Yeast protein extracts and hydrolysates production and characterization for application in the food industry as flavour enhancers

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Proenol
investigar, produzir, aconselhar

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Resumo

Atualmente, os consumidores priorizam cada vez mais a saúde e a consciência ambiental. Esta mudança de mentalidade e procura por fontes alimentares sustentáveis têm levado a um aumento em alternativas amigas do ambiente e à base de plantas. Neste contexto, a proteína de levedura surge como uma solução promissora e versátil para muitos dos desafios enfrentados pela indústria alimentar, oferecendo não só benefícios nutricionais, como também propriedades funcionais em aplicações alimentares. Este trabalho investiga o potencial de extratos proteicos de levedura como fonte natural do sabor umami, com o principal objetivo de desenvolver um potenciador de sabor umami pela hidrólise de extratos proteicos de levedura, para ser industrialmente produzido e comercializado pela Poenol S.A., uma empresa de biotecnologia e enologia.

A pesquisa foca-se na otimização do processo de hidrólise, tendo em conta as limitações industriais, e na caracterização dos hidrolisados obtidos. A reação de hidrólise foi acompanhada por eletroforese e cromatografia líquida de alta performance, e os hidrolisados foram analisados por espetrometria de massa. O uso de ferramentas de previsão (UMPred-FRL e iBitter-Fuse) foi crucial para a análise dos perfis de sabor umami e amargo dos péptidos. A aceitação dos potenciais produtos finais por parte dos consumidores foi avaliada por testes sensoriais, e uma análise analítica comparativa com produtos comerciais no mercado foi realizada.

Este trabalho demonstrou que a hidrólise é um processo viável para o desenvolvimento de um potenciador de sabor umami. Os resultados obtidos relativamente à caracterização de potenciais productos finais foram satisfatórios, revelando uma alta presença de péptidos umami e mostrando um potencial competitivo com o mercado atual. O feedback dos consumidores após a prova foi positivo. Este estudo originou potenciadores de sabor umami promissores, demonstrando o sucesso de esforços colaborativos entre a academia e a indústria alimentar

Palavras-chave: Extrato proteico de levedura, hidrólise, potenciador de sabor, umami, amargo

Abstract

Today's consumers are increasingly prioritizing health and environmental consciousness. This shift in mentality and demand for sustainable food sources has led to a rise in plant-based and eco-friendly alternatives. In this context, yeast protein arises as a promising and versatile solution to many of the challenges faced by the food industry, offering both nutritional benefits and functional properties in food applications. This work investigates the potential of yeast protein extracts (YPE) as a natural source of umami flavour, with the main goal of developing an umami flavour enhancer by the hydrolysis of YPE to be industrially produced and commercialized by Proenol S.A., a biotechnology and oenology company.

Research focus on the optimization of the hydrolysis process, considering industrial limitations, and on the further characterization of the obtained hydrolysates. The hydrolysis reaction was monitored by electrophoresis and high-performance liquid chromatography, and the hydrolysates were analysed through tandem mass spectrometry. The use of prediction tools (UMPred-FRL and iBitter-Fuse) was crucial for the assessment of umami and bitter profiles of peptides. Consumer's acceptance of potential products was evaluated by sensory tests, and an analytical comparative analysis with existing products on the market was performed.

This work demonstrated that enzymatic hydrolysis is a viable process for developing an umami flavour enhancer. The results obtained for the characterization of potential final products were satisfactory, revealing high presence of umami peptides, and showing competitive potential in the current market. The consumer's feedback upon tasting was positive. This study resulted in promising umami flavour enhancers, highlighting the success of collaborative efforts between academia and the food industry.

Keywords: Yeast protein extract, hydrolysis, flavour enhancer, umami, bitter

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List of Abbreviations

AAC	Amino acid composition
AAI	Amino acid indices
ACC	Balanced accuracy
APAAC	Amphiphilic pseudo-amino acid composition
CTDC	Composition
CTDD	Distribution
CTDT	Transition
DIAAS	Digestible essential amino acid score
DPC	Dipeptide composition
DTT	Dithiothreitol
EFSA	European Food Safety Authority
ET	Extremely randomized trees
FAO	Food and Agriculture Organization of the United Nations
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database
FDA	U.S. Food and Drug administration
GA-SAR	Genetic Algorithm with Self-Assessment Report
GRAS	Generally Recognized as Safe
HPLC	High performance liquid chromatography
KNN	k-nearest neighbour
LC-MS/MS	Liquid chromatography – tandem mass spectrometry
LC-Q-TOF-MS/MS	Liquid chromatography and quadrupole-time-of-flight - tandem mass spectrometry
LR	Logistic regression
MCC	Matthews correlation coefficient
MSG	Monosodium glutamate
PAAC	Pseudo-amino acid composition
PDCAAS	Protein digestibility corrected amino acid score
PLS	Partial least squares
RF	Random forest
SCP	Single cell protein
SDS	Sodium dodecyl sulphate
SEC	Size exclusion chromatography
Sn	Sensitivity

Sp	Specificity
SPE	Solid-phase extraction
SVM	Support vector machine
TCA	Trichloroacetic acid
UPLC-MS/MS	Ultra performance liquid chromatography - tandem mass spectrometry
WHO	World Health Organization
YPE	Yeast protein extract

I. Introduction

1. Contextualization

Interest in yeast proteins has been growing due to the major necessity to discover new and sustainable sources of protein and integrate them in food products, as alternative to the traditional protein sources, such as meat, fish and dairy. This is of utmost importance due to the fast population growth, implemented policies, and the consumers' search for innovative products. According to FAOSTAT, human global population will grow to 9.1 billion by 2050, reflecting a great need for a better distribution of food and nutrition, as well as a decrease of land used for raising livestock and agriculture (FAO, 2009).

Single cell protein (SCP), being of microbial origin, is seen as a potential solution; the same happens with yeast proteins, which focus on the utilization of yeast as a protein source, rather than protein synthetized from various microorganisms, such as bacteria, fungi and algae (Bratosin et al., 2021). These are sustainable and healthier alternatives, with numerous advantages, in comparison to traditional sources.

The use of microorganisms allows a high efficiency at converting various organic materials and substrates, including agricultural waste and industrial byproducts into proteins, reducing the environmental impact as a new sustainable source. For this very reason, the cultivation of microorganisms for proteins production has the potential to reduce the carbon footprint of food industry, as well as greenhouse gas emissions, deforestation and water usage, associated with traditional livestock farming (Fasolin et al., 2019).

Yeast, as a type of fungus, is an accessible microorganism, presenting therefore the beforementioned advantages. Furthermore, yeast is described as a source of highly nutritious protein, due to its presence of essential and non-essential amino acids, vitamins and minerals (Ma et al., 2023).

Yeasts have been widely used in food for hundreds of years, for example in the brewing process of wine or beer, and for baking bread. More recently, it has also been used by the pharmaceutical industry. With the exploration of novel sources of protein gaining momentum, researchers are continuously studying innovative ways to produce, process and incorporate SCP and yeast protein into a variety of products. These can be used not only as a substitute of meat, but also with other applications in mind, such as fining agents, flavour enhancers, etc. Hand-in-hand with this development comes the

evaluation of food security of these novel protein sources, which is a critical challenge, as well as the education on the topic, critical to consumer acceptance.

2. Yeast extracts nutritional composition

So that the use of yeast protein can be consumed as human food or animal feed, it must meet nutritional requirements regarding protein and amino acid content, digestibility of said protein, among other parameters established by regulatory entities, namely immunogenic potential. Furthermore, it is necessary that the microorganism used are considered non-pathogenic and non-toxic for the consumer.

Yeast protein extracts (YPE), as the name indicates, contain the protein fraction of yeast cells, as well as minerals, lipids, and vitamins. The nutritional content depends mainly on the yeast species, growth conditions, production method, cell growth stage and the post-fermentation technological processing.

Protein content in yeast cells normally reaches 40% to 60% (on dry basis), an amount similar or even higher than traditional protein sources such as meat, soybean or milk. Due to a high protein percentage, yeast extracts contain a broad amino acid profile, including semi-essential and essential amino acids (Agboola et al., 2022). Microbial protein is deficient on sulphur-containing amino acids, such as methionine and cysteine, and rich in lysine, which presence is limited in plant protein (Jach et al., 2022). The FAO/WHO committee recommends a reference value of 40% of essential amino acids, which is close to the amount existing in yeast proteins (Ma et al., 2023; WHO/FAO/UNU, 2007). Furthermore, the protein digestibility corrected amino acid score (PDCAAS), which takes into account the digestibility of protein, presents high values for YPEs, indicating this protein as high-quality (S. Wang et al., 2023). These values show us that indeed, yeast protein is a promising alternative source of protein.

Besides protein, microbial extracts are able to provide lipids, carbohydrates, vitamins and minerals, among other important compounds (Kurcz et al., 2018). *Saccharomyces cerevisiae*, for example, has been described as a good source of vitamin from the B group, and *Yarrowia lipolytica* is a source of vitamin E and is capable of assimilating vitamin B12 into its own cells (Jach et al., 2022).

All things considered, yeast extracts appear to have a favourable nutritional profile, optimal for human consumption. It is worth saying that even more dietary value can be added though genetic engineering, but this requires a well-thought of and clear

regulation, and a shift in consumers' perception, since genetically modified organisms are usually perceived as negative.

3. Yeast extracts digestibility

The digestibility and absorption of protein plays a major role in its actual nutritional value and efficacy in meeting the body's requirements. Factors such as the source of protein, processing conditions, structure and presence/interaction with other components present can influence its digestibility. YPEs are generally noted for their high digestibility and efficient absorption and utilization of amino acids and nucleotides, especially when hydrolysed, since the smaller size of peptides facilitates and quickens its absorption (Zeng et al., 2022).

PDCAAS and digestible essential amino acid score (DIAAS) are two values used to assess the bioavailability of amino acids, considering protein digestion (FAO, 2013). PDCAAS evaluates the quality of protein, considering its amino acid profile in relation to human requirements and its digestibility; its value ranges from 0 to 1.0, being the latter an indication that the protein after digestion provides 100% or more of all the amino acids required by the body. DIAAS considers the digestibility of individual amino acids rather than the overall protein digestibility, being therefore more complex and harder to calculate; in this case, values are not truncated at a maximum value, allowing a better differentiation between high-quality proteins. Animal proteins generally have PDCAAS close to 1.0, while plants tend to have lower values due to its lower digestibility and lack of specific amino acids. As for YPEs, PDCAAS can reach high values, ranging from 0.82 to 0.90. As for DIAAS, milk and casein present values higher than 100, and YPE can reach 97 (Zeng et al., 2022). FAO recommends that for a protein to be considered high quality, the DIAAS score should be higher than 75, and for it to be considered excellent quality, the score should be higher than 100 (FAO, 2013).

Wang et al. studied the digestibility of a yeast protein concentrate, in comparison to a whey protein concentrate (S. Wang et al., 2023). It was concluded that yeast concentrate is a slow-digestive protein, with whey protein reaching higher values of *in vitro* digestibility faster. A higher resistance to digestion was observed in pepsin digestion, and it might be explained by the presence of β-glucans in yeast concentrate, and by a denser protein microstructure, in relation to a looser from whey protein. Yeast concentrate shows a higher content of ordered β-sheets, associated with a more stable conformation and, consequently, to a reduced accessibility of enzymes. Furthermore,

yeast protein concentrate particles tend to aggregate and therefore, once more, deter access from the proteolytic enzymes.

The digestibility and absorption of proteins are critical determinants of their nutritional value. YPEs have demonstrated high digestibility and efficient amino acid utilization, particularly when hydrolysed. Compared to animal and plant proteins, YPEs offer competitive PDCAAS and DIAAS scores, highlighting their potential as a high-quality protein. Studies on the structural factors and compounds interaction affecting protein digestibility emphasizes the need for targeted processing techniques that optimize the nutritional benefits of YPEs. This positions yeast protein as a promising source in functional foods and dietary supplements, capable of meeting the dietary protein needs of various populations.

4. Food matrix applications

More than just a nutritional purpose, yeast proteins present several attributes that can be used in food matrixes to reach a given characteristic. Several applications of yeast protein are already known and used, but its full potential may yet not be known.

4.1. Fining agents

Fining agents are used in the wine industry to promote clarification and stabilization of wines, contributing to elevated levels of brilliance, a pleasant colour and a balanced mouthfeel. Animal and mineral sourced fining agents have been greatly used but raise health and environmental issues that yeast protein-based products are able to surpass.

Studies have shown that yeast derivatives can be used as fining agents successfully, leading to a decrease of turbidity and an improvement in the organoleptic profile of wines (Bonilla et al., 2001; Dupin et al., 2000; Iturmendi et al., 2010). When treated with cytoplasmatic yeast proteins, compared to treatment with commercial fining agents, wines are brighter, clearer, more stable and present a more desirable colour (Fernandes et al., 2015). Furthermore, it was proven that the outcome of YPE application is achieved by its interaction with polyphenols (Francisco et al., 2021), reason why the extent of this interaction is fundamental to the effect in astringency, bitterness, colour, or other organoleptic properties of wine.

Despite all these positive results, further research is necessary in relation to the influence of the composition of wines, as well as the composition of the YPE used, so the application of yeast protein as a fining agent can become widely implemented and targeted for specific applications in the wine industry.

4.2. Supplements

With the increasing demand of protein globally, alternative sources have been put in focus. With the problems associated with both animal protein and plant protein (allergens, rising number of vegan population, environmental and agricultural pressures, etc.), microbial protein arises as a potential solution, capable of overcoming these effects and mitigate these issues. With its rich nutritional content and sustainable and economical production, yeast protein can be used to supplement the diet of both humans and animals alike. The European Food Safety Authority (EFSA) has declared *Yarrowia lipolytica* yeast safe as a food ingredient in a number of food categories, in foods for special medical purposes and in foods for total diet replacement for weight control, stating that this yeast does not raise any safety concerns and does not present any nutritional disadvantage (EFSA Panel on Nutrition et al., 2023), after having it authorized to be used as a dietary supplement a few years before. *Saccharomyces cerevisiae* has a status of Generally Recognized as Safe (GRAS) by regulatory agencies and has been used as a bakers' and brewers' yeast for thousands of years, having a long history of safe consumption. After fermentation, the yeast can be recovered and reused for alternative ends in the food industry thanks to the high nutritional and technological value it presents, contributing to a circular economy.

During World Wars I and II, microbial protein was used in food, being mainly incorporated into sausages and soup. Nowadays, it is used as a fodder supplement in animal feed, but also in ready to eat meals, baked products, as emulsifying agents, seasoning in vegetarian food, etc. for human consumption (Silva et al., 2021; Srividya et al., 2013; Torreggiani et al., 2023). The addition of yeast protein in different food products can also contribute to the decrease of malnutrition issues, especially present on developing countries.

With the continuous studies on yeast protein and its nutritional content, new applications can be developed and the use of yeast as a protein supplement can reach its full potential, with the knowledge of all its advantages.

4.3. Natural flavouring

YPEs are used not only for its nutritional and molecular properties, but also for its organoleptic potential. Yeast extracts can provide sweet, salty, spicy, meaty, savoury and/or umami flavour, which makes them suitable for use as flavouring and taste masking agents in diverse products (Tomé, 2021).

These different flavour sensations are determined by the presence of different components such as amino acids, peptides, nucleotides and several other flavouring molecules, which can act either synergistically to increase a flavour, or antagonistically to mask other (Methven et al., 2014). The composition of these same extracts is in turn determined by the production conditions and processes: for example, a study performed on yeast extracts found that the temperature of Maillard reaction has a great influence on the taste profile, with a temperature of 110ºC being optimal for umami tasting peptide generation (Alim et al., 2019). For this very reason, production methods are adjusted to achieve the goal in mind, and studies are continuously performed to try and correlate production processes, optimal conditions for generation of flavouring peptides, physicochemical properties and conditions, protein structure and flavour properties (Hau et al., 1997; Lin et al., 2014). Unfortunately, these topics and the relationships between them remain unclear, sometimes with contradictory conclusions, requiring further investigation.

One of the most desirable flavours to obtain using yeast extracts is umami. Umami is associated mostly with glutamate, but also aspartate residues, being mostly induced with the use of several glutamate salts, especially monosodium glutamate (MSG) and 5'-mononucleotides, such as inosine-5'-monophosphate. However, when consumed excessively, MSG has been associated with an increase of metabolic disorders, and even though these findings are somewhat controversial, efforts to substitute it have arose (Mondal et al., 2018; Obayashi & Nagamura, 2016; Shimada et al., 2015). In this line of thought, yeast extracts have been increasingly studied. The protein extracts have a content of about 5% w/w of free glutamate, and 10% w/w of total glutamate, with a capacity to induce umami taste when used at amounts below 1% in foods. This is a smaller amount of free glutamate than the required to obtain the same flavour using pure MSG (Tomé, 2021).

4.4. Health considerations

Hand-in-hand with the introduction of novel protein sources into human diet, a study of their behaviour and impact on human health is necessary.

The first aspect we have to take into account is the nucleic acid yield on yeast proteins. The recommended dose of protein for adults is 46 g/day for women and 52 g/day for men, while the recommended dose of daily nucleic acids intake by adult humans is 2 g/day (Coelho et al., 2020). This presents a problem since nucleic acid percentage in yeast proteins can round the 3% - 12% (Diaz-Bustamante et al., 2023), causing the consumed amount to greatly exceed the recommended daily dosage.

A high intake of nucleic acids leads to a purine excess after breakdown, which in turn can lead to gout, kidney stones, hypertension, diabetes, etc. (de Oliveira & Burini, 2012). For this very reason, it is fundamental to find ways to decrease the level of nucleic acids in yeast proteins for them to be implemented safely in human diet. Several methods are implemented with this goal in mind: chemical treatment, thermal shock, activation of endogenous ribonucleases, and addition of exogenous ones (Castro et al., 1971; Jach et al., 2022; Yang et al., 1979). The assessment of which methods are safer, more efficient and more environmentally friendly are necessary for companies to choose the best alternative.

Digestibility varies depending on the protein or hydrolysate in study. Furthermore, this very digestibility can influence the effect that the protein exerts on consumers' health, which can be either positive (e.g. nutraceutical compounds) or negative (e.g. allergenicity).

Food allergies' incidence have been substantially growing, especially in infants. *In vitro* digestion assays are usually performed as an indicative as to whether a protein is a possible allergen since the stability in the gastrointestinal tract is a major indicator of allergenicity (Astwood et al., 1996). But this information is not sufficient to take conclusions. Each assessment needs to be studied individually since there are allergens that aren't resistant to gastric fluids (Fu et al., 2002). Moreover, the digestion process can lead to the creation of neoepitopes or increase the concentration of a given epitope, and even lead to an influence in allergenicity potential due to conformational changes.

Besides digestion, food processing can also influence the allergenicity of proteins. Several emergent technologies have been showed to reduce potential allergenicity, such as high-pressure processing, pulsed electric field, gamma irradiation and enzymatic,

non-thermal treatments (Chizoba Ekezie et al., 2018; Rahaman et al., 2016). The influence of these processes on allergenicity is dependent on the alterations they cause to the food structure, and to its digestibility. Once again further studies are required, and an individual consideration of each case is necessary.

Protein and peptides toxicity directly impact consumers' health. Modification of proteins, either chemical, non-chemical, or by mutation, can lead to toxic peptides or derivatives, or even carcinogenic substances (Fasolin et al., 2019). This can happen due to unskilful handling of the microorganism, or inadequate processing. Cytotoxicity is studied by the assessment of necrosis, apoptosis, cell cycle disturbances, and detection of enzymes produced in living cells or released after death. The parameter studied depends not only on the cell type, but also on the injury caused and the agent responsible for it. Furthermore, substances like heavy metals are also relevant and should be taken into account when assessing safety for human consumption. Studies done on two strains of yeasts, *S. cerevisiae* and *Y. lipolytica*, concluded that these did not show excessive amounts of cadmium, mercury or arsenic (Michalik et al., 2014).

All these aspects demonstrate the need for continuous studies on safety assessment on yeasts that are not used as a protein source so far. However, there are several yeasts that are GRAS by FDA (U.S. Food and Drug Administration) and EFSA, namely *Saccharomyces cerevisiae*, *Yarrowia lipolytica*, *Candida utilis* among others, which may be a sign that others can be consumed without major implications.

5. Yeast protein hydrolysates

In addition to the possibilities presented using YPEs, more value can be obtained by hydrolysis. Protein hydrolysis can yield a number of peptides with great value for technological applications, flavour modulation and bioactivity. This allows a greater exploitation of yeast and a wider variety of products obtained from it.

5.1. Hydrolysis techniques

5.1.1. Chemical hydrolysis

Chemical hydrolysis can be used to break down proteins into peptides and amino acids in an economical way. It consists on the use of temperature and optionally strong acids or bases. While the use of these solvents can expedite the process, and even allow lower

temperatures, which in turn reduces energy costs, it may also cause the formation of undesirable byproducts or side reactions and safety problems. Furthermore, when opting for chemical hydrolysis, the cleavage of proteins is non-specific, making it challenging to obtain peptides with defined properties and difficult to achieve repeatability of results. However, this technique can be used in a wide range of proteins, without being limited by the type of protein or its source. Taking it all into consideration, chemical hydrolysis is generally more suited for versatile hydrolysis, especially when specific peptide sequences are not critical.

Acid hydrolysis was performed by Sari et. al for glutamic acid production from wheat by-products. In this protocol, 6M HCl containing 1% (w/v) phenol was added to wheat. The sample was then flushed with nitrogen, sealed and incubated at 110ºC for 24h. For a less harsh condition, wheat was also hydrolysed with the presence of 1M HCl, for 48h, at 95ºC. The latter, despite being a greener and less harsh alternative, did not hydrolyse wheat gluten protein completely (Sari et al., 2014).

Another example is a flash hydrolysis, used by Thakkar et al. for nutrient recovery. In this study, different percentages of yeast were hydrolysed at temperatures between 160ºC and 280ºC for 10 ± 2 seconds. The protein was then cooled and recovered after separation of the solid and liquid fractions (Thakkar et al., 2021) In this case, by using a much higher temperature, it is possible to not only lower the time of incubation, but also skip the use of a strong acid or base.

5.1.2. Enzymatic hydrolysis

Alternatively to chemical hydrolysis, it is possible to perform enzymatic hydrolysis on yeast proteins. The use of enzymes allows specificity, since there are enzymes who only target specific peptide bonds. This means that it is easier to predict and control the peptides obtained, and repeatability of results is expected. Since the hydrolysis relies on the catalytic activity of enzymes, the use of strong acids and bases is not necessary, allowing for milder and safer reaction conditions. However, the process itself is much more easily affected by changes in temperature and pH, requiring careful optimization. Furthermore, the choice of enzyme must be attentively made since because of its specificity, a specific enzyme may not hydrolyse a given protein. Enzymatic hydrolysis is usually preferred due to its selectivity and predictability, but its main disadvantage, the high cost, often leads to a second thought of whether its use is worth the economical downside. The decision on which hydrolysis method to use must be made considering

the desired characteristics of the final product, as well as economic feasibility and sustainability considerations.

The conditions used in the hydrolysis are established by the enzyme´s data sheet provided by the producer. The enzyme should be selected according to the objective in mind, considering efficiency, hydrolysates obtained and costs.

