agriloop

high-value products from agricultural residues through sustainable chains

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Deliverable D2.1

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LIST OF ABBREVIATIONS

AAE, Ascorbic acid equivalent
AAPPC, alcohol extracted peanut protein concentrate
ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
APTES, 3-aminopropyl)triethoxysilane
B, Bromelain
BCA, bicinchoninic acid
BSA, bovine serum albumin
BSG, brewery spent grain
CFS, cell free supernatant
CFU, colony forming unit
ChCl/LA, choline chloride/lactic acid
COM, cutin oligomeric mixtures
DFP, defatted peanut flour
diOHC1616, hydroxyhexadecanoic acid
DMSO, dimethyl sulfoxide
DPF, defatted peanut flour
DPPH, 2,2-diphenyl-1-picrylhydrazyl
DSC, differential scanning calorimetry
DTS, defatted tomato seeds
EC, emulsifying capacity
FC, apple pomace from concentrated juice
FD, freeze drying
FS, feedstock
FTIR, Fourier transform infrared
g d.m, grams of dry mass
GC-FID, gas chromatography with flame ionization detector
GC-MS, gas chromatography with mass spectrometer detector
gDW, grams of dry weight
gFW, grams of fresh weight
HBA, hydrogen bond acceptor
HBD, hydrogen bond donor
HPD, heat pump drying
IAPPC, combined isoelectric precipitation and alcohol extraction peanut protein concentrate
ICP-MS, inductively coupled plasma mass spectrometry
ID, industrial dried pomace
IL, ionic liquid
IPPPC, isoelectrically precipitated peanut protein concentrate
LC-MS/MS, liquid chromatography with tandem mass spectrometry
Men/LA, menthol/lactic acid
NADES, natural deep eutectic solvents
ND, not digested
NFC, apple pomace from not concentrated juice
NMR, nuclear magnetic resonance
P, Protamex
PPC, peanut protein concentrate
PPI, protein by alkaline solubilization and acid precipitation
RT, retention time
S/L, solid to liquid ratio
SD, standard deviation
SDS-PAGE, sodium dodecyl sulphate - polyAcrylamide gel electrophoresi
SEC, size exclusion chromatography
SNPs, silica nanoparticles
T, Trypsin
TD, thermally digested
TFS, tribo flow system
TP, tomato peels
TS, tomato seeds
UF, ultrafiltration
UPLC-MS, ultra-high performance liquid chromatography with mass spectrometry
V, Viscozyme
WTP, whole tomato pomace

1. Executive Summary

AgriLoop WP2 specific objective is to develop integrated flexible upscalable extraction processes for added-value biochemicals with targeted structures/functionalities. A first set of data for the optimization of green extraction process of proteins, cutin (plant polyester), polyphenols, carotenoids and fiber is here described. Data include the efforts of European and Chinese partners in view to strengthen the EU-China scientific and technological cooperation in line with new trends in circular agricultural development such as green, low-carbon, and innovation in the pretreatment, separation and valorization of agro-industrial plant-based residues.
Different agro-industrial residues were used and in particular in Europe tomato pomace, grape pomace, brewery spent grain, and in China peanut cake meal, peanut skin, apple pomace and potato waste.
European partners carried out the extraction of proteins from tomato pomace, grape pomace and brewery spent grain by means of proteases, cell wall hydrolytic enzymes and microbial digestions. The best extraction conditions were optimized and samples (i.e., tomato seeds and brewery spent grain), yielding the highest protein amount, were selected for further experiments. Trials by comparing free and immobilized enzymes were also carried out. Trypsin protease was successfully immobilized and its protein extraction capacity tested. For peanut cake meal (in Chinese side), the influence of different pre-treatment methods on the functional characteristics (i.e., solubility, emulsification, water/oil binding capacity, gelation) of extracted proteins was studied, and extraction processes via alcohol precipitation optimized.
Cutin extraction process was optimized by European partners on tomato peels, obtained after flotation pre-processing of whole tomato pomace, by using two different extraction strategies: (1) ionic liquid-assisted extraction, to preserve cutin polymeric backbone, and (2) alkaline hydrolysis to obtain cutin monomers. The cutin extraction process via ionic liquids was based on a standard suberin extraction protocol which was further optimized within AgriLoop project to meet new key sustainability criteria (i.e., reduction of processing time, usage of Generally Recognized As Safe (GRAS) solvents, recyclability of the cholinium hexanoate catalyst and of the ethanol solvent). The alkaline hydrolysis of tomato peels produced a fraction rich in cutin monomers which will be used within AgriLoop to build new elastomers or copolyesters.
Polyphenol extraction was carried out on tomato peels, seeds and grape pomace on EU side, and on peanut skin and apple pomace on CN side. Various NADES (Natural Deep Eutectic Solvents) were tested under different conditions. A mixture of choline chloride and lactic acid proved to be the most efficient in extracting polyphenols from tomato, grape and peanut residues. Apple pomace samples from apple juice production were pre-treated by different drying methods and the content of phenolic compounds was studied.

Being less polar than polyphenols, tomato peel carotenoids were extracted with an apolar NADES composed of menthol and lactic acid.
The proximate composition of potato starch processing residues and the optimization of dietary fiber extraction technology were carried out, and the dietary fibers characterized for their molecular composition.

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.

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.

Within this project, WP2 objectives are (1) to develop an integrated flexible up-scalable extraction process for proteins, polyesters, polyphenols, carotenoids and fibers 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.

In connection with the specific objective 1 of WP2 EU-CN, Deliverable 2.1 presents a first set of data from European and Chinese efforts related to the optimization of green, efficient, technologically advanced and sustainable extraction processes of proteins, cutin (plant polyester), polyphenols, carotenoids and fibers from different agro-industrial residues, and on preliminary techno-functional and bioactivity assessments of the collected extracts. These include in Europe tomato pomace, grape pomace, brewery spent grain, and in China peanut cake meal, peanut skin, apple pomace and potato residues.

Prior to extraction, different residue pre-treatment methods were studied: flotation to separate tomato peels from tomato skins, dry fractionation by grinding and electrostatic separation with the aim of enriching residues fractions in specific molecules (e.g., phenols or polyesters) of interest, different drying methods (hot air, heat pump drying, freeze drying).

Agro-industrial residues are rich in proteins and peptides that can be extracted and further valorized in several industrial fields[2], making culture practices more profitable and reducing human dependency on animal products. Currently available plant proteins are mainly extracted from legume and cereal seed feedstocks and are often poorly characterized for their technological (e.g., emulsification or gelation properties), nutritional and biological activities and potential risks (e.g., allergenic potential)[3,4].

Enzymatic hydrolysis, by using different proteases under mild conditions (pH 6–8), is an efficient method that, after optimization of the digestion conditions, preserves the nutrional functionality of extracted proteins[5]. Protein enzymatic extraction, via enzymes and/or microorganisms, can also lead to the production of peptide hydrolysates and may be useful in modulating the biological and functional properties of recovered peptides[5]. Protein recovery by enzymatic hydrolysis (both by free and immobilised enzymes) on agri-residues, has also been widely studied by using plant or animal proteases in combination with carbohydrolases, to promote the solubilization of proteins from cell wall components[2] and is applied within AgriLoop project. In addition, the protein extraction from peanut cake meal based on alcohol precipitation is also carried out.

[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.

[2] del Mar Contreras et. (2029). Protein extraction from agri-food residues for integration in biorefinery: potential techniques and current status. Bioresour. Technol. 2019, 280, 459–477.

[3] Prandi et al. (2021). Targeting the nutritional value of proteins from legumes by-products through mild extraction technologies. Front. Nutr. 8, https://doi.org/10.3389/fnut.

[4] Tassoni et al. (2020). State-of-the-art production chains for peas, beans and chickpeas -Valorization of agro-industrial residues and applications of derived extracts. Molecules 25, 1383.

[5] Ferri et al. (2022). Looking for peptides from rice starch processing by-product: bioreactor production, anti-tyrosinase and anti-inflammatory activity, and in silico putative taste assessment. Front. Plant Sci. 13:929918. doi: 10.3389/fpls.2022.929918

Available polyesters from plant sources are currently obtained by methods that perform nonspecific hydrolyses, releasing their composing building blocks[6]. An innovative eco-sustainable extraction of plant polyesters, namely cutin and suberin, has been developed based on the use of biocompatible ionic liquids. This method allows the preservation of the polymeric backbones of the polyesters[7,8,9]. For the extraction of cutin and suberin the same ionic liquid is used: cutin is recovered as the insoluble portion, and suberin as the soluble portion that can be subsequently precipitated. In either case, the process preserves the native properties of the plant polyester barrier, i.e., antimicrobial, antibiofouling, selective permeabilities, and probably other yet unexplored but relevant properties. Nonetheless, the onepot extraction of plant polyesters from agricultural residues requires optimization following optimal design criteria: catalyst and solvent recyclability/reusability, process scalability, eco & cost-sustainability which is performed withing AgriLoop. Another cutin valorisation strategy consists in the cutin alkaline high-yield hydrolysis to obtain cutin monomers hydrolysed from the tomato peel-fractions. The two alkaline processes described in the literature do not give quantitative evaluation of the extracted cutin monomers[10,1], while during AgriLoop, more accurate characterization methods is developed. Cutin monomers obtained are used to rebuild either cutin-like self-standing elastomers or co-polyester (WP4, T4.1).

In the laboratory and on an industrial scale, the extraction of polyphenols and carotenoids often relies on organic solvents that are harmful to human health and the environment, such as methanol, acetone, hexane and their mixtures[11]. In WP2, the extraction of polyphenols and carotenoids is developed using NADES (natural deep eutectic solvents). These solvents are eutectic mixtures of two or three natural components (sugars, polyols, organic acids), interacting by hydrogen bonding, which have a melting point lower than that of each component. Thanks to their complex matrix of hydrogen bonds, NADES acquire interesting extraction properties that can be adapted by changing the composition or adjusting the proportions of the mixture. In addition, these solvents can act as stabilizers for the extracted molecules. In Agriloop, the extraction of phenolic compounds from tomato, grape, apple and peanut residues is carried out using eutectic solvents and is compared with extraction using traditional solvents.. Reaction conditions are optimized and identified polyphenols are quantified by UPLC. The same procedure was applied to extract carotenoids from tomato peels with less polar NADES composed of menthol and lactic acid. Finally, the composition analysis and characterization of potato dietary fibers are carried out.

In this interim report D2.1, work has focused on extracting proteins, polyesters, polyphenols, carotenoids and fibers in separate, individual processes in order to optimize them and identify their bottlenecks. The next steps will involve the development and implementation of a cascading extraction approach for the various target compounds aimed at a zero-waste processing strategy for the initial biomass.

[6] Marc et al. (2021). Bioinspired co-polyesters of hydroxy-fatty acids extracted from tomato peel agro-wastes and glycerol with tunable mechanical, thermal and barrier properties. Ind. Crops Prod., 170, 113718

[7] Correia et al. (2020). The molecular structure and multifunctionality of the cryptic plant polymer suberin. Materials Today Bio., 5, 100039.

[8] Escórcio et al. (2022). Finding a needle in a haystack: producing antimicrobial cutin-derived oligomers from tomato pomace. ACS Sust. Chem. Eng., 10, 34, 11415– 11427.

[9] Moreira et al. (2020). An ionic liquid extraction that preserves the molecular structure of cutin shown by nuclear magnetic resonance. Plant Physiol., 184, 592–606.

[10] Cigognigni et al. (2014). Extraction method of a polyester polymer or cutin from the wasted tomato peels and polyester polymer so extracted. CHIESA Virginio [IT]/[IT]CONSERVAS MARTINETE S.A. [ES]/[ES]. Patent N. WO-2015028299-A1

[11] Dzah et al. (2020). The effects of ultrasound assisted extraction on yield, antioxidant, anticancer and antimicrobial activity of polyphenol extracts: A review. Food Bioscience, 35, 100547.

3. Tomato pomace

3.1. Feedstock pre-treatment and preliminary characterization

Tomato pomace was acquired by TomaPaint partner (TOMA) from one of the main Italian tomato processor company based in Parma and was stored during the tomato season 2023 in a trench system storage under anaerobic conditions.

3.1.1. Flotation and drying (TOMA)

The flotation system in place at TOMA facilities, was used to separate the tomato seeds from the tomato peels, starting from whole tomato pomace (Figure 1). The floatation system is composed by a screw conveyor that can receive 2500 kg/h of tomato pomace. Through a mechanical shovel the pomace was transferred to the separation tank by means of the dosing screw. The separation tank with a 10m^3 of capacity uses flotation as a technology for separating the skins from the seeds: 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.

Figure 1: Tomato pomace flotation system at TOMA site.

A first sample of tomato pomace, was prepared into tomato seeds and tomato peels and sent to the partners under frozen conditions (-20°C). The samples were obtained within a 4 batches production. In total 16 kg of tomato pomace, 69 kg of tomato peels and 37kg of tomato seeds were delivered in February 2023. 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, giving respectively average humidity results of 69.3/30.7 kg water/kg dry matter for total pomace, 72.1/27.9 kg water/kg dry matter for seeds, and 81.7/18.3 kg water/kg dry matter for peels.

Figure 2: Feedstock drying system by using biogas at TOMA site.

In October 2023 samples of pomace, floated peels and seeds samples were also dried by a drying system realized at TomaPaint plant (Figure 2), that uses biogas combustion heat to increase environmental sustainability (drying at air temperature of 70°C). The total amount of dried tomato pomace was 7.5 kg, while the total amount of

dried peels was 2 kg. In addition, a sample of wet seeds was lyophilized and sent to the partners with average 94.6/5.4 kg water/kg dry matter for peels and 74.4/25.6 kg water/kg dry matter for seeds.

3.1.2. Microbial analysis (TOMA, ITQB-NOVA)

After flotation of tomato by-products, stored at room temperature in anaerobic conditions, and separation of peels from seeds, microbiological analysis was performed on the floating peels by TOMA by using the method EN ISO 4833-1:2013[12]. The average total microbial count (30°C) was of $1.0 \cdot 10^7$CFU/g while the lactic acid bacteria were $1.3 \cdot 10^6$ CFU/g. The values remained below the maximum allowed law limit[13] for food products.

At ITQB-NOVA tomato seeds and peels underwent microbial analysis following the ISO 6887:2017[14] protocol for microbiology of the food chain. Initially, samples were either thawed for up to 3-hours at room temperature or dried and milled, preceded by 1-hour pre-soaking in a peptone salt solution. Subsequently, 10 g of the sample was mixed with 90 mL of peptone salt solution, homogenized using an orbital shaker, and left to settle for 15 minutes to allow sedimentation of particles. The maximum allowable time from homogenization to plating was 45 minutes. Plate Count Agar was used for enumerating total viable bacteria (through CFUs, colony forming units), while Sabouraud Dextrose Agar served for the detection and enumeration of yeasts and fungi.
The results showed both bacterial and fungal contamination. Thawed tomato peels exhibited higher contamination levels compared to thawed seeds, with counts ranging from ranging from 10^2 to 10^3 CFU/g. Additionally, the process of drying and milling the samples resulted in a reduction of microbial load (below 30 CFUs/g). Moreover, the extracted cutin, obtained after ionic liquid (IL) processing, showed no microbial contamination.

Conclusions

Regarding the isolation of cutin the apparent microbial contamination of the raw material is eliminated by the ionic liquid process. Therefore, any cascading process derived from this operation will be devoid also of bacterial contamination. Besides during the drying process implemented at TOMA, the temperature and the final low water content (<10%) of the samples are conditions unfavorable to microorganismes growth and survival, which should solve the issue of tomato feedstock microbial contamination.

Next steps

The replacement of the ionic liquid by choline base eutectic solvents is under development. This will require testing if the microbial contaminants are also eliminated after the treatment.

3.1.3. Elemental and metal analysis (UNIBO-BIGEA)

After flotation process (Section 3.1.1, TOMA), at UNIBO-BIGEA samples of whole tomato pomace, tomato seeds and tomato peels were dried (48h, 80°C) and the amount of dry weight (DW) resulted to be respectively 30.1%, 24.6%, 20.1% of fresh weight (FW) and comparable with data reported by TOMA (Section 3.1.1).
Biological triplicates of dried samples were subjected to elemental analysis to assess the presence of contaminating heavy metals and of metalloids. Elemental quantification was carried out by pre-digesting samples (about 0.4 g of dry weight of powder, gDW) at room temperature with 2 mL 70% (v/v) HNO_3 for 1 day, followed by digestion for 1 h at 70°C and 1 h at 125°C according to Huang and Schulte[15]. After digestion, samples were

[12] EN ISO 4833-1:2013. Microbiology of the food chain - Horizontal method for the enumeration of microorganisms - Part 1: Colony count at 30 degrees C by the pour plate technique (ISO 4833-1:2013)

[13] COMMISSION REGULATION (EC) No 2073/2005 of 15 November 2005 "on microbiological criteria for foodstuffs" and following updates (Reg. CE 1441/07)

[14] EN ISO 6887-1:2017. Microbiology of the food chain - Preparation of test samples, initial suspension and decimal dilutions for microbiological examination - Part 1: General rules for the preparation of the initial suspension and decimal dilutions (ISO 6887-1:2017)

[15] Huang and Schulte (1985). Digestion of plant tissue for analysis by ICP emission spectroscopy. Comm Soil Sci Plant Anal**,** *16*, 943-958.

brought up to 20 mL with deionised water. Five replicates of reference material (Apple leaves, NIST® SRM® 1515) and blanks (only 70% v/v HNO_3) were subjected to the same procedure and added to samples as quality controls. Elemental concentration analysis was performed by inductively coupled plasma mass spectrometry (ICP-MS) by an Elan 9000 DRCe detector (Perkin Elmer, Waltham, Massachusetts, USA) (Table 1).

Table 1: Elemental analysis of whole tomato pomace, and of tomato seeds and peels obtained after flotation process. Data (mg/kgDW, ppm) are the means of 3 replicates ± standard deviation (SD).

Element	Whole pomace (mg/kgDW)	Seeds (mg/kgDW)	Peels (mg/kgDW)
Ag	0.287 ± 0.003	0.280 ± 0.004	0.280 ± 0.004
Al	24.008 ± 1.114	29.475 ± 1.043	22.083 ± 9.230
As	0.999 ± 0.039	1.124 ± 0.037	1.410 ± 0.020
B	11.725 ± 0.104	8.867 ± 0.093	5.758 ± 0.172
Ba	3.254 ± 0.036	2.598 ± 0.032	3.720 ± 0.076
Be	0.465 ± 0.000	0.464 ± 0.000	0.464 ± 0.000
Ca	2305.000 ± 34.785	1264.167 ± 24.983	2574.167 ± 55.445
Cd	0.698 ± 0.023	0.693 ± 0.007	0.653 ± 0.004
Co	0.799 ± 0.026	0.777 ± 0.004	0.900 ± 0.125
Cr	1.408 ± 0.118	1.623 ± 0.057	1.523 ± 0.166
Cu	12.717 ± 0.398	18.825 ± 0.129	6.625 ± 0.157
Fe	124.642 ± 2.775	146.575 ± 13.969	178.183 ± 75.462
Hg	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
K	6900.000 ± 176.068	2637.500 ± 69.408	1945.000 ± 168.967
Li	1.223 ± 0.003	1.195 ± 0.000	1.200 ± 0.000
Mg	1918.333 ± 39.200	1016.667 ± 12.111	1050.833 ± 46.949
Mn	35.850 ± 0.512	20.575 ± 0.151	29.250 ± 0.740
Mo	0.463 ± 0.007	0.843 ± 0.015	0.067 ± 0.008
Na	130.083 ± 3.368	189.750 ± 11.991	421.583 ± 74.483
Ni	1.985 ± 0.227	3.514 ± 0.892	5.942 ± 4.869
P	3632.500 ± 71.885	2370.000 ± 36.332	910.833 ± 93.831
Pb	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
S	1596.667 ± 31.728	1990.000 ± 22.136	887.500 ± 18.641
Sb	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
Se	1.464 ± 0.068	1.574 ± 0.035	1.665 ± 0.034
Si	17.358 ± 0.976	14.683 ± 0.705	20.183 ± 1.396
Sn	0.000 ± 0.000	0.000 ± 0.000	0.012 ± 0.030
Sr	8.500 ± 0.141	6.308 ± 0.220	8.958 ± 0.139
Ti	0.361 ± 0.074	0.087 ± 0.123	0.150 ± 0.034
Tl	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000
V	1.039 ± 0.004	1.065 ± 0.003	1.004 ± 0.007
Zn	23.008 ± 0.427	20.392 ± 0.183	14.342 ± 0.472

Conclusions and next steps

Data on elemental analysis did not evidence specific or anormal contaminations of the tomato pomace feedstocks in specific relation to maximum law limits[16],[17] of heavy metals dangerous for human health (such as Cd, Cr, Pb, Hg) in foodstuffs. Elemental and metal analysis will be repeated in finally selected samples from different extractions.

