Tryptophan-containing milk protein-derived dipeptides inhibit xanthine oxidase

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Abstract

Of twelve dipeptides tested, only the Trp containing peptides Val-Trp and its reverse peptide Trp-Val showed a xanthine oxidase (XO) inhibitory activity. Studies with Val and Trp revealed that XO inhibition was mainly attributed to the Trp residue. No significant difference ($P \geq 0.05$) was found for the XO inhibitory potency (IC50) values for Trp, Val-Trp and Trp-Val, which were about 200 times higher than that for Allopurinol. Lineweaver and Burke analysis demonstrated that Trp, Val-Trp and Trp-Val were non-competitive inhibitors while Allopurinol was a competitive inhibitor. Of the different milk-protein substrates hydrolyzed with gastro-intestinal enzyme activities, only lactoferrin (LF) hydrolyzates displayed XO inhibition. Peptides present in a LF hydrolyzate (GLF-240 min) were adsorbed onto activated carbon followed by subsequent desorption with stepwise elution using acetonitrile (ACN). Separation and detection of Trp containing peptides within the different fractions were achieved using RP-HPLC coupled with fluorescence detection. The desorbed fractions displayed different XO inhibitory properties, with no inhibition in the unbound fraction and highest inhibition in fractions eluted with 30, 40 and 70 % ACN. The fraction eluting at 40 % ACN was significantly more potent (19.1 ± 2.3 % inhibition at 1.25 mg mL$^{-1}$) than the GLF-240 min hydrolyzate (13.4 ± 0.4 % inhibition at 1.25 mg mL$^{-1}$), showing the potential for enrichment of the bioactive peptides on fractionation with activated carbon.

Key words: Xanthine oxidase inhibition, tryptophan, bioactive peptides, lactoferrin, activated carbon, antioxidant
1. Introduction

Cardiovascular and renal conditions have been reported as secondary diseases in individuals suffering from type 2 diabetes and insulin resistance syndrome [32, 38]. A strong link between hyperuricemia, and the development of atherosclerosis, hypertension and insulin resistance has been reported, and uric acid has been described as a marker of both cardiovascular and renal disease risk [25, 38]. In vivo, uric acid is produced following oxidation of the aldehyde groups in xanthine or hypoxanthine by XO [68]. This reaction also generates superoxide radicals, which are responsible for the production of other compounds such as H$_2$O$_2$, hydroxyl and peroxyl radicals [87]. The generation of oxidants in endothelial cells can result in endothelial injury. Although uric acid is considered as an antioxidant in the early stages of atherosclerosis, it can become a pro-oxidant at a later stage in the progression of this condition. This change from anti- to pro-oxidant activity occurring in an oxidative environment depleted in antioxidants has been described as the urate redox shuttle [38]. Different synthetic XO inhibitors have been developed to control the formation of uric acid in vivo [68]. 1,5-Dihydro-4H-pyrazolo[3,4-d]pyrimidin-4-one (Allopurinol), an allosteric XO inhibitor, is used in the clinical management of hyperuricemia and gout [68]. Despite Allopurinol’s potency, adverse side effects involving oxypurinol have been reported [87]. These include the development of fever, rash and Allopurinol hypersensitivity syndrome which can be fatal [5, 50, 68]. Therefore, the development of natural XO inhibitors has been proposed as a means of regulating XO activity in vivo. In order to reinforce endogenous antioxidant defense systems, supplementation of anti-oxidants through dietary intake has been proposed to counteract oxidative stress [33, 83]. Excess of dietary purine rich foods has been associated with an increased risk of gout [44]. However, Choi et al. [10] suggested that the consumption of moderate amounts of purine rich vegetables and proteins was not associated with an increased risk in gout. Changes in the diet have been suggested as a means of reducing the incidence of gout in humans. These include consumption of fruits, vegetables and low-fat dairy products and a decrease in meat, seafood and beer intake [44]. Different natural compounds, mainly plant extracts have been used for their XO inhibitory properties [50, 65, 79, 85, 90, 92]. The most common natural XO inhibitors described in the literature are flavonoids, which have a relatively wide range of IC$_{50}$ values ranging between 0.75 and >40.00 μM [87]. Milk proteins and milk-derived peptides have been associated with health promoting and disease risk reducing activities, notably in the modulation of diverse conditions associated with the metabolic syndrome. These include the management of cardiovascular disease with angiotensin converting enzyme (ACE) inhibitory, antithrombotic, anti-inflammatory, mineral binding and
anti-oxidant peptide activities [6, 12, 23, 49]. Milk proteins, peptide sequences encrypted within the primary structures of the milk proteins and amino acids have also been associated with the regulation of postprandial glycaemia and insulin secretion in normoglycaemic and type 2 diabetic subjects [24, 26, 56, 57, 61, 72]. Different studies have demonstrated the anti-oxidative role of milk proteins and peptides [19, 53, 70, 75, 83]. A fraction from human milk enriched in Trp has been identified for its high scavenging activity in vitro [84]. However, when evaluated in a Caco-2 cell culture, this fraction or Trp itself behaved as a pro-oxidant [20]. It has been reported that whey protein hydrolyzates can help decrease oxidative stress by their radical scavenging activity and by their ability to increase the production of antioxidant enzymes in vivo [40, 54, 70]. Similarly, caseins and casein-derived peptides have been associated with radical-scavenging properties in vitro [8, 27, 47, 83]. Antioxidant activity of sodium casein hydrolyzates has been demonstrated in cell culture of human Jurkat cells with an increase in cellular catalase activity and different effects on GSH depending on the hydrolyzate studied [69]. Milk and dairy products have also been associated with the prevention of gout in humans as they can reduce serum urate [10, 11, 17]. Various fractions of milk have been tested in a cell culture model of THP-1 cells for their anti-inflammatory properties [16]. Glycomacropeptide was shown to inhibit Interleukin 1β gene and protein expression of THP-1 cells and a milk fat extract (G600) could reduce the production of IL8 in response to monosodium urate monohydrate crystals [16]. These in vitro findings were confirmed in an in vivo mice model of gout where feeding with GMP and G600 resulted in an anti-inflammatory effect [16]. Recently, Dalbeth et al. [15] showed that ingestion of skim milk powder enriched with G600 and GMP could reduce the frequency of gout flares with patients. As Trp shows structural similarities with Allopurinol, this amino acid appeared like an interesting candidate as a potential natural inhibitor of XO. The aim of this study was to explore the potential of Trp and Trp containing dipeptides to inhibit XO. Other non Trp containing dipeptides were also screened to their XO inhibitory potential. Milk proteins are a good source of Trp. Therefore, the enzymatic release of Trp and Trp containing peptides was carried out using different milk protein substrates and XO inhibition by milk protein hydrolyzates was also investigated.

