



HAL
open science

Identification, production and bioactivity of casein phosphopeptides – A review

Mathie Tenenbaum, Barbara Deracinois, Camille Dugardin, Aurélie Matéos, Audrey Romelard, Julie Auger, Audrey Boulier, Rozenn Ravallec, Christophe Flahaut, Benoit Cudennec

► To cite this version:

Mathie Tenenbaum, Barbara Deracinois, Camille Dugardin, Aurélie Matéos, Audrey Romelard, et al.. Identification, production and bioactivity of casein phosphopeptides – A review. *Food Research International*, 2022, 157, pp.111360. 10.1016/j.foodres.2022.111360 . hal-03685317

HAL Id: hal-03685317

<https://univ-artois.hal.science/hal-03685317>

Submitted on 22 Jul 2024

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.



Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Identification, production and bioactivity of casein phosphopeptides – a review

Mathie Tenenbaum¹, Barbara Deracinois¹, Camille Dugardin¹, Aurélie Matéos¹, Audrey Romelard², Julie Auger², Audrey Boulier², Rozenn Ravallec¹, Christophe Flahaut¹ and Benoit Cudennec¹

¹UMR Transfrontalière BioEcoAgro N° 1158, Univ. Lille, INRAE, Univ. Liège, UPJV, YNCREA, Univ. Artois, Univ. Littoral Côte d'Opale, ICV – Institut Charles Viollette, F-59000 Lille, France

²Ingredia S.A. 51 Av. Lobbedez - CS 60946, 62033 Arras Cedex - France

* CORRESPONDING AUTHOR FOOTNOTE:

E-mail address: mathie.tenenbaum@univ-lille.fr ; benoit.cudennec@univ-lille.fr

Postal address: UMR-t 1158 Bâtiment SN2, Cité Scientifique - 59655 Villeneuve d'Ascq, France

Keywords: casein phosphopeptide, bioactive peptide, analytical characterization, peptide released, mineral-chelating peptide, mineral bioavailability

Abbreviations:

Collision-induced dissociation; CID
Casein Phosphopeptide; CPP
Electron transfer induced dissociation; ETD
Electrospray ionization; ESI
Gastrointestinal; GI
Mass spectrometry; MS
Matrix-assisted laser desorption/ionisation ; MALDI
Post-translational modifications; PTMs
Reverse phase-high pressure liquid chromatography; RP-HPLC
Simulated gastrointestinal digestion; SGID
Trifluoroacetic acid; TFA

Abstract

Milk and dairy products are significant sources of proteins and peptides impacting human health. In this way, the interest in CPPs, bioactive phosphorylated peptides resulting from the hydrolysis of caseins, has grown in the past years. CPPs were mainly studied for their capacity to chelate and increase the bioavailability of essential minerals involved in multiple physiological processes. Moreover, CPPs harbour interesting antioxidant and anti-inflammatory properties. Recent *in vivo* and *in vitro* studies demonstrated that these different roles are strongly linked to the intrinsic properties of CPPs and CPP concentrate preparations.

This review first comments on the different methods of CPP analytical characterization, focusing on recent techniques. Then, the CPP release occurring during the gastrointestinal digestion was reviewed, followed by the different CPP obtention processes and their impact on their physicochemical characteristics. Finally, the different bioactive roles attributed to CPPs, including mineral chelating properties, are discussed.

We show that CPPs have a promising role in treating various pathologies, notably to compensate for deficiencies in certain nutrients and an anti-oxidant and anti-inflammatory role. Nevertheless, the mechanisms by which CPPs exert their role remain to be elucidated, and this requires precise characterization of CPPs.

This work highlights the key parameters to be considered to study and produce CPPs and the different ways to be investigated in the future to elucidate their roles *in vivo* and characterize their potential for human health.

Introduction

Dairy proteins have been widely characterized for their benefits on human health by impacting cardiovascular, digestive, immune, and nervous systems (Muro Urista et al., 2011). Milk proteins are divided into two fractions: caseins and whey proteins. In ruminant milk, caseins represent 80% (w/v) of the total milk protein content and exist in four forms (α_{S1} -, α_{S2} -, β -, and κ -casein), encoded by four different genes and their different variants, which bind together to form micelles (Głąb & Boratyński, 2017). The amino acid sequence alignment and the comparison of phosphorylation sites of goat, sheep, and cow caseins show only a few differences, but casein micelles differ in composition and molecular mass between ruminant dairy species (Ginger & Grigor, 1999; Roy et al., 2020). However, the amounts of the different four forms vary between species; bovine milk caseins are composed of equal amounts of β -casein and α_{S1} -casein, which is superior to α_{S2} -casein, and κ -casein amounts, whereas buffalo and camel milk contain more β -casein than $\alpha_{S1/2}$ -caseins and κ -casein (Roy et al., 2020).

Regarding the variation between the different casein forms, there is little homology in the amino acid sequences, phosphorus, and carbohydrate content, but they are all amphiphilic. Intramolecular bonds do not stabilize these proteins, and their flexibility facilitates their digestion and hydrolysis (Głąb & Boratyński, 2017). In other words, caseins are random coil proteins without spatially well-organized secondary and tertiary structures, which confer them a significant susceptibility to proteolytic cleavages. Casein phosphopeptides (CPPs) have been identified from caseins hydrolysates obtained *in vitro* with enzymatic hydrolysis, during *in vitro* and *in vivo* gastrointestinal (GI) digestions, and during milk and cheese fermentations (Pinto et al., 2012).

No physiological role is assigned explicitly to caseins themselves, but the biological activities of peptides released during their GI digestion have been subjected to numerous studies. Indeed, peptides encrypted in the native proteins are inactive, but their release during hydrolysis leads to bioactive peptides (Muro Urista et al., 2011). Following the ingestion and GI digestion of caseins, CPPs could interact with the intestinal epithelium or cross the gut barrier to exert their bioactivities (Miquel, Ángel Gómez, et al., 2006). CPPs are a group of bioactive peptides involved in numerous biological activities and worldwide used as nutritional supplements. The presence of a phosphate group on serine (pSer) or threonine (pThr) residues characterizes CPPs, but no phosphotyrosine (pTyr) has been experimentally detected in the bovine caseins (Ginger & Grigor, 1999). The principal sources of CPPs are the α_{S1} -, α_{S2} - and β -casein, which contain in the cow 9, 12, 5 phosphoserine residues in their native form, respectively (Ginger & Grigor, 1999). The κ -casein, which contains three phosphoserine residues and one phosphothreonine residue (Huang et al., 2014), leads to

fewer released CPPs (Holt, 1992). Note that, instead of other dairy animal species (Matéos et al., 2010), the occupancy of the phosphorylation sites is total (100%) in bovine and, therefore, each CPP contains one or more phosphate groups, called mono-, di-, tri-, quadri- or quinti-phosphopeptides.

CPPs are attractive bioactive peptides due to their physicochemical properties. Their stability at acid pH during digestion and their good solubility confer them growing interest. Moreover, since these phosphorylated peptides are anionic, they chelate interesting nutritional minerals such as calcium and iron (FitzGerald, 1998). However, other bioactivities of interest have been recently attributed to CPPs.

CPP-enriched products are commercially available and widely studied for their beneficial effects. In addition, numerous studies are currently conducted to demonstrate their potential as functional food ingredients to prevent diseases such as osteoporosis and anaemia (Ahn & Je, 2019; Delshadian et al., 2018).

The present review focuses first on the different analytical methods used to, qualitatively and quantitatively, characterize CPPs. Then, the heterogeneity of CPPs obtained from caseins with the different established methods, going from GI digestion to *in vitro* hydrolysis of caseins, is reviewed. Next, their mineral chelating capacities focusing on calcium and iron and their impacts on health, their antioxidant, and other biological effects are discussed.

2. Analytical characterization of CPPs: qualitative and quantitative aspects

The lactating mammary gland synthesizes and secretes large amounts of phosphoproteins that are mainly associated with the casein fraction of milk. The biosynthesis of casein occurs in two steps: synthesis of the polypeptide chain, followed by phosphate addition to the nascent casein by an ATP-dependent protein kinase localized in the Golgi apparatus. The inorganic phosphate of blood serves as building materials for casein, and the final product appears in milk as a colloidal-sized particle, the casein micelle (Bingham et al., 1972; Bingham & Farrell, 1977). The casein micelle has a spherical appearance with a hairy appearance on the surface (Dalglish et al., 2004). Cellular phosphorylation of amino acids is a post-translational modification (PTM), an enzymatically-mediated etherification reaction, with H₂O elimination, between a hydroxyl group of phosphate and a lateral hydroxyl group of only three amino acids: serine (to form pSer or pS), threonine (to form pThr or pT) and tyrosine (to form pTyr or pY). The bovine milk caseins do not contain phosphotyrosine. They contain mainly phosphoserine residues and a few phosphothreonine residues (Ginger & Grigor, 1999).

Specific phosphorylation of caseins in the mammary gland is required to obtain the physicochemical properties of casein micelle. Indeed, the phosphate content of k-casein must be low to form a stable casein micelle (Yoshikawa et al., 1981). However, even if divalent ions such as Mg²⁺, Ca²⁺, or Mn²⁺ stimulate the protein-kinase activity involved in the addition of phosphate, the cellular mechanisms, the cellular rules, and the regulatory mechanisms that control the phosphate addition on the nascent protein remain largely unknown. Nevertheless, *in vitro* experiments of dephosphorylation-rephosphorylation of κ -casein versus κ -lactalbumin have demonstrated the impact of the protein folding on the rephosphorylation yield of proteins (Bingham et al., 1988). Therefore, the neighbouring amino acids may be essential in determining which serine/threonine residues in casein are phosphorylated.

Today, bioinformatic prediction of phosphorylation site occupancy is a helpful tool to many international research teams involved in understanding cellular communication mechanisms, and NetPhos 3.1 Server is a reference bioinformatics tool in this field (Blom et al., 1999, 2004). Submission of the amino acid sequences of casein precursors to NetPhos 3.1 (access online, June 2021) returns a total number of 91 potential phosphorylation sites displaying a NetPhos 3.1 score superior to the theoretical threshold of 0.5 (Figure 1B) for both bovine caseins: 21 (14 pSer, 4 pThr and 3 pTyr) potential phosphorylation sites for α_{S1} -casein (CASA1), 29 (16 pSer, 10 pThr and 3 pTyr) for α_{S2} -casein (CASA2), 19 (12 pSer, 7 pThr and

0 pTyr) for β -casein (CASB) and 22 (12 pSer, 9 pThr and 1 pTyr) for κ -casein (CASK). Among all the potential phosphorylation sites, one phosphorylation site was predicted in the signal peptide of both α_{S1} - and β -caseins. Three potential phosphotyrosines are predicted from the α_{S1} - and α_{S2} -casein amino acid precursors, while zero and only one for the β - and κ -casein amino acid precursors, respectively. However, experimentally-reported phosphorylation sites or those suspected to be phosphorylated (by similarity with other species where experimentally-reported phosphorylation sites have been published (Huang et al., 2014; Imanishi et al., 2007; Kappeler et al., 1998; Larsen et al., 2005; Mercier et al., 1976; Minikiewicz et al., 1996; Ribadeau Dumas et al., 1972; Wu et al., 2005) and listed in UniprotKB), are only 29 (Figure 1B, red, bold, underlined and dash-underlined case). Therefore, close to 70% of potential phosphorylation sites predicted may be not biologically phosphorylated in reality. Note that the phosphorylation, deduced by similarity and reported in UniprotKB, of $^{187}\text{pSer}$ for κ -casein is not predicted by NetPhos 3.1.

In any case, due to their biological functions, accurate and complete characterization of PTMs, overall, but especially phosphorylation sites are of prime importance to understand the structure(s) - function(s) relationships of biomolecules. Prior to analytical, sample preparation is primordial to enrich/purify in phosphopeptides embedded in a mixture of non-phosphopeptides (Fíla & Honys, 2012; Qiu et al., 2020). The analytical methods of phosphorylation site characterization have not ceased to progress since their advent. However, two epochs, separated by the advent of mass spectrometry, stand out clearly. The analytical methods corresponding to these two epochs are reviewed below and referenced in table 1.

2.1. Conventional methods (old methods)

The structure of the micellar caseins, where phosphate and calcium form colloidal calcium phosphate bridges, has been historically used for assessing the CPP yields by calcic precipitation with additional divalent ions. In 1996, Adamson and Reynolds reported the characterization of CPPs by high-performance liquid chromatography (HPLC) following the addition of 10% calcium chloride (Adamson & Reynolds, 1996). A simple precipitation yield calculation can be used to estimate the rate of CPPs initially present in a sample. This precipitation technique is only an enrichment step, which gives it the advantage of being fast and inexpensive but the disadvantage of lacking precision as to the percentage of CPPs calculated. Today, the National Food Safety Standard of the People's Republic of China related to the food nutrition enhancers CPPs (GB 31617-2014) uses this principle with barium chloride for evaluating the percentage of CPPs. In 2012, Mirza et al. reported a novel method for phosphopeptide enrichment using lanthanide phosphate co-precipitation. They

demonstrated, by MALDI-TOF-MS analysis, that erbium ions were found to be most efficient for precipitating phosphopeptides (Mirza et al., 2012).

The chromatography principle is also a commonly used technique to pre-purify phosphopeptides. Different chromatographic stationary phases interact by non-covalent linkages with the phosphopeptides. For example, the hydroxyapatite stationary phase retains the phosphopeptides preferentially due to its strong interaction with phosphate (Fonslow et al., 2012; Mamone et al., 2010). The negative charge of the phosphate group can also be targeted by anion exchange chromatography (Ellegård et al., 1999; Hardman et al., 2019). Electrostatic repulsion-hydrophilic interaction chromatography (ERLIC) (Alpert et al., 2010), which is a subset of hydrophilic interaction chromatography (HILIC), has been used for characterizing the glycopeptides and phosphopeptides. Several comparative studies demonstrated that ERLIC is an unequivocally superior alternative since many more phosphopeptides identification is possible (Alpert et al., 2015; Tabang et al., 2021; Yeh et al., 2019). Further analysis of the heterogeneity of the precipitated or pre-purified CPPs can then be carried out by liquid chromatography conventional analysis such as HPLC coupled with UV detection (Adamson & Reynolds, 1995; McDonagh & FitzGerald, 1998). However, a lack of a specific detection method for phosphopeptides due to the UV-based detection mode (detecting all the phosphorylated and non-phosphorylated peptides) is the main drawback to evaluating phosphopeptide yields.

2.2. Modern methods

The identification, characterization, and quantification of phosphopeptides have gained significant advances from the advent of mass spectrometry (MS). However, due to close chemical structures, unmodified peptides and post-translationally modified peptides could harbour the same physicochemical features (molecular mass, net charge and hydrophobicity/hydrophilicity ranges). Fortunately, the atomic structure of phosphate confers a particular affinity for specific support, permitting phosphopeptides to bind. Overall, the phosphocaseins have served as phosphoprotein models to develop methodologic approaches already used today.

2.2.1 CPP ionization

The two main ionization sources in MS, used to characterize phosphopeptides have been, over the last decades, mainly the matrix-assisted laser desorption/ionization (MALDI) and the electrospray ionization (ESI). Today, ESI is the method of choice due to its fitting with molecular separation techniques, such as chromatography and capillary electrophoresis. However, the mass spectrometry analysis of purified mono- and multi-phosphorylated

peptides and negative than positive ionization modes have been reported through several analytical optimization studies both from MALDI (Annan & Carr, 1996; Asara & Allison, 1999; Hou et al., 2010; Jagannadham & Nagaraj, 2008; Kjellström & Jensen, 2004) and ESI (Boersema et al., 2009; Busman et al., 1996) sources. For example, MALDI analysis of phosphopeptides has been demonstrated to be better sensitive and efficient when the co-crystallization between the MALDI matrix and the sample analytes is performed using phosphoric acid as an acidifying agent when the analysis is performed in negative ionization mode (Kjellström & Jensen, 2004). In the same way, Hou *et al.* recently published their works on optimizing a MALDI matrix to enhance the detection of phosphopeptides from MALDI source (Hou et al., 2010). Concomitantly, the ESI analysis by infusion of phosphorylated peptides is optimized when the analyses are performed with phosphoric acid (0.1–1.0%) to the sample solution (Kim et al., 2004).

Although their physicochemical features, especially their net charge and molecular mass, are closed, the ionization efficiency of peptides and phosphopeptides in mixture favours peptides in positive ion detection mode (Zou et al., 2013). Their relatively low stoichiometry reinforces this suppression ionization phenomenon affecting the phosphopeptides, the whole peptidome analyzed, and their number of phosphorylation sites. In addition, the multi-phosphorylated peptides generally have a lower positive ionization efficiency than monophosphorylated ones: in other terms, the more the phosphorylation, the lower the ionization in the positive mode (Zou et al., 2013). However, it would be wrong to think that the charge inversion of ionization mode leads to reverse the suppression ionization mechanisms (Kawasue et al., 2021; Knight et al., 2003; Poot et al., 2006), which remained misunderstood.

Since the net charge of phosphate is a hurdle to phosphopeptide MS-based analysis, various authors suggested that phosphopeptide dephosphorylation could be a suitable strategy. Further, it will be possible to identify them through dephosphorylated/phosphorylated comparative analyses. Dephosphorylation can be performed chemically or enzymatically, and dephosphorylation is total or partial (Mann et al., 2002; Matéos et al., 2009; Steen & Mann, 2002). The chemical dephosphorylation of phosphopeptides is a beta-elimination mechanism (Steen & Mann, 2002) and is often combined with the addition of specific chemical groups by a Michael substitution mechanism (Mann et al., 2002; Steen & Mann, 2002). Evidently, the specific chemical groups that replace the phosphate at the original place of phosphorylation are chosen i) to enhance the MS-based detection/identification of these modified phosphopeptides and/or ii) to bring a binding specificity to enhance the phosphopeptide enrichment and numerous chemical groups have been reported (Furukawa

et al., 2011; McLachlin & Chait, 2003), including isobaric labelling and fluorinated derivatives (Linke et al., 2015).

