high-value products from agricultural residues through sustainable chains

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DEM	Demonstrator, Pilot, Prototype	☐
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Abbreviations

AA, ascorbic acid
ABTS, 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid
AP, apple pomace
BSA, bovine serum albumin
ChCl-CA, Choline chloride: Citric acid
ChLA, Choline chloride: Lactic acid
CH_3CN, acetonitrile
COM, cutin oligomeric mixtures
DES, deep eutectic solvent
DTS, defatted tomato seeds
EtOH, ethanol
FTIR, Fourier transform infra-red spectroscopy
GalA, Galacturonic Acid
gDW, g Dry Weight
gFW, g Fresh Weight
HPLC, high performance liquid chromatography
IL, ionic liquid
KA, kojic acid
MenLA, Menthol: Lactic acid
MenThy, Menthol: Thymol
MeOH, methanol
MS, mass spectrometry
MTBE, methyl *tert*-butyl ether
NADES, natural deep eutectic solvent
NMR, nuclear magnetic resonance
RGP, Red grape pomace
TP, tomato peels
TS, tomato seeds
WTP, whole tomato pomace

1. Executive Summary

AgriLoop WP2 specific objective is to develop integrated flexible scalable extraction processes for added-value biochemicals with targeted structures/functionalities. This deliverable D2.3 presents an update to the characterisation of the compounds obtained in the extraction processes, such as proteins, the plant polyester cutin, polyphenols, carotenoids and fibres, complementing information that has been presented before in D2.1. Different agro-industrial residues were used; in Europe: tomato pomace, grape pomace, and brewery spent grain, and in China peanut, potato and apple residues. The data encompass collaborative efforts between European and Chinese partners aimed at strengthening EU-China scientific and technological cooperation. This initiative aligns with emerging trends in circular agricultural development, focusing on green and low-carbon approaches, as well as innovations in the pre-treatment, separation, and valorisation of agro-industrial plant-based residues. Although using different sources of residues due to the economical industry on each partner's country, the families of compounds retrieved in the multiple strategies developed are mostly coincident. Moreover, the potential translation of the technologies applied to one residue to a different one will be tested as well as, targeting biorefinery concepts that are inherently flexible.

A fraction with high content and moderate purity of globulin was obtained from peanut residues by the Chinese partner IFST-CAAS, while UNIBO and FHNW successfully obtained peptides by enzymatic digestion in Europe. More, the small molecular weight peptides obtained by UNIBO-BIGEA showed antioxidant and anti-browning activities. In the case of the peptides obtained using immobilized enzymes at FHNW, ACE and DPP-IV inhibitory activities were recorded, as well as anti-hypertension and anti-diabetic properties.

At ITQB NOVA, the plant polyester cutin was successfully isolated using the natural deep eutectic solvent (NADES) Choline chloride: Lactic acid (ChLA), showing similar spectral signature to cutin obtained using the ionic liquid (IL) cholinium hexanoate previously established. This IL was also able to isolate the cutin from red grape pomace. Furthermore, anti-microbial activity against *S. aureus* was recorded for cutin oligomeric mixtures (COMs) generated from the IL-cutin extracted from TOMAPAINT pomace source. The COMs potential usage in the development of antimicrobial materials and coatings is under development in WP4.

The NADES ChLA allowed the researchers at INRAE-IATE to extract the four main phenolic compounds from tomato peels, with a predominance of flavonoids. Therefore, ChLA is likely suitable as the extractant for moving towards the cascading approach. The usage of an alternative hydrophobic NADES allowed the simultaneous extraction of phenolics and carotenoids from tomato peels. The identification and quantification of condensed tannins present in grape pomace required a depolymerisation step developed in ChLA.

From potato residues, researchers at IFST-CAAS were able to isolate dietary fibres using a high hydrostatic pressure cellulase method, improving the physico-chemical and functional properties of the product. The same partner reported deviations on the galacturonic acid content and degree of esterification according to the used method for the isolation of pectin from apple pomace.

2. Introduction

The agricultural plant residues, contains a plethora of proteins, phytochemicals, and others functional compounds able to bring health and well-being benefits with multiple applications in the food, feed, pharmacy, materials or cosmetic industries[1]. Today the main challenges to optimize the extraction of added-value molecules from agricultural residues, are to tackle a multitude of parameters and integrate scientific advances in the larger picture both for upstream and downstream.

The technical core of the project is structured around two additional pillars: one dedicated to update the recovery approach of highly functional native molecules from residual plant biomass; and another one, to open and tailor the bioconversion schemes, both pillars aiming to overcome feedstock, processes and end-products related limitations.

AgriLoop project aims at developing safe and sustainable-by-design bioconversion processes to extend the value of agricultural (solid or liquid) residues (from plant or animal), through the production of high added-value bioproducts for food, feed and materials applications. This will include developing an integrated and scalable cascading biorefinery approach for the extraction of a different range of molecules in the optimal processing order to ensure maximum extraction yield, while preserving the native functionalities, increasing the eco-efficiency of the processing routes and tailoring to the just necessary the end-products to well-defined usages and post-usage fate (end-of-life options).

Within this project, WP2 objectives are (1) to develop an integrated flexible up-scalable extraction process for proteins, polyesters, polyphenols, carotenoids and fibres with targeted structures/functionalities; (2) to characterize extracts chemical, physical and biological properties within a knowledge-based identification of potential applications; (3) to build predictive data-driven models on structure / functionality relationships and a data library for optimal extraction design; (4) carrying out the pilot-scale or industrial production of high-value food components.

Integrated sustainable extraction process are being adapted / optimised for the treatment of a wide range of agri-residues, aiming at the extraction of different families of bioactive compounds, namely proteins/peptides, polyesters (cutin and suberin), polyphenols/carotenoids and dietary fibres. The development of the extraction process was described in D2.1 and is currently under further optimization, particularly of the cascading strategies. Specific proteolytic enzymes were selected according to the targeted peptides (length, biological or techno-mechanical properties, availability of reactive residues). The extraction of plant polyesters, cutin and suberin, with preserved esterified polymeric backbone and hence preserved native barrier properties, was achieved by exploring both biocompatible ionic liquid and NADES formulations. Finally, the development of polyphenol and carotenoid extractions based on natural deep eutectic solvents (NADES) which are biodegradable, non-toxic, water miscible, with good thermal stability, is being explored. The fine structural characterisation of the different extracts will allow the development of a data-driven model predicting the molecules functional properties from their chemical structures. Particularly, this deliverable (D2.3) concerns the objective identified at Task 2.2 of the WP2, providing a view of the status of the characterisation of the plant extracts. Namely, making evidence of the physical-chemical characterisation, safety assessment, and biological properties of proteins/peptides, polyesters,

[1] Castro-Muñoz et al. (2022). A comprehensive review on current and emerging technologies toward the valorization of bio-based wastes and by products from foods. Compr Rev Food Sci Food Saf., 21, 46–105.

polyphenols, carotenoids, and fibres from tomato pomace, grape pomace, apple pomace, brewery spend grain, peanut residue, and potato residue.

Results from a battery of tests on the chemistry, safety, structural and functional properties of the purified plant compounds will allow application of stringent decision-criteria; any no-go decision, *i.e.* the residue or its leftovers which cannot undergo further extractions, will be channelled and possibly used in fermentative-dependent process to secure full circularity of the integrate process.

This document is organized by raw materials, as sources for the extraction of bio-compounds of interest, with a second level representing the different families of compounds retrieved from each source; finally, a division by extraction method / partner is used to state the main findings for the characterisation of the different extracts.

3. Tomato Pomace

3.1 Proteins

3.1.1 By enzymatic extraction (UNIBO-BIGEA)

3.1.1.1 Further extraction optimization

Among the different tomato pomace streams (D2.1), tomato seeds (TS) supplied by TOMA were selected for protein extraction at UNIBO-BIGEA. The enzymatic digestion protocol employed is outlined in D2.1 (section 3.2.1).

Further extraction optimization tests were performed to try to further improve peptide/protein yield compared to the results presented in D2.1 section 3.2.1. The enzymatic protocol was applied to not ground fresh tomato seeds (fTS) and defatted tomato seeds (dTS). The later were obtained by a novel pre-treatment process (different from that described in D2.1). For fTS, the seeds were first autoclaved. However, since the seeds remained intact after autoclaving, a sonication step was introduced to help break out the outer seed layer, and the two-step digestion protocol (see D2.1 section 3.2.1) was applied. Two controls were used: a non-digested control (ND) and a thermally digested control (TD) (i.e., without any enzyme addition). For fTS, the solid/liquid (S/L) ratio based on dry weight (DW) was changed from 1:5 to 1:10 to facilitate stirring. Protein yield was measured using the Lowry assay[2].

Protein yield in fTS digestates was approximately 40% lower than the one initially obtained in D2.1 from dried and grounded TS. This is likely because the pre-treatment—sonication plus cell-wall enzyme-based hydrolysis—did not sufficiently break down the seeds which would be required for achieving higher protein extraction yields. Considering that the autoclaving step and the bigger S/L ratio, increases, both cost and environmental impact without increasing the protein extraction yield, this new procedure was discarded.

The dried TS were defatted using 100% (v/v) ethyl-acetate (S/L 1:10 w/v) with stirring for 30 minutes at room temperature. The selection of the non-polar solvent ethyl-acetate was made as this solvent as a minor environmental impact respect to chloroform or hexane and is allowed for food applications[3]. The dTS were recovered by centrifugation, then grounded. Controls were performed with non-defatted TS

, and both ND and DT were analysed. Protein yield was determined using the Lowry[2] assay. The protein extraction yield, using proteases, obtained from dTS was not significantly higher than that of non-defatted TS: 228.7 mg BSA eq/gDW for TS and 231.8 mg BSA eq/gDW for dTS minor not significant difference. Given that the extraction yields are comparable but this method requires using the organic solvent ethyl-acetate, our option is to resort to the defatting process reported in D2.1.

With the goal of obtaining a purer protein pellet for subsequent enzymatic digestion, an alternative initial extraction method was also tested. Alkaline (0.1 M NaOH, pH 11) or neutral (0.05 M phosphate buffer, pH 7.2) solubilization (24°C, 3 hours, 1:5 S/L) followed by acidic precipitation (6N HCl in case of alkaline solubilization or 85% phosphoric acid in case of neutral solubilization, pH 3) was performed on dried TS samples. Two types of samples were used: samples dried in-house by UNIBO-BIGEA and samples dried by

[2] Lowry et al. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.
[3] Calvo et al (2007). Influence of extraction with ethanol or ethyl acetate on the yield of lycopene, β-carotene, phytoene and phytofluene from tomato peel powder. Eur. Food Res. Technol. 224, 567–571

TOMA (D2.1) to evaluate the impact of pre-drying the seeds before shipment. Protein concentrations in supernatants and resuspended pellet was assessed using the Lowry assay[2]. The results showed in Figure 1 indicate minimal differences between pre-dried and laboratory dried TS. Moreover, alkaline solubilization proved to be more effective than neutral solubilization. However, a significant portion of peptides remain unprecipitated, likely due to their low molecular weight.

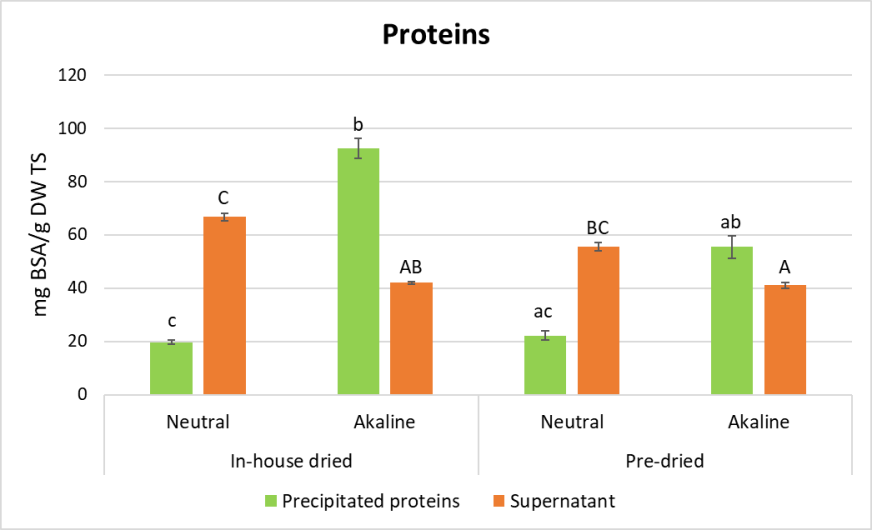


Figure 1: Protein content (mg BSA equivalents/gDW tomato seeds) in supernatants and resuspended pellet after alkaline or neutral solubilization followed by acidic precipitation from tomato seeds (TS) pre-dried by TOMA or in-house dried. Lowercase and capital letters indicate statistically significant difference respectively among precipitated protein and supernatant samples determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). BSA, bovine serum albumin. Data are the mean (n=4) ± SD.

Only the protein pellet obtained through alkaline solubilization followed by acidic precipitation was subjected to the protease protocol (5% (w/w) enzyme/sample gFW ratio, 60 °C, 2 h, 1:5 S/L ratio, pH 7, 100 rpm), as the neutral solubilization followed by acidic precipitation did not yield a significant amount of precipitated proteins. Before the digestion, the pellet was washed 3 times with milli-Q water with a S/L ratio of 1:15 to remove excess salts produced during the previous treatment.

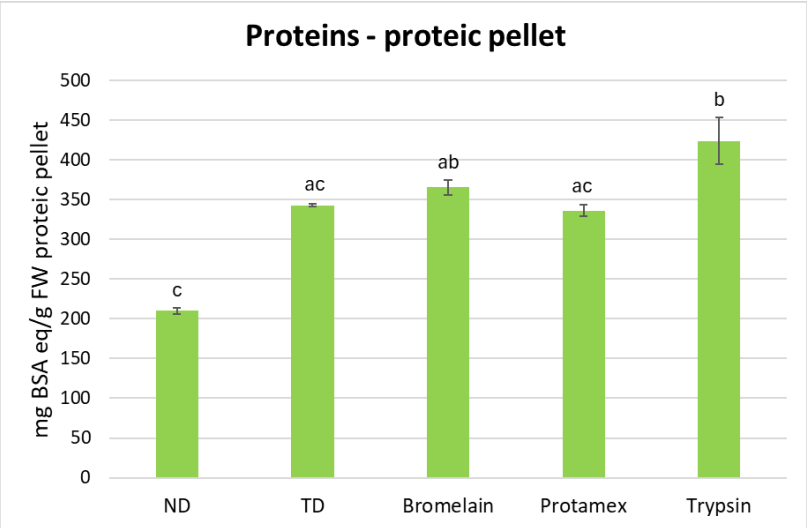


Figure 2: Protein content (mg BSA equivalents/gFW protein pellet) in supernatant fractions of tomato seed protein pellets after alkaline/acid extraction/precipitation digested with selected proteases. Letters indicate statistically significant difference among samples determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). BSA, bovine serum albumin; ND, not digested control; TD, thermally digested control. Data are the mean (n=4) ± SD.

The highest yield was obtained with Trypsin, while treatments with Bromelain and Protamex produced results comparable to the TD control (Figure 2). However, the obtained pellet represents less than 10% of fresh weight of the original feedstock and requires multiple water washes to remove impurities leading to an additional use of water. Furthermore, the costs and environmental impacts of the alkaline extraction and acidic precipitation are noteworthy due to the nature of the chemicals employed (NaOH and HCl). Considering these factors, direct protease treatment on dried tomato seeds reported before (D2.1) was selected as the more sustainable option.

Finally, to further reduce the environmental impact of the previously selected enzymatic extraction process (D2.1, section 3.2.1), considering the optimal temperature working range of the enzymes, the incubation temperature was lowered from 60 °C to 37 °C, while all other parameters remained unchanged.

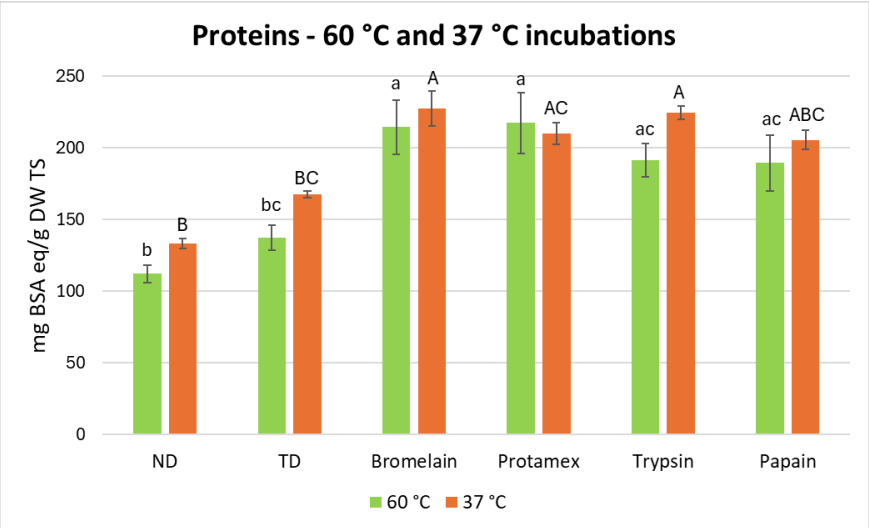


Figure 3: Protein content (mg BSA equivalents/gDW sample) in tomato seeds (TS) supernatants following selected protease treatment at two different incubation temperatures (60 °C and 37 °C). Protein yields obtained at 60°C were previously reported in D.2.1 (section 3.2.1) and are here repeated for comparison. Lowercase and capital letters indicate statistically significant difference respectively among precipitated protein and supernatant samples determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). BSA, bovine serum albumin; ND, not digested control; TD, thermally digested control. Data are the mean (n=4) ± SD.

Results showed, generally, comparable protein concentration in the supernatants of digestates at 60 °C and 37 °C, and in some cases higher (i.e., Trypsin) for the lower temperature (Figure 3). Given this result, the lower incubation temperature was selected for the updated protocol ensuring energy savings.

After choosing the final incubation temperature, TS were also subjected to sonication, hydration or a combination of both, before enzymatic digestion. However, protein yields were comparable across all pre-treatments, leading to the exclusion of these additional steps in the final protocol.

The final enzymatic protein extraction protocol from tomato seeds is the following: TS are dried and grounded; the digestion conditions are 5% (w/w) enzyme/sample gDW ratio, 37 °C, 2 hours, 1:5 S/L ratio (2 gDW tomato seeds/10 mL of milli-Q water), pH 7, 100 rpm. The enzymes chosen for the final protocol are Trypsin from porcine pancreas (Sigma-Aldrich, Milan, Italy) and Protamex® (Novozymes A/S, Denmark).

SDS-PAGE gels were performed (16% v/v acrylamide) (35 µl of digestate per well) with Blue Coomassie staining on all obtained hydrolysates, confirming again the presence of small molecular weight peptides (< 20 kDa) (Figure 4).