2. Materials and methods

2.2. Reagents

The synthetic dipeptides Val-Trp, Trp-Val, Ala-Leu, Asp-Lys, Glu-Lys, Gly-Leu, Ser-Leu and Val-Ala were obtained from Bachem (Bubendorf, Switzerland) while Phe-Leu, His-Leu and Gly-Gln, Trp and Val were obtained from Sigma-Aldrich (Dublin, Ireland). Sodium phosphate
monobasic, sodium phosphate dibasic, ethylenediamine tetraacetic acid (EDTA), hydroxylamine phosphate, xanthine, Allopurinol, bovine xanthine oxidase (0.1-0.4 units/mg protein), sodium phosphate monobasic dihydrate, sodium phosphate dibasic dihydrate, sodium dodecyl sulfate (SDS), leucine, trifluoroacetic acid (TFA) and activated carbon were obtained from Sigma Aldrich (Dublin, Ireland). 2,4,6-Trinitrobenzenesulfonic acid (TNBS) was obtained from Pierce Biotechnology (Medical Supply, Dublin, Ireland). Hydrochloric acid, sodium hydroxide, HPLC grade water and acetonitrile (ACN) were from VWR (Dublin, Ireland). Different milk protein substrates were hydrolyzed including: whey protein isolate (WPI - 88.3 % (w/w) protein, Carbery Milk Products, Ballineen, Ireland) and its associated 0.2 μm retentate (WPI Ret – 68.1 % (w/w) protein, Carbery Milk Products, Ballineen, Ireland), sodium caseinate (NaCN – 90.4 % (w/w) protein, Kerry Ingredients, Lisowel, Ireland), and Lactoferrin (LF - 87.0 % (w/w) protein) obtained from a commercial supplier. Enzymatic hydrolysis of LF was carried out with two food-grade commercial preparation of pancreatic (P - PTN 6.0S, Novozymes, Bagsvaerd, Denmark) and gastric (G - BC pepsin, Biocatalysts, Wales, UK) enzyme activities.

2.2. Methods

2.2.1. Hydrolysis of milk protein substrates

Two different food-grade gastro-intestinal enzymes: gastric enzyme preparation (G - BC pepsin) and a pancreatic enzyme preparation (P - PTN 6.0S) which have been identified for their ability to release Trp or Trp containing dipeptides were used to carry out hydrolysis of lactoferrin (LF). The starting LF substrate was suspended at 10 % (w/w) on a protein basis in water and dispersed under agitation at 50°C for 1 h using an overhead stirrer (Heidolph RZR 1, Germany). In the first instance, LF was hydrolyzed using PTN 6.0S with an enzyme to substrate ratio (E:S) of 2 % (w/w), where hydrolysis was conducted at 50°C, pH7.0 and hydrolyzate samples (PLF) were withdrawn at different time intervals (60, 120, 180 and 240 min). Secondly, LF was hydrolyzed at 50°C with BC pepsin for 240 min (GLF-240min) at an E:S of 3 % (w/w) , pH 3.0 followed by a 4 h hydrolysis with PTN 6.0S (E:S of 2 % (w/w), pH 7.0). Hydrolyzate samples (GPLF) were withdrawn at 60, 120, 180 and 240 min. The protein solutions were adjusted to pH 3.0 on hydrolysis with G and to pH 7.0 on hydrolysis with P using aqueous solutions of HCl 10.0 N or NaOH 2.0 N, respectively. A control sample was removed from the protein dispersion and maintained at 50°C for the duration of the hydrolysis reaction. Hydrolysis was carried out at a constant pH of 3.0 or 7.0 using a pH Stat (Titramo 843, Tiamo 1.4 Metrohm, Dublin, Ireland). The enzyme was inactivated by heat treatment of the hydrolyzate samples at 90°C for 20 min. Hydrolyzates were freeze-dried (FreeZone 18L, Labconco, Kansas City, U.S.A.) and stored at -
20°C until further analysis.

2.2.2. Determination of the degree of hydrolysis

The degree of hydrolysis (DH) was determined using the TNBS (trinitrobenzenesulfonic acid) method following the procedure of Adler-Nissen [4] as modified by Spellman et al. [80]. A 5 % (w/v) TNBS in methanol solution was diluted in 0.2125 M sodium phosphate buffer, pH 8.2 to a concentration of 0.1 % (w/v). The test hydrolyzate samples were diluted in 1 % (w/v) aqueous SDS solution. Sample aliquots were analyzed in triplicate. Test tubes were incubated at 50°C for 60 min in the dark. The reaction was stopped by the addition of 0.1 M HCl. Absorbance values were measured at 340 nm (Shimadzu UV mini 1240, Kyoto, Japan) which allowed determination of the DH using the following formula:

\[
DH = 100 \times \frac{(AN_2 - AN_1)}{Npb}
\]

With AN$_1$, the amino nitrogen content of the unhydrolyzed protein (mg g$^{-1}$ protein), AN$_2$, the amino nitrogen content of the hydrolyzate (mg g$^{-1}$ protein) and Npb the nitrogen content of the peptide bonds in the protein substrate (123.3 for whey proteins and 112.1 for casein).