In 2008, Tan *et al.* reported an original method based on the dephosphorylation activity of cerium oxide attributed to Lewis acid and nucleophile activation mechanisms (Tan et al., 2008). The dephosphorylation activity of cerium oxide (CeO₂), independent of the temperature, which occurs in 10 min, was evaluated by two standard phosphopeptides and enzymatic digests of phosphoprotein α -caseins and β -casein. This dephosphorylation property of CeO₂ has been recently used through a multiplexed quantitative approach using a metal-organic framework (MOF)-templated porous CeO₂ and a magnetite/Ceria codecorated titanoniobate nanosheets (Furukawa et al., 2011; Min et al., 2015). Concomitantly, enzymatic dephosphorylation enhances cleavage specificity compared to chemical methods and permits modulating the dephosphorylation degree to reach different yields. Enzymatic dephosphorylation is either performed in acid or basic conditions, and both of them can be performed consequently (Deracinois et al., 2021; Matéos et al., 2009; Wang et al., 2008). At the beginning of the 21st century, a chemical and combined enzymatic approach presented chemical modifications of pSer and pThr, transforming them into lysine analogues (aminoethylcysteine and β -methylaminoethylcysteine, respectively), which are then cleaved with a lysine-specific protease to map sites of phosphorylation (Knight et al., 2003).

2.2.2 MS/MS-fragmentation of CPPs

MS-based detection of phosphopeptides and the location of phosphate on the amino acid backbone of peptides gained for the advent of MS/MS fragmentation methods (Boersema et al., 2009; Mann et al., 2002; Potel et al., 2019). Whatever the ion source used (MALDI or ESI), the first MS experiments dedicated to phosphopeptide studies revealed a very abundant fragmentation process corresponding to the neutral loss of 98 or 80 Da (owing to H₃PO₄ or HPO₃ loss, respectively) at moderate collision offset potential (Annan & Carr, 1996; Beck et al., 2001; Busman et al., 1996). In the same way, MS/MS fragmentation methods using higher collision energies, such as collision-induced dissociation (CID), infrared multiphoton dissociation (IRMPD) and higher-energy collisional dissociation (HCD, even if this latter MS/MS fragmentation method generates less neutral loss), leads mainly to this neutral loss of 98 and 80 Da (Flora & Muddiman, 2001; Palumbo et al., 2008; Potel et al., 2019; Tholey et al., 1999; Y. Zhang et al., 2009) which can seriously hamper identification and phosphosite determination. Ultraviolet photodissociation (UVPD) has also been reported for characterizing phosphopeptides. The molecular fragmentation resulting from UVPD depends on the wavelength (157, 193, 220 and 266 nm) of photons used. The functionality of UVPD for sequencing both peptide-cations and -anions enabled monophosphorylated to

pentaphosphorylated peptide characterization. Recently, Robinson et al. concluded, through their comparative study using either HCD or UPVD, that UPVD could be combined with HCD to improve the overall depth of coverage compared to each method alone (Robinson, 2016). To obtain more sequence information from peptides showing an extensive neutral loss in their CID spectra, such as peptide backbone fragments revealing the amino acid sequence and locating the phosphate group, the use of a second step of activation (MS^3 and multistage activation) to activate the ions produced by the neutral loss, is a convenient solution mainly reported for extensive cellular phosphorylation studies (Boersema et al., 2009) but never reported for milk phosphopeptides. On the contrary, the electron driven dissociation methods (electron-capture dissociation (ECD) and electron-transfer induced dissociation (ETD)) induce more extensive fragmentation of the peptide backbone than the collision mentioned above methods, providing better sequence coverage without loss of phosphoric acid, phosphate or water illustrating its applicability for identification of phosphorylated residues (Molina et al., 2007; Thingholm et al., 2010). This method allows direct assignment of phosphorylation sites. ETD and CID combination has also been reported (Molina et al., 2007), and MS/MS fragmentation methods have been applied to study the phosphorylation of intact caseins (B. Chen et al., 2017; J. Chen et al., 2019).

Mass analysers and detectors' instrumental performances (resolution power, accuracy, sensitivity...) play a crucial role in characterizing the phosphopeptides. Therefore, very high-resolution mass spectrometers, based on the Fourier transform-ion cyclotron resonance (FT-ICR), have been reported to allow a quasi-entire characterization of phosphopeptides without the need for prior liquid chromatography separation (Paris et al., 2020).

2.2.3 CPP purification

As reported mainly, chromatography separation techniques enhance the identification yield of phosphopeptides, although the hydrophobicity-based co-elution of peptides and phosphopeptides leads to the same suppression ionization phenomena even if it occurs to a less extent. Today the phosphorylation state of abundant animal proteins such as milk caseins is not fully elucidated. Indeed, among all bovine milk caseins, 31 phosphorylation sites are referenced, based on the literature or by similarity with other animal species, in UniprotKB/Swiss-Prot (September 1, 2021): (α_{S1} -Casein : pS56, pS61, pS63, pS79, pS81, pS82, pS83, pS90 and pS130; α_{S2} -Casein: pS23, pS24, pS25, pS28, pS46, pS71, pS72, pS73, pS76, pS144, pS146, pS150 and pS158; β -Casein: pS30, pS32, pS33, pS34 and pS50; κ -Casein: pS148, pT166, pS170 and pS187 (by similarity for this latter). Thus, the most extensive study dedicated to the phosphorylation of bovine caseins is undoubtedly the study reported by Fang *et al.*. They investigated the variation in phosphorylation degree of

α_S -caseins among milk of individual cows and the relationships among different phosphorylation isoforms of α_S -caseins through the analysis of morning milk samples from 529 French Montbéliarde cows using liquid chromatography (LC) coupled with ESI-MS (Fang et al., 2016). Capillary electrophoresis was also evaluated for its capability to analyze phosphopeptides. Ultra-low flow capillary electrophoresis was reported to significantly improve the detection sensitivity of phosphopeptides in equal sample load and equal sample concentration conditions while providing complementary data to LC-MS (Heemskerk et al., 2012). Furthermore, Dong *et al.* reported the site-specific separation and detection of phosphopeptide isomers with pH-mediated stacking capillary electrophoresis coupled to ESI-MS (Dong et al., 2013).

The stoichiometry of phosphopeptides is low compared to peptides when they are in a mixture obtained by the hydrolysis of one or several proteins. Various approaches were developed for the phosphopeptide enrichment step based on the molecular affinity permitting phosphopeptide concentration. Metal affinity capture (MAC) coupled to ESI-MS/MS allows for selective detection and identification of phosphopeptides in a complex mixture (Blacken et al., 2006, 2008). Briefly, phosphopeptides are captured in solution as ternary complexes with Fe^{3+} or Ga^{3+} , electrosprayed as doubly or triply charged positive ions, and the gas-phase complexes uniformly dissociate to produce a common ion that is used as a specific marker in precursor ion scans. Concomitantly, several authors have reported the concentration of phosphopeptides using an immobilized version of the MAC method with different metal ions (TiO_2 , Ti^{4+} , Fe^{3+} , Ga^{3+} and Zr^{4+}) named immobilized metal ion affinity chromatography (IMAC) (Arribas Diez et al., 2021; Bian et al., 2014; Capriotti et al., 2018; Salimi et al., 2017; Zhu & FitzGerald, 2012). Concomitantly, Thingholm *et al.* demonstrated the efficiency of titanium dioxide (TiO_2) used with an optimized solution containing 2,5-dihydroxybenzoic acid (DHB) or phthalic acid solution, acetonitrile and trifluoroacetic acid (TFA) (Thingholm et al., 2008; Thingholm & Larsen, 2016) for the phosphopeptide enrichment. This advantageous property of metal ions (e.g. TiO_2) to interact with the phosphopeptides was also used in combination with graphene for the analysis of phosphopeptides by surface-enhanced laser desorption/ionization (SELDI) (Tang et al., 2012). Several evolutions of the metal-based capture of phosphopeptides have been reported in the following years, such as the metal oxide affinity chromatography (MOAC) (Kyono et al., 2008; Sugiyama et al., 2007) and the polymer-based metal-ion affinity capture (PolyMAC) (Iliuk et al., 2010; Tao et al., 2005). Recently, Rui *et al.* reported a new set of different metal ions (Ti^{4+} , Zr^{4+} , Fe^{3+} , Tb^{3+} , Tm^{3+} and Ho^{3+})-immobilized magnetic nanoparticles using tetrakis(4-carboxylphenyl) porphyrin ((TCPP)) and 1,4,7,10-tetraazacyclododecane N,N',N'',N'''-tetraacetic acid (DOTA) to form $Fe_3O_4@TCPP-DOTA-Tb$ or Ti nanoparticles to capture and enrich the phosphopeptides

(Zhai et al., 2015). A multitude of metal-functionalized materials, such as poly(glycidyl propargyl ether/divinyl benzene (poly(GPE/DVB); amphiphilic polyamidoamine (PAMAM); movable magnetic supraparticule (MSP); ATP-magnetic nanoparticule (MNPs); inorganic nanofiber; polydopamine (PDA) have been reported (He et al., 2022; Jiang et al., 2018; Krenkova et al., 2016; Ma et al., 2014; Saeed et al., 2013; L. Zhang et al., 2015). The last generation of functionalized materials with excellent phosphopeptide enrichment ability are bifunctional surfaces that trap both glycopeptides and phosphopeptides (L. Cao et al., 2020, p.; He et al., 2022; Lu et al., 2020).

However, for several methods, the metal ions cause the non-elution of strongly fixed multi-phosphopeptides and interferences, such as the metal contamination of chromatographic column and non-specific interactions with some peptides. To overcome these drawbacks, several authors have proposed solutions such as the sequential elution of phosphopeptides (Sun et al., 2014), the use of a phosphopeptide elution microgradient (Ondrej et al., 2020) and the use of additives, like ethylenediaminetetraacetic acid (EDTA), citrate or a tetraphosphorylated peptide: pSpSpSpS (Seidler et al., 2011), and lactic acid (Sugiyama et al., 2007). In the same way, methods that do not use metal ions are free of these interferences. In that respect, the two-dimensional LC-MS/MS using either strong cation exchange chromatography (Beausoleil et al., 2004) or mixed bed anion-cation exchange chromatography (Motoyama et al., 2007) in the first separation dimension and reverse phase-LC-MS/MS in the second one has also evidenced as an efficient approach for the detection and identification of phosphopeptides.

Finally, modern high-throughput and high content biological research are performed with advanced high-resolution instrumentation and complex, time-consuming protocols, which, as a whole, pose a challenge for routine implementation in analytical labs.

3. CPP formation

CPPs are endogenously present at a low concentration in milk. Some authors attribute this to the activity of different enzymes present in milk, like plasmin and cathepsin (Baum et al., 2013; Gaucher et al., 2008). However, as discussed in this part, CPPs are mainly produced during the different steps of GI digestion of caseins, during fermentation, or *in vitro* by enzymatic hydrolysis.

3.1. CPP release during GI digestion

3.1.1. *In vivo*

Investigating the release of CPPs *in vivo* during GI digestion is tricky due to endogenous proteins and their derived peptides in the different compartments of the GI tract. However,

this has already been investigated in rats, pigs, and humans (Bouzerzour et al., 2012; Chabance et al., 1998; Hirayama et al., 1992).

Thus, in piglets, peptide profiles obtained after infant formula at the different digestion steps revealed that caseins were massively hydrolyzed in the stomach, although peptides principally from β -casein were also found in the jejunum and only three peptides from β -casein in the ileum (Bouzerzour et al., 2012). Another study conducted in mini-pig showed hydrolysis kinetics of various milk matrices and identified different poly-phosphopeptides issued from α_{S1} , α_{S2} , β , κ -casein in duodenal effluents (Barbé et al., 2014).

CPP release during *in vivo* digestion has also been undertaken in humans. Indeed, in 1998, it was shown that CPPs were found in the stomach and the duodenum after milk and yoghurt ingestion (Chabance et al., 1998). A few years later, Meisel *et al.* showed that CPPs produced during bovine casein digestion can be found even down in the ileum (Meisel et al., 2003). More recently, it has been shown that CPPs were present in the jejunum after casein ingestion (Boutrou et al., 2013). Interestingly, a clinical study revealed that CPPs could pass the GI barrier as they were found in the plasma of different subjects after cheese ingestion, supporting the potential bioactive roles of CPPs (Caira et al., 2016).

3.1.2. *In vitro* simulated gastrointestinal digestion (SGID)

Research on peptides released during GI digestion can also be undertaken *in vitro* with simulated gastrointestinal digestion (SGID), which is currently used in food and nutritional sciences. In this way, static and semi-dynamic SGID protocols have been recently standardized, leading to a better knowledge of *in vivo* mechanisms (Brodkorb et al., 2019; Guerra et al., 2012; Ménard et al., 2014; Minekus et al., 2014). This method facilitates identifying the panel of CPP sequences that can be released during digestion.

In 2010, Picariello *et al.* investigated CPP release profile after SGID, using pepsin for the gastric phase and trypsin, chymotrypsin, elastase and carboxypeptidase for the intestinal phase. Results showed that caseins had been fully digested and led to the identification of CPPs, most from β -casein, which survived to the different steps of the digestive system (Picariello et al., 2010). Next, Miquel *et al.* investigated the CPP profiles after gastric and intestinal digestion phases using porcine pepsin and pancreatin, a mix of diverse enzymes including trypsin, amylase, lipase, ribonuclease, and other proteases. They found 19 CPPs principally from α_{S1} -casein and only four from α_{S2} , five from β -casein and none from κ -casein, which has only one phosphate group as illustrated in figure 1C (Miquel, Ángel Gómez, et al., 2006).

These *in vitro* investigations also highlighted the importance of the different digestive enzymes. Indeed, the comparison of the intestinal CPP profiles of digested Grana Padano cheese obtained with two different static SGID protocols using either trypsin, chymotrypsin and lipase or pancreatin during the intestinal digestion phase (Cattaneo et al., 2017) showed that the number of CPPs released with pancreatin was higher, emphasizing the role of all pancreatic enzymes in casein hydrolysis. In contrast, Cruz-Herta *et al.* found 44 and 37 CPPs after casein tryptic hydrolysis and SGID using a mix of pepsin and pancreatic enzymes, respectively. Furthermore, only a few differences were evidenced between the CPP profiles (Cruz-Huerta et al., 2015). The contradictory aspect of these outcomes could be attributed to the variable matrices and SGID parameters used in the different studies, making comparison difficult.

In these different works, the action of gastric and intestinal enzymes on CPP release has mainly been highlighted, but the action of the intestinal brush border membrane enzymes was not often considered. Among these enzymes, there are endopeptidases, aminopeptidases, carboxypeptidases, phosphatases, and the dipeptidyl peptidase-IV (Maestracci, 1976). Currently, only a few studies investigate the role of these enzymes on casein hydrolysis (Boutrou et al., 2010; Yeung et al., 2001). However, it has been shown that intestinal alkaline phosphatase, an enzyme present on the apical microvilli of brush border membrane, can dephosphorylate clustered phosphoserine residues present in whole casein suspension or isolated caseins. Moreover, this dephosphorylation could occur under the action of other digestive enzymes like pepsin and pancreatic enzymes (Li-Chan & Nakai, 1989). Another study confirms that phosphorylation sites and mineral binding seem to limit the hydrolysis of CPPs by intestinal brush border membrane enzymes (Boutrou et al., 2010). Picariello *et al.* showed that specific peptides released after SGID of caseins could resist an incubation step with intestinal brush border membrane enzymes (Picariello et al., 2010).

In addition, regarding the bioactive potential of these phosphopeptides, CPP-enriched products are commercialized for healthcare application with oral administration. In this context, some studies compared the CPP profiles of those CPP-enriched products with that of caseins after digestion. Thus, Garcia-Nebot *et al.* compared either milk or CPP concentrate, both added to a fruit beverage, before and after SGID. Firstly, they noticed that SGID has very few impacts on CPPs already present in the initial sample and on the phosphorylated residues. Secondly, the quantity of CPPs after digestion was higher in the fruit beverage supplemented with CPP concentrate than the one supplemented with milk (García-Nebot, Alegría, Barberá, Contreras, et al., 2010). A recent study published in 2020 also compared CPP profiles of CPP concentrate preparation and caseins and found they were pretty similar after SGID. The number of identified CPPs was thus approximatively the

same as for di- and tri-phosphorylated CPP amounts and for α_{S1} -, α_{S2} - and β -casein precursors. Only fewer monophosphorylated forms of CPPs were found in CPP concentrate preparation digestion samples (Bottani et al., 2020).

3.1.3. Comparison between *in vivo* and *in vitro* gastrointestinal digestions

Recently, many research teams investigated the discrepancy between CPPs released during SGID and *in vivo* GI digestion. In 2018, Sanchón *et al.* compared peptide profiles of human jejunal and intestinal samples of SGID milk digestion using the consensual INFOGEST protocol (Sanchón et al., 2018). They evidenced similitudes between *in vivo* and *in vitro* digests for global peptide profiles, but certain sequence regions were more or less represented. Regarding phosphorylated peptides, most of β -CPPs found in the jejunum were also present after SGID, and for α_{S1} -CPPs, principally α_{S1} -casein (42-52) region was found *in vivo* and *in vitro*.

In the same way, CPPs corresponding to β -casein (33-39) and β -casein (33-51) identified after milk-based fruit beverage SGID in the Garcia-Nebot *et al.* work were found or correspond partially to the sequence of those identified *in vivo* in the study of Chabance *et al.* (Chabance et al., 1998; García-Nebot, Alegría, Barberá, Contreras, et al., 2010). For peptides released after α_{S1} -casein and α_{S2} -casein digestion, some phosphopeptides identified *in vitro* were previously identified in clinical studies: α_{S1} -casein (73-79), α_{S1} -casein (110-119) and α_{S2} -casein (138-146) (Boutrou et al., 2013; García-Nebot, Alegría, Barberá, Contreras, et al., 2010).

Another study published in 2019 showed no significant difference between peptides released either by static INFOGEST SGID or dynamic *in vitro* protocol from Menard *et al.* 2014 and pig digestion and supported the fact that SGID can give a good approximation of peptide pattern obtained *in vivo* (Egger et al., 2019). Similar results were obtained with cheese digestion since Masotti *et al.* found that some phosphorylated peptides from β -, α_{S1} -, and α_{S2} -caseins, obtained after the *in vitro* digestion of Trentingrana PDO cheese, were found in human and mini-pig digestive tracts after GI digestion (Barbé et al., 2014; Boutrou et al., 2013; Masotti et al., 2018).

The analysis of CPPs *in vivo* and *in vitro* during the different steps of SGID revealed that CPPs were able to survive the different digestion steps and were, therefore, good candidates for a bioactive role. However, these studies showed that the various protocols used impacted the quantity and properties of the CPPs formed or already present.