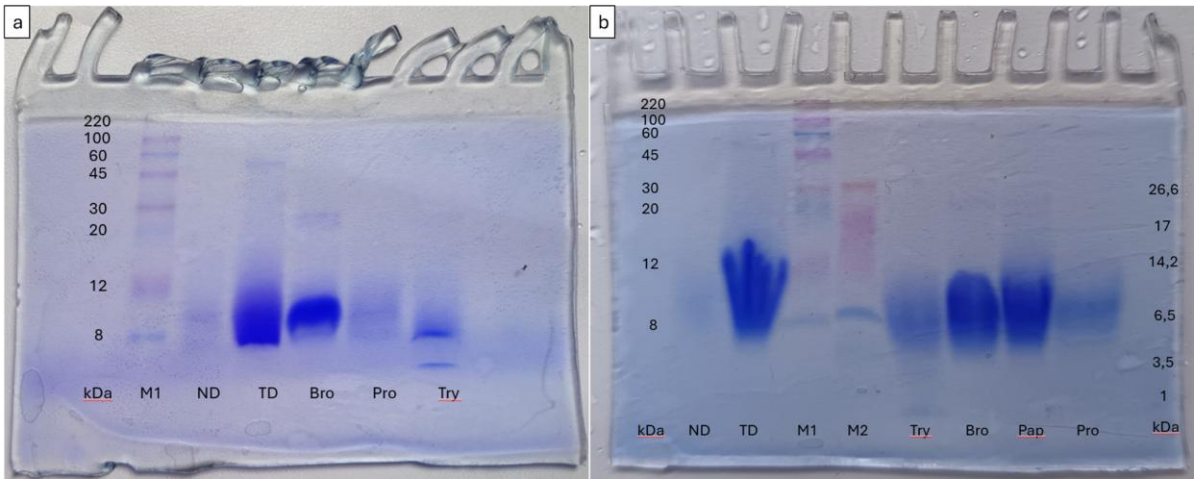


Figure 4: SDS-PAGE gels (16% v/v acrylamide) on tomato seeds digestates (35 µl per well) following selected protease treatment at 60 °C (a) and at 37 °C (b). M1 = marker 8-220 kDa, M2 = marker 1-26,6 kDa, ND = nondigested, TD = thermally digested, Bro = Bromelain, Pro = Protamex, Try = Trypsin, Pap = Papain.

3.1.1.2 Peptide fractions biological activities

Antioxidant and anti-browning activities determination were performed on peptide fractions (supernatants after protease digestions) by applying respectively the ABTS (2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) colorimetric assay[4,5] and the anti-tyrosinase y assay[6,7]. Antioxidant and anti-browning activities were evaluated, as these are crucial characteristics for the potential application of tomato peptide fractions in food and feed formulations. These properties play a significant role in enhancing the stability, nutritional value, and shelf life of food products by preventing oxidative degradation and enzymatic browning. Assessing these functional attributes helps determine the effectiveness of tomato peptide fractions in preserving food quality, making them suitable candidates for incorporation into natural antioxidant systems in the food and feed industries.

The ABTS assay was first performed on the samples treated with proteases or cell-wall hydrolytic enzymes with a 5% (w/w) enzyme/sample gDW ratio, as well as their corresponding controls (see D2.1 section 3.2.1), including samples treated with enzymes that were not initially selected for the final protocol (Figure 5).

The treatments with proteases produced digestates with comparable antioxidant activity, except for Pancreatin that exhibit approximately 50% higher antioxidant (Figure 5). All digestates presented similar protein yields with those derived from Pancreatin showing for some samples even slightly lower values (see D2.1 section 3.2.1). Pancreatin consist of a mix of enzymes including proteases, lipases and glucosidases. Therefore, the high antioxidant activity of these samples may be due to the release of other compounds, for instance reducing sugars, though this requires further investigation. Treatments with cell-wall hydrolytic enzymes exhibited minimal antioxidant activity (Figure 5), further supporting the use of proteases alone as the most effective approach.

[4] Ferri M, Gianotti A, Tassoni A. Optimisation of assay conditions for the determination of antioxidant capacity and polyphenols in cereal food components. J Food Comp Anal. 2013; 30(2): 94–101.
[5] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26, 1231–1237
[6] Masmoto Y, Iida S, Kubo M. Inhibitory effect of Chinese crude drugs on tyrosinase. Planta Med. 1980; 40: 361–365.
[7] Ferri M, Graen-Heedfeld J, Bretz K, Guillon F, Michelini E, Calabretta MM, et al. (2017) Peptide fractions obtained from rice by-products by means of an environment-friendly process show in vitro health-related bioactivities. PLoS ONE 12(1): e0170954.

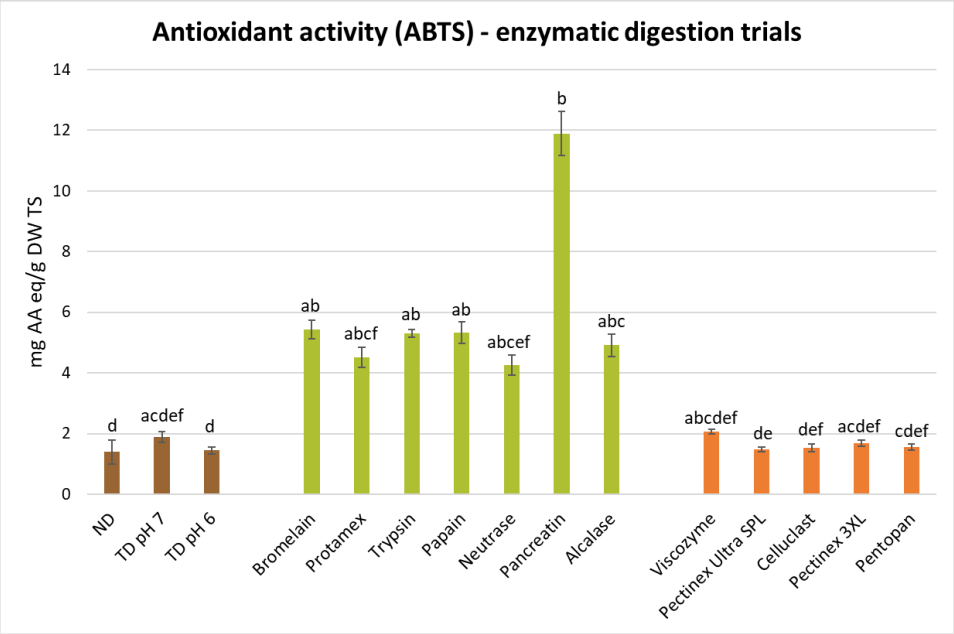


Figure 5. Antioxidant activity (mg AA equivalents/gDW sample) in tomato seeds (TS) supernatants following enzymatic digestions. Letters indicate statistically significant difference among samples determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). AA, ascorbic acid; ND, not digested control; TD, thermally digested control. Data are the mean (n=4) ± SD.

ABTS assay was performed on digestates from protease-incubation performed at 37 °C (detailed above in Figure 3) and 60 °C (Figure 6).

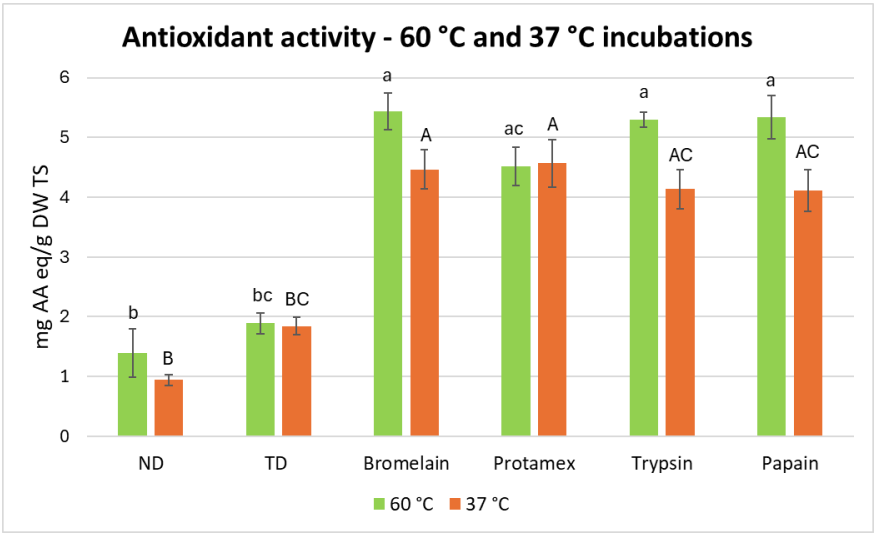


Figure 6: Antioxidant activity (mg AA equivalents/gDW sample) in tomato seeds (TS) supernatants following selected protease treatment at two different incubation temperatures (60 °C and 37 °C). Lowercase and capital letters indicate statistically significant difference respectively among samples from 60 °C and 37°C incubations determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). AA, ascorbic acid; ND, not digested control; TD, thermally digested control. Data are the mean (n=4) ± SD.

The antioxidant activity of digestates obtained at 37 °C were similar or only 20%-lower compared to their counterparts obtained at 60 °C (Figure 6). However, considering both the protein yield (Figure 3) and the energy spending, the 37 °C incubation is considered a better choice. Protease-digested samples were subjected to the anti-tyrosinase activity assay (Figure 7).

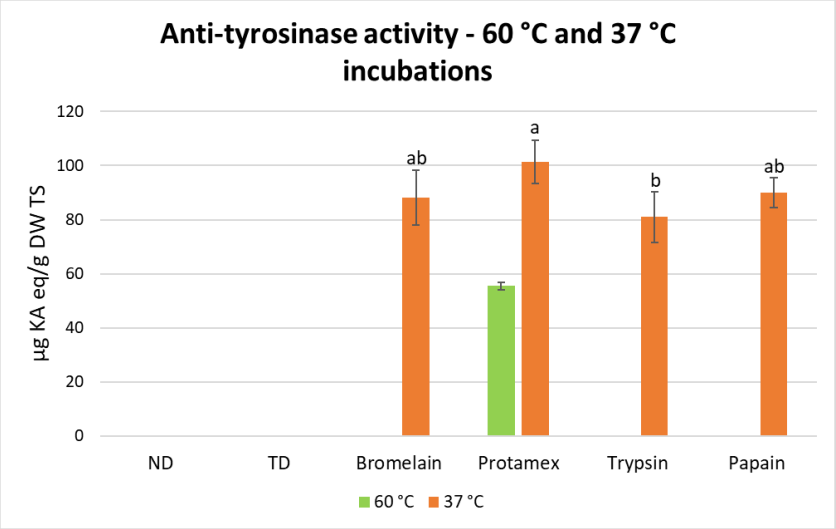


Figure 7: Anti-tyrosinase activity (mg KA equivalents/gDW sample) in tomato seeds (TS) supernatants following selected protease treatment at two different incubation temperatures (60 °C and 37 °C). ND and TD controls and Bromelain, Trypsin and Papain treatments at 60 °C activity was below the detectable activity level of the assay. Letters indicate statistically significant difference among samples from 37 °C incubations determined by ANOVA test followed by post-hoc Tukey test ($p < 0.05$). KA, Kojic acid; ND, not digested control; TD, thermally digested control. Data are the mean (n=2) ± SD.

At 60 °C, digestion with all proteases (Bromelain, Trypsin, Papain, Alcalase, Pancreatin and Neutrase (D2.1, section 3.2.1) resulted in undetectable levels of anti-tyrosinase activity, except Protamex that showed an activity of 55.44 µg KA eq/g DW TS (Figure 7) leading to 32% tyrosinase inhibition relatively to the positive water control (data not shown). At 37 °C, the selected protease digested samples exhibited an average activity of 90.12 µg KA eq/g DW TS (Figure 7) with a tyrosinase inhibition of around 24% with respect to the positive water control (data not shown). At this temperature, Protamex hydrolysates showed the highest anti-tyrosinase activity (Figure 7). The anti-tyrosinase and antioxidant activities showed an opposing trend with temperature.

Conclusions

The tests done to further optimize the protein enzymatic extraction protocol from dried and ground tomato seed supplied by TOMA (previously reported in D2.1), did not lead to substantial improvement of the protein extraction yield. Specifically, the use of fTS or dTS did not drastically improve peptide extraction yield and would require additional steps that are environmentally impactful. Furthermore, alkaline and neutral solubilization followed by acidic precipitation before enzymatic digestion, were deemed unnecessary as a large quantity of peptides did not precipitate, besides increasing environmental and economic costs.

To further decrease the environmental impact and costs, the incubation temperature was lowered to 37 °C as the protein yield was similar or even slightly better (i.e., trypsin treatment) compared to those obtained at 60 °C. Antioxidant and anti-tyrosinase activities were detected and were respectively higher in digestates obtained at 60 °C and in those obtained at 37 °C.

Next steps

An evaluation of protein content in selected digestates using the Kjeldahl method[8] is planned. Following this, a scale-up of the process up to 2 L by using a bioreactor will be carried out. For the scale-up, the feedstock that will be used is the dried and ground TS sent by INRAE in November 2024. Next steps will include trials of protein fractionation by using filters with different cut-offs, as well as peptide sequencing of the obtained sub-fractions. Moreover, approaches to target the cascading extraction of polyphenols and/or polyesters will be developed through collaboration with INRAE_IATE and ITQB NOVA.

Bottlenecks and deviations

The main bottleneck encountered is the overall low protein yield with respect to previously detected total protein content of the seeds using the Kjeldahl method, which was 26,1% (w/w) (see D2.1, section 3.2.1). Trials with different pre-treatments of the feedstock and incubation conditions were anyhow unsuccessful in improving protein yield.

3.1.2 By immobilized enzymes extraction (FHNW)

3.1.2.1 Protein extraction and peptide preparation for LC-MS-MS analysis

Proteins and peptides were analysed and characterized via LC-MS-MS analysis by using a Q TOF LC MS system from Agilent. Briefly, after extraction process (see MS8 report), the supernatant which contains the proteins and peptides of interest was used to determine the quantities of proteins using BSA assay. The concentration was adjusted by diluting to 0.01µg *per* µL when needed. To complete full digestion of proteins and characterize as many peptides fragments as possible, a trypsin digestion step was performed using the rapid trypsin digestion kit from Promega (see protocol, for VA1060 and VA1061 products on Promega website). The resulting samples were subjected to a desalting step using Pierce C18 spin tips columns (see procedure for 10615555 products on ThermoFisher website) to eliminate all the impurities (salts, detergents, *etc.*). The cleaned sample was then dried using a speed vacuum and resuspended in water with 0.01% formic acid. Peptide's content was measured using BSA assay and adjusted if necessary to 0.001 µg *per* µL. Subsequently, samples were directly analysed.

3.1.2.2 LC-MS-MS analysis and data preparation

A C18, AdvanceBio Peptide Mapping 120Å, 2.1 x 150 mm, 2.7 µm column (Agilent) was used for the analysis. The mobile phase consisted of water with 0.1% formic acid (phase A) and CH_3CN with 0.1% formic acid (phase B). The elution gradient was: 0-1 min, 2.5% B; 2-90 min, 35% B; 90-94 min, 95% B; 94.5 min, 2.5% B. The flow rate was 0.4 mL/min.

[8] Official Methods of Analysis (1995) 16th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, sec. 33.2.11, Method 991.20

The HPLC (Agilent technology 1290 Infinity II) was online coupled to a Q-TOF-MS mass spectrometer (Agilent Accurate 6500 series). A duty cycle of the mass spectrometer consists in the acquisition of a MS spectrum and the subsequent fragmentation of the twenty ions with the highest intensity. All spectra of the 95 minutes run were processed with Mascot Distiller. The search parameters for the database search were: "trypsin" as cleavage agent, a tolerance on the mass measurement of 100 ppm in the MS mode and 0.7 Da for MS/MS analysis. Glycosyl P, pyroglutamine for N-terminal glutamine, oxidation of methionine and proline were used as variable modification and carbamidomethyl as a fixed modification.

The MS/MS spectra were used for database searches. For automated interpretation, database searches were performed using Mascot Distiller interface (Version 2.5.0.5, from Matrix Science). Mascot searches were done against the *Solanum lycopersicum* database from Uniprot.

3.1.2.3 Data interpretation

Each tomato seed extracts previously treated rather with bromelain or trypsin immobilized enzymes were analysed.

Following the analysis steps as described above more than one hundred proteins came out as being present among the identified peptides.

PeptideRanker was used to predict the bioactivity of all the peptides collected. The relative bioactivity is expressed in the form of prediction scores. Peptides were considered to possess potential bioactivity using 0.5 as the threshold value. Based on the prediction, potential ACE-inhibitory peptides, DPP-IV inhibitory peptides and antimicrobial peptides were detected. BIOPEP database was used as a second screening method for potential bioactivity. Searches of similar sequences previously identified having ACE inhibitory activity, DPP-IV inhibitory activity and antimicrobial activity were performed.

To explore further the potential bioactivity of some of these peptides, twelve of the most interesting peptides with the highest bioactivity prediction score with both Peptide Ranker and BIOPEP were ordered for testing activities. The following peptide sequences were selected:

- ADVYNPR
- GQNPVFPR
- SGLWVNPR
- PGVPGPIQR
- EMACPHLGR
- QSLPGLCR
- FFQGPGGR
- FGQFFEAR
- LAADDFR
- AENLPGLCR
- NQLDLTFR
- YGFNVWDR

The ACE inhibitory activity of the individual peptide was tested with a concentration of peptides of 50 µM using an *in vitro* method based on fluorescence signal caused by enzyme catalysed kinetic reaction by using an assay kit (see procedure for CS0002-1KT product on Sigma website[9]). A similar test for testing DPP-IV inhibitory activity was also performed with a concentration of peptides of 50 µM by using also an *in vitro* method based on fluorescence with an assay kit (see procedure for ab13308 product on Abcam website[10]).

[9] https://www.sigmaaldrich.com/deepweb/assets/sigmaaldrich/product/documents/248/077/cs0002bul-mk.pdf?srsltid=AfmBOopxsGcRp_qj2e-SjIKXAz0bCBKCHwXS63y65Ry1J7hGCLgSrwSm

[10] https://www.abcam.com/ps/products/133/ab133081/documents/ab133081%20Dipeptidyl%20peptidase%20IV%20(DPP4)%20Inhibitor%20Screening%20Assay%20Kit-protocol%20v2a%20(website).pdf?srsltid=AfmBOorSRgswib6dQxvY76AYiKj6MQnPceW-0fDoxo2mILvg2sKnjKf3

In vitro tests showed some inhibition of ACE as well as DPP-IV for the different tested peptides. Results are summarized in Table 1.

Table1. Summary of the different % of inhibition for ACE and DPP-IV for the twelve ordered peptides.

PEPTIDE SEQUENCE	% INHIBITION OF ACE	% INHIBITION OF DPP-IV
ADVYNPR	29	22
GQNPVFPR	5	0
SGLWVNPR	18	0
PGVPGPIQR	0	20
EMACPHLGR	0	0
QSLPGLCR	30	13
FFQGPGGR	35	0
FGQFFEAR	3	0
LAADDFR	0	60
AENLPGLCR	0	12
NQLDLTFR	6	5
YGFNVWDR	36	55

Conclusions

Some interesting peptides which show ACE inhibitory activities as well as DPP-IV inhibitory activities were found into the different tomato extracts. These peptides are promising source for reducing hypertension as well as for their anti-diabetic properties, respectively.

Next steps

Fractionation, isolation and quantification of those peptides into tomato mix extracts are currently under investigation.

The next steps would also include some antimicrobial and antifungal tests for the various tomato extracts. Digestion of proteins into peptides with other proteases (e.g., those tested by UNIBO_BIGEA) and conditions will also be tested to assess whether other peptides or fractions contain potentially interesting bioactivity. Finally, the number of runs that can be done with the immobilised enzymes will be accessed as this impacts the economics of the process, for example immobilise laccases could be used six times (personal communication).

Bottlenecks

The price of pure trypsin for the digestion of proteins is a critical criterion for the potential use of the peptides of interest for nutraceutical applications. The production price of those interesting peptides might be too high for food application. Alternative solutions for proteins digestion need to be further investigated.

3.1.3 By extraction and hydrolysis through microbial routes (UNIBO-DICAM)

The main task of UNIBO-DICAM is to produce bioactive hydrolysates from tomato pomace through microbial routes, exploiting bacterial isolates obtained from non-conventional environments (*i.e.*, desert and sea). The hydrolysis of tomato byproducts *i.e.* whole tomato pomace (seed plus peel) (WTP), tomato peel (TP), tomato seeds (TS) and defatted tomato seeds (DTS) was assayed using ten bacterial isolates. After bacterial growth, bioactivities (antioxidant and antibacterial) as well as extracellular hydrolytic enzymes production were evaluated on cell free supernatant (CFS). Furthermore, assays for the recovery of

antioxidant compounds and proteases from the most promising isolate (R50)—exhibiting highest antioxidant and proteolytic activities—were performed using different approaches. The results obtained have been reported in D2.1.