3.1.4. Fractionation (INRAE_IATE)

INRAE_IATE's fractionation platform (PLANET) is equipped with various grinding and separation devices enabling the production of fractions of different compositions, in a dry environment (without the use of solvents). Biomass behavior during grinding and separation is highly dependent on tissue morphology and physico-chemical properties.

First, the tomato peels and seeds received from TOMA (after flotation Section 3.1.1) were dried in an oven at 60°C for 24 h, and successively ground in SM300 knife mill (2 mm sieve), and UPZ impact mill (0.5 mm sieve). After reaching a particle size distribution D50 ~ 200 µm (meaning that the volume of particles less than 200 µm account for 50%), the powders underwent electrostatic separation (TFS, Tribo Flow System), to produce fractions of different compositions.

Electrostatic separation is based on charging biomass particles by rubbing them against the device walls or against each other. Depending on their composition and properties, particles acquire different charges (positive or negative) and can be separated by being attracted to electrodes of opposite polarity.

Due to the fatty nature of tomato seeds, they could not be ground in the impact mill because of the agglomeration that occurred. After defatting by hexane oil extraction, the particles produced by the knife mill were separated directly in the TFS. The results related to the dry fractionation of tomato peels and seeds are summarized in Table 2.

Table 2: Dry fractionation results of tomato peels and seeds by means of knife mill (SM300), impact mill (UPZ) and tribo flow system (TFS). (+), Positive fraction; (-), negative fraction; D50, median particles diameter.

	Moisture*	**SM300**	**UPZ**	**TFS**	
Tomato peels	3.7 %	Yield[#]: 98.0 % D50: 775 µm	Yield[#]: 96.0 % D50: 237 µm	Yield[#] (+): 71.5 % D50 (+): 180 µm	Yield[#] (-): 28.0 % D50 (-): 195 µm
Tomato seeds	4.2 %	Yield[#]: 95.0 % D50: 500 µm	-	Yield[#] (+): 51.6%. D50 (+): 400 µm	Yield[#] (-): 44.8% D50 (-): 435 µm

* Moisture after drying at 60°C.

[#] Yield = ($M_{\text{ground powder}}/M_{\text{initial biomass}}$) *100

Conclusions and next steps

The positive and negative fractions of tomato peels and seeds were sent to UNIBO, ITQB and INRAE_BIA for protein and cutin quantification and extraction tests. Polyphenols and carotenoids content of these fractions is currently being determined.

According to the results on different compound extractions, the maintaining of the electrostatic separation step will be discussed.

Bottlenecks

The presence of oil in tomato seeds prevents proper grinding and efficient separation of the different fractions. As a first solution, delipidation by hexane extraction was carried out which facilitated grinding. However, the use

[16] Commission Regulation (EC) No 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance)

[17] Commission Regulation (EC) No 629/2008 of 2 July 2008 amending Regulation (EC) No 1881/2006 setting maximum levels for certain contaminants in foodstuffs (Text with EEA relevance)

of such an organic solvent is not in line with the project's objectives. An alternative solution involving actors capable of generating defatted tomato seeds is currently under discussion.

3.2. Protein extraction

3.2.1. Enzymatic extraction (UNIBO-BIGEA)

At UNIBO-BiGEA, the tomato feedstocks were provided by TOMA partner under frozen conditions and they included: whole tomato pomace (WTP), and tomato seeds (TS), tomato peels (TP) after flotation process (Section 3.1.1). Samples were dried (48h at 80°C) and ground down to a fine powder. Samples were stored at -10°C degrees until use. All tests were conducted on dried and ground samples.
Initially, the Kjeldahl method[18] was used to assess the protein content in each feedstock. The analysis was carried out on the WTP as well as on TS and TP and their positive and negative fractions obtained through electrostatic fractionation by INRAE_IATE (Section 3.1.4, Table 2) and that were composed of concentrated feedstock particles of around 200 um (positive) or 400 um (negative)(Figure 3).

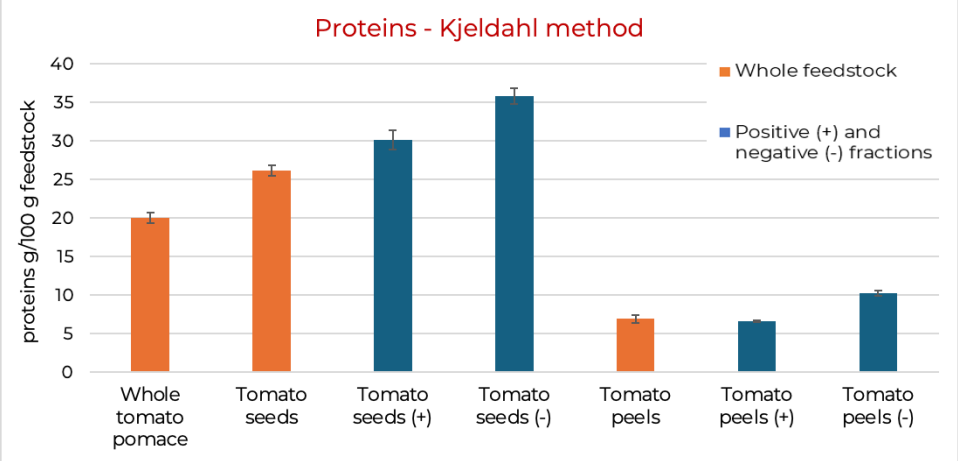


Figure 3: Protein content (g/100 gDW feedstock) in tomato samples and their positive and negative subfractions. WTP, whole tomato pomace; TS tomato seeds; TP, tomato peels. Data are the mean (n=3) data ± SD.

Data pointed out in WTP a protein content (total organic N + ammonium) of about 20.0% (w/w) while total TS exhibited a higher concentration with values of 26.1% (w/w), and 35.8% and 30.1% respectively for the negative and positive fractions. Total TP showed lower protein levels with percentages of 6.9% (w/w), 10.2% for the negative fraction and 6.5% for the positive fraction.

Considering these outcomes, initial enzymatic extraction trials were only performed on tomato seeds (TS) due to the expected higher protein yield.

Protein enzymatic extractions were performed using: a) **seven commercial proteases** Papain from *Carica papaya* (Sigma-Aldrich, Milan, Italy), Bromelain from pineapple stem (Sigma-Aldrich, Milan, Italy), Neutrase® (Novozymes A/S, Denmark), Protamex® (Novozymes A/S, Denmark), Pancreatin from porcine pancreas (Sigma-Aldrich, Milan, Italy), Trypsin from porcine pancreas (Sigma-Aldrich, Milan, Italy) and Alcalase® (Novozymes A/S, Denmark); b) **five commercial plant cell wall hydrolytic enzymes** Viscozyme® (Novozymes A/S, Denmark), Pectinex® Ultra SP-L (Novozymes A/S, Denmark), Cellulase from *Trichoderma reesei* (Sigma-Aldrich, Milan, Italy), Pectinex® XXL (Novozymes A/S, Denmark), Pentopan® (Novozymes A/S, Denmark).

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

The primary goal of this phase was to identify at least two proteases and one plant cell wall hydrolytic enzyme with better efficiency in extracting proteins from TS. The extraction procedure involved different enzyme/sample g dry weight ratios (1%, 2% and 5% w/w) at a temperature of 60 °C for 2 hours, with a solid liquid (S/L) ratio of 1:5 (2 gDW of TS and 10 mL of milli-Q water), 100 rpm shaking. pH was adjusted to 7 for extractions with proteases and to 6 for extractions with plant cell wall hydrolytic enzymes. Two different controls were performed: non-digested control (ND) where tomato seed samples were added with milli-Q water (1:5 S/L) and then promptly subjected to centrifugation and separation of the supernatant; thermally digested control (TD) where sample aliquots were subjected to the same procedure as the enzyme-treated samples but without any enzyme addition. Proteins in supernatants were quantified by Lowry assay[19].

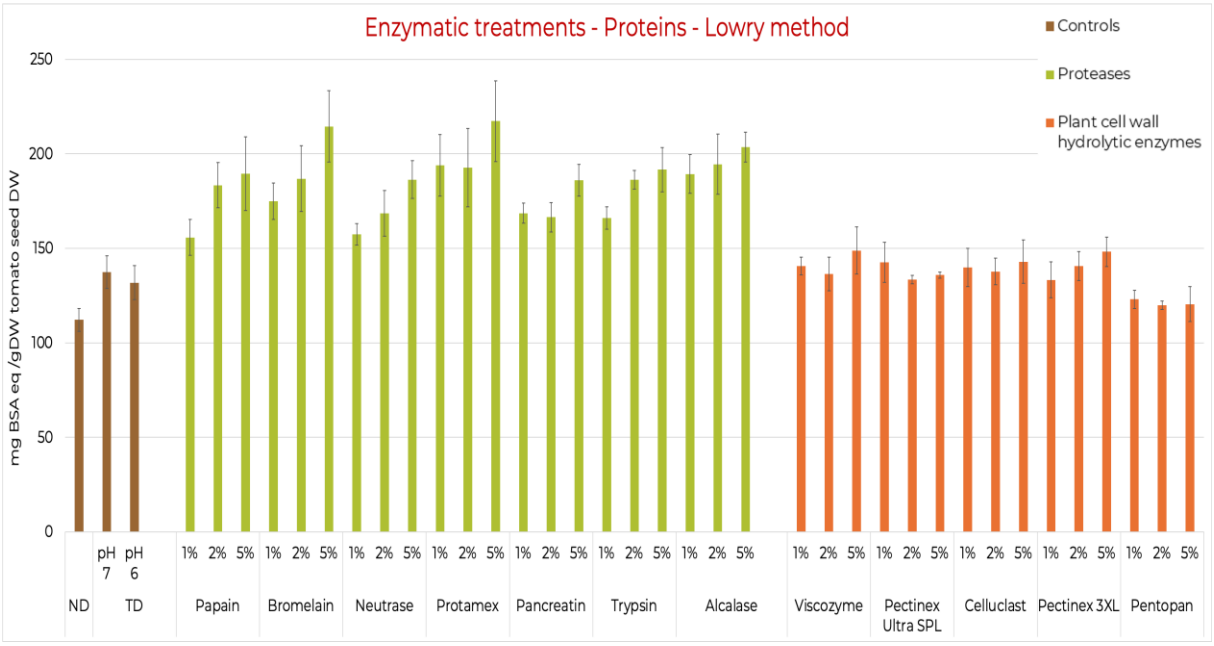


Figure 4: Protein content (mg BSA equivalents/gDW sample) in tomato seed supernatants following enzymatic treatment at different enzyme/sample gDW ratios (1%, 2% and 5% w/w) and in not digested (ND) and thermally digested (TD) controls. BSA, bovine serum albumin. Data are the mean (n=4) ± SD.

All proteases were able to enhance protein release compared to both ND and TD controls, with a slight increase in the presence of higher enzyme/gDW sample percentages. On the contrary, plant cell wall hydrolytic enzymes did not exhibit a notable increase in protein release (Figure 4), under the tested conditions.

The proteases Bromelain (B) and Protamex (P) were chosen for further steps due to their higher efficiency, together with Trypsin (T) which was recommended by FHNW following preliminary trials on enzyme immobilization. Among plant cell wall hydrolytic enzymes Viscozyme (V) was selected. Given best results achieved previous trails (Figure 4), in following optimization steps it was decided to use all enzymes at a 5% (w/w) enzyme/gDW sample ratio.

Once the most effective enzymes were identified, a two-step combined digestion approach was employed to explore potential enhancements in protein extraction yield. Initially, the cell walls were hydrolysed by using Viscozyme (V) under the same conditions as previously described. Subsequently, one of the selected proteases was added and the sample incubated again under identical conditions. Viscozyme (V) only treatment and thermally digested control (TD) under the same conditions previously reported, were also performed for

[19] Lowry et al. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

comparison (Figure 5). Proteins were quantified by Lowry assay[4] and extracts are actually under quantification by Kjeldhal method.

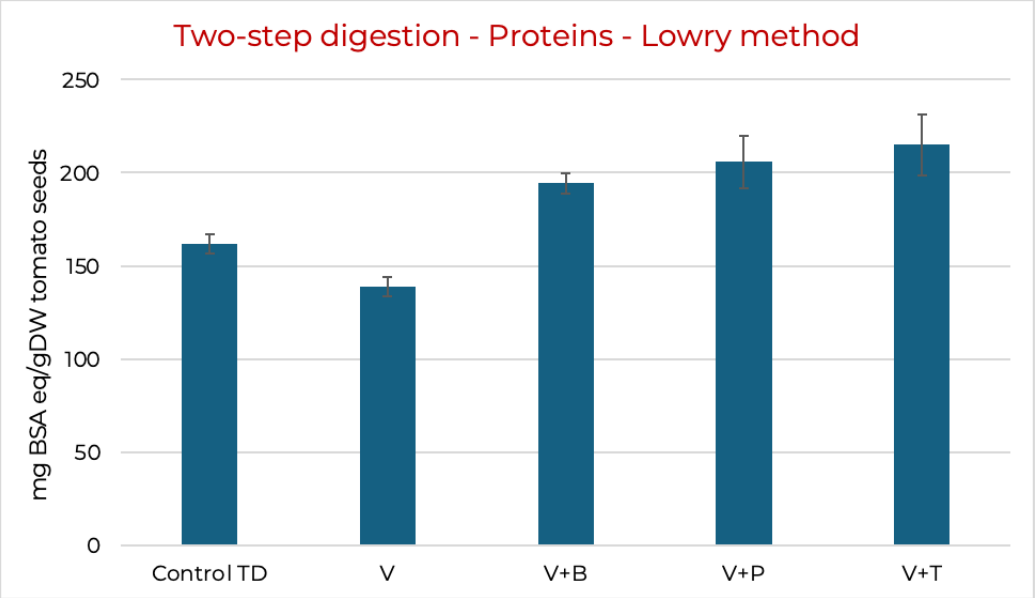


Figure 5: Protein content (mg BSA equivalents/gDW sample) in tomato seed supernatants following a two-step digestion with cell wall hydrolytic enzymes and proteases both at 5% (w/w) enzyme/gDW sample ratio. BSA, bovine serum albumin; TD, thermally digested control; V, Viscozyme, B, Bromelain; P, Protamex; T, Trypsin. Data are the mean (n=4) ± SD.

The addition of proteases resulted in an increased protein yield compared to both Viscozyme only treatment and TD control. Overall, the addition of Viscozyme cell wall hydrolytic enzyme did not appear to enhance the final protein release beyond what was achieved with proteases alone with an average of 205 mg BSA eq/gDW in two-step combined treatments (Figure 5) respect to an average of 208 mg BSA eq/gDW indigestions with 5% (w/w) of selected proteases (Figure 4).

Given the obtained results and also to improve the environmental and economic sustainability of the extraction process, it was decided to use only proteases for the extraction of the proteins from tomato samples. The final optimized processing conditions were: 5% (w/w) enzyme/sample gDW ratio, 60°C, 2 hours incubation, 1:5 S/L ratio (2 g of sample and 10 mL of milli-Q water), pH 7, 100 rpm shaking.

Subsequently, the protocol involving only the protease treatment was extended to the other tomato feedstocks (WTP and TP), along with the positive and negative fractions of TP and TS (Figure 6). This phase aimed at determining the best tomato feedstock for protein extraction and, consequentially, the one to be used in further steps of the AgriLoop project (Tasks 2.2 and 4.1).
After protein quantification by Lowry assay, overall, protease treatment proved effective in extracting proteins compared to controls (ND and TD), particularly showcasing a significant difference in TS (both total and + or - fractions) (Figure 6). Higher protein yields were achieved in total tomato seeds, **suggesting that non-fractionated TS are the most suitable choice for subsequent protein extraction trials** and that fractionation process does not efficiently enough concentrate proteins in either positive or negative fractions.

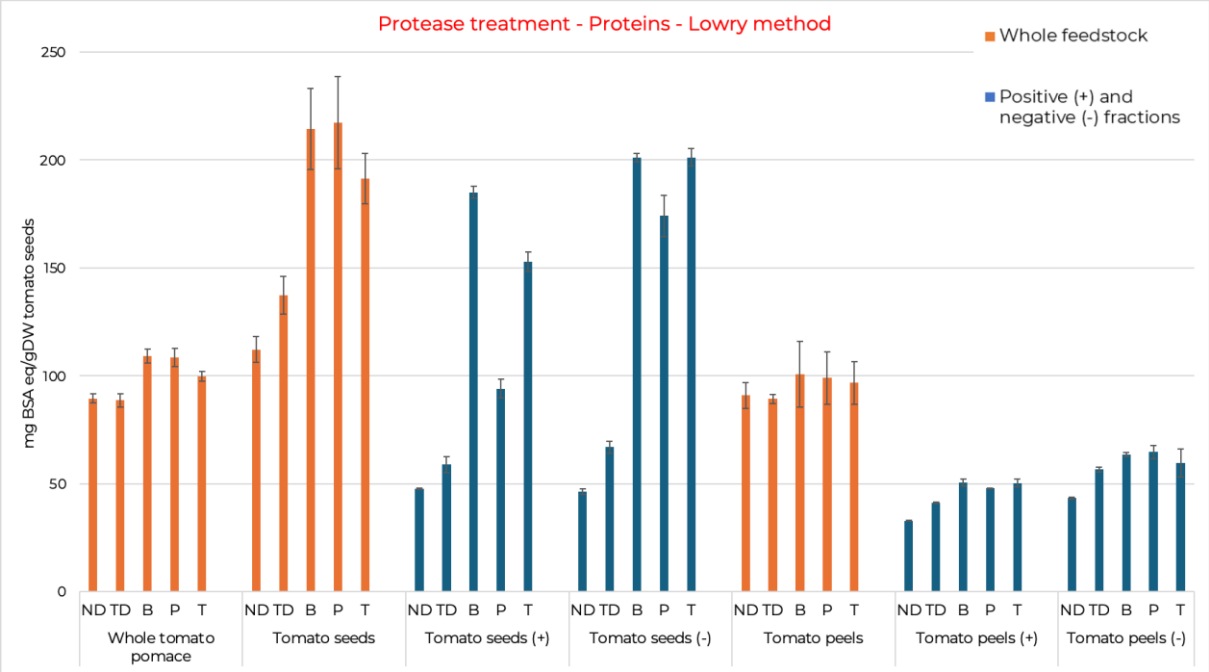


Figure 6: Protein content (mg BSA equivalents/gDW sample) in tomato supernatants following selected protease treatments. BSA, bovine serum albumin; ND, not digested control; TD, thermally digested control; B, Bromelain; P, Protamex; T, Trypsin. Data are the mean (n=4) ± SD.