3.2. Production of CPPs during ripening and fermentation

Besides digestion, CPPs are also naturally released in various dairy products during milk fermentation. Indeed, during cheese ripening and milk fermentation, there is significant proteolysis due to the action of several microorganism proteinases and peptidases, leading to CPP release. Several studies showed that CPPs are present in diverse cheeses (Ebner et al., 2015; Raja & Arunachalam, 2011; Savastano et al., 2019). For instance, CPP formation has been evidenced during ripening of Herrgard or Grana Padano, Comté and Parmigiano Reggiano cheeses (Ardö et al., 2007; Sousa et al., 2001). Moreover, some studies reveal that various bioactive peptides can be formed during milk fermentation due to lactic bacteria, like *Lactobacillus helveticus* (Narva et al., 2004; Raveschot et al., 2018). In this way, the investigation of β , α_{s1} , and α_{s2} -caseins hydrolysis by different *Streptococcus thermophilus* strains revealed CPP release (Miclo et al., 2012).

A study showed that cheese SGID produced phosphopeptides mainly released from α_{s1} , α_{s2} and β -casein (Masotti et al., 2018). Another team evidenced that 1, 13 and 58 phosphorylated peptides from α_{s1} , α_{s2} and β -casein, respectively, are present in Beaufort cheese before SGID and 17, 23 and 39 after SGID. 19 CPPs, principally from β -casein, are present before and after Beaufort cheese digestion, and 60 new CPPs are formed (Adt et al., 2011).

3.3. CPP released by enzymatic hydrolysis

Even if CPPs are produced during milk and cheese digestion, the casein *in vitro* enzymatic hydrolysis allows the production of commercial products enriched in CPPs in large quantities. *In vitro* CPP obtention with purified enzymes has been studied in several works. The results showed that CPPs could be released *in vitro* using various digestive enzymes, principally gastric and pancreatic. After 10 minutes to 2 hours of hydrolysis by pepsin or pancreatin, the intact caseins started to disappear (Atallah et al., 2020; Picariello et al., 2010; Su et al., 2007). The authors principally investigated the hydrolytic action of these enzymes on α -, β -casein, and sodium caseinate. It is important to note that the matrices are essential; the enzyme does not impact the whole casein complex and isolated casein in the same manner.

Regarding gastric enzyme, Klaus Raith's group showed the effects of pepsin on CPP release (Schmelzer et al., 2004, 2007). Others evidenced the action of trypsin, a pancreatic peptidase, on different casein hydrolysis during the previous century and of the tandem trypsin/chymotrypsin (Adamson & Reynolds, 1995; Deutsch et al., 2000; Tauzin et al., 2003). According to the different caseins, these enzymes have privileged cleavage sites; for example, regarding the α_{s2} -casein, the C-terminal region is more accessible by trypsin than the N-terminal region and the medium part of the protein (Tauzin et al., 2003). Some other studies also determined the peptide profiles obtained after double hydrolysis of caseins, first with pepsin and then with trypsin (Carreira et al., 2004; ONO et al., 1998). This double

digestion can lead to a higher peptide release (ONO et al., 1998). Other proteases from diverse origins can also be used for bioactive peptide delivery, like plasmin and Alcalase (Adamson & Reynolds, 1996; Ferranti et al., 2004).

Diverse parameters can impact the CPP yield and profile. The choice of the enzyme for *in vitro* CPP production is crucial because the degree of hydrolysis is linked to the type of enzyme and impacts the CPP profile. For example, a purified enzyme can release fewer CPPs than the pancreatic enzyme cocktail. Indeed, the CPP yield (percentage (w/w) of original protein) after caseinate sodium hydrolysis is 8.3% with trypsin and 11.6% with pancreatin, while it is 16% with Bioprotease N100L (McDonagh & FitzGerald, 1998). Nevertheless, the choice of enzyme cocktail also influences CPP yield: 16% with Bioprotease N100L and only 3.4% with Protease DS. In the same study, it has been demonstrated that enzymes can influence CPP hydrophobicity; Alcalase hydrolysis increases CPP hydrophobicity compared to proteinase hydrolysis (McDonagh & FitzGerald, 1998). Moreover, enzyme choice is not the only parameter to consider; the reaction time can influence qualitative and quantitative CPP profiles. For instance, degrees of hydrolysis were obtained: 1.5%, 3%, 6%, 12%, 16%, 20%, 25%, 28% and 28.5% with different reaction time: 2.5, 5, 10 and 30 min, 1, 2, 6, 12 and 24 h, respectively (Su et al., 2007). Furthermore, it has been shown that a short time of hydrolysis (5 minutes) privileged hydrophobic peptide release and a long time of hydrolysis (> 6 hours) can negatively impact the presence of CPPs containing the interesting cluster sequence. Pancreatin hydrolysis time can also impact the phosphorylated level of CPPs released (Su et al., 2007).

Many parameters must be taken into account to establish a protocol allowing the obtention of precise and required CPP profiles. Therefore, to get industrially reproducible products to be commercialized, it is essential to study each parameter during the industrial process and standardise the protocols

4. CPP bioactive actions

Numerous studies evidenced the benefits of casein-derived peptides in preventing deficiencies and pathologies. Among them, CPPs and their physiological actions were extensively studied. The still-growing evidence of their impact on health is discussed in this part.

4.1. Mineral chelation

Caseins can bind different divalent cations, thus decreasing their bioavailability. This phenomenon is reduced by proteolysis occurring during GI digestion, which leads to CPP release. One of the famous roles of CPPs is their ability to sequester divalent metal ions, including iron, calcium and zinc. Indeed, CPPs can prevent cation precipitation through alkaline pH in the small intestine (FitzGerald, 1998; Zidane et al., 2012). Generally, mineral chelation by CPPs increases bioavailability and mineral solubility, making them the subject of numerous studies on their bioactive roles (Figure 2).

This mineral chelation property comes from the negatively charged amino acid sequence characterized by three phosphoserine residues and two glutamic acid residues (Ferraretto et al., 2003; Miquel, Alegría, et al., 2006). Therefore, this acidic polar sequence, resistant to hydrolysis, was investigated in SGID CPP qualitative profiles (Cruz-Huerta et al., 2015; Masotti et al., 2018). In 2006, Miquel *et al.* found that 50 % of CPPs released after infant formula digestion contained this cluster, which seems essential for calcium binding. Other phosphoserine residues in CPPs could be involved for iron and zinc (Miquel, Ángel Gómez, et al., 2006). After cheese digestion, 40 % of tri-phosphorylated CPPs contained the cluster sequence (Masotti et al., 2018). In these different studies, the digestion protocol used seems to influence the number of CPPs harbouring this cluster (Masotti et al., 2018), just like the dairy matrices. Indeed, Garcia-Nebot *et al.* found a difference in the number of CPPs containing the cluster sequences between the pool of CPPs-enriched fruit beverage digest and the milk one (García-Nebot, Alegría, Barberá, Contreras, et al., 2010).

4.1.1. Calcium

Calcium is an essential mineral involved in diverse physiological processes, especially in bone tissue maintenance and integrity; 99% of the body's calcium is in the skeleton. Many diseases are due to a calcium deficiency. The GI tract is the only gate of calcium entry, and the mechanisms involved in calcium uptake through the intestinal barrier are therefore intensively investigated.

The CPP's positive impact on calcium uptake has been mainly studied in rats and to a lesser extent in humans. Naito's group showed in rats that CPPs promoted calcium absorption in

intestinal ligated segments and increased femur calcium deposition. These results are primarily due to the effect of CPPs on calcium solubility (Sato et al., 1986, 1991). Kuwata and al. demonstrated that a mixture of β -casein (1-25) and β -casein (1-28) increased calcium absorption in young ovariectomized rats with calcium deficiency (Tsuchita et al., 1993), and a calcium-bound CPP mixture increased bone mineral density of excised femur in aged ovariectomized rats (Tsuchita et al., 1996). In 2001, the same group observed that the CPPs released after bovine casein enzymatic hydrolysis led to a significant increase in calcium absorption and calcium level in the femur and tibia of young rats fed with milk supplemented with calcium (Tsuchita et al., 2001).

In humans, Heany *et al.* found that CPPs (87.5 mg) daily co-ingested with calcium (250 mg) significantly increased calcium absorption in postmenopausal women with a low basal calcium intake (Heaney et al., 1994). Furthermore, in healthy young people fed with single test meals, which consisted of rice-based cereal complemented with 1 or 2 g of CPPs and respectively 69 mg and 138 mg of labelled calcium, the total calcium absorption was significantly improved (Hansen et al., 1997). Nevertheless, in the same study, when young people received whole grain-cereal bread meals supplemented with different concentrations of CPPs and calcium, the absolute quantity of calcium absorbed was unaffected by CPPs.

Nevertheless, some studies obtained opposite results showing that CPPs did not impact or could even decrease calcium uptake. For example, Bennet *et al.* showed that increasing the CPP concentration in protein-free meals did not affect or reduce calcium absorption and deposition in the femur of 9-week-old rats (Bennett et al., 2000). These different results highlight the importance of the protocol procedure and show that numerous parameters can influence CPP bioactivities like CPP concentration and meal preparation, CPPs physicochemical properties and CPPs/Calcium ratio used. The authors of this work tried to explain these conflicting results with several assumptions. Firstly, the CPP concentrations: in their work, high CPP concentrations (100 to 500 g.kg⁻¹) were used (Bennett et al., 2000), while another study on rats showed that CPP supplementation increased calcium absorption even though lower doses (0.7 to 3.5 g.kg⁻¹) (Saito et al., 1998). So, low and high amounts of CPPs may exert an opposite effect. Secondly, meal preparation can also impact: Hansen *et al.* showed in healthy young people that CPP addition to a rice-based infant cereal meal increased calcium absorption significantly while CPP addition to a whole-grain infant cereal did not (Hansen et al., 1997). Thirdly, CPP preparation can also influence their physicochemical properties, as discussed above, impacting the interaction with calcium in the GI tract. Recent work on Caco-2 cell monolayer supports the differences observed in calcium uptake between different CPP fractions, comprising CPPs from α - or β -casein, with the fact that they cannot induce calcium uptake with the same intensity (Y. Cao et al., 2017).

Interestingly, the effect of α_{s1} -casein (59-79) 5P is less pronounced than the β -casein (1-25) 4P (Ferraretto et al., 2003; Gravaghi et al., 2007). The CPPs/calcium is another essential concern pointed out in numerous studies. Indeed, in humans, Heaney *et al.* and Hansen *et al.* used CPPs/Calcium ratios of 0.35 and 14.5, respectively (Hansen et al., 1997; Heaney et al., 1994). In 2002, Erba *et al.* tested different CPPs/Calcium ratios in isolated rat ileum and found that calcium uptake increased when the ratio ranged from 5 to 15 and that the calcium concentration had no effect (Erba et al., 2002). The impact of CPPs/Calcium ratios has been shown *in vitro*, using the HT-29 cell model to determine optimal CPP and calcium concentrations for a higher calcium uptake (Gravaghi et al., 2007). Results showed that, for a mixture of CPPs and β -casein (1–25) 4P, the optimal conditions for bioactive effect were an initial calcium concentration of 4 mmol.L⁻¹ and a CPP mixture concentration of 1280 μ mol.L⁻¹ or a β -casein (1–25) 4P concentration of 200 μ mol.L⁻¹ (Gravaghi et al., 2007).

Thus, it would be interesting to investigate further the link between the effect of CPPs on calcium transport and their origin and sequence. Unravelling this link will determine the mechanism of CPP's action more precisely and concentrate nutraceutical preparations in specific CPPs. Thanks to previous investigations, it is admitted that CPPs induce intestinal calcium absorption *in vivo*. However, only a few studies investigated the CPP role in calcium uptake in humans, and more investigations are needed to understand the mechanisms involved.

In this way, through *in vitro* studies, some important parameters and mechanisms involved in CPP's role in calcium bioavailability have been identified. The role of CPPs on extracellular calcium uptake has mainly been investigated *in vitro* using Caco-2 and HT-29 intestinal human cell lines (Cosentino et al., 2010). With these models, Anita Ferraretto's group determined some factors involved in the action of CPPs on intestinal calcium uptake. Indeed, when using synthetic β -casein (1-25) 4P and α_{s1} -casein (59-79) 5P truncated forms, they demonstrated that some elements of CPP primary structure are essential for calcium-uptake like phosphorylation, the acidic motif and the N-terminal region. Interestingly, the C-terminal region did not impact calcium uptake (Ferraretto et al., 2003). The acidic motif seems essential but not sufficient to improve calcium uptake. These results also showed that the supramolecular structure is critical. Due to their negative charged phosphorylated serine and the positively charged divalent calcium, CPPs can form aggregates. In this way, a study using laser light scattering investigated the relative amounts of CPP aggregates (Gravaghi et al., 2007). First, results showed that CPPs could form themselves aggregates and secondly, the CPP aggregate level fitted with the dose-response CPP-induced calcium uptake, highlighting the importance of aggregation in the intestinal calcium uptake (Gravaghi et al., 2007). In 2015, Perego *et al.* investigated the impact of the SGID on the aggregate structure

using pepsin and pancreatin and evidenced that gastric pH altered aggregates which were quickly reformed at intestinal pH (Perego et al., 2015). The cell differentiation state was an important parameter to be considered. Indeed, the differentiation protocol using vitamin D3 led to increased calcium uptake in intestinal HT-29 and Caco-2 cell models (Cosentino et al., 2010). Interestingly, according to the cell differentiation state, CPPs could protect intestinal cells from the toxic effect of calcium excess and favour their proliferation. Ferraretto's group proposed a CPP anticancer role with these different findings since they can also induce apoptosis in undifferentiated tumour cells (Cosentino et al., 2010; Perego et al., 2012).

Some investigations elucidated essential factors regarding the mechanisms of CPP action on intestinal calcium uptake. Calcium seems to pass the intestinal barrier through two pathways: the intracellular one, which involves different transport proteins, like channels and receptors, and the paracellular one involving tight junctions. CPPs do not alter tight junctions regarding the paracellular pathway as membrane permeability was not changed. Furthermore, CPPs seem not to modify vitamin D receptor (VDR) expression, which is known to regulate the expression of many genes involved in calcium homeostasis (Colombini et al., 2013). On the other hand, it has been shown in HT-29 and osteoblast-like cells that CPPs could interact with the depolarizing voltage-operated L-type calcium channels (Donida et al., 2009; Perego et al., 2012). Several investigations in HT-29 or Caco-2 cells provided different results: firstly, differentiated HT-29 cells seemed to be more responsive to CPPs than differentiated Caco-2 cells. Secondly, a pre-treatment with the active form of vitamin D gave a higher response in Caco-2 than in HT-29 cells (Cosentino et al., 2010). Regarding the intracellular calcium transport, in HT-29 cells, the L-type calcium channels seemed to be involved in the process, whereas in Caco-2 cells, its expression is controversial. In the same way, the TRPV-6 channel appeared to be the main calcium channel involved in CPPs-mediated calcium uptake in the Caco-2 cell model, whereas siRNA against TRPV6 had no effect in HT-29 cells (Perego et al., 2012, 2013).

These different results could also be explained by the different genotypes and morphologies of these models. HT-29 cells are assimilated to the ileum region, while Caco-2 cells to the duodenum one, despite being isolated from the colon. These findings suggest that CPP actions are time- and localization-dependent and that more than one transporter is involved in the CPP-mediated intestinal calcium uptake (Cosentino et al., 2010; Perego et al., 2012, 2013).

As discussed in the *in vivo* paragraph of this section, the increase of calcium uptake by CPPs can improve calcium bioavailability for bones and modulate cell bone activity. In different *in vivo* models, the impact of CPPs on bone weight and calcium content has been identified.

Indeed, in rats with ectopic bones, it has been shown that CPPs decreased osteoclastic bone resorption and seemed to be involved in cartilage calcification (Matsui et al., 1994). Other studies in rats evidenced that CPPs prevented bone loss in young and aged rats (Tsuchita et al., 1993, 1996). In chicken models, CPPs could reduce the incidence of tibial dyschondroplasia and increase bone calcification in rachitic chicks (Mykkänen & Wasserman, 1980; Parkinson & Cransberg, 2004). *In vitro* experiments confirmed these results, showing that CPPs from bovine casein hydrolysate stimulated calcium uptake in human primary osteoblast-like cells. CPPs were also able to modulate these cells' proliferation and apoptotic levels (Donida et al., 2009). Other *in vitro* and *in vivo* studies obtained similar results by showing that CPPs modulated alkaline phosphatase expression and activity, an enzyme involved in bone and cartilage calcification, suggesting that CPPs can be involved in osteoblast differentiation (Donida et al., 2009; Matsui et al., 1994).

4.1.2. Iron

Iron is one of the essential minerals found in food. Therefore, iron deficiency is a source of anaemia and can affect the cognitive and socio-emotional development of the child's brain. Nowadays, it is the best-known nutritional deficiency (Gupta & Gupta, 2014). Consequently, iron-enriched food and beverages are developed.

The particularity of the binding between iron and CPPs is its strength. Indeed this covalent binding is more robust than those with other mineral elements. It has been observed that CPPs could bind more ferrous ions than milk proteins and the iron CPP complex is more resistant to enzymatic digestion; this effect comes from a higher concentration of phosphoserine-rich residues issued from caseinate tryptic digestion (Boutrou et al., 2010; Smialowska et al., 2017). Indeed, some studies have shown that phosphoserine residues are essential because their dephosphorylation decreases the iron-binding capacity of CPPs (Bernos et al., 1997).

The CPP iron chelation phenomenon has been well documented by Saïd Bouhallab and Dominique Bouglé's team, thanks to *in vivo* and *in vitro* studies. In 1997, a first investigation showed that CPPs obtained after β -casein digestion improved Fe bioavailability in rats (Aït-oukhatar et al., 1997). Regarding the mechanisms involved, they hypothesized that CPPs could pass with linked Fe ions through the epithelial membrane by passive and active pathways and endocytose (Pérès et al., 1999). Thanks to CPP-Fe complex radiolabeling experiments, they then evidenced the bioactive role of CPPs regarding iron bioavailability. Indeed, iron is better absorbed by the different organs involved in Fe metabolism like bone marrow, liver, and spleen than inorganic salts when bound to CPPs (Ait-Oukhatar et al., 2002).