San Martin et al. used different enzyme mixes from Novozyme, performing the hydrolysis at temperature 55ºC and pH 6, with a percentage of enzyme of 1% enzyme/protein. In this case, yeast protein was hydrolysed with the goal of being included in aquaculture feed. For this very reason, the mixes used were rich in proteases, cellulases and peptidases to modulate flavour, reducing bitterness. The effectiveness of the different enzyme mixes hydrolysis was then assessed by SDS-PAGE (San Martin et al., 2020). In (San Martin et al., 2021) the same hydrolysis conditions were performed, and the reaction was stopped by temperature at 90ºC for 15 minutes.

On a study of characterization of yeast protein enzymatic hydrolysis, Mirzaei et. al used trypsin and chymotrypsin. Each enzyme was added to the substrate at a ratio of 1:10 (w/w), and the reaction occurred for 5 hours, at a temperature of 37ºC and pH 7.8 (Mirzaei et al., 2015).

5.2. Bioactive peptides

Bioactive and bio-functional peptides are those who cause a beneficial effect on the consumer and may lead to health benefits, namely in digestive, immune, cardiovascular and nervous human systems (Mora et al., 2019). The action of these small peptides depends on its amino acid composition and sequence, and is generally very specific (Oliveira et al., 2022).

Commercially available bioactive peptides are, for the most part, chemically synthesized, but natural sources, such as animal, plant and microbial protein, have been studied to try to shift the norm. Furthermore, it has been found that several foods have bioactive peptides in their composition, which are released during digestion, an aspect which can be of extreme importance when talking about functional foods (Oliveira et al., 2022).

Yeast cells are known to generate bioactive peptides. These remain inactive while kept inserted in a protein, but once they are released by hydrolysis during processing or digestion, they become active and are able to exert a positive effect after crossing the gastrointestinal barrier (Mora et al., 2019). Therefore, physical, chemical and enzymatic

extraction processes have been optimized in order to obtain the highest yield of bioactive molecules at the lowest price possible (Amorim et al., 2016; Podpora et al., 2015). *S. cerevisiae* protein extracts can be obtained as a brewing by-product (spent yeast) and consequently be an inexpensive source of biologically significant peptides. Bioactive peptides from this species have been linked to antihypertensive, antioxidant, antidiabetic and antimicrobial activities, as well as to the prevention and treatment of different diseases (Al-sahlany et al., 2020; Gorouhi & Maibach, 2009; Kanauchi et al., 2005; Oliveira et al., 2022; Sklirou et al., 2015). These peptides can be used in the food industry for animal feed or human supplements, for example, but also in the cosmetic sector.

The advantages of the use of spent yeast as a source of bio-functional peptides goes beyond the economic ones, since it can mean a circular economy and exploitation of by-products that could otherwise be wasted.

The demand for bioactive peptides, especially for application in nutraceuticals (and even cosmeceuticals) is growing at an ever increasing pace, which in turn raises the demand for further studies on the extraction and purification of said peptides.

5.3. Natural flavouring

YPEs have the potential to elicit flavour, namely umami, not only due to the aspartate and glutamate residues, but also the interaction between the different components present.

Numerous peptides obtained from hydrolysis of different food ingredients and products, especially those containing at least one glutamyl or aspartyl residue were described to induce umami taste (Qi et al., 2022; Zhang et al., 2017), even though the conclusions are somewhat controversial. The umami taste is determined by the peptides present (some are unable to provide an umami taste on their own, but can enhance that very taste provided by others), by the number of amino acids associated with umami taste present in the peptide, as well as their position in the chain, e.g. when amino acids responsible for umami taste are closer to the N-terminus, a stronger umami taste is detected (Alim et al., 2019).

Since yeast proteins have a relatively high content of umami tasting amino acids, the same happens for the peptides generated after hydrolysis. Studies found that peptides with a molecular weight smaller than 1 kDa have a more intense taste perception, namely a stronger umami taste (Liu et al., 2015; H. Wang et al., 2023). Wang et al. also

determined that aspartic acid plays a key role in umami tasting peptides, since it is present in every peptide identified. Other amino acids such as valine, arginine, proline and leucine also appear to be present in high frequencies in umami tasting peptides (H. Wang et al., 2023).

In Table 1 some peptides discovered to be associated with umami or kokumi taste are presented. Kokumi is a natural flavour enhancer, normally described as a complex sensation that contributes to the mouthfeel of foods, promoting the fullness and thickness of flavour in the mouth. It is interesting to note that some peptides are identified as having different taste properties in different studies: for example, Glu-Leu is described as having umami and kokumi flavour in one study, and kokumi and bitter in another. This may be due to the difference in the extraction process, analysis, or even due to the solvent or matrix in which it is studied. Aligned with this hypothesis are the results that prove that the flavour sensation and its perception depend on the food matrix in which the peptides are present. This is shown for example in the study by Liu et. al in which the same peptides show different properties depending on whether they are in water or chicken broth (Liu et al., 2015). This may be due to the synergistic effect of peptides.

Table 1 - Examples of peptides identified as having an umami or kokumi-like flavour.

Peptide	Taste (or flavour) property	Perception threshold	Information on the process	Reference
Pro-Ala-Ala	Umami	10.25 mg/L	Yeast extracts were submitted to a thermal treatment, the fraction under 1 kDa was separated through ultrafiltration, purified and analysed	(Alim et al., 2019)
Pro-Glu-Thr	Umami	4.78 mg/L		
Glu-Asn	Umami	0.75 mg/L		
Glu-Ala	Umami	0.82 mg/L		
Glu-Leu	Umami and kokumi	1.52 mg/L		
Gly-Gly-Tyr	Umami and bitter	8.2 mg/L		
Leu-Glu	- Bitter in water - Bitter and kokumi sensation in blank chicken broth	- 2.4 mmol/L in water - 0.3 mmol/L in blank chicken broth	A yeast extract paste was filtrated and purified, the kokumi peptides were identified by LC-Q-TOF-MS/MS,	(Liu et al., 2015)

Glu-Leu	- Bitter in water - Bitter and kokumi sensation in blank chicken broth	- 4.8 mmol/L in water - 0.6 mmol/L in blank chicken broth	and the samples were further analysed chemically, and by a sensory panel	
Glu-Val	- Tasteless in water - Kokumi sensation in blank chicken broth	- less than 20.2 mmol/L in water - 0.6 mmol/L in blank chicken broth		
Asp-Trp-Thr-Asp-Asp-Val-Glu-Ala-Arg	Strong umami, salty and sour	0.07 ± 0.01 mM	Umami fractions of yeast extract were separated and purified, peptides identified by Nano UPLC-MS/MS, and analysed by a sensory evaluation panel and by electronic tongue, and further validated	(H. Wang et al., 2023)
Val-Gln-Pro-Glu-Asp-Asn-Lys-Gly-Val-Phe-Gln	Umami, salty and astringent	0.61 ± 0.01 mM		

The results presented show that further studies are necessary to figure out how the different factors, such as nature of the food matrix, dilution, interaction with other peptides, etc., may affect the flavouring sensation of both proteins and hydrolysed peptides.

6. Umami and Bitter computational prediction tools

Given the importance and promising applications of flavour enhancers in the food industry, the need to predict a peptide's characteristics in a fast and economical way is

growing. Traditional methods for identifying the potential of a peptide to present a given flavour are time-consuming and costly, and therefore new technologies have surfaced recently. In the last few years, computational tools based on machine learning have been continuously studied and improved. Nowadays, a wide pool of algorithms that allow the characterization of peptides and proteins are available to reach various goals, for example to predict anti-microbial properties, anti-inflammatory properties, anticancer activity, binding activity and even flavour enhancement potential based on the peptide sequence. UMPred-FRL and iBitter-Fuse are two predicting tools able to estimate the probability of a given peptide presenting umami and bitter potential, respectively.

6.1. UMPred-FRL

UMPred-FRL is a machine learning-based approach, available online, for predicting umami peptides, based on its amino acid sequence (Phasit Charoenkwan et al., 2021). This computational tool uses feature representation learning (FRL), combining a total of six different algorithms with seven feature encoding methods to develop a meta-predictor.

The authors used benchmark datasets containing a total of 140 umami peptides and 304 non-umami peptides, which were divided into a training set (UMP-TR) with 80% of the umami and non-umami peptides, and an independent test set (UMP-IND) with the remaining 20% of the peptides. These benchmark datasets, derived from literature and databases, were essential for training the model and later evaluating its performance.

Feature extraction was then performed on the UMP-TR and UMP-IND using seven different encoding schemes to represent peptide sequences in various feature vectors: amino acid composition (AAC), dipeptide composition (DPC), pseudo-amino acid composition (PAAC), amphiphilic pseudo-amino acid composition (APAAC), and composition (CTDC), transition (CTDT) and distribution (CTDD). Each encoding scheme takes into account different characteristics of peptide sequences to capture a wide range of properties. AAC considers the frequency of each amino acid in the sequence, resulting in a 20-dimensional vector that reflects the overall presence of the 20 standard amino acids. DPC goes a step further considering the frequency of each dipeptide, producing a 400-dimensional vector that captures local sequence order information. PAAC adds factors related to physicochemical properties to the amino acid sequence, providing more detailed information beyond the composition. APAAC extends this approach by considering the amphiphilic properties of amino acids, and how these properties are

distributed along the peptide. CTDC focuses on the global composition of defined structural or physicochemical properties of amino acids, dividing them into groups; it gives a numerical representation of these different groups in the sequence. CTDT is related with CTDC, since it captures the number of transitions between these groups, informing how the different groups are positioned relative to each other. Finally, CTDD focuses on the positional distribution of these groups along the length of the peptide sequence, providing information on where in the sequence particular properties are located.

The authors then combined each of these seven feature descriptors with six machine learning algorithms: extremely randomized trees (ET), k-nearest neighbour (KNN), logistic regression (LR), partial least squares (PLS), random forest (RF), and support vector machine (SVM). This combination allowed the creation of 42 baseline models, which served as the foundation to the generation of new features that integrate diverse sources of information and leverage the strengths and complementary information provided by each baseline model.

These new features were then evaluated, measuring accuracy, sensitivity and Matthews correlation coefficient of the predictor, and the ones who performed better were selected and combined. The performance of the final predictor was validated using cross-validation on the UMP-TR and an independent test on the UMP-IND, showing that UMPred-FRL outperformed existing methods in terms of balanced accuracy (ACC) with a value of 0.888, sensitivity (Sn) with 0.786, specificity (Sp) with 0.934 and Matthews correlation coefficient (MCC) with a value of 0.735 on the independent test dataset. The superior performance of UMPred-FRL is attributed to its use of feature representation learning and the integration of diverse feature descriptors and machine learning algorithms. This comprehensive approach allowed UMPred-FRL to be more accurate and robust in predicting umami peptides compared to previous methods.

6.2. iBitter-Fuse

The predictor iBitter-Fuse is able to identify bitter peptides (P. Charoenkwan et al., 2021). This model integrates multiple feature encoding schemes to capture comprehensive information about peptide sequences, including compositional data and physicochemical properties. The benchmark dataset includes 640 peptide sequences, evenly split into 320 bitter peptides and 320 non-bitter peptides. This dataset was divided into a training

set, with 256 bitter and 256 non-bitter peptides, and an independent test set, with 64 bitter and 64 non-bitter peptides.

Feature extraction was performed using several encoding schemes: AAC, DPC, PAAC, APAAC, and amino acid indices (AAI), with each one capturing different aspects of the peptide sequences. AAI consists of numerical values representing various biochemical properties of amino acids.

The extracted features were then used to construct several machine learning models, including extremely randomized trees (ET), k-nearest neighbour (KNN), logistic regression (LR), partial least squares (PLS), random forest (RF), and support vector machine (SVM). This resulted in a total of 42 baseline models.

To optimize the final model, the authors employed a customized genetic algorithm known as GA-SAR (Genetic Algorithm with Self-Assessment Report). This algorithm evaluates different subsets of the features repeatedly to determine which combinations provide the best predictive performance, helping identify the most informative features and fine-tune the SVM parameters. The features are selected and refined based on their relevance to the prediction task, ensuring that the final model uses a minimal number of highly informative features.

The performance of iBitter-Fuse was evaluated using both 10-fold cross-validation and independent test datasets. The model demonstrated superior performance compared to existing methods, including iBitter-SCM and BERT4Bitter. The evaluation metrics used included accuracy (ACC), sensitivity (Sn), specificity (Sp), and Matthews correlation coefficient (MCC). iBitter-Fuse achieved an ACC of 0.930, Sn of 0.938, Sp of 0.922, and MCC of 0.859 on the independent test dataset, indicating its high predictive capability and robustness.

It is then concluded that iBitter-Fuse is a powerful and accurate tool for the identification of bitter peptides. Its integration of multiple feature encoding schemes and the use of the GA-SAR algorithm for optimization have resulted in a model that outperforms other existing predictors. The iBitter-Fuse web server, available online, provides an accessible platform for high-throughput identification of bitter peptides, aiding in the discovery and design of new peptides for various applications.

7. Objective and study design

Yeast proteins show a plethora of applications in the food industry. The number of possible uses broadens when the exploitation of hydrolysates is also considered. The use of yeast protein and peptides as supplements, fining agents, bioactive peptides and flavour enhancers are only a few examples of the possibilities. The outcome of their use depends on a variety of factors, which need further research and optimization.

Overall, the investigation of yeast proteins and hydrolysates is a dynamic and rapidly evolving field, representing a promising area for further research and day-to-day application.

In this work, different YPEs are characterized and hydrolysed with the main goal of developing an umami flavour enhancer. The hydrolysis conditions are optimized with this goal in mind, and the hydrolysates are analytically and sensorially characterized to try and prove the viability of its production and commercialization.

This work is developed in partnership with Proenol S.A., a company specialized in the use of yeast for the food industry. The key objective is for the umami flavour enhancer to be developed and further commercialized by Proenol S.A., and therefore industry conditions, as well as scale-up processes, are considered throughout this work.

II. Materials and Methods

1. Reagents

All reagents used were of analytical grade. Milli-Q EQ 7000 ultrapure water purification system (Merck Millipore), equipped with a Millipak 0.22 µm hydrophilic 17 membrane filter. Copper sulphate, potassium sulphate, phenol, glucose, trichloroacetic acid, urea, dithiothreitol, tris base, sodium azide, sodium phosphate monobasic and sodium phosphate dibasic were purchased from Sigma-Aldrich. Sulfuric acid, hydrochloric acid, acetic acid, formic acid and acetonitrile were purchased from LabChem. Sodium hydroxide and methanol were purchased from Honeywell. Coomassie Blue R-250 dye was purchased form Bio-Rad. Acrylamide/bis-Acrylamide (37:5:1 solution), 10× Tris-Glycine buffer, 5× SDS-Page loading buffer and Nzycolour protein marker II were purchased from NZYTech. Enzymes A, B and C, and YPE extracts were supplied by Proenol S.A..

2. Yeast protein extracts characterization

2.1. YPE samples

YPEs vary in content depending on various factors, including yeast species and processing conditions. A total of 9 extracts of *Saccharomyces cerevisiae*, (Divino (dry), Divino neutral (dry), SP1 (dry), SP2 (dry), SP1 + SP2 (dry), EPLc (dry), Divino (liquid), SP1 (liquid) and SP1 + SP2 (liquid)), donated by Proenol S.A., were characterized based on its protein and carbohydrates content, and further tested for umami potential after hydrolysis. These extracts differ from each other depending on the processing techniques and type of purification from which they pass (Figure 1). Therefore, its content in cell components also differs from each other. One of the YPEs is submitted to a neutralizing process, which decreases the intensity of the yeasts' smell and flavour of the extract.

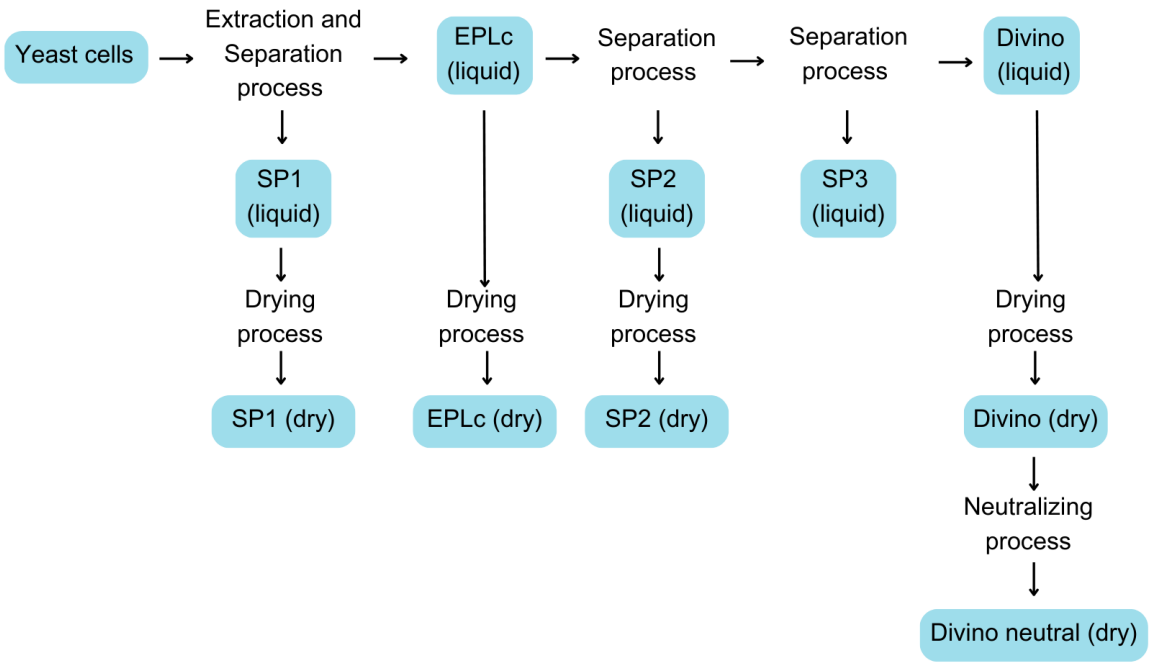


Figure 1 - Processing scheme of the various YPEs.

2.2. Protein quantification

The Kjeldahl method (Kjeldahl, 1883) was used to quantify protein in YPEs. This method consists in a three-step protocol: digestion, distillation and titration. The YPE is digested, converting the nitrogen in the sample to ammonium sulphate. The addition of sodium hydroxide while heating transforms the ammonium sulphate into volatile ammonia gas, which in turn is distilled and trapped in the form of ammonium borate in an excess boric acid solution. This solution is then titrated with a standard hydrochloric acid and the total nitrogen in the sample is estimated.

In this study, the samples were digested in a VELP Scientifica DK 6 digestion unit at 420ºC for 1 hour in the presence of copper sulphate, potassium sulphate and sulfuric acid. Following digestion, the samples were distilled in vapor in a VELP Scientifica UDK 129 distillation unit for 7 min with sodium hydroxide and nitrogen was trapped in a 4% boric acid solution. Afterwards, a titration was made with standardized hydrochloric acid 1 mol/L, using bromocresol green as a pH indicator. The percentage of protein was obtained multiplying the percentage of nitrogen (Equation 1) by the conversion factor of

6.25. A blank was performed using deionized water. Assays were performed in independent triplicates.

$$\%_{nitrogen} = \left(\frac{V_{HCl} \times C_{HCL} \times 14}{m_{sample}} \times 100\right) - blank \qquad \text{(Equation 1)}$$

V_{HCL} corresponds to the volume, in mL, of HCl used in the titration. C_{HCl} corresponds to the concentration of HCl used in the titration, in mol/mL. m_{sample} corresponds to the mass, in g, of the sample.

Results were analysed and presented using GraphPad Prism 9.0 software. For multiple group comparisons, the 2-way ANOVA with Tukey post-test was performed. Statistically significance was labelled with letters, in which different letters correspond to a significant difference of $P < 0.05$.

2.3. Carbohydrates quantification

Carbohydrates in YPEs were quantified through the DuBois method (DuBois et al., 1956). This method is sometimes referred as the phenol-sulphuric method, since the samples are incubated in the presence of a phenol solution and concentrated sulphuric acid. The polysaccharides are hydrolysed into monosaccharides, which are in turn dehydrated to produce furfural or hydroxymethylfurfural. These compounds then react with phenol to form a yellow-coloured complex, whose absorbance is measured at a wavelength of 490 nm.

The samples were diluted in sulfuric acid 98% and phenol solution 50 mg/mL and incubated at 98ºC for 30 min. After cooling at room temperature, the samples were transferred to a 96 well plate and the absorbance at 490 nm was recorded in a spectrophotometer Bio-Tek Power Wave XS. The calibration curve was obtained using glucose as standard at known concentrations (0.00 mg/mL, 0.02 mg/mL, 0.04 mg/mL, 0.08 mg/mL, 0.10 mg/mL, 0.12 mg/mL and 0.20 mg/mL). Assays were performed in triplicate and replicated independently thrice.

Results were analysed and presented using GraphPad Prism 9.0 software. For multiple group comparisons, the 2-way ANOVA with Tukey post-test was performed. Statistically significance was labelled with letters, in which different letters correspond to a significant difference of $P < 0.05$.

3. Yeast protein extracts hydrolysis

3.1 Chemical hydrolysis

Hydrolysis was performed at a protein concentration of 4.30 mg/mL, in water, at 50 ºC or 70ºC. Aliquots were collected and analysed at different time-points (from 0h to 32h). Samples were then precipitated with trichloroacetic acid (TCA) and centrifuged for 10 min at 9580 g at a temperature of 4 ºC. The supernatant was analysed through HPLC (Section II 4.2) and HPLC-SEC (Section II 4.3) after filtration with 0.45 µm filters. The pellets were stored for further SDS-PAGE analysis (Section II 4.1).

3.2. Enzymatic hydrolysis

Initial optimization – protocol 1

Hydrolysis was performed for 30 min at a protein concentration of 4.30 mg/mL in Tris 0.1 M pH 8, with one of two different proteases at different enzymatic activities: enzyme A at 0.1 U/g, 0.8 U/g and 50 U/g and enzyme B at 0.3 U/g, 0.5 U/g and 0.8 U/g, at three different temperatures (37ºC, 50ºC and 70ºC). Hydrolysis was stopped by incubation at 95ºC for 5 min. Samples were then precipitated with TCA and centrifuged for 10 min at 9580 g at a temperature of 4 ºC. The pellets were stored for further SDS-PAGE analysis (Section II 4.1), while the supernatants were analysed through HPLC and HPLC-SEC after filtration with 0.45µm filters (as described in Sections II 4.2 and 4.3, respectively).

Initial optimization – protocol 2

Hydrolysis was performed at YPE concentrations of 4 mg/mL, 6 mg/mL and 10 mg/mL, which corresponds to a protein concentration of 2.65 mg/mL, 3.98 mg/mL and 6.63 mg/mL, respectively. Enzyme A, at 50 U/g, and enzyme B, at 0.8 U/g, were added in different volumes (20 µL and 30 µL), and the reaction occurred at 50ºC for 4h or 24h. Hydrolysis was stopped by incubation at 95ºC for 5 min. Samples were then precipitated with TCA and centrifuged for 10 min at 9580 g at a temperature of 4 ºC. The pellets were discarded, and the supernatants followed peptide quantification (Section II 4.4) before and after SPE (Section II 4.5), followed by LC-MS/MS analysis (Section II 4.6).

Final optimization

Hydrolysis was performed at a protein concentration of 10 mg/mL, upon a pre-treatment for inactivation of the YPE endogenous enzymes at 65 ºC for 15 min. Enzyme C was added at 0.5% (v/w) to the YPE at pH 8.5, and after 45 min of reaction, when the pH has

dropped to 7.0-7.5, 0.2% (v/w) of enzyme A was added. The reaction occurred at 55 ºC, for a total of 24h, and aliquots were collected and analysed at different time-points. The hydrolysis was stopped at 90 ºC for 10 min. Hydrolysates were dried. Hydrolysis following this procedure were performed at pilot scale at Proenol S.A..