During this reporting period, the activities performed dealt with evaluating the thermal stability of the bioactivities (of the three best performing isolates R50, N1 and L100) and performing first assays for the scale-up of the hydrolysate production process in a 3 L fermenter by isolate R50, using DTS as substrate.

The results of stability studies, performed on CFS recovered after 72 h incubation, showed that antimicrobial activity was lost after thermal treatment (autoclaving). This result suggests that the antimicrobial compounds are sensitive to high temperatures, therefore possibly proteins that have been denatured after autoclaving. On the contrary, the antioxidant properties were mostly preserved after the autoclaving (Figure 8), i.e., antioxidant activity of autoclaved hydrolysates was about 90% of that of non-autoclaved ones, representing a proper sterilization method for the antioxidant hydrolysates.

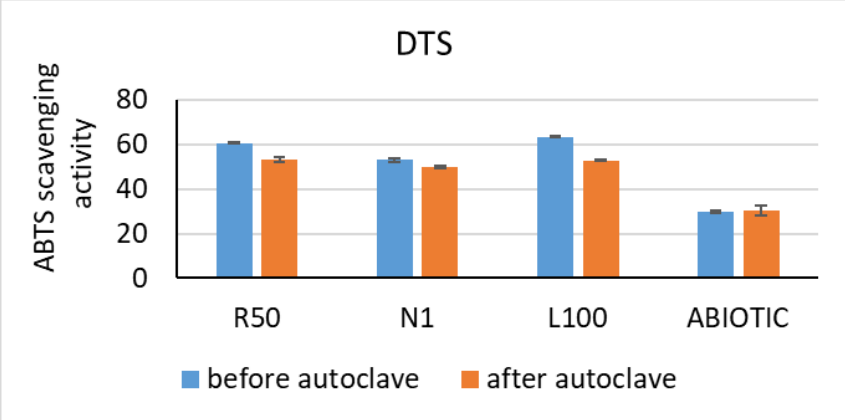


Figure 8: Antioxidant activity of the DTS hydrolysates obtained after 72 h incubation with microorganisms and subjected or not to autoclaving (121 °C, 20 min) expressed as ABTS scavenging activity (%).

Regarding the scale-up of DTS hydrolysis, a preculture of isolate R50 was inoculated into a 3 L STR bioreactor (working volume of 1.5 L of growth medium supplemented with 1% (w/v) of DTS). Samples were aseptically withdrawn after 0, 5, 24, 30 and 48 h incubation for bacterial cell counts and bioactivities evaluation.

The microbial growth was observed up to 24 h, after which the bacterial culture entered stationary phase. The antioxidant activity of the hydrolysate increased over time reaching the highest value of ABTS inhibition of 48±0.007% after 48 h incubation. This value corresponds to 21% improvement/increase of the initial antioxidant activity (of ≈27%) measured at T0 (Figure 9). The antioxidant activity of the hydrolysate produced in bioreactor is relatively lower than that previously obtained in flask after the same incubation time, which was reported in D2.1. Indeed, in flask, an antioxidant activity of 58.80% was recorded after 48 h incubation. However, it is important to notice that under flask conditions, the sample recovered at T0 exhibited a higher antioxidant activity (of about 38%) compared to the one measured here. Thus, the differences between flask and bioreactor results could be due to a change of substrate properties after a long storage period. This is confirmed by the additional flask trial performed as control. In this experiment, the antioxidant activity increased over time, reaching 55.2±0.005% after 48 h which is slightly lower

compared to the activity recorded at the same sampling point in earlier trials (58.80%), performed with the fresh substrate.

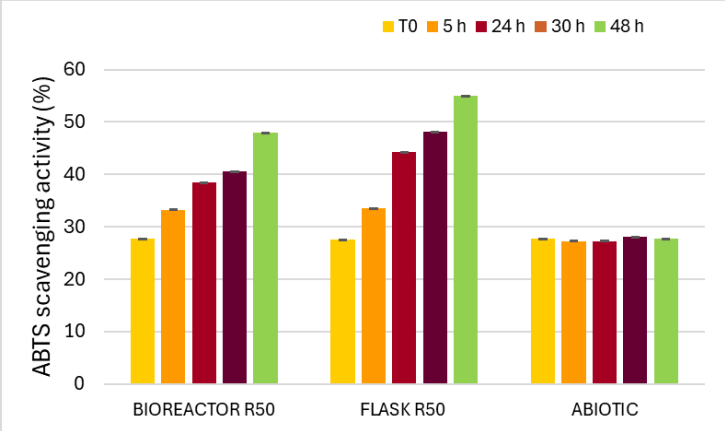


Figure 9. Antioxidant activity time course of CFS of isolate R50 in bioreactor and flask expressed as ABTS scavenging activity (%).

The antimicrobial activity against *Staphylococcus aureus* of the DTS hydrolysates obtained by R50 in the bioreactor was monitored over time. The abiotic negative control never exhibited inhibitory activity. The CFS of R50 showed antimicrobial activity between 5-48 h and the inhibition halo diameters decreased at higher time points. The inhibition diameters observed in the bioreactor are comparable to those obtained in the flask trials. The qualitative production of proteases and cellulases by R50 in the bioreactor was tested over time. The cellulase and protease production was detected between 24-48 h.

Conclusions

Three bacterial isolates (N1, L100 and R50) have been previously selected as most promising based on their bioactivities evaluated under standard conditions. After thermal treatment, antimicrobial activity was lost while antioxidant activity was shown to be highly stable to autoclaving (sterilization), which is an important property from hydrolysates safety and microbiological stability viewpoints. Taking into consideration also enzymes (proteases) production, the isolate R50 was selected and used for upscale experiments aiming at the production of active hydrolysates in 3 L bioreactor. The results of first trials using DTS as substrate showed that it was possible to produce a hydrolysate having antioxidant, antimicrobial as well enzymatic activities with values like those obtained in flask.

Next steps

Other assays to produce bioactive hydrolysates will be performed using tomato seeds (without defatting) as substrate to overcome the defatting step. Moreover, the next steps will concentrate on the recovery and further characterisation of antimicrobial and/or antioxidant compounds produced in a 3 L fermenter.

3.2 Cutin

3.2.1 TOMA

TomaPaint partner (TOMA) provided Tomato pomace collected from one of the main Italian tomato processor companies based in Parma during the tomato seasons 2023 and 2024. The tomato pomaces were used for the extraction process both fresh and after storing in a trench system storage under anaerobic conditions.

3.2.1.1 Flotation and drying

Tomato pomace is composed of seeds and skins in almost equal percentages. The flotation system in place at TOMA facilities was used to separate the tomato seeds from the tomato peels, starting from whole tomato pomace. Given their specific weight, the skins tend to float on the water surface at 30 °C, while the seeds tend to settle on the bottom.

The samples for the partners were obtained within several batches of production. Each type of samples has been supplied fresh (16 kg of tomato pomace, 69 kg of tomato peels and 37 kg of tomato seeds) and dried (11.50 kg of tomato pomace, 10 kg of tomato peels and 4 kg of tomato seeds). In addition, a sample of wet seeds (0.6 kg) was lyophilized and sent to the partners.

3.2.1.2 Sample analyses

Humidity analysis on total pomace prior flotation and on seed and peel fractions after flotation, was performed at TOMA by means of a thermogravimetric balance at 105 °C. Proteins analysis was performed on peel and seed fractions. The results on wet and dried samples are reported in the Table 2.

Table 2. Humidity and proteins analysis on wet and dried pomace, peels and seeds.

	Pomace		**Peels**		**Seeds**	
	Wet	Dried	Wet	Dried	Wet	Dried
Humidity, % Thermobalance 105 °C	70.86±2.57	3.26±0.13	76.87±0.55	4.10±0.90	72.12±2.15	5.20±0.28
Proteins %, Reports Istisan 96/34 pag. 13	-	-	2.10	7.20	6.35	19.11

In the Table 3 below the results of the chemical analyses performed on two batches (2023 and 2024) of the tomato pomace such as (peels +seeds) are reported.

Table 3. Chemical analysis.

Parameter	Test Method	Value 2023	Value 2024	M.U.
Humidity	Reports Istisan 96/34 pag. 7 met.B	80.27	70.27	g/100 g
Dry residue	Reports Istisan 96/34 pag. 7 met.B	19.73	29.73	g/100 g
Proteins	Reports Istisan 96/34 pag. 13	2.20	4.39	g/100 g
Ashes	Reports Istisan 96/34 pag. 77	0.14	1.11	g/100 g
Total Lipids	Reports Istisan 96/34 pag. 41	1.60	3.90	g/100 g
Total Dietary Fibre	AOAC 985.29	15.69	18.92	g/100 g
Sugars	P-PRO-ALI-32	0.06	0.32	g/100 g of glucose
Total Polyphenols (expressed as gallic acid)	Spectrophotometric method-Folin-Ciocalteau	-	19.4	mg/100g

3.2.2 INRAE-BIA

All the main findings until now were reported and discussed in Deliverable D2.1, where the relevant information is available for consultation.

3.2.3 ITQB-NOVA

At ITQB-NOVA, the focus has been on the peels fraction, with isolation of cutin and its subsequent mild hydrolysis to mixtures of oligomers. The extraction process has been done using two classes of solvents: Ionic Liquids (IL), represented by Cholinium hexanoate, and Natural Deep Eutectic Solvents (NADES), in this case using Choline chloride and Lactic acid (ChLA) in a 1:2 molar ratio[11]. The cutin hydrolysis is then achieved using 20 mL of 1 M NaOH in methanol/water (1:1, v/v) at 90 °C for 1 hour without stirring for each 0.5 g of cutin.

The use of the NADES ChLA has allowed the concomitant isolation of cutin from tomato peels in high yield (*ca.* 80%), and of a phenol-rich extract. This constitutes a good indication for the cascading approach for selective and consecutive extraction/isolation of valuable natural compounds. Additionally, results point to the efficacy of the process lowering both time and temperature during the extraction. Moreover, while the use of IL requires introducing 80 mL of ethanol (EtOH) *per* gram of starting material to the mixture for filtration, using the NADES allowed the reduction of this solvent to 20 mL *per* gram. Note that the ethanol is recovered by distillation and can be reused.

Previously, the group at ITQB NOVA has already reported the characterisation of cutin isolated from tomato peels using nuclear magnetic resonance (NMR) spectroscopy [12, 13], namely identifying some of the predominant signals using Heteronuclear Single Quantum Coherence (HSQC) technique, which correlates

[11] Vorobyova et al. (2022). Extraction of phenolic compounds from tomato pomace using choline chloride–based deep eutectic solvents. J. Food Measur. Charact., 16, 1087-1104.

[12] Moreira, C. J.S., Bento, A., Pais, J., Petit, J., Escórcio, R., Correia, V. G., Pinheiro, A., Halinski, L. P., Mykhaylyk, O. O., Rothan, C., Silva Pereira, C. (2020) Plant Physiol. 184, 592–606.

[13] Artur Bento, Carlos J. S. Moreira, Vanessa G. Correia, Rita Escórcio, Ruben Rodrigues, Ana S. Tomé, Nathalie Geneix, Johann Petit, Bénédicte Bakan, Christophe Rothan, Oleksandr O. Mykhaylyk, and Cristina Silva Pereira (2021) ACS Sustainable Chem. Eng., 9, 15780−15792.

the ^{1}H and ^{13}C direct-chemically bounded (see D2.1, section 3.3.3). Therefore, analyses of the HSQC spectra of samples of cryomilled cutin obtained with either the IL or ChLA (at 100 °C and at 60 °C, respectively) were done to check for possible differences between the products. Although the general pattern is maintained throughout the samples, it is observed that the signals are more fainted when using the NADES, as well as there are other signals present, possibly due to entrapped compounds not fully removed in the isolation process. Nevertheless, the spectra show the main characteristic signals of cutin, as observed in Figure 10. Comparison of the spectrum of the product obtained with the NADES at 100 °C (b) with that at 60 °C (c), one can conclude on the efficacy of the isolation process at lower temperatures, as the spectral signatures are mostly the same.

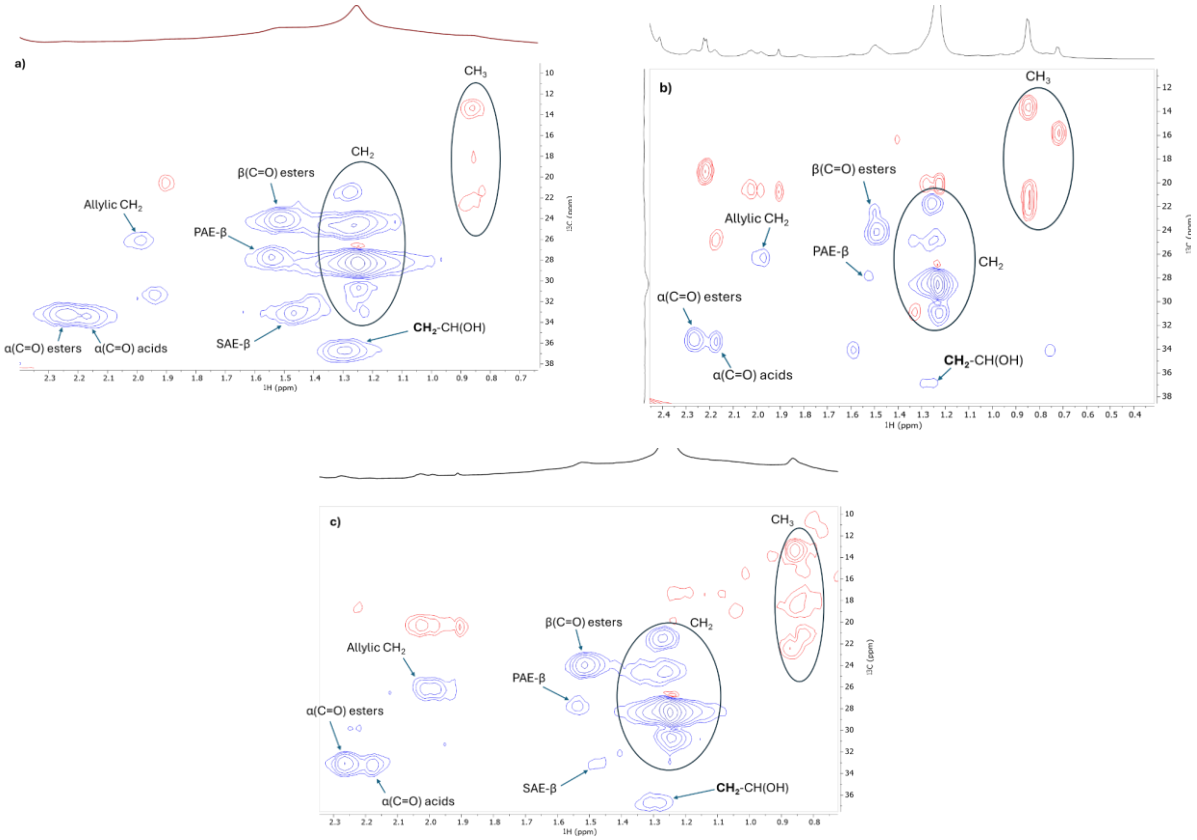


Figure 10: Characterisation of the aliphatic region of cutin from tomato peels by ^{1}H–^{13}C HSQC nuclear magnetic resonance spectroscopy. The cutin was obtained under different conditions: (a) 10× IL; 2 h; 100 °C; (b) 10× ChLA; 2 h; 100 °C; and (c) 10× ChLA for 1 h at 60 °C. (Note: the blue signals correspond to methyl – CH_3, while the red signals correspond to methylene – CH_2 – or methine – CH – groups).

All spectral signatures show the distinct regions of the methyl and methylene groups. Moreover, it is always possible to assign the characteristic signal of the primary aliphatic esters (PAE-β), while the signal for the secondary aliphatic esters (SAE-β) is not clearly identified in the product obtained using the NADES at 100 °C for 2 h. Nevertheless, it is possible to identify the existence of both ester and acid groups in all the obtained solids, as well as the presence of allylic CH_2 groups.

There is also a superimposition of the Infra-Red spectroscopy (FTIR) signatures for both cutin samples, as shown in Figure 11. The spectra display the characteristic bands for the stretching vibrations of methyl, methylene, carbonyl and ester groups (as identified in the figure).

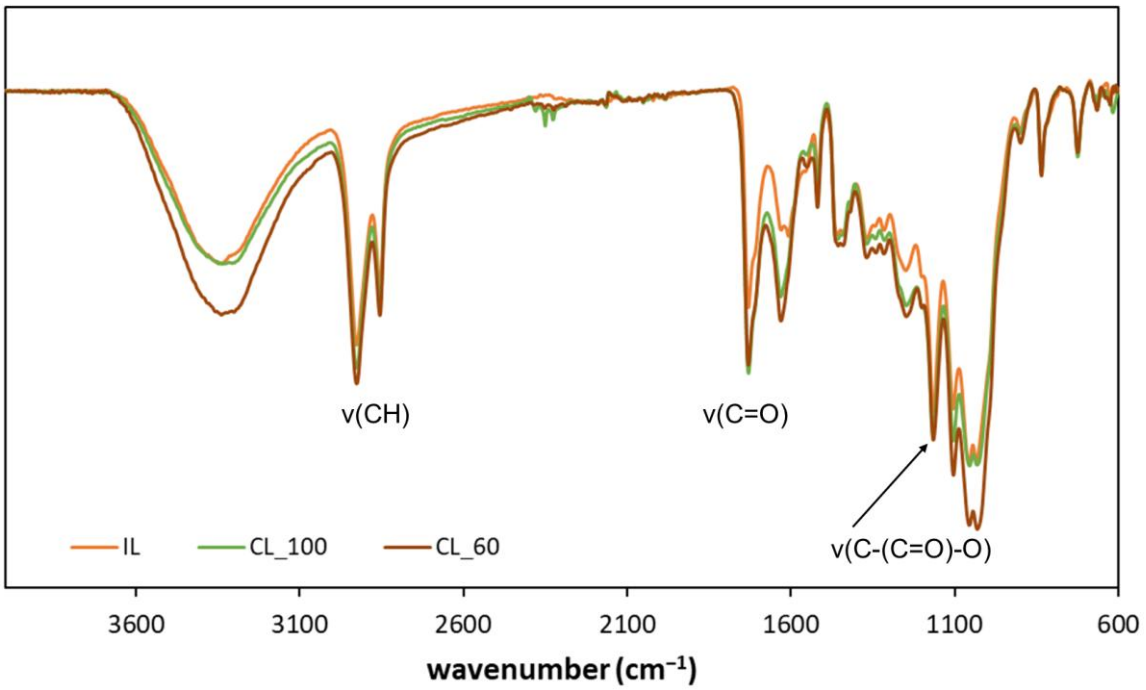


Figure 11: Infra-red absorption (FTIR) spectra of the cutin isolated with IL 100 °C for 2 h (orange), ChLA at 100 °C for 2 h (green), and ChLA at 60 °C for 1 h (brown), obtained using Attenuated Total Reflectance (ATR) sampling technique.