Nevertheless, as for calcium, the effects of CPPs on iron transport, retention and uptake appear controversial in numerous works, which could be explained by the many parameters involved, for instance, the inorganic salt forms used and the different physicochemical properties of CPPs.

For inorganic salt forms used, Kibangou *et al.* demonstrated, in a first study, that Fe link to β -casein (1-25) increased total iron uptake in Caco-2 cells and a model of rat duodenal loop compared to Fe gluconate (Kibangou, Bouhallab, Henry, et al., 2005). Nevertheless, they showed no difference in a second study when Fe- β -casein (1-25) total uptake was compared with iron sulfate total uptake in Caco-2 cells (Kibangou, Bouhallab, Bureau, et al., 2005). In the same way, Yeung *et al.* found that CPPs decreased relative iron availability with iron citrate in Caco-2 cells (Yeung et al., 2002).

Regarding the physicochemical properties of CPPs, in a first *in vivo* work, Bouhallab and Bouglé used β -casein (1-25), a N-terminal CPP characterized by four phosphoserines residues and good resistance to hydrolysis and FeSO_4 . They showed that CPPs increased iron bioavailability in young rat model (Aït-oukhatar et al., 1997). However, other investigations compared α -CPPs to β -casein CPPs and determined the importance of the nature and origin of CPPs on iron-binding capacity and bioavailability. Comparison between different casein digest fractions in a perfused rat duodenal loop system showed that iron bound to CPPs from β -casein displayed a better absorption than whole CPPs or α -casein CPPs and that the β -casein (1-25) CPPs was the best and this observation is consistent with those obtained for calcium (Bouhallab et al., 2002). In 2005, Kibangou *et al.* showed an opposite effect between CPPs from α or β -casein on Caco-2 cells; Fe- β -casein (1-25) increased while Fe- α_{s1} casein (59-79) decreased iron net absorption (Kibangou, Bouhallab, Bureau, et al., 2005). Moreover, in 2010, Garcia-Nebot *et al.* found a slight effect of a CPP-added fruit beverage on iron uptake in the Caco-2 model, which could be explained by the higher proportion of CPPs from α -casein than from β -casein in their mix (García-Nebot, Alegría, Barberá, Clemente, et al., 2010). These different effects could be explained because CPPs derived from α -casein could bind iron ions stronger than those from β -casein. Besides iron uptake and bioavailability, some researchers investigated the CPP impact on ferritin expression, a protein implicated in iron storage. They evidenced differences between a pool of CPPs and an isolated CPP. Indeed, in Caco-2 cells, β - and α -casein-issued CPP pools did not affect ferritin synthesis, but specific isolated CPP induced its synthesis (1-25) (García-Nebot et al., 2013).

Regarding the mechanisms involved in the increased iron bioavailability, numerous studies showed that phosphatase alkaline action led to an increase in iron-free ion concentration,

which decreased its absorption by the intestinal barrier. Therefore, CPPs could protect iron, indicating that the CPP-Fe complex can be a good candidate for iron delivery (Ani-Kibangou et al., 2005). However, contradictory results showed that phosphatase alkaline hydrolyzed phosphoester bond in phosphoserine residues inhibiting iron chelation and allowing its absorption. Nevertheless, a mix of CPPs was used in this work, and α -CPPs could release iron ions inhibiting its absorption and decreasing iron bioavailability (Yeung et al., 2001).

Taken together, these different *in vivo* and *in vitro* results suggest the importance of CPPs on iron bioavailability and that β - and α -CPPs could exert an opposite role.

However, the mechanisms involved in the role of CPPs on iron transport remain to be studied. As with calcium, it is not clear whether CPPs promote intracellular or paracellular transport or both. For example, CPPs could facilitate the passage of minerals by crossing the intestinal barrier and releasing the ions into the blood compartment. On the other hand, CPPs could activate the cellular transport machinery of the different ions, as shown for calcium. However, the different minerals do not enter the cell by the same mechanisms. Several studies are therefore needed to identify the mechanisms involved. Once these mechanisms are identified and the precise role of the different released CPPs, it will be possible to adjust various preparations.

4.1.3. Other minerals

Although CPP effects on calcium and iron absorption have been extensively studied, CPPs can also chelate other divalent ions such as magnesium and zinc. Recently, it has been shown that CPPs increased magnesium transport through Caco-2 cell monolayer and that this effect was amplified when magnesium was co-injected with calcium (Y. Cao et al., 2017). Moreover, a diet supplemented with magnesium and CPPs positively impacted femur length, serum parathyroid hormone level, and urinary deoxypyridinoline in rats (Liu et al., 2017). Numerous studies also investigated the role of CPPs on zinc bioavailability, but the results obtained were contradictory, and further studies are needed (Miquel & Farré, 2007).

4.1.4. Mineral chelation: antioxidant activity

Due to their ability to bind divalent metals, some authors identified an antioxidant action of the CPPs. For instance, we indicated above that CPPs can form a stable complex with iron ions and that this phenomenon led to a decrease in ROS formation in connection with the Fenton reaction. In 2004, Chiu *et al.* found that CPPs issued from tryptic digestion decreased the formation of secondary oxidation products and stabilized free radicals in an aqueous solution (Chiu & Kitts, 2003). CPP antioxidant properties have also been identified in oil-in-water solutions, in which they inhibited lipid oxidation (Díaz et al., 2003). Equivalent effects

were shown with phosphatidylcholine liposome model systems and beef homogenates (Díaz & Decker, 2004; Kansci et al., 2004). In 2008, *in vitro* investigation in Caco-2 cells showed that CPPs had no impact on mitochondrial activities involved in reactive oxygen species (ROS) metabolism. However, they regulated the activity of the enzymes involved in ROS metabolism. Interestingly, CPPs exerted more effects than non-digested milk (Laparra et al., 2008). In recent work, authors investigated after SGID the CPP antioxidant capacity in intestinal cells and osteoblasts. Results showed that in the presence of an agent able to induce peroxy radicals or FeCl₂, CPPs exerted an antioxidant activity in intestinal and osteoblasts cells (Bottani et al., 2020).

4.2. Other roles

Even if CPPs are primarily known for their mineral chelation properties, other investigations indicated that CPPs might impact the regulation of the immune system. Hata *et al.* demonstrated that CPPs enhanced spleen cell proliferation and immunoglobulin production. They used the β -casein (1-28), which is rich in phosphoserine residues, and showed that phosphoserine residues are essential for the immunomodulatory effect (Otani et al., 2001). In addition, CPPs exert a mitogenic effect *in vitro*: β -casein (1-28) incubation with different culture cell lines of B and T cells and monocytes induced proliferation and IgA production (Kawahara et al., 2004). Furthermore, CPPs induced anti-inflammatory cytokine expression and production in spleen cells, including Interleukin 6 and 10 (Mullaiselvan et al., 2020; Tobita et al., 2006). The stimulation of Interleukin 6 production by CPPs in CD19+ cells involves toll-like receptor 4 (Tobita et al., 2006). Interestingly, the same group demonstrated that CPPs induced Interleukin 6 and TNF alpha mRNA expression in intestinal Caco-2 cells (Kawahara & Otani, 2004; Kitts & Nakamura, 2006).

Other studies showed that CPPs exert antimicrobial effects. Indeed, in 2010, Arunachalam *et al.* tested CPP antimicrobial activity against different pathogens and found a positive effect *against Escherichia coli* and some *Pseudomonas* species (Raja & Arunachalam, 2011). More recently, Mullaiselvan *et al.* confirmed this activity on four pathogens: *Escherichia coli*, *Bacillus cereus*, *Staphylococcus aureus* and *Salmonella enterica* (Mullaiselvan et al., 2020). However, the mechanisms involved remain unravelled.

5. Conclusion

Bioactive peptide preparations are of interest for their use as functional ingredients in nutraceutical, pharmaceutical and cosmetic domains for different applications in preventing and managing several chronic diseases. Among them, chelating peptides were the subject of many studies, and in these past years, CPPs have emerged as promising candidates for pharmaceutical and nutraceutical products due to their resistance to digestive enzymes and their good bioavailability.

Since the recent advances in mass spectrometry, powerful methods lead possible to identify, characterize, and quantify phosphopeptides produced during hydrolysis in various more or less complex environments such as the gastrointestinal tract.

CPPs, which are encrypted in caseins, are naturally produced during gastrointestinal digestion. The study of their release, using *in vitro* simulated digestion protocols, enforces our knowledge of what could happen *in vivo* and better understands the specific role of the various enzymes implicated in caseins digestion.

The characterization of the CPPs released *in vitro* shows that the method used impacts the peptide sequence, physicochemical properties and yield. However, these factors are involved in the bioactive role of CPPs. The hydrolysis protocol affects the proportion of CPPs derived from α_{s1} , α_{s2} and β -caseins, as well as the presence or absence of peptides containing the cluster sequence pSpSpSGG. These different parameters are strongly linked to CPP bioactivities, and their control makes it possible to obtain a particular and more or less pronounced effect. Therefore, it is crucial to master and expand knowledge on *in vitro* hydrolysis in the presence of exogenous enzymes and separation techniques to obtain preparations richer in CPPs and with controlled and directed profiles to target specific applications and pathologies. In addition, the conclusions of the studies carried out with a pool of CPPs are not the same as those carried out with an isolated CPP, so it would be critical to study more precisely the role of the different CPPs, identified after digestion, and their possible synergetic or antagonist effects. The development of techniques allowing the isolation and concentration of these CPPs could be an asset in the pharmaceutical field.

Funding: This work has been carried out in the framework of the joint laboratory between UMR-t BioEcoAgro 1158 and the Ingredia project (Allinpep) funded by the Hauts-de-France region (FEDER) and ALIBIOTECH project funded by the European Union, French State, and the French Region of Hauts-de-France.

Author Contributions: MT participated in all steps of preparation of this manuscript. BD, CF and BC participated in the redaction and in the editing of the manuscript and revised it. CD, AM, AR, JA, AB and RR, participated in the editing of the manuscript and revised it.

6. References

- Adamson, N. J., & Reynolds, E. C. (1995). Characterization of tryptic casein phosphopeptides prepared under industrially relevant conditions. *Biotechnology and Bioengineering*, *45*(3), 196-204. <https://doi.org/10.1002/bit.260450303>
- Adamson, N. J., & Reynolds, E. C. (1996). Characterization of casein phosphopeptides prepared using alcalase : Determination of enzyme specificity. *Enzyme and Microbial Technology*, *19*(3), 202-207. [https://doi.org/10.1016/0141-0229\(95\)00232-4](https://doi.org/10.1016/0141-0229(95)00232-4)
- Adt, I., Dupas, C., Boutrou, R., Oulahal, N., Noel, C., Mollé, D., Jouvét, T., & Degraeve, P. (2011). Identification of caseinophosphopeptides generated through in vitro gastro-intestinal digestion of Beaufort cheese. *International Dairy Journal*, *21*(3), 129-134. <https://doi.org/10.1016/j.idairyj.2010.10.002>
- Ahn, C.-B., & Je, J.-Y. (2019). Bone health-promoting bioactive peptides. *Journal of Food Biochemistry*, *43*(1), e12529. <https://doi.org/10.1111/jfbc.12529>
- Aït-oukhatar, N., Bouhallab, S., Bureau, F., Arhan, P., Maubois, J.-L., Drosdowsky, M. A., & Bouglé, D. L. (1997). Bioavailability of caseinophosphopeptide bound iron in the young rat. *The Journal of Nutritional Biochemistry*, *8*(4), 190-194. [https://doi.org/10.1016/S0955-2863\(97\)00002-8](https://doi.org/10.1016/S0955-2863(97)00002-8)
- Ait-Oukhatar, N., Peres, J. M., Bouhallab, S., Neuville, D., Bureau, F., Bouvard, G., Arhan, P., & Bouglé, D. (2002). Bioavailability of caseinophosphopeptide-bound iron. *The Journal of Laboratory and Clinical Medicine*, *140*(4), 290-294. <https://doi.org/10.1067/mlc.2002.128146>
- Alpert, A. J., Hudecz, O., & Mechtler, K. (2015). Anion-exchange chromatography of phosphopeptides : Weak anion exchange versus strong anion exchange and anion-exchange chromatography versus electrostatic repulsion-hydrophilic interaction chromatography. *Analytical Chemistry*, *87*(9), 4704-4711. <https://doi.org/10.1021/ac504420c>

- Alpert, A. J., Petritis, K., Kangas, L., Smith, R. D., Mechtler, K., Mitulović, G., Mohammed, S., & Heck, A. J. R. (2010). Peptide Orientation Affects Selectivity in Ion-Exchange Chromatography. *Analytical Chemistry*, *82*(12), 5253-5259. <https://doi.org/10.1021/ac100651k>
- Ani-Kibangou, B., Bouhallab, S., Mollé, D., Henry, G., Bureau, F., Neuville, D., Arhan, P., & Bouglé, D. (2005). Improved absorption of caseinophosphopeptide-bound iron : Role of alkaline phosphatase. *The Journal of Nutritional Biochemistry*, *16*(7), 398-401. <https://doi.org/10.1016/j.jnutbio.2005.02.008>
- Annan, R. S., & Carr, S. A. (1996). Phosphopeptide Analysis by Matrix-Assisted Laser Desorption Time-of-Flight Mass Spectrometry. *Analytical Chemistry*, *68*(19), 3413-3421. <https://doi.org/10.1021/ac960221g>
- Ardö, Y., Lilbæk, H., Kristiansen, K. R., Zakora, M., & Otte, J. (2007). Identification of large phosphopeptides from β -casein that characteristically accumulate during ripening of the semi-hard cheese Herrgård. *International Dairy Journal*, *17*(5), 513-524. <https://doi.org/10.1016/j.idairyj.2006.06.027>
- Arribas Diez, I., Govender, I., Naicker, P., Stoychev, S., Jordaan, J., & Jensen, O. N. (2021). Zirconium(IV)-IMAC Revisited : Improved Performance and Phosphoproteome Coverage by Magnetic Microparticles for Phosphopeptide Affinity Enrichment. *Journal of Proteome Research*, *20*(1), 453-462. <https://doi.org/10.1021/acs.jproteome.0c00508>
- Asara, J. M., & Allison, J. (1999). Enhanced detection of phosphopeptides in matrix-assisted laser desorption/ionization mass spectrometry using ammonium salts. *Journal of the American Society for Mass Spectrometry*, *10*(1), 35-44. [https://doi.org/10.1016/S1044-0305\(98\)00129-9](https://doi.org/10.1016/S1044-0305(98)00129-9)
- Atallah, N., Deracinois, B., Boulier, A., Baniel, A., Jouan-Rimbaud Bouveresse, D., Ravallec, R., Flahaut, C., & Cudennec, B. (2020). In Vitro Assessment of the Impact of Industrial Processes on the Gastrointestinal Digestion of Milk Protein Matrices Using the INFOGEST Protocol. *Foods*, *9*(11). <https://doi.org/10.3390/foods9111580>

- Barbé, F., Le Feunteun, S., Rémond, D., Ménard, O., Jardin, J., Henry, G., Laroche, B., & Dupont, D. (2014). Tracking the in vivo release of bioactive peptides in the gut during digestion : Mass spectrometry peptidomic characterization of effluents collected in the gut of dairy matrix fed mini-pigs. *Food Research International*, *63*, 147-156.
<https://doi.org/10.1016/j.foodres.2014.02.015>
- Baum, F., Ebner, J., & Pischetsrieder, M. (2013). Identification of multiphosphorylated peptides in milk. *Journal of Agricultural and Food Chemistry*, *61*(38), 9110-9117.
<https://doi.org/10.1021/jf401865q>
- Beausoleil, S. A., Jedrychowski, M., Schwartz, D., Elias, J. E., Villén, J., Li, J., Cohn, M. A., Cantley, L. C., & Gygi, S. P. (2004). Large-scale characterization of HeLa cell nuclear phosphoproteins. *Proceedings of the National Academy of Sciences*, *101*(33), 12130-12135.
<https://doi.org/10.1073/pnas.0404720101>
- Beck, A., Deeg, M., Moeschel, K., Schmidt, E. K., Schleicher, E. D., Voelter, W., Häring, H. U., & Lehmann, R. (2001). Alkaline liquid chromatography/electrospray ionization skimmer collision-induced dissociation mass spectrometry for phosphopeptide screening. *Rapid Communications in Mass Spectrometry*, *15*(23), 2324-2333. <https://doi.org/10.1002/rcm.511>
- Bennett, T., Desmond, A., Harrington, M., McDonagh, D., FitzGerald, R., Flynn, A., & Cashman, K. D. (2000). The effect of high intakes of casein and casein phosphopeptide on calcium absorption in the rat. *The British Journal of Nutrition*, *83*(6), 673-680.
<https://doi.org/10.1017/s0007114500000854>
- Bernos, E., Girardet, J. M., Humbert, G., & Linden, G. (1997). Role of the O-phosphoserine clusters in the interaction of the bovine milk alpha s1-, beta-, kappa-caseins and the PP3 component with immobilized iron (III) ions. *Biochimica Et Biophysica Acta*, *1337*(1), 149-159.
[https://doi.org/10.1016/s0167-4838\(96\)00159-8](https://doi.org/10.1016/s0167-4838(96)00159-8)
- Bian, Y., Song, C., Cheng, K., Dong, M., Wang, F., Huang, J., Sun, D., Wang, L., Ye, M., & Zou, H. (2014). An enzyme assisted RP-RPLC approach for in-depth analysis of human liver