Samples were then resuspended in water and centrifuged at 9580 g for 10 min, the supernatant followed peptide quantification (Section II 4.4) before and after SPE (Section II 4.5), followed by LC-MS/MS analysis (Section II 4.6).

4. Hydrolysates analysis and characterization

4.1. SDS-PAGE gel

This technique leverages the denaturing properties of SDS (sodium dodecyl sulfate), a detergent that binds to proteins and acquires them with a negative charge, proportional to the protein's length. This charge is strong enough to mask the intrinsic charge of the protein, and since this is denatured, proteins are separated based on size rather than natural charge or shape. The migration in the gel is influenced by the pore size of the gel, which is in turn determined by the concentration of acrylamide. When the samples are loaded into the gel, and electric field is applied and the proteins migrate towards the anode, with the smaller proteins migrating faster in the gel.

Hydrolysis pellets were resuspended in a DTT (dithiothreitol) 200mM, urea 8M solution and loading buffer to a final concentration of 2.5 mg/mL. Lastly, the sample proteins were incubated with continuous shaking at 95ºC for 10 min for denaturation to occur and analysed through SDS-PAGE.

SDS gels were prepared with a 4% acrylamide percentage in the stacking gel and 12% in the running or separating gel. A total of 50 ug of protein was loaded per well into the gel and a NZY Colour Protein Marker II from NZYtech with molecular weights ranging from 11 kDa to 245 kDa was used for comparison for the estimation of molecular weights. The electrophoresis was conducted using a Bio-Rad PowerPac Basic Power Supply at a voltage of 100 V until the samples reached the running gel and 150 V for the rest of the run, using Tris-Glycine Buffer with 0.1% SDS as running buffer. Gel slabs were then incubated in a fixation solution of 10% acetic acid and 40% methanol in water, stained for 15 min with a Bio-Rad Coomassie Blue R-250 dye-based reagent, and afterwards destained with a decolorizing solution of 10% acetic acid and 20% methanol in water to remove extra dye. The gel slabs were then washed in water and photographed.

4.2. High performance liquid chromatography technique

High performance liquid chromatography (HPLC) analysis of YPE hydrolysates were performed on a Thermo Scientific Vanquish – photo diode array detector (VH-D10-A) equipment. Samples were diluted 1:14 fold and 25 µL of each sample were injected into the system equipped with an Agilent Poroshell 120 EC-18 column (250 x 4.6 mm i.d., 2.7 µm). Detection was performed at 214 nm and 280 nm using as solvent A 0.2% formic acid in water, and solvent B 0.2% formic acid in acetonitrile at a flow of 0.3 mL/min. The gradient is presented in table 2.

Table 2 - Flow gradient of HPLC-DAD analysis.

Time (min)	SOLVENT A (%)	SOLVENT B (%)
0	98.0	2.0
30	90.0	10.0
45	50.0	50.0
55	2.0	98.0
65	2.0	98.0
68	98.0	2.0
80	98.0	2.0

4.3. Size exclusion chromatography

The molecular weight of the different peptides was estimated through size exclusion chromatography (SEC). Analysis was conducted on a Thermo Scientific Vanquish – photo diode array detector (VH-D10-A) equipment. Separation of undiluted samples (15 µL) was performed in a Yarra SEC-2000 Phenomenex column (3000 x 7.8 mm i.d., 3 µm). Detection was performed at 214 nm and 280 nm. Phosphate buffer 0.1 M with 0.025% sodium azide at pH 6.8 was used as mobile phase in an isocratic method of 30 min at a flow of 1 mL/min.

4.4. Peptide Quantification

The Thermo Scientific™ Pierce™ Quantitative Colorimetric Peptide Assay was used to quantify the peptides in enzymatic hydrolysates (performed at laboratorial and pilot scale) before and after SPE. The samples were incubated with the reagent mix at 37ºC

for 15 min, after which the absorbance was measured at 480 nm in a plate spectrophotometer Bio-Tek Power Wave XS. A calibration curve was performed using a peptide standard at different concentrations (1 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.125 mg/mL, 0.063 mg/mL, 0.031 mg/mL, 0.016 mg/mL and 0 mg/mL). Each sample was analysed at two different concentrations, and triplicates were performed.

4.5. Solid phase extraction

Purification of the YPE hydrolysates (obtained at laboratorial and pilot scale hydrolysis) by solid-phase extraction (SPE) was performed using C18 (Waters™ Oasis Prime HLB cartridges) reverse phase silica gel as stationary phase. C18 cartridges were activated with methanol (1 column volume) and washed with H_2O 0.1% formic acid (2 column volumes). The sample was diluted and passed through the cartridge, and after washing the column with 1 column volume of H_2O 0.1% formic acid, peptides were eluted in 300 µL of a 50/50 solution of acetonitrile and methanol. Samples were then lyophilized and resuspended before quantifying peptide concentration through the protocol described in Section II 4.4.

4.6. Liquid Chromatography-Tandem Mass Spectrometry

Liquid chromatography – tandem mass spectrometry (LC-MS/MS) is widely used for the qualitative and quantitative analysis of complex biological mixtures by combining the separation capabilities of liquid chromatography and the specificity of mass spectrometry. Following the separation of molecules based on their chemical properties by liquid chromatography, these are ionized and detected based on their mass to charge (m/z) ratio. This technique allows for the selection and fragmentation of ions, providing structural information that is crucial for identifying the amino acid sequence of peptides.

Selected YPE hydrolysates were analysed through LC-MS/MS following column chromatography purification. 10 µL of each sample at a concentration of 0.033 mg/mL were separated on a Thermo Fischer Scientific RSLC Nano System Ultimate 3000, using a Thermo Fischer Scientific PepMap RSLC C18 column (2 µm particle size and dimensions 75 µm i.d. x 15 mm). The samples were eluted at a flow rate of 0.3 µL/min, using 0.1% (v/v) formic acid in water as solvent A and 0.1% (v/v) formic acid in acetonitrile as solvent B, following the gradient presented in Table 3. The column temperature was maintained at 35ºC.

Mass spectrometric analysis was performed on a Thermo Fischer Scientific Orbitrap Q Exactive HF mass spectrometer, controlled by Tune Application 2.11 and Xcalibur 4.4.16.14. The electrospray ionization source was operated in positive ion mode with a capillary voltage set to 1.9 kV. The capillary temperature was 275ºC. The resolution of MS scan was 120000. Data-dependent MS/MS was performed using higher-energy collisional dissociation with nitrogen as the collision gas, at energy settings of 27, 30, 35 V. The m/z range for MS acquisition was 300-1500 Da.

MS data handling software (Xcalibur QualBrowser software, Thermo Fischer Scientific) was used to search for predicted metabolites based on their m/z and MS/MS values. Regarding the analysis conditions in Proteome Discoverer (v. 2.5.0.500), raw files were processed and analysed with a precursor mass range of 300 to 5000 Da, a peptide length between 4 and 150 amino acids, a precursor ion tolerance of 10 ppm, and a fragment ion tolerance of 0.02 Da. The database used for this analysis was exported from Swiss-Prot, containing 6650 reviewed proteins specific to *Saccharomyces cerevisiae* (accessed on July 2023). The profiling of hydrolysates considered dynamic modifications such as methionine oxidation (M) and pyroglutamination (Q). Sequences identified with confidence below high, rank 1, or with absolute mass deviations greater than 5 ppm were excluded from further analysis.

Table 3 - Flow gradient of HPLC-MS/MS analysis.

Time (min)	Solvent A (%)	Solvent B (%)
0	85.0	15.0
90	1.0	99.0

4.7. Umami and bitter peptides prediction

The UMPred-FRL (Phasit Charoenkwan et al., 2021) and iBitter-Fuse (P. Charoenkwan et al., 2021) prediction tools for prediction of umami and bitter peptides, respectively, were used to analyse the peptides identified by HPLC-MS/MS with high confidence. The sequence of the various peptides of each hydrolysis sample was uploaded into the prediction tools, available freely at http://pmlabstack.pythonanywhere.com/UMPred-FRL (accessed in May 2024) and http://camt.pythonanywhere.com/iBitter-Fuse (accessed in May 2024). If the probability calculated for each of the properties is higher or equal to 0.5, the peptide is identified as umami or bitter, and if it is lower, then the peptide is identified as non-umami or non-bitter, depending on the characteristic in analysis.

4.8. Sensory analysis

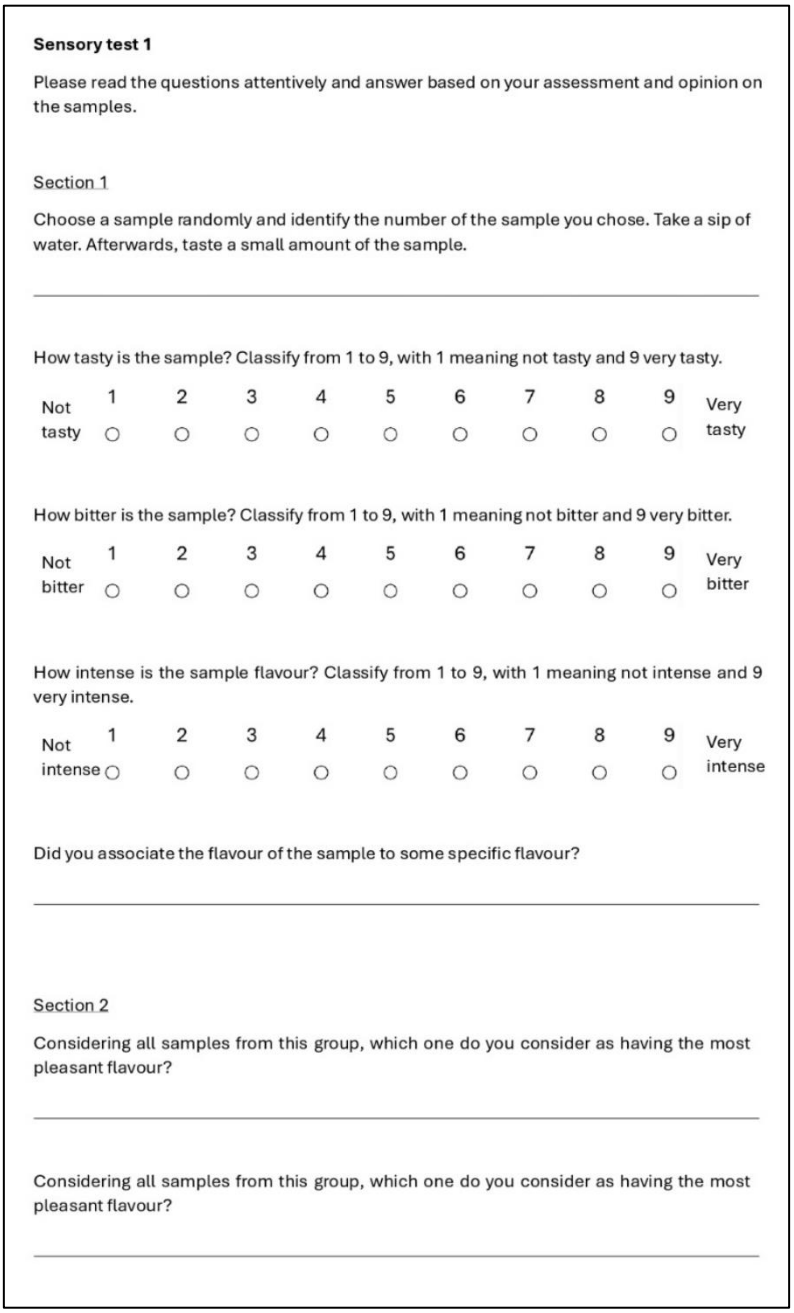
Hydrolysates obtained from pilot scale production, performed by Proenol S.A., were subjected to a sensory analysis. Samples were separated into three groups of 5. Each group of samples was presented to a taster panel in random order, and it was asked that each taster answer the following questionnaire (Figure 2). Controls of undigested YPE were included with each group of samples. Tastings of each group were performed at different days, by the same taste panel.

Results were analysed and presented using GraphPad Prism 9.0 software. For multiple group comparisons, the 2-way ANOVA with Tukey post-test was performed. Statistically significance was labelled with letters, in which different letters correspond to a significant difference of $P < 0.05$.

Two of the hydrolysis conditions which the hydrolysates were considered the tastier (considering answers to questionnaire 1) were replicated by Proenol S.A. to verify the repeatability of the process. The original samples (reference and product A) and the repeated ones (product B) were presented to the panel in a discrimination Duo Trio test. This test consists of presenting 2 products and a reference product and asking the panellist to identify which one is most similar to the reference product. The panellists were asked to answer the following questionnaire (Figure 3). Statistical analysis followed the binomial test, in which the p-value is calculated through equation 2. Significant difference is detected between the two samples if the p-value is less than 0.05, and no significant difference is detected when the p-value is equal or greater than 0.05.

$$P\ (X \geq k) = \sum_{i=k}^{n} \binom{n}{i} p^i\ (1-p)^{n-i} \qquad \text{Equation 2}$$

X corresponds to the random variable representing the number of correct identifications. k is the number of observed successes. n is the total number of trials (total number of tasters in the panel). p is the probability of correct identifications under the null hypothesis (0.5).



Sensory test 1

Please read the questions attentively and answer based on your assessment and opinion on the samples.

Section 1

Choose a sample randomly and identify the number of the sample you chose. Take a sip of water. Afterwards, taste a small amount of the sample.

_____

How tasty is the sample? Classify from 1 to 9, with 1 meaning not tasty and 9 very tasty.

	1	2	3	4	5	6	7	8	9	
Not tasty	○	○	○	○	○	○	○	○	○	Very tasty

How bitter is the sample? Classify from 1 to 9, with 1 meaning not bitter and 9 very bitter.

	1	2	3	4	5	6	7	8	9	
Not bitter	○	○	○	○	○	○	○	○	○	Very bitter

How intense is the sample flavour? Classify from 1 to 9, with 1 meaning not intense and 9 very intense.

	1	2	3	4	5	6	7	8	9	
Not intense	○	○	○	○	○	○	○	○	○	Very intense

Did you associate the flavour of the sample to some specific flavour?

_____

Section 2

Considering all samples from this group, which one do you consider as having the most pleasant flavour?

_____

Considering all samples from this group, which one do you consider as having the most pleasant flavour?

_____

Figure 2 - Sensory analysis questionnaire for the sensory characterization of hydrolysates. Each taster responded these questions for each group of 6 samples, being section 1 repeated six times, one for each sample in the group. Section 2 allows a more global distinction between samples.

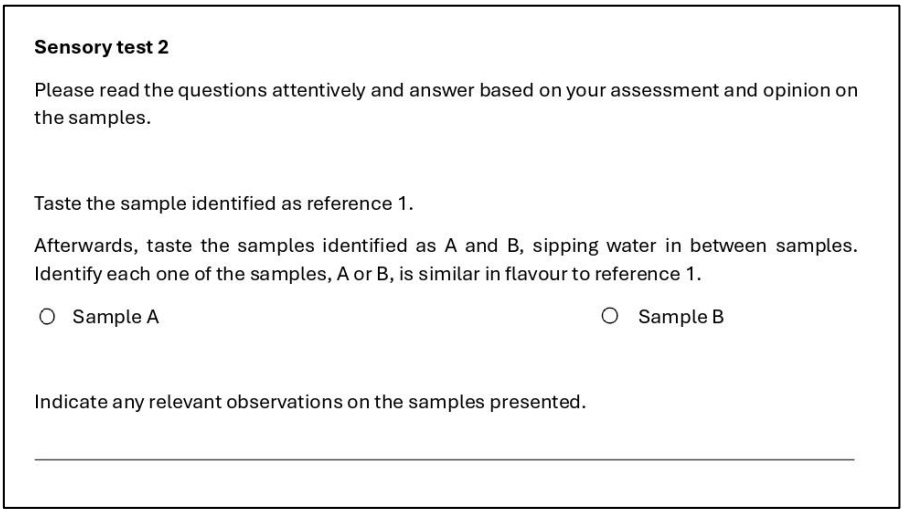


Figure 3 - Sensory analysis questionnaire for assessing the repeatability of the hydrolysis process. Each taster responded these questions 2 times, each one for a different set of samples corresponding to the selected hydrolysis conditions.

III. Results and Discussion

1. Yeast protein extract characterization

The 9 YPEs in study are obtained through different processing techniques and types of purification. Therefore, its cellular contents differ from each other. For this very reason, the first step of our study was to characterize these YPEs in protein and carbohydrates content.

1.1. Protein quantification

The protein content of each YPE was estimated through the Kjeldahl method. This method consists in a three-step protocol: digestion, distillation and titration. A nitrogen-to-protein conversion factor is taken into account depending on the type of protein being analysed. For YPEs, the conversion factor of 6.25 was considered, since no other values have been described as being more accurate. This conversion factor is calculated assuming that all nitrogen present in the sample is in the form of protein and therefore the presence of other forms of nitrogenous species such as nucleic acids can contribute to the inaccuracy of this estimation. This technique quantifies total protein, opposed to soluble protein, which is of special importance since the samples in study present several solubility issues.

The values of protein content of each YPE are presented in Table 4. It is important to note that values for protein content of liquid samples refer to the percentage of protein in dry weight, since these samples were lyophilized before protein content determination.

Table 4 - Protein percentage in the YPEs analysed, obtained by the Kjeldahl method. Data are means ± SD of three independent experiments.

YPE	% Protein
Divino (dry)	66.28 ± 2.24
Divino neutral (dry)	68.81 ± 0.37
SP1 (dry)	29.52 ± 0.63
SP2 (dry)	46.66 ± 1.72
SP1 + SP2 (dry)	38.61 ± 0.53
EPLc (dry)	56.94 ± 0.60
Divino (liquid)	71.25 ± 0.35
SP1 (liquid)	29.70 ± 2.63
SP1 + SP2 (liquid)	34.63 ± 0.31

The YPE Divino is the extract that has passed through more purification steps and therefore it is natural that this is the YPE with the highest protein content. We can observe that the YPEs Divino and Divino neutral have very similar protein content, which is expected since the neutralization process does not separate or eliminate protein from the sample. It is also possible to observe that the protein content of SP1 is the lowest. We know from Proenol S.A. that this subproduct corresponds mainly to yeast cell walls components. In these, proteins are mostly linked to polysaccharides, forming mannoproteins, and therefore its expected values fall under a low to moderate range. It is also interesting to note that the extract SP1 + SP2 has a protein content of approximately 69%. This value is the same as the result of the mean of the protein content values of SP1 and SP2, leading us to believe that the contributions of both subproducts is the same to the SP1 + SP2 extract.

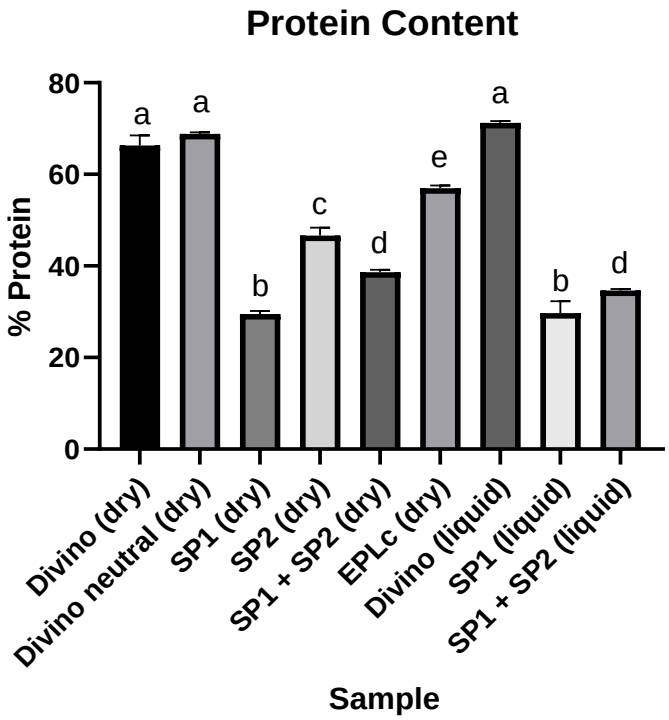


Figure 4 - Protein content in the YPEs analysed, obtained by the Kjeldahl method. Data are means ± SD of three independent experiments. Different letters above the columns represent statistical significance (P<0.05).

Moreover, it is possible to conclude that the physical state of the YPE and the neutralization process do not significantly influence the protein content of a given YPE, shown by the statistical comparison of values of extracts in its dry and liquid form, presented in Figure 4.

1.2. Carbohydrates quantification

The DuBois method was employed to quantify the carbohydrates content in each YPE. This method reacts with all carbohydrates without further specificity, allowing for a broader quantification. Each set of samples must be accompanied be a calibration curve (Figure 5), created by plotting the absorbance values of the standard solutions against their known concentrations.

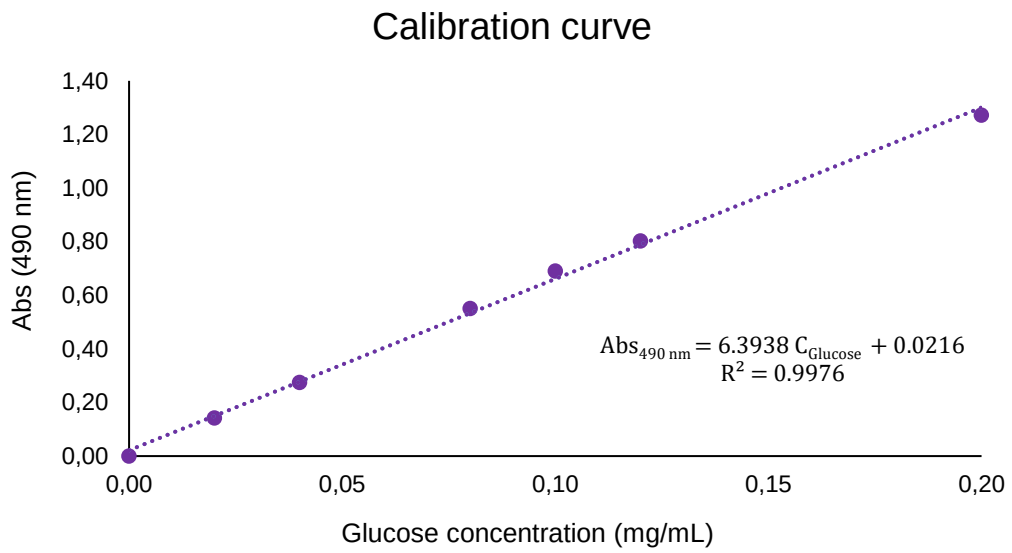


Figure 5 - Calibration curve for the DuBois method. Absorbance (490 nm) in relation to the concentration (mg/mL) of glucose standard solutions.

The values calculated for carbohydrates content of each YPE are presented in Table 5 and Figure 6.

Table 5 - Carbohydrates percentage in the YPEs analysed, obtained by the DuBois method. Data are means ± SD of three independent experiments.