2.2.3. Reversed-phase ultra-performance liquid chromatography of LF hydrolyzates

LF and LF hydrolyzates were analyzed by liquid chromatography using an ultra-performance liquid chromatograph (UPLC Acquity - Waters, Dublin, Ireland). Solvent A was 0.1 % (v/v) TFA in HPLC grade water and solvent B was 0.1 % (v/v) TFA in 80 % HPLC grade ACN. Freeze-dried hydrolyzate samples were resuspended in mobile phase A at 0.5 % (w/v) and filtered through 0.2 μm cellulose acetate syringe filters (Millipore, Carrigtwohill, Ireland). Separation of the peptides and individual milk proteins was carried out at 30°C, using a 2.1 x 100 mm, 1.7 μm Acquity UPLC C18 BEH column mounted with a 0.2 μm inline filter (Waters, Dublin, Ireland). The flow rate was set at 0.3 mL min$^{-1}$. Peptides and proteins were eluted using a linear gradient: 0–0.28 min: 100 % A; 0.28–45 min: 100–20 % A; 45–46 min: 20-0 % A; 46–48 min 0 % A; 48-49 min: 0-100 % A; 49-51 min 100 % A. The absorbance of the eluent was monitored at 214 nm.

2.2.4. Fractionation of LF hydrolyzates with activated carbon

Activated carbon was used to extract hydrophobic peptides [51] from the LF hydrolyzates (GLF-240min). A custom-made solid-phase extraction (SPE) cartridge containing activated carbon was
produced by placing 10 g activated carbon in a 50 mL syringe (13 × 3 cm I.D. - Phenomenex, Cheshire, UK) fitted with frits at both ends to retain the activated carbon. The freeze-dried lactoferrin hydrolyzate (GLF-240min) was redispersed at 5% (w/v) in HPLC-grade water. A volume of 20 mL of this solution was slowly applied at the top of the syringe containing the activated carbon in order to allow adsorption of the peptides. The activated carbon was washed with water to remove any unbound material. Peptides were then eluted stepwise using 10, 20, 30, 40, 50, 60, 70 and 100 % (v/v) ACN as described by Herraiz and Casal [41]. The different fractions were dried in a solvent evaporator (Genevac, EZ-2 Plus, Genevac Ltd., Ipswich, UK).

2.2.5. Fluorescence detection of tryptophan

The detection of Trp and Trp-containing residues was carried out following Kema et al. [45] by HPLC and fluorescence detection (2475 multi λ fluo detector, Waters, Dublin, Ireland) of indole groups at excitation and emission wavelengths of 280 and 340 nm, respectively. Peptide separation in the GLF-240 min hydrolyzate was carried out using a Jupiter C18 column (250 x 4.6 mm I.D., 5.0 µm particle size) attached to a C18 (4 x 3 mm I.D.) guard column (Phenomenex, Cheshire, UK). Hydrolyzate samples were resuspended in mobile phase A at 0.5 % (w/v) and filtered through 0.2 µm cellulose acetate syringe filters (Millipore, Carrigtwohill, Ireland). The flow rate was set at 1 mL min⁻¹ and the gradient was: 0–4 min: 100 % A; 4–59 min: 100–20 % A; 59–60 min: 20-0 % A; 60–70 min 0 % A; 70-75 min: 0-100 % A; 75-90 min 100 % A.

2.2.6. Xanthine oxidase inhibition assay

The test samples (dipeptides and hydrolyzates) were dispersed in HPLC grade water at concentrations ranging from 1.25×10⁻³ to 12.5 mg mL⁻¹. The XO inhibition assay was carried out following the method of Noro et al. [65]. The test samples (50 µL) were pipetted on a 96 well microplate (Sarstedt, Dublin, Ireland) containing EDTA (final concentration 12.5 µM ), hydroxylamine phosphate (final concentration 25 µM) and xanthine (final concentration 0.125 mM). The reaction was initiated by adding 50 µL of XO (0.1 U mL⁻¹). Each sample was analyzed in quadruplicate. The microplate was incubated at 37 °C for 30 min in a microplate reader (Biotek Synergy HT, Winoosky, VT, USA), absorbance of the uric acid formed was monitored at 290 nm. The XO IC₅₀ values (concentration of active compound required to observe 50 % XO inhibition) were determined by plotting the percentage inhibition as a function of the concentration of test compound. The mode of inhibition of the different compounds (Allopurinol, Trp, Trp-Val and Val-Trp) was investigated using Lineweaver and Burk analysis [65]. This was
achieved by measuring the initial rate of the reaction at different xanthine concentrations between 0.0125 and 0.1250 mM without inhibitors and in the presence of inhibitors (Allopurinol, Trp, Trp-Val and Val-Trp) at their IC50 concentrations. Km and Vmax values were deducted from the double reciprocal plots [79].

2.2.7. Statistical analysis

Means comparison was carried out with a Student Newman-Keuls test using SPSS (version 9, SPSS Inc., Chicago, IL, USA) at a significance level $P < 0.05$. For the Lineweaver and Burke plots, experimental data were fitted by linear regression with SPSS.

3. Results

3.1. Xanthine oxidase inhibitory activity of dipeptides

The amino acid Trp presents structural similarities with Allopurinol, a potent XO inhibitor. For this reason, two Trp containing dipeptides (Val-Trp and its reverse peptide Trp-Val) were studied for their ability to inhibit XO. The XO inhibitory potential of ten other dipeptides was also studied. Of these twelve dipeptides, eight sequences (Table 1) could be released from bovine milk proteins following the hydrolytic action of different gastrointestinal enzymes (pepsin, trypsin and chymotrypsin activities) as determined using the Peptide Cutter program [21]. Of the twelve dipeptides, two (Val-Trp: 37.2 ± 2.2 % and Trp-Val: 36.7 ± 11.2 %) showed significant XO inhibitory properties (Table 1). In order to better understand the origin of the inhibitory activity, the individual amino acids present in those two dipeptides were also evaluated for XO inhibitory activity (Table 1). Val showed a very low level of inhibition (5.3 ± 2.8 %). In contrast, Trp inhibited XO by 70.3 ± 1.1 % at a concentration 0.25 mg mL⁻¹. Inhibition curves as a function of Trp, Val-Trp, Trp-Val or Allopurinol (positive control) concentration are shown in Fig. 1. The IC50 for Allopurinol (5.5 ± 0.7 μM) was around 200 times lower than that of Trp, Val-Val or Trp-Val. The IC50 value for Allopurinol found herein was of the same order, 2.4 μM, as previously reported in the literature [42]. The IC50 values for Trp and the two dipeptides (Val-Trp and Trp-Val) were not significantly different ($P ≥ 0.05$). Lineweaver and Burk analysis was used to determine the mode of inhibition by Allopurinol, Trp, Val-Trp and Trp-Val (Fig. 2). The double reciprocal plots allowed determination of Km and Vmax values in the presence or absence of inhibitors. Statistical analysis showed that there was no significant difference in Vmax ($P ≥ 0.05$) without inhibitors (0.033 ± 0.012 Abs units min⁻¹) and in the presence of Allopurinol (0.031 ±
0.017 Abs units min⁻¹), whereas Vmax differences were observed for Trp (0.014 ± 0.004 Abs units min⁻¹), Val-Trp (0.015 ± 0.006 Abs units min⁻¹) and Trp-Val (0.017 ± 0.003 Abs units min⁻¹). Km values were not significantly different (P ≥ 0.05) without inhibitor (0.024 ± 0.033 mM) and with Trp (0.014 ± 0.014 mM), Val-Trp (0.022 ± 0.009 mM) and Trp-Val (0.015 ± 0.017 mM) whereas significant differences were seen in the presence of Allopurinol (0.143 ± 0.031 mM). These results suggest different types of inhibition with Allopurinol being a competitive inhibitor, whereas Trp, Val-Trp and Trp-Val behaved like non-competitive inhibitors of XO.