The solids obtained using the different conditions were analysed for carbohydrates content[14] and ability to reduce Fe(III) to Fe(II) – Ferric Reducing Antioxidant Power (FRAP)[15]. Firstly, an extraction of the water-soluble compounds still present in the solids was achieved by warming a 100 mg sample of each solid with 10 mL of distilled water at 50 °C for 1 h. The FRAP assay was done directly with these extracts, while a strong hydrolysis with 4.0 M HCl (100 °C, 30 min) was done to further quantify the total sugars in each sample. The hydrolysates were then submitted to a phenol-sulfuric acid mixture (100 °C, 30 min) and quantified by UV-Visible absorption spectroscopy, using solutions of glucose with known concentration to build the calibration. The results, which are shown in Table 4, demonstrate that the solids obtained using the NADES are richer in sugar and display higher reducing power compared to that obtained using IL. Nonetheless, the values are all in the same range, pointing to the efficacy of the isolation processes.

Table 4. Total sugar content (in glucose equivalents) and FRAP (in Fe(II) equivalents) of the water-soluble fraction of the solids obtained in the isolation of cutin using IL, and NADES at 100 °C and 60 °C.

	IL	ChLA@100 °C	ChLA@60 °C
Glucose eq. (mg/ g solid)	7 ± 2	15 ± 3	27 ± 5
Fe(II) eq. (µM)	16 ± 8	34 ± 5	26 ± 6

[14] Pragna Rao and Thillaisthanam N. Pattabiraman (1989) Analytical Biochemistry 181,18-22.

[15] Iris F. F. Benzie and J. J. Strain (1996) Analytical Biochemistry 239, 70–76.

The solids obtained in the isolation procedures were also submitted to a strong alkaline hydrolysis (20 mL of 1.5 M KOH in MeOH per gram of cutin, stir overnight at room temperature) to find the monomer(s) that constitutes the polymer. In all cases, NMR analyses established the 9(10),16–dihydroxy hexadecanoic acid as the major monomer, as shown in Figure 12.

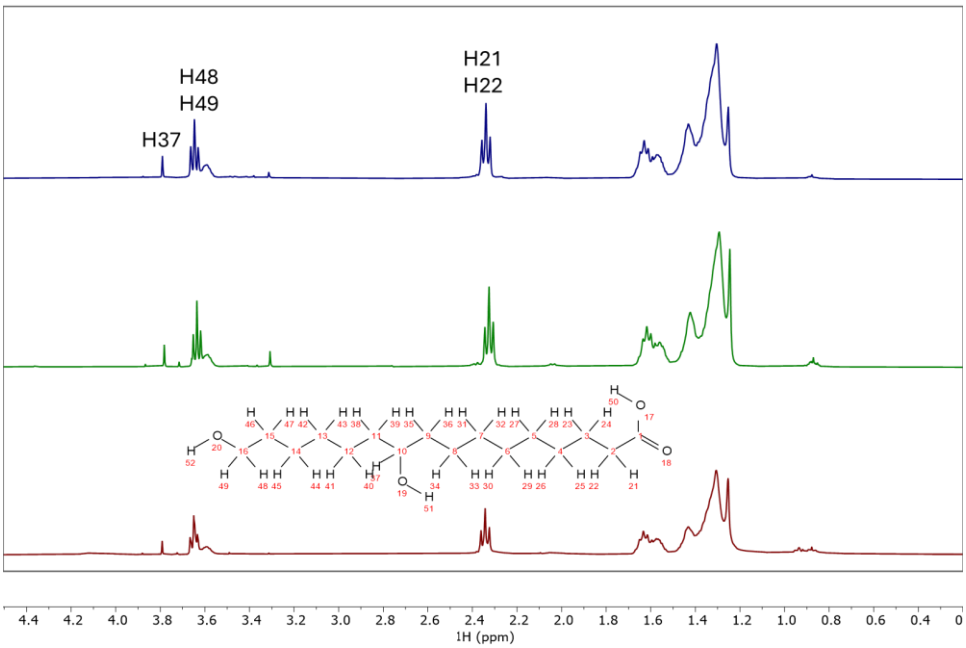


Figure 12. Overlay of the ^{1}H NMR spectra (in $CDCl_3$) of the product of alkaline hydrolysis of the solids obtained using (from top to bottom) IL, ChLA (100 °C, 2 h), and ChLA (60 °C, 1 h).

Cutin extracted with IL underwent mild hydrolysis to create oligomer mixtures. The mass balance analysis of the process demonstrates that mild hydrolysis of the cutin produced per hydrolytic round yields 57 % of cutin oligomeric mixtures (COMs). Previous data indicated that COMs generated from cutin contained antimicrobial activity against two models of bacteria: *S. aureus* and *E. coli*[16]. The antimicrobial activity of the cutin and COMs generated from TOMAPAINT pomace source were tested as following: *S. aureus* cells were exposed for 24 h to 1mg/mL of COMs at 37 °C under orbital agitation (100 rpm). After 24 h, the antimicrobial activity was evaluated through colony-forming units (CFU).

[16] Rita Escórcio, Artur Bento, Ana S. Tomé, Vanessa G. Correia, Ruben Rodrigues, Carlos J. S. Moreira, Didier Marion, Bénédicte Bakan, and Cristina Silva Pereira (2022) *ACS Sustainable Chem. Eng.* 10, 11415-11427.

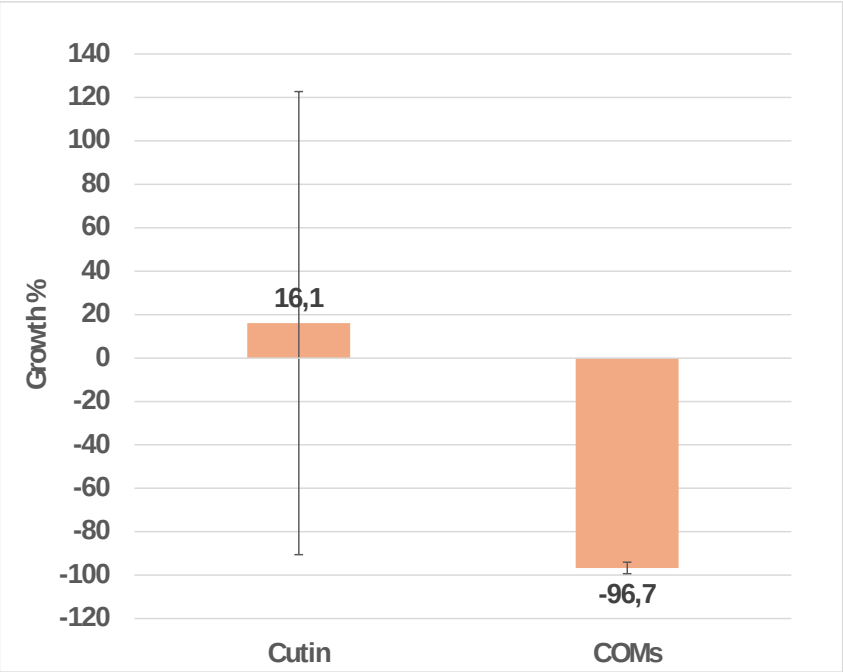


Figure 13. Antimicrobial activity results of *S. aureus* exposed to cutin and COMs in a concentration of 1mg/mL.

The antimicrobial results show that COMs from this source have the capacity of conferring antimicrobial activity, killing around 96 % of bacterial cells. Cutin results are due to the highly insoluble and heterogeneous nature of the polymer resulting in a high standard deviation.

Conclusions

It was possible to demonstrate the possibility of replacing IL by the NADES, ChLA (1:2) for isolation of cutin from tomato peel fraction. Spectral characterisation of the obtained solids revealed matching signatures, pointing to similar products in all the studied processes. In this respect, advantages are identified using the later as it enables the simultaneous extraction of polyphenols, as well as it provides a method that economizes time, energy, and solvents.

Next steps

Further characterisation of the cutin obtained with the NADES is planned (*e.g.*, determination of melting temperature). Next steps also include the mild hydrolysis of this cutin, to obtain oligomers and testing them against relevant microorganisms. Regarding cutin extracted using IL, the antimicrobial tests need to be performed in *E. coli*. The mild hydrolysis of the cutin isolated with the NADES to produce bioactive oligomeric mixtures, will be also accessed. Phenolics are known to act as cross-linking agents during the polycondensation of cutin monomers. Therefore, the monomeric composition of cutin recovered through the cascading extraction of phenolics and cutin will also be analysed by INRAE BIA.

Bottlenecks

Not identified at this stage.

3.3 Polyphenols (INRAE_IATE)

As reported in deliverable D2.1, extraction of phenolic compounds from tomato peels was carried out using the natural deep eutectic solvent NADES composed of choline chloride and lactic acid (molar ratio 1:2) with 10 wt.% H_2O (ChLA). Conventional extraction in methanol (MeOH) was used as a control.

After extraction, the phenolic amount and composition of tomato peels were assessed by HPLC-DAS-MS.

3.3.1. Phenolic extract pre-treatment

Without pre-treating the extract after separation from the plant residue, precipitation occurs when the extract comes into contact with the HPLC elution solvent, leading to column plugging. To avoid this issue, the phenolic extract (3 mL) was mixed with 9 mL of acetonitrile (one of the elution solvents of HPLC), vortexed thoroughly and left at room temperature for 10 min. The mixture was then centrifuged at 1900 g for 2 min, and the supernatant was collected and concentrated under vacuum prior to HPLC injection.

The loss of polyphenols during this pre-treatment step was assessed by comparing the yield before and after treatment and was found to be ≤5%.

3.3.2. HPLC-DAD-MS analysis conditions

Phenolic compounds identification and quantification was performed with UHPLC-DAD-MA. ThermoFisher UHPLC UltiMate 3000, equipped with a photodiode array detector (DAD) set at 280 nm. Waters C18 column was 2.1 mm x 100 mm, HSS T3 with particle size of 1.8 µm. For elution, solvents used at 0.55 mL/min flow rate were A (99.9% H2O and 0.1% HCCOH v/v), and B (100% CH_3CN) with the gradient as follow: from 0 to 7 min 99.9%-60% A, from 7 to 10 min, 60%-1% A; from 10 to12 min 1% A, from 12 to 13 min, 1%-99.9% A.

The identification of phenolic compounds by mass spectrometry was carried out at Laboratoire de Mesure Physique (LMP, University of Montpellier), using an Orbitrap ID-X (Thermo) equipped with an ESI source. The mass spectrum was recorded in positive and negative modes, ranging from 100 to 1000 Da. The spray voltage was set to 3500V, while the vaporization and ion transfer tube temperatures were 400°C and 350°C, respectively. The flow rates for the Sheath gas, Aux gas, and Sweep gas were set to 60, 15, and 2 (arbitrary units), respectively. MS/MS spectra were recorded in HCD mode with a fixed collision energy of 30%. The resolution of the Orbitrap analyzer was set to 60,000 for MS mode and 30,000 for MS/MS mode.

Quantification was carried out through calibration curves of external commercial standards of the identified phenolics.

3.3.3. Identification and quantification of phenolic compounds in tomato peels

The HPLC-MS results of the tomato peels extract are presented in Figure 14 and Table 5.

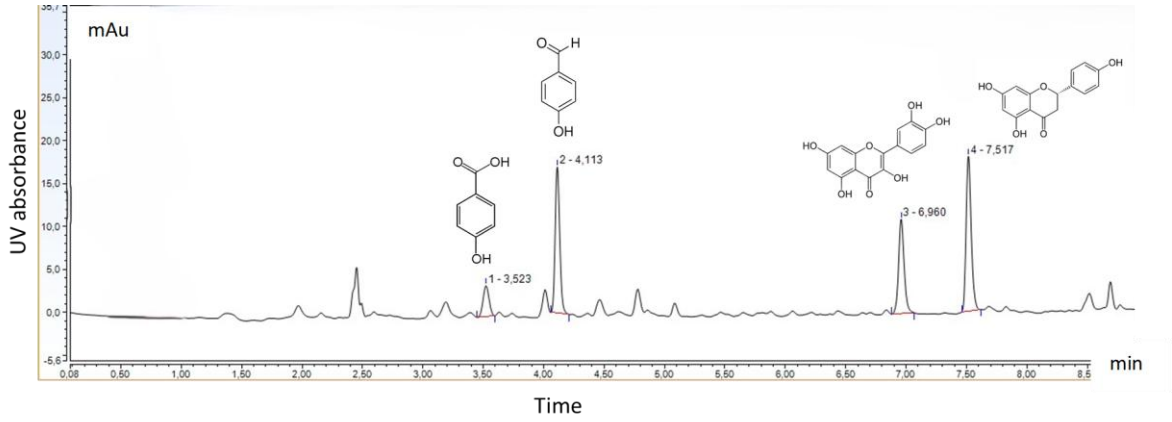


Figure 14: HPLC chromatogram at 280 nm of phenolic compounds in tomato peels, extracted with ChLA NADES.

Table 5: Characterisation of the main phenolic compounds of the tomato peels extract using their spectral characteristic in HPLC–DAD-MS.

Peak	m/z In negative mode $[M-H]^-$	Fragments MS2	Compound	Content µg/g in ChLA	Content µg/g in MeOH
1	137.02	93.03	4-hydroxybenzoic acid	30.27±1.90	27.80±0.51
2	121.03	92.02	4-hydroxybenzaldehyde	36.20±1.41	36.36±0.92
3	301	193, 179, 151, 107	Quercetin	88.54±2.79	74.18±1.42
4	271	179, 153, 147, 125, 74	Naringenin	89.22±3.99	73.08±1.58
				Total 244.24±10.02	**Total** 211.42±4.53

The predominant phenolic compounds in peels extract were phenolic acid (4-hydroxybenzoic acid), aldehyde (4-hydroxybenzaldehyde), flavonol (quercetin) and flavanone (naringenin) (Figure 14). 4-hydroxybenzoic acid was identified with m/z 137.02 (in negative ion mode), and MS2 fragmentation at m/z 93.03, corresponding to the loss of carboxylic group (CO_2). 4-Hydroxybenzaldehyde was identified with m/z 121.03, with a MS2 fragment of 92.02, characteristic COH ion for aldehydes. Quercetin was assigned to m/z 301, with MS2 fragments at m/z 193, 179, 151 and 107, consistent with fragments reported by Hong et al.[17] in negative ion mode. Naringenin was identified at m/z 271, with MS2 fragments at m/z 179, 153, 147, 125, 74, which distinguishes it from its isomeric form—naringenin chalcone (Table 5). It has been reported that naringenin chalcone is the predominant flavonoid in raw tomatoes, whereas naringenin is scarcely detected. However, during tomato processing, naringenin chalcone undergoes transformation due to heat and pH, leading to an increased concentration of naringenin[18]. The highest phenolic content was obtained

[17] Hong, Y.; Liao, X.; Chen, Z., Determination of bioactive components in the fruits of Cercis chinensis Bunge by HPLC-MS/MS and quality evaluation by principal components and hierarchical cluster analyses. Journal of Pharmaceutical Analysis 2021, 11 (4), 465-471.

[18] Wu, X.; Yu, L.; Pehrsson, P. R., Are processed tomato products as nutritious as fresh tomatoes? Scoping review on the effects of industrial processing on nutrients and bioactive compounds in tomatoes. Advances in Nutrition 2022, 13 (1), 138-151.

after extraction in ChLA NADES with 244.24±10.02 µg/g compared to MeOH with 211.42±4.53 µg/g (Table 5).

Conclusions

Extraction of tomato peels with choline chloride-based NADES gave a higher phenolic content than with the conventional solvent methanol. Four main compounds were identified in the extract, by HPLC-DAD-MS, with a majority of flavonoids quercetin and naringenin, which is consistent with literature results[19].

Next steps

Characterisation of the antioxidant activity of phenolic compounds extracted from tomato peels is in progress. Moreover, cascading approaches to target the concomitant extraction of cutin are under development through collaboration with ITQB NOVA. The resulting cutin will be also analysed by INRAE BIA as a raw material to produce monomers for the fabrication of materials.

Bottlenecks

The issue of precipitate formation in the HPLC column has been resolved. For the time being, no bottlenecks have been identified concerning the chemical characterisation of phenolic compounds in tomato peels.

3.4 Carotenoids (INRAE_IATE)

After literature review and preliminary experiment, two hydrophobic NADES were selected for the extraction of carotenoids from tomato peels: menthol:lactic acid (MenLA) at a 4:1 molar ratio and menthol:thymol (MenThy) at a 1:1 molar ratio. These were compared against a conventional organic solvent mixture (hexane:ethanol:acetone, 1:1:1) used as a reference.

3.4.1. HPLC analysis of carotenoids in tomato peels

Carotenoid analysis was carried out using the same UHPLC system and column used to characterize phenolic compounds. However, the DAD detector was set at 450 nm and 470 nm, suitable for carotenoid detection. Solvents for carotenoid elution were A (methanol) and B (methyl *tert*-butyl ether, MTBE), with a flow rate of 0.5 ml/min. The gradient program was as follows: From 0 to 2 min 99.9%-60% A, from 2 to 8 min 60%-1% A, from 8 to 9 min 1% A, from 9 to 10 min 1%-99.9% A.

The carotenoids present in the extract were identified by comparison with a range of commercial standards. Quantification was carried out using calibration curves for the carotenoid standards identified.

[19] Wu, X.; Yu, L.; Pehrsson, P. R., Are processed tomato products as nutritious as fresh tomatoes? Scoping review on the effects of industrial processing on nutrients and bioactive compounds in tomatoes. Advances in Nutrition 2022, 13 (1), 138-151.

3.4.2. Identification and quantification of carotenoids in tomato peels

As shown in Figure 15, the two main carotenoids identified in tomato peels were lycopene and β-carotene.

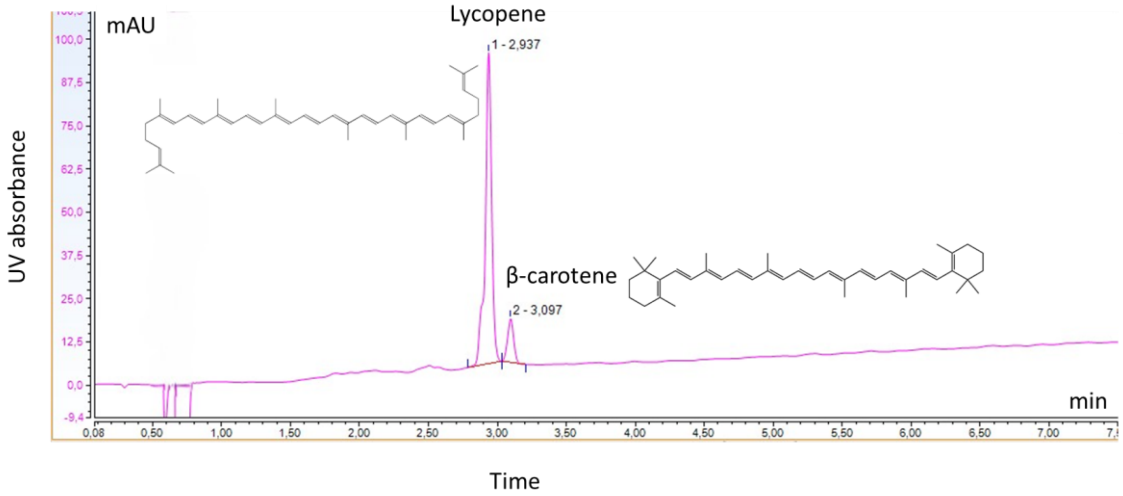


Figure 15: HPLC chromatogram at 450 nm of carotenoids in tomato peels, extracted with MenLA NADES.