Selected fractions of previous experiments are actually under analysis with Kjeldhal technique to verify the degree of purify of obtained extracts.

Trials actually on-going aim at protein extraction by applying on TS alkaline or neutral solubilization (24°C, 3 hours, 1:5 S/L) followed by acidic precipitation (pH 4). Alkaline extraction was performed with 0.1 M NaOH at pH 11, while neutral extraction was performed with 0.05 M phosphate buffer at pH 7.2. TS directly dried by TOMA were also tested during this step. Preliminary results show comparable yields between alkaline extraction and neutral extraction, with minimal disparity observed between pre-dried and laboratory-dried TS.

Conclusions

Tomato seeds seemed to be the most suitable source of proteins among the 3 tomato feedstocks. Whole tomato pomace seems less useful for protein and peptide recovery, given its higher complexity and the risk of having several interferent molecules that could impair extraction process and contaminate the final the final protein product. Tomato peels gave lower protein yields with respect to seeds, and it was agreed to reconsider them for protein recovery in a second or third step of the cascading approach starting from the residues of other extractions (i.e., after carotenoid extraction from INRAE_IATE or cutin extraction from ITQB-NOVA).
Best protocol for enzymatic extraction of proteins/peptides from tomato seeds was set up and released proteins and peptides are in the liquid fraction. The use of a plant cell wall hydrolytic enzyme (2 steps treatment) didn't improve the final release with respect to the use of proteases alone.

Next steps

The study of a preliminary protein precipitation step (alkaline or neutral solubilization and acid precipitation) before enzymatic treatment is in progress, with the aim to obtain purer protein/peptide final extracts. Next steps will be SDS-PAGE gel analyses, to verify molecular weight distribution in the extracts, and peptide sequencing. Also the cost of the selected enzymes will be evaluated in view of a more economical sustainable process.

Bottlenecks and deviations

In relation to protein extraction the major bottlenecks encountered during protein extraction from the 3 tomato feedstocks, was the initial microbial contamination. In fact, the first batch of tomato industrial residues was sent frozen from TOMA to UNIBO, but the fresh material storage in TOMA and/or the not maintenance of a proper low temperature during delivery, did not preserve tomato feedstock from microbial growth. In fact, the initial materials received by UNIBO were smelly, on the contrary to those sent by successively TOMA in dried form. No deviations were experimented respect to the DOA.

3.2.2. Immobilized enzymes extraction (FHNW)

3.2.2.1. Pre-processing of samples

Whole tomato pomace, and peels and seeds after flotation (Section 3.1.1) were obtained frozen by TOMA partner. Additionally, peels and seeds were also tested in the dried form after lyophilization or after drying by a biogas-powered system performed by TOMA.

A cryomill machine (Retsch) was then used to grind all dried feedstock in a small homogenous powder (grinding at 30Hz for 5 minutes). Then the water amount in each sample was determined on approximately 5 gFW of each primary feedstock put in an oven at 105°C for 3 hours. Frozen seeds and peels showed respectively to have a dry matter content of 30.1% and 16.6% comparable with the data from TOMA and UNIBO (Sections 3.1.1, 3.1.3).

Then, based on the water amount, each feedstock was weighted at a biomass/solvent ratio from 1/5 to 1/20 for further proteins and peptides extractions. The amount of solvent corresponded to the amount of water present in the sample plus the addition of the specific buffer.

3.2.2.2. Free enzymes extraction

In order to enable the maximum extraction yield of proteins, different biomass/solvent ratios were tested, as using an excess amount of solvent compared to biomass is the driving force for the protein to go into solution[20]. Different buffers (i.e., acetate buffer pH 5.5 and Tris-HCl buffer at pH 7.0 and 8.0, respectively) were chosen based on the added enzymes and their optimal pH for proteins/peptides extraction. Cell wall degrading enzymes provided from Novozymes were used to degrade pectin and release proteins: Pectinex UC, Pectinex USP, Celluclast and Viscozyme in enzyme/solvent (v/v) ratio of 3%, 1% and 0.05%, at a pH 5.5. Protein hydrolytic enzymes as bromelain, papain and trypsin were also tested at a concentration of 10, 5, 2 and 1 U/mL of solvent respectively, at pH 8. Different temperatures and conditions were tested: 37°C from 9 to 36 hours and 50°C for 2 hours. The enzymes which were the most active, were then tested in parallel via two digestions: first a cell wall digestion was performed followed by a alkaline treatment with 0.1M NaOH to have the optimal pH for the digestion with the hydrolytic enzyme, then a second digestion was performed. After the digestion, the protein content of the supernatant was analyzed via BCA assay[21] (Thermo Fisher).

Concerning the tomato residues, it was directly observed that the frozen ones, were subjected to undesired fermentations which had already begun to change the sample composition, leading to a lowered extraction amount of proteins as confirmed by the BCA assay. Peels and seed dried by TOMA via biogas heater or lyophilization process were also tested with the best protein yield obtained on lyophilized samples. However this process is also the most cost intensive one. Therefore, the discontinuation of lyophilization in the workflow was decided at the 1st AgriLoop annual meeting to focus on peels an seeds dried from the biogas system from TOMA. Pectinex UC, Pectinex USP as well as Cellclast independently used did not show a real impact on protein release. Viscozyme (at 3% enzyme solvent v/v ratio) was the cell wall degrading enzyme showing the best result with a protein release of 12% for seeds and 7% for peels (Figure 7).

[20] Sari et al. (2015). Towards plant protein refinery: review on protein extraction using alkali and potential enzymatic assistance, Biotech. J., 10 (8), 1138-1157

[21] Walker J. M. (1994). The bicinchoninic acid (BCA) assay for protein quantitation. Methods in molecular biology (Clifton, N.J.), 32, 5–8.

Mixing Pectinex UC, Pectinex USP and viscozyme showed a higher release of proteins with a protein content of 15% and 12% for seeds and peels respectively. However, it also created a much more complex mixture of proteins, as the preparations of Novozymes are already mixes of proteins and peptides. In fact, after mixing Pectinex UC, Pectinex USP and Viscozyme, it was not possibly to identify or even see the seed and peel proteins with SDS-PAGE. In this case, theses fractions were not used for in gel digestion, but used directly for in solution digestion and further LC-MS-MS analysis. On the opposite, when Viscozyme was used alone for protein extraction, the seed and peel proteins bands were very well separated and able to be further used for in gel digestion and further LC-MS/MS analysis.

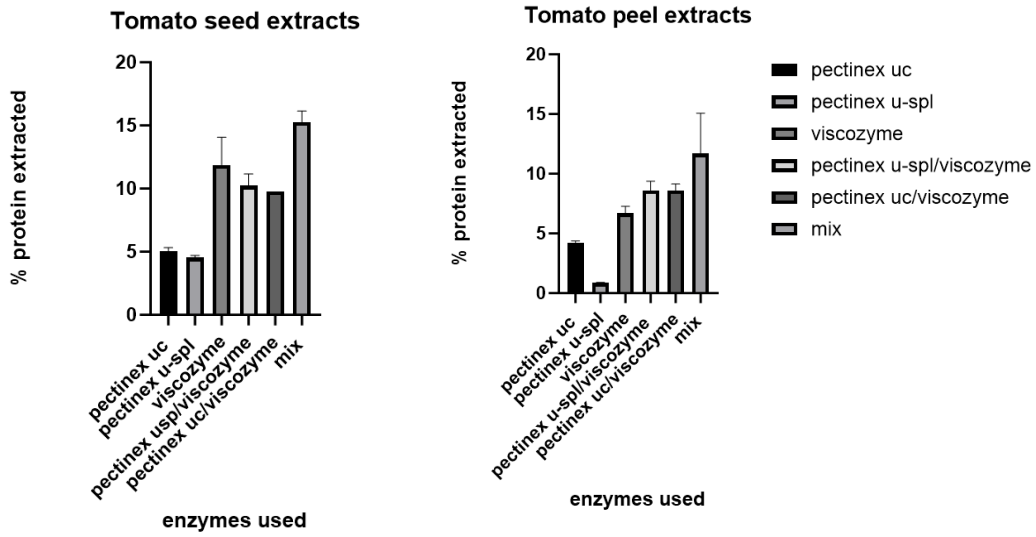


Figure 7: Percentage of proteins extracted (based on the BCA assay) (g/gDW of tomato seed or peel) with 3% (v/v) of commercial enzyme preparation of Novoymes after 10 hours incubation time at 37°C. Data are the mean (n=3) ± SD.

3.2.2.3. Comparison of proteins/peptides extraction via free and immobilized enzymes.

Both free and immobilized enzymes have been reported to convert food waste into valuable bioactive molecules such as peptides or proteins. Enzyme immobilization onto a solid support presents some advantages over free enzymes such as possible reusability, more stability compared to free enzymes as well as more resistance to temperature and pH fluctuations[22,23]. Indeed, many free enzymes are stable at pH 37°C but start to lose stability at more elevated temperatures. This loss can be alleviated via immobilization. In this case, higher temperatures allow the use of eutectic mixtures of choline chloride or urea, which are biodegradable and easy to remove compared to a use at a lower temperature where they are more viscous[24]. Furthermore, the use of some substrates also affects the stability of free enzymes by denaturing them, which can be prevented via immobilization.

In this study, some immobilization trials by using silica nanoparticles functionalized with papain or trypsin were made to determine the amount of proteins that can be extracted in comparison to free papain or trypsin.

[22]Bilal and Iqbal (2019). Sustainable bioconversion of food waste into high-value products by immobilized enzymes to meet bio-economy challenges and opportunities – A review. Food Res. Int., 123, 226–240.

[23] Andler and Goddard (2018). Transforming food waste: how immobilized enzymes can valorize waste streams into revenue streams. Npj Sci. Food, 2(1), 19.

[24] Yazid, (2017). Proteases from protein-rich waste: production by SSF, downstream, immobilisation onto nanoparticles and application on protein hydrolysis, PhD Thesis, Autonomous University of Barcelona.

Silica nanoparticles were produced using the Stöber method[25]. The complete process of the bioconjugation method is visible in Figure 8.

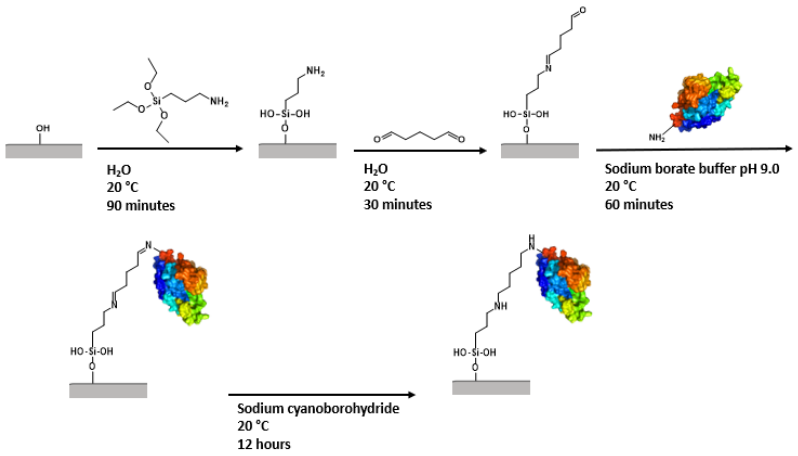


Figure 8: Bioconjugation method used to immobilize the selected enzyme (papain or trypsin) into silica nanoparticles.

Then, to see how much enzyme was immobilized onto the nanoparticles, the immobilization was quantified by using BCA protein quantification assay. For that the reaction suspension was centrifuged and the enzyme content quantified in the supernatant. By knowing the amount of enzyme in the supernatant and the initial concentration of the enzyme, it was calculated the amount of enzyme immobilized on the particles with the following formula:

$$\text{Immobilization efficiency } (\%) = \frac{\text{initial enzyme } \left(\frac{\mu g}{ml}\right) - \text{supernatant } \left(\frac{\mu g}{ml}\right)}{\text{initial enzyme } \left(\frac{\mu g}{ml}\right)} * 100$$

The immobilization efficiency depends on the enzyme used and is not consistent over experiments. This is why the immobilisation efficiency needs to be measured after each synthesis to be sure to use the same quantities of immobilized enzymes and free enzymes to have an according comparison of each method. The immobilization technique into silica nanoparticles was used for trypsin and papain. It was not possible to immobilize papain properly on silica nanoparticles, as immobilized enzyme showed self digestion. Consequently it was used only trypsin both as a free and immobilized enzyme to see the difference in protein yield extracted via these two methods. A little more proteins were extracted with the free trypsin (0.7% compared with the immobilized one) (Figure 9). This can be explained by the fact, that immobilized trypsin could have been stuck into the feedstocks and not able to digest proteins as free trypsin. The use of combined free enzyme digestion (i.e., viscozyme 3%+ trypsin 10U) was beneficial for a slightly more protein release (up to 0.8%) respect to the same treatment with immobilized trypsin (Figure 9). These results took into account the standard deviation error.

[25] Stoeber, Fink, and Bohn (1968). Controlled growth of monodisperse silica spheres in the micron size range. J. Colloid Interface Sci., 26, 62.

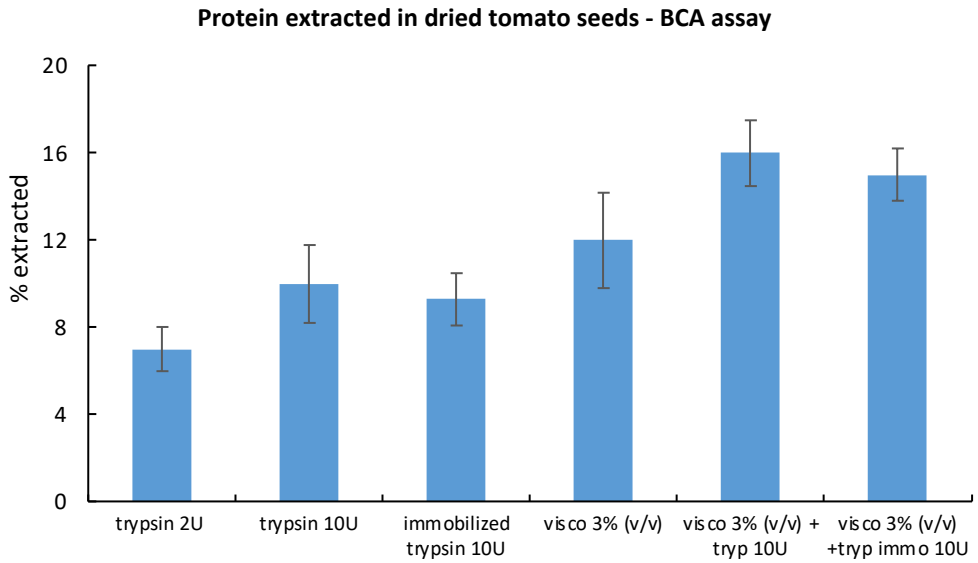


Figure 9: Percentage (g/gDW) of protein extracted via proteolytic treatment with trypsin (free or immobilized), Viscozyme hydrolysis (3% v/v), or combined treatment (viscozyme + trypsin) of tomato seeds (dried by biogas heater) after 10 hours incubation time at 37°C.

Conclusions

The extraction of proteins seemed to work the best with viscozyme and trypsin especially mixed together (viscozyme 3% and trypsin 10U/mL). Up to 16% of proteins were extracted from tomato seeds. Immobilization technique via silica nanoparticles was achieved with trypsin but not with papain and showed slightly less performance that free trypsin which can be explained by the fact that immobilized enzymes do not have so much free access to the feedstocks. Nevertheless, the standard deviation is higher with free enzymes (up to 4 %) compared to immobilized one (up to %), which made the difference observed really relative.

Next steps

The next steps and further analysis include the identification of the proteins and peptides released from the tomato seeds and peels. For that, an LC-MS-MS method will be used by using a database search via the Mascot interface with a *S. lycopersicum* database from Phytozyme.

Furthermore, another immobilization technique for trypsin, papain and bromelain with be tested. involving the creation of magnetic nanoparticles functionalizing with enzyme. This will allow to remove the immobilized enzyme from the feedstocks and to reuse it.

Bottlenecks

Further investigation need to be realized concerning the accessibility to the substrate of immobilized enzymes.

3.2.3. Protein 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. For this purpose, an innovative research approach was followed by testing bacteria isolates from unconventional environments (i.e., desert and sea) since there have been very few studies on their use for the purposes of AgriLoop compared to most available studies that used bacteria deriving from conventional environment such as soil.

Ten of such bacterial isolates were incubated in liquid mineral medium supplemented with 1% (w/v) of tomato by-products, used as only carbon and nitrogen source. The substrates tested were whole tomato pomace (seed plus peel) (WTP), tomato peel (TP), tomato seeds (TS) and defatted tomato seeds (DTS) dried overnight at 50°C

and grinded before use. First, the cultures were incubated up to 72 h, and at different sampling time points microbial growth and bioactivities (antioxidant, antimicrobial and extracellular hydrolytic enzymatic activities) were evaluated. Then, on the most active isolate assays for the separation of the enzymes and bioactive compounds were performed through size exclusion chromatography (SEC) and ultrafiltration (UF). Finally, the recovered concentrated partially purified enzymes were used for the production of bioactive hydrolysate from DTS.

With regard to bacterial growth, all isolates were able to grow on all substrates, reaching up to ~9 log CFU/mL from an initial bacterial count of ~6.5log CFU/mL after 24-48 h incubation. The antioxidant activity was evaluated on cell free supernatant (CFS) by ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) antiradical activity (%)[26]. In all substrates the abiotic sample showed a basal antioxidant activity of 30-35% (0.12-0,15 Ascorbic Acid Equivalent, AAE g/L). With the exception of isolate R4, hydrolysates of all isolates caused an increase of the ABTS radical inhibition compared to the non-inoculated sterile samples used as abiotic controls after 48-72h incubation. Specifically, the best substrates were shown to be TS and DTS and the highest antioxidant activity of up to 74.96±5.3% was recorded, after 72h incubation, in the CFS of three isolates (R50, L100, N1) when DTS was used for growth. The lowest antioxidant activity was observed in the CFS recovered after growth with TP as substrate even when the incubation was prolonged to 7 days (Figure 10).