YPE	% Carbohydrates
Divino (dry)	11.47 ± 2.61
Divino neutral (dry)	7.29 ± 1.53
SP1 (dry)	59.93 ± 12.99
SP2 (dry)	8.19 ± 2.06
SP1 + SP2 (dry)	34.18 ± 7.84
EPLc (dry)	8.29 ± 1.56
Divino (liquid)	11.90 ± 1.41
SP1 (liquid)	47.26 ± 8.38
SP1 + SP2 (liquid)	44.82 ± 11.71

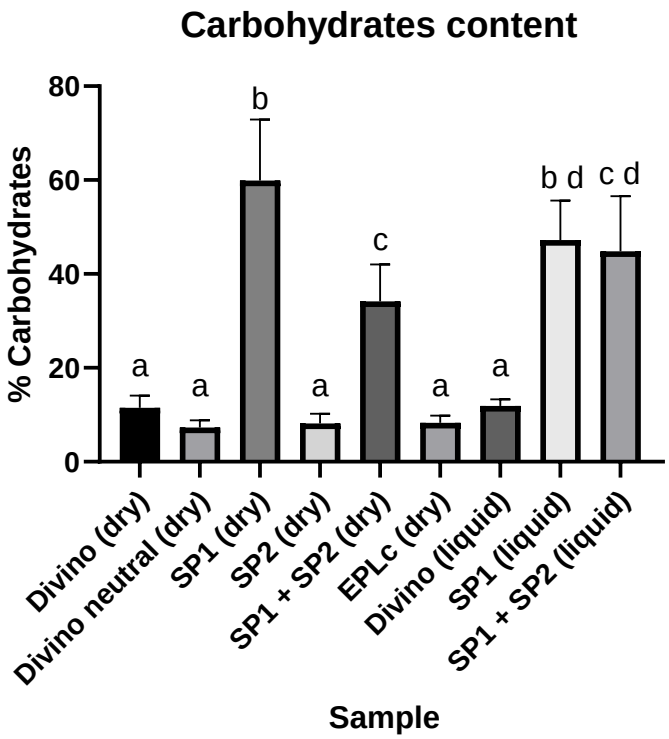


Figure 6 - Carbohydrate content in the YPEs analysed, obtained by the DuBois method. Data are means ± SD of three independent experiments. Different letters above the columns represent statistical significance (P<0.05).

The subproduct SP1, in both the dry and liquid forms, presents the highest carbohydrates content, followed by SP1 + SP2 and then, with lower contents, the remaining YPEs. The high content of carbohydrates of SP1 can be explained by the fact that this extract is composed, in the most part, by cell wall components, which in turn tend to be rich in carbohydrates, mainly on the form of glucans and mannans. The rest of the YPEs present lower values of carbohydrates content since these components are separated in the early stages of the purification process (Figure 1, Section II 2.1).

Considering the results from protein and carbohydrates percentage, it is possible to observe that these compounds make up around 80% of the extracts, with the remaining 20% corresponding to all other cellular components, such as nucleic acids, lipids, vitamins and minerals, secondary metabolites, etc., not quantified in this work.

2. Yeast Protein Extract hydrolysis – Initial optimization

A process of hydrolysis was optimized in order to break the proteins present in the different YPEs into smaller size peptides, since it is known that peptides smaller than 1

kDa are more likely to induce an umami flavour. Chemical and enzymatic hydrolysis are the two most frequent methods of hydrolysis, and both were tested in this work for the different extracts.

Chemical hydrolysis was conducted at a fixed protein concentration of 4.30 mg/mL, considering the protein content values obtained by Kjeldahl (Section III 1.1). Each YPE was resuspended or diluted in water to the desired final concentration, and aliquots of 1 mL were prepared. One of these aliquots serves as control as un-hydrolysed YPE. The remaining aliquots were heated at 50ºC or 70ºC, for 8h, 12h, 24h or 32h. The maximum binomial time-temperature to be studied were selected upon contacting Proenol S.A. to inquire about economically and technologically viable conditions, since the end goal is to replicate this protocol industrially. With the maximum temperature value set at 80ºC, tests were performed at both 50ºC and 70ºC, with agitation. Even though this temperature is considered a little low for this type of processes, it is important to note that it is a range in which autolysis is described, which can aid the reaction. Times were tested from 8h to 32h to accompany the development of the hydrolysis reaction.

Enzymatic hydrolysis was also performed on the different YPEs, using enzyme A and enzyme B separately. In order to compare both methods, the reaction was also performed at a normalized protein concentration of 4.30 mg/mL in Tris 0.1 M HCl pH 8, based on Kjeldahl results (Section III 1.1). Enzyme A was added to the YPE at an enzymatic activity of 50 U/g, 0.8 U/g and 0.1 U/g, while enzyme B was added at an enzymatic activity of 0.8 U/g, 0.5 U/g and 0.3 U/g. The hydrolysis was performed for each enzyme activity at three temperatures: 37ºC, 50ºC and 70ºC, all within the temperature working range of these enzymes, for 30 min with agitation. The reaction was stopped by incubation at 95ºC for 5 min. For these hydrolysis, three controls were considered: enzyme A or enzyme B without YPE, and YPE without the addition of either enzyme.

After the reaction was stopped, the samples were precipitated with 10% TCA in ice, which leads to the precipitation of high molecular weight proteins (un-hydrolysed proteins), centrifuged, and the pellet and supernatant were stored at -20ºC separately for further analysis.

The optimization of hydrolysis and analysis of hydrolysates was initially performed on YPE Divino, and only then on the other YPEs. The next sections present results only of YPE Divino hydrolysis as an example. Results obtained for other YPEs were similar, unless mentioned otherwise.

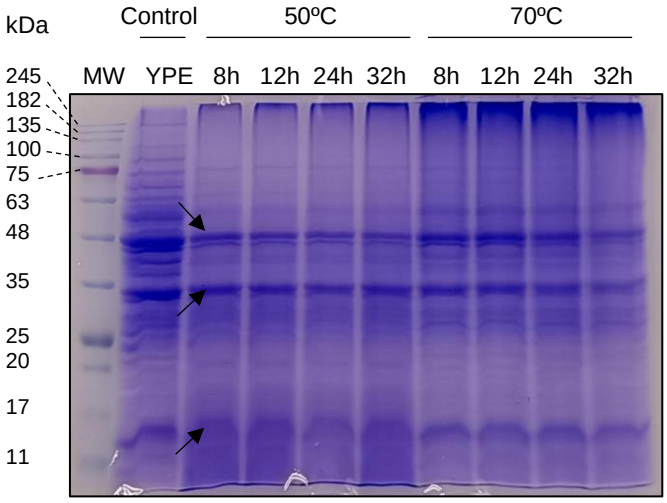
2.1. Electrophoresis analysis

As a preliminary analysis of the hydrolysation efficiency of the different YPEs, an SDS-PAGE electrophoresis, used for the separation and analysis of proteins based on its molecular weight, was performed.

The pellets of the different conditions were resuspended in loading buffer and a DTT urea solution, and denatured before loading into the gels. This technique allows us to visualize high molecular weight proteins, being therefore, in this case, an indirect analysis, since we can take conclusions based on the intensity and number of the bands that correspond to higher molecular weight proteins after hydrolysis, instead of small proteins and peptides. This way, SDS-PAGE provides us a visual representation of the hydrolysis process by illustrating the breakdown of intact protein, unlike other methods, that will be addressed posteriorly, that focus on the characterization of the hydrolysed peptides and small proteins.

In Figure 7 we can observe the gel slabs of the chemical and enzymatic hydrolysis of YPE Divino, as an example.

I. Chemical hydrolysis



II. Enzymatic Hydrolysis

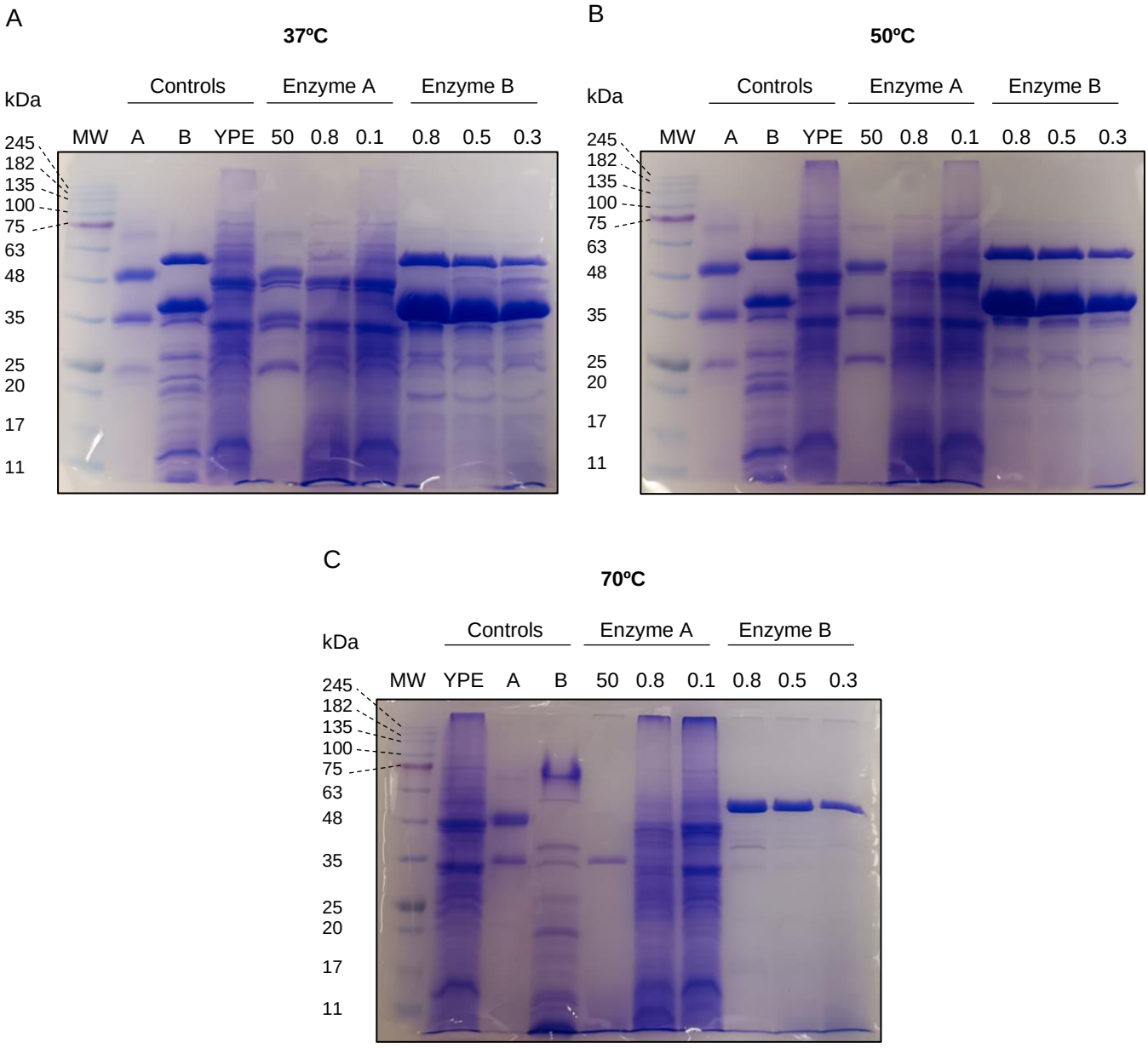


Figure 7 - SDS-PAGE analysis of YPE Divino hydrolysis. The pellets of control samples and hydrolysates of different conditions were loaded into 4% (stacking) and 12% (separating) acrylamide gels. 50 µg of protein were loaded for each condition. Gels were stained with Bio-Rad Coomassie Blue R-250 dye-based reagent. Lane 1 is the molecular marker. (I) Chemical hydrolysis performed at a protein concentration of 4.30 mg/mL for 8h, 12h, 24h and 32h at 50ºC and 70ºC, with the respective control at 0h. (II) Enzymatic hydrolysis performed at a protein concentration of 4.30 mg/mL with two different enzymes at different enzymatic activities: enzyme A at 50 U/g, 0.8 U/g and 0.1 U/g and enzyme B at 0.8 U/g, 0.5 U/g and 0.3 U/g, at 37ºC (II. A), 50ºC (II. B) and 70ºC (II. C), with controls of enzymes A and B and unhydrolyzed YPE.

In Figure 7 I. it is possible to observe that the hydrolysis is indeed happening, since there is a decrease in the number of bands and their intensity in hydrolysis conditions with a higher reaction time, when compared to the control. Efficient hydrolysis is observable for proteins with a molecular weight between 63 kDa and 245 kDa, however, there are

proteins that are resistant to hydrolysis in the conditions tested (marked by the arrows). On top of this, it is observable that the lanes corresponding to the hydrolysis performed at 70ºC appear to be more intense than the control and the ones corresponding to the hydrolysis at 50ºC. This result was not expected, since a higher temperature usually translates into a more extensive reaction. However, we can see that at the top of the lanes corresponding to the 50ºC hydrolysis, more smear is present than at lanes corresponding to the 70ºC hydrolysis. This detail leads us to believe that a problem in sample solubility may have occurred: the dissolution of the YPE in water may have worked better for the control and 50ºC hydrolysis, while the dissolution of the same YPE in water may not have been as efficient for the 70ºC hydrolysis initial stock. Another possible explanation is the occurrence of protein aggregation events, which leads to structures with high molecular weight.

For enzymatic hydrolysis (Figure 7 II.), the same initial YPE stock at 4.30 mg/mL of protein was used for the different conditions. Comparing the different conditions of enzymatic hydrolysis, it is possible to observe that the conditions corresponding to hydrolysis with enzyme B present a significantly lower number of bands. As for enzyme A, there is a clear outperformance at an enzymatic activity of 50 U/g, compared to 0.8 U/g and 0.1 U/g. It is interesting to note that in the presence of enzyme A 50 U/g and enzyme B in any of the three enzymatic activities tested, the YPE appears to be hydrolysed to a great extent, with almost all the bands visible corresponding to the enzyme control. It is also possible to see that a higher temperature appears to cause a more efficient hydrolysis, since there is a reduction of the number of bands in the different lanes with the increase of temperature. Taking all this into consideration, it is possible to conclude that, for both enzymes A and B the hydrolysis was more efficient at higher enzymatic activities (with the hydrolysis with enzyme A at 0.1 U/g appearing to be practically inexistent). Additionally, for the same enzymatic activity, higher temperatures lead to more extensive hydrolysis, with this effect being more prominent for enzyme B and less pronounced for enzyme A. Overall, enzyme B appears to be more efficient.

Comparing the I and II gels, the enzymatic hydrolysis appears to be more efficient. This result is beneficial for the company since despite the added cost of enzymes, there is a considerable energy saving in comparison to the chemical hydrolysis. Furthermore, as described above, enzymatic hydrolysis results in more repeatable outcomes.

2.2. High Pressure Liquid Chromatography

HPLC offers high resolution, sensitivity and accuracy, serving as a critical tool for characterizing and quantifying different types of compounds. Here, HPLC was used to obtain a peptide profile for each hydrolysis to be able to monitor the progress of the hydrolysis reaction and to optimize the separation conditions necessary for the LC-MS/MS analysis.

The chromatographic conditions were optimized to achieve the most efficient separation of the YPE hydrolysates with a reverse-phase C18 column. The supernatants of hydrolysates were injected into the HPLC equipment after centrifugation, filtration and dilution.

In Figure 8 the chromatogram of the unhydrolyzed YPE Divino is presented. This condition is used as control for the different hydrolysis conditions studied. Figures 9, 10 and 11 present the chromatograms resulting from the analysis of YPE Divino hydrolysates, as an example, after chemical and enzymatic hydrolysis with enzyme A and enzyme B, respectively.

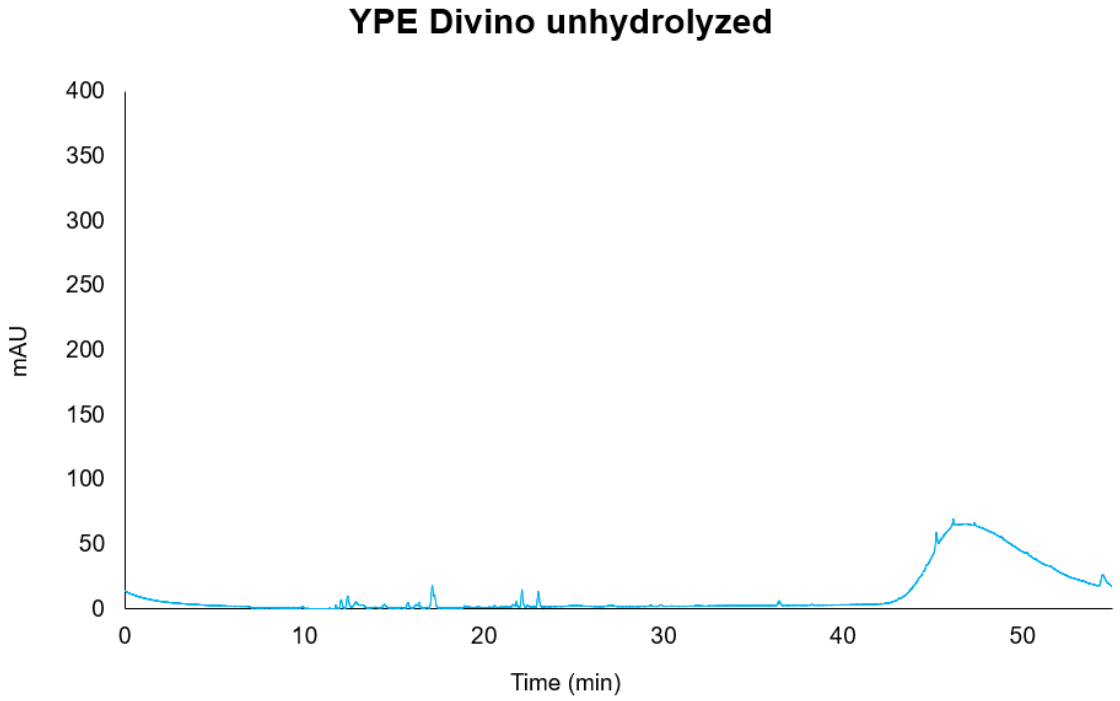


Figure 8 - Chromatographic profile (280 nm) of YPE Divino unhydrolyzed, obtained by HPLC-DAD. 25uL of the sample were injected into a C18 column. Condition used as control.

The unhydrolyzed YPE Divino chromatogram shows a low number of peaks, indicating that the protein extract remains in its native form, with limited presence of smaller

peptides or amino acids. This observation aligns with the fact that the hydrolysate was precipitated and only the supernatant was injected into the HPLC, meaning that the proteins with the highest molecular weight weren't analysed. Consequently, the overall signal of the chromatogram is low, consistent with the absence of hydrolysis.

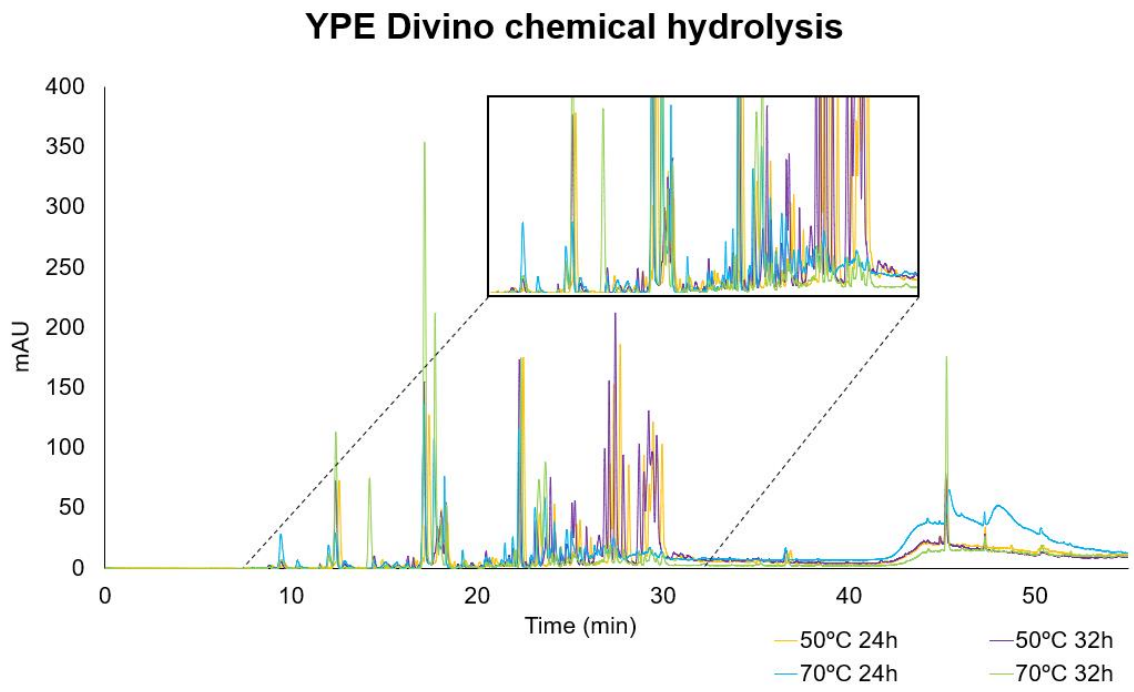


Figure 9 - Chromatographic profile (280 nm) of YPE Divino hydrolysates, following chemical hydrolysis, obtained by HPLC-DAD. 25uL of each sample were injected into a C18 column.

From the chromatograms in Figure 9 we can observe the progression of the hydrolysis reaction. Even though the specific proteins and/or peptides corresponding to each peak cannot be identified directly, it is possible to see that certain peaks tend to decrease in intensity with the advancement of hydrolysis, while others tend to increase. We can then suppose that the peaks eluted around 25 to 30 minutes are proteins with a higher molecular weight. Initially, these proteins appear to increase in quantity compared to the control, possibly due to the formation of intermediate products during the early stages of hydrolysis. As hydrolysis progresses, these high molecular weight proteins are further degraded, as indicated by a reduction in peak intensity with increasing reaction time and temperature.

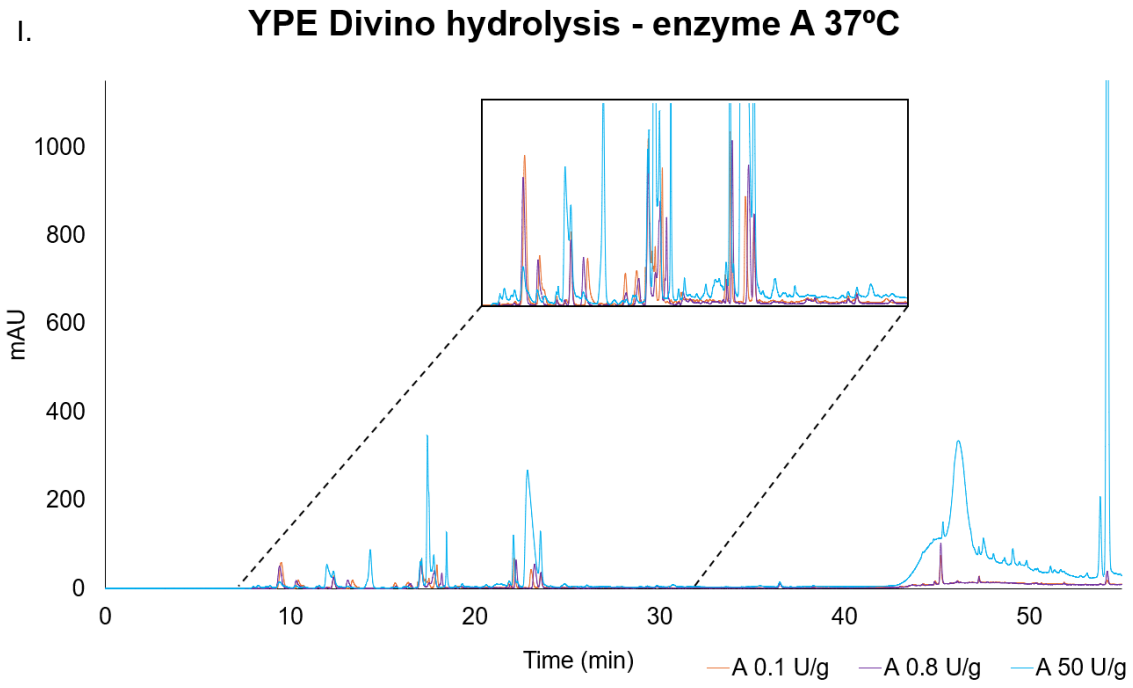
Contrarily, the peaks eluting around 10 to 25 minutes and 40 to 50 minutes are likely indicative of smaller molecular weight proteins or peptides, since these peaks tend to appear in higher quantities with a higher reaction time and temperature, which is consistent with the expected breakdown of larger proteins into smaller fragments.