- phosphoproteome. *Journal of Proteomics*, 96, 253-262.
<https://doi.org/10.1016/j.jprot.2013.11.014>
- Bingham, E. W., & Farrell, H. M. (1977). Phosphorylation of Casein by the Lactating Mammary Gland : A Review. *Journal of Dairy Science*, 60(8), 1199-1207. [https://doi.org/10.3168/jds.S0022-0302\(77\)84011-3](https://doi.org/10.3168/jds.S0022-0302(77)84011-3)
- Bingham, E. W., Farrell, H. M., & Basch, J. J. (1972). Phosphorylation of Casein : ROLE OF THE GOLGI APPARATUS. *Journal of Biological Chemistry*, 247(24), 8193-8194.
[https://doi.org/10.1016/S0021-9258\(20\)81827-4](https://doi.org/10.1016/S0021-9258(20)81827-4)
- Bingham, E. W., Parris, N., & Farrell, H. M. (1988). Phosphorylation of β -Casein and α -Lactalbumin by Casein Kinase from Lactating Bovine Mammary Gland. *Journal of Dairy Science*, 71(2), 324-336. [https://doi.org/10.3168/jds.S0022-0302\(88\)79561-2](https://doi.org/10.3168/jds.S0022-0302(88)79561-2)
- Blacken, G. R., Gelb, M. H., & Tureček, F. (2006). Metal Affinity Capture Tandem Mass Spectrometry for the Selective Detection of Phosphopeptides. *Analytical Chemistry*, 78(17), 6065-6073.
<https://doi.org/10.1021/ac060509y>
- Blacken, G. R., Sadílek, M., & Tureček, F. (2008). Gallium metal affinity capture tandem mass spectrometry for the selective detection of phosphopeptides in complex mixtures. *Journal of Mass Spectrometry*, 43(8), 1072-1080. <https://doi.org/10.1002/jms.1387>
- Blom, N., Gammeltoft, S., & Brunak, S. (1999). Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *Journal of Molecular Biology*, 294(5), 1351-1362.
<https://doi.org/10.1006/jmbi.1999.3310>
- Blom, N., Sicheritz-Pontén, T., Gupta, R., Gammeltoft, S., & Brunak, S. (2004). Prediction of post-translational glycosylation and phosphorylation of proteins from the amino acid sequence. *Proteomics*, 4(6), 1633-1649. <https://doi.org/10.1002/pmic.200300771>
- Boersema, P. J., Mohammed, S., & Heck, A. J. R. (2009). Phosphopeptide fragmentation and analysis by mass spectrometry. *Journal of Mass Spectrometry*, 44(6), 861-878.
<https://doi.org/10.1002/jms.1599>

- Bottani, M., Cattaneo, S., Pica, V., Stuknyté, M., De Noni, I., & Ferraretto, A. (2020). In vitro antioxidant properties of digests of hydrolyzed casein and caseinophosphopeptide preparations in cell models of human intestine and osteoblasts. *Journal of Functional Foods*, *64*, 103673. <https://doi.org/10.1016/j.jff.2019.103673>
- Bouhallab, S., Cinga, V., Aít-Oukhatar, N., Bureau, F., Neuville, D., Arhan, P., Maubois, J.-L., & Bouglé, D. (2002). Influence of Various Phosphopeptides of Caseins on Iron Absorption. *Journal of Agricultural and Food Chemistry*, *50*(24), 7127-7130. <https://doi.org/10.1021/jf025554v>
- Boutrou, R., Coirre, E., Jardin, J., & Léonil, J. (2010). Phosphorylation and coordination bond of mineral inhibit the hydrolysis of the beta-casein (1-25) peptide by intestinal brush-border membrane enzymes. *Journal of Agricultural and Food Chemistry*, *58*(13), 7955-7961. <https://doi.org/10.1021/jf100568r>
- Boutrou, R., Gaudichon, C., Dupont, D., Jardin, J., Airinei, G., Marsset-Baglieri, A., Benamouzig, R., Tomé, D., & Leonil, J. (2013). Sequential release of milk protein-derived bioactive peptides in the jejunum in healthy humans. *The American Journal of Clinical Nutrition*, *97*(6), 1314-1323. <https://doi.org/10.3945/ajcn.112.055202>
- Bouzerzour, K., Morgan, F., Cuinet, I., Bonhomme, C., Jardin, J., Le Huërou-Luron, I., & Dupont, D. (2012). In vivo digestion of infant formula in piglets : Protein digestion kinetics and release of bioactive peptides. *The British Journal of Nutrition*, *108*(12), 2105-2114. <https://doi.org/10.1017/S000711451200027X>
- Brodkorb, A., Egger, L., Alminger, M., Alvito, P., Assunção, R., Ballance, S., Bohn, T., Bourlieu-Lacanal, C., Boutrou, R., Carrière, F., Clemente, A., Corredig, M., Dupont, D., Dufour, C., Edwards, C., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., ... Recio, I. (2019). INFOGEST static in vitro simulation of gastrointestinal food digestion. *Nature Protocols*, *14*(4), 991-1014. <https://doi.org/10.1038/s41596-018-0119-1>
- Busman, M., Schey, K. L., Oatis, J. E., & Knapp, D. R. (1996). Identification of phosphorylation sites in phosphopeptides by positive and negative mode electrospray ionization-tandem mass

- spectrometry. *Journal of the American Society for Mass Spectrometry*, 7(3), 243-249.
[https://doi.org/10.1016/1044-0305\(95\)00675-3](https://doi.org/10.1016/1044-0305(95)00675-3)
- Caira, S., Pinto, G., Vitaglione, P., Dal Piaz, F., Ferranti, P., & Addeo, F. (2016). Identification of casein peptides in plasma of subjects after a cheese-enriched diet. *Food Research International*, 84, 108-112. <https://doi.org/10.1016/j.foodres.2016.03.023>
- Cao, L., Zhao, Y., Chu, Z., Zhang, X., & Zhang, W. (2020). Core-shell magnetic bimetallic MOF material for synergistic enrichment of phosphopeptides. *Talanta*, 206, 120165.
<https://doi.org/10.1016/j.talanta.2019.120165>
- Cao, Y., Miao, J., Liu, G., Luo, Z., Xia, Z., Liu, F., Yao, M., Cao, X., Sun, S., Lin, Y., Lan, Y., & Xiao, H. (2017). Bioactive Peptides Isolated from Casein Phosphopeptides Enhance Calcium and Magnesium Uptake in Caco-2 Cell Monolayers. *Journal of Agricultural and Food Chemistry*, 65(11), 2307-2314. <https://doi.org/10.1021/acs.jafc.6b05711>
- Capriotti, A. L., Cavaliere, C., Ferraris, F., Gianotti, V., Laus, M., Piovesana, S., Sparnacci, K., Zenezini Chiozzi, R., & Laganà, A. (2018). New Ti-IMAC magnetic polymeric nanoparticles for phosphopeptide enrichment from complex real samples. *Talanta*, 178, 274-281.
<https://doi.org/10.1016/j.talanta.2017.09.010>
- Carreira, R. L., Marco, L. M. de, Dias, D. R., Morais, H. A., & Silvestre, M. P. C. (2004). *Analysis of peptide profiles of casein hydrolysates prepared with pepsin, trypsin and subtilisin*.
- Cattaneo, S., Stuknytė, M., Ferraretto, A., & De Noni, I. (2017). Impact of the in vitro gastrointestinal digestion protocol on casein phosphopeptide profile of Grana Padano cheese digestates. *LWT*, 77, 356-361. <https://doi.org/10.1016/j.lwt.2016.11.069>
- Chabance, B., Marteau, P., Rambaud, J. C., Migliore-Samour, D., Boynard, M., Perrotin, P., Guillet, R., Jollès, P., & Fiat, A. M. (1998). Casein peptide release and passage to the blood in humans during digestion of milk or yogurt. *Biochimie*, 80(2), 155-165. [https://doi.org/10.1016/s0300-9084\(98\)80022-9](https://doi.org/10.1016/s0300-9084(98)80022-9)

- Chen, B., Hwang, L., Ochowicz, W., Lin, Z., Guardado-Alvarez, T. M., Cai, W., Xiu, L., Dani, K., Colah, C., Jin, S., & Ge, Y. (2017). Coupling functionalized cobalt ferrite nanoparticle enrichment with online LC/MS/MS for top-down phosphoproteomics. *Chemical Science*, 8(6), 4306-4311.
<https://doi.org/10.1039/C6SC05435H>
- Chen, J., Shiyarov, P., & Green, K. B. (2019). Top-down mass spectrometry of intact phosphorylated β -casein : Correlation between the precursor charge state and internal fragments. *Journal of Mass Spectrometry*, 54(6), 527-539. <https://doi.org/10.1002/jms.4364>
- Chiu, S. C. K., & Kitts, D. D. (2003). Antioxidant Characterization of Caseinophosphopeptides from Bovine Milk. In *Nutraceutical Beverages* (Vol. 871, p. 279-289). American Chemical Society.
<https://doi.org/10.1021/bk-2004-0871.ch021>
- Colombini, A., Perego, S., Ardoino, I., Marasco, E., Lombardi, G., Fiorilli, A., Biganzoli, E., Tettamanti, G., & Ferraretto, A. (2013). Evaluation of a possible direct effect by casein phosphopeptides on paracellular and vitamin D controlled transcellular calcium transport mechanisms in intestinal human HT-29 and Caco2 cell lines. *Food & Function*, 4(8), 1195-1203.
<https://doi.org/10.1039/c3fo60099h>
- Cosentino, S., Gravaghi, C., Donetti, E., Donida, B. M., Lombardi, G., Bedoni, M., Fiorilli, A., Tettamanti, G., & Ferraretto, A. (2010). Caseinophosphopeptide-induced calcium uptake in human intestinal cell lines HT-29 and Caco2 is correlated to cellular differentiation. *The Journal of Nutritional Biochemistry*, 21(3), 247-254.
<https://doi.org/10.1016/j.jnutbio.2008.12.016>
- Cruz-Huerta, E., García-Nebot, M. J., Miralles, B., Recio, I., & Amigo, L. (2015). Caseinophosphopeptides released after tryptic hydrolysis versus simulated gastrointestinal digestion of a casein-derived by-product. *Food Chemistry*, 168, 648-655.
<https://doi.org/10.1016/j.foodchem.2014.07.090>

- Dalgleish, D. G., Spagnuolo, P. A., & Douglas Goff, H. (2004). A possible structure of the casein micelle based on high-resolution field-emission scanning electron microscopy. *International Dairy Journal*, 14(12), 1025-1031. <https://doi.org/10.1016/j.idairyj.2004.04.008>
- Delshadian, Z., Mortazavian, A. M., Tabarzad, M., Hosseini, S. M., Mohammadi, R., Rouhi, M., Salami, M., & Khosravi-Darani, K. (2018). Optimisation of experimental conditions for binding of divalent iron to bioactive casein phosphopeptides. *International Journal of Food Science & Technology*, 53(3), 784-793. <https://doi.org/10.1111/ijfs.13654>
- Deracinois, B., Matéos, A., Romelard, A., Boulier, A., Auger, J., Baniel, A., Ravallec, R., & Flahaut, C. (2021). Partial-, Double-Enzymatic Dephosphorylation and EndoGluC Hydrolysis as an Original Approach to Enhancing Identification of Casein Phosphopeptides (CPPs) by Mass Spectrometry. *Foods*, 10(9), 2134. <https://doi.org/10.3390/foods10092134>
- Deutsch, S.-M., Molle, D., Gagnaire, V., Piot, M., Atlan, D., & Lortal, S. (2000). Hydrolysis of Sequenced β -Casein Peptides Provides New Insight into Peptidase Activity from Thermophilic Lactic Acid Bacteria and Highlights Intrinsic Resistance of Phosphopeptides. *Applied and Environmental Microbiology*, 66(12), 5360-5367.
- Díaz, M., & Decker, E. A. (2004). Antioxidant Mechanisms of Caseinophosphopeptides and Casein Hydrolysates and Their Application in Ground Beef. *Journal of Agricultural and Food Chemistry*, 52(26), 8208-8213. <https://doi.org/10.1021/jf048869e>
- Díaz, M., Dunn, C. M., McClements, D. J., & Decker, E. A. (2003). Use of Caseinophosphopeptides as Natural Antioxidants in Oil-in-Water Emulsions. *Journal of Agricultural and Food Chemistry*, 51(8), 2365-2370. <https://doi.org/10.1021/jf025984l>
- Dong, Y.-M., Chien, K.-Y., Chen, J.-T., Lin, S.-J., Wang, T.-C. V., & Yu, J.-S. (2013). Site-specific separation and detection of phosphopeptide isomers with pH-mediated stacking capillary electrophoresis-electrospray ionization-tandem mass spectrometry. *Journal of Separation Science*, 36(9-10), 1582-1589. <https://doi.org/10.1002/jssc.201300054>

- Donida, B. M., Mrak, E., Gravaghi, C., Villa, I., Cosentino, S., Zacchi, E., Perego, S., Rubinacci, A., Fiorilli, A., Tettamanti, G., & Ferraretto, A. (2009). Casein phosphopeptides promote calcium uptake and modulate the differentiation pathway in human primary osteoblast-like cells. *Peptides*, *30*(12), 2233-2241. <https://doi.org/10.1016/j.peptides.2009.08.003>
- Ebner, J., Aşçı Arslan, A., Fedorova, M., Hoffmann, R., Küçükçetin, A., & Pischetsrieder, M. (2015). Peptide profiling of bovine kefir reveals 236 unique peptides released from caseins during its production by starter culture or kefir grains. *Journal of Proteomics*, *117*, 41-57. <https://doi.org/10.1016/j.jprot.2015.01.005>
- Egger, L., Ménard, O., Baumann, C., Duerr, D., Schlegel, P., Stoll, P., Vergères, G., Dupont, D., & Portmann, R. (2019). Digestion of milk proteins : Comparing static and dynamic in vitro digestion systems with in vivo data. *Food Research International*, *118*, 32-39. <https://doi.org/10.1016/j.foodres.2017.12.049>
- Ellegård, K. H., Gammelgård-Larsen, C., Sørensen, E. S., & Fedosov, S. (1999). Process scale chromatographic isolation, characterization and identification of tryptic bioactive casein phosphopeptides. *International Dairy Journal*, *9*(9), 639-652. [https://doi.org/10.1016/S0958-6946\(99\)00135-1](https://doi.org/10.1016/S0958-6946(99)00135-1)
- Erba, D., Ciappellano, S., & Testolin, G. (2002). Effect of the ratio of casein phosphopeptides to calcium (w/w) on passive calcium transport in the distal small intestine of rats. *Nutrition (Burbank, Los Angeles County, Calif.)*, *18*(9), 743-746. [https://doi.org/10.1016/s0899-9007\(02\)00829-8](https://doi.org/10.1016/s0899-9007(02)00829-8)
- Fang, Z. H., Visker, M. H. P. W., Miranda, G., Delacroix-Buchet, A., Bovenhuis, H., & Martin, P. (2016). The relationships among bovine α S-casein phosphorylation isoforms suggest different phosphorylation pathways. *Journal of Dairy Science*, *99*(10), 8168-8177. <https://doi.org/10.3168/jds.2016-11250>
- Ferranti, P., Traisci, M. V., Picariello, G., Nasi, A., Boschi, V., Siervo, M., Falconi, C., Chianese, L., & Addeo, F. (2004). Casein proteolysis in human milk : Tracing the pattern of casein breakdown

- and the formation of potential bioactive peptides. *Journal of Dairy Research*, 71(1), 74-87.
<https://doi.org/10.1017/S0022029903006599>
- Ferraretto, A., Gravaghi, C., Fiorilli, A., & Tettamanti, G. (2003). Casein-derived bioactive phosphopeptides : Role of phosphorylation and primary structure in promoting calcium uptake by HT-29 tumor cells. *FEBS Letters*, 551(1-3), 92-98. [https://doi.org/10.1016/s0014-5793\(03\)00741-5](https://doi.org/10.1016/s0014-5793(03)00741-5)
- Fíla, J., & Honys, D. (2012). Enrichment techniques employed in phosphoproteomics. *Amino Acids*, 43(3), 1025-1047. <https://doi.org/10.1007/s00726-011-1111-z>
- FitzGerald, R. J. (1998). Potential Uses of Caseinophosphopeptides. *International Dairy Journal*, 8(5), 451-457. [https://doi.org/10.1016/S0958-6946\(98\)00068-5](https://doi.org/10.1016/S0958-6946(98)00068-5)
- Flora, J. W., & Muddiman, D. C. (2001). Selective, Sensitive, and Rapid Phosphopeptide Identification in Enzymatic Digests Using ESI-FTICR-MS with Infrared Multiphoton Dissociation. *Analytical Chemistry*, 73(14), 3305-3311. <https://doi.org/10.1021/ac010333u>
- Fonslow, B. R., Niessen, S. M., Singh, M., Wong, C. C., Xu, T., Carvalho, P. C., Choi, J., Park, S. K., & Yates, J. R. (2012). Single-step inline hydroxyapatite enrichment facilitates identification and quantitation of phosphopeptides from mass-limited proteomes with MudPIT. *Journal of Proteome Research*, 11(5), 2697-2709. <https://doi.org/10.1021/pr300200x>
- Furukawa, J., Fujitani, N., Araki, K., Takegawa, Y., Kodama, K., & Shinohara, Y. (2011). A Versatile Method for Analysis of Serine/Threonine Posttranslational Modifications by β -Elimination in the Presence of Pyrazolone Analogues. *Analytical Chemistry*, 83(23), 9060-9067.
<https://doi.org/10.1021/ac2019848>
- García-Nebot, M. J., Alegría, A., Barberá, R., Clemente, G., & Romero, F. (2010). Addition of milk or caseinophosphopeptides to fruit beverages to improve iron bioavailability? *Food Chemistry*, 119(1), 141-148. <https://doi.org/10.1016/j.foodchem.2009.06.005>
- García-Nebot, M. J., Alegría, A., Barberá, R., Contreras, M. del M., & Recio, I. (2010). Milk versus caseinophosphopeptides added to fruit beverage : Resistance and release from simulated