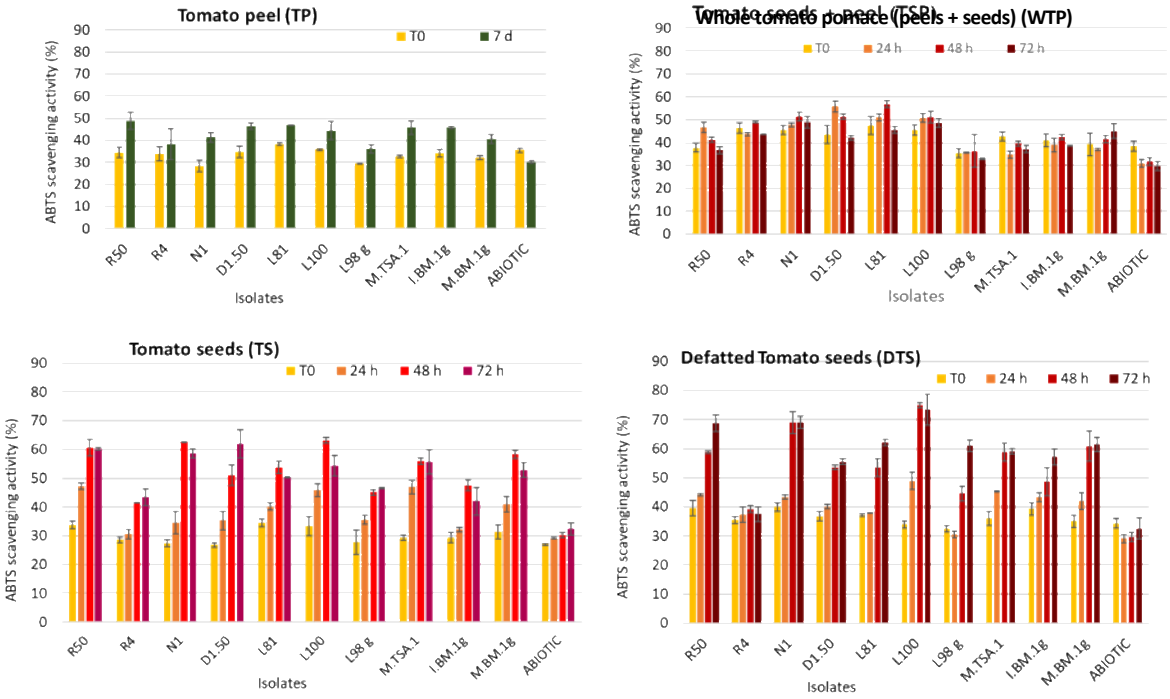


Figure 10: Antioxidant activity of the tomato byproduct hydrolysates after incubation with microorganisms expressed as ABTS scavenging activity (%).

The antimicrobial activity was monitored in the CFS of the 10 isolates by well diffusion assay[27] towards two indicator bacterial species *Staphylococcus aureus* DSM 1104 and *Escherichia coli* DSM 11034. The results showed that antimicrobial activity was recorded, only against *S. aureus* DSM 1104, in the CFS of isolates grown with the different substrates with DTS being the most suitable one allowing the detection of the activity from 6/10 isolates tested (among them isolate R50).

[26] Re et al. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Rad. Biol. Med., 26, 1231-1237.

[27] Cherif et al., (2008). Characterization and partial purification of entomocin 110, a newly identified bacteriocin from Bacillus thuringiensis subsp. Entomocidus HD110, Microbiol. Res., 163, 684-692.

Moreover, the CFS recovered after bacterial growth in the presence of the different substrates were subjected to a qualitative screening for the production of different extracellular hydrolytic enzymes using agar media plates supplemented with skim milk, pectin, carboxymethyl-cellulose sodium salt or xylan. All isolates could produce proteases and cellulases, and most could secrete pectinases; while only a few isolates showed low xylanase activity. The proteolytic activity was also quantified at 72 h incubation, through a continuous assay using Suc-Ala-Ala-Pro-Phe-pNA as substrate[28] allowing the selection of isolate R50 expressing the highest proteolytic activity of 12.52±0.87 U/mL after growth on DTS as substrate.

Based on the previous results, the isolate R50 which showed both the highest bioactivities and proteolytic activity was selected for enzymes and bioactive compounds separation/recovery. The separation of enzymes from bioactive compounds was attempted at first through gel-filtration chromatography with a Sephadex G-50 column. Then with an ultrafiltration approach, using 3 kDa Amicon Ultra-15 Centrifugal Filter Devices. As for the purification through chromatography, the soluble proteins, via NanoDrop, were quantified and peaked around the fractions 35-45 (Figure 11a). Then, the enzymes were qualitatively screened, and cellulase and proteases were mainly present between the fraction 8-37, while the antioxidant activity peaked in the fractions 38-46 with the highest (61.14%) in fractions 40 and 41 (Figure 11b), therefore it was possible to separate the extracellular hydrolytic enzymes from the antioxidant compounds.

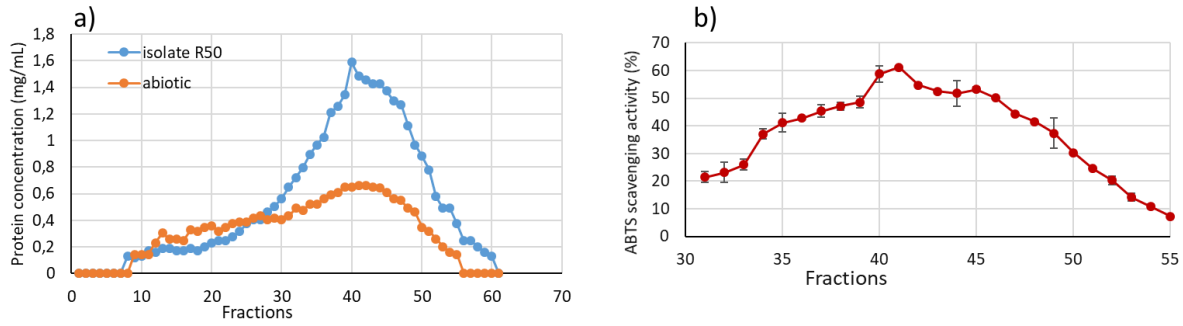


Figure 11: Sephadex G-50 chromatography purification of R50 DTS hydrolysate, a) soluble protein concentration (mg/mL) of fractions 0-60 measured by NanoDrop; b) antioxidant activity of fractions 31-55, expressed as ABTS scavenging activity (%), the ABTS scavenging activity (%) of the CFS not subjected to fractionation was 62,2%.

As for the concentration of the enzyme via ultrafiltration, the CFS of R50 was concentrated by 4.2X fold. The cellulolytic and proteolytic activities were only detected only in the retentate, while no activity was present in the permeate. The proteolytic activity was then quantified, and it confirmed the concentration of the enzymes in the retentate (from 7.9±0.3 U/mL in the CFS to 31.0±0.3 U/mL of proteolytic activity in the retentate) highlighting that there was no loss of proteases activity after ultrafiltration process. The antioxidant activity redistributed from the CFS (64.6±2.2% antioxidant activity) between both the permeate (48.5±2.2%) and retentate (75.4±3.1%), therefore the antioxidant activity may be due to peptides/compounds both smaller and bigger than 3 kDa (Figure 12a). Hence through the ultrafiltration process using 3 kDa filters, it was possible to recover and concentrate the extracellular enzymes but not all antioxidant compounds.

[28] Zanaroli et al., (2011). Selection of commercial hydrolytic enzymes with potential antifouling activity in marine environments, Enzyme and Microbial Technology, 49, 574-579.

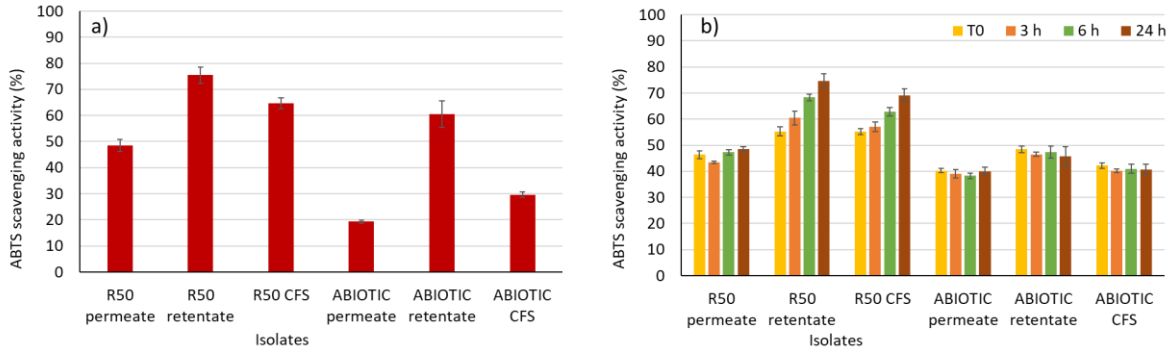


Figure 12: Ultrafiltration of R50 DTS hydrolysate: a) antioxidant activity of the hydrolysate before (CFS) and after ultrafiltration (permeate and retentate), expressed as ABTS scavenging activity (%), b) antioxidant activity of DTS hydrolysate obtained using crude or ultrafiltrate enzymes, expressed as ABTS scavenging activity (%).

Finally, the cocktail of the produced extracellular enzymes, both crude (CSF) and partially purified (recovered by ultrafiltration), was used for the enzymatic hydrolysis of the DTS (Figure 12b). The reaction consisted of 1% (w/v) of DTS, 750 µL of buffer Tris-HCl at pH 7.6 (100mM), and 250 µL of CFS, retentate or permeate sample; and it took place in a water bath, at 45 °C. The increase of antioxidant activity, compared to abiotic conditions, was recorded only in R50 retentate and CFS, from around 55±1.7% of activity to respectively 68.3±1.26% and 62.9±1.62% in 6 h; and 74.7±2.6% and 69.1±2.4% in 24 h, with respectively 19.5±2.6% and 13.9±2.4% of increase of activity.

Conclusions

Out of 10 bacterial isolates deriving from marine and desert environments, the isolate R50 was selected based on its ability to produce a tomato seed hydrolysate rich in bioactivities and enzymes. It was also possible to partially recover the enzyme and bioactive compounds from the hydrolysate.

Next steps

The next steps will concentrate on the better recovery and characterization of these compounds; but also, a scale-up of the process in a 3 Lt fermenter. Moreover, other assays for the production of bioactive hydrolysates and the recovery of bioactive compounds will be performed using TS as substrate in order to overcome the defatting step which requires the use of hexane. Furthermore, other defatting approaches will be attempted.

3.3. Cutin extraction

3.3.1. TOMA cutin extraction

During TOMA process the cutin extraction takes place after the separation of the peels through floatation. Cutin was extracted by thermal treatment at high temperature in an alkaline solution that was followed by separation of exhausted peels by centrifugation[29]. Then cutin was precipitated by acidification and separated from the liquid supernatant by means of another centrifuge. The cutin obtained is a solid paste of brown colour with humidity content of about 30% (w/w).

3.3.2. INRAE_BIA cutin extraction

The alkaline hydrolysis was conducted at INRAE-BIA on the peel-enriched fraction produced at the industrial scale from tomato pomaces and provided by TOMA. As a control, a peel-enriched fraction produced from another tomato pomace (UNIPROLEDI, Bergerac, France) at the pilot lab scale was used (hereafter referred as French pomaces).

[29] Cigognigni et al. (2014). Extraction method of a polyester polymer or cutin from the wasted tomato peels and polyester polymer so extracted. CHIESA Virginio [IT]/[IT]CONSERVAS MARTINETE S.A. [ES]/[ES]. Patent N. WO-2015028299-A1

Both pomaces were air-dried before alkaline hydrolysis (the cheapest and more efficient way, according to the hydrophobic feature of the peels).

The peels were dipped directly into a solution of KOH ethanol bath (5% w/w) for 24 h without heating (room temperature) and compared with the cutin monomers obtained by TOMA (using a 5% w/w) hot NaOH-water depolymerization process. The KOH ethanol hydrolysis time can be reduced to overnight period with stirring at room temperature. After filtration, the ethanolic extract was concentrated, enabling the recycling of the ethanol solvent. After the addition of water and acidification, the precipitation of the cutin monomers (hydroxyfatty acids) was triggered. The acidification process was initially conducted with HCl[30]. Considering the incompatibility problems with stainless steel, we checked that acidification with different acids (e.g., sulphuric acid) was also compatible with the process.

The cutin monomer yield obtained from TOMA peels fraction by KOH ethanol depolymerization was on average 45.5 % w/w of the initial peel fraction. The yield is quite comparable to those obtained from UNIPROLEDI pomace-peel fractions. The slight differences may be due to co-extracted materials (seeds, pulp) in the industrial-scale peel fraction. Nevertheless, the cutin monomer yield from TOMA pomaces is still high and corresponds to more than 65% (w/w) of the extractable cutin present in the peel fraction, assuming that tomato cutin accounts for a similar w:w of the tomato peels[31].

The extent of cutin depolymerization was further checked by FTIR (Fourier Transform InfraRed) spectrometric analysis, before and after alkaline hydrolysis. Indeed, cutin polyester highlighted typical FTIR signature dominated by methylene (2916 cm^{-1}, 2850 cm^{-1}), esterified carbonyl (1730 cm^{-1}) and ester (1166 cm^{-1}) peaks that are drastically reduced after alkaline hydrolysis (Figure 13).

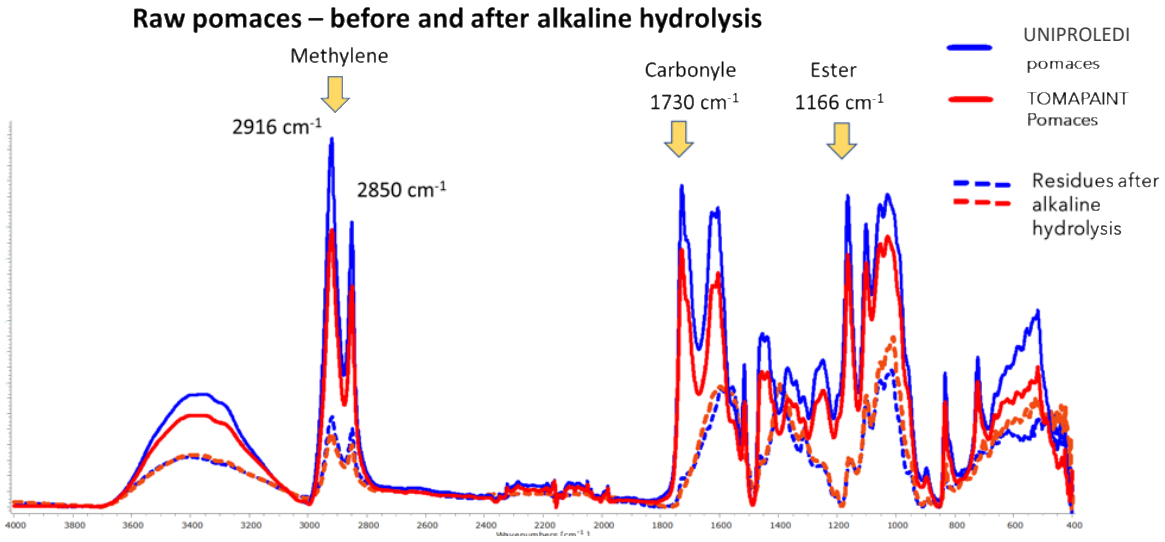


Figure 13: Infra-red spectra of two sources of tomato pomaces (TOMA and UNIPROLEDI pomaces).

The lipid composition of the cutin monomer fractions obtained was further characterized by GC-MS and quantified by GC-FID (Flame ionization detection) analyses using heptadecanoic acid (C17) as external standard. For these characterizations, a cutin monomer fraction from TOMA obtained by alkaline hydrolyses conducted in water (instead of ethanol) was also included (Table 3).

Production of cutin monomers by KOH-EtOH process

[30] Marc et al. (2021). Bioinspired co-polyesters of hydroxy-fatty acids extracted from tomato peel agro-wastes and glycerol with tunable mechanical, thermal and barrier properties. Ind. Crops Products, 170, 113718.

[31] Osman et al. (1999). Preparation, isolation, and characterization of cutin monomers and oligomers from tomato peels. J. Agric. Food Chem. 47, 799–802.

- The depolymerization process (KOH ethanol) applied to two different peels sources yielded fractions with almost the same lipid composition. This data was in full agreement with the literature [32] i.e., a quite homogeneous composition, mainly consisting in (9)10-16-hydroxyhexadecanoic acid (diOHC16). The presence of non-hydroxylated fatty acids in the industrial-scaled tomato peel fraction, is probably due minor seed residues.
- Using, the same source of industrial-scaled peel fraction (provided by TOMA) alkaline hydrolysis in water and ethanol led to the same relative lipid/monomer composition per g of cutin monomer, with a slight increase in *p*-coumaric acid yield. This result is congruent with INRAE-BIA previous unpublished results.

Comparing the NaOH-water and KOH-EtOH processes

- The KOH-ethanol depolymerization process applied to two industrial pomaces sources yielded a diOHC16 rich fraction, whatever the pomace source.
- Conversely, comparing the water and ethanol alkaline process and using the same peel fraction led to a significantly reduced (60%) cutin monomer amounts. This lower yield of hydroxyfatty acids for the same mass of monomer extract could have several origins (i.e., higher co-extracted molecules) or the presence of oligomers extracted through the water hydrolysis process (and not in the ethanol process).

Table 3: Cutin monomers quantification from two different sources of tomato pomaces (TOMA and control LabScale Bergerac) and two alkaline hydrolysis processes (KOH-ethanol or NaOH-water).

	KOH EtOH Hydrolysis (INRAE BIA process)		NaOH water Hydrolysis (TOMAPAINT process)
	2023-07	2023-06	
	BERGERAC	**TOMAPAINT**	**TOMAPAINT**
%	Peel pomaces)	peels fraction	monomers
p coumaric acid	0,3%	1,0%	3,1%
palmitic acid	0,8%	1,9%	2,7%
linoleic acid	0,2%	1,9%	3,7%
oleic acid	0,1%	0,6%	1,3%
stearic acid	0,1%	0,4%	0,5%
16 hydro-hexadecanoic acid	3,3%	3,3%	3,0%
16, 10(9)-hydroxyhexadecanoic acid	93,7%	89,8%	85,1%
hydroxyhexadecandioic acid	1,4%	1,1%	0,5%

Conclusions

The depolymerization of cutin in EtOH is robust and gives the maximum yield in cutin monomers. The depolymerization of cutin in water led to a similar mass yield of extract, although containing more phenolics and less monomers.

[32] Marc et al. (2021). Bioinspired co-polyesters of hydroxy-fatty acids extracted from tomato peel agro-wastes and glycerol with tunable mechanical, thermal and barrier properties. Ind. Crops Products, 170, 113718.

Next steps

Inclusion of this depolymerization process in a cascade biorefinery strategy, in particular after the phenolic extractions by natural deep eutectic solvents (NADES) of tomato and grape pomaces.

Bottlenecks

The use of ethanol on an industrial scale requires an ATEX-type installation.

3.3.3. ITQB-NOVA cutin extraction

3.3.3.1. Extraction process

Tomato pomace, tomato peels and tomato seeds were dried 1 week at 60°C, grinded using a Retsch ZM200 electric grinder (granulometry 0.5 mm; 10000 rpm) and stored at room temperature.

Initially, cutin-rich materials, where the cutin polymeric matrix remains largely unaltered, were extracted with cholinium hexanoate from tomato pomace or tomato peels. The samples and the ionic liquid were mixed (1:10 w/w) and stirred for 2 h at 100°C. The reaction was stopped by the addition of dimethyl sulfoxide (DMSO) 80 mL per g of cutin. The polymer was recovered by filtration using a nylon membrane filter (0.45 µm) and then washed with an excess of deionized water. The samples were dried at 40 °C in an oven and stored at room temperature.

This cutin extraction process was reformulated to improve both sustainability and recyclability. The new design uses 80 mL of ethanol per g of cutin instead of DMSO. The ethanol can be recovered by distillation (up to 80-90 %) and re-used in cutin extractions. The ionic liquid can be also recycled and re-used (n.b. only two subsequent extractions were tested). The production of cutin-rich materials is being up-scaled from 20 g to 40 g of initial biomass.