Among the tested solvents, MenThy demonstrated the highest efficiency in carotenoid extraction, yielding 601.76 ± 7.50 µg/g DW, of which lycopene accounted for 326.62 ± 3.49 µg/g DW. These results align with reported literature values for lycopene content in tomato peels[20]. Conventional solvent (Hexane: ethanol: acetone) extracted about 10% less carotenoids than MenThy (Table 6). In addition to carotenoid extraction, both MenLA and MenThy NADES facilitated the co-extraction of phenolic compounds (Table 6). MenLA extracted 215.29 ± 2.89 µg/g DW of phenolics, demonstrating comparable efficiency to ChLA (244.24 ± 10.02 µg/g DW). However, the proportion of flavonoids (quercetin and naringenin) in the MenLA extract was lower than that in ChLA, accounting for 65% (vs 73% in ChLA). While MenThy (1:1) was less efficient overall in extracting phenolic compounds compared to MenLA, it exhibited greater selectivity for flavonoids, particularly quercetin. The MenThy extract contained 78% flavonoids, with quercetin reaching a concentration of 94 µg/g DW. Despite the lower extraction efficiency of MenThy, the carotenoids showed better stability over time in this solvent compared to MenLA.

Table 6: Carotenoids and phenolic compounds contents (µg/g DW) extracted from tomato peels by MenLA and MenThy.

	Polyphenols (µg/gDW)					Carotenoids (µg/gDW)		
Solvent	*p*-hydroxybenzoic acid	*p*-hydroxybenzaldehyde	quercetine	naringenine	Total polyphenol	lycopene	β-carotene	Total carotenoids
MenLA	38.60±1.61	36.10±0.49	60.14±2.34	80.45±0.13	215.29±2.89	201.04±25.35	203.66±3.05	404.70±25.53
MenThy	20.08±0.34	18.85±0.17	94.72±2.24	50.12±1.50	183.76±3.73	326.62±3.49	275.14±6.64	601.76±7.50
Hex:Ac:Et	44.94±8.16	37.94±1.40	33.08±5.49	62.64±1.95	178.61±10.12	282.85±0.73	266.81±5.03	549.66±5.09

[20] Lazzarini, C.; Casadei, E.; Valli, E.; Tura, M.; Ragni, L.; Bendini, A.; Gallina Toschi, T., Sustainable drying and green deep eutectic extraction of carotenoids from tomato pomace. *Foods* **2022,** *11* (3), 405.

3.4.3. Separation of carotenoids and phenolic compounds as a prerequisite for their HPLC characterisation

Phenolic compounds and carotenoids exhibit significant differences in polarity, making it impossible to analyse both groups of compounds using the same HPLC elution system. As both MenThy and MenLA extracts contain carotenoids and phenolic compounds, it was necessary to separate these compounds prior to HPLC analysis.

To this end, the solvent ChLA was added to the solution of phenolics and carotenoids in MenThy in a 3:1 (v/v) ratio. The mixture was stirred for 60 minutes at 60°C, facilitating the transfer of phenolic compounds into the polar phase (ChLA). After separation, the phenolic compounds were analysed and quantified using their respective HPLC methods.

Conclusions

Extraction of carotenoids from tomato peels with hydrophobic NADES (MenLA and MenThy) led to co-extraction of phenolic compounds. A separation method based on liquid-liquid extraction of phenolic compounds in polar ChLA NADES was developed to enable HPLC characterisation of the two groups of bioactive molecules.

Next steps

The single step extraction/separation of phenolic compounds and carotenoids using a NADES biphasic system is under development. The synergistic effects of carotenoids and phenolic compounds on the antioxidant activity of the mixture will be studied.

Bottlenecks

No bottlenecks were identified for the characterisation of carotenoids from tomato peels. However, the low stability of lycopene during extraction and storage, particularly in acidic solvents, means that the use of neutral NADES for carotenoid extraction will have to be considered, even if yields are reduced.

4. Grape Pomace

4.1 Proteins (UNIBO-BIGEA)

As disclosed in D2.1 section 4.2, UNIBO-BIGEA did not performed any further activity in the recover of proteins from this source to report here in D2.3. However, the future collaboration on the cascading approach will allow the endeavour of new efforts to accomplish this objective.

4.2 Cutin (ITQB-NOVA)

At ITQB-NOVA the extraction process of grape pomace (containing peels and seeds of grapes) to obtain cutin has been done using Ionic Liquids (IL), represented by Cholinium hexanoate. The use of the IL has allowed the isolation of cutin from grape pomace with a yield of *ca.* 45% (Table 7) largely dependent of the % of seed content in the pomace. Contrarily to tomato, grape cuticle contains another polymer as a backbone—cutan—that can influence the extraction yield and subsequent hydrolysis to produce COMs. Cutan is a lipid polyether, resistant to hydrolysis, composed of C4–33 long-chain n-alkenes and n-alkanes.[21,22,23]

The characterisation of total sugar content in grape cutin followed the same steps as point 3.2.3. The results show a similar content of polysaccharides compared to tomato pomace samples, being a good indication that IL process is suitable to extracted cutin from grape pomace.

Table 7. Comparison of the extraction yields and total sugar content (in glucose equivalents) obtained in the isolation of cutin using IL in grape pomace and tomato pomace.

	Cutin extracted from:	
	Grape Pomace	**Tomato Pomace**
Yields	≈45.5%	≈53%
Polysaccharides (ng/mg of cutin)	0.64 ± 0.16 ng	0.47 ± 0.19 ng

Following the polymer characterisation, we resorted to the NMR characterisation of some of the archetypal signals of cutin (see D2.1, section 3.3.3)[8,9] using the HSQC technique (Figure 16). All spectral signatures show the distinct regions of the methyl and methylene groups. Moreover, it's possible to assign the characteristic signal of the primary aliphatic esters (PAE-β), while the signal for the secondary aliphatic esters (SAE-β) is not present. The loss of this signal deserves further investigation, since it's a hallmark NMR signal of tomato cutin that comprises primary and secondary esters in its polymeric structure. Nevertheless,

[21] M. Pollard, F. Beisson, Y. Li and J. B. Ohlrogge, *Trends in Plant Science*, 2008, **13**, 236–246.
[22] A. P. Deshmukh, A. J. Simpson, C. M. Hadad and P. G. Hatcher, *Organic Geochemistry*, 2005, **36**, 1072–1085.
[23] S. Schouten, P. Moerkerken, F. Gelin, M. Baas, J. W. de Leeuw and J. S. S. Damsté, *Phytochemistry*, 1998, **49**, 987–993.

it is possible to identify the existence of ester and acid groups in all the obtained solids, as well as the presence of allylic CH_2 groups.

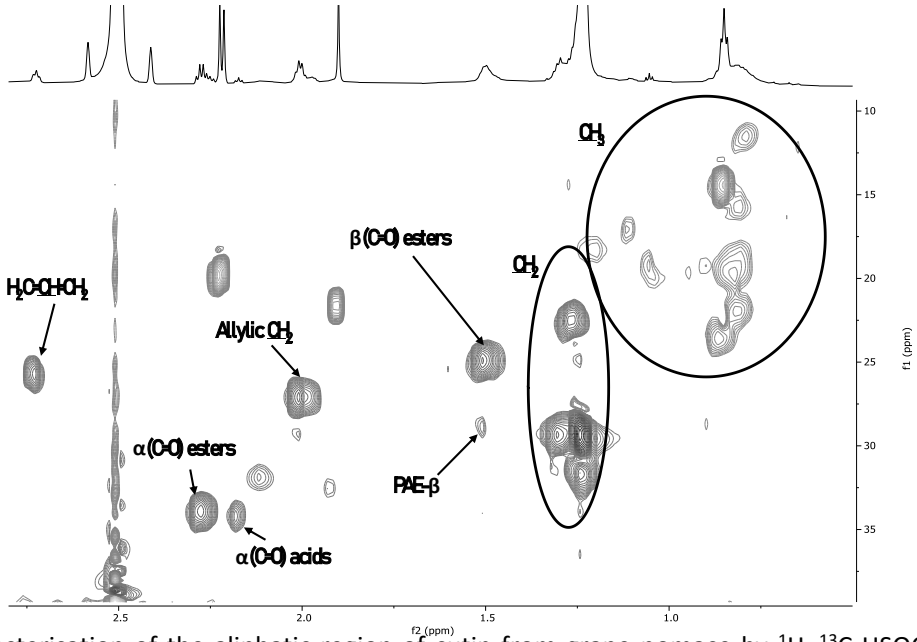


Figure 16: Characterisation of the aliphatic region of cutin from grape pomace by $^{1}H–^{13}C$ HSQC nuclear magnetic resonance spectroscopy.

Conclusions

It was possible to demonstrate the usage of IL for isolation of cutin from grape pomace. Spectral characterisation of the obtained cutin revealed matching signatures, although the absence of secondary aliphatic esters needs further investigation.

Next steps

Further chemical detailed characterisation will be done. Cutin and COMs generated from grape pomace will be tested against the two model bacteria, *S. aureus* and *E. coli*.

Bottlenecks

Cutan presence in the cutin isolated from the grape pomace can hinder the yields of COMs and alter the antimicrobial activity, regardless that this is yet to be tested.

4.3 Polyphenols (INRAE_IATE)

Red grape pomace (RGP) is rich in polyphenols, particularly condensed tannins, also known as procyanidins. Procyanidins are polymers composed of monomeric units of flavanols, mainly epicatechin and catechin,

linked by C4-C8 bonds. These compounds form a complex mixture of polymers with varying subunits, isomeric forms and degrees of polymerization, making them difficult to analyse by HPLC[24].

As described in D2.1, polyphenols were extracted from RGP provided by Grap'Sud using both a conventional organic solvent (50:50 aqueous ethanol) and a NADES (choline chloride/lactic acid, ChLA, 2:1 molar ratio, with 10% water). Extracts were filtered and injected in HPLC-MS under the same conditions as for tomato phenolics. Figure 17 shows the chromatogram profile at 280 nm of the ChLA extract of red grape pomace.

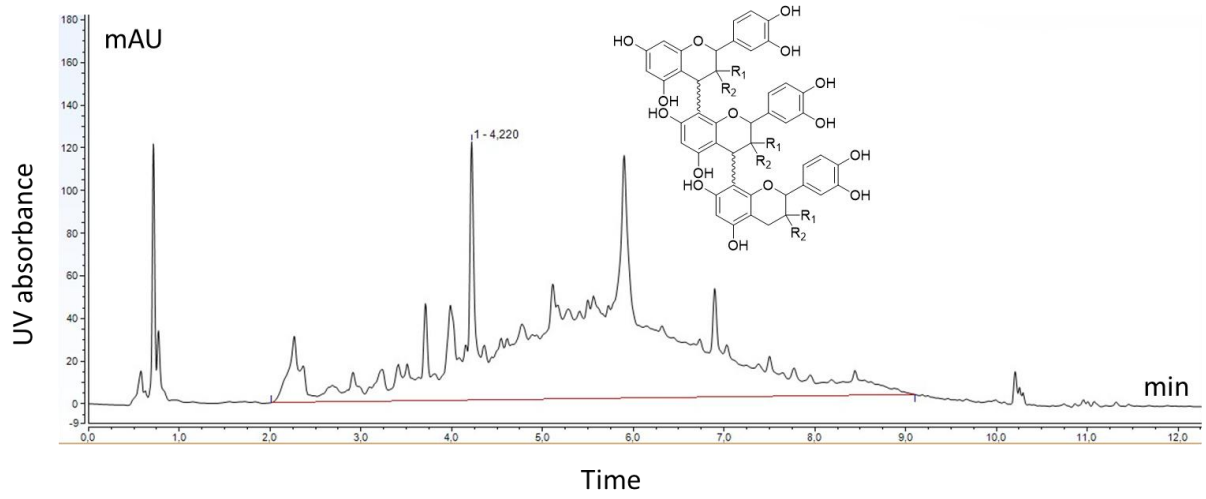


Figure 17: HPLC chromatogram at 280 nm of grape pomace polyphenols, extracted with ChLA NADES.

As shown in Figure 17, a large bump appears on the chromatogram, reflecting the very poor separation of procyanidins on the HPLC column, due to their very high structural complexity. Hence, a depolymerisation pre-treatment is required.

4.3.1. Depolymerisation of procyanidins from red grape pomace as a prerequisite for their HPLC characterisation

Depolymerisation is generally achieved by acid-catalysed cleavage of the interflavan bonds connecting the subunits, in the presence of a nucleophilic reagent. During this process, the extending subunits are trapped by the nucleophile, while the terminal subunits are rearranged into neutral monomeric flavanols (Figure 18).

[24] Khanal, R.; Howard, L.; Prior, R., Procyanidin content of grape seed and pomace, and total anthocyanin content of grape pomace as affected by extrusion processing. Journal of food science 2009, 74 (6), H174-H182.

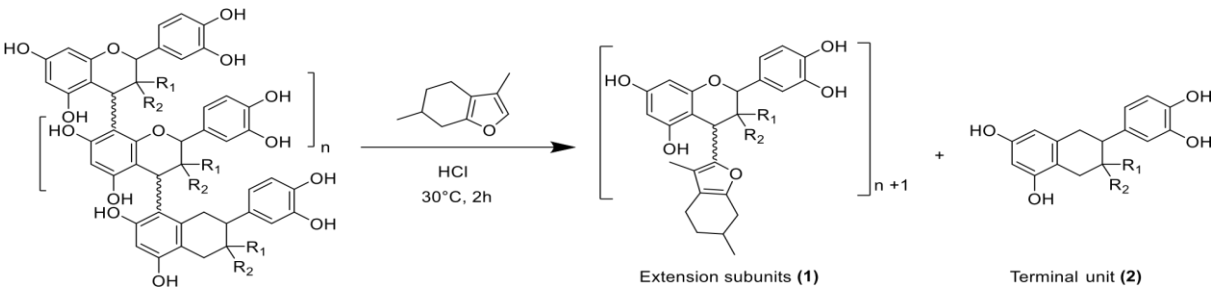


Figure 18: Depolymerisation reaction of procyanidins in the presence of menthofuran as nucleophile.

Based on the work of Billerach et al[25], a depolymerization reaction for grape polyphenols was developed for the first time in NADES. After extraction in ChLA, 1 mL of the extract was mixed with 1 mL HCl (0.3 M in ethanol) and 1 mL menthofuran solution (400 g/L in ethanol). The mixture was stirred at 30 °C for 2 hours, filtered and injected into a UHPLC-MS system under the same conditions described above (section 3.3.2). The chromatographic profile of grape procyanidins at 280 nm, after depolymerisation, is shown in Figure 19.

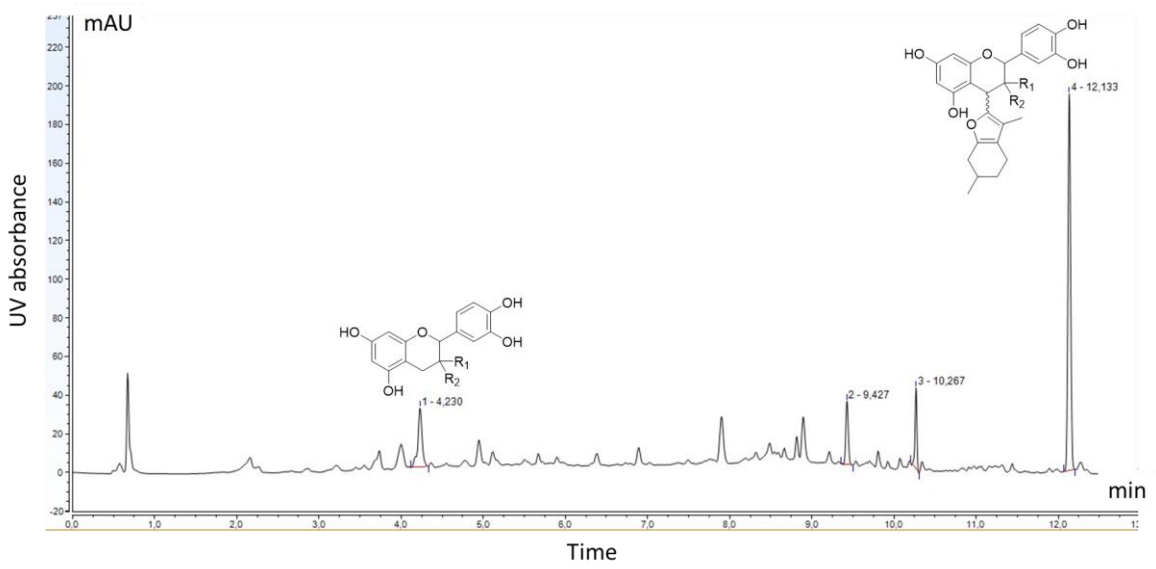


Figure 19: HPLC chromatogram at 280 nm of grape pomace polyphenols, extracted with ChLA NADES, after depolymerisation in the presence of menthofuran.

As shown in Figure 19, the highest peak corresponds to the extension subunits, while the terminal unit appears in a more polar region. If the extension unit has the same UV response coefficient, quantification of polyphenols in RGP extracts will be possible.

[25] Billerach, G.; Roumeas, L.; Dubreucq, E.; Fulcrand, H., Furanolysis with menthofuran: A new depolymerization method for analyzing condensed tannins. *Journal of agricultural and food chemistry* **2019,** *68* (10), 2917-2926.

Conclusions

Condensed tannins (procyanidins) present in red grape pomace have a complex chemical structure that prevents them from being accurately identified and quantified by HPLC. For this, a preliminary depolymerisation step is required. For the first time, this reaction was developed in the NADES solvent. However, the nucleophile (menthofuran) was used in very large excess.

Next steps

The depolymerisation reaction will be optimized by (1) reducing the nucleophile concentration; (2) attempting simultaneous extraction/depolymerisation in NADES.

Bottlenecks and deviations

No bottlenecks identified for the characterisation of polyphenols from red grape pomace.

5. Brewery spent grain

5.1 Protein (UNIBO-BIGEA)

The enzymatic digestion protocol employed on brewery spent grain (BSG) supplied by UGENT and the obtained protein yield were previously outlined in D2.1 (section 5.1).

With the goal of obtaining a purer protein hydrolysate fraction, an alternative extraction method was tested. Alkaline (0.1 M NaOH, pH 11) or neutral (0.05 M phosphate buffer, pH 7.2) solubilization (24 °C, 3 h, 1:5 S/L) followed by acidic precipitation (6N HCl in case of alkaline solubilization or 85% phosphoric acid in case of neutral solubilization, pH 3) was performed on BSG. Protein concentrations in supernatants and resuspended pellet were assessed using the Lowry assay[2] (Figure 20).

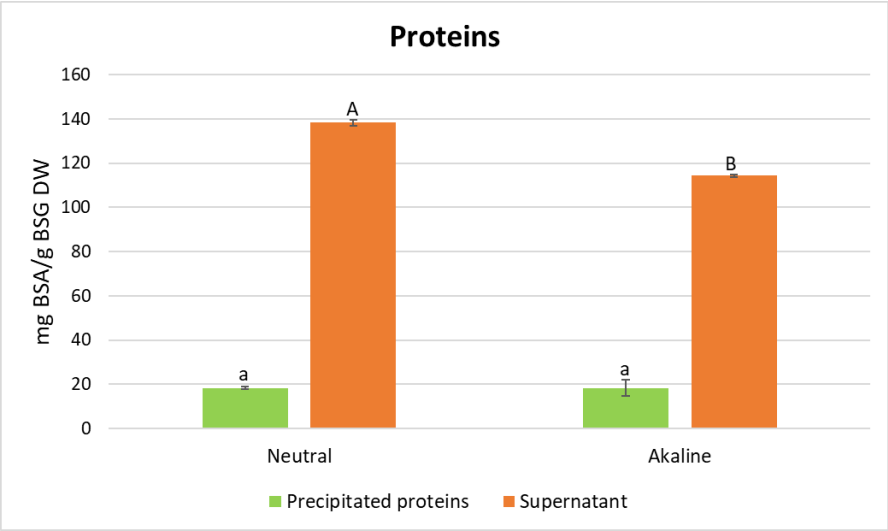


Figure 20: Protein content (mg BSA equivalents/gDW sample) in brewery spent grain (BSG) supernatants and resuspended pellets after alkaline or neutral solubilization followed by acidic precipitation. Lowercase and capital letters indicate statistically significant difference respectively among precipitated protein and supernatant samples determined by t test ($p > 0.05$). BSA, bovine serum albumin. Data are the mean (n=4) ± SD.