3.2. XO inhibition of the milk protein hydrolyzates and physicochemical characteristics of LF hydrolyzates

The release of Trp and Trp containing peptides from different milk protein substrates was carried out by enzymatic hydrolysis. Fifteen milk protein hydrolyzates generated with food-grade gastrointestinal enzyme preparations using three milk protein substrates (WPI, WPI Ret and NaCN) were initially evaluated. None of the 15 hydrolyzates tested inhibited XO (data not shown), suggesting that complex mixtures of milk proteins did not allow the generation of potent hydrolyzates which inhibit XO. A more targeted approach was therefore followed using LF, a Trp rich starting substrate, which was hydrolyzed with two food-grade enzyme preparations, i.e., with gastric (G) and/or pancreatic activities (P). In addition, the dipeptide Val-Trp can in theory be released from LF when hydrolyzed with gastrointestinal enzyme activities [21]. During the initial screen, all LF hydrolyzates showed similar XO inhibition values when tested at 12.5 mg mL⁻¹, apart from unhydrolyzed LF (Table 3) which had low XO inhibitory activity. The physicochemical characteristics of LF hydrolyzates were studied by determination of degree of hydrolysis (Fig. 3) and by RP-UPLC (Fig. 4). For the PLF series, the DH obtained on 1 h of hydrolysis was 21.4 ± 1.5 %, and increased to 26.3 ± 1.2 % after 4 h hydrolysis (Fig. 3). In contrast, a relatively low DH (5.2 ± 0.3 %) was obtained when LF was hydrolyzed with the gastric enzyme G (GLF-240 min). DH significantly increased to 13.9 ± 1.3 % after 1 h hydrolysis for GLF-240 min with the pancreatic enzyme P. Surprisingly, the DH obtained with a combination of enzymes G and P (GPLF series) was lower than that of hydrolyzates generated with enzyme P alone (PLF series). In agreement with the relatively high DH, LF (Fig. 4a) was substantially hydrolyzed in PLF-60 min (Fig. 4b). Very similar profiles were obtained for PLF-60 min (Fig. 4b) and PLF-240 min (Fig. 4c). The peptide profiles of GLF-240 min hydrolyzate (Fig. 4d) and GPLF-240 min (Fig. 4e) were significantly different as expected due to differences in the cleavage specificity of the enzymes used.
3.3. XO inhibition by LF hydrolyzate fractions

Hydrolyzate GLF-240 min was fractionated using activated carbon in order to selectively retain hydrophobic peptides [2, 51]. Elution from activated carbon with ethanol has been carried out after extraction of peptides from a casein hydrolyzate [1]. In our study, different acetonitrile concentrations were used to elute peptides adsorbed on the activated carbon as described previously with different commercial SPE cartridges by Herriaz and Casal [41]. The 9 fractions eluted from the activated carbon cartridge, including the unbound fraction, were all assayed at 1.25 mg mL\(^{-1}\) for XO inhibitory activity (Table 4). XO inhibition was not found with the unbound fraction. The highest XO inhibition was observed in the fraction eluted at 40 % ACN (19.1 ± 2.3 %), this was significantly higher inhibition (\(P < 0.05\)) than that of GLF-240 min hydrolyzate (13.4 ± 0.4 %).

Separation and detection of peptides with indole groups was carried out by RP-HPLC and fluorescence detection with excitation at 280 nm and emission at 340 nm [29, 45]. The chromatograms obtained for the most potent fractions (30, 40 and 70 % ACN) and GLF-240 min hydrolyzate are shown in Fig. 5. No peaks were detected in the unbound fractions and relatively small peaks could be seen on elution with up to 30 % ACN (data not shown). Peptide peaks eluting (between 25 and 30 min) in the same region as Trp, Val-Trp and Trp-Val were seen in the most potent fractions (Fig. 5). Surprisingly, comparison of the RP-HPLC profiles of GLF-240 min hydrolyzate and its fractions, revealed that a relatively large number of peaks were not eluted from activated carbon using stepwise desorption with ACN. Mass balance analysis of the extraction procedure showed that only 28.0 % (w/w) of GLF-240 min hydrolyzate material was recovered after elution with ACN.