The yields of extraction using cholinium hexanoate from tomato pomace is 53% while the yields of extraction from tomato peels is around 80%.

The production of cutin-rich materials in lab-scale is continuously performed, and the current produced stocks are around 46.2 g of cutin from tomato pomace and 29.3 g of cutin from tomato peels.

The use of NADES to extract cutin will also be explored, using the ones recommended by INRAE_IATE (choline chloride/lactic acid and menthol/lactic acid). If successful, this procedure will allow for the recovery of both cutin and polyphenols in the same batch.

The production of cutin oligomeric mixtures (COM) through mild hydrolyses of the cutin-rich materials aims to generate a broader diversity of cutin structural units for the development of functional ingredients/materials. These oligomers range from small dimers/trimers to larger polymeric units. Cutin-rich materials were mixed with 20 mL of 1 M NaOH in methanol/water (1:1, v/v) at 90°C for 1 hour without stirring. Each mixture was cooled to room temperature and centrifuged (4 °C, 30 minutes, 4000g) to obtain the nonhydrolyzed cutin fraction (pellet). The supernatant was acidified to pH 3–3.5 with HCl 37% and centrifuged (4°C, 30 minutes, 4000g). The precipitate (P) was recovered, and the supernatant was extracted three times by dichloromethane/water partition to obtain the soluble hydrolysates (S); sodium sulfate anhydrous was added to remove traces of water. The organic phase was concentrated under a nitrogen flux and stored at room temperature, for further analysis. The current produced stock is around 2-3g of COM(P) and 344 mg of COM(S).

Some materials produced by ITQB-NOVA in WP2 were already sent to WP4 to build materials and specifically extracted cutin from peels (9.5 g), COM(P) (2.4 g).

3.3.3.2. Samples characterization

To better understand the pomace chemical signature, the crude sample was fractionated into peels and seeds and the resulting ^{13}C- ^{1}H HSQC NMR spectra of the cryogenic milled fractions are depicted in Figures 14 and 15, respectively.

The peels spectra included assignments related to primary aliphatic esters (PAE) PAE-α/PAE-β and secondary aliphatic esters (SAE) SAE-α/SAE-β (Figs. 14-16 A aliphatic regions; Figs. 14-16 B glycerol CH-acyl regions). These signals together with the chemical signature of the aliphatic region confirmed, as expected, that peels are the source of cutin in the pomace. Also, secondary free hydroxyl groups (−CH–(OH)−) were assigned in peels found in tomato cutin. The triacylglycerol (TAG) signal is also present and we hypothesize that there's still some contamination after the process in the peel fraction, since this signal is characteristic from the seed fraction[33].

Figure 16 displays the ^{13}C- ^{1}H HSQC spectra of the cutin-rich material extracted from the tomato peels. The presence of aliphatics (CH_2 and CH_3), α and β(C═O) esters, primary and secondary free hydroxyl groups (CH_2CH_2OH and CH_2–CH–(OH)), PAE and SAE (both α- and β-configurations) in the spectrum matches the chemical fingerprint observed herein in the peels' spectrum, and that were previously reported before for cutin isolated from peels using the same process[34,35].

[33] Escórcio et al. (2022). Finding a needle in a haystack: producing antimicrobial cutin-derived oligomers from tomato pomace. ACS Sustainable Chemistry & Engineering 2022 10 (34), 11415-11427

[34] Bento et al. (2021). Quantification of structure–property relationships for plant polyesters reveals suberin and cutin idiosyncrasies. ACS Sustainable Chemistry & Engineering 2021 9 (47), 15780-15792

[35] Moreira et al. (2020). An ionic liquid extraction that preserves the molecular structure of cutin shown by nuclear magnetic resonance. Plant Physiol. 184 (2), 592–606

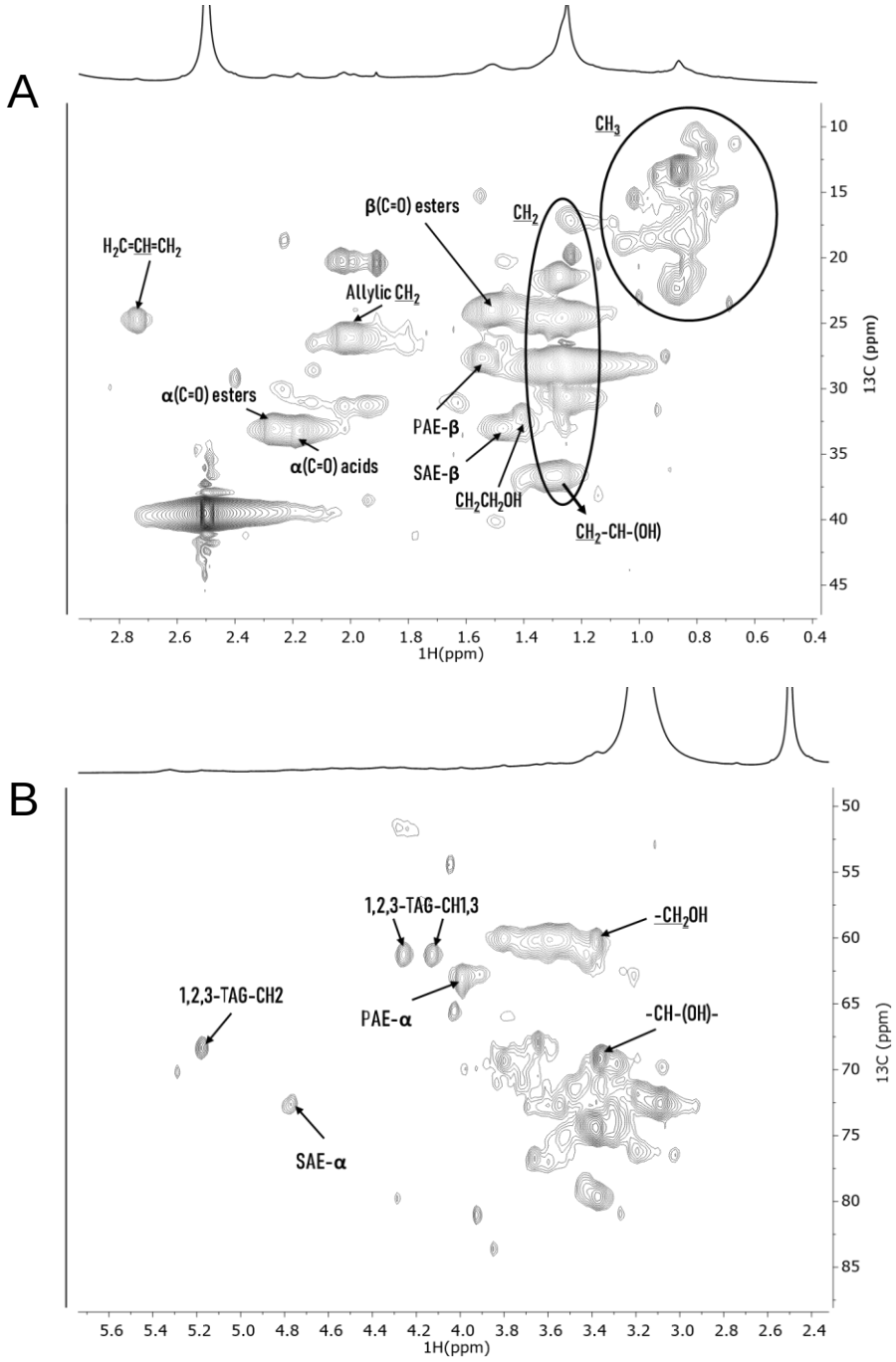


Figure 14: NMR spectral characterization of the peels fraction. 13C- 1H HSQC spectral characterization differentiating the aliphatic (subfigure A) and glycerol CH-acyl (subfigure B) regions. PAE and SAE: primary and secondary aliphatic esters, respectively; TAG: triacylglycerol. Some assignments (unlabeled) are uncertain or unidentified.

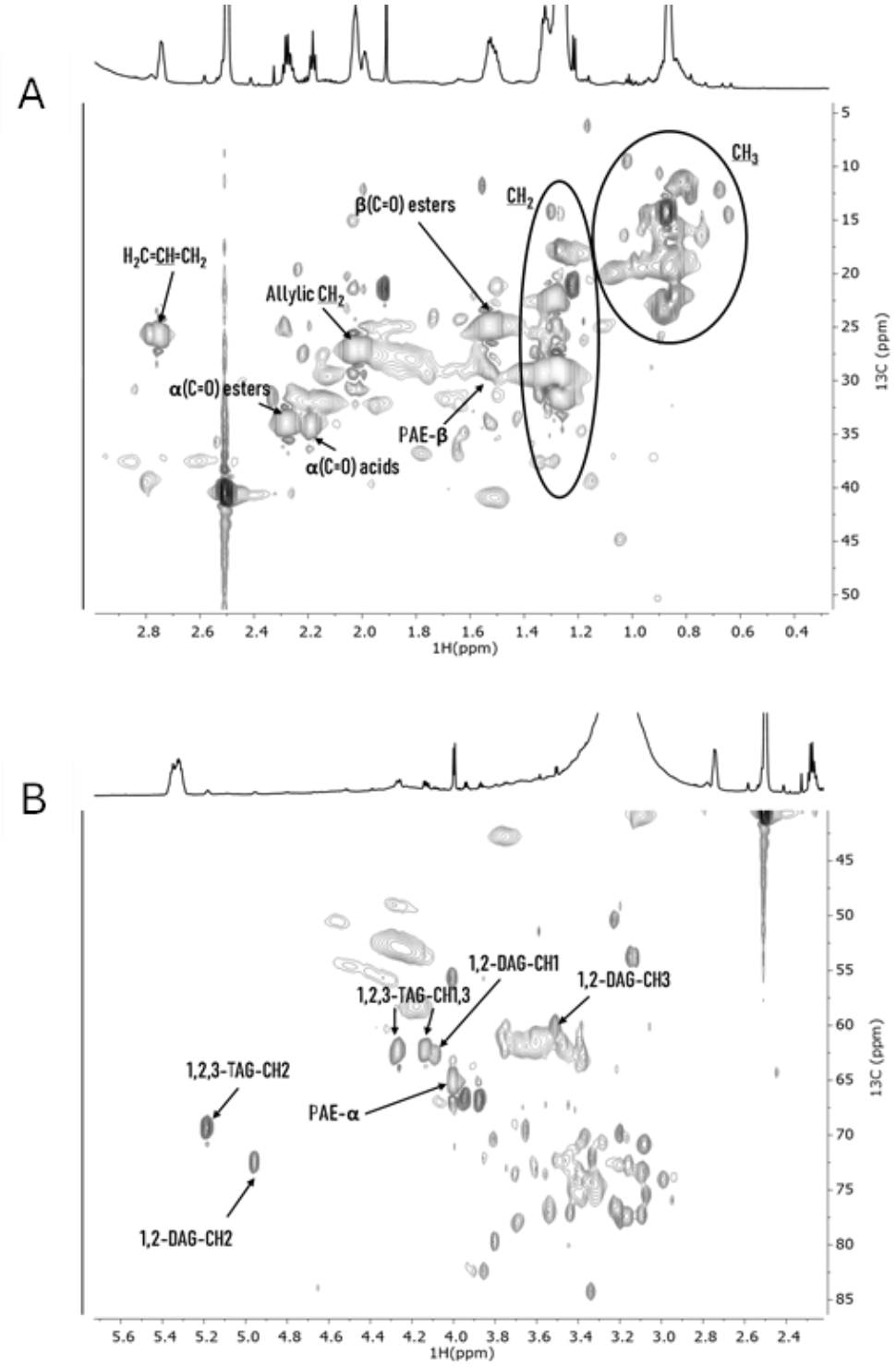


Figure 15: NMR spectral characterization of the seeds fraction. 13C- 1H HSQC spectral characterization differentiating the aliphatic (subfigure A) and glycerol CH-acyl (subfigure B) regions. PAE and SAE: primary and secondary aliphatic esters, respectively; TAG: triacylglycerol. Some assignments (unlabeled) are uncertain or unidentified.

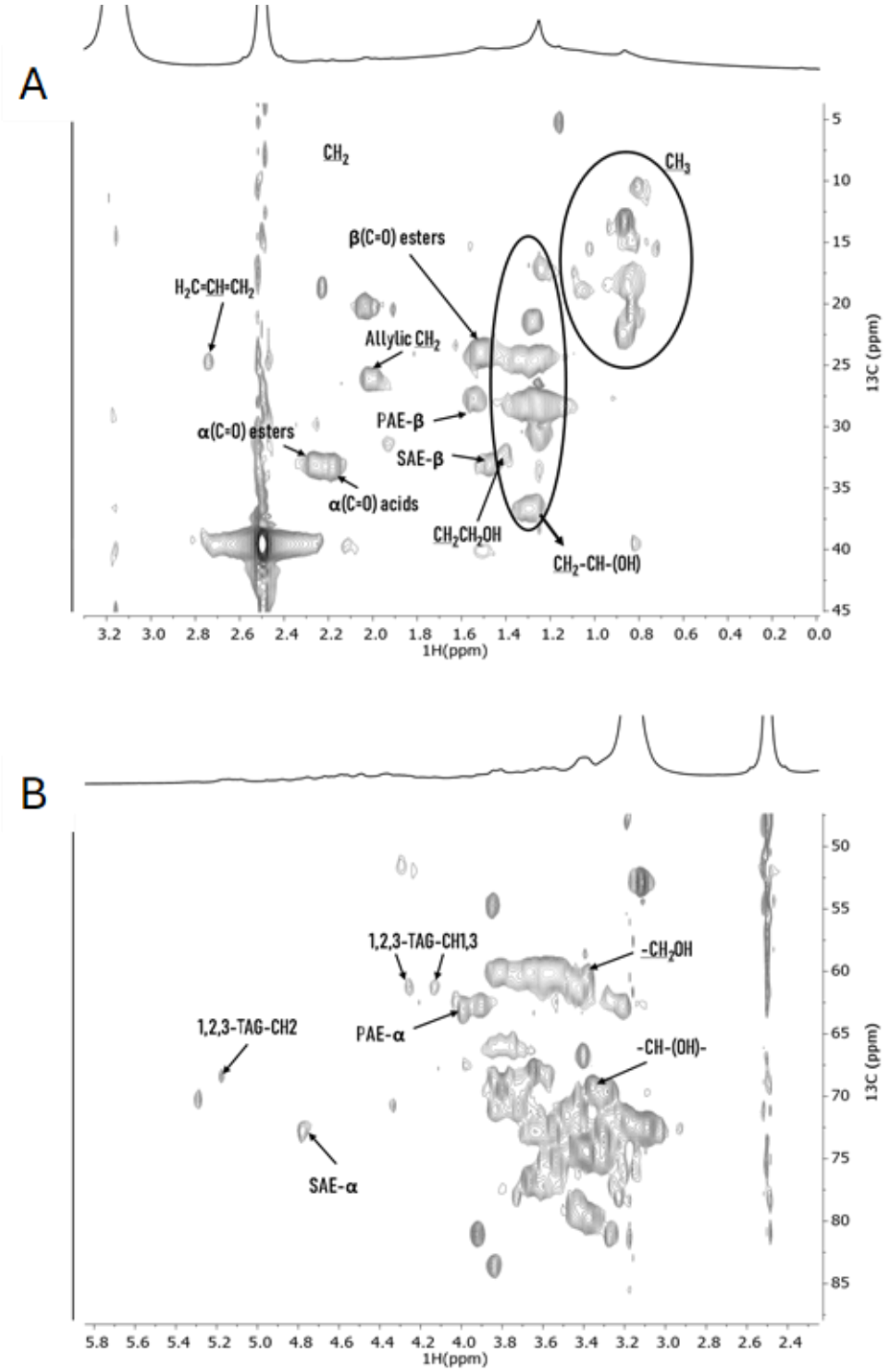
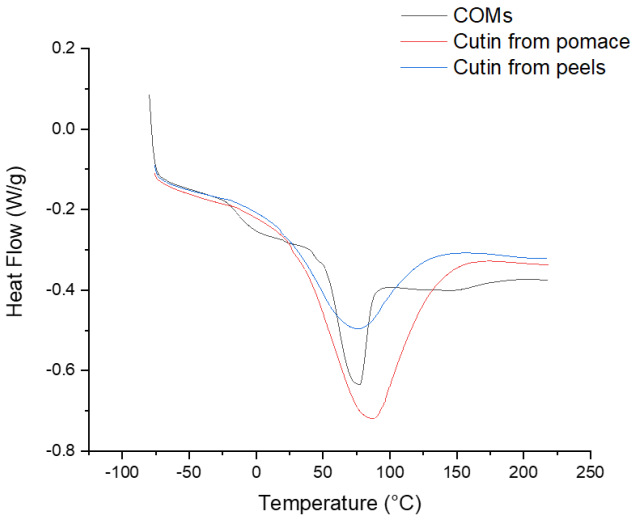


Figure 16: NMR spectral characterization of the cutin from tomato peels. 13C- 1H HSQC spectral characterization differentiating the aliphatic (subfigure A) and glycerol CH-acyl (subfigure B) regions. PAE and SAE: primary and secondary aliphatic esters, respectively; TAG: triacylglycerol. Some assignments (unlabeled) are uncertain or unidentified**.**

Other physico-chemical properties of cutin from tomato peels and COM(P) samples, like the melting temperatures, were determined. The thermal analysis of cutin-rich materials (derived from pomace and tomato peels) and the produced COM(P) reveal reduced heterogeneity in the oligomers compared to either cutin (i.e., less broad melting point) (Figure 17). The data are useful to integrate in WP4 when designing frugal materials.



Sample	Melting Temperature (°C)
Cutin from Tomato Pomace	86.7
Cutin from Tomato Peels	75.7
COM(P)	77.1

Figure 17: Differential scanning calorimetry (DSC) thermograms of the cutin extracted from tomato pomace and tomato peels, and COM(P).

The cutin from peels and the oligomers COM(P) were also characterized for their antimicrobial properties (Figure 18). Initially, a model bacterial *S. aureus* (a Gram+ bacterium) was selected. *S. aureus* NCTC8325 cells (5×10^5 cells/mL) in Mueller–Hinton broth (MHB) media were exposed to both samples at a concentration of 1000 µg/mL (24 h, 37 °C, with continuous agitation).

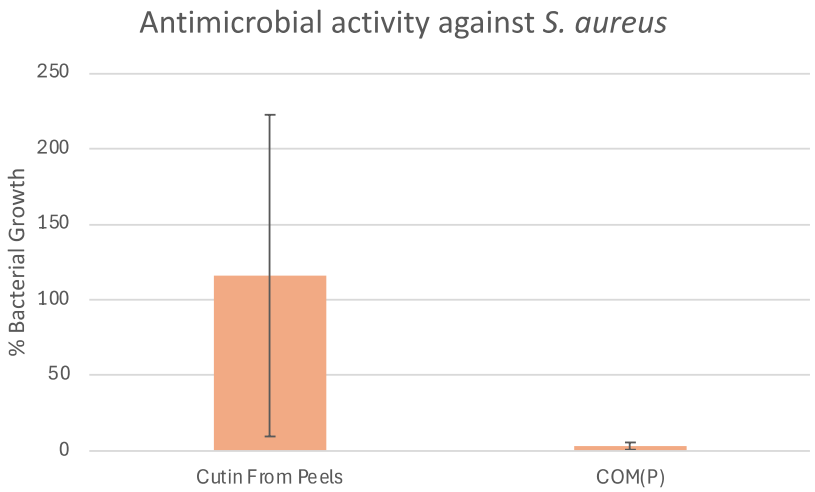


Figure 18: Antimicrobial activity of cutin rich materials from tomato peels and cutin oligomeric mixtures (COM(P)) when exposed to *S. aureus* (Gram +).