- gastrointestinal digestion. *Peptides*, 31(4), 555-561.
<https://doi.org/10.1016/j.peptides.2009.12.021>
- García-Nebot, M. J., Barberá, R., & Alegría, A. (2013). Iron and zinc bioavailability in Caco-2 cells : Influence of caseinophosphopeptides. *Food Chemistry*, 138(2-3), 1298-1303.
<https://doi.org/10.1016/j.foodchem.2012.10.113>
- Gaucher, I., Mollé, D., Gagnaire, V., & Gaucheron, F. (2008). Effects of storage temperature on physico-chemical characteristics of semi-skimmed UHT milk. *Food Hydrocolloids*, 22(1), 130-143. <https://doi.org/10.1016/j.foodhyd.2007.04.007>
- Ginger, M. R., & Grigor, M. R. (1999). Comparative aspects of milk caseins. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, 124(2), 133-145.
[https://doi.org/10.1016/S0305-0491\(99\)00110-8](https://doi.org/10.1016/S0305-0491(99)00110-8)
- Głąb, T. K., & Boratyński, J. (2017). Potential of Casein as a Carrier for Biologically Active Agents. *Topics in Current Chemistry (Cham)*, 375(4), 71. <https://doi.org/10.1007/s41061-017-0158-z>
- Gravaghi, C., Del Favero, E., Cantu', L., Donetti, E., Bedoni, M., Fiorilli, A., Tettamanti, G., & Ferraretto, A. (2007). Casein phosphopeptide promotion of calcium uptake in HT-29 cells— Relationship between biological activity and supramolecular structure. *The FEBS Journal*, 274(19), 4999-5011. <https://doi.org/10.1111/j.1742-4658.2007.06015.x>
- Guerra, A., Etienne-Mesmin, L., Livrelli, V., Denis, S., Blanquet-Diot, S., & Alric, M. (2012). Relevance and challenges in modeling human gastric and small intestinal digestion. *Trends in Biotechnology*, 30(11), 591-600. <https://doi.org/10.1016/j.tibtech.2012.08.001>
- Gupta, U. C., & Gupta, S. C. (2014). Sources and Deficiency Diseases of Mineral Nutrients in Human Health and Nutrition : A Review. *Pedosphere*, 24(1), 13-38. [https://doi.org/10.1016/S1002-0160\(13\)60077-6](https://doi.org/10.1016/S1002-0160(13)60077-6)
- Hansen, M., Sandström, B., Jensen, M., & Sørensen, S. S. (1997). Casein phosphopeptides improve zinc and calcium absorption from rice-based but not from whole-grain infant cereal. *Journal*

- of Pediatric Gastroenterology and Nutrition*, 24(1), 56-62. <https://doi.org/10.1097/00005176-199701000-00014>
- Hardman, G., Perkins, S., Brownridge, P. J., Clarke, C. J., Byrne, D. P., Campbell, A. E., Kalyuzhnyy, A., Myall, A., Evers, P. A., Jones, A. R., & Evers, C. E. (2019). Strong anion exchange-mediated phosphoproteomics reveals extensive human non-canonical phosphorylation. *The EMBO Journal*, 38(21), e100847. <https://doi.org/10.15252/emj.2018100847>
- He, Y., Zheng, Q., Huang, H., Ji, Y., & Lin, Z. (2022). Synergistic synthesis of hydrophilic hollow zirconium organic frameworks for simultaneous recognition and capture of phosphorylated and glycosylated peptides. *Analytica Chimica Acta*, 1198, 339552. <https://doi.org/10.1016/j.aca.2022.339552>
- Heaney, R. P., Saito, Y., & Orimo, H. (1994). Effect of caseinphosphopeptide on absorbability of co-ingested calcium in normal postmenopausal women. *Journal of Bone and Mineral Metabolism*, 12(1), 77-81. <https://doi.org/10.1007/BF02383413>
- Heemskerk, A. A. M., Busnel, J.-M., Schoenmaker, B., Derks, R. J. E., Klychnikov, O., Hensbergen, P. J., Deelder, A. M., & Mayboroda, O. A. (2012). Ultra-Low Flow Electrospray Ionization-Mass Spectrometry for Improved Ionization Efficiency in Phosphoproteomics. *Analytical Chemistry*, 84(10), 4552-4559. <https://doi.org/10.1021/ac300641x>
- Hirayama, M., Toyota, K., Hidaka, H., & Naito, H. (1992). Phosphopeptides in Rat Intestinal Digests after Ingesting Casein Phosphopeptides. *Bioscience, Biotechnology, and Biochemistry*, 56(7), 1128-1129. <https://doi.org/10.1271/bbb.56.1128>
- Holt, C. (1992). Structure and Stability of Bovine Casein Micelles. In C. B. Anfinsen, F. M. Richards, J. T. Edsall, & D. S. Eisenberg (Éds.), *Advances in Protein Chemistry* (Vol. 43, p. 63-151). Academic Press. [https://doi.org/10.1016/S0065-3233\(08\)60554-9](https://doi.org/10.1016/S0065-3233(08)60554-9)
- Hou, J., Xie, Z., Xue, P., Cui, Z., Chen, X., Li, J., Cai, T., Wu, P., & Yang, F. (2010). Enhanced MALDI-TOF MS Analysis of Phosphopeptides Using an Optimized DHAP/DAHC Matrix. *Journal of Biomedicine and Biotechnology*, 2010, e759690. <https://doi.org/10.1155/2010/759690>

- Huang, L.-J., Lin, J.-H., Tsai, J.-H., Chu, Y.-Y., Chen, Y.-W., Chen, S.-L., & Chen, S.-H. (2014). Identification of protein O-glycosylation site and corresponding glycans using liquid chromatography-tandem mass spectrometry via mapping accurate mass and retention time shift. *Journal of Chromatography. A*, *1371*, 136-145.
<https://doi.org/10.1016/j.chroma.2014.10.046>
- Iliuk, A. B., Martin, V. A., Alicie, B. M., Geahlen, R. L., & Tao, W. A. (2010). In-depth Analyses of Kinase-dependent Tyrosine Phosphoproteomes Based on Metal Ion-functionalized Soluble Nanopolymers*. *Molecular & Cellular Proteomics*, *9*(10), 2162-2172.
<https://doi.org/10.1074/mcp.M110.000091>
- Imanishi, S. Y., Kochin, V., Ferraris, S. E., de Thonel, A., Pallari, H.-M., Corthals, G. L., & Eriksson, J. E. (2007). Reference-facilitated phosphoproteomics : Fast and reliable phosphopeptide validation by microLC-ESI-Q-TOF MS/MS. *Molecular & Cellular Proteomics: MCP*, *6*(8), 1380-1391. <https://doi.org/10.1074/mcp.M600480-MCP200>
- Jagannadham, M. V., & Nagaraj, R. (2008). Detecting the site of phosphorylation in phosphopeptides without loss of phosphate group using MALDI TOF mass spectrometry. *Analytical Chemistry Insights*, *3*, 21-29. <https://doi.org/10.4137/aci.s497>
- Jiang, J., Sun, X., Li, Y., Deng, C., & Duan, G. (2018). Facile synthesis of Fe₃O₄@PDA core-shell microspheres functionalized with various metal ions : A systematic comparison of commonly-used metal ions for IMAC enrichment. *Talanta*, *178*, 600-607.
<https://doi.org/10.1016/j.talanta.2017.09.071>
- Kansci, G., Genot, C., Meynier, A., Gaucheron, F., & Chobert, J.-M. (2004). β -Caseinophosphopeptide (f1-25) confers on β -casein tryptic hydrolysate an antioxidant activity during iron/ascorbate-induced oxidation of liposomes. *Le Lait*, *84*(5), 449-462.
<https://doi.org/10.1051/lait:2004019>
- Kappeler, S., Farah, Z., & Puhani, Z. (1998). Sequence analysis of Camelus dromedarius milk caseins. *Journal of Dairy Research*, *65*(2), 209-222. <https://doi.org/10.1017/S0022029997002847>

- Kawahara, T., Katayama, D., & Otani, H. (2004). Effect of beta-casein (1-28) on proliferative responses and secretory functions of human immunocompetent cell lines. *Bioscience, Biotechnology, and Biochemistry*, 68(10), 2091-2095. <https://doi.org/10.1271/bbb.68.2091>
- Kawahara, T., & Otani, H. (2004). Stimulatory effects of casein phosphopeptide (CPP-III) on mRNA expression of cytokines in Caco-2 cells. *Bioscience, Biotechnology, and Biochemistry*, 68(8), 1779-1781. <https://doi.org/10.1271/bbb.68.1779>
- Kawasue, S., Sakaguchi, Y., Koga, R., Hayama, T., Yoshida, H., & Nohta, H. (2021). Multiple phosphorylated protein selective analysis via fluoruous derivatization and liquid chromatography-electrospray ionization-mass spectrometry analysis. *Analytical Biochemistry*, 628, 114247. <https://doi.org/10.1016/j.ab.2021.114247>
- Kibangou, I. B., Bouhallab, S., Bureau, F., Guerin, P., Allouche, S., Arhan, P., & Bouglé, D. (2005). Influence of Two Forms of Caseinophosphopeptide on Iron Bioavailability. In B. Koletzko, P. Dodds, H. Akerblom, & M. Ashwell (Éds.), *Early Nutrition and its Later Consequences : New Opportunities* (p. 221-222). Springer Netherlands. https://doi.org/10.1007/1-4020-3535-7_60
- Kibangou, I. B., Bouhallab, S., Henry, G., Bureau, F., Allouche, S., Blais, A., Guérin, P., Arhan, P., & Bouglé, D. L. (2005). Milk proteins and iron absorption : Contrasting effects of different caseinophosphopeptides. *Pediatric Research*, 58(4), 731-734. <https://doi.org/10.1203/01.PDR.0000180555.27710.46>
- Kim, J., Camp II, D. G., & Smith, R. D. (2004). Improved detection of multi-phosphorylated peptides in the presence of phosphoric acid in liquid chromatography/mass spectrometry. *Journal of Mass Spectrometry*, 39(2), 208-215. <https://doi.org/10.1002/jms.593>
- Kitts, D. D., & Nakamura, S. (2006). Calcium-enriched casein phosphopeptide stimulates release of IL-6 cytokine in human epithelial intestinal cell line. *The Journal of Dairy Research*, 73(1), 44-48. <https://doi.org/10.1017/S0022029905001330>

- Kjellström, S., & Jensen, O. N. (2004). Phosphoric Acid as a Matrix Additive for MALDI MS Analysis of Phosphopeptides and Phosphoproteins. *Analytical Chemistry*, 76(17), 5109-5117.
<https://doi.org/10.1021/ac0400257>
- Knight, Z. A., Schilling, B., Row, R. H., Kenski, D. M., Gibson, B. W., & Shokat, K. M. (2003). Phosphospecific proteolysis for mapping sites of protein phosphorylation. *Nature Biotechnology*, 21(9), 1047-1054. <https://doi.org/10.1038/nbt863>
- Krenkova, J., Moravkova, J., Buk, J., & Foret, F. (2016). Phosphopeptide enrichment with inorganic nanofibers prepared by forcespinning technology. *Journal of Chromatography A*, 1427, 8-15.
<https://doi.org/10.1016/j.chroma.2015.12.022>
- Kyono, Y., Sugiyama, N., Imami, K., Tomita, M., & Ishihama, Y. (2008). Successive and Selective Release of Phosphorylated Peptides Captured by Hydroxy Acid-Modified Metal Oxide Chromatography. *Journal of Proteome Research*, 7(10), 4585-4593.
<https://doi.org/10.1021/pr800305y>
- Laparra, J. M., Alegría, A., Barberá, R., & Farré, R. (2008). Antioxidant effect of casein phosphopeptides compared with fruit beverages supplemented with skimmed milk against H₂O₂-induced oxidative stress in Caco-2 cells. *Food Research International*, 41(7), 773-779.
<https://doi.org/10.1016/j.foodres.2008.06.002>
- Larsen, M. R., Thingholm, T. E., Jensen, O. N., Roepstorff, P., & Jørgensen, T. J. D. (2005). Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide Microcolumns *. *Molecular & Cellular Proteomics*, 4(7), 873-886.
<https://doi.org/10.1074/mcp.T500007-MCP200>
- Li-Chan, E., & Nakai, S. (1989). Enzymic dephosphorylation of bovine casein to improve acid clotting properties and digestibility for infant formula. *Journal of Dairy Research*, 56(3), 381-390.
<https://doi.org/10.1017/S0022029900028843>

- Linke, D., Koudelka, T., Becker, A., & Tholey, A. (2015). Identification and relative quantification of phosphopeptides by a combination of multi-protease digestion and isobaric labeling. *Rapid Communications in Mass Spectrometry*, 29(10), 919-926. <https://doi.org/10.1002/rcm.7185>
- Liu, G., Miao, J., Sun, S., Luo, Z., Xia, Z., Guo, B., Liu, F., Chen, Y., Ying, D., Miao, B., & Cao, Y. (2017). The effect of dietary magnesium and caseinphosphopeptides on bone metabolism in rats. *Food & Function*, 8(12), 4487-4495. <https://doi.org/10.1039/c7fo00842b>
- Lu, Q., Chen, C., Xiong, Y., Li, G., Zhang, X., Zhang, Y., Wang, D., Zhu, Z., Li, X., Qing, G., Sun, T., & Liang, X. (2020). High-Efficiency Phosphopeptide and Glycopeptide Simultaneous Enrichment by Hydrogen Bond-based Bifunctional Smart Polymer. *Analytical Chemistry*, 92(9), 6269-6277. <https://doi.org/10.1021/acs.analchem.9b02643>
- Ma, W.-F., Zhang, C., Zhang, Y.-T., Yu, M., Guo, J., Zhang, Y., Lu, H.-J., & Wang, C.-C. (2014). Magnetic MSP@ZrO₂ Microspheres with Yolk-Shell Structure : Designed Synthesis and Application in Highly Selective Enrichment of Phosphopeptides. *Langmuir*, 30(22), 6602-6611. <https://doi.org/10.1021/la501381v>
- Maestracci, D. (1976). Enzymic solubilization of the human intestinal brush border membrane enzymes. *Biochimica Et Biophysica Acta*, 433(3), 469-481. [https://doi.org/10.1016/0005-2736\(76\)90274-1](https://doi.org/10.1016/0005-2736(76)90274-1)
- Mamone, G., Picariello, G., Ferranti, P., & Addeo, F. (2010). Hydroxyapatite affinity chromatography for the highly selective enrichment of mono- and multi-phosphorylated peptides in phosphoproteome analysis. *PROTEOMICS*, 10(3), 380-393. <https://doi.org/10.1002/pmic.200800710>
- Mann, M., Ong, S.-E., Grønborg, M., Steen, H., Jensen, O. N., & Pandey, A. (2002). Analysis of protein phosphorylation using mass spectrometry : Deciphering the phosphoproteome. *Trends in Biotechnology*, 20(6), 261-268. [https://doi.org/10.1016/S0167-7799\(02\)01944-3](https://doi.org/10.1016/S0167-7799(02)01944-3)

- Masotti, F., Cattaneo, S., Stuknytė, M., & De Noni, I. (2018). Assessment of casein phosphopeptide profile in in vitro digestates of Trentingrana PDO cheese. *European Food Research and Technology*, 244(3), 513-521. <https://doi.org/10.1007/s00217-017-2970-7>
- Matéos, A., Girardet, J.-M., Mollé, D., Corbier, C., Gaillard, J.-L., & Miclo, L. (2010). Identification of phosphorylation sites of equine β -casein isoforms. *Rapid Communications in Mass Spectrometry*, 24(11), 1533-1542. <https://doi.org/10.1002/rcm.4552>
- Matéos, A., Miclo, L., Mollé, D., Dary, A., Girardet, J.-M., & Gaillard, J.-L. (2009). Equine α S1-casein : Characterization of alternative splicing isoforms and determination of phosphorylation levels. *Journal of Dairy Science*, 92(8), 3604-3615. <https://doi.org/10.3168/jds.2009-2125>
- Matsui, T., Yano, H., Awano, T., Harumoto, T., & Saito, Y. (1994). The influences of casein phosphopeptides on metabolism of ectopic bone induced by decalcified bone matrix implantation in rats. *Journal of Nutritional Science and Vitaminology*, 40(2), 137-145. <https://doi.org/10.3177/jnsv.40.137>
- McDonagh, D., & FitzGerald, R. J. (1998). Production of caseinophosphopeptides (CPPs) from sodium caseinate using a range of commercial protease preparations. *International Dairy Journal*, 8(1), 39-45. [https://doi.org/10.1016/S0958-6946\(98\)00019-3](https://doi.org/10.1016/S0958-6946(98)00019-3)
- McLachlin, D. T., & Chait, B. T. (2003). Improved β -Elimination-Based Affinity Purification Strategy for Enrichment of Phosphopeptides. *Analytical Chemistry*, 75(24), 6826-6836. <https://doi.org/10.1021/ac034989u>
- Meisel, H., Meisel, H., Fairweather-Tait, S., FitzGerald, R. J., Hartmann, R., Lane, C. N., McDonagh, D., Teucher, B., & Wal, J. M. (2003). Detection of caseinophosphopeptides in the distal ileostomy fluid of human subjects. *British Journal of Nutrition*, 89(3), 351-358. <https://doi.org/10.1079/BJN2002803>
- Ménard, O., Cattenoz, T., Guillemin, H., Souchon, I., Deglaire, A., Dupont, D., & Picque, D. (2014). Validation of a new in vitro dynamic system to simulate infant digestion. *Food Chemistry*, 145, 1039-1045. <https://doi.org/10.1016/j.foodchem.2013.09.036>

- Mercier, J. C., Addeo, F., & Pélissier, J. P. (1976). [Primary structure of the casein macropeptide of caprine kappa casein]. *Biochimie*, 58(11-12), 1303-1310.
- Miclo, L., Roux, É., Genay, M., Brusseau, É., Poirson, C., Jameh, N., Perrin, C., & Dary, A. (2012). Variability of Hydrolysis of β -, α 1-, and α 2-Caseins by 10 Strains of *Streptococcus thermophilus* and Resulting Bioactive Peptides. *Journal of Agricultural and Food Chemistry*, 60(2), 554-565. <https://doi.org/10.1021/jf202176d>
- Min, Q., Li, S., Chen, X., Abdel-Halim, E. S., Jiang, L.-P., & Zhu, J.-J. (2015). Magnetite/Ceria-Codecorated Titanoniobate Nanosheet : A 2D Catalytic Nanoprobe for Efficient Enrichment and Programmed Dephosphorylation of Phosphopeptides. *ACS Applied Materials & Interfaces*, 7(18), 9563-9572. <https://doi.org/10.1021/acsami.5b01006>
- Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, R., Corredig, M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes, U., Macierzanka, A., Mackie, A., ... Brodkorb, A. (2014). A standardised static in vitro digestion method suitable for food—An international consensus. *Food & Function*, 5(6), 1113-1124. <https://doi.org/10.1039/c3fo60702j>
- Minikiewicz, P., Slangen, C. J., Lagerwerf, F. M., Haverkamp, J., Rollema, H. S., & Visser, S. (1996). Reversed-phase high-performance liquid chromatographic separation of bovine kappa-casein macropeptide and characterization of isolated fractions. *Journal of Chromatography. A*, 743(1), 123-135. [https://doi.org/10.1016/0021-9673\(96\)00122-7](https://doi.org/10.1016/0021-9673(96)00122-7)
- Miquel, E., Alegría, A., Barberá, R., & Farré, R. (2006). Casein phosphopeptides released by simulated gastrointestinal digestion of infant formulas and their potential role in mineral binding. *International Dairy Journal*, 16(9), 992-1000. <https://doi.org/10.1016/j.idairyj.2005.10.010>
- Miquel, E., Ángel Gómez, J., Alegría, A., Barberá, R., Farré, R., & Recio, I. (2006). Identification of casein phosphopeptides after simulated gastrointestinal digestion by tandem mass spectrometry. *European Food Research and Technology*, 222(1), 48-53. <https://doi.org/10.1007/s00217-005-0023-0>