The results showed in Figure 20 indicated minimal differences between alkaline and neutral solubilization based on the protein concentration in the precipitated protein pellet. However, the quantity of protein/peptide pellet produced was low because a significant portion did not precipitate, likely due to a low molecular weight. These peptides were likely produced during previous beer malting processes from which the by-product BSG originates. This treatment was therefore deemed as unnecessary.

To have an overlook on the antioxidant activity and on the anti-browning activity of the extracts, ABTS [26,27] and anti-tyrosinase activity assays[28,29] were performed. Both assays were performed on samples treated with previously selected proteases and digestion protocol with a 5% (w/w) enzyme/sample gDW ratio, 2h at 60°C, and their controls (see D2.1 section 5.1).

Samples treated with proteases exhibited over 5-fold higher antioxidant activity compared to the ND control, and also slightly higher activity than the TD control (Figure 21). Samples treated with proteases showed an inhibition of the tyrosinase activity of about -51% with respect to the water positive control, like the TD control (-52%). This suggests that the compounds responsible for anti-tyrosinase activity were already extracted with water.

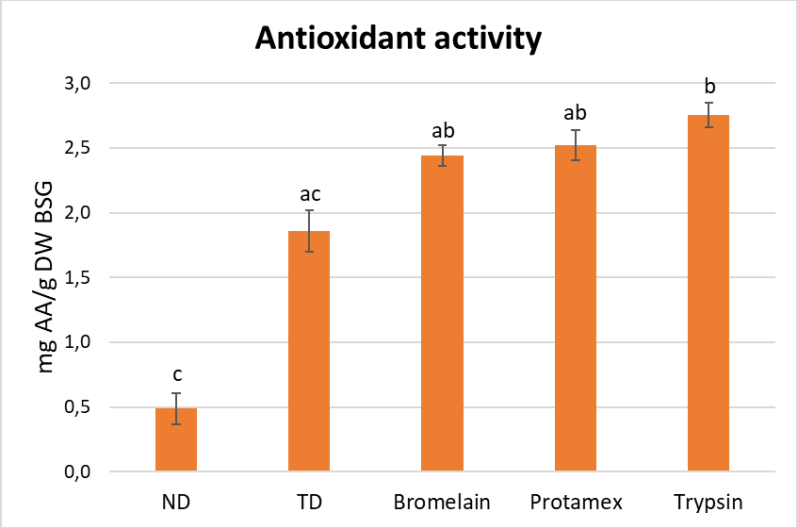


Figure 21: Antioxidant activity (mg AA equivalents/gDW sample) in brewery spent grain (BSG) supernatants following selected protease treatment. Letters indicate statistically significant difference among samples determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). AA, ascorbic acid; ND, not digested control; TD, thermally digested control. Data are the mean (n=4) ± SD.

[26] Ferri M, Gianotti A, Tassoni A. Optimisation of assay conditions for the determination of antioxidant capacity and polyphenols in cereal food components. J Food Comp Anal. 2013; 30(2): 94–101.
[27] Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. Free Radical Biology and Medicine 26, 1231–1237.
[28] Masmoto Y, Iida S, Kubo M. Inhibitory effect of Chinese crude drugs on tyrosinase. Planta Med. 1980; 40: 361–365.
[29] Ferri M, Graen-Heedfeld J, Bretz K, Guillon F, Michelini E, Calabretta MM, et al. (2017) Peptide fractions obtained from rice by-products by means of an environment-friendly process show in vitro health-related bioactivities. PLoS ONE 12(1): e0170954

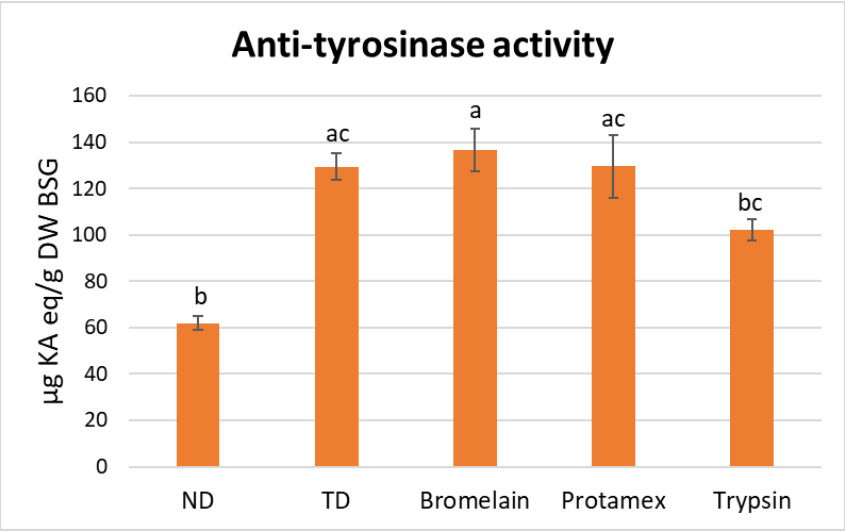


Figure 22: Anti-tyrosinase activity (µg KA equivalents/gDW BSG) in brewery spent grain (BSG) supernatants following selected protease treatment. Letters indicate statistically significant difference among samples determined by Kruskal-Wallis test followed by post-hoc Dunn test ($p < 0.05$). KA, kojic acid; ND, not digested control; TD, thermally digested control. Data are the mean (n=2) ± SD.

Conclusions

Alkaline and neutral extractions followed by acidic precipitation were ruled unnecessary since most proteins were found in the liquid fraction and did not precipitate.

BSG hydrolysates obtained after direct enzymatic extraction with selected proteases and their controls, showed both antioxidant and anti-tyrosinase activities. However, while the antioxidant activity is slightly increased in samples treated with proteases compared to the TD control, the anti-tyrosinase activity is comparable. Further analysis to determine other compounds in the extracts are needed.

Next steps

Next steps will include the determination of polyphenols and reducing sugars content to verify the presence of other active compounds in the BSG hydrolysates. Additionally, SDS-PAGE gel analyses to verify molecular weight distribution in the extracts are planned.

Due to the beer production process, specifically the malting step, peptides seem to be already produced and present in the BSG feedstock. Comparison of these samples to their thermally digested controls suggest that the use of enzymes is unnecessary but this is to be further evaluated.

Bottlenecks and deviations

No deviations to be reported.

6. Peanut residues

6.1 Proteins

6.1.1 One-factor experiment on the preparation of peanut protein fractions by cold precipitation method

(1) Effect of material-liquid ratio on the extraction rate of peanut globulin

The effect of the feed-liquid ratio on the extraction rate of peanut globulin was investigated under the conditions of solution pH 7.5, phosphate buffer solution concentration of 0.2 mol/L, and cold settling time of 16h. With the increase of the feed-liquid ratio, the extraction rate of peanut globulin showed a tendency of increasing and then decreasing, and the extraction rate reached the maximum when the feed-liquid ratio was 4:10. This may be due to the fact that the supernatant extracted from the phosphate buffer solution was placed at low temperature, and both larger and smaller feed-liquid ratios were unfavourable to the cold precipitation of peanut globulin, which led to the low rate of peanut globulin extraction.

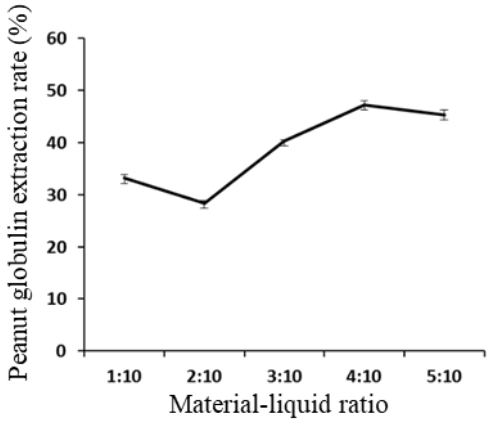


Figure 23: Effect of material-liquid ratio on the extraction rate of peanut globulin.

(2) Effect of solution pH on the extraction rate of peanut globulin

The effect of solution pH on the extraction rate of peanut globulin was investigated under the conditions of material-liquid ratio of 4:10, phosphate buffer solution concentration of 0.2 mol/L, and cold settling time of 16h. With the increase of pH, the protein solubilization increased, and the extraction rate of peanut globulin gradually increased, reaching 35% at pH 7.5. Further increase in pH would influence the functional properties of the protein. Therefore, the extraction of peanut globulin was selected at solution pH 7.5.

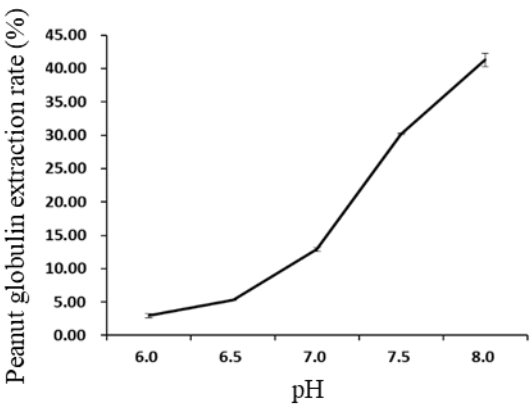


Figure 24: Effect of solution pH on the extraction rate of peanut globulin.

(3) Effect of phosphate buffer solution concentration on the extraction rate of peanut globulin

The effect of phosphate buffer solution concentration on the extraction rate of peanut globulin was investigated under the conditions of material-liquid ratio of 4:10, solution pH 7.5 and cold settling time of 16 h. At 0.1–0.3 mol/L, with the increase of phosphate buffer solution concentration, the extraction rate of peanut globulin showed an increasing trend from 12% to 40%. However, above 0.3 mol/L, the extraction rate of peanut globulin reaches a plateau. Therefore, the concentration of phosphate buffer solution was selected as 0.3 mol/L.

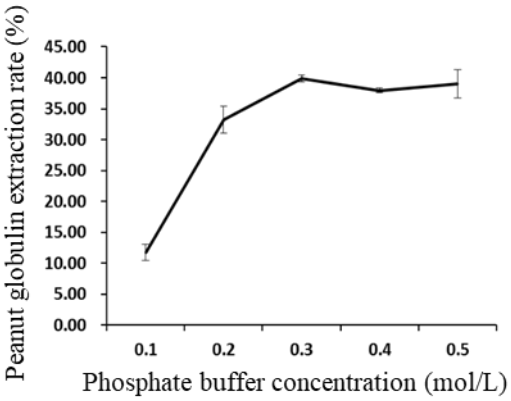


Figure 25: Effect of phosphate buffer solution concentration on peanut globulin extraction rate.

(4) Effect of cold settling time on the extraction rate of peanut globulin

The effect of cold settling time on the extraction rate of peanut globulin was investigated under the conditions of material-liquid ratio of 4:10, solution pH 7.5, and phosphate buffer solution concentration of 0.2 mol/L. The extracted peanut globulin was extracted by the cold settling time at a concentration of 0.2 mol/L. In the range of 0~4h, the extraction rate of peanut globulin increased sharply with the prolongation of cold settling time. After 4 h of cold sinking, the extraction rate of peanut globulin tends to stabilize. These

results suggest that above this time, the cold sinking of peanut globulin reaches a dynamic equilibrium. Therefore, the cold-sinking time was chosen to be 4 h.

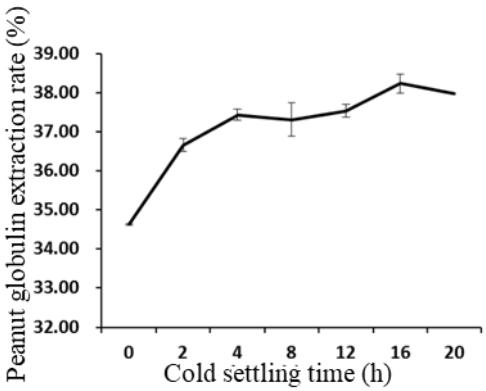


Figure 26: Effect of cold settling time on the extraction rate of peanut globulin.

(5) Determination of the number of leaching times of peanut defatted powder

The total protein extraction rate of peanut protein was used as an index to determine the optimal number of leaching times. Under the condition of material-liquid ratio of 4:10, phosphate buffer solution concentration of 0.2 mol/L, and solution pH of 7.5, the number of leaching times were taken as 1, 2, 3, 4, and 5 times. The experimental results showed that the extraction rate of peanut protein increased rapidly from one extraction to two extractions with an increase of 16.70%. However, the rate of increase slowed down considerably and levelled off after more than two times. This suggest that the peanut protein dissolution rate reached a dynamic equilibrium and after the second leaching it has basically been all dissolved. More leaching times would increase the amount of phosphate buffer solution, hence the cost, therefore not conducive to industrial production. Accordingly, the number of times of leaching selected was two. Based on this result an orthogonal test was carried out.

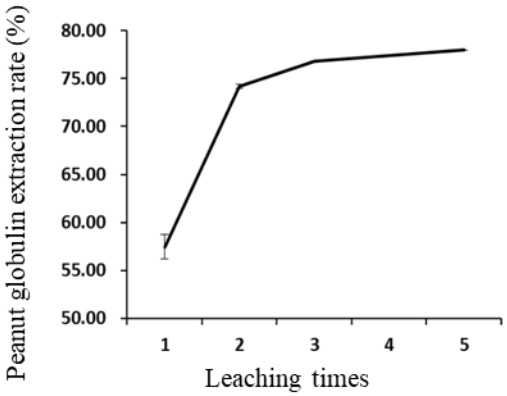


Figure 27: Effect of the number of immersions on the total protein extraction rate of peanut.

6.1.2 Orthogonal optimization of peanut protein components prepared by cold precipitation method

Peanut globulin was extracted by leaching peanut defatted powder twice. On the basis of single factor, L9 (43) four-factor three-level orthogonal experimental design was carried out. The ensuing results were then analysed by Duncan's new complex polar deviation method to analyse the significance of the effects of each factor. The results are shown in Table 8. The larger the extreme deviation of each factor, the more significant is its effect on the extraction rate of peanut globulin. The order of the influence of each factor on the extraction rate of peanut globulin was as follows: D (cold settling time) > A (material-liquid ratio) > C (concentration of phosphate buffer solution) > B (pH of the solution). The optimal combination was A3B3C3D2, i.e., the material-liquid ratio was 5:10, the solution pH was 7.9, the phosphate buffer solution concentration was 0.4 mol/L, and the cold settling time was 4 h.

Table 8: Orthogonal test polar analysis table

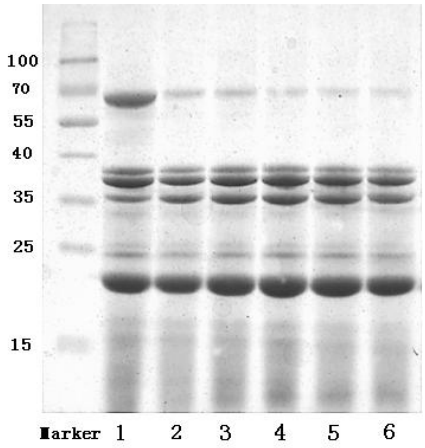
	A (material-liquid ratio)	B (pH)	C (phosphate buffer concentration)	D (cold settling time)	peanut globulin extraction rate /%
1	1	1	1	1	25.34
2	1	2	2	2	44.73
3	1	3	3	3	45.47
4	2	1	2	3	52.29
5	2	2	3	1	36.38
6	2	3	1	2	50.77
7	3	1	3	2	54.71
8	3	2	1	3	52.42
9	3	3	2	1	37.54
K1	115.54	132.34	128.53	99.26	
K2	139.44	133.53	134.56	150.21	
K3	144.67	133.78	136.56	150.18	
k1	38.51	44.11	42.84	33.09	
k2	46.48	44.51	44.85	50.07	
k3	48.22	44.59	45.52	50.06	
R	9.71	0.48	2.68	16.98	
optimum level	A3	B3	C3	D2	
order of priority	D（cold settling time）＞A（material-liquid ratio）＞C（phosphate buffer concentration）＞B（pH）				

6.1.3 Purity studies on peanut protein components

(1) Effect of the number of cold precipitations in phosphate buffer solution on the purity of peanut globulin fractions

The peanut globulin samples were prepared by vacuum freeze-drying through different times of cold precipitation and subjected to SDS-PAGE electrophoresis. The purity of the peanut globulin fractions obtained from different times of extraction was determined by optical density analysis as shown in the table 9. The results demonstrated that the purity of peanut globulin was 77.97% after one cold precipitation,

increasing only 2.03% to 80.00% after five cold precipitations. However, this difference was found insignificant after DPS significance analysis (p>0.05).



1 - Peanut isolate protein; 2 - Peanut globulin prepared by first cold precipitation; 3 - Peanut globulin prepared by second cold precipitation; 4 - Peanut globulin prepared by third cold precipitation; 5 - Peanut globulin prepared by fourth cold precipitation; 6 - Peanut globulin prepared by fifth cold precipitation

Figure 28: SDS-PAGE profiles of peanut globulin prepared by different number of cold deposition times

Table 9: Purity of peanut globulin fractions prepared by different number of cold precipitation times

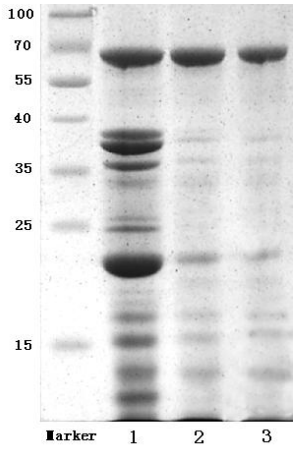
cold sink count	component purity (%)
first	77.97±1.53[a]
twice	77.70±0.61[a]
three times	79.13±0.55[a]
four times	78.63±0.83[a]
five times	80.00±0.30[a]

Note: Fraction purity is the proportion of peanut protein fractions to total protein. a, b, c indicates different levels in the same row, significance P < 0.05

(2) Different ways of preparing companion peanut globulin

Vacuum freeze-dried samples of companion peanut globulin, were subjected to SDS-PAGE electrophoresis, and the purity of the components was determined by optical density scanning analysis, as shown in Table

10. The data showed that the purity of the components of companion peanut globulin prepared by direct lyophilization of the supernatant was 64.6%, with a protein content of 28.88%, while that of the companion peanut globulin prepared by acid precipitation followed by lyophilization had a component purity of 64.1%, with a protein content of 84.52%. After DPS significance analysis, it was found that there was no significant difference in the purity of the fractions of peanut globulin prepared in different ways. The protein content of peanut globulin prepared by direct freeze-drying of supernatant was lower, mainly because more polysaccharides, fibres and other impurities were present. Considering the two factors, the preparation of peanut globulin by acid precipitation followed by drying was chosen.



1-peanut isolate protein; 2-acid precipitation followed by freeze-drying to prepare companion peanut globulin; 3-direct freeze-drying to prepare companion peanut globulin

Figure 29: SDS-PAGE profiles of companion peanut globulin prepared in different ways

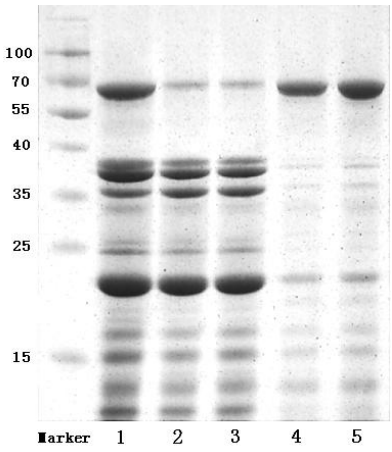
Table 10: Purity and protein content of companion peanut globulin fractions prepared in different ways

preparation method	protein content (%)	component purity (%)
direct freeze drying	28.88±0.07[a]	64.6±0.26[a]
freeze-drying after acid precipitation	84.52±2.08[b]	64.1±0.17[a]

Note: Fraction purity is the proportion of peanut protein fractions to total protein. a, b indicates different levels in the same column, significance P < 0.05

(3) Preparation of peanut globulin and companion peanut globulin by different drying methods

The peanut globulin was extracted under the optimal process conditions, and the supernatant was dried after acid precipitation to prepare the companion peanut globulin. The samples of peanut globulin and companion peanut globulin were prepared by two drying methods, i.e., vacuum freeze-drying and spray-drying. The purity of peanut globulin fractions prepared by vacuum freeze-drying and spray-drying was 76.40% and 74.30%, respectively, whereas for the companion peanut globulin fractions values were 64.10% and 62.73%, respectively. After DPS significance analysis, it was found that there was a significant difference in the purity of peanut protein fractions prepared by different drying methods (P<0.05).