The results demonstrate that the cutin-rich materials walled diverse bactericidal esterified oligomeric structures that when released are capable to kill up to 97% of bacteria. However, the cutin-rich materials have no bactericidal effect (Figure 18). Further tests will be done in *E. coli*, a Gram negative bacterium.

To optimize the methodologies and evaluate some of the constrains for cascading between polyester-phenols-proteins we have analyzed samples obtained from traditional medicinal plants which were under study at UNIBO. The collected data were integrated in a manuscript (under proofing); the project will be acknowledged despite that none of the tested plant materials are under study by the consortium.

Conclusions

Cutin-rich polymeric materials can be recovered from either the crude pomace or the peel fraction of the pomace. Their chemistries are compared with minor contamination by compounds comprising triacylglycerol in the materials obtained from the pomace. Both materials are therefore suitable to generate smaller oligomers. The larger oligomers (COMP) were active against a model Gram+ bacterium.

Next steps

The activity of the larger and smaller oligomers against model Gram+ and Gram- bacteria will be tested. The interest of these tests is to identify bactericidal structures which can be used to develop antimicrobial materials in WP4. Once cutin-rich materials are produced using the eutectic solvents (under development), these samples will be processed to generate COMs as means to evaluate if the "quality" of the polymer as a raw material to produce bioactive oligomers is preserved.

Bottlenecks

None anticipated, but the processes are time-consuming.

3.4. Polyphenols extraction (INRAE_IATE)

The extraction of polyphenols from biomass is widely described in the literature[36]. In general, extraction is carried out using organic solvents such as methanol, ethanol, acetone and aqueous solutions of some of these. The use of these hazardous solvents was avoided, and polyphenols were extracted using biobased, biodegradable solvents, namely NADES (Natural Deep Eutectic Solvents). NADES are eutectic mixtures of 2 or 3 natural components that interact by hydrogen bonding, and whose melting point is lower than that of each component. These solvents are prepared by mixing the various components until a liquid phase is obtained.

Following a literature review[37], at INRAE_IATE choline chloride/lactic acid (1:2, mol:mol) with 10% H_2O (v/v) (ChCl/LA) was selected as the most suitable solvent for extracting tomato polyphenols (peels and seeds). Conventional extraction in methanol (MeOH) was used as a reference. Biomass suspensions (100g/L) in the respective solvents were heated at 60°C for 60 min.

The main polyphenols in tomato residues were identified and quantified by UPLC-MS analysis, using acidified water and acetonitrile as UPLC elution solvents[38]. Chromatograms of peels and seeds extracts, in both solvents at 280 nm UV wavelength are shown in Figure 19. Extraction with MeOH allowed the extraction of apolar compounds (retention time (RT) between 9 and 12 min), with a m/z 559 in positive mode. However, their identification was not possible (Fig. 19).

[36] Sridhar et al. (2021). Techniques and modeling of polyphenol extraction from food: a review. Environ. Chem. Lett. 19, 3409-3443.

[37] 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.

[38] Bonnenfant et al. (2022). Biobased and biodegradable polymers in a circular economy context: Understanding quercetin and gallic acid impacts on PHBV thermal properties. Polymer Degradation and Stability, 201, 109975.

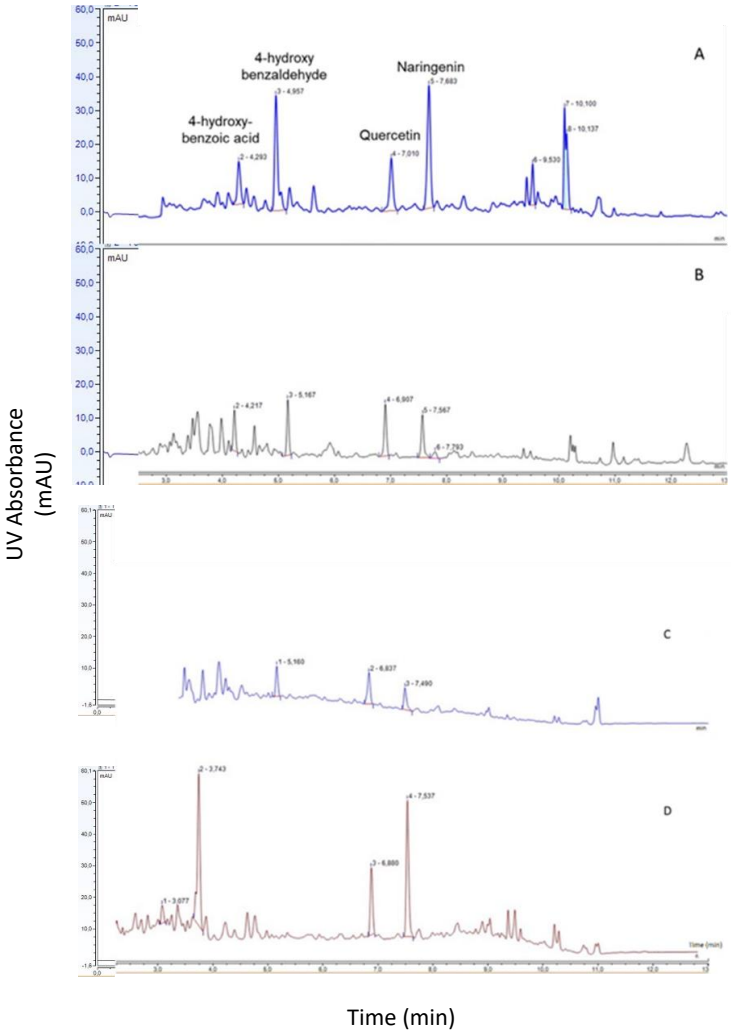


Figure 19: Chromatogram profiles at 280 nm wavelength, of (A) tomato peels extract in MeOH, (B) tomato seeds extract in MeOH, (C) tomato seed extract in ChCl/LA/, (D) tomato peels extract in choline chloride/lactic acid (ChCl/LA). shift in retention time of 4-hydroxybenzaldehyde was observed between peels and seeds extract in NADES. This is due to the interactions between eutectic solvents and some compounds.

For tomato seeds, the same profile of polyphenols composition was observed with lower peaks intensity. No difference in the extraction profiles of tomato residues was observed in ChCl/LA (except the reduction of apolar compounds). The 4 main polyphenols identified in tomato peels and seeds were 4-hydroxy benzoic acid, 4-hydroxybenzaldehyde, quercetin and naringenin (Table 4). As naringenin and naringenin chalcone are isomers of the same molecular weight, they cannot be distinguished by mass spectrometry. Thus, one of them is present in tomato residues. Further studies are required for precise identification. For the time being, this compound will be referred to as naringenin.
The polyphenol content in tomato peels and seeds is shown in Table 4.

Table 4: Polyphenols of tomato peels and seeds extracts in ChCl/LA. RT, retention time.

	Polyphenol	**RT (min)**	**m/z (-)**
Peels/seeds	4-hydroxy benzoic acid	4.29	137.0244
	4-hydroxy benzaldehyde	4.96	121.1193
	Quercetin	7.01	301.0352
	Naringenin/chalcone	7.68	271.0611

In order to improve the extraction yield of the polyphenols of tomato peels, tests were carried out on:

1- *Impact of the extraction time*

100 g of peels in 1 L of solvent were extracted at 60°C, for different times: 60, 90, 120 and 150 min. No modification in polyphenols content was detected for longer extraction times (*result not shown*).

2- *Impact of successive extractions on the same biomass*

Tomato peels at 100 g/L in solvent were extracted a first time at 60°C for 60 min. After centrifugation, the residual biomass was extracted again with fresh solvent under the same conditions. This operation was repeated 2 times. At the third time, no more polyphenols were extracted.

3- *Impact of biomass concentration*

To avoid extraction solvent saturation, suspension of tomato peels at 66 g/L in both solvents was heated at 60°C, for 90 min. Tomato peels polyphenols content, in both solvents under the different conditions cited above (Figure 20).

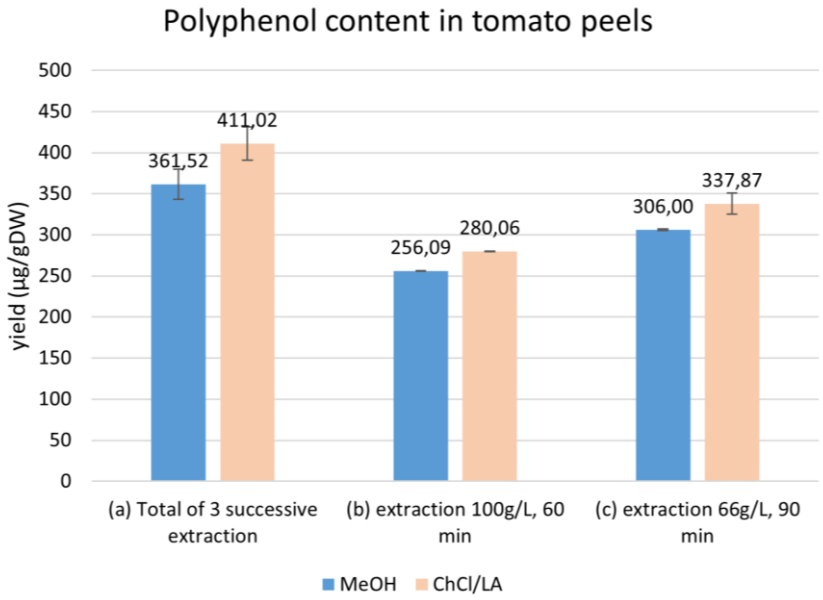


Figure 20: Tomato peels polyphenol content (µg/gDW of peels), in MeOH and ChCl/LA, at 60°C, after (a) 3 successive extractions, (b) one extraction at 100 g/L for 60 min, (c) one extraction at 66 g/L for 90 min. Data are the mean (n=3) ± SD.

As shown in Figure 20, under all conditions, the polyphenol content of tomato peels was higher with NADES extraction. In the case of biomass depletion, by successive extractions, the polyphenol content in NADES was 411.02±20.44 µg/gDW. This represents the maximum extractable polyphenol content when tomato peels are at 100 g/L in solvent. For a single extraction cycle with the same biomass concentration, 70% (280.05 ±0.24 µg/gDW) of the polyphenols are extracted in NADES. Dilution of the biomass suspension to 66 g/L, and heating for 90 min, extracted 75% (305.88±12.88 µg/gDW) of the maximum polyphenol content.

In the case of tomato seeds, 148.41±5.62 µg/gDW of polyphenols was obtained (without any optimization of the extraction reaction).

For future extractions, tomato peels will be extracted at 66 g/L, for 90 minutes, at 60°C, with an additional extraction. Optimization of tomato seeds extraction will be carried out.

In tomato peels and seeds, quercetin and naringenin were present in highest content compared to p-hydroxy benzoic acid and its aldehyde. Indeed, after 60 min extraction at 100 g/L biomass suspension, in ChCl/LA, quercentin was extracted at 116.72±4.49 µg/gDW, naringenin at 109.02±15.16 µg/gDW, while 4-hydroxy benzaldehyde was at 54.31±8.40 µg/gDW (Figure 21). For tomato seeds, quercetin and naringenin were extracted at 89.64±7.94 µg/gDW, and 58.77±3.31 µg/gDW, respectively, while the amount of 4-hydroxybenzaldehyde was negligeable. The quantification of 4-hydroxybenzoic acid is in progress.

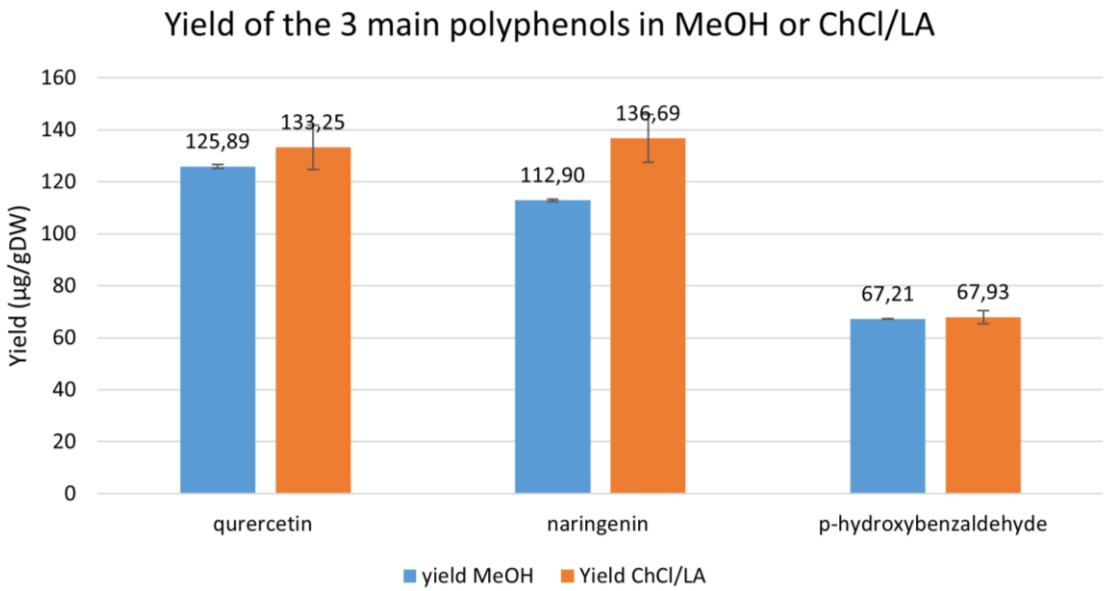


Figure 21: Tomato peels polyphenols (quercetin, naringenin, 4-hydroxy benzaldehyde) content (µg/gDW), in MeOH and ChCl/LA. Data are the mean (n=3) ± SD.

In order to study the composition of the extracts in more detail, the methanolic extract was dried under vacuum and the residue weighed (this is not possible for the NADES extract at present, due to its very low volatility). 6300 µg of extract were obtained from 1 g of peels. HPLC quantification revealed the presence of 280 µg of polyphenols in this extract, meaning that components other than polyphenols were extracted with methanol. In order to explore the overall composition of the extract, an elemental analysis is in progress.

Conclusions

The eutectic solvent choline chloride/lactic acid was used to extract polyphenols from tomato peels and seeds. The same polyphenols (naringenin, quercetin and 4-hydroxy benzaldehyde) were recovered in both residues, with a lower yield in seeds. Compared with the conventional methanol-based extraction method, NADES gave a 14% higher yield.

Next steps

Identification of the composition of the ChCl/LA tomato residue extract (other compounds present in the extracts).
Exchange of tomato residues between partners after their respective extractions.

Bottlenecks

At room temperature, deposits and crystals form in NADES extracts after a certain time. These lead to clogging of the HPLC column. This problem needs to be resolved before continuing with the experiments.

3.5. Carotenoids extraction (INRAE_IATE)

Carotenoids are tetraterpene derivatives and natural pigments found in different plants. The two main carotenoids present in tomato peels are lycopene and β-carotene.
As with polyphenols, carotenoid extraction was performed with NADES and compared with conventional extraction in organic solvents.

Based on a literature review[39], the NADES used for carotenoid extraction was chosen as menthol/lactic acid (8:1 mol:mol) (Men/LA). Conventional reference extraction was performed in hexane/ethanol/acetone (2:1:1 v/v/v), with a biomass concentration of 100 g/L, at 60°C for 60 min. These conditions were selected after testing several conditions of time and temperature.

Identification and quantification of tomato peel carotenoids were carried out by UPLC-MS analysis using methanol and methyl-terbutyl ether as UPLC elution solvents. Chromatogram profiles at 470 nm wavelength of tomato peels extracts for Men/LA and conventional extractions are displayed in Figure 22.

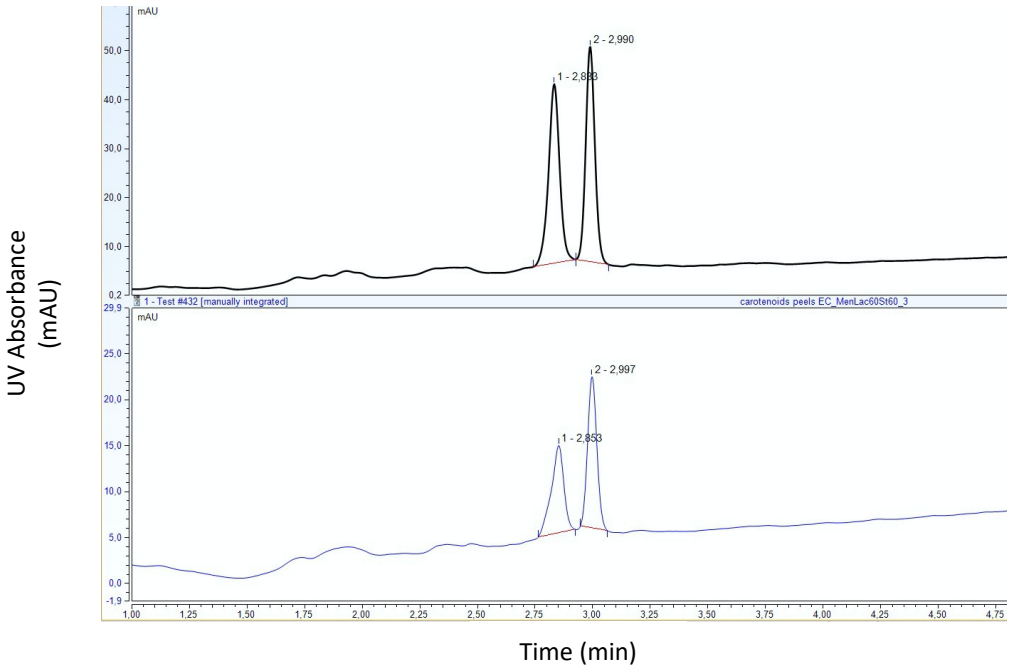


Figure 22: Chromatogram at 470 nm of tomato peels carotenoids. (a) Hexane/ethanol/acetone extraction. (b) Men/LA extraction.

Lycopene and β–carotene content in tomato peels is summarized in Table 5.

Table 5: Carotenoids (µg/gDW) in tomato peels extract. Men, menthol, LA, lactic acid; RT, retention time. Data are the mean (n=3) ± SD.

Carotenoid	RT (min)	m/z (+)	µg/gDW Hexane/ethanol/acetone	µg/gDW Men/LA
Lycopene	2.83	537	22.22 ± 0.075	8.13±0.22
β–carotene	2.98	537	65.0± 0.228	68.18±0.62

For carotenoids too, two successive Men/LA extractions were performed on the plant matrix. In the second extraction11.8% more lycopene and 18.7% more β-carotene were extracted.

At first sight, carotenoid extraction seems less efficient in Men/LA. This is not the final result, and optimizations of the quantification method will be carried out.

According to the literature[40], the lycopene content of tomato skin varies from 5 to 70 µg/g (our results are in line with these estimates).

To check whether coextraction exists in NADES solvents, the presence of polyphenols was investigated in the Men/LA extract, and conversely, the presence of carotenoids was verified in the ChCl/LA extract, using the corresponding UPLC characterization methods. No coextractions were noticed in both cases.

[39] Silva et al. (2019). Sustainable approach for lycopene extraction from tomato processing by-product using hydrophobic eutectic solvents. J. Food Sci. Technol., 56, 1649-1654.