- Miquel, E., & Farré, R. (2007). Effects and future trends of casein phosphopeptides on zinc bioavailability. *Trends in Food Science & Technology*, 18(3), 139-143.
<https://doi.org/10.1016/j.tifs.2006.11.004>
- Mirza, M. R., Rainer, M., Güzel, Y., Choudhary, I. M., & Bonn, G. K. (2012). A novel strategy for phosphopeptide enrichment using lanthanide phosphate co-precipitation. *Analytical and Bioanalytical Chemistry*, 404(3), 853-862. <https://doi.org/10.1007/s00216-012-6215-0>
- Molina, H., Horn, D. M., Tang, N., Mathivanan, S., & Pandey, A. (2007). Global proteomic profiling of phosphopeptides using electron transfer dissociation tandem mass spectrometry. *Proceedings of the National Academy of Sciences*, 104(7), 2199-2204.
<https://doi.org/10.1073/pnas.0611217104>
- Motoyama, A., Xu, T., Ruse, C. I., Wohlschlegel, J. A., & Yates, J. R. (2007). Anion and Cation Mixed-Bed Ion Exchange for Enhanced Multidimensional Separations of Peptides and Phosphopeptides. *Analytical Chemistry*, 79(10), 3623-3634.
<https://doi.org/10.1021/ac062292d>
- Mullaiselvan, I., Kanagaraj, V., Sekar, S., Dharmar, B., & Sambandan, R. (2020). Studies on Immunomodulatory Effect of Casein Phospho Peptide Isolated from Cultured Dairy Product. *International Research Journal of Pure and Applied Chemistry*, 28-40.
<https://doi.org/10.9734/irjpac/2020/v21i2330300>
- Muro Urista, C., Álvarez Fernández, R., Riera Rodríguez, F., Arana Cuenca, A., & Téllez Jurado, A. (2011). Review : Production and functionality of active peptides from milk. *Food Science and Technology International = Ciencia Y Tecnologia De Los Alimentos Internacional*, 17(4), 293-317. <https://doi.org/10.1177/1082013211398801>
- Mykkänen, H. M., & Wasserman, R. H. (1980). Enhanced absorption of calcium by casein phosphopeptides in rachitic and normal chicks. *The Journal of Nutrition*, 110(11), 2141-2148.
<https://doi.org/10.1093/jn/110.11.2141>

- Narva, M., Nevala, R., Poussa, T., & Korpela, R. (2004). The effect of *Lactobacillus helveticus* fermented milk on acute changes in calcium metabolism in postmenopausal women. *European Journal of Nutrition*, *43*(2), 61-68. <https://doi.org/10.1007/s00394-004-0441-y>
- Ondrej, M., Rehulka, P., Rehulkova, H., Kupcik, R., & Tichy, A. (2020). Fractionation of Enriched Phosphopeptides Using pH/Acetonitrile-Gradient-Reversed-Phase Microcolumn Separation in Combination with LC–MS/MS Analysis. *International Journal of Molecular Sciences*, *21*(11), 3971. <https://doi.org/10.3390/ijms21113971>
- ONO, T., TAKAGI, Y., & KUNISHI, I. (1998). Casein Phosphopeptides from Casein Micelles by Successive Digestion with Pepsin and Trypsin. *Bioscience, Biotechnology, and Biochemistry*, *62*(1), 16-21. <https://doi.org/10.1271/bbb.62.16>
- Otani, H., Watanabe, T., & Tashiro, Y. (2001). Effects of bovine beta-casein (1-28) and its chemically synthesized partial fragments on proliferative responses and immunoglobulin production in mouse spleen cell cultures. *Bioscience, Biotechnology, and Biochemistry*, *65*(11), 2489-2495. <https://doi.org/10.1271/bbb.65.2489>
- Palumbo, A. M., Tepe, J. J., & Reid, G. E. (2008). Mechanistic Insights into the Multistage Gas-Phase Fragmentation Behavior of Phosphoserine- and Phosphothreonine-Containing Peptides. *Journal of Proteome Research*, *7*(2), 771-779. <https://doi.org/10.1021/pr0705136>
- Paris, J., Morgan, T. E., Wootton, C. A., Barrow, M. P., O'Hara, J., & O'Connor, P. B. (2020). Facile Determination of Phosphorylation Sites in Peptides Using Two-Dimensional Mass Spectrometry. *Analytical Chemistry*, *92*(10), 6817-6821. <https://doi.org/10.1021/acs.analchem.0c00884>
- Parkinson, G. B., & Cransberg, P. H. (2004). Effect of casein phosphopeptide and 25-hydroxycholecalciferol on tibial dyschondroplasia in growing broiler chickens. *British Poultry Science*, *45*(6), 802-806. <https://doi.org/10.1080/00071660400012733>
- Perego, S., Cosentino, S., Fiorilli, A., Tettamanti, G., & Ferraretto, A. (2012). Casein phosphopeptides modulate proliferation and apoptosis in HT-29 cell line through their interaction with

- voltage-operated L-type calcium channels. *The Journal of Nutritional Biochemistry*, 23(7), 808-816. <https://doi.org/10.1016/j.jnutbio.2011.04.004>
- Perego, S., Del Favero, E., De Luca, P., Dal Piaz, F., Fiorilli, A., Cantu', L., & Ferraretto, A. (2015). Calcium bioaccessibility and uptake by human intestinal like cells following in vitro digestion of casein phosphopeptide-calcium aggregates. *Food & Function*, 6(6), 1796-1807. <https://doi.org/10.1039/c4fo00672k>
- Perego, S., Zabeo, A., Marasco, E., Giussani, P., Fiorilli, A., Tettamanti, G., & Ferraretto, A. (2013). Casein phosphopeptides modulate calcium uptake and apoptosis in Caco2 cells through their interaction with the TRPV6 calcium channel. *Journal of Functional Foods*, 5(2), 847-857. <https://doi.org/10.1016/j.jff.2013.01.032>
- Pérès, J. M., Bouhallab, S., Bureau, F., Neuville, D., Maubois, J. L., Devroede, G., Arhan, P., & Bouglé, D. (1999). Mechanisms of absorption of caseinophosphopeptide bound iron. *The Journal of Nutritional Biochemistry*, 10(4), 215-222. [https://doi.org/10.1016/s0955-2863\(98\)00101-6](https://doi.org/10.1016/s0955-2863(98)00101-6)
- Picariello, G., Ferranti, P., Fierro, O., Mamone, G., Caira, S., Di Luccia, A., Monica, S., & Addeo, F. (2010). Peptides surviving the simulated gastrointestinal digestion of milk proteins : Biological and toxicological implications. *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, 878(3-4), 295-308. <https://doi.org/10.1016/j.jchromb.2009.11.033>
- Pinto, G., Caira, S., Cuollo, M., Lilla, S., Chianese, L., & Addeo, F. (2012). Bioactive Casein Phosphopeptides in Dairy Products as Nutraceuticals for Functional Foods. *Milk Protein*. <https://doi.org/10.5772/50725>
- Poot, A. J., Ruijter, E., Nuijens, T., Dirksen, E. H. C., Heck, A. J. R., Slijper, M., Rijkers, D. T. S., & Liskamp, R. M. J. (2006). Selective enrichment of Ser-/Thr-phosphorylated peptides in the presence of Ser-/Thr-glycosylated peptides. *PROTEOMICS*, 6(24), 6394-6399. <https://doi.org/10.1002/pmic.200600373>

- Potel, C. M., Lemeer, S., & Heck, A. J. R. (2019). Phosphopeptide Fragmentation and Site Localization by Mass Spectrometry : An Update. *Analytical Chemistry*, *91*(1), 126-141.
<https://doi.org/10.1021/acs.analchem.8b04746>
- Qiu, W., Evans, C. A., Landels, A., Pham, T. K., & Wright, P. C. (2020). Phosphopeptide enrichment for phosphoproteomic analysis—A tutorial and review of novel materials. *Analytica Chimica Acta*, *1129*, 158-180. <https://doi.org/10.1016/j.aca.2020.04.053>
- Raja, R. B., & Arunachalam, K. D. (2011). Anti-genotoxic potential of casein phosphopeptides (CPPs) : A class of fermented milk peptides against low background radiation and prevention of cancer in radiation workers. *Toxicology and Industrial Health*, *27*(10), 867-872.
<https://doi.org/10.1177/0748233711407244>
- Raveschot, C., Cudennec, B., Coutte, F., Flahaut, C., Fremont, M., Drider, D., & Dhulster, P. (2018). Production of Bioactive Peptides by Lactobacillus Species : From Gene to Application. *Frontiers in Microbiology*, *9*, 2354. <https://doi.org/10.3389/fmicb.2018.02354>
- Ribadeau Dumas, B., Brignon, G., Grosclaude, F., & Mercier, J. C. (1972). [Primary structure of bovine beta casein. Complete sequence]. *European Journal of Biochemistry*, *25*(3), 505-514.
<https://doi.org/10.1111/j.1432-1033.1972.tb01722.x>
- Robinson, M. R. (2016). *Advancement of photodissociation mass spectrometry methods for the analysis of protein post-translational modifications* [Thesis].
<https://doi.org/10.15781/T2XW48F40>
- Roy, D., Ye, A., Moughan, P. J., & Singh, H. (2020). Composition, Structure, and Digestive Dynamics of Milk From Different Species—A Review. *Frontiers in Nutrition*, *7*, 577759.
<https://doi.org/10.3389/fnut.2020.577759>
- Saeed, A., Najam-ul-Haq, M., Jabeen, F., & Svec, F. (2013). High Affinity Phosphopeptides Enrichment and Desalting of Biological Materials on Newly Engineered Poly(Glycidyl Propargyl Ether/Divinyl Benzene). *Analytical Chemistry*, *85*(19), 8979-8986.
<https://doi.org/10.1021/ac4015484>

- Saito, Y., Lee, Y. S., & Kimura, S. (1998). Minimum effective dose of casein phosphopeptides (CPP) for enhancement of calcium absorption in growing rats. *International Journal for Vitamin and Nutrition Research. Internationale Zeitschrift Fur Vitamin- Und Ernährungsforschung. Journal International De Vitaminologie Et De Nutrition*, 68(5), 335-340.
- Salimi, K., Usta, D. D., Çelikbıçak, Ö., Pinar, A., Salih, B., & Tuncel, A. (2017). Ti(IV) carrying polydopamine-coated, monodisperse-porous SiO₂ microspheres with stable magnetic properties for highly selective enrichment of phosphopeptides. *Colloids and Surfaces B: Biointerfaces*, 153, 280-290. <https://doi.org/10.1016/j.colsurfb.2017.02.028>
- Sanchón, J., Fernández-Tomé, S., Miralles, B., Hernández-Ledesma, B., Tomé, D., Gaudichon, C., & Recio, I. (2018). Protein degradation and peptide release from milk proteins in human jejunum. Comparison with in vitro gastrointestinal simulation. *Food Chemistry*, 239, 486-494. <https://doi.org/10.1016/j.foodchem.2017.06.134>
- Sato, R., Noguchi, T., & Naito, H. (1986). Casein Phosphopeptide (CPP) Enhances Calcium Absorption from the Ligated Segment of Rat Small Intestine. *Journal of Nutritional Science and Vitaminology*, 32(1), 67-76. <https://doi.org/10.3177/jnsv.32.67>
- Sato, R., Shindo, M., Gunshin, H., Noguchi, T., & Naito, H. (1991). Characterization of phosphopeptide derived from bovine β -casein : An inhibitor to intra-intestinal precipitation of calcium phosphate. *Biochimica et Biophysica Acta (BBA) - Protein Structure and Molecular Enzymology*, 1077(3), 413-415. [https://doi.org/10.1016/0167-4838\(91\)90559-I](https://doi.org/10.1016/0167-4838(91)90559-I)
- Savastano, M. L., Liu, Y., Mels, J., Dittrich, D., Haus, S., Gensberger-Reigl, S., & Pischetsrieder, M. (2019). Profiling of Multiphosphorylated Peptides in Kefir and Their Release During Simulated Gastrointestinal Digestion. *ACS Omega*, 4(5), 7963-7970. <https://doi.org/10.1021/acsomega.8b03105>
- Schmelzer, C. E. H., Schöps, R., Reynell, L., Ulbrich-Hofmann, R., Neubert, R. H. H., & Raith, K. (2007). Peptic digestion of β -casein : Time course and fate of possible bioactive peptides. *Journal of Chromatography A*, 1166(1), 108-115. <https://doi.org/10.1016/j.chroma.2007.08.015>

- Schmelzer, C. E. H., Schöps, R., Ulbrich-Hofmann, R., Neubert, R. H. H., & Raith, K. (2004). Mass spectrometric characterization of peptides derived by peptic cleavage of bovine β -casein. *Journal of Chromatography A*, *1055*(1), 87-92. <https://doi.org/10.1016/j.chroma.2004.09.003>
- Seidler, J., Zinn, N., Haaf, E., Boehm, M. E., Winter, D., Schlosser, A., & Lehmann, W. D. (2011). Metal ion-mobilizing additives for comprehensive detection of femtomole amounts of phosphopeptides by reversed phase LC-MS. *Amino Acids*, *41*(2), 311-320. <https://doi.org/10.1007/s00726-010-0647-7>
- Smialowska, A., Matia-Merino, L., & Carr, A. J. (2017). Assessing the iron chelation capacity of goat casein digest isolates. *Journal of Dairy Science*, *100*(4), 2553-2563. <https://doi.org/10.3168/jds.2016-12090>
- Sousa, M. J., Ardö, Y., & McSweeney, P. L. H. (2001). Advances in the study of proteolysis during cheese ripening. *International Dairy Journal*, *11*(4), 327-345. [https://doi.org/10.1016/S0958-6946\(01\)00062-0](https://doi.org/10.1016/S0958-6946(01)00062-0)
- Steen, H., & Mann, M. (2002). A new derivatization strategy for the analysis of phosphopeptides by precursor ion scanning in positive ion mode. *Journal of the American Society for Mass Spectrometry*, *13*(8), 996-1003. [https://doi.org/10.1016/S1044-0305\(02\)00415-4](https://doi.org/10.1016/S1044-0305(02)00415-4)
- Su, R., Qi, W., He, Z., Yuan, S., & Zhang, Y. (2007). Pancreatic hydrolysis of bovine casein : Identification and release kinetics of phosphopeptides. *Food Chemistry*, *104*(1), 276-286. <https://doi.org/10.1016/j.foodchem.2006.11.039>
- Sugiyama, N., Masuda, T., Shinoda, K., Nakamura, A., Tomita, M., & Ishihama, Y. (2007). Phosphopeptide enrichment by aliphatic hydroxy acid-modified metal oxide chromatography for nano-LC-MS/MS in proteomics applications. *Molecular & Cellular Proteomics: MCP*, *6*(6), 1103-1109. <https://doi.org/10.1074/mcp.T600060-MCP200>
- Sun, Z., Hamilton, K. L., & Reardon, K. F. (2014). Evaluation of quantitative performance of sequential immobilized metal affinity chromatographic enrichment for phosphopeptides. *Analytical Biochemistry*, *445*, 30-37. <https://doi.org/10.1016/j.ab.2013.09.025>

- Tabang, D. N., Cui, Y., Tremmel, D. M., Ford, M., Li, Z., Sackett, S. D., Odorico, J. S., & Li, L. (2021). Analysis of pancreatic extracellular matrix protein post-translational modifications via electrostatic repulsion-hydrophilic interaction chromatography coupled with mass spectrometry. *Molecular Omics*, 17(5), 652-664. <https://doi.org/10.1039/D1MO00104C>
- Tan, F., Zhang, Y., Wang, J., Wei, J., Cai, Y., & Qian, X. (2008). An efficient method for dephosphorylation of phosphopeptides by cerium oxide. *Journal of Mass Spectrometry*, 43(5), 628-632. <https://doi.org/10.1002/jms.1362>
- Tang, L. A. L., Wang, J., Lim, T. K., Bi, X., Lee, W. C., Lin, Q., Chang, Y.-T., Lim, C. T., & Loh, K. P. (2012). High-Performance Graphene-Titania Platform for Detection of Phosphopeptides in Cancer Cells. *Analytical Chemistry*, 84(15), 6693-6700. <https://doi.org/10.1021/ac301119r>
- Tao, W. A., Wollscheid, B., O'Brien, R., Eng, J. K., Li, X., Bodenmiller, B., Watts, J. D., Hood, L., & Aebersold, R. (2005). Quantitative phosphoproteome analysis using a dendrimer conjugation chemistry and tandem mass spectrometry. *Nature Methods*, 2(8), 591-598. <https://doi.org/10.1038/nmeth776>
- Tauzin, J., Miclo, L., Roth, S., Mollé, D., & Gaillard, J.-L. (2003). Tryptic hydrolysis of bovine α S2-casein : Identification and release kinetics of peptides. *International Dairy Journal*, 13(1), 15-27. [https://doi.org/10.1016/S0958-6946\(02\)00127-9](https://doi.org/10.1016/S0958-6946(02)00127-9)
- Thingholm, T. E., & Larsen, M. R. (2016). The Use of Titanium Dioxide for Selective Enrichment of Phosphorylated Peptides. In L. von Stechow (Éd.), *Phospho-Proteomics : Methods and Protocols* (p. 135-146). Springer. https://doi.org/10.1007/978-1-4939-3049-4_9
- Thingholm, T. E., Larsen, M. R., Ingrell, C. R., Kassem, M., & Jensen, O. N. (2008). TiO₂-Based Phosphoproteomic Analysis of the Plasma Membrane and the Effects of Phosphatase Inhibitor Treatment. *Journal of Proteome Research*, 7(8), 3304-3313. <https://doi.org/10.1021/pr800099y>