1 - Peanut isolate protein; 2 - Freeze-drying to prepare peanut globulin; 3 - Spray-drying to prepare peanut globulin; 4 - Freeze-drying to prepare companion peanut globulin; 5 - Spray-drying to prepare companion peanut globulin

Figure 30: SDS-PAGE profiles of peanut protein fractions prepared by different drying methods

Table 11: Purity of peanut protein fractions prepared by different drying methods

sample	component purity/%
freeze-dried globulin	76.40±0.53[a]
spray-dried globulin	74.30±0.70[b]
freeze-dried accompanying globulin	64.10±0.17[c]
spray-dried accompanying globulin	62.73±0.15[d]

Note: Fraction purity is the proportion of peanut protein fractions to total protein. a, b, c, d indicate different levels in the same column, significance $P < 0.05$

Conclusions

The optimal process conditions for the separation and preparation of peanut protein fractions by cold precipitation was established: material-liquid ratio of 5:10, solution pH 7.1, phosphate buffer solution concentration of 0.4 mol/L, and cold precipitation time of 4 h. The extraction rate of peanut globulin under these conditions was 53.60%. Using supernatant acid precipitation followed by drying, it was observed that the purity of the companion peanut globulin fraction obtained was 64.1%, with a protein content of 84.52%. The purity of the fractions of the samples prepared by spray drying were all lower than that of the samples prepared by freeze drying ($P<0.01$).

Next steps

Next, we will modify the extracted peanut proteins with different methods to enhance their gelation.

6.2 Polyphenols

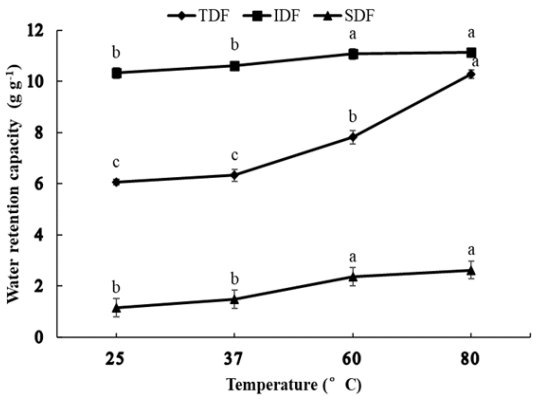
No additional data to report at this stage.

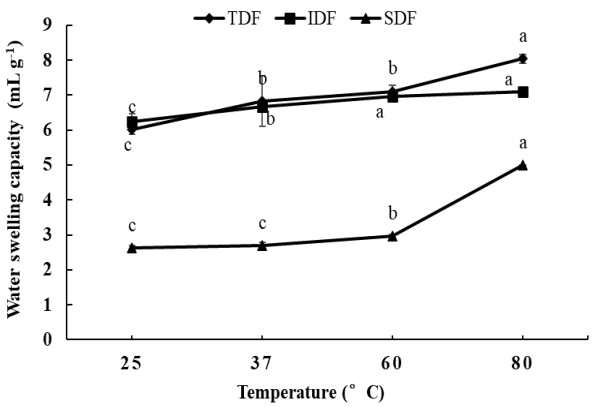
7. Potato residues

7.1 Dietary fibres

7.1.1 Physico-chemical properties of potato dietary fibre

The effects of different temperatures, pH, and NaCl concentrations on the water retention capacity, water swelling capacity, and solubility of total dietary fibre, insoluble dietary fibre, soluble dietary fibre extracted from potato residues were studied. The results in Figure 31 show that with the increase of temperature, the water retention capacity, water swelling capacity, and solubility of total dietary fibre and insoluble dietary fibre increased. The first two parameters increase in the soluble dietary fibre as well, but solubility decreases then increase. With the increase of pH (Figure 32), the water retention capacity and water swelling capacity of total dietary fibre and insoluble dietary fibre increased, while their solubility decreased. The water retention capacity of soluble dietary fibre decreased, while its water absorption, swelling, and solubility increased. As the concentration of NaCl increased (Figure 33), the water retention capacity, water swelling capacity, and solubility of total dietary fibre and insoluble dietary fibre decreased. The water retention capacity of soluble dietary fibre was improved, while its water absorption, swelling, and solubility were reduced. In addition, the water retention capacity of potato TDF under different temperature, pH, and NaCl concentration was higher than that of TDF extracted from grapefruit (2.09 $g.g^{-1}$), citrus (1.65 $g.g^{-1}$), apple (1.87 $g.g^{-1}$), and banana (1.71 $g.g^{-1}$), while lower than that of TDF extracted from mango (11.40 $g.g^{-1}$) and coconut (10.71 $g.g^{-1}$). The water swelling capacity of potato TDF under different temperature, pH, and NaCl concentration was higher than that of TDF extracted from pea (5.26 $mL.g^{-1}$) and chickpea (4.28 $mL.g^{-1}$), while similar to that of TDF extracted from some edible seaweed, such as kelp, bok choy, etc. (5.7-10.5 $mL.g^{-1}$).





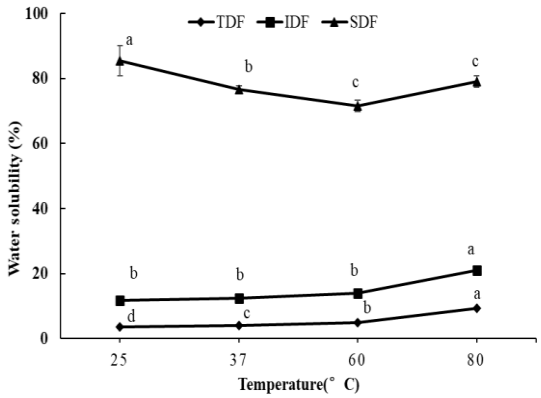


Figure 31: The effect of different temperature on the water retention capacity, water swelling capacity, and water solubility of total, insoluble, and soluble dietary fibre extracted from potato residues

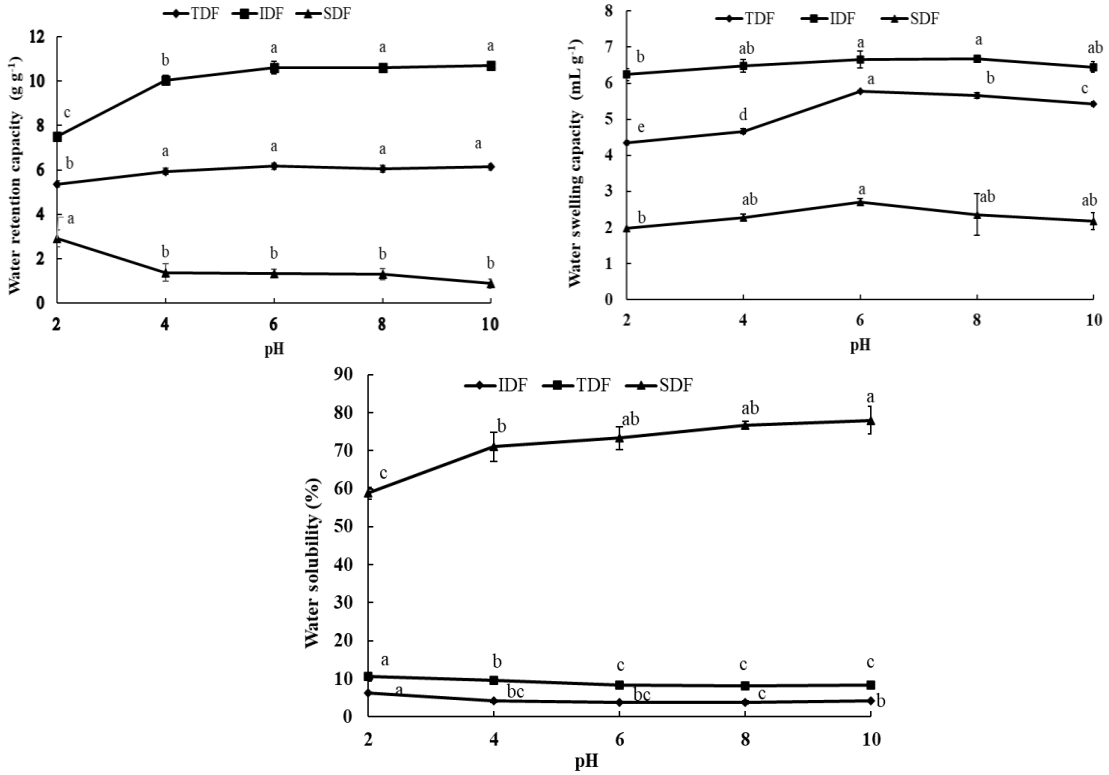


Figure 32: The effect of different pH on the water retention capacity, water swelling capacity, and water solubility of total, insoluble, and soluble dietary fibre extracted from potato residues.

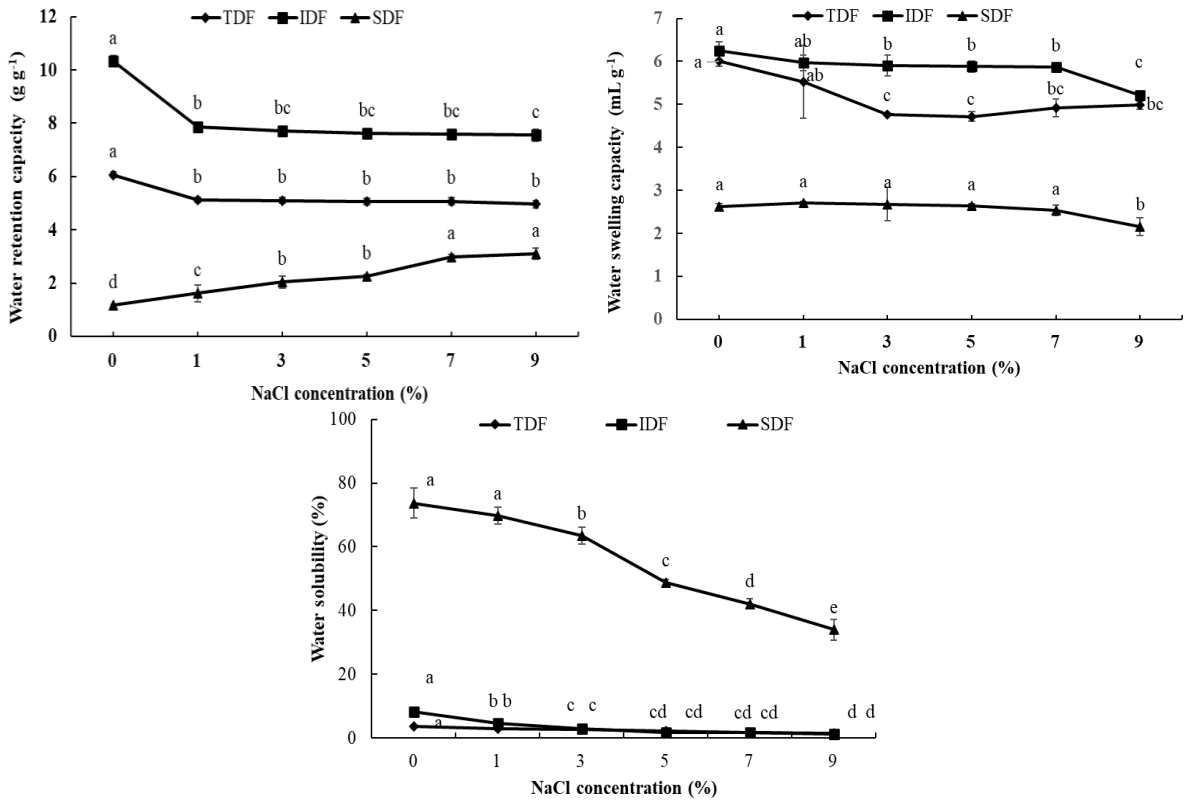


Figure 33: The effect of different NaCl concentration on the water retention capacity, water swelling capacity, and water solubility of total, insoluble, and soluble dietary fibre extracted from potato residues.

7.1.2 Functional properties of potato dietary fibre

Total dietary fibre and insoluble dietary fibre extracted from potato residues exhibited a high fat absorption capacity, and soluble dietary fibre showed a higher adsorption capacity for glucose, cholesterol, and α-amylase. Furthermore, compared with pH 2, potato dietary fibre displayed a higher cholesterol absorption capacity at pH 7, indicating that dietary fibre could better play a role in adsorbing cholesterol in the intestine.

Table 12: Functional characteristics of total dietary fibre, insoluble dietary fibre, and soluble dietary fibre extracted from potato residues.

Samples	OHC(g/g)	GAC (mmol/g)			CBC (mg/g)		α-AAIR (%)
		50	100	200	pH2	pH7	
TDF	1.25±0.09^{a}	3.81±0.16^{ab}	6.82±0.47^{ab}	14.14±0.16^{ab}	2.39±0.23^{b}	3.71±0.39^{b}	11.40±2.99^{b}
IDF	1.29±0.06^{a}	3.54±0.12^{b}	6.41±0.29^{b}	13.76±0.34^{b}	1.33±0.39^{c}	2.12±0.79^{c}	3.86±1.85^{c}
SDF	0.88±0.04^{b}	4.02±0.20^{a}	7.51±0.44^{a}	14.42±0.19^{a}	3.57±0.60^{a}	5.15±0.23^{a}	17.89±1.39^{a}

Note: OHC, oil holding capacity; GAC, glucose absorption capacity; CBC, cholesterol binding capacity; α-AAIR, α-amylase activity inhibition ratio. TDF, total dietary fibre; IDF, insoluble dietary fibre; SDF, soluble dietary fibre.

7.1.3 Optimization of potato dietary fibre modification

The optimal process parameters for high hydrostatic pressure assisted cellulase modification of potato dietary fibre were determined through single factor experiments: high hydrostatic pressure of 400 MPa, cellulase addition of 210 U/g. Compared with unmodified potato dietary fibre, the content of soluble components in potato dietary fibre increased by 59.30% after high hydrostatic pressure assisted cellulase modification (Figure 34-35).

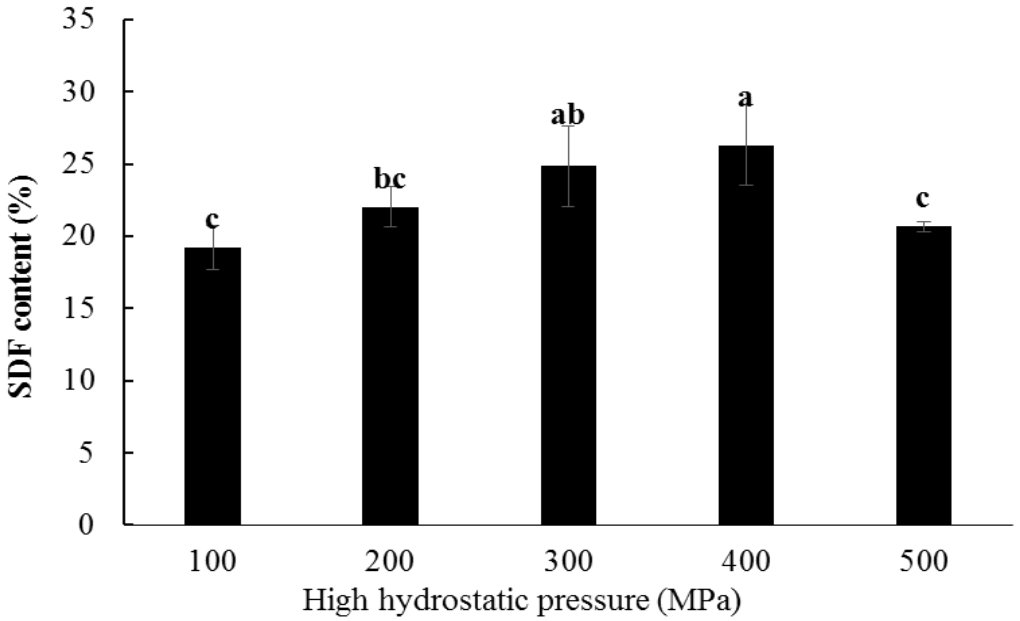


Figure 34: The effect of different high hydrostatic pressure on soluble dietary fibre content in potatoes.

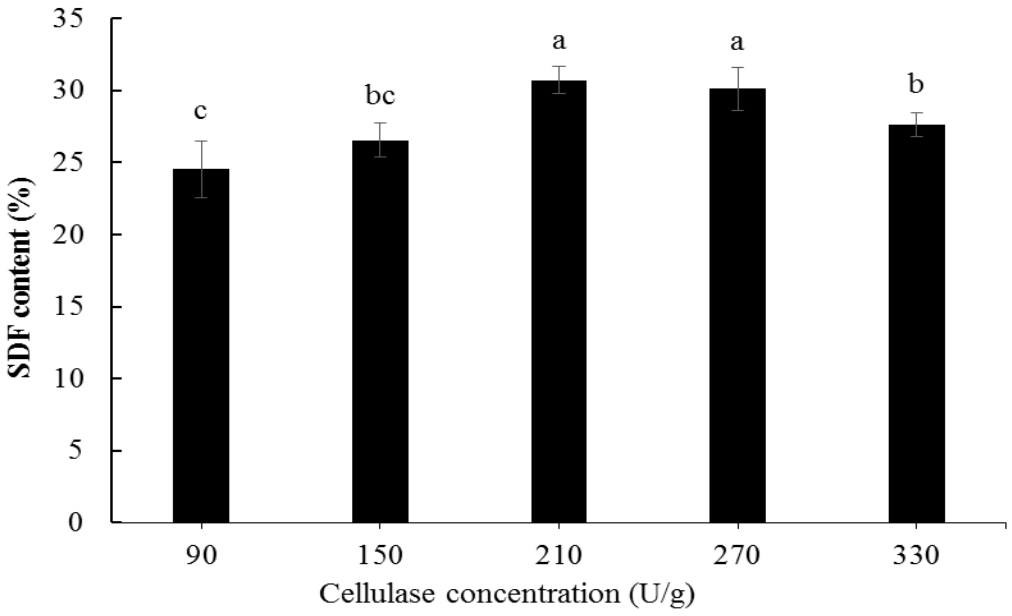


Figure 35: The effect of different cellulase addition amount on soluble dietary fibre content in potatoes.

7.1.4 Physico-chemical properties of unmodified and modified potato dietary fibre

Compared with unmodified potato dietary fibre, treatment using high hydrostatic pressure, cellulase, and their combination, lead to improved physico-chemical properties of potato dietary fibre. After high hydrostatic pressure modification, the potato dietary fibre exhibited the highest water retention capacity, furthered when combined with cellulase that resulted in the potato dietary fibre with the highest water swelling capacity and solubility (Table 13).