[40] Inbaraj (2008). Tomatoes and tomato products. Book, CRC Press, p 32.

Conclusions

Carotenoids (lycopene and β–carotene) were extracted from tomato peels using a menthol/lactic acid eutectic solvent. A UPLC-based identification and quantification method was developed. Comparison between NADES extractions and conventional organic solvents showed the advantage of organic solvents in terms of extraction yield. These are preliminary results. The quantification of carotenoids will be optimized.

Next steps

The optimization of the UPLC-based quantification of lycopene and β–carotene will be performed.
The order of extraction of polyphenols and carotenoids from tomato peels will be studied in terms of extraction yield and extract composition.

Bottlenecks

No identified bottlenecks for this extraction.

4. Grape pomace

4.1. Feedstock pre-treatment and fractionation (INRAE_IATE)

The grape pomace was supplied by Grap'Sud (France). It is a mixture of peels, seeds and stems (without pre-separation) from the vinification of red grapes. The material was received at INRAE_IATE with a moisture content of 60%, and oven-dried at 60°C for 4 days, to achieve a residual moisture content of 6%. As with the tomato residues, the red grape pomace (RGP) was first ground in a knife mill and an impact mill, before being electrostatically separated under the same conditions.
Results related to RGP dry fractionation are summarized in Table 6.

Table 6: Dry fractionation results of red grape pomace (RGP) after treatment by means of knife mill (SM300), impact mill (UPZ) and tribo flow system (TFS). (+), Positive fraction; (-), negative fraction; D50, median particles diameter.

	SM300	**UPZ**	**TFS**	
Grape pomace	Yield*: 97.0 % D50: 335 µm	Yield*: 89.0 % D50: 190 µm	Yield* (+): 46.0 % D50 (+): 131 µm	Yield* (-): 38.0 % D50 (-): 150 µm

* Yield = ($M_{ground\ powder}/M_{initial\ biomass}$) x 100

Red grape pomace fractions were sent to UNIBO-BIGEA, ITQB-NOVA and INRAE_BIA, for characterization of proteins, cutin and suberin.

4.2. Protein extraction (UNIBO-BIGEA)

4.2.1. Samples initial characterization

At UNIBO, whole red grape pomace was received dried from INRAE_IATE, together with positive (+) and negative (-) subfractions (Table 6) obtained after electrostatic fractionation (Section 3.1.4). Samples were ground and stored at -20°C until further analysis.
An initial protein quantification in dried and grounded samples of grape samples was conducted by Kjeldahl method[41]. Results showed that the whole grape pomace sample contains about 14.0% (w/w) of proteins (total organic N + ammonium), while positive and negative fractions contain respectively 10.0% and 12.1% (w/w) protein contents. As previously reported for tomato samples (Section 3.2) also for grape pomace it was not evidenced a specific enrichment of the proteins between the 2 charged subfractions.

4.2.2. Enzymatic extraction

Proteins were then extracted from whole grape pomace and subfractions following the same procedure outlined in Section 3.2, and using previously selected protease enzymes Bromelain (B), Protamex (P) and Trypsin (T) at the same extraction conditions: 5% (w/w) enzyme/grape pomace gDW ratio, 60°C, 2 hours incubation, 1:5 S/L ratio (2 gDW of sample and 10 mL of milli-Q water), pH 7, 100 rpm shaking.

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

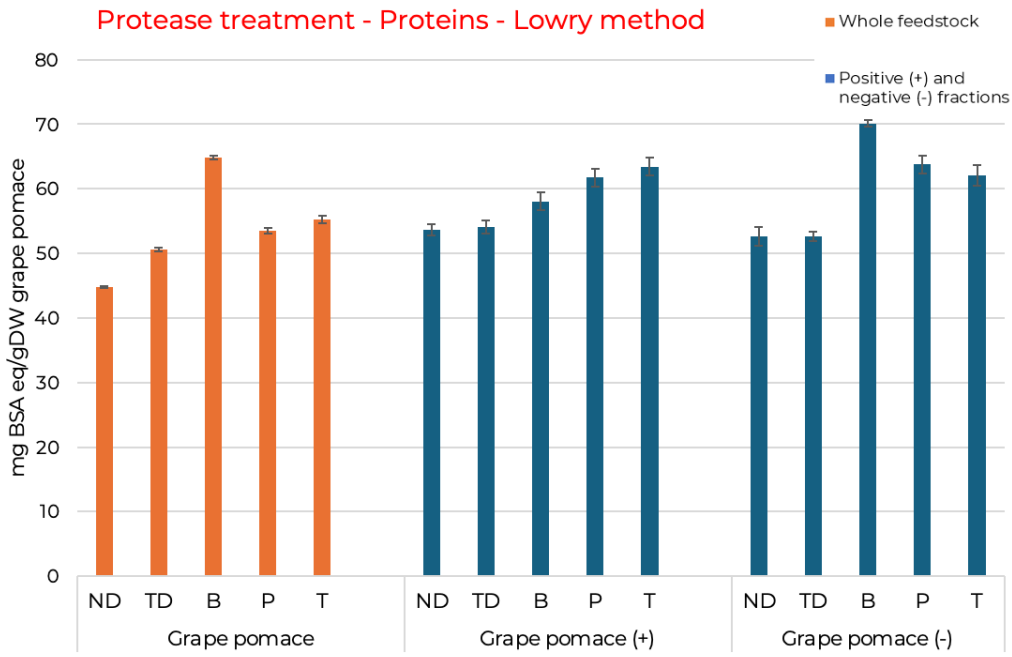


Figure 23: Protein content (mg BSA equivalents/gDW sample) in red grape pomace supernatants following protease treatment. BSA, bovine serum albumin; ND, not digested control, TD, thermally digested control, B, Bromelain; P, Protamex; T, Trypsin. Data are the mean (n=4) ± SD.

After comparison with ND and TD controls data pointed out (Figure 23) small amounts of proteins released after protease digestions of the 3 different feedstocks with very little differences among total grape pomace and positive or negative subfractions. Among the tested enzymes bromelain seemed to be the most efficient especially for total grape pomace and negative subfraction. Overall released proteins were on average 3.5-fold lower than those obtained from tomato seeds (Figure 4).

Conclusions and next steps

Given the previous data, in agreement with other partners of WP2 it was decided to reconsider grape pomace samples for protein recovery in a second or third step of the cascading extraction approach therefore starting from the residues of other extractions (i.e., after polyphenol extraction from INRAE-IATE or cutin extraction from ITQB-NOVA).

4.3. Cutin extraction (ITQB-NOVA)

Cutin-rich materials were extracted from grape pomace using cholinium hexanoate (mixed (1:10 w/w) and stirred for 2 h at 100 °C) at ITQB-NOVA. The reaction was stopped by the addition of ethanol 80 mL per g of cutin. The polymer was recovered by filtration using a nylon membrane filter (0.45 µm) and then washed with an excess of deionized water. The samples are currently drying at 40 °C in an oven.

Conclusions

The analysis of the insoluble rich material obtained from grape is pending, therefore one cannot yet anticipate the yield of the extraction nor the chemistry of the obtained material.

Next steps

To conclude the characterisation of the insoluble material obtained from grape, and possibly also the extractable soluble constituents which may have interest for the extraction of phenolics. To integrate the NADES in this process if relevant. After all test are conclude, the selection of raw materials to proceed will balance extraction

yield, bioactivity and possibility of integration in a cascading approach for the recovery of phenolics and/or proteins.

Bottlenecks

None identified so far but access to grape pomace was delayed.

4.4. Polyphenols extraction (INRAE_IATE)

Red grape pomace is rich in various classes of polyphenols, including stilbenes (such as resveratrol), phenolic acids (gallic acid) and flavonoids (epicatechin), but procyanidins (also known as condensed tannins) are the main class of polyphenols present in grapes. Grape seeds contain 60% of total grape polyphenols, and 50-70% of procyanidins, most of which remain in the pomace[42].

Procyanidins are condensed tannins composed of flavanol monomeric units (epicatechin and catechin), linked together by C4-C8 links. they are a complex mixture of polymers with different subunits, numerous isomeric forms and different degrees of polymerization, making their analysis by HPLC rather difficult.

Extraction of polyphenols from red grape pomace (RGP) from Grap'Sud was carried out in a conventional organic solvent (50:50 aqueous ethanol) and in a NADES (choline chloride/lactic acid, ChCl/LA, 2:1 mol:mol, with 10% H_2O). Both extractions were carried out at 60°C for 60 min. Extracts were then analyzed by UPLC-MS, under the same conditions as for tomato polyphenols.

Figure 24 shows the 280 nm chromatogram profile of the aqueous ethanol extracts of RGP polyphenols. The same profile was observed in ChCl/LA.

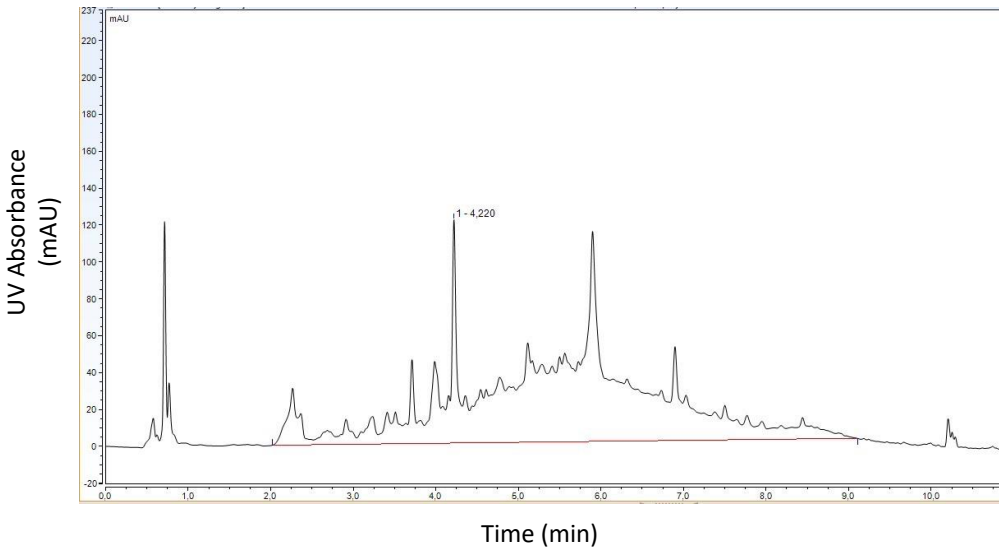


Figure 24: Chromatograms at 280 nm wavelength of polyphenol extracts of red grape pomace in H_2O:ethanol (50:50).

Procyanidins are poorly separated on chromatographic columns due to their high complexity. This is reflected in their chromatogram profile, where a large hump shape is observed without any peak separation. Consequently, a preliminary depolymerization step of these procyanidines is necessary for their analysis by UPLC.

[42] Monrad et al. (2010). Subcritical solvent extraction of procyanidins from dried red grape pomace. J. Agric. Food Chem. 58, 4014-4021.

Depolymerization is generally achieved by acid-catalyzed cleavage of the interflavan bonds linking the subunits, in the presence of a nucleophilic reagent. All extension subunits are trapped by the nucleophile, while the terminal units are rearranged to neutral monomeric flavanol (Figure 25).

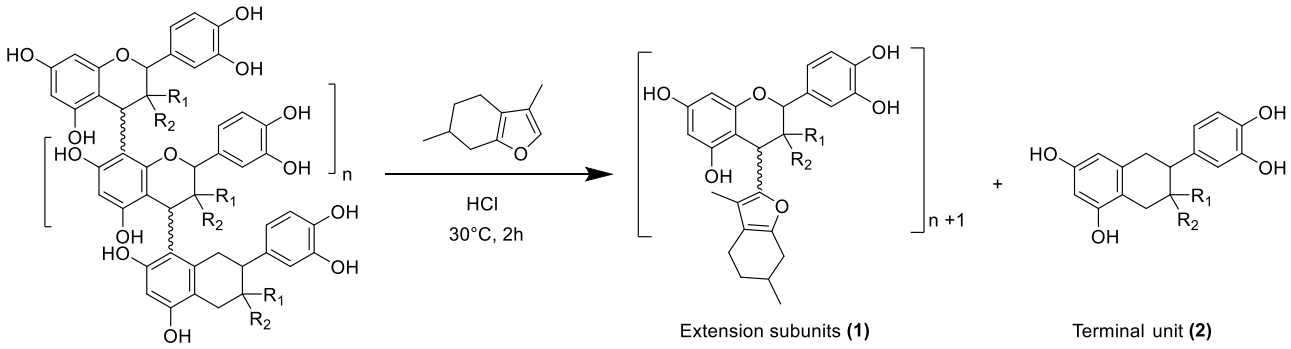


Figure 25: Depolymerization of red grape pomace procyanidins in the presence of menthofuran.

After extraction, the depolymerization reaction of red grape pomace was catalyzed by HCl ethanolic solution, in the presence of menthofuran as nucleophile, under the same conditions described by Billerach et al[43].
Figure 26 displays the chromatograms profile of depolymerized RGP extract in both aqueous EtOH 50:50 (v/v), and ChCl/LA.

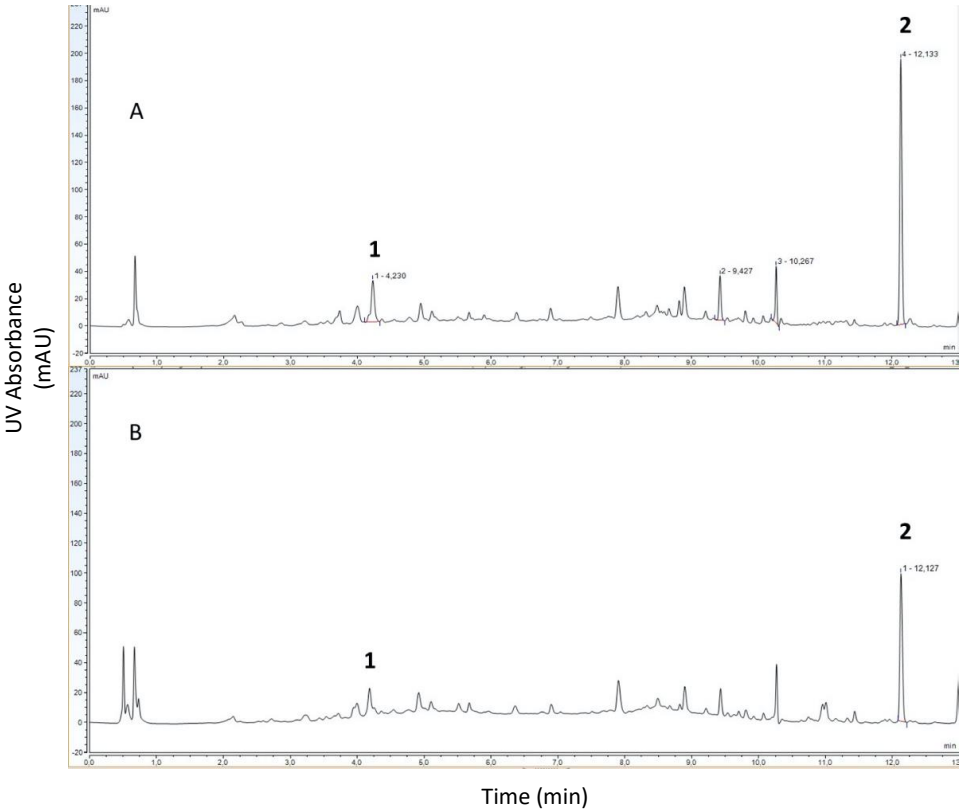


Figure 26: Chromatogram at 280 nm wavelength of depolymerized red grape pomace extract in (A) aqueous EtOH (50:50), and (B) ChCl/LA. (1) represent the peak of the extension subunits, and (2) is the peak of the terminal unit.

[43] Billerach et al. (2020). Furanolysis with mentho-furan: a new depolymerization method for analyzing condensed tannins. J. Agric. Food Chem., 68, 2917-2926.

As depicted in Figure 26, the highest peak corresponds to the extension subunits, while terminal unit (peak 2), appears in more polar region. By considering that extension unit has the same response coefficient in UV, quantification of polyphenols in RGP extracts will be carried out. It is important to note that the procyanidins depolymerization in NADES was never performed before.

Conclusions

Grape pomace polyphenols are mainly composed of procyanidins, flavonoid-based polymers with different degrees of polymerization. Procyanidins were extracted from grape pomace using a choline chloride/lactic acid eutectic solvent. However, their polymeric structure did not allow accurate identification and quantification by UPLC. For this purpose, a depolymerization reaction was carried out in NADES, in the presence of menthofuran and the monomers composing the procyanidins will be identified and their content assessed.

Next steps

Optimization of the depolymerization reaction of RGP in ChCl/LA and monomers quantification.

Bottlenecks and deviations

In this case, too, deposits and crystals form in the NADES extracts over time. These lead to clogging of the HPLC column. This problem is currently being resolved.

5. Brewery spent grain

Brewery spent grain (BSG) samples were received dried from UGENT partner (WP3).

5.1. Protein enzymatic extraction (UNIBO-BIGEA)

BSG samples were ground to a fine powder and stored at 4°C until further analysis.
An initial protein quantification in dried and ground samples of BSG was conducted using the Kjeldahl method[44]. Results showed a content of 28.0% (w/w) of proteins (total organic N + ammonium).
Afterwards, proteins were extracted through enzymatic digestion following the procedure outlined in paragraph 3.2, using previously selected proteases: Bromelain (B), Protamex (P) and Trypsin (T). The extraction conditions remained consistent with those described earlier: 5% (w/w) enzyme/BSG gDW ratio, 60°C, 2 hours incubation, 1:5 S/L ratio (2 g of sample and 10 mL of milli-Q water), pH 7, 100 rpm shaking.

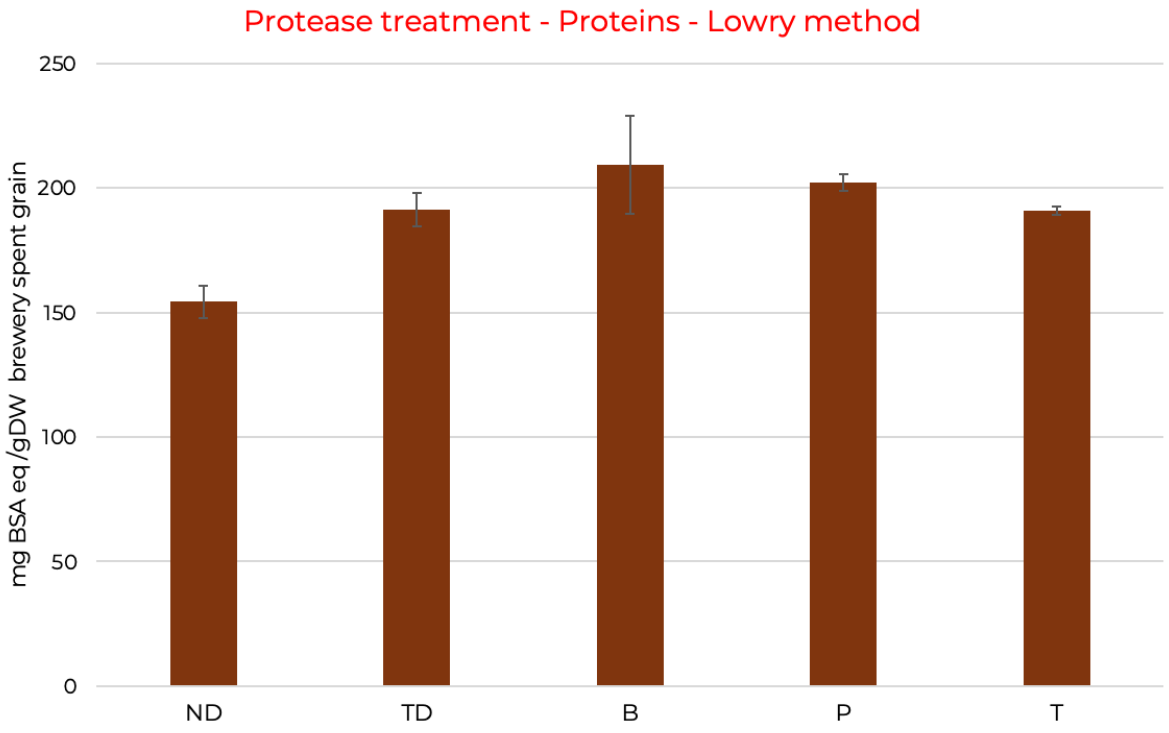


Figure 27: Protein content (mg BSA equivalents/gDW sample) in brewery spent grain supernatants following protease treatment. BSA, bovine serum albumin; ND, not digested control, TD, thermally digested control, B, Bromelain; P, Protamex; T, Trypsin. Data are the mean (n=4) ± SD.