- Thingholm, T. E., Palmisano, G., Kjeldsen, F., & Larsen, M. R. (2010). Undesirable Charge-Enhancement of Isobaric Tagged Phosphopeptides Leads to Reduced Identification Efficiency. *Journal of Proteome Research*, 9(8), 4045-4052. <https://doi.org/10.1021/pr100230q>
- Tooley, A., Reed, J., & Lehmann, W. D. (1999). Electrospray tandem mass spectrometric studies of phosphopeptides and phosphopeptide analogues. *Journal of Mass Spectrometry*, 34(2), 117-123. [https://doi.org/10.1002/\(SICI\)1096-9888\(199902\)34:2<117::AID-JMS769>3.0.CO;2-V](https://doi.org/10.1002/(SICI)1096-9888(199902)34:2<117::AID-JMS769>3.0.CO;2-V)
- Tobita, K., Kawahara, T., & Otani, H. (2006). Bovine beta-casein (1-28), a casein phosphopeptide, enhances proliferation and IL-6 expression of mouse CD19+ cells via Toll-like receptor 4. *Journal of Agricultural and Food Chemistry*, 54(21), 8013-8017. <https://doi.org/10.1021/jf0610864>
- Tsuchita, H., Goto, T., Shimizu, T., Yonehara, Y., & Kuwata, T. (1996). Dietary casein phosphopeptides prevent bone loss in aged ovariectomized rats. *The Journal of Nutrition*, 126(1), 86-93. <https://doi.org/10.1093/jn/126.1.86>
- Tsuchita, H., Sekiguchi, I., Kuwata, T., Igarashi, C., & Ezawa, I. (1993). The effect of casein phosphopeptides on calcium utilization in young ovariectomized rats. *Zeitschrift Für Ernährungswissenschaft*, 32(2), 121-130. <https://doi.org/10.1007/BF01614755>
- Tsuchita, H., Suzuki, T., & Kuwata, T. (2001). The effect of casein phosphopeptides on calcium absorption from calcium-fortified milk in growing rats. *The British Journal of Nutrition*, 85(1), 5-10. <https://doi.org/10.1079/bjn2000206>
- Wang, H., Duan, J., Zhang, L., Liang, Z., Zhang, W., & Zhang, Y. (2008). Characterization of multi-phosphopeptides by μ HPLC-ESI-MS/MS with alkaline phosphatase treatment. *Journal of Separation Science*, 31(3), 480-487. <https://doi.org/10.1002/jssc.200700445>
- Wu, S.-L., Kim, J., Hancock, W. S., & Karger, B. (2005). Extended Range Proteomic Analysis (ERPA) : A new and sensitive LC-MS platform for high sequence coverage of complex proteins with extensive post-translational modifications-comprehensive analysis of beta-casein and

- epidermal growth factor receptor (EGFR). *Journal of Proteome Research*, 4(4), 1155-1170.
<https://doi.org/10.1021/pr050113n>
- Yeh, T.-T., Ho, M.-Y., Chen, W.-Y., Hsu, Y.-C., Ku, W.-C., Tseng, H.-W., Chen, S.-T., & Chen, S.-F. (2019). Comparison of different fractionation strategies for in-depth phosphoproteomics by liquid chromatography tandem mass spectrometry. *Analytical and Bioanalytical Chemistry*, 411(15), 3417-3424. <https://doi.org/10.1007/s00216-019-01823-0>
- Yeung, A. C., Glahn, R. P., & Miller, D. D. (2001). Dephosphorylation of sodium caseinate, enzymatically hydrolyzed casein and casein phosphopeptides by intestinal alkaline phosphatase : Implications for iron availability. *The Journal of Nutritional Biochemistry*, 12(5), 292-299. [https://doi.org/10.1016/s0955-2863\(01\)00141-3](https://doi.org/10.1016/s0955-2863(01)00141-3)
- Yeung, A. C., Glahn, R. P., & Miller, D. D. (2002). Effects of Iron Source on Iron Availability from Casein and Casein Phosphopeptides. *Journal of Food Science*, 67(3), 1271-1275.
<https://doi.org/10.1111/j.1365-2621.2002.tb09489.x>
- Yoshikawa, M., Sasaki, R., & Chiba, H. (1981). Effects of Chemical Phosphorylation of Bovine Casein Components on the Properties Related to Casein Micelle Formation. *Agricultural and Biological Chemistry*, 45(4), 909-914. <https://doi.org/10.1080/00021369.1981.10864626>
- Zhai, R., Tian, F., Xue, R., Jiao, F., Hao, F., Zhang, Y., & Qian, X. (2015). Metal ion-immobilized magnetic nanoparticles for global enrichment and identification of phosphopeptides by mass spectrometry. *RSC Advances*, 6(2), 1670-1677. <https://doi.org/10.1039/C5RA22006H>
- Zhang, L., Liang, Z., Zhang, L., Zhang, Y., & Shao, S. (2015). Facile synthesis of gallium ions immobilized and adenosine functionalized magnetic nanoparticles with high selectivity for multi-phosphopeptides. *Analytica Chimica Acta*, 900, 46-55.
<https://doi.org/10.1016/j.aca.2015.10.012>
- Zhang, Y., Ficarro, S. B., Li, S., & Marto, J. A. (2009). Optimized orbitrap HCD for quantitative analysis of phosphopeptides. *Journal of the American Society for Mass Spectrometry*, 20(8), 1425-1434. <https://doi.org/10.1016/j.jasms.2009.03.019>

Zhu, Y.-S., & FitzGerald, R. J. (2012). Caseinophosphopeptide enrichment and identification.

International Journal of Food Science & Technology, 47(10), 2235-2242.

<https://doi.org/10.1111/j.1365-2621.2012.03094.x>

Zidane, F., Matéos, A., Cakir-Kiefer, C., Miclo, L., Rahuel-Clermont, S., Girardet, J.-M., & Corbier, C.

(2012). Binding of divalent metal ions to 1–25 β -caseinophosphopeptide : An isothermal titration calorimetry study. *Food Chemistry*, 132(1), 391-398.

<https://doi.org/10.1016/j.foodchem.2011.11.006>

Zou, Y., Jiang, W., Zou, L., Li, X., & Liang, X. (2013). [Ionization suppression effect of phosphopeptides

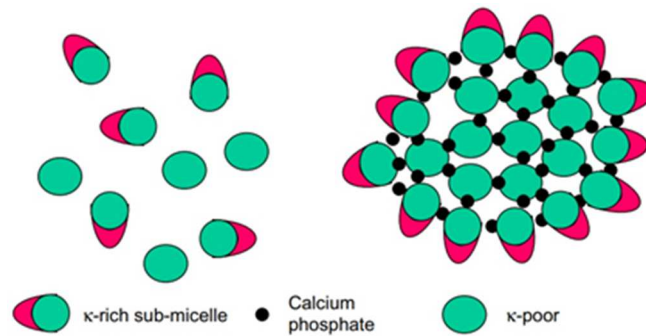
in nano-electrospray ionization quadrupole time-of-flight mass spectrometry]. *Se pu =*

Chinese journal of chromatography, 31(4), 367-371.

<https://doi.org/10.3724/sp.j.1123.2012.11024>

Figure 1

A



B

>sp|P02662|CASA1_BOVIN **Alpha-S1-casein** OS=Bos taurus OX=9913 GN=CSN1S1 PE=1 SV=2

```
MKLLIILCLVAVALARPKHPKIKHGLPQEVLENENLLRFFVAPFPEVFGKE # 50
KVNELSKDIGSESSTEDQAMEDIKQMEAESISSSEEEIVPNSVEQKHIQKED # 100
VPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPM # 150
IGVNQELAYFYPELFRQFYQLDAYPSGAWYYVPLGTQYTDAPSFSDI PNP # 200
IGSENSEKTTMPLW # 214
```

```
.....T.....
...S...S.S.T.....S.SSS.....S.....
..S.....Y.....S.....S.....
.....Y.....T.Y.....S.S.....
.....S.T.....
```

>sp|P02666|CASB_BOVIN **Beta-casein** OS=Bos taurus OX=9913 GN=CSN2 PE=1 SV=2

```
MKVLILACLVALALARELEELNVPGEIVESLSSSEESITRINKKIEKFS* # 50
EEQQQTEDELQDKIHPPAQTSLVYPPFGPIPNSLPQNIPLTQTPVVVP # 100
PFLQPEVMGVSKEAMAPKHKEMPFPKYVPEPFTESQSLTLTVDENLHL # 150
PLPLLQSWMHQPHQPLPPTVMFPQSVLSLSQSKVLPVPQKAVPYPQRDM # 200
PIQAFLLYQEPVLGPRVGRFPPIIV # 224
```

```
.....S.SSS...S.....S
...T.....T.....S.....T.T.....
.....S.S.T.T.....
.....T.....S.S.S.....
```

>sp|P02663|CASA2_BOVIN **Alpha-S2-casein** OS=Bos taurus OX=9913 GN=CSN1S2 PE=1 SV=2

```
MKFFIFTCLLAVALAKNTMEHVSSSEESIISQETKQEKMAINP$KENL # 50
CSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDKHYQKALNEI # 100
NQFYQKFPQYLQYLYQGPIVLNPDQVKNRNPITPTLNREQL$T$EENS$ # 150
KKTVDMESTEVEFTKTKLTEEKNRLNFKKISQRYQK$FALPQYLKTVYQ # 200
HQBAMKFWIQPKTKVIPYVRYL # 222
```

```
.....SSS.S.S.TY.....S...
..T.....YS.SS.S$.....T.....
...Y.....T.T.....$T$S...S
...ST...T...T.....S.....T...
.....
```

>sp|P02668|CASK_BOVIN **Kappa-casein** OS=Bos taurus OX=9913 GN=CSN3 PE=1 SV=1

```
MMKSFFLVVTILALTLPLFLGAQEQNQEQPIRCEKDERFFSDKIAYIPIQ # 50
YVLSRYPSYGLNYQQKPVVALINNQLFLPYPYAKPAAVRSPAQILQWQVL # 100
SNTVPAKSCQAQPTTMRHPHLSFMAI$PKKNQDKTEIPTINTIA$GE # 150
PTSTPTTEAVESTVATLED$PEVIESPPEINTVQVT$TAV # 190
```

```
...S.....S.....
.....S.....Y.....S.....
S.T...S...TT.....S.....S
.TST..T...ST..T..$S...S...T.....
```

Figure 1. Macromolecular organization, primary peptide sequences and phosphosites of bovine milk caseins. A and B) schematic representation of micellar caseins showing the intrinsic molecular association and organization of micellar caseins: A) Figure for Horne *et al.* (2006): The submicellar model. This latter illustrates the two kinds of submicelles: the submicelles in the heart of the micellar caseins are hydrophobes, while those at the periphery of the micellar caseins are hydrophiles. The submicelles are linked together through colloidal calcium phosphate bridges. B) Amino acid sequences of casein precursors extracted from UniprotKB (access on line, June 2021) highlighting the experimentally-reported phosphorylation sites (red, bold and underlined case: 1] for CASA1; 2,3] for CASA2); 4-6] for CASB and 7-10] for CASK) or referenced by similarity (red, bold and dash-underlined case) and the potential phosphorylation sites predicted using NetPhos 3.1 (access on line, June 2021) where normal case reports the potential phosphorylation sites which were not experimentally confirmed

until today. * β -casein phosphoserine in variant A1, A2, A3, B, E, F, G and H; # alternance between phosphorylation and O-glycosylation. Note that the phosphorylation, deduced by similarity, of $^{187}\text{pSer}$ from CASK is not predicted by NetPhos 3.1.

Figure 2

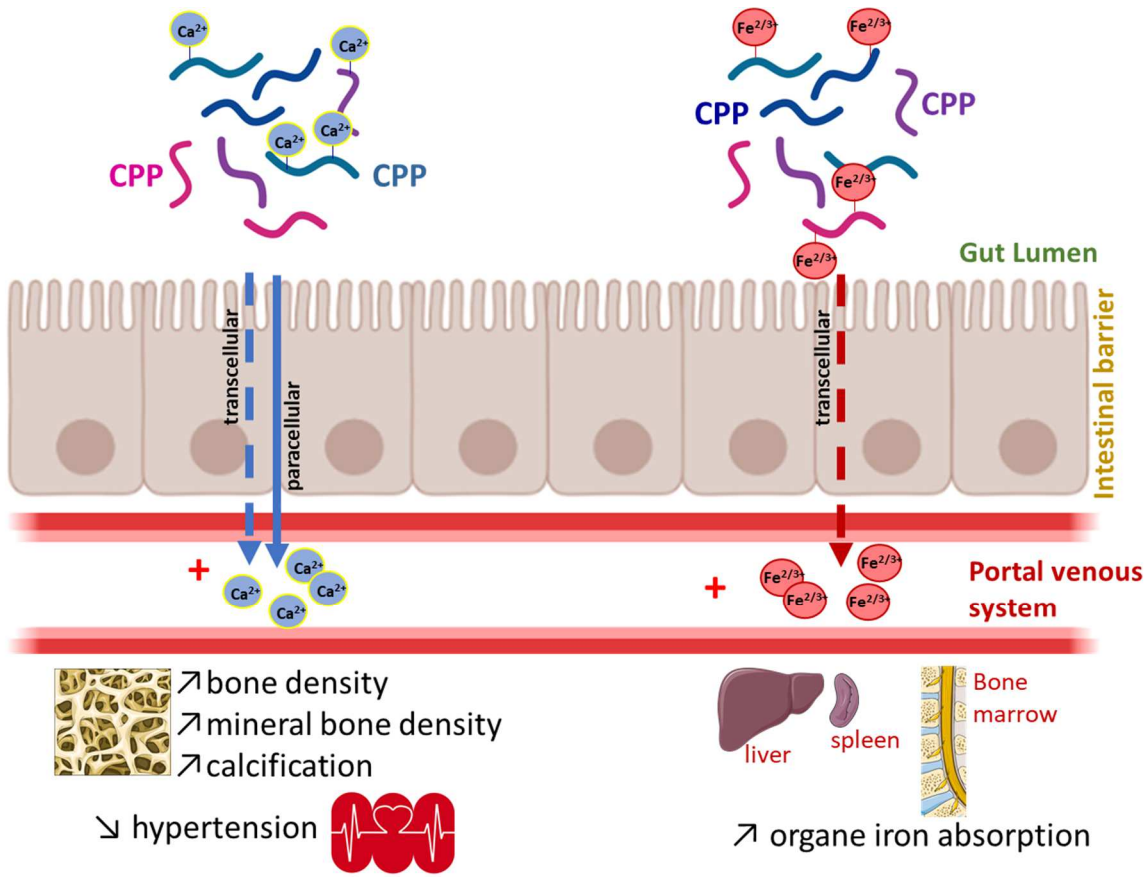


Figure 2. Schematic model of CPP bioactive roles. During digestion, different CPPs are released through the action of digestive enzymes. In the gastrointestinal tract, these different phosphorylated peptides chelate minerals, including calcium and iron ions. This chelation leads to an increased mineral passage through the intestinal barrier by transepithelial and/or paracellular pathways. On the one hand, increasing calcium blood concentration is involved in bone density, mineral bone density and calcification increase, and hypertension reduction. On the other hand, increased iron absorption by CPPS leads to better iron absorption by different organs like the liver, spleen and bone marrow.

		Methods	Benefits	Drawbacks	References
Conventional methods (old methods)	Sample preparation	Selective calcic precipitation with divalent ions	Quick and inexpensive method	Lack of specificity	Adamson & Reynolds, 1995
		Prefractionation by chromatography (hydroxyapatite, anion or cation exchange)	Phosphopeptide enrichment		Ellegård et al, 1999 ; Mamone et al, 2010 ; Meisel et al., 2003
	Sample analysis	Measure of a precipitation yield	Quantitative evaluation	Lack of precision (under or over estimation)	Adamson & Reynolds, 1995
		Liquid chromatography with UV detection	Coarse evaluation of the CPP heterogeneity	Uncertainty on the detection of unphosphorylated or phosphorylated peptides	Adamson & Reynolds, 1995 , McDonagh & FitzGerald, 1998
Modern methods	Sample preparation	Capture (IMAC / Phos-Tag / MOAC)	Sensibility / Phosphopeptide enrichment / Commonly worldwide used/ Well documented	Background interferences / non elution of strongly fixed multiphosphopeptides	Blacken et al, 2006, 2008 ; Leitner, 2010 ; Matéos et al., 2010 ; Zhu & FitzGerald, 2012
		Dephosphorylation strategy	Identification of multi- and long-phosphorylated peptides	Multi-step protocol	Deracinois et al., 2021; Kawasue et al, 2021; Linke et al., 2015; Matéos et al., 2009; McLachlin & Chait, 2003; Steen & Mann, 2002; Tan et al., 2008 ; Wang et al., 2008
	Sample analysis (HPLC-MS)	MALDI source	Rapid visualization of the sample heterogeneity	For analysis of low molecular heterogeneity samples	Cosentino et al., 2010; Ferraretto et al., 2003; Annan & Carr, 1996; Asara & Allison, 1999; Hou et al., 2010; Jagannadham & Nagaraj, 2008; Kjellström & Jensen, 2004
		ESI source	One-line association with LC or CE / soft ionisation	Soft ionisation / Ionisation suppression phenomena	Boersema et al., 2009; Busman et al., 1996; Deracinois et al., 2021; Kawasue et al., 2021; Matéos et al., 2010; Zhu & FitzGerald, 2010; Zhu & FitzGerald, 2012
		ESI positive ion mode	Ease of processing using proteomics software	Low positive ionisation efficiency due to the negative charge of phosphate group / ionisation suppression	Zhu & FitzGerald, 2010; Zhu & FitzGerald, 2012; Deracinois et al., 2021; Cosentino et al., 2010 Cosentino et al 2010; Matéos et al., 2010;
		ESI negative ion mode	Better phosphopeptide ionisation efficiency	Non conventionnal in peptidomics studies	Kjellström & Jensen, 2004
		Fragmentation CID	More available in the laboratories than ETD / Phosphopeptide identification by neutral loss	Difficulties to map with accuracy the phosphosite if more than 2 sites are present in the peptide	Deracinois et al., 2021; Zhu & FitzGerald, 2010; Zhu & FitzGerald, 2012
		Fragmentation ECD / ETD	Phosphosite accurate localization / phosphopeptide mapping / Identification of CID-labile post-translational modifications	Less available in the laboratories than CID	Molina et al., 2007; Thingholm et al., 2010

Table 1 : Analytical methods for CPP characterization

Graphical abstract:

