and 8-PN, respectively (Fig. 3A and B). The correlation of urinary xanthohumol concentrations was not included in this study, because, first of all, the levels in the patient samples were too low to get detected with our developed ELISA. Secondly, it has been reported before that xanthohumol has a very poor bioavailability [11,30] and therefore this ELISA will not be directly applicable in the clinical field. Furthermore, serum correlations were also not investigated, because the amount of serum from the study was limited and the spiking of pooled blank serum would not have given an added value to our study.

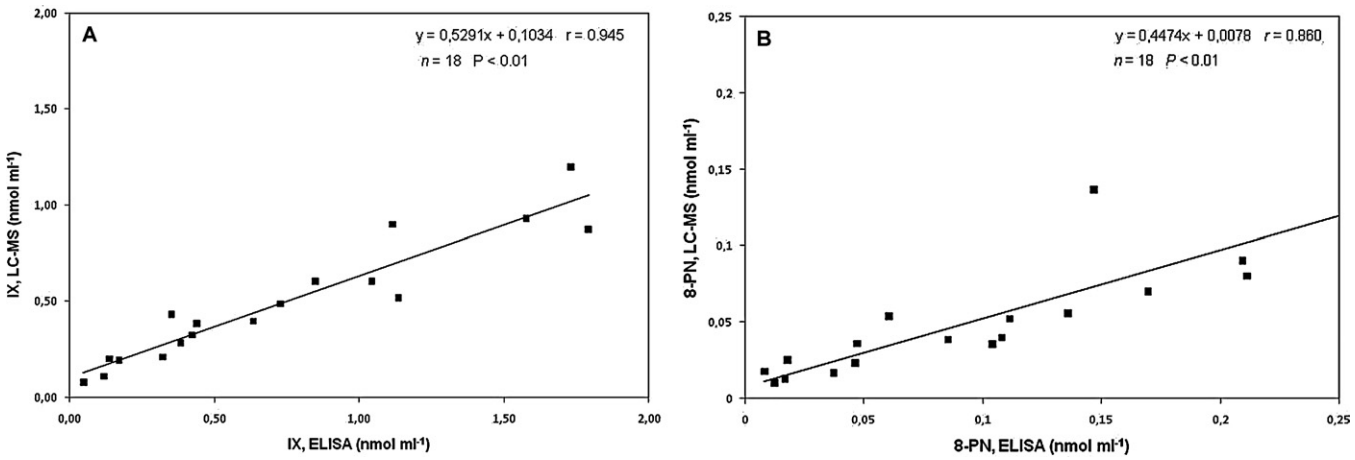

Fig. 3. (A) Correlation graph of significant linear correlation (Pearson's correlation coefficient) between urinary IX concentrations measured with the validated LC–MS method and the competitive ELISA. (B) Correlation graph of significant linear correlation (Pearson's correlation coefficient) between urinary 8-PN concentrations measured with the validated LC–MS method and the competitive ELISA.

Table 8
Comparative table of indirect competitive ELISA and LC/APCI–MS method.

Parameter	Indirect competitive ELISA	LC/APCI–MS method
Sample prep (after hydrolysis of conjugates)	Urine and serum: LLE	Urine: LLE (diethyl ether) Serum: SPE (C18)
LOD	X: 14.5 ng/mL IX: 5.6 ng/mL 8-PN: 4.4 ng/mL	Urine/serum: X: 0.6/1.4 ng/mL IX: 0.2/1.5 ng/mL 8-PN: 0.4/1.5 ng/mL
LLOQ	X: 41.0 ng/mL IX: 13.9 ng/mL 8-PN: 17.1 ng/mL	Urine/serum: X: 1.9/4.8 ng/mL IX: 0.8/5.0 ng/mL 8-PN: 1.2/4.8 ng/mL
Accuracy (%RE) (mean of three QC levels)	Urine/serum: X: 6.2/8.0 IX: 5.3/3.2 8-PN: 5.1/5.6	Urine/serum: X: 10.4/5.1 IX: 3.3/10.6 8-PN: 10.1/7.4
Precision (%CV) (mean of three QC levels)	Inter-assay (urine/serum): X: 2.3/8.9 IX: 6.2/6.1 8-PN: 7.2/6.1 Intra-assay (urine/serum): X: 1.9/1.4 IX: 2.4/1.6 8-PN: 0.7/2.4	Inter-assay (urine/serum): X: 16.3/10.1 IX: 10.7/12.4 8-PN: 14.4/9.7 Intra-assay (urine/serum): X: 7.1/5.2 IX: 5.2/5.6 8-PN: 6.0/3.9

4. Conclusion

In conclusion, three monoclonal antibodies were successfully developed against the hop-derived prenylflavonoids X, IX and 8-PN. They were integrated in an indirect competitive ELISA, which was validated for the measurement of the respective compounds in urine and serum samples. This ELISA technique requires a short sample preparation that consists of enzymatic hydrolysis and subsequent solvent extraction with diethyl ether, to reduce matrix effects and avoid potential over- or underestimation. The sensitivity of this assay, although acceptable, is not so high as with our established LC–MS method, as shown in a comparative table (Table 8), but can be further improved by changing the type of immunoassay. In addition, all three antibodies showed no substantial cross-reactivity with related compounds and both accuracy and precision of the measurements were within the criteria of acceptance. Therefore, this ELISA may serve as a reliable, simple and cost effective tool and as a promising alternative for chromatographic methods in intervention trials that require the assessment of the exposure to hop-derived prenylflavonoids. Additionally, these monoclonal antibodies may be valuable for other applications such as time-resolved fluoroimmunoassay, immunocyto- and histochemistry to reveal molecular mechanisms of X, IX and 8-PN and the subcellular localization and tissue distribution of these compounds.

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