These results also demonstrate that a higher temperature and a longer hydrolysis time leads to a more extensive protein degradation. However, the difference in protein breakdown between 24h and 32h at 70ºC is less pronounced compared to the difference observed at 50ºC, suggesting that the majority of hydrolysis at 70ºC may occur within the first 24 hours, implying that extending the hydrolysis beyond this period may not significantly enhance the efficiency of protein degradation.

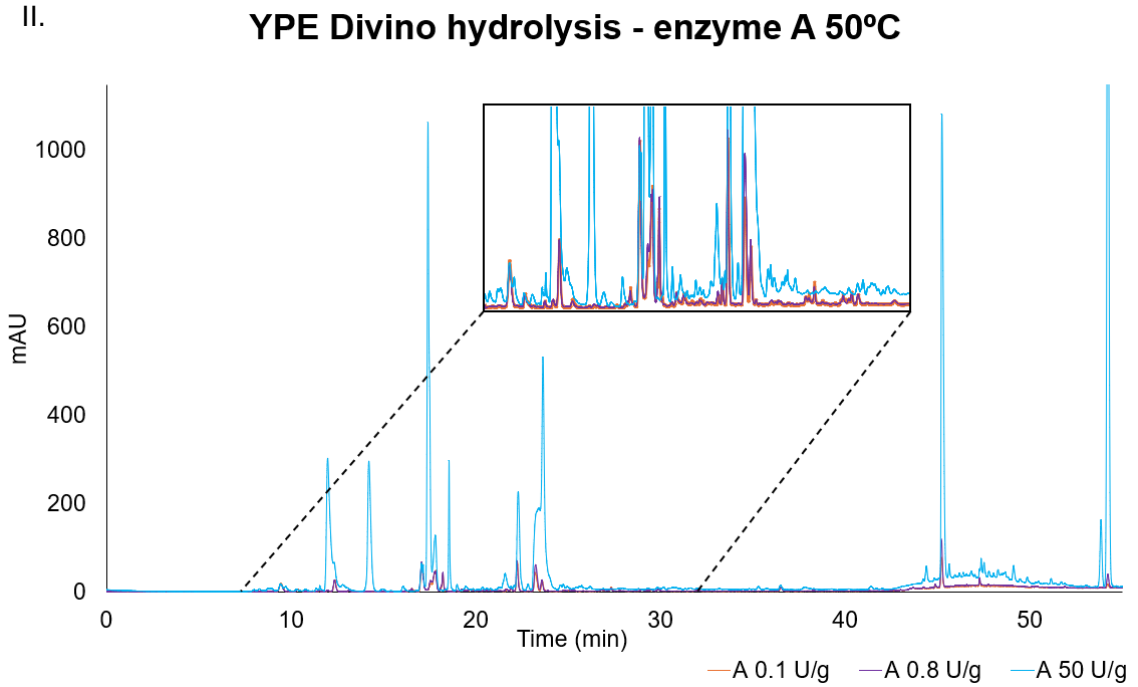
These observations can be correlated to the SDS-PAGE gels. In the electrophoresis analysis it was concluded that a higher reaction time leads to a decrease of high molecular weight proteins. With the chromatograms, we see the increase of smaller molecular weight proteins and/or peptides, which are produced by the breakdown of high molecular weight proteins. However, this same relation is not observed for the 70ºC hydrolysis, since in the SDS-PAGE gels the lanes appear to be more intense for these conditions when compared to the 50ºC hydrolysis, but in the chromatograms presented we also see an increase in smaller molecular weight proteins and/or peptides.

In spite of these observations, the interpretation of these chromatograms alone does not give us sufficient data to take further conclusions.

I.



II.



III.

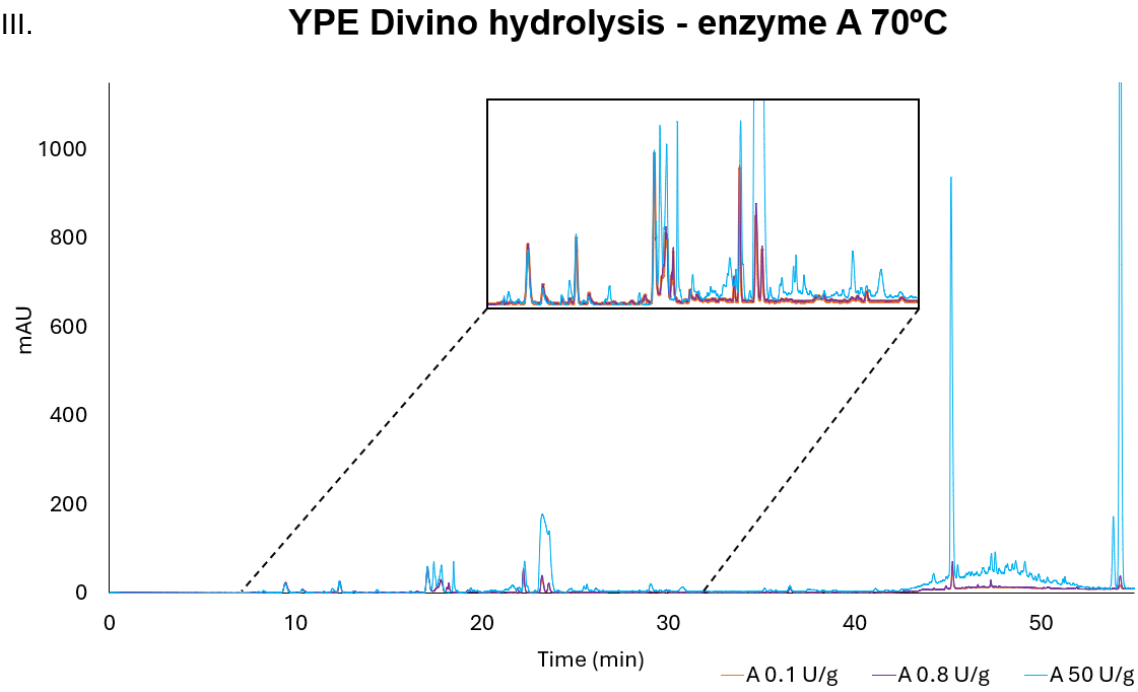
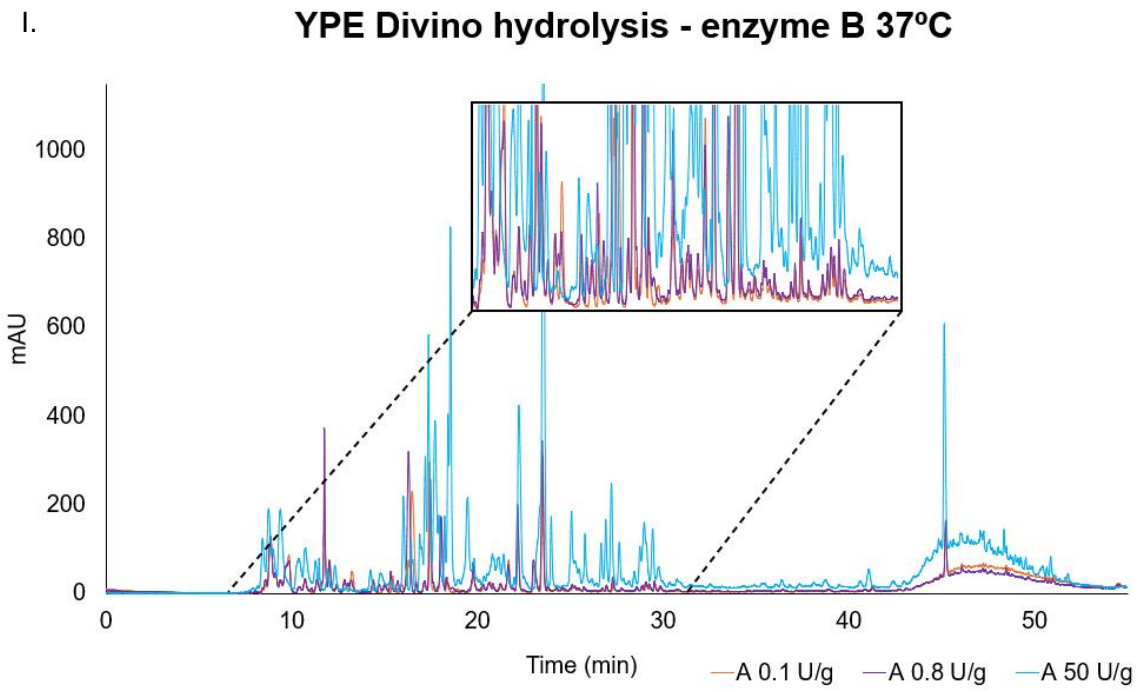


Figure 10 - Chromatographic profile (280 nm) of YPE Divino enzymatic hydrolysates, following hydrolysis with enzyme A, obtained by HPLC-DAD. 25uL of each sample were injected into a C18 column. (I) Hydrolysis at 37ºC. (II) Hydrolysis at 50ºC. (III) Hydrolysis at 70ºC.

The enzyme A was used in three largely different enzymatic activities, which translates in an evident difference in the hydrolysis extent. It is clear by the observation of the

profiles depicted in Figure 10 that a higher enzymatic activity results in a more extensive reaction, since the height of the peaks increases significantly. Furthermore, it was concluded that even though the enzyme is active at a low temperature of 37ºC, a temperature of 50ºC seems to be optimal for the hydrolysis. At this temperature, a significant increase in peak intensities is observed across all enzymatic activities, compared to the 37ºC conditions. However, at 70ºC, while the enzyme still retains significant activity, there is a slight decrease in overall peak intensities compared to the 50ºC conditions, especially at 0.1 U/g and 0.8 U/g. For this very reason, using a temperature of 70ºC instead of 50ºC is considered a waste of energy, since it doesn´t translate in better results.

I.



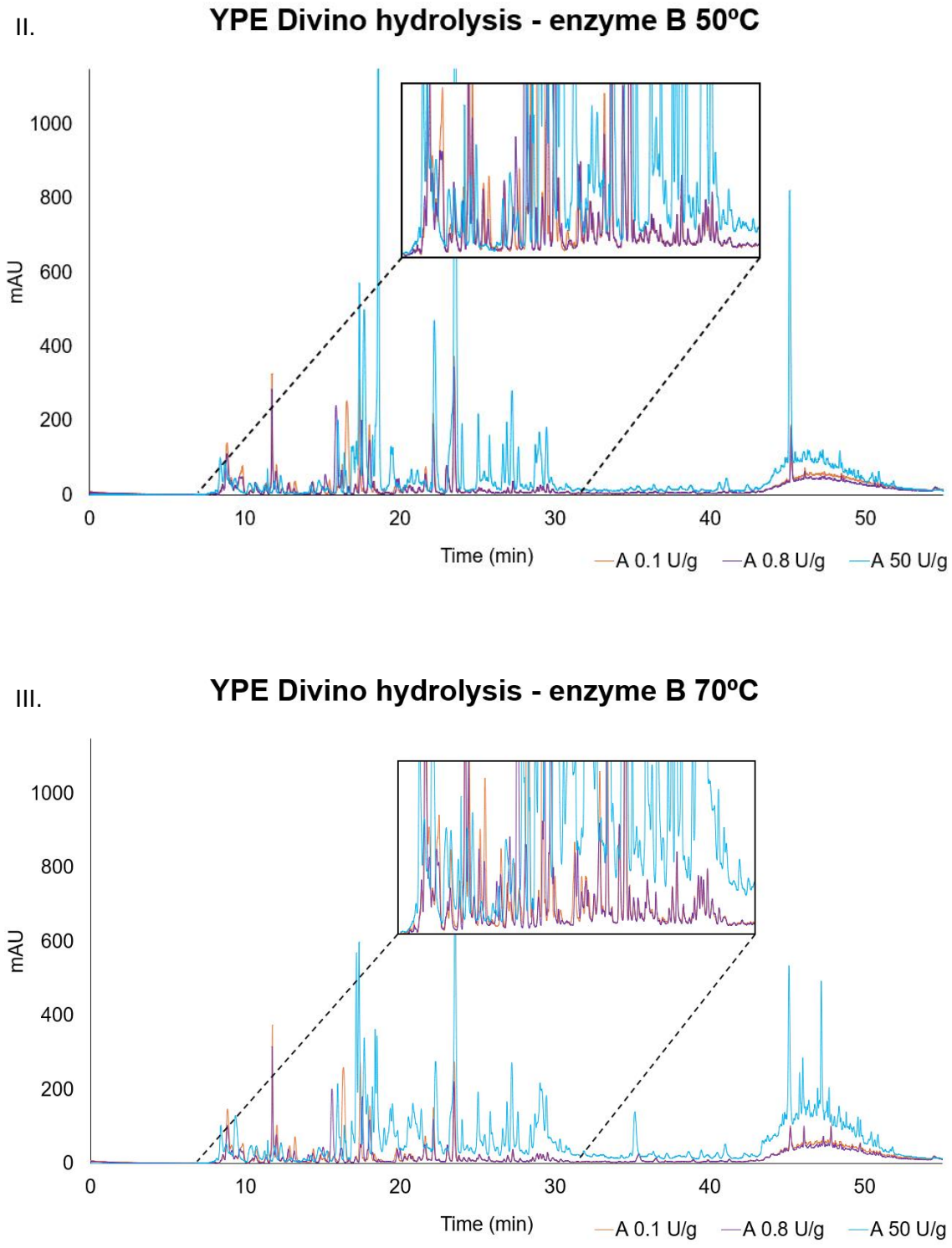


Figure 11 - Chromatographic profile (280 nm) of YPE Divino enzymatic hydrolysates, following hydrolysis with enzyme B, obtained by HPLC-DAD. 25uL of each sample were injected into a C18 column. (I) Hydrolysis with enzyme B at a 0.3 U/g activity. (II) Hydrolysis with enzyme B at a 0.5 U/g activity. (III) Hydrolysis with enzyme B at a 0.8 U/g activity.

Hydrolysis using enzyme B shows substantial peaks within the 5 to 30 minutes range, even at 37ºC. This suggests that even at a relatively low temperature, enzyme B is active and effective at hydrolysing the YPE. As observed with enzyme A, peak intensities

increase with higher enzymatic activities of enzyme B, indicating a more extensive breakdown of proteins into smaller peptides and amino acids.

At 50ºC, the chromatograms exhibit similar retention times for the peaks but with higher intensities compared to those observed at the 37°C conditions. This indicates that increasing the temperature to 50°C enhances the enzymatic activity, leading to a more efficient hydrolysis. The fact that more pronounced peaks are observed across all enzymatic activities suggests that 50ºC is closer to the optimal temperature for enzyme B, leading to a more complete and efficient protein breakdown.

However, at 70°C, the chromatographic profile changes noticeably. While some peaks are still present, particularly at higher enzymatic activities, the overall intensity is reduced compared to the 50°C condition. This could suggest that 70°C may exceed the optimal temperature for enzyme B, potentially leading to partial denaturation or decreased activity of the enzyme. Consequently, the efficiency of hydrolysis is reduced at this temperature, resulting in less extensive protein breakdown.

2.3. High Pressure Liquid Chromatography - Size Exclusion Chromatography

HPLC-SEC allows the separation of molecules based on their size or hydrodynamic volume, being particularly valuable in our study for assessing molecular weight distribution of hydrolysates in the different hydrolysis conditions, since this is an important factor in the development of an umami flavour enhancer.

As standards for this technique, three molecules of different sizes were used: a peptide of 3 kDa, and two proteins of different sizes (casein with 20 kDa (Vincent et al., 2016) and bovine serum albumin (BSA) with 66.5 kDa (Babcock & Brancaleon, 2013)). The use of these molecules allows us to correlate elution time to molecular weight. Samples pass through a porous stationary phase, in which larger molecules elute from the column earlier, as they are not small enough to enter the pores and thus travel through a shorter path. On the contrary, smaller molecules enter the pores, passing through a longer path and therefore elute later. Knowing that molecules of a specific molecular weight are being eluted at a specific time, it is possible to conclude that any molecule being eluted earlier is bigger that our standard, and any molecule being eluted later is smaller than our standard.

Figure 12 represents the chromatograms of the standards used for the interpretation of chromatograms of HPLC-SEC of unhydrolyzed YPE (Figure 13), chemical (Figure 14) and enzymatic hydrolysates, obtained with enzyme A (Figure 15) and enzyme B reaction (Figure 16).

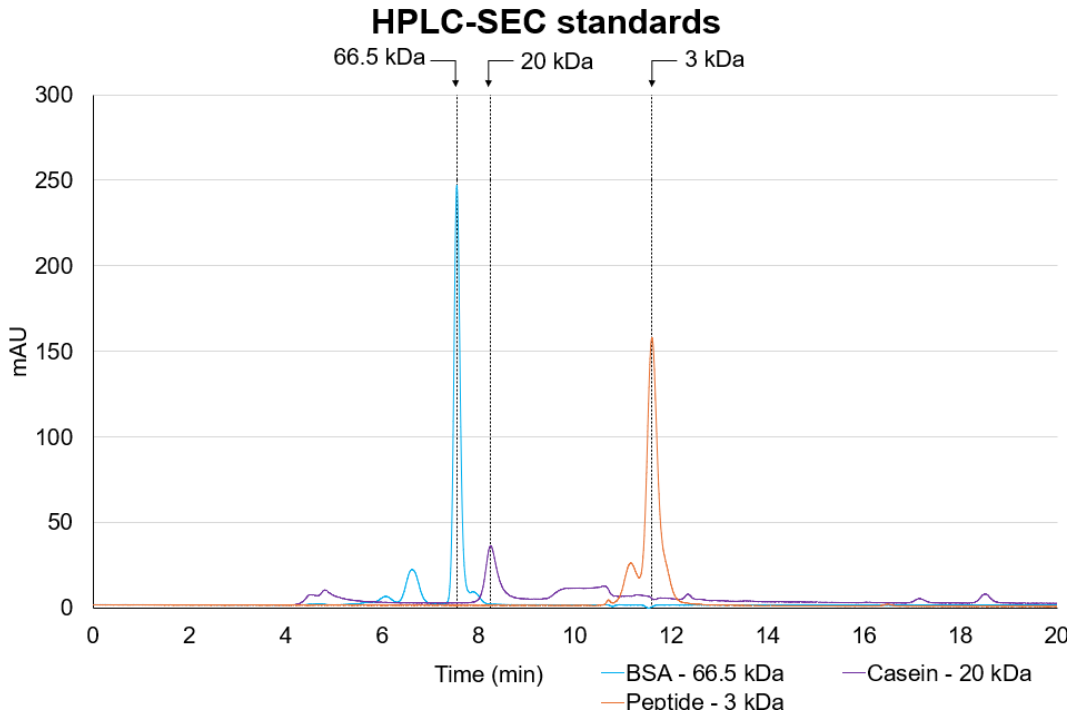


Figure 12 - Chromatographic profile (280 nm) of molecular weight standards for HPLC-SEC.

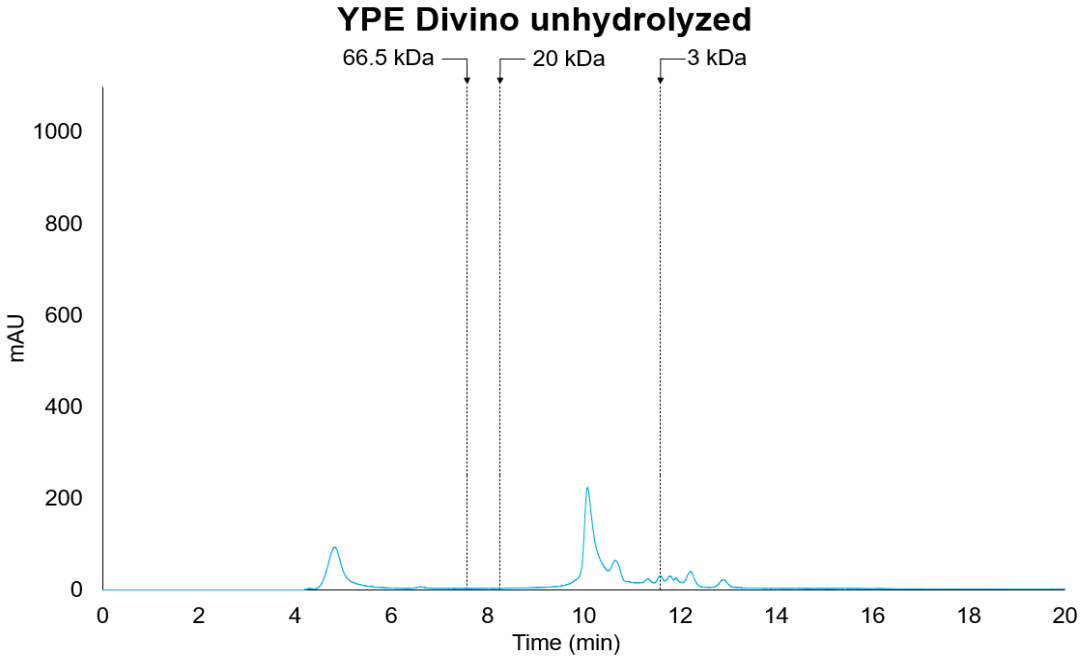


Figure 13 - Chromatographic profile (280 nm) of YPE Divino unhydrolyzed, obtained by HPLC-SEC. Condition used as control. Vertical lines represent the elution time of molecules with molecular weight of standards.

The chromatogram of the unhydrolyzed YPE Divino shows minimal peaks, with low intensities. The proteins correspondent to these peaks are being mainly eluted before the 12 minutes mark, which corresponds to the 3 kDa standard. This leads us to conclude that the unhydrolyzed YPE presents in its majority proteins bigger than this.

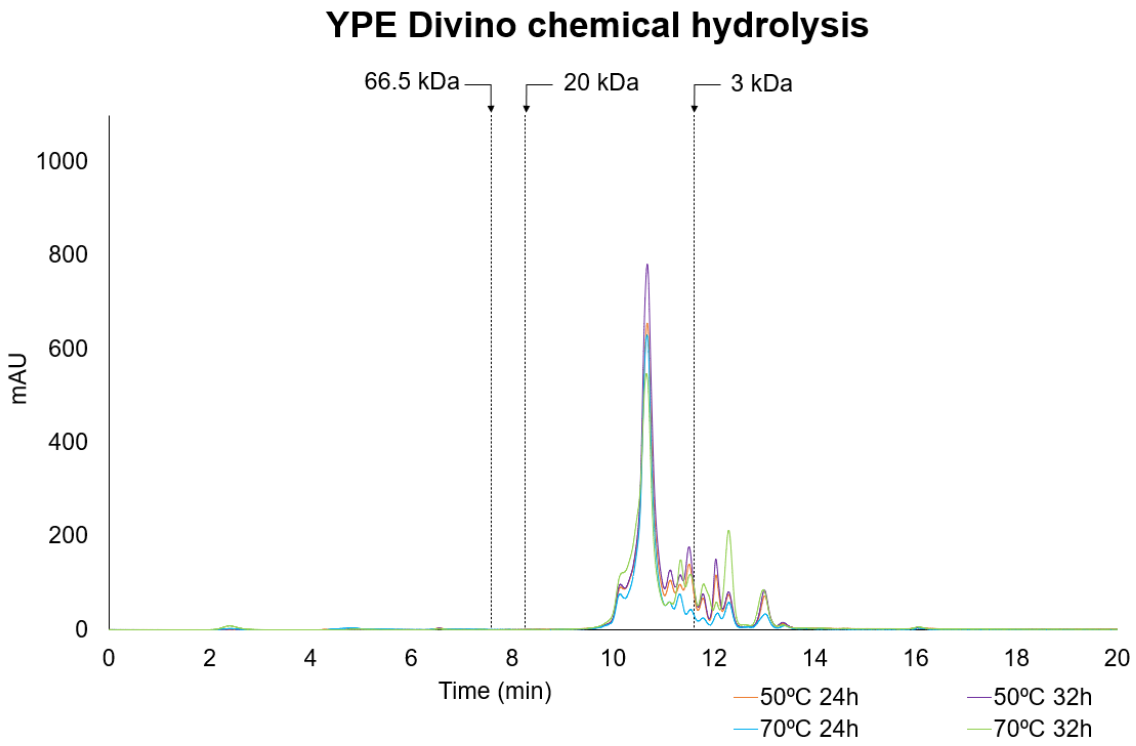
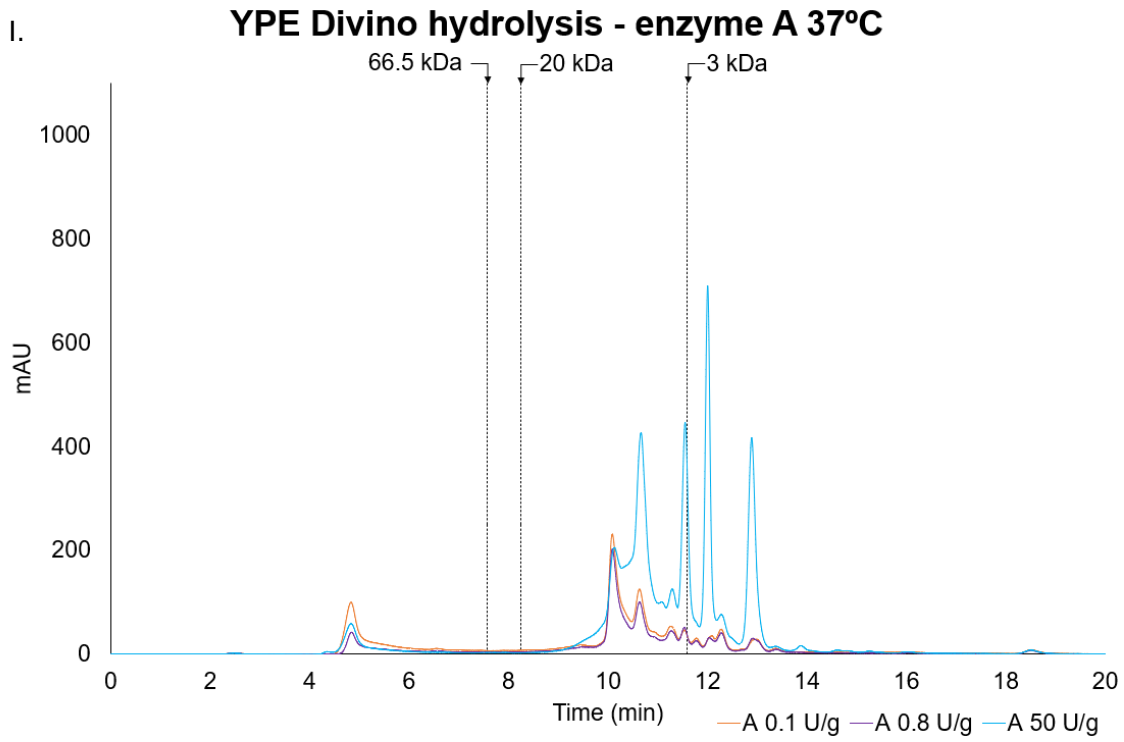


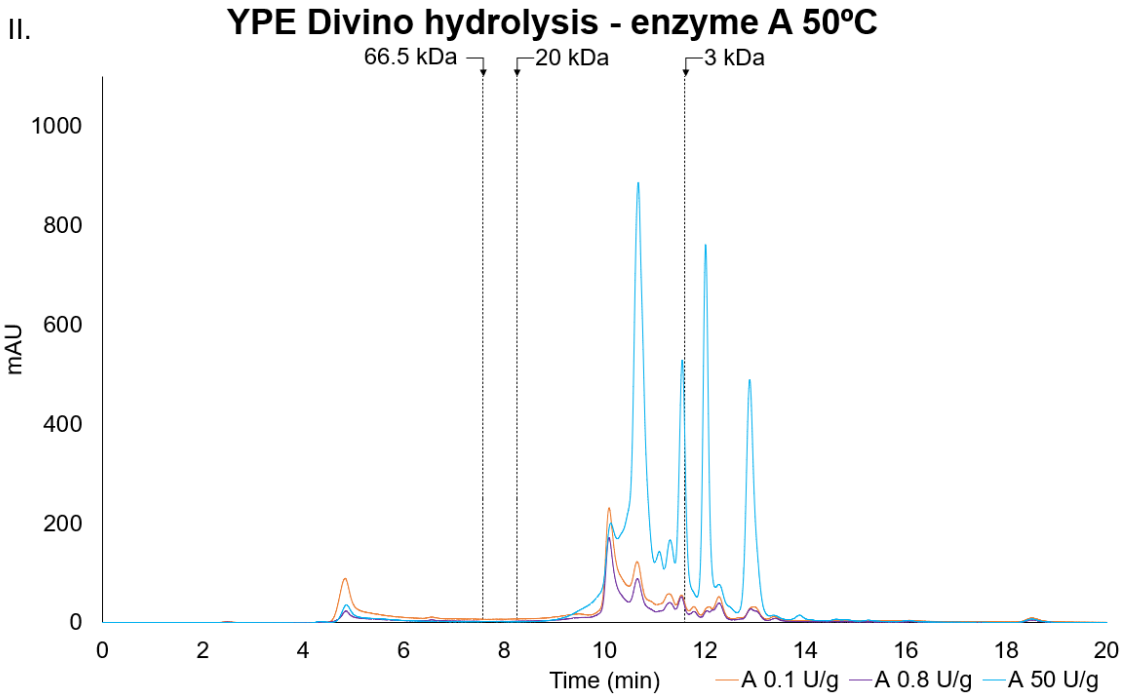
Figure 14 - Chromatographic profile (280 nm) of YPE Divino chemical hydrolysates, obtained by HPLC-SEC. Vertical lines represent the elution time of molecules with molecular weight of standards.

In figure 14 it is possible to observe that the hydrolysis time effect at 50ºC does not have a strong impact, since chromatograms of hydrolysis at 50ºC for 24h and 32h appear to be similar, without significant differences. This leads us to believe that at 50ºC the hydrolysis reaches a plateau at around 24h, an information that was not observable by the HPLC analysis (Section III 2.2). However, time does seem to have an influence on hydrolysis efficiency at 70ºC, with the 32h condition resulting in higher intensities in peaks corresponding to peptides smaller than 3 kDa. Interestingly, hydrolysis at 50ºC, at both time points, appears to produce a bigger quantity of smaller peptides, when compared to hydrolysis at 70ºC for 24h.

I.


YPE Divino hydrolysis - enzyme A 37ºC
66.5 kDa
20 kDa
3 kDa
mAU
Time (min)
A 0.1 U/g
A 0.8 U/g
A 50 U/g

II.


YPE Divino hydrolysis - enzyme A 50ºC
66.5 kDa
20 kDa
3 kDa
mAU
Time (min)
A 0.1 U/g
A 0.8 U/g
A 50 U/g

III.

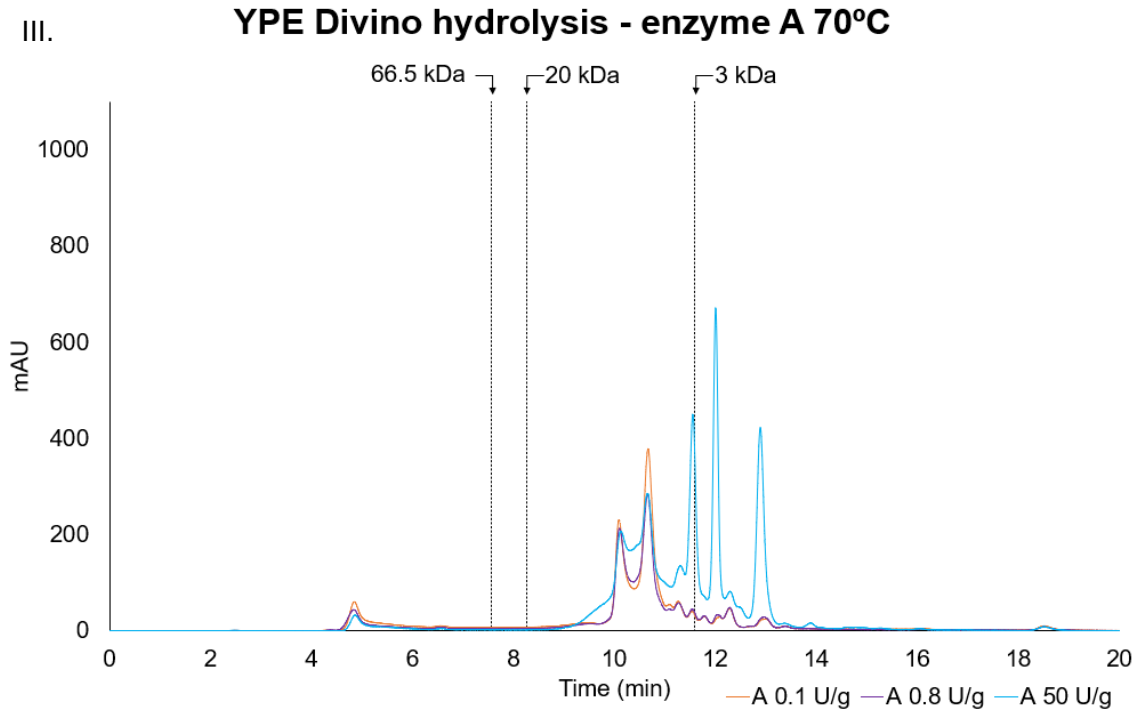
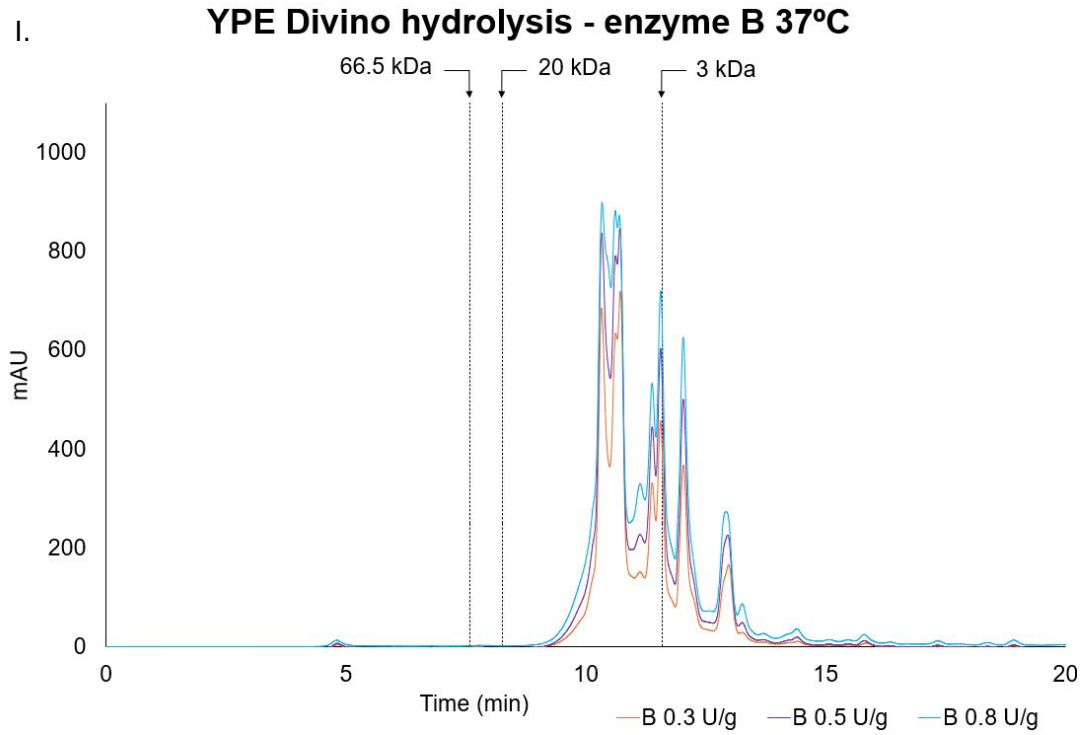


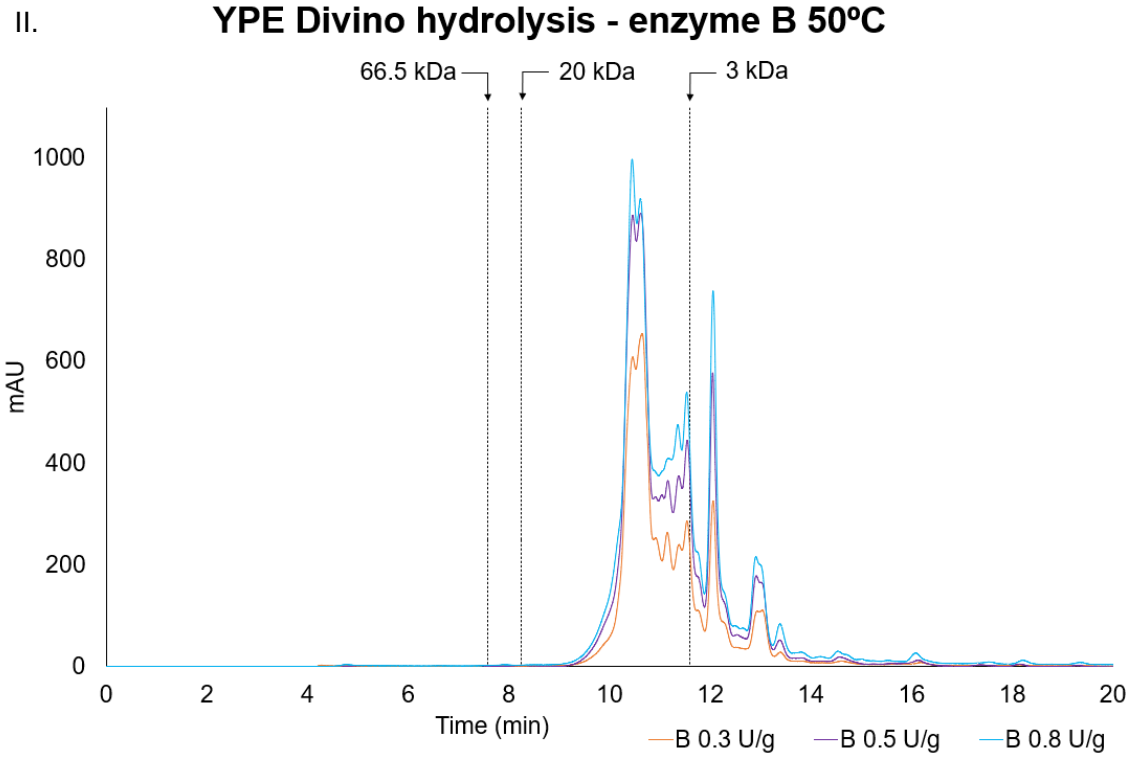
Figure 15 - Chromatographic profile (280 nm) of YPE Divino enzymatic hydrolysates, following hydrolysis with enzyme A, obtained by HPLC-SEC. Vertical lines represent the elution time of molecules with molecular weight of standards. (I) Hydrolysis at 37ºC. (II) Hydrolysis at 50ºC. (III) Hydrolysis at 70ºC.

The chromatographic profiles of hydrolysates obtained through enzyme A reaction are similar at the three different temperatures tested. We can, however, conclude that even though peak intensities increase in the 50ºC conditions when compared to the 37ºC conditions, a decrease in overall peak intensities happens at the 70ºC conditions. These conclusions align to the preliminary observations made on the HPLC-DAD analysis.

I.


YPE Divino hydrolysis - enzyme B 37ºC
66.5 kDa
20 kDa
3 kDa
mAU
1000
800
600
400
200
0
0
5
10
15
20
Time (min)
B 0.3 U/g
B 0.5 U/g
B 0.8 U/g

II.


YPE Divino hydrolysis - enzyme B 50ºC
66.5 kDa
20 kDa
3 kDa
mAU
1000
800
600
400
200
0
0
2
4
6
8
10
12
14
16
18
20
Time (min)
B 0.3 U/g
B 0.5 U/g
B 0.8 U/g

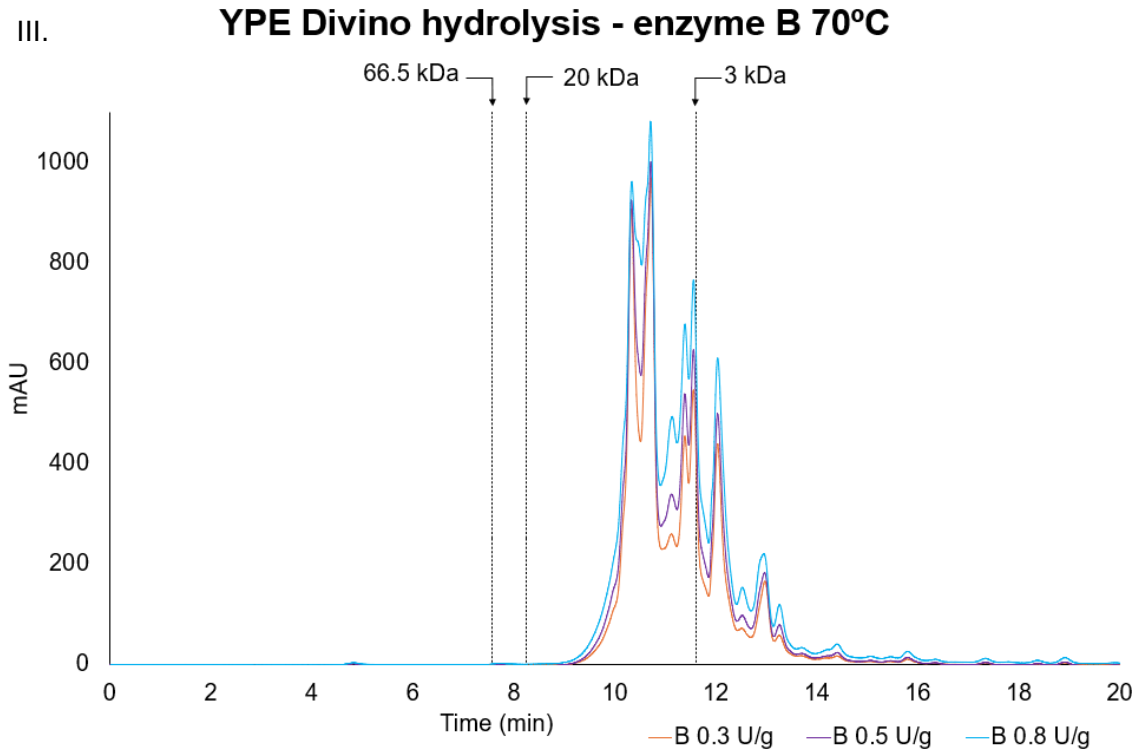


Figure 16 - Chromatographic profile (280 nm) of YPE Divino chemical hydrolysates, following hydrolysis with enzyme B, obtained by HPLC-SEC. Vertical lines represent the elution time of molecules with molecular weight of standards. (I) Hydrolysis at 37ºC. (II) Hydrolysis at 50ºC. (III) Hydrolysis at 70ºC.

Similarly to the observed with enzyme A, enzyme B appears to be more efficient at 50ºC. However, enzyme B appears to maintain its hydrolytic activity better than enzyme A, especially at higher enzymatic activity, since the difference in peak intensities at 50ºC and 70ºC for enzyme B is not as pronounced as the observed for enzyme A. It is important to note, however, that enzyme A produces a broader range of smaller peptides, with the peaks corresponding to peptides smaller than 3 kDa being more intense on the enzyme A conditions when compared to the enzyme B conditions.

HPLC-SEC chromatograms of enzymatic hydrolysis allow us to conclude that 50ºC and a higher enzymatic activity (50 U/g for enzyme A and 0.8 U/g for enzyme B) promotes the formation of smaller peptides. These conditions seem, therefore, to be the ones more effective for the hydrolysis to occur more extensively and thus may be the ones that allow the generation of peptides with a greater umami intensity.

It was interesting to note that, although dry and liquid samples present similar profiles after enzyme A hydrolysis, the same does not happen for enzyme B hydrolysis. In this case, dry samples present similar profiles amongst themselves, and the same happens for liquid samples, but these profiles are not similar amongst samples in different states, with liquid samples presenting smaller peptides. This result may be beneficial for the

company Proenol S.A. since the hydrolysis is more economically and energetically beneficial when conducted on liquid samples.

Upon discussing the obtained results of chemical hydrolysis vs. enzymatic hydrolysis with Proenol S.A., it was decided that the chemical hydrolysis protocols were to be dropped, since this technique is described as having an inferior repeatability compared to the enzymatic hydrolysis. Since the main goal of this study is to produce an umami flavour enhancer that can be commercialized by Proenol S.A., it is of great importance that the final product is consistent in different batches and the results are reproduceable over time. For this reason, the following experiments are performed only on samples of enzymatic hydrolysates.

2.4. Liquid Chromatography - Tandem Mass Spectrometry and use of prediction tools

In our study, LC-MS/MS is particularly valuable for the identification and characterization of the peptides present in the hydrolysates.

Up to this point in the study, all analysis regarding enzymatic hydrolysis were performed on hydrolysates obtained following the protocol described on Section II 3.2 initial optimization – protocol 1. However, this protocol was found not to be viable for a large scale production by Proenol S.A. due to the high enzyme volume that was being used. For this very reason, the protocol for enzymatic hydrolysis was altered to fit into the economic requirements, and the following experiments were performed only on YPE Divino.

Three different YPE concentrations were tested (4 mg/mL, 6 mg/mL and 10 mg/mL, which correspond to a protein concentration of 2.65 mg/mL, 3.98 mg/mL and 6.63 mg/mL, respectively), with different volumes of enzyme (20 µL and 30 µL) of the two enzymes in study (enzyme A at 50 U/g and enzyme B at 0.8 U/g), at different times of hydrolysis (4h and 24h) at 50ºC. After precipitation and centrifugation, the supernatant was analysed by a colorimetric assay to quantify the peptide concentration, and then passed through a C18 column in order to eliminate impurities. After repeating the peptide quantification, the samples were diluted and injected into the LC-MS/MS following the procedure indicated in Section II 4.6. Afterwards, the results were analysed using the Proteome Discoverer software. This results in a list of peptides, identified with various degrees of confidence by the software (low, medium and high), present in each sample.