4. Discussion

Consumption of low-fat dairy products has been associated with a reduced risk for developing gout [17, 44]. Ingestion of intact milk [18] or milk proteins [25] was shown to reduce serum urate concentration in an acute manner in healthy subjects. This was correlated with the low purine concentration of milk and an acute increase in the renal excretion of uric acid (uricosuric effect) and xanthine following the protein load [17, 25]. The acute uricosuric effect has been explained by multi-factorial parameters including a higher uric acid clearance with serum amino acid concentration increase and the concomitant clearance of urea and uric acid [25]. These different studies have demonstrated the positive effects of milk and particularly milk proteins on the regulation of uric acid in humans. Very little work has been carried out on the effect of milk protein hydrolyzates on the management of gout. Garell et al. [25] have shown that uric acid
clearance was greater following the consumption of lactalbumin compared to caseins. This has been linked with the faster rate of digestion of lactalbumin compared to caseins, yielding an increase in serum urea and branched chain amino acids. These results suggest that the hydrolysis of milk proteins with gastro-intestinal enzymes may help reducing high uric acid concentrations in the serum of humans. It has been suggested that depending on their concentration, milk proteins could act either as activators or inhibitors of milk XO [43]. Little or no data is available in the literature describing XO inhibition by peptides and food protein hydrolyzates. To our knowledge, no other mention of Trp, Val-Trp, Trp-Val and lactoferrin hydrolyzates as potential inhibitors of XO has previously been reported in the literature. Nevertheless, these compounds have been associated with other bioactive properties. Trp has been described for its central role as a precursor of biomolecules such as melatonin, serotonin and dynorphin [22, 39] and for its antioxidant properties [48, 60]. Similarly, Val-Trp has been associated with various bioactive properties including angiotensin-converting enzyme (ACE) inhibition [6, 37, 64, 67, 74, 89, 91, 93, 94] and for α-glucosidase inhibition [58, 66]. The reverse peptide Trp-Val has also been described as an ACE inhibitor [67] and an anti-oxidant [31]. The amino acid Val did not show XO inhibition in contrast to Trp (Table 1). These results demonstrate that XO inhibition by the dipeptides was mainly due to the Trp residue. A Trp rich containing fraction from human mother’s milk has previously been described for its antioxidant properties in vitro [84]. Certain amino acid structures have been correlated with high antioxidant properties. These include amino acids with sulfur residues (Cys and Met) and aromatic structures (Trp, Tyr, His and Phe) [19, 48, 60, 73, 75]. Phenol and indole groups in amino acids can act as potential hydrogen donors [70, 73, 75]. The radicals formed with phenol and indole amino acids are relatively stable, allowing inhibition of oxidation propagation [73]. Of the twelve dipeptides studied, only peptides containing a Trp residue displayed XO inhibitory activity (Table 1). Phe and His containing dipeptides did not inhibit XO activity, suggesting that antioxidant activity may not directly correlate with XO inhibitory properties. Comparison of the molecular structure of Trp, Allopurinol and xanthine reveals many similarities between those 3 compounds all of which possess a C6 and a C5 ring structure. Inhibitors of XO have been found notably in the class of purine analogs eventhough a xanthine-like structure has not necessarily been correlated with high inhibitory activity [68]. Indeed, a non-purine competitive XO inhibitor such as FYX-051 has been reported as a more potent inhibitory drug than Allopurinol [59]. Allopurinol was around 200 times more potent than Trp, Val–Trp or Trp-Val (Table 2). Similar differences in potency have been reported when comparing drugs to dietary peptides. For ACE inhibition, IC₅₀ values of milk-derived peptides have been shown to be a hundred fold greater than Captopril [46, 63]. There was no significant difference (P ≥ 0.05) between the IC₅₀ (µM)
observed for Trp, Val-Trp and Trp-Val, indicating that the individual amino acid Trp had the same potency as the dipeptides (Table 2). In contrast, other studies showed differences in bioactivities for those two dipeptides with Val-Trp having an IC\textsubscript{50} value 200 times less than Trp-Val for ACE inhibition [67]. This was explained by the fact that Val-Trp and Trp-Val had a different mechanism of ACE inhibition, with Val-Trp being a non-competitive and Trp-Val a competitive inhibitor of ACE [67]. Similarly, Suetsuna and Chen [82] found that the antioxidant activity of dipeptides depended on the C or N terminal position of certain amino acid residues and they reported a stronger antioxidant activity for Trp-Lys compared to Lys-Trp. Our results showed no effect of the position of the Trp residue in the dipeptide on XO inhibition, suggesting that the N or C-terminal location of Trp does not affect its bioactivity. Amino acids have been reported to display a lower anti-oxidant activity compared to peptides as they have a lower radical scavenging activity [19]. In contrast, we found that Trp displayed a similar XO IC\textsubscript{50} (\mu M) to the dipeptides Val-Trp and its reverse peptide Trp-Val. In order to further interpret the XO inhibition observed with Trp, Val-Trp and Trp-Val, Lineweaver and Burk analysis was carried out in the absence (control) and presence of inhibitors (Allopurinol, Trp, Val Trp or Trp Val) (Fig. 2). The Km and Vmax values extrapolated from Lineweaver and Burk plots were determined. Allopurinol has been reported as a competitive inhibitor of XO [68, 85]. Trp, Trp-Val and Val-Trp are non-competitive inhibitors. Different polyphenolic compounds have also been reported as non-competitive inhibitors of XO [79, 85, 86]. However, their precise mode of inhibition has yet to be fully determined [79]

No XO inhibition was observed with hydrolyzates of complex protein substrates such as WPI, WPI Ret and NaCN. This may be due to the fact that even though the bioactive sequences are present within these protein substrates, their abundance may be too low to observe any bioactivity. For this reason, different individual milk protein substrates were studied to determine their Trp composition. Following analysis with Peptide Cutter [21], we found that Trp and a wide range of Trp containing peptide sequences could be released from LF with gastro-intestinal enzymes, including the dipeptide Val-Trp. LF has been described as a multifunctional milk protein displaying a range of bioactivities. Intact and hydrolyzed lactoferrin have mainly been reported as antimicrobial [7, 9, 12, 28, 35, 52], anti-inflammatory [14, 28, 36] opioid antagonist [12] and antioxidant agents [71]. Intact LF did not inhibit XO (Table 3). In contrast to unhydrolyzed LF, LF hydrolyzates displayed a relatively high XO inhibition (> 80% - Table 3). Hydrolysis can affect bioactivity by releasing specific peptide sequences which were previously entrapped within the protein structure [19]. Free amino acids were quantified (Trp, Gln and Asn not determined) as per Mounier et al. [62]. Relatively low amounts of free amino acids were
released in the hydrolyzates with values of 0.22, 0.08 and 0.46 g/100g determined in PLF-240 min, GLF-240 min and GPLF-240 min, respectively. This result suggested that the LF hydrolyzates mainly contained peptides. The most abundant free amino acids released were Tyr, Phe, His, Lys and Arg. These amino acids could theoretically be released by gastrointestinal enzyme activities (ExPaSy) with high cleavage probability for the peptide bonds (> 70 %). In the LF sequence, the aforementioned amino acids, with the exception of His, were positioned less than 5 amino acids upstream or downstream of a Trp residue. Free Trp and Trp containing di- or tripeptides in the proximity of Tyr, Phe, Lys and Arg residues could theoretically be released from LF by gastrointestinal enzyme digestion (ExPaSy). For example, Trp, Gln-Trp or Trp-Gln-Trp may be released (probability of cleavage > 90 %) following the cleavage of Arg residues at positions 40 and 44. For the PLF hydrolyzate series, no improvement in XO inhibition could be seen beyond 1 h hydrolysis, suggesting that the bioactive peptides were released during the early stages of hydrolysis. This was supported by the fact that very little difference in the RP profiles of PLF-60 min and PLF-240 min could be seen (Fig. 4), suggesting that the peptide composition in both hydrolyzates was very similar. The DH values also showed that hydrolysis occurred to a limited extent between 1 and 4 h with a DH increase of 4.9 % within this time period (Fig. 3). Similarly, there was no significant difference (P ≥ 0.05) in XO inhibition between GLF-240 min and the GPLF hydrolyzate series (Table 3). These results suggest that the bioactive peptides were released during the first stage of hydrolysis following hydrolysis with enzyme G. In this case, there was no benefit in combining the two enzymes G and P in order to improve bioactive peptide release. The RP profiles of GLF-240 min and GPLF-240 min samples (Fig. 4) differed. This is in agreement with the different cleavage specificity of the enzyme activities present in enzymes G and P. Despite differences in the peptide profile and the DH, no difference in XO inhibition was seen (Table 3). Combination of the enzyme preparations G and P mimics gastrointestinal digestion. Interestingly, the GLF-240 min hydrolyzate did not lose its XO inhibitory properties following the hydrolytic action of enzyme P, suggesting that the bioactive peptides may be of a relatively low molecular mass, which further supports our hypothesis that Trp and Trp containing dipeptides within these hydrolyzates may be responsible for XO inhibition. Extrapolation of these in vitro results to in vivo may suggest that bioactive components in the GLF-240 min hydrolyzate would have the potential to survive gastro-intestinal digestion. Comparison of the DH values for PLF and GPLF series revealed unexpected results, with DH values of the GPLF series being significantly lower than that of the PLF (Fig. 3). Pre-digestion of LF with enzyme G should in theory have allowed a better access for enzyme P to the peptide bonds, resulting in higher DH values. A possible explanation for the lower DH values observed in the GPLF series may come from an inhibition of enzyme P by peptides present in the GLF-240
min hydrolyzate. Inhibition of lactic acid bacteria proteolytic enzymes by milk-derived peptides has been described previously [81]. Furthermore, sodium caseinate hydrolyzates have been shown to inhibit trypsin activity [34].

Trp is characterized as a relatively hydrophobic amino acid, therefore, isolation of hydrophobic peptides from GLF-240 min hydrolyzate was attempted. It has been reported that activated carbon could selectively adsorb Trp and Phe amino acids or peptides containing these residues during debittering of milk protein hydrolyzates [13]. Activated carbon has been successfully used for the removal of Phe from food-protein hydrolyzates, notably to develop low-Phe content foods for phenylketonuric patients [55, 77]. Other uses of activated carbon have been to selectively prepare dipeptide mixtures with a high Fischer ratio (branched chain amino acids/ aromatic amino acids) with the low Fisher ratio peptides being adsorbed on the activated carbon [2]. Activated carbon therefore appeared like an interesting adsorbent for the binding of hydrophobic components of the GLF-240 min hydrolyzate. The specific fluorescence detection of indole groups [45] was utilized in order to selectively detect Trp containing peptides in these fractions.

Binding of the GLF-240 min components to the activated carbon cartridge was achieved. Surprisingly, the extraction procedure with activated carbon and ACN elution only allowed recovery of 28.0 % (w/w) of GLF-240 min hydrolyzate, suggesting strong binding on the activated carbon. Only 4 % (w/w) of the GLF-240 min hydrolyzate was found in the unbound fraction. In contrast with previous results where peptides from a casein hydrolyzate were extracted with activated carbon, a yield of nearly 30 % (at pH 6.5) was obtained in the unbound fraction [1]. These differences may arise from the particle size of the activated carbon used, with smaller particle size resulting in a larger surface area of the adsorbent and a higher adsorption capacity [78]. The different fractions collected displayed XO inhibition, with highest activity seen in the 30, 40 and 70 % ACN fraction (Table 4). The fact that bioactivity was found in several fractions suggests that the bioactivity arises from different compounds present in this hydrolyzate. This is further reinforced by the differences seen in the RP-HPLC profile of the fractions (Fig. 5). Some of the peptide peaks seen in the 30, 40 and 70 % ACN eluted within the same time interval as Trp, Val-Trp and Trp-Val (Fig. 5), which may indicate that the bioactivity within these fractions comes from one or a combination of these compounds. The different hydrolyzates (PLF-240 min, GLF-240 min and GPLF-240 min) were spiked with Trp, Val-Trp and Trp-Val and increase in peaks corresponding to the retention times of these compounds was seen (data not shown), reinforcing the assumption that these components were responsible for the XO inhibition. XO inhibition in the 40 % ACN fraction was higher than that of GLF-240 min hydrolyzate, suggesting that a more potent fraction has been isolated with activated carbon. Further characterization of the fractions is needed to elucidate the identity of the bioactive...
peptides.

Previous studies have demonstrated the antioxidant potential of Trp and a Trp rich fraction from human mother’s milk *in vitro* [84]. However, these results did not translate in a similar effect in a cell culture model of Caco 2 cells, showing a prooxidant activity of Trp and the Trp rich fraction which was dose-dependent [20]. The pro-oxidant effect has been attributed to intracellular oxidation products coming from the interaction of oxidized Trp with other cellular components [20]. However the mechanism of Trp described in this paper is different as it involves inhibition of an enzyme and not scavenging of free-radicals. Additional confirmation of *in vivo* XO inhibition with Trp, Val-Trp, Trp-Val and LF hydrolyzates is needed [84]. Food-grade hydrolyzates and dipeptides could have potential as a natural alternative to drugs for XO inhibition. In addition, side effects associated with drugs such as Allopurinol should not occur with food-derived protein hydrolyzates. It has been demonstrated that protein breakdown following enzyme hydrolysis results in a greater increase in plasma amino acids and di-peptides compared to unhydrolyzed proteins *in vivo* [61, 88]. In addition, amino acid uptake decreases with hydrolyzates containing higher peptide chain length [30]. Nevertheless, di-peptide uptake has been shown to be greater than that of amino acids [3]. XO is found *in vivo* in the plasma and in different tissues including the liver, gut, lung, kidney, brain and heart [68]. Therefore, bioactive components with XO inhibitory potential must be bioavailable to display their activity *in vivo*. Inhibition of XO by dipeptides is therefore highly relevant in this regard. In addition, Trp and Trp containing dipeptides are relatively hydrophobic in nature, which should allow them to permeate through the intestinal layer. Indeed, hydrophobic peptides have been associated with a high trans-epithelial permeation through a caco-2 cell monolayer [76]. Hydrolysis with a combination of gastric and pancreatic enzyme activities showed that the LF hydrolyzates were still bioactive, suggesting that these compounds may survive gastro-intestinal digestion and possibly reach the target organs in the body.
Acknowledgements

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Conflicts of interests

The authors declare that they have no conflict of interest.
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Table captions

**Table 1** Xanthine oxidase (XO) inhibitory activity of milk protein derived dipeptides and amino acids when tested at 0.25 mg mL\(^{-1}\).

**Table 2** Inhibitory concentration inducing 50 % inhibition (IC\(_{50}\)) for Xanthine oxidase with Allopurinol, milk derived dipeptides (Val-Trp and Trp-Val) and Trp.

**Table 3** Xanthine oxidase inhibitory activity of lactoferrin (LF) and lactoferrin hydrolyzates when tested at 12.5 mg mL\(^{-1}\).

**Table 4** Xanthine oxidase inhibitory activity of lactoferrin (LF) hydrolyzed with BC pepsin (preparation G) for 240 min (GLF- 240 min) and fractions obtained on elution from activated carbon with different acetonitrile (ACN) concentrations. All fractions were tested at 1.25 mg mL\(^{-1}\).
Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>XO inhibition (%)(^a)</th>
<th>Fragments released by \textit{in silico} gastrointestinal hydrolysis of milk protein substrates(^b)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly-Leu</td>
<td>2.2 ± 2.3</td>
<td>(\kappa)-cas f(39-40), (\beta)-Lg (f9-10), (\alpha)-La f(51-52), BSA f(27-28), LF f(15-16), LF f(491-492)</td>
<td></td>
</tr>
<tr>
<td>Ala-Leu</td>
<td>-5.1 ± 2.6</td>
<td>(\alpha_{s2})-cas f(81-82), (\alpha_{s2})-cas f(175-176), (\beta)-Lg f(139-140), (\alpha)-La f(109-110), BSA f(215-216), BSA f(411-412), BSA f(533-534), BSA f(587-588), LF f(61-62), LF f(635-636)</td>
<td></td>
</tr>
<tr>
<td>Ser-Leu</td>
<td>-13.8 ± 2.8</td>
<td>(\beta)-cas f(57-58), (\beta)-cas f(124-125), (\beta)-cas f(164-165), (\beta)-(\beta)-Lg f(21-22), (\beta)-Lg f(116-117), BSA f(434-435), BSA f(459-460), LF f(469-470)</td>
<td></td>
</tr>
<tr>
<td>Ser-Phe</td>
<td>-13.6 ± 2.8</td>
<td>(\kappa)-cas f(104-105), (\beta)-Lg f(150-151), LF f(304-305)</td>
<td></td>
</tr>
<tr>
<td>Asp-Lys</td>
<td>-7.4 ± 3.9</td>
<td>(\beta)-Lg f(137-138), (\alpha)-La f(97-98), BSA f(380-381), LF f(472-473), LF f(532-533)</td>
<td></td>
</tr>
<tr>
<td>Glu-Lys</td>
<td>-4.1 ± 3.6</td>
<td>(\beta)-Lg f(134-135)</td>
<td></td>
</tr>
<tr>
<td>Val-Ala</td>
<td>-19.4 ± 2.2</td>
<td>BSA f(557-558)</td>
<td></td>
</tr>
<tr>
<td>Val-Trp</td>
<td>37.2 ± 2.2</td>
<td>LF f(567-568)</td>
<td></td>
</tr>
<tr>
<td>Phe-Leu</td>
<td>0.2 ± 3.8</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>His-Leu</td>
<td>5.9 ± 2.1</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Gly-Gln</td>
<td>6.6 ± 5.7</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Trp-Val</td>
<td>36.7 ± 11.2</td>
<td>na</td>
<td></td>
</tr>
<tr>
<td>Val</td>
<td>5.3 ± 2.8</td>
<td>(\kappa)-cas (31), (\beta)-Lg (92, 94), BSA (29, 194), LF (83, 403)</td>
<td></td>
</tr>
<tr>
<td>Trp</td>
<td>70.3 ± 1.1</td>
<td>(\beta)-Lg (61), (\alpha)-La (104), BSA (140), LF (27, 41), (\alpha_{s1})-cas (199), (\alpha_{s2})-cas (109, 193),</td>
<td></td>
</tr>
</tbody>
</table>
\( ^a \) Values represent mean ± confidence interval \((P = 0.05)\) for quadruplicate determination

\( ^b \) Prediction of dipeptide fragments released by gastro-intestinal digestion of milk proteins using the Peptide Cutter program (ExPaSy)

na: not applicable
Table 2

<table>
<thead>
<tr>
<th>Compound</th>
<th>XO IC$_{50}$ (µM)*</th>
<th>XO IC$_{50}$ (µg mL$^{-1}$)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Allopurinol</td>
<td>5.5 ± 0.1$^a$</td>
<td>0.75 ± 0.01$^a$</td>
</tr>
<tr>
<td>Trp</td>
<td>1259.8 ± 84.5$^b$</td>
<td>257.27 ± 17.26$^b$</td>
</tr>
<tr>
<td>Val-Trp</td>
<td>1195.7 ± 131.5$^b$</td>
<td>362.77 ± 39.89$^c$</td>
</tr>
<tr>
<td>Trp-Val</td>
<td>1301.7 ± 336.7$^b$</td>
<td>394.94 ± 102.15$^c$</td>
</tr>
<tr>
<td>PLF-240 min</td>
<td>na</td>
<td>4339.21 ± 325.35$^d$</td>
</tr>
<tr>
<td>GPLF-240 min</td>
<td>na</td>
<td>5238.28 ± 2058.73$^d$</td>
</tr>
</tbody>
</table>

*Values represent mean IC$_{50}$ values ± confidence interval (P = 0.05) for quadruplicate determination. Values with different superscript letters are significantly different (P < 0.05). na: not applicable
<table>
<thead>
<tr>
<th>Hydrolyzate</th>
<th>XO inhibition (%)&lt;sup&gt;#{}&lt;/sup&gt;</th>
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<tr>
<td>LF</td>
<td>20.9 ± 23.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLF-60 min</td>
<td>90.4 ± 3.8&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLF-120 min</td>
<td>88.0 ± 4.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLF-180 min</td>
<td>87.7 ± 5.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>PLF-240 min</td>
<td>89.9 ± 1.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GLF-240 min</td>
<td>83.8 ± 3.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPLF-60 min</td>
<td>85.3 ± 3.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPLF-120 min</td>
<td>88.5 ± 3.4&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPLF-180 min</td>
<td>88.2 ± 2.9&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>GPLF-240 min</td>
<td>84.6 ± 2.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>PLF: LF hydrolyzed with pancreatic preparation P; GLF-240 min: LF hydrolyzed with gastric preparation G for 240 min; GPLF: GLF-240 min hydrolyzed with pancreatic enzyme P

<sup>b</sup>Mean values ± confidence interval (P = 0.05) for quadruplicate determination. Values with different superscript letters are significantly different (P < 0.05).
**Table 4**

<table>
<thead>
<tr>
<th>Fraction</th>
<th>XO inhibition (%) *</th>
</tr>
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<tbody>
<tr>
<td>unbound</td>
<td>-0.5 ± 1.7 a</td>
</tr>
<tr>
<td>10 % ACN</td>
<td>5.5 ± 1.5 b</td>
</tr>
<tr>
<td>20 % ACN</td>
<td>7.4 ± 2.2 b, c</td>
</tr>
<tr>
<td>30 % ACN</td>
<td>10.5 ± 2.4 b, c</td>
</tr>
<tr>
<td>40 % ACN</td>
<td>19.1 ± 2.3 d</td>
</tr>
<tr>
<td>50 % ACN</td>
<td>4.1 ± 1.6 b</td>
</tr>
<tr>
<td>60 % ACN</td>
<td>5.9 ± 2.0 b</td>
</tr>
<tr>
<td>70 % ACN</td>
<td>13.0 ± 0.5 c</td>
</tr>
<tr>
<td>100 % ACN</td>
<td>7.4 ± 0.1 b, c</td>
</tr>
<tr>
<td>GLF-240 min</td>
<td>13.4 ± 0.4 c</td>
</tr>
<tr>
<td>Allopurinol 0.003 mg mL⁻¹</td>
<td>45.7 ± 5.4 e</td>
</tr>
</tbody>
</table>

*Mean values ± confidence interval (P = 0.05) for six determinations. Values with different superscript letters are significantly different (P < 0.05)
Figure captions

Fig. 1 Xanthine oxidase inhibition as a function of log_{10}(inhibitor concentration) (a) Allopurinol, (b) Trp (c) Val-Trp and (d) Trp-Val. Values represent mean ± confidence interval ($P = 0.05$)

Fig. 2 Lineweaver and Burke plots of xanthine oxidase inhibitors with (a) Allopurinol, (b) Trp and (c) Val-Trp and Trp-Val. Each point represents the average of 6 values

Fig. 3 Degree of hydrolysis (DH) of lactoferrin (LF) hydrolyzates as a function of digestion time (mean of 3 values ± confidence interval ($P = 0.05$)). PLF: LF hydrolyzed with pancreatic enzyme P; GLF-240 min: LF hydrolyzed with gastric enzyme G for 240min; GPLF: GLF-240 min hydrolyzed with pancreatic enzyme P. Values with different letters are significantly different ($P < 0.05$).

Fig. 4 Reversed-phase ultra-performance liquid chromatographic (RP-UPLC) profile of lactoferrin (LF) hydrolyzates, dipeptides (Val-Trp and Trp Val) and Trp. (a) unhydrolyzed Lactoferrin (b) PLF-60 min (c) PLF-240 min (d) GLF-240min (e) GPLF-240 min (f) overlay of Trp, Val-Trp and Trp-Val

Fig. 5 Reversed-phase high-performance liquid chromatographic (RP-HPLC) profiles with fluorescence detection of LF hydrolyzed with gastric enzyme G for 240min (GLF-240 min) and associated fractions, dipeptides (Val-Trp and Trp-Val) and Trp. (a) GLF-240min (b) fraction eluted with 30 % ACN (c) fraction eluted with 40 % ACN (d) fraction eluted with 70 % ACN from the activated carbon cartridge and (e) overlay of Trp, Trp-Val and Val-Trp
Fig. 1
Fig. 3
Fig. 4
Fig. 5

(a) Relative fluorescence units vs. retention time (min)

(b) Relative fluorescence units vs. retention time (min)

(c) Relative fluorescence units vs. retention time (min)

(d) Relative fluorescence units vs. retention time (min)

(e) Relative fluorescence units vs. retention time (min)

1 - Trp
2 - Trp-Val
3 - Val-Trp
Supplementary data

The figures below represent the UPLC profile of the different hydrolysates obtained at 240 min. For clarity, only the region of interest (retention time between 0 and 12 min) has been displayed. The UPLC profiles of the hydrolysate and the hydrolysate spiked with Trp, Val-Trp or Trp-Val were overlaid to show where these compounds may elute.
GPLF-240 min + Trp

- Hydrolyzate
- Hydrolyzate spiked

GPLF-240 min + Val-Trp

GPLF-240 min + Trp-Val

Retention time (min)