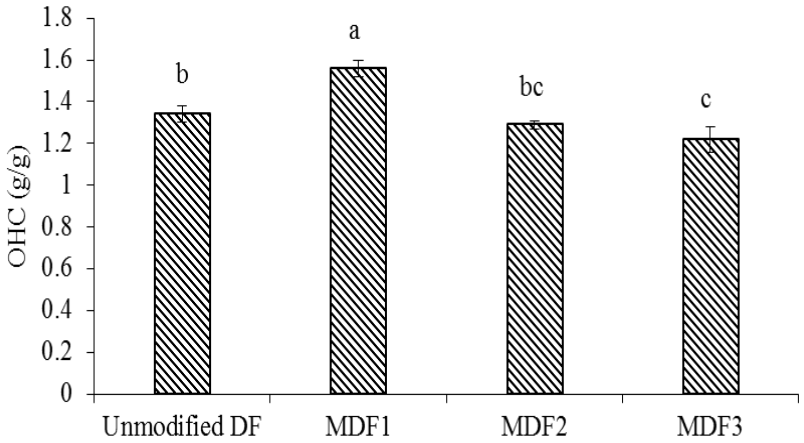
Table 13: Physico-chemical properties of unmodified and modified potato dietary fibre

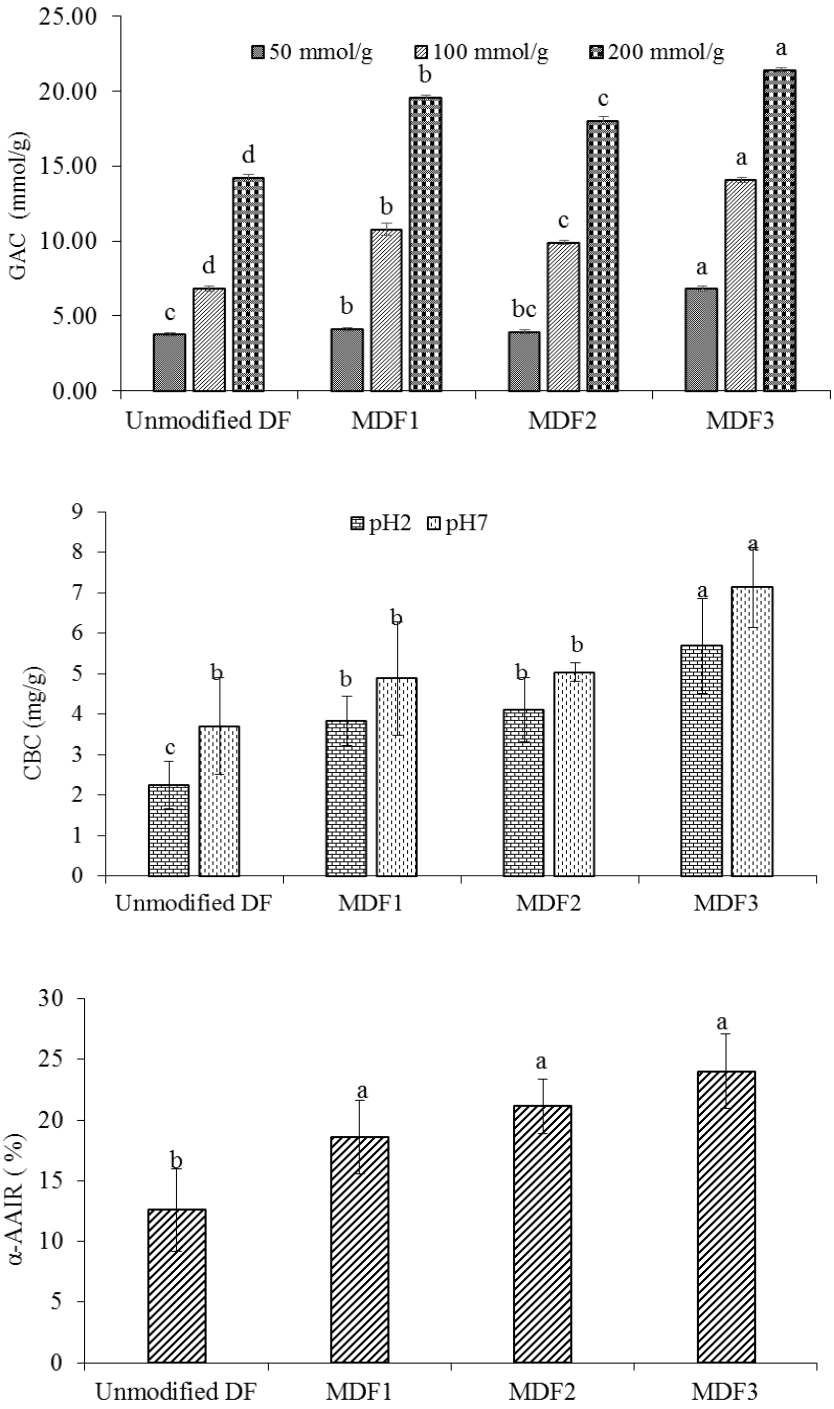
Samples	Water retention capacity (g/g)	Water swelling capacity (mL/g)	Solubility (%)
Unmodified	5.41±0.16^{cd}	5.12±0.02^{d}	4.62±1.04^{c}
MDF1	6.96±0.03^{c}	5.79±0.09^{c}	5.58±0.41^{bc}
MDF2	7.95±0.05^{b}	6.60±0.24^{b}	5.69±0.67^{bc}
MDF3	8.43±0.02^{a}	7.65±0.06^{a}	6.71±1.13^{a}

Note: MDF1, high hydrostatic pressure modified dietary fibre; MDF2, cellulase modified dietary fibre; MDF3, high hydrostatic pressure assisted cellulase modified dietary fibre.

7.1.5 Functional properties of unmodified and modified potato dietary fibre

High hydrostatic pressure, cellulase, and their combination could all improve the functional characteristics of potato dietary fibre. Among them, after high hydrostatic pressure modification, potato dietary fibre exhibited the highest oil holding capacity. After high hydrostatic pressure assisted cellulase modification, potato dietary fibre displayed the strongest adsorption capacity for glucose, cholesterol, and α-amylase (Figure 36).





Note: MDF1, high hydrostatic pressure modified dietary fibre; MDF2, cellulase modified dietary fibre; MDF3, high hydrostatic pressure assisted cellulase modified dietary fibre. OHC, oil holding capacity; GAC, glucose absorption capacity; CBC, cholesterol binding capacity; α-AAIR, α-amylase activity inhibition ratio. TDF, total dietary fibre; IDF, insoluble dietary fibre; SDF, soluble dietary fibre.

Figure 36: Functional properties of unmodified and modified potato dietary fibre

Conclusions

A key technology for the targeted enzymatic hydrolysis of potato dietary fibre using enzymes, as well as the modification of potato dietary fibre using a high hydrostatic pressure assisted cellulase method was

established, 59.30% soluble dietary fiber was increased after modification. Meanwhile, modification improved the physico-chemical and functional properties of potato dietary fibre significantly, thus broadening the application in the food and health products industry.

Next steps

(1) Principal component analysis, Person correlation analysis, artificial neural network, etc., will be used to calculate the correlation probability between modified potato dietary fibre and biological activities, its key functional groups and sites will be also investigated, thus revealing the correlation mechanism between structure and biological activities.

(2) Jointly publish articles with EU side. For this aim, the potential usage of potato residues (upon extraction of the dietary fibre) suitability as raw material to isolate polyesters will be tested (ITQB NOVA).

8. Apple Pomace

8.1 Apple pomace pectin extracted with deep eutectic solvent (DES) (IFST-CAAS)

Using ultrasonic-assisted water extraction as a control, Figure 37 shows that most deep eutectic solvents (DES) achieved a higher pectin extraction yield compared to traditional water extraction. Notably, DES with a choline chloride to citric acid molar ratio (ChCl-CA) of 1:2 exhibited the highest yield, prompting further optimization. The effects of ChCl-CA water content, liquid-solid ratio, temperature, extraction time, and ultrasonic power on pectin yield were investigated through single-factor experiments and response surface methodology. As shown in Figure 38, DES extraction achieved a pectin yield 7.99 ± 0.60% higher than ultrasonic-assisted water extraction. In particular, ChCl-CA produced a yield increase of 17.95 ± 0.77%. The optimal extraction conditions for ChCl-CA were determined to be a liquid-solid ratio of 40 mL/g, a temperature of 81°C, an extraction time of 40 min, and an ultrasonic power of 100 W. Under these conditions, the predicted pectin yield was 25.16 ± 0.17%. Further characterisation of the pectin's properties is ongoing.

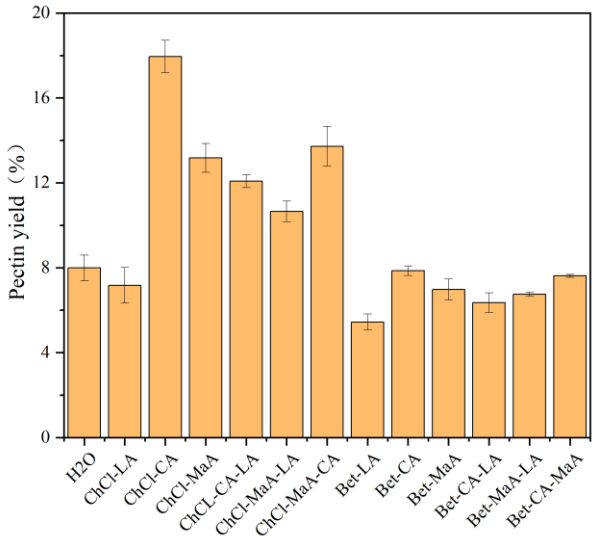


Figure 37: The extraction yield of pectin using ultrasonic-assisted extraction with different DES (water as control).

8.2 Apple pomace pectin extracted from wet extraction

This study investigates a novel approach to pectin extraction from wet (fresh) apple pomace using innovative methods such as microwave-assisted, ultrasound-assisted, enzymatic treatments, and their combinations. High-speed shearing was applied as a pretreatment step for all extractions except the untreated samples to facilitate solution preparation. Citric acid was utilized as the solvent in all methods except enzymatic extraction. For comparison, dried apple pomace was used as a control.

8.2.1 The yield of pectin

Table 14 presents the yields of pectin obtained using different extraction methods. Enzymatic extractions exhibited the lowest yields, with cellulase, xylanase, and their mixture producing yields of 6%, 4%, and 7%, respectively. This outcome is likely due to the mild extraction environment of enzymatic processes, which are insufficient to effectively disrupt the cell wall structure of apple pomace, thereby limiting extraction efficiency. However, combining enzymatic extraction with ultrasound-assisted, microwave-assisted, or their sequential methods significantly improved the yields. The highest yield, 19%, was achieved with the enzymatic-microwave-ultrasound combination (EmUM). This enhancement can be attributed to the enzymatic pretreatment breaking down the structure, making the subsequent physical field extractions more effective at cell wall disruption, thereby increasing yield substantially.

For standard wet extraction, yields were relatively low. When ultrasound or microwave-assisted methods were applied, the yields increased. This can be explained by the ability of physical field processes to rupture plant tissues. Additionally, the sequence of microwave and ultrasound treatments influenced the yield. Microwave processing disrupts the plant matrix, causing a structural collapse that may reduce the cavitation effect of ultrasound, thereby impacting the extraction efficiency. The dried apple pomace exhibited excellent yields, which can be attributed to its smaller particle size after superfine grinding. The increased surface area of these particles likely improved the extraction efficiency.

Table 14: The abbreviation of different extraction and its yield.

Extraction technique	Abbreviation	Yield (%)
Dried-Nontreatment	D-NT	15%
Wet-Nontreatment	W-NT	7%
Wet-High speed shearing	W-HSS	9%
Wet- Microwave-assisted extraction	W-MAE	12%
Wet- Ultrasound assisted extraction	W-UAE	13%
Wet- Ultrasound-microwave assisted extraction	W-UMAE	16%
Wet- Microwave-Ultrasound assisted extraction	W-MUAE	13%
Enzyme-assisted extraction (Cellulase)	Ec	6%
Enzyme-assisted extraction (Xylanase)	Ep	4%
Enzyme-assisted extraction (Mixture)	Em	7%
Enzyme-microwave assisted extraction (Mixture)	EmM	15%
Enzyme-ultrasound assisted extraction (Mixture)	EmU	17%
Enzyme-assisted ultrasound-microwave extraction	EmUM	19%

8.2.2 The characterisation of pectin from different extraction

The highest GalA content (49.46%) was observed in pectin extracted using microwave-assisted extraction (MAE). In contrast, enzymatic extraction produced pectin with the lowest GalA content, particularly in EmM (enzymatic-ultrasound-assisted extraction), which contained only 18.28% GalA. It is noteworthy that enzymatic preparations resulted in a lower degree of esterification (DE) and reduced GalA and protein content, with all DE values below 10%. The protein content also showed significant variation in enzymatic extractions, with Em exhibiting the lowest protein content (3.5%) and Ep the highest (8.3%). In comparison, pectin obtained through other extraction methods had DE values exceeding 70%, with D-NT achieving the highest DE at 94%. Further characterisation and functional analysis of the extracted pectin will be conducted in future experiments.

Table 15: Degree of Esterification, Galacturonic Acid Content, and Protein Content of Pectin Samples

Sample	Degree of esterification (DE)	GalA（%）	Protein (%)
D-NT	94%	46.38	6.4
W-NT	74%	42.71	6.6
W-HSS	92%	41.17	6.8
W-MAE	86%	49.42	6.4
W-UAE	85%	45.28	7.7
W-UMAE	78%	43.03	7.3
W-MUAE	87%	47.25	7.1
Ec	4%	38.85	5.0
Ep	7%	28.10	8.3
Em	9%	45.84	3.5
EmM	82%	22.98	4.6
EmU	74%	18.28	5.8
EmUM	85%	26.07	4.7

Conclusions

The findings indicate that while the drying method had no significant effect on the extraction rate of pectin, it influenced both the galacturonic acid content and the degree of esterification. Additionally, ultrasonic-assisted deep eutectic solvent extraction demonstrated a higher pectin yield compared to traditional water-based extraction methods. The development of a novel wet extraction process highlights its potential for improving pectin extraction efficiency and broadening its industrial applications.

9. Conclusion

Key-achievements reported on D2.3

Proteins - The final peptide extraction protocol from dried tomato seeds was set up. Small molecular weight peptides were obtained. The fractions showed antioxidant and anti-browning activities.

Peptides obtained thought digestion with immobilized enzymes, some showed ACE and DPP-IV inhibitory activities as well as promising properties for reducing hypertension and to act as anti-diabetic.

The optimal process conditions for the separation and preparation of peanut protein fractions by cold precipitation were established. Supernatant acid precipitation followed by drying was used to prepare the companion peanut globulin, with a purity of 64.1% and a protein content of 84.52%. Freeze drying treatment ensured the highest purity (p<0.01).

Plant polyester cutin – Successful isolation of cutin in its polymeric form was achieved with the NADES ChLA, as demonstrated by its nuclear magnetic resonance spectral similarities with that of cutin obtained using ionic liquid. This process supports a first cascading approach for the co-isolation of polyphenols and cutin (under validation). The 9(10),16–dihydroxy hexadecanoic acid was accessed as the major monomer of the polymer through NMR. The antimicrobial activity of the cutin and COMs generated from TOMAPAINT pomace source were tested against *S. aureus* cells, concluding that COMs have promising anti-microbial activity. Furthermore, it was possible to isolate cutin from grape pomace using the same IL as for tomato sources, regardless that the absence of the archetypal secondary aliphatic esters deserves further analysis.

Polyphenols and carotenoids - Extraction of tomato peels with choline chloride-based NADES gave a higher phenolic content than with the conventional solvent methanol. Four main compounds were identified in the extract, by HPLC-DAD-MS, as being phenolic acid (4-hydroxybenzoic acid), aldehyde (4-hydroxybenzaldehyde), flavonol (quercetin) and flavanone (naringenin).

The hydrophobic NADES (MenLA and MenThy) led to co-extraction of phenolic compounds and carotenoids from tomato peel. A separation method based on liquid-liquid extraction of phenolic compounds in polar ChLA NADES was developed to enable HPLC characterisation of the two groups of bioactive molecules. The two main carotenoids identified in tomato peels were lycopene and β-carotene.

The same processes were applied successfully in grape pomace. A preliminary depolymerisation step using menthofuran as nucleophile was required to analyse the condensed tannins (procyanidins) present in red grape pomace by HPLC.

Dietary fibres - A high hydrostatic pressure assisted cellulase method was established for the targeted enzymatic hydrolysis of potato dietary fibre. Meanwhile, further modifications improved the physico-chemical and functional properties of potato dietary fibre significantly.

Pectin - The drying method had no significant effect on the extraction rate of pectin, but it influenced both the galacturonic acid content and the degree of esterification. Additionally, ultrasonic-assisted deep eutectic solvent extraction demonstrated a higher pectin yield compared to traditional water-based extraction methods.

Data Management Plan follow-up

N°	Dataset name	Open Data	Closed Data	Means of dissemination	Maximum delay before access	Data set access
1	WP2.2_FHNW	collection of preferences (about valorisation pathways) and their associated justifications associated with an anonymized stakeholder		Scientific and popularized publications, conferences, various dissemination and capacity-building events	Once published and at the latest 2 years after the end of the project	
2	WP2.3_UNIBO-BIGEA_2	Data on biological activity analysis of proteins from tomato and BSG feedstocks		Data in progress not yet completed	Once published and at the latest 2 years after the end of the project	Not yet available
3	WP2.1_2.2_ITQB_NOVA	Data on extraction of cutin. Chemical data NMR, GC-MS and elemental analysis data; extraction yields for each raw material; microscopy data (SEM, TEM); specific (target) structural identification of chemicals/polymeric structures; thermal properties of plant polyesters.	Functional properties	Publications, conferences, Webinars	Once published	Not yet available
4	WP2.3_INRAE_IATE	Data on structural characterisation of phenolic compounds and carotenoids from tomato and grape feedstock	Structural characterisation	Publications, conferences	Once published	Not yet available
5	WP2.1_IFST-CAAS_HH	----------	Extraction technology, properties of functional components	publications, conferences	Once published	Not published
6	WP2.2_IFST-CAAS_AMS	----------	Extraction technology, properties of functional components	publications, conferences	Once published	Not published
7	WP2.3_IFST-CAAS_MMM	----------	Extraction technology, properties of functional components	publications, conferences	Once published	Not published
8	WP2.4_IFST-CAAS_XJ	----------	Extraction technology, properties of functional components	publications, conferences	Once published	Not published

This table above sums up the main information regarding the data produced for this deliverable, where is it stored and are the specific rules to respect concerning access, publication and FAIR principles.

N°	Dataset name	Owner	Name of the current contact	IPR issues	Use of third-party	Restrictions on data sharing (Y/N)
1	WP2.2_FHNW	FHNW	Aurelie Hoeffel	Require data transfer contract with chinese partners for closed data	no	no
2	WP2.3_UNIBO-BIGEA_2	UNIBO	Annalisa Tassoni	no	no	No (after publishing)
3	WP2.1_ITQB_NOVA	ITQB NOVA	Cristina Silva Pereira	Require data transfer contract with chinese partners for closed data	no	No (after publishing or patenting)
4	WP2.3_INRAE-IATE	INRAE-IATE	Chahinez Aouf	Require data transfer contract with chinese partners for closed data	No	No (after publishing or patenting)
5	WP2.1_IFST-CAAS_HH	IFST-CAAS	Hui Hu	Require data transfer contract with european partners for closed data	No	Yes
6	WP2.2_IFST-CAAS_AMS	IFST-CAA	Aimin Shi	Require data transfer contract with european partners for closed data	No	Yes
7	WP2.3_IFST-CAAS_MMN	IFST-CAA	Mengmei Ma	Require data transfer contract with european partners for closed data	No	Yes
8	WP2.4_IFST-CAAS_XJ	IFST-CAA	Xin Jin	Require data transfer contract with european partners for closed data	No	Yes

This table above sums up the main information regarding potential Intellectual property protection or GDPR issues.