Only the ones identified with a high degree of confidence were considered for further analysis. The peptide molecular weight distribution of the different samples is presented in Figure 17. Additionally, the list of amino acid sequences of the peptides identified with a high degree of confidence were then uploaded to the UMPred-FRL and iBitter-Fuse prediction tools for identification of umami and bitter peptides, respectively. These prediction tools estimate a value for the probability of a peptide being umami or bitter. If that probability is equal or higher to 0.5, then the peptide is identified as umami or bitter, depending on the tool being used. Table 5 presents a summary of the conclusions retrieved from the analysis by Proteome Discoverer and the prediction tools.

In Table 5 different data, relevant to the umami potential and flavour inducing capability, are presented. The number of peptides identified with high confidence can provide information on either the extent of hydrolysis or the homogeneity of the peptides present in the sample. It is necessary to be aware that this value does not necessarily represent the sample's diversity accurately, since the hydrolysates can contain peptides that were not identified by the software with high confidence but are indeed present. As described before, glutamate residues are often associated with the umami flavour. Therefore, even though a higher number of these residues, either in absolute value or relative to the total number of peptides identified, does not necessarily result in a more intense umami flavour, it can be a strong indicative of the flavour enhancer's potential. Then, the data obtained form the prediction tools is presented. The number of umami and bitter peptides, when analysed with the average probabilities of the peptides presenting either one of these flavours, are probably the most relevant results. Two values of average probability are displayed: the total average probability corresponding to the average of the umami probability of all peptides identified with high confidence in the sample, and the specific average probability corresponding to the average of the umami probability of only the peptides identified as being umami. This means that the first one gives a probability of a random peptide from the sample being umami, while the second one represents the probability of a random peptide identified as umami being, in fact, umami. The same logic follows the bitter probabilities.

In figure 17, each violin represents the range and frequency of peptide weight. The wider sections of each plot indicate a higher frequency of peptides if that particular weight, while narrower sections indicate lower frequencies. It is observable in Figure 17 that conditions of hydrolysis of 4 mg of YPE Divino with 30 µL of enzyme A for 24h, 6mg of Divino with either 20 µL of enzyme A for 24h or 30 µL of enzyme A for 4h, and 10 mg of Divino with 30 µL of enzyme A for 24h originate peptides with a relatively small weight,

with median values around or below 1 kDa. This is an interesting and promising result, since, as described before, smaller peptides are generally more associated with umami flavour. It is also possible to observe that the hydrolysis of 6mg of YPE Divino with 20 µL of enzyme A for 24h results in the most homogenous and consistent peptide weight. However, the hydrolysis of 6 mg of YPE Divino with 30 µL of either enzyme A or B (with or without stronger agitation) for 24h results in a much less consistent peptide weight, with a lower frequency of more varied weights.

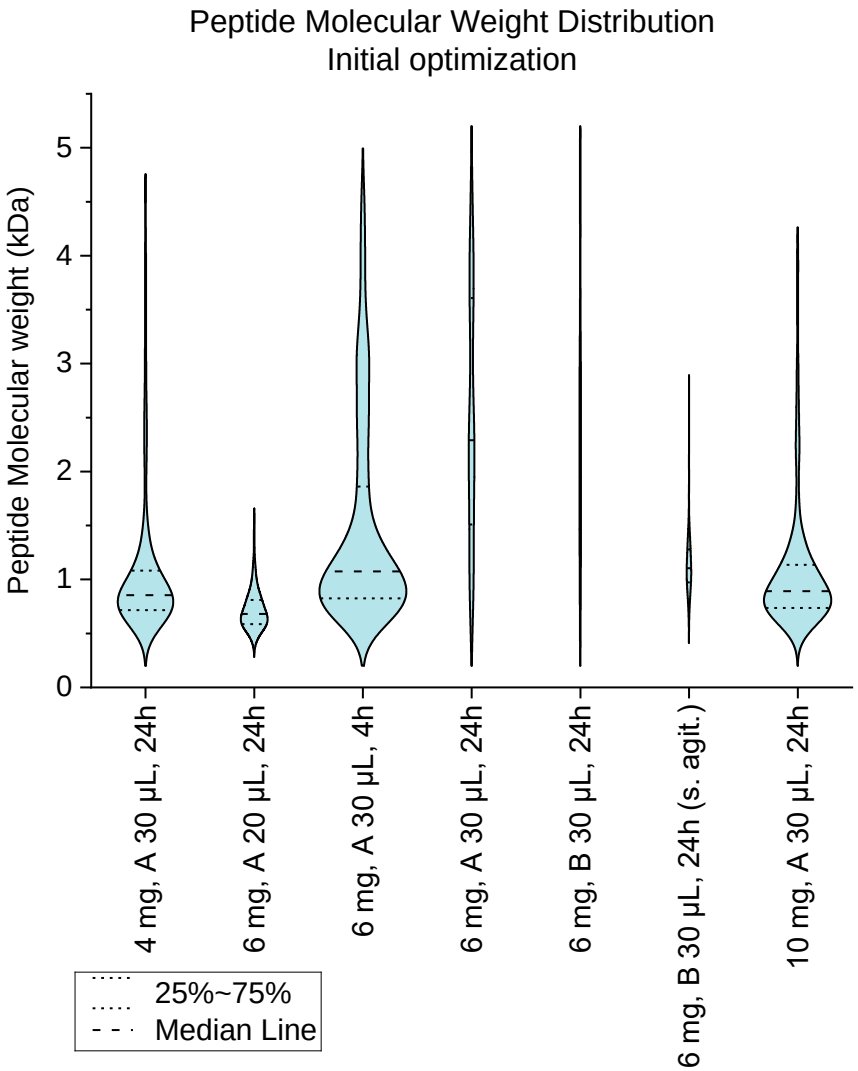


Figure 17 - Peptide size distribution of hydrolysates from initial optimization, protocol 2. Only the peptides identified with high confidence were considered.

Table 6 - Summary of the results of initial optimization samples, obtained by LC-MS/MS, UMPred-FRL and iBitter-Fuse relevant to the development of a comercial umami flavour enhancer. Values of general probability correspond to the mean of the umami / bitter probability of all peptides identified with high confidence in the sample. Values of specific probability correspond to the umami / bitter probability of only the peptides identified as being umami / bitter.

Sample	Nº of peptides (high confidence)	Nº of glutamates	Nº of glutamates per peptide	Nº of umami peptides	% Umami peptides	Average umami probability (total)	Average umami probability (specific)	Nº of bitter peptides	% Bitter peptides	Average bitter probability (total)	Average bitter probability (specific)	Nº of peptides both umami and bitter	% peptides both umami and bitter
39. 4 mg, A 30 µL, 24h	1819	1414	0.78	740	40.7	0.44	0.77	756	41.6	0.45	0.80	292	16.1
37. 6 mg, A 20 µL, 24h	879	319	0.36	263	29.9	0.35	0.77	411	46.8	0.49	0.79	92	10.5
70. 6 mg, A 30 µL, 4h	2851	2492	0.87	1523	53.4	0.52	0.79	1047	36.7	0.41	0.78	513	18.0
40. 6 mg, A 30 µL, 24h	187	320	1.71	134	71.7	0.67	0.84	71	38.0	0.44	0.79	42	22.5
46. 6 mg, B 30 µL, 24h	49	65	1.33	32	65.3	0.63	0.84	13	26.5	0.40	0.74	8	16.3
6 mg, B 30 µL, 24h (stronger agitation)	150	201	1.34	56	37.3	0.41	0.79	77	51.3	0.56	0.80	30	20.0
41. 10 mg, A 30 µL, 24h	2207	1592	0.72	937	42.4	0.44	0.77	846	38.3	0.43	0.79	333	15.1

Comparing the conditions of 4 mg, 6 mg and 10 mg of YPE Divino hydrolysis with 30 µL of enzyme A, for 24h, we can observe that even though the condition of hydrolysis is 6 mg of YPE Divino is the one where fewer peptides were identified with high confidence, it is, of the three conditions, the one where we find a higher percentage of umami peptides. This means that, even if the hydrolysis is not as extensive in this condition, it is the one where the peptides formed have a higher chance of enhancing the umami flavour, and therefore, being more important for the final product. On top of this, out of the three conditions, this is also the one where a lower percentage of bitter peptides is found. This can be either an advantageous or disadvantageous characteristic depending on the food matrix in which the final flavour enhancer is used, since even though the bitter flavour is usually unwanted, its presence in certain foods is indispensable, such as in cheeses.

This result leads us to the comparation of the hydrolysis of 6 mg of YPE Divino for 24h, with 20 µL versus 30 µL of enzyme A. Once again, the condition in which 30 µL of enzyme A were added to the reaction has a lower number of identified peptides with high confidence, but presents a higher percentage of umami peptides, which in turn present higher probabilities of enhancing this flavour. Furthermore, this condition also presents a lower percentage of bitter peptides, which is commonly a good characteristic. In this type of situation, it is necessary for Proenol S.A. to access if producing a more intense flavour enhancer outweighs the higher economical cost of the enzyme.

Looking now at the effect of reaction time, we can compare the conditions of 6mg of YPE Divino hydrolysis with 30 µL of enzyme A, for 4h or 24h. Here, we can see that once again, the 24h condition presents a higher percentage of umami peptides even though the number of total identified peptides with high confidence is lower. Here, however, the 24h condition also leads to the formation of more bitter peptides. Similarly to the previous comparison, the evaluation of whether or not higher economical expense of energy is compensated by a more intense flavour enhancer.

Another important comparison is the type of enzyme used. Until now, both enzyme A and enzyme B have been used separately to try and conclude which one may be more efficient for this study. Here, we can see that enzyme A, in the same conditions as enzyme B, leads to the formation of more umami peptides. This result was expected since enzyme A is described in literature to boost the formation of these type of peptides. However, this enzyme also leads to a higher percentage of bitter peptides, which may be disadvantageous for the final product.

It is important to note, however, that the low number of peptides identified in the sample YPE Divino 6mg with 30 µL of enzyme A for 24h may be a protocol or software error and does not demonstrate the reality of the variety of peptides existent in the sample. Another explanation for this result is that this condition leads to the formation of fewer different peptides, and a higher homogeneity in the sample.

Finally, the effect of a stronger agitation was studied. Even though all hydrolysis experiments were conducted under constant agitation, a precipitation was seen at the bottom of the laboratory tubes at the end of the reaction. For this very reason, an additional condition was performed. Comparing these, it is clear the impact that a stronger agitation has on hydrolysis, since three times more peptides were identified with high confidence. However, this does not necessarily translates in a higher percentage of umami peptides, since the contrary is seen. This means that even though hydrolysis is important to the process, and needs to occur, an excessive agitation may lead to a poorer final product.

Taking all this into account, the condition of 6 mg of YPE Divino hydrolysis with 30 µL of enzyme A, for 24h seems to be the more interesting and promising one.

3. Yeast Protein Extract hydrolysis – Final optimization

Despite the initial optimization and the selection of a more promising condition, further optimization into the process was performed. When the previous results were shared with Proenol S.A., the company decided to contact the enzyme supplier for further information on the best use conditions of the enzymes in study for umami peptides production. With this goal in mind, the enzyme supplier provided a protocol from which had been obtained good results with other substrates. In this line of work, a new hydrolysis protocol was performed.

Enzyme A was used in combination with enzyme C, either in its native (C1) or pure (C2) form. Enzyme B was dropped altogether. Hydrolysis was performed upon a pre-treatment for inactivation of YPE endogenous enzymes, with the addition of enzymes C and A at different points of hydrolysis and different reaction pH. The reaction occurred at 55ºC for a total of 24h, and stopped by incubation at 95ºC. Hydrolysates were then treated and analysed as described before.

Another alteration on this set of hydrolysis were the YPEs in which the reaction was performed. Until now, a total of 9 extracts were analysed, but from this point on only

YPEs Divino and EPLc on its liquid form were hydrolysed, for a number of reasons. First of all, these two extracts present the highest protein contents (Section III 1.1). This means that the same mass of one of these extracts, in comparison to any other, contains a higher mass of protein and therefore, has a higher probability of containing a higher quantity of peptides post-hydrolysis. In addition, the production and use of these two extracts is industrially more economic (Figure 1). The use of liquid YPEs allows the company to skip the drying step of production, eliminating the energy costs associated with this stage. On top of this, EPLc results of the extraction of SP1 to the initial lysed yeast cells stock, meaning that most steps of separation and purification aren't necessary, eliminating once again the costs associated with these steps and decreasing the number of wasted products (SP2 and SP3).

Taking all this into consideration, Proenol S.A. produced 15 potential products in pilot-scale: Divino, Enzymes C1 + A, 16h; Divino, Enzymes C1 + A, 16h (without inactivation of endogenous enzymes); EPLc, Enzymes C1 + A, 16h; Divino, Enzymes C1 + A, 20h; EPLc, Enzymes C1 + A, 20h; Divino, Enzymes C1 + A, 25h; EPLc, Enzymes C1 + A, 25h; EPLc, Enzymes C2 + A, 10h; Divino, Enzymes C2 + A, 16h; EPLc, Enzymes C2 + A, 16h; EPLc, Enzyme A,16h; Divino, Enzyme C1, 10h; EPLc, Enzyme C1, 10h; EPLc, Enzyme C1, 16h; Divino, Enzyme C2, 10h. The potential products followed sensory analysis. Each extract was also purified by SPE (Section II 4.5) for further LC-MS/MS analysis.

3.1. Sensory analysis

The 15 potential products produced by Proenol S.A. were submitted to a sensory analysis to assess the most accepted and preferred samples, through an affective test. The samples were numbered and separated into three groups, with each one containing a control sample. The samples were presented to tasters identified only by numbers, in a random order.The samples were numbered as follows:

Group A

1 – Divino, Enzyme C1, 10h
2 – EPLc, Enzymes C1 + A, 20h
3 – Control (unhydrolyzed Divino)
4 – Divino, Enzymes C1 + A, 25h
5 – EPLc, Enzymes C1 + A, 25h
6 – EPLc, Enzyme C1, 16h

Group B

7 – Divino, Enzymes C2 + A, 16h
8 – EPLc, Enzymes C1 + A, 16h
9 – EPLc, Enzymes C2 + A, 10h
10 – EPLc, Enzymes C2 + A, 16h
11 – Control (unhydrolyzed EPLc)
12 – Divino, Enzymes C1 + A, 20h

Group C

13 – Divino, Enzyme C2, 10h
14 – Divino, Enzymes C1 + A, 16h
15 – EPLc, Enzyme A,16h
16 – EPLc, Enzyme C1, 10h
17 – Divino, Enzymes C1 + A, 16h (without inactivation of endogenous enzymes)
18 – Control (unhydrolyzed Divino)

Tasters were asked to fill out the provided questionnaire (Figure 2, Section II 4.8). Figures 18, 10 and 20 present the results regarding tastiness, bitterness and intensity of the samples, respectively. Figure 21 summarizes the results for the second section of the test, regarding potential products' general pleasant and unpleasantness.

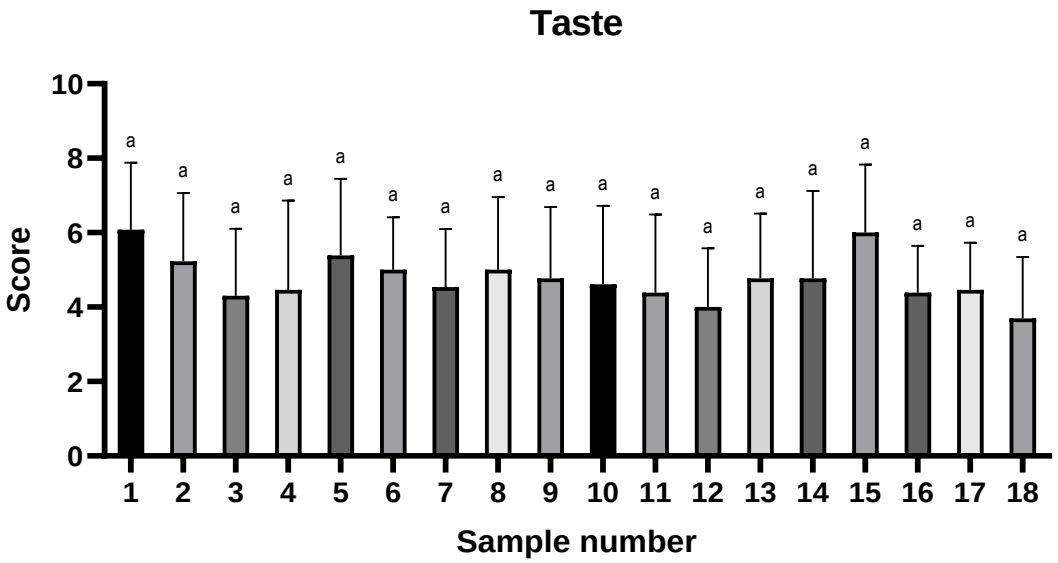


Figure 18 - Sensory analysis results on the tastiness of samples. Samples are separated into the three tasting groups, with each tasting being performed on different days. Different letters above the columns represent statistical significance ($P<0.05$).

According to the responses from the taste panel none of the samples is significantly different from the rest, regarding overall tastiness.

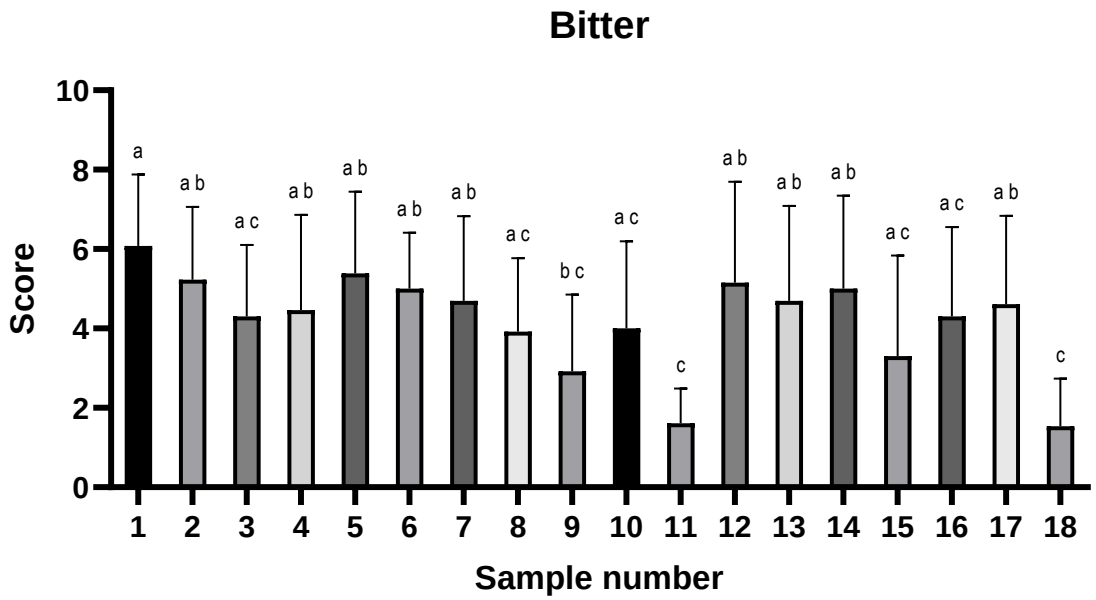


Figure 19 - Sensory analysis results on the bitterness of samples. Samples are separated into the three tasting groups, with each tasting being performed on different days. Different letters above the columns represent statistical significance ($P<0.05$).

Regarding bitterness, samples present more significant differences. It is possible to observe that samples 11 and 18, both sample controls are considered the less bitter from all of the samples. However, it is interesting to note that even though samples 3 and 18 both correspond to unhydrolyzed Divino, the taste panel identified sample 18 as being more bitter than sample 18. This difference is, nevertheless, not significantly different, as presented in Figure 19. It is possible to conclude that samples 3, 8, 9, 10, 11, 15, 16 and 18 do not appear to show significant differences in bitterness perception, with low values. This can mean that the hydrolysis conditions corresponding to these samples produce low amounts of bitter peptides, or bitter peptides with low intensities or high thresholds.

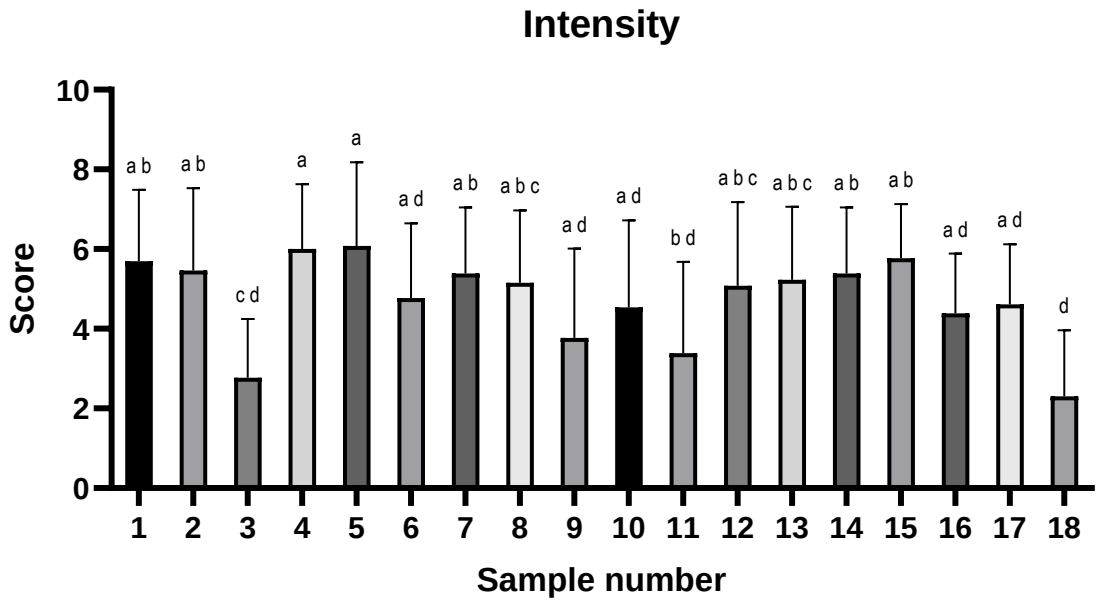


Figure 20 - Sensory analysis results on the flavour intensity of samples. Samples are separated into the three tasting groups, with each tasting being performed on different days. Different letters above the columns represent statistical significance (P<0.05).

Flavour intensity can be an unwanted characteristic in a food ingredient, since it may obscure other tastes and not allow the consumer to enjoy the experience. However, intensity may be a commercial advantage, since it may mean that a lower amount of product is able to induce the same flavour intensity as a lower amount of other product.

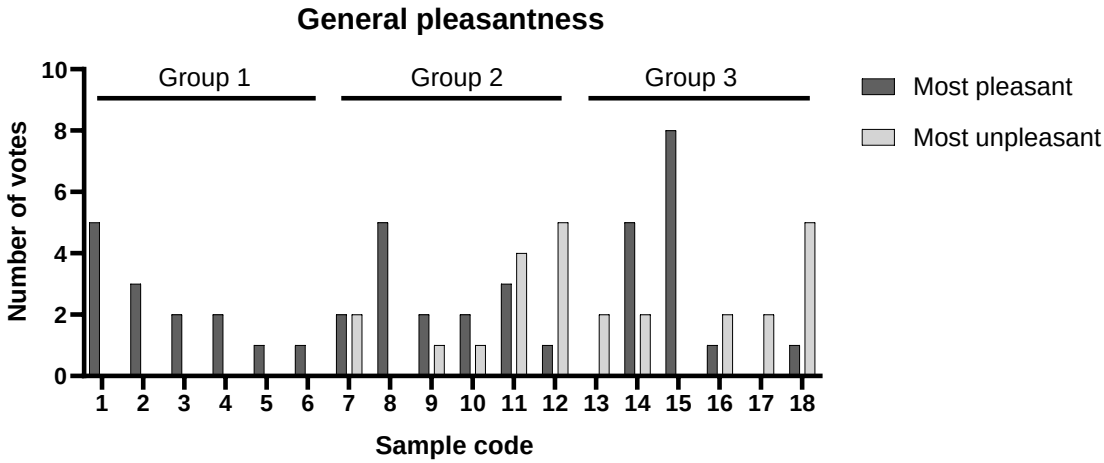


Figure 21 - Sensory analysis results on the pleasant and unpleasantness of samples. Samples are separated into the three tasting groups, with each tasting being performed on different days. Group 1 does not present data for the most unpleasant samples since this question was only added afterwards.

Samples 1, 8, 14 and 15 are the most picked as most pleasant overall. In addition, samples 8 and 15 were not picked as the most unpleasant sample by anyone on the

taste panel. Contrarily, controls 11 and 18, and sample 12 were the most picked as most unpleasant samples. This leads us to believe that the hydrolysis process does indeed increase the pleasantness of the YPE.

Upon discussing these results with Proenol S.A., samples 8, 14 and 15 were selected for further analysis.

With these samples selected, the following step was to assess the sensory repeatability of the process. For this very reason, Proenol S.A. reproduced the hydrolysis following two conditions: EPLc hydrolysed with enzymes C1 and A for 16h (sample 8) and EPLc hydrolysed with only enzyme A for 16h (sample 15). These conditions, studied to assess the repeatability of the process, were numbered as 19 and 20, respectively. One of the most used tests to conclude whether two products have significant and noticeable differences is the Duo-Trio test. In this study, samples 8 and 15 were presented to the taste panel as the reference, and samples 8 and 19, and 15 and 20, respectively, were presented as the products under analysis. The tasting panel was asked to identify which one of the products is similar to the reference.

The primary method to analyse duo-trio test data is the binomial test, which evaluates whether the observed proportion of correct identifications of the product similar to the reference (correct answers) is significantly different from what would be expected by chance.

For the test assessing significant differences between samples 8 and 19, a total of six answers out of eight were incorrect. Considering this data, the p-value is 0.965, a value much greater than 0.05, and therefore there is no evidence to suggest a perceptible difference between these two samples.

For the comparison between samples 15 and 20, only one answer out of eight was incorrect. This results in a p-value of 0.035, which indicates a statistically difference between the samples.

From this test, it is possible to conclude that while hydrolysis of EPLc with enzymes C1 and A for 16h is repeatable, the same does not happen with EPLc hydrolysis with enzyme A for 16h. This non-repeatability may be due to differences in process conditions between the two batches, or due to the enzyme activity varying and not being predictable even under the same conditions. To make a more informed conclusion, further testing on new batches, produced under the same conditions, is necessary.

3.2. Liquid Chromatography - Tandem Mass Spectrometry and use of prediction tools

Upon selection of potential products 8, 14 and 15, these were analysed through LC-MS/MS combined with the prediction tools UMPred-FRL and iBiter-Fuse, as described in Sections II 4.6 and 4.7. The analysis followed the same logic and steps described in Section III 2.4.

For the production and success of the umami flavour enhancer produced by Proenol S.A., two factors are of major importance. First of all, the repeatability of the process; different batches must result in the same product, with predictable results. This characteristic is studied through the comparison of potential products 8 and 19, and 15 and 20. In addition, the product must be able to compete to other commercial umami flavour enhancers, already established in the market. For this very reason, in parallel to the analysis of the hydrolysates produced by Proenol S.A., three commercial products (CPa, CPc and CPs) were analysed.

Figure 22 shows the weight distribution of the peptides identified with a high degree of confidence of hydrolysates 8, 14, 15, 19, 20, CPa, CPc and CPs. These peptides were uploaded to the prediction tools software and results are summarized in Table 6.

In terms of peptide molecular weight distribution, it is possible to observe in Figure 22 that the second batch of hydrolysates production by Proenol S.A. does not show repeatability, since there are noticeable differences between samples 8 and 19, and 15 and 20. However, it is necessary to note that samples 8 and 19 present similar median values, and the major difference is seen in molecular weigh frequency rather than range. It is also possible to observe that the use of enzyme A alone (samples 15 and 20) results in a clearly different weight distribution when compared to the simultaneous use of enzymes C1 and A (samples 8, 19 and 14). In the commercial products, it is also possible to observe a clear distinction in weight distribution between CPa and CPc and CPs. Further conclusions can be made by the analysis of Table 6 data.

Comparing the values for the three previously selected samples (8, 14 and 15) showcased in Table 6, it is possible to conclude that EPLc hydrolysis with enzyme A for 16h (sample 15) results in a higher percentage of umami peptides and a lower percentage of bitter peptides. Furthermore, it is observable that peptides from this sample present higher umami probabilities and lower bitter probabilities, when compared to the hydrolysates from Divino and EPLc with enzymes C1 and A for 16 h (samples 8 and 14). This leads us to believe that the use of enzyme C1 may be impairing

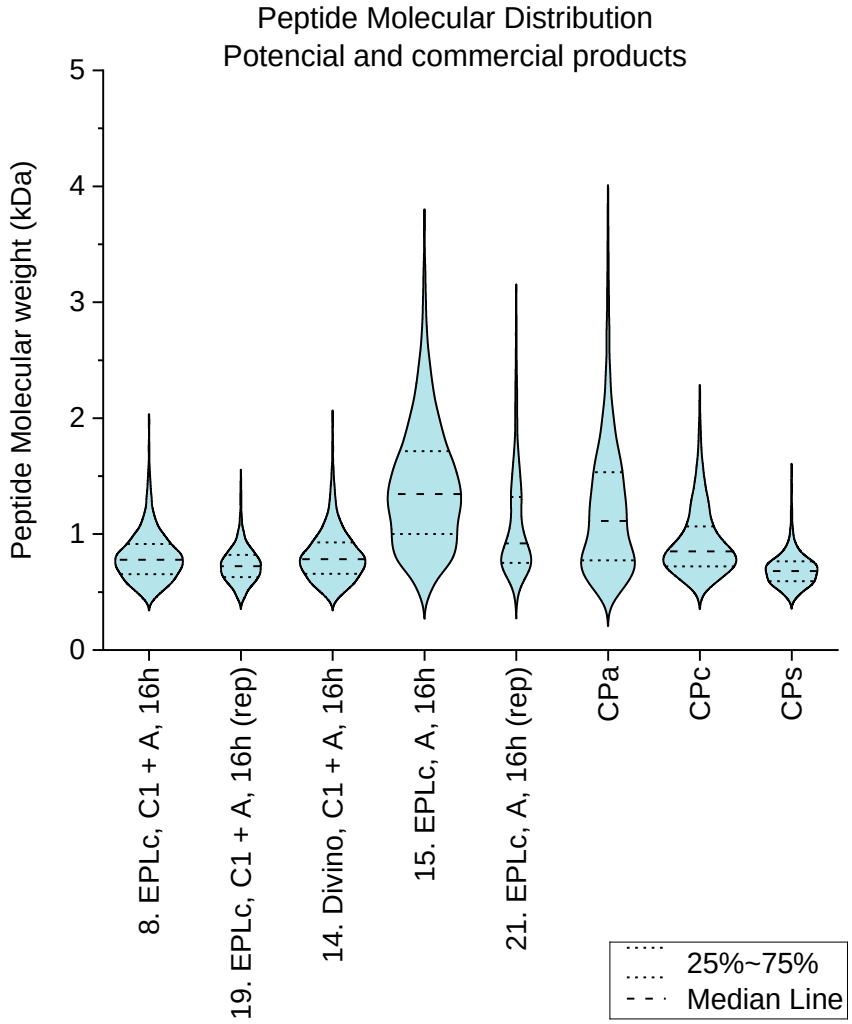


Figure 22 - Peptide size distribution of Proenol S.A. potential products and commercial products. Only the peptides identified with high confidence were considered.

the enzyme A activity on producing umami peptides, and that enzyme C1 may be responsible for the formation of bitter peptides. From this analysis alone, sample 15 seems to be the more promising one. However, it is important to note that a higher umami probability does not translate in a more intense umami flavour and therefore, threshold values and sensorial intensity perception needs to be evaluated and considered.

Regarding the repeatability of the hydrolysates obtained with the action of enzymes C1 and A on EPLc for 16h, comparison of samples 8 and 19 is crucial. The data shows that even though the number of peptides identified with high confidence, the number of glutamates and the number of peptides identified as being umami are bigger for sample

Table 7 - Summary of the results of selected potential products, obtained by LC-MS/MS, UMPred-FRL and iBitter-Fuse relevant to the development of a commercial umami flavour enhancer. Values of general probability correspond to the mean of the umami / bitter probability of all peptides identified with high confidence in the sample. Values of specific probability correspond to the umami / bitter probability of only the peptides identified as being umami / bitter.

Sample	Nº of peptides (high confidence)	Nº of glutamates	Nº of glutamates per peptide	Nº of umami peptides	% Umami peptides	Average umami probability (total)	Average umami probability (specific)	Nº of bitter peptides	% Bitter peptides	Average bitter probability (total)	Average bitter probability (specific)	Nº of peptides both umami and bitter	% peptides both umami and bitter
8. EPLc, C1 + A, 16h	5765	4171	0.72	2249	39.0	0.42	0.77	3127	54.2	0.55	0.81	1271	22.0
14. Divino, C1 + A, 16h	5587	4043	0.72	2224	39.8	0.42	0.77	3062	54.8	0.55	0.82	1257	22.5
15. EPLc, A,16h	6262	8041	1.28	3283	52.4	0.52	0.79	2742	43.8	0.47	0.78	1412	22.5
19. EPLc, C1 + A, 16h (rep)	3409	1774	0.52	1331	39.0	0.41	0.77	1412	41.4	0.45	0.80	547	16.0
20. EPLc, A, 16h (rep)	2562	1755	0.69	1178	46.0	0.47	0.78	797	31.1	0.37	0.78	338	13.2
CPa	4580	5029	1.10	2108	46.0	0.47	0.78	2104	45.9	0.48	0.79	878	19.2
CPc	6231	2846	0.46	2212	35.5	0.39	0.78	2425	38.9	0.43	0.77	820	13.2
CPs	4425	1744	0.39	1563	35.3	0.38	0.77	1910	43.2	0.45	0.79	620	14.0

8 than sample 19, the percentage of umami peptides and their probability is similar in both samples. These results may be caused by a problem in the sample analysis, which resulted in the incapability of the software to identify peptides as accurately. Whether or not this is true, the similarity in values of umami peptides percentage and probability may be indicative that there is no noticeable difference in umami sensation between the two samples, which aligns with the sensory analysis conclusions (Section III 3.1). However, a bigger difference is observed between samples 8 and 19 on values regarding bitter peptides. This leads to the conclusion that bitter peptides may not be as influential for the general taste and flavour of these samples as the umami peptides.

The two samples corresponding to EPLc hydrolysis with enzyme A for 16h (samples 15 and 20) present several differences. Number of glutamates residue per peptide, percentages of umami and bitter peptides, as well as umami and bitter probabilities values are lower for sample 20 than sample 15. Just as the previous comparison, this may be due to a problem in sample analysis, since the total number of peptides identified for sample 20 is also significantly lower than for sample 15. However, when these results are combined with the sensory test results, it seems clear that the hydrolysis process is not, indeed, repeatable between these two samples and further testing and optimization is necessary.

Considering the three commercial products analysed, it is clear that CPa presents the highest glutamate presence, the highest umami peptide percentage, and the highest umami probabilities. The percentage of bitter peptides and bitter probability is also the highest, which, as mentioned before, can be beneficial when the taste enhancer is added to particular foods. Commercial products CPc and CPs present values regarding umami peptides lower than the samples produced by Proenol S.A., which is a very promising result and indicates that the potential products developed in this study may be able to compete with products already established on the market. However, only sample 15 surpassed values regarding umami peptides of CPa.

Overall, EPLc hydrolysis with enzyme A for 16h appears to be the most promising of conditions for an umami flavour enhancer.

3.3. Analytical comparison to commercial products on the market

The data presented in Table 6 allows us to take conclusions on the number of peptides associated with umami or bitter flavour. However, several other factors are fundamental to the enhancing of these flavours. The intensity of the flavour of each peptide, their

threshold and their abundance in the final product are all decisive to the flavour induced. As an attempt to calculate the abundance of different peptides in the sample, their relative abundance was estimated through the areas of corresponding peaks, in relation to the total area of the chromatograms of each selected sample, and commercial products for comparison.

The relative abundance was estimated for the umami non bitter peptides with higher umami probability of samples 8, 14 and 15 (Tables 7, 8 and 9, respectively), as well as for all umami non bitter peptides present simultaneously in the three commercial products and at least one of the selected samples (8, 14 and/or 15) (Table 10). The values for relative abundance are normalized to protein concentration, since all samples were injected in the LC-MS/MS equipment at a normalized protein concentration of 0.033 mg/mL (Sections II 4.4 and 4.6). However, it is important for the future commercialization of the product to compare the potential Proenol S.A. products to the commercial ones already on the market normalized to the extract concentration, since a higher abundance of umami non bitter peptides in a hydrolysate can result in a less quantity being needed to create the same result and therefore, the product can be advertised as being of higher quality and purity. The conversion between the relative abundance values can be calculated through the protein percentage of each extract, which is in turn calculated based on the dilution performed for peptide quantification (Section II 4.4.).

Table 8 - Relative abundance of the non-bitter peptides with the twenty highest umami probabilities, present in sample 8. nq stands for non-quantifiable.

8. EPLc C1 + A 16h

Peptide Sequence	**Umami probability**	**Relative abundance (*10^{-3} %)**
DVSWVD	0.988	6.15
SKDSML	0.988	0.04
IDSTGVF	0.987	nq
TVDNPF	0.986	2.09
VDVPTGW	0.986	0.45
DSGKTMY	0.986	0.23
IDNML	0.985	14.44
SLGVEPV	0.983	0.24
LSGSPL	0.982	0.04
SKPTYPGTE	0.981	0.23
TPFPRTE	0.981	0.41
QDFDKV	0.981	0.08
VDNPFAN	0.979	0.03
WGDDIPT	0.979	0.15
LPSASLP	0.979	0.18
QSIEDR	0.979	1.24
QDSDIM	0.979	0.006
KDEEAW	0.978	2.41
GVEDVYTQ	0.977	nq
KDTGYSC	0.977	2.55
TOTAL		30.98

Table 9 - Relative abundance of the non-bitter peptides with the twenty highest umami probabilities, present in sample 14. nq stands for non-quantifiable.

14. Divino C1 + A 16h

Peptide Sequence	Umami probability	Relative abundance (*10^{-3} %)
NDSIY	0.990	1.79
DVSWVD	0.988	5.73
IDDFNSW	0.988	0.20
IDSTGVF	0.987	0.13
KDTEP	0.987	2.37
TVDNPF	0.986	0.03
VDVPTGW	0.986	nq
AVSTIEP	0.982	0.80
SKPTYPGTE	0.981	0.59
QDFDKV	0.981	nq
IDDYLN	0.980	1.17
VDNPFAN	0.979	1.51
WGDDIPT	0.979	5.87
THKVE	0.979	5.70
KDEEAW	0.978	0.16
GVEDVYTQ	0.977	0.03
ISPNSPL	0.977	0.61
VDTAPY	0.976	0.50
ISNTPL	0.976	3.45
VPWEPVD	0.975	0.56
TOTAL		31.20

Table 10 - Relative abundance of the non-bitter peptides with the twenty highest umami probabilities, present in sample 15. nq stands for non-quantifiable.

15. EPLc A 16h

Peptide Sequence	Umami probability	Relative abundance (*10^{-3} %)
ISWYDNEYGYSARV	0.991	0.48
VEKYFPGLLRATNE	0.989	0.99
KDTEP	0.987	0.52
SDKSKWLTGVE	0.986	3.47
LENLRYHIE	0.985	2.46
VDSTGVFKE	0.983	7.30
LPEIYEKMEKGQIVGRY	0.982	4.03
AGITTVKGGTGKIVEY	0.982	0.15
VVGLSTLPEIYEK	0.982	0.14
SWNGIDPIDRGKA	0.981	0.60
NGSWNGIDPIDRGKA	0.980	1.34
FPTEIVGKRV	0.980	1.31
FKKVLENTEIGDSIF	0.980	0.86
GPENDMGIKY	0.979	0.64
NDPFITNDY	0.979	0.98
QSIEDR	0.979	nq
VDSTGVFKELDTA	0.978	2.14
YEHVTF	0.978	0.14
TLPEIYEKMEKGQIVGRY	0.977	0.39
VDSTGVFKELDT	0.977	0.65
TOTAL		28.59

The top 20 umami non bitter peptides represent a total of 30.98x10^{-3} % of total protein in sample 8, 31.20x10^{-3} % in sample 14 and 28.59x10^{-3} % in sample 15. It is interesting to note that ten peptides are a part of the top twenty of samples 8 and 14, simultaneously, while the top twenty of sample 15 only shares one peptide with the top twenty of either one of the previously mentioned samples. This shows, once again, that hydrolysis with only enzyme A results in a much distinct peptide profile, when compared to the use of both enzymes C1 and A.

Table 11 - Relative abundance of umami non bitter peptides present simultaneously in selected samples and commercial products. nq stands for non-quantifiable.

Peptide Sequence	**8 (*10^{-3} %)** EPLc C1 + A 16h	**14 (*10^{-3} %)** Divino C1 + A 16h	**15 (*10^{-3} %)** EPLc A 16h	**CPa (*10^{-3} %)**	**CPc (*10^{-3} %)**	**CPs (*10^{-3} %)**
IDSTGVF	nq	0.13	---	4.47	5.81	4.43
NVNDVIAPA	3.49	1.79	---	nq	0.09	0.43
NGSLISP	208.26	23.86	nq	18.31	252.59	25.40
PANLPWGSS	0.36	0.15	0.01	0.02	36.82	15.97
QKAVDDF	0.04	0.01	0.03	1.41	0.01	0.66
DVTPVPSDSTRKK	---	0.24	---	3.96	1.11	2.00
TEDSVLK	nq	---	0.04	1.49	1.82	3.92
NDPFITN	0.08	0.03	0.25	0.56	74.01	2.81
ATVPTGGAS	nq	0.12	nq	1.26	5.75	0.42
QTPKVE	nq	nq	---	1.64	5.64	2.97
VPTVDVS	7.66	6.50	1.37	0.25	9.07	15.73
AAVPAAGPA	---	---	nq	0.67	0.57	1.07
VDFNVPLDGK	12.78	21.27	0.47	0.008	16.90	nq

GISNEGQNASIK	0.001	0.02	2.36	0.53	1.29	2.23
GQDSLTPQ	3.38	2.22	---	nq	0.27	0.35
TOTAL (normalized to protein concentration)	236.05	56.33		29.95	410.07	75.32
				32.42	409.36	73.40
			4.54	24.51	398.83	68.21
↓						
% Protein in hydrolysate	44.73	51.70	36.34	26.10	30.50	50.00
↓						
TOTAL (normalized to extract concentration)	105.59	29.12		10.88	125.07	37.66
				8.43	124.85	36.70
			1.65	6.37	121.64	34.11

By analysing the values of relative abundance normalized to protein, in Table 10, it is possible to observe that sample 15 is the one which has the least amount of umami non bitter peptides in common with the commercial products, and these peptides are present in the lowest relative abundance. This may be an indication, once again, of the distinct peptide profile that the use of enzyme A alone produces, when compared to either samples 8 and 14, or commercial products. It is also possible to observe that CPc has the biggest contribution in relative abundance of peptides in common with the other samples, followed by sample 8. However, when the data is normalized to the extract concentration, this difference decreases, with sample 8 following CPc much more closely.

This data gives an indication of the contribution of high umami probability peptides, but it is necessary to take into account that a high probability does not necessarily translate in a high flavour intensity or in a low perception threshold. This fact, allied to the presence of high umami probability or intensity peptides in a given sample and not present in the rest, can result in a sample with lower relative abundance of the peptides presented in Table 10 being, in actuality, the one who has a higher umami enhancement effect.

However, even with these limitations in mind, it is possible to conclude that the potential Proenol S.A. products can be competitive to commercial products already on the market, leading us to believe that the flavour enhancers developed in this study can be successful for the main goal.

IV. Conclusion

The initial YPEs characterization revealed significant differences in the extracts' composition, relevant to the effectiveness of the hydrolysis process and especially to the final peptide yield after hydrolysis.

Initial chemical and enzymatic hydrolysis optimization, with the last one performed with enzymes A and B independently, at different enzyme activities or concentrations, was proved to be effective. SDS-PAGE allowed the visual observation of the disappearance of high molecular proteins, while HPLC and HPLC-SEC analysis allowed the observation of small weight proteins and peptides production. Chemical hydrolysis was, however, disregarded from further analysis due to the lower peptide yield and higher unpredictability in results. Regarding enzymatic hydrolysis, it was concluded that enzyme A produces a broader range of peptides, and LC-MS/MS allied to the use of prediction tools allowed to conclude that this enzyme also produces a higher number of umami peptides, which aligns to the literature. In the initial optimization (protocol 2) the condition of hydrolysis with 30 µL of enzyme A for 24h was selected as the most promising.

In the final phase of hydrolysis optimization, the hydrolysis conditions were altered to better take advantage of the enzymes' capabilities and to better adapt to industrial conditions. In this stage, the use of enzymes C1, C2 and A were tested alone and simultaneously. Out of a wide range of conditions, varying in enzyme used, time of reaction and YPE substrate, three conditions were selected through sensory analysis: EPLc C1 + A 16h, Divino C1 + A 16h and EPLc A 16h. These tests revealed consumers' acceptance towards the potential products, with samples showing significant differences in terms of bitterness, flavour intensity and overall pleasantness. This data is crucial for the success of the product after being launched. Although these three samples were selected by a tasting panel, it was revealed by sensory testes and LC-MS/MS analysis allied with the prediction tools use that a problem in the repeatability of the process of the sample EPLc A 16h may exist. To assess with certainty if this problem was a one-time error or if it is something that will systematically influence the repeatability of the process, making the final product unpredictable and unreliable, further tests are needed.

Comparison to the commercial products through LC-MS/MS allied once again to the use of prediction tools allows us to conclude that the samples selected show competitive potential. This result is of extreme importance for Proenol S.A., who can make an informed decision on whether to launch the product to the market, with a better predictability of its success.

Samples EPLc C1 + A 16h, Divino C1 + A 16h and EPLc A 16h show different strengths and disadvantages, and only further testing will help decide which one may be the more promising flavour enhancer. Further sensory tests, this time with the hydrolysates already added to a food matrix may be an interesting approach to make a better distinction between the three options. Besides, economic considerations should be taken into account, since a balance between enzyme and energy costs, among others, and efficiency of the process, intensity and purity of the final product needs to be considered.

In the end, it is possible to conclude that the production of an umami flavour enhancer was a success, with the production and commercialization by Proenol S.A. being a viable and promising path.

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