Non-digested (ND) samples refer to BSG with the addition of milli-Q water, followed by immediate centrifugation and separation of solid from liquid, while thermally digested (TD) samples represent aliquots that underwent the same incubation as the protease-treated samples but without the addition of enzymes.
Proteins were quantified by Lowry assay. All three proteases were able to increase protein release with respect to ND (Figure 27). Protein yield is promising, and thus, the feedstock has been selected to proceed with further steps.

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

Additionally, alkaline and neutral solubilization (24 °C, 3 hours, 1:5 S/L) with acidic precipitation (pH 3.5) was tested as well as a protein isolation method. Alkaline extraction involved the use of 0.1 M NaOH at pH 11, while neutral extraction was performed with 0.05 M phosphate buffer at pH 7.2, as described in section 3.2. Preliminary results show comparable yields between alkaline and neutral extraction. However, a noteworthy fraction of peptides, likely originating from previous fermentation processes, appear to remain unprecipitated.

Conclusions

Optimisation of protein extraction from brewery spent grain is in still progress. Both enzymatic treatment (feedstock resuspended in water with solid/liquid ratio 1:5 and incubated 2 hours at 60°C with 5% w/w of Bromelain or Protamex or Trypsin) and protein precipitation (alkaline or neutral solubilisation and acidic precipitation) processes studied for tomato seeds were successfully applied to this feedstock, reaching similar protein/peptide yields.

Next steps

Next steps will be SDS-PAGE gel analyses, to verify molecular weight distribution in the extracts, and peptide sequencing.

Bottlenecks and deviations

According to results obtained up to now, brewery spent grain seems to already contain peptides that originate during the initial steps of beer production process. It can be a positive deviation from the project, making not necessary the use of commercial enzyme. On the other hand, peptides are more difficult than proteins to be precipitated.

6. AGRILOOP Chinese project research progress

6.1. Peanut residues pre-treatment and preliminary characterization for protein extraction (IFST-CAAS)

Peanut residues were provided to IFST-CAAS by SJCOF peanut transformation company. Among these, peanut meal is the product from peanut kernels that are pressed to extract oil. Defatted peanut flour (DPF) is the product of peanut cake meal that has been crushed and defatted.

6.1.1. Analysis of peanut cake meal composition

The basic composition of peanut meal was determined according to national standard method[45], and the total sugar content was determined by the sulfuric acid-phenol method. Data shown in Table 7.

Table 7: Content of basic composition of peanut meal (% of residue wet weight). Values are means (n=3) ± SD.

Moisture (%)	Crude fiber (%)	Protein (%)	Fat (%)	Total sugar (%)	Ash content (%)
4.54±0.04	3.64±0.00	47.28±0.70	3.40±0.06	32.45±0.50	8.78±0.02

6.1.2. Peanut cake meal pretreatment and protein extraction

6.1.2.1. Extraction process of peanut protein

Peanut proteins were isolated from defatted peanut flour (DPF) by isoelectric precipitation, alcohol precipitation, isoelectric precipitation combined with alcohol precipitation and protein isolation (Figure 28). Peanut protein concentrate (PPC) powders were evaluated for proximate composition and functional properties (protein solubility, water holding/oil binding capacity, emulsifying capacity and stability, foaming capacity and gel property).

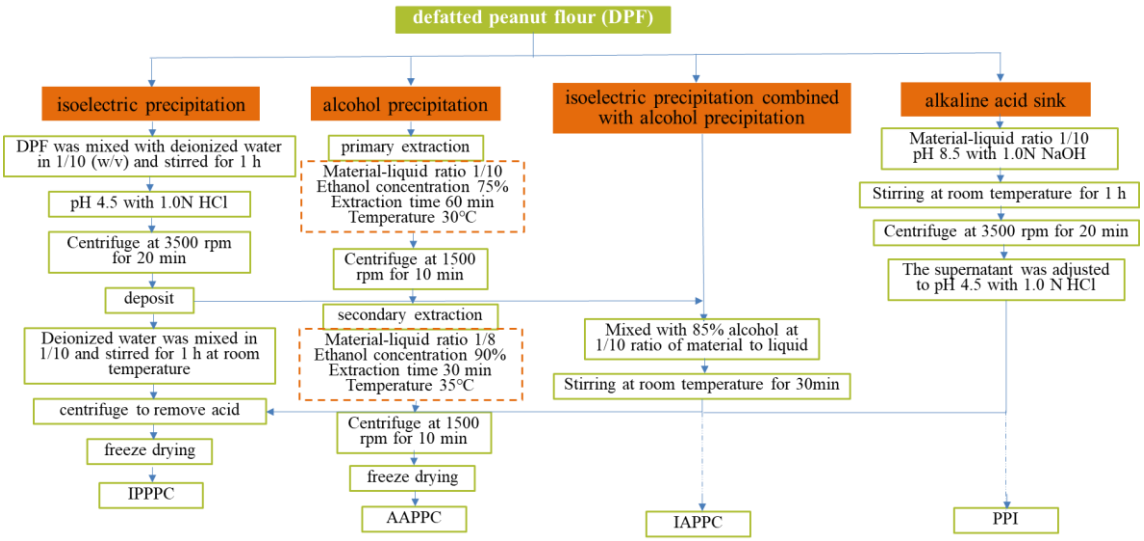


Figure 28: Protein extraction process from peanut meal.

[45] GB 5009.5-2016

6.1.2.2. Chemical composition of peanut protein products

The protein content of peanut protein products from different treatments was significantly higher than that of DPF, while the values of crude fat, total sugar and ash were significantly lower than those of DPF (Table 8).

Table 8: The content of basic composition of peanut meal. All values are means (n=3) ± SD. Means within levers with different letter (a, b, c, d, e) are significantly different ($p < 0.05$). Control DPF: defatted peanut flour; IPPPC: isoelectrically precipitated peanut protein concentrate; AAPPC: alcohol extracted peanut protein concentrate; IAPPC: combined isoelectric precipitation and alcohol extraction peanut protein concentrate; PPI: protein by alkaline solubilization and acid precipitation.

Products	Protein (dry basis %)	Fat (%)	Total sugar (%)	Ash content (%)
DPF	55.88±1.07^{d}	1.50±0.01^{a}	25.14±0.04^{a}	4.85±0.16^{a}
IPPPC	72.35±2.33^{b}	1.13±0.01^{b}	17.97±0.25^{b}	3.05±0.20^{b}
AAPPC	69.04±0.34^{c}	0.70±0.01^{d}	16.06±0.17^{d}	2.06±0.19^{c}
IAPPC	71.49±0.89^{b}	0.84±0.01^{c}	16.46±0.08^{c}	2.03±0.14^{c}
PPI	96.65±0.26^{a}	0.20±0.01^{e}	0.36±0.01^{e}	2.22±0.01c

Among them, the protein content of PPI was the highest, while its ash content was second only to that of DPF followed by isoelectrically precipitated peanut protein concentrate (IPPPC) and combined isoelectric precipitation and alcohol extraction peanut protein concentrate (IAPPC), and the protein content of alcohol extracted peanut protein concentrate (AAPPC) was only 69.04±0.34%, but its crude fat and total sugar contents were significantly lower than those of DPF, IPPPC and IAPPC, indicating that the second alcoholic extraction process could effectively remove lipids and soluble sugars from the peanut protein.

6.1.2.3. Effect of different methods and pH on the solubility of peanut protein products

Most of the functional properties of proteins (proteolysis, water/oil holding, emulsification and emulsion stability, foaming and foam stability, gel properties) influence the organoleptic quality of the food, especially in terms of texture, but also play an important role in the preparation of the food components, the physical properties of the food during processing or storage. So we tested these properties to evaluate the proteins.
The solubility of peanut protein in the range of pH 2.0-12.0 was assessed (Figure 29).

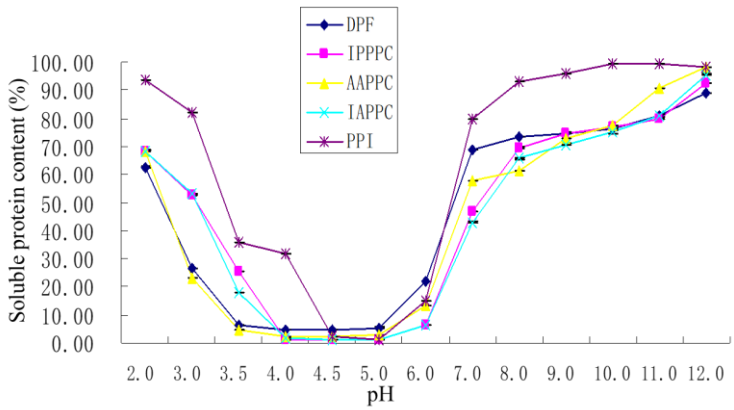


Figure 29: Effects of pH on peanut protein products solubility. Control DPF: defatted peanut flour; IPPPC: isoelectrically precipitated peanut protein concentrate; AAPPC: alcohol extracted peanut protein concentrate; IAPPC: combined isoelectric precipitation and alcohol extraction peanut protein concentrate; PPI: protein by alkaline solubilization and acid precipitation. Data are the mean (n=3) ± SD.

Data showed a trend of decreasing and then increasing with the increase of pH, the solubility decreased to the lowest at pH 4.0-5.0, and the protein solubility increased with the continued increase of pH, and the solubility of protein reached a high point at pH 8.5, and then the solubility of protein continued to increase with the continued increase of pH, and its solubility did not change significantly. When the pH of the solution system was 7.0, the solubility was PPI, AAPPC, IPPPC and IAPPC in descending order.

6.1.2.4. Effect of different methods and pH on the emulsifying activity and emulsification stability of peanut protein products

In Figure 30 the emulsification capacity of peanut proteins obtained from different treatments that all showed a trend of decreasing and then increasing with increasing pH. The turbidimetric method[46] was adopted. Three replications of the experiment were done.

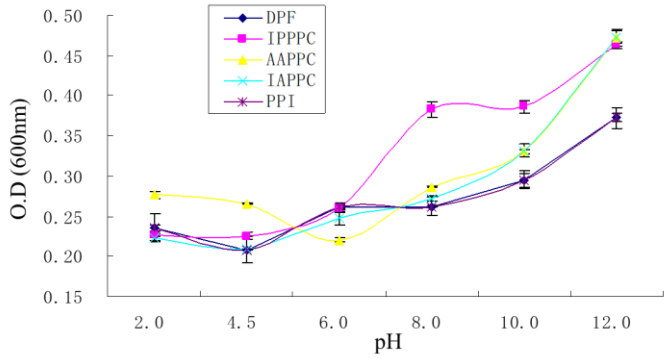


Figure 30: Effects of pH on emulsifying capacity (EC) of peanut protein products. Control DPF: defatted peanut flour; IPPPC: isoelectrically precipitated peanut protein concentrate; AAPPC: alcohol extracted peanut protein concentrate; IAPPC: combined isoelectric precipitation and alcohol extraction peanut protein concentrate; PPI: protein by alkaline solubilization and acid precipitation; O.D., optical density. Data are the mean (n=3) ± SD.

When pH 2.0-4.5, the emulsification capacity showed a decreasing trend, when pH 4.5 (except AAPPC), the emulsification capacity dropped to the lowest, and AAPPC only dropped to the lowest when pH 6.0; with the further increase of pH peanut proteins emulsification capacity all showed a trend of gradual increase in pH 6.0-10.0, the emulsification capacity of IPPPC was significantly higher than the emulsification capacity of other peanut proteins, and reached the maximum when pH increased to 12.

Table 9: Effect of pH on emulsifying stability (min) of peanut protein products. Control DPF: defatted peanut flour; IPPPC: isoelectrically precipitated peanut protein concentrate; AAPPC: alcohol extracted peanut protein concentrate; IAPPC: combined isoelectric precipitation and alcohol extraction peanut protein concentrate; PPI: protein by alkaline solubilization and acid precipitation. Means within levers with different letter (a, b, c, d, e) are significantly different ($p < 0.05$). Data are the mean (n=3) ± SD.

Products	pH 2.0	pH 4.5	pH 6.0	pH 8.0	pH 10.0	pH 12.0
DPF	50.68±1.55^{c}	43.36±1.57^{d}	54.04±3.21^{c}	60.04±0.62^{b}	64.94±0.70^{a}	31.72±1.38^{e}
IPPPC	37.80±1.01^{b}	23.92±1.45^{d}	33.54±3.19^{c}	37.58±0.49^{b}	34.54±1.26^{a}	20.46±0.64^{d}
AAPPC	34.50±1.18^{a}	28.37±2.77^{bc}	29.58±1.59^{b}	30.10±0.23^{b}	36.95±0.65^{a}	24.79±1.25^{c}

[46] McClements et al (2022). Proposed methods for testing and comparing the emulsifying properties of proteins from animal, plant, and alternative sources. Colloids Interfaces, 6, 19.

IAPPC	40.49±0.81[b]	34.44±1.48[b]	37.77±0.06[b]	40.02±3.20[b]	46.43±3.57[a]	35.27±5.48[b]
PPI	16.71±0.40[c]	14.89±0.19[d]	19.18±0.48[b]	20.45±0.19[b]	24.31±1.34[a]	20.36±0.39[b]

As can be seen from the Table 9, there was a significant difference in the emulsification stability between the peanut proteins obtained from different treatments. At the same pH, the emulsification stability of IPPPPC, AAPPC, IAPPC and PPI was significantly lower compared with that of DPF. In particular, the emulsification stability of PPI was the worst. At pH 2.0-4.5, the emulsification stability of peanut protein showed a decreasing trend, and at pH 4.5, the emulsification stability was the worst; then, the emulsification stability gradually increased, and at pH 10.0, the emulsification stability was the best, especially the best for AAPPC, and the worst for PPI; and at pH 10-12, the emulsification stability was significantly reduced.

6.1.2.5. Effect of different methods on the emulsification stability of peanut protein products

Different treatments significantly affected the water/oil binding capacity of peanut proteins (Figure 31).

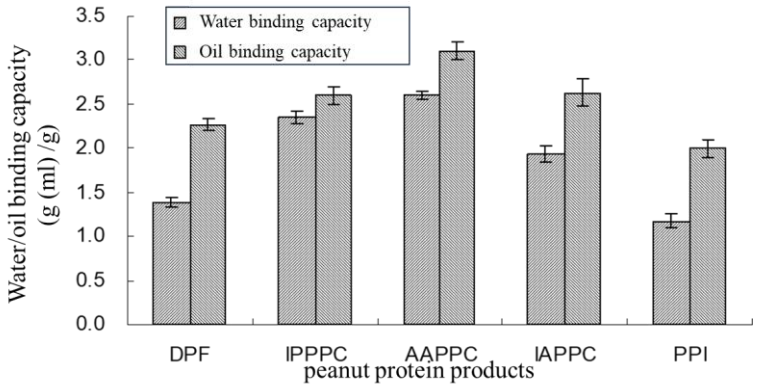


Figure 31: Water holding capacities and oil binding capacities of peanut protein products. Control DPF: defatted peanut flour; IPPPC: isoelectrically precipitated peanut protein concentrate; AAPPC: alcohol extracted peanut protein concentrate; IAPPC: combined isoelectric precipitation and alcohol extraction peanut protein concentrate; PPI: protein by alkaline solubilization and acid precipitation. Data are the mean (n=3) ± SD.

The water/oil binding capacity of IPPPPC, AAPPC and IAPPC were significantly increased compared to DPF, with AAPPC having the best water/oil binding capacity, while the water/oil binding capacity of PPI were significantly decreased.

6.1.2.6. Effect of different methods on the gel property of peanut protein products

The hardness, resilience and cohesion of the PPI gels were significantly different from the rest of the protein products, which may be due to the low content of protein powder in the gel system that fails to form a gel, or it may be due to its poor water/oil binding capacity (Table 10).

Table 10: Gel property of peanut protein products. Control DPF: defatted peanut flour; IPPPC: isoelectrically precipitated peanut protein concentrate; AAPPC: alcohol extracted peanut protein concentrate; IAPPC: combined isoelectric precipitation and alcohol extraction peanut protein concentrate; PPI: protein by alkaline

solubilization and acid precipitation. Means within levers with different letter (a, b, c, d, e) are significantly different ($p < 0.05$). Data are the mean (n=3) ± SD.

Products	Hardness （g）	Resilience*	Cohesion**
DPF	56.70±2.80^{b}	0.90±0.02^{a}	0.56±0.01^{a}
IPPPC	64.96±2.93^{a}	0.86±0.03^{a}	0.52±0.02^{ab}
AAPPC	69.46±3.98^{a}	0.90±0.03^{a}	0.53±0.02^{ab}
IAPPC	57.47±0.58^{b}	0.88±0.03^{a}	0.51±0.02^{b}
PPI	16.43±0.50^{d}	0.58±0.02^{c}	0.48±0.01^{c}

*Cohesion: the relative resistance after the first compression deformation to the second compression deformation, expressed on the curve as the ratio of the positive work done by the two compressions (Area 2/Area 1),
**Resilience: the ratio of the elasticity released by the sample on return from the first compression to the energy dissipated by the probe during compression, expressed on the curve as the ratio of (Area 5/Area 4).

The gel hardness, resilience and adhesion of IPPPC, AAPPC and IAPPC were good, with AAPPC being the best, suggesting that alcohol improves the properties of protein gels.

Conclusions

(1) The functional properties of peanut such as solubility and emulsifying capacity and stability were significantly influenced by pH.
(2) PPI had a solubility profile similar with their emulsifying capacity and stability profile, with minimum solubility observed at 4.0~5.0 and maximum solubility at 8.5 and higher.
(3) AAPPC had better functional properties particularly water holding/oil binding capacity and gel property. Alkali solution and isoelectric precipitation reduced all functional properties of defatted peanut flour while other products had the reverse effect.

Results obtained from this study suggest that the functional properties of peanut protein prepared by alcohol precipitation (AAPPC) were better than that of other peanut protein concentrates by isoelectric precipitation, isoelectric precipitation combined with alcohol precipitation and protein isolation respectively.

Next steps

Optimize the parameters of the alcohol extraction process through the one-way experiment of alcohol extraction of peanut protein and the second orthogonal rotary combination experiment, and determine the alcohol extraction process.

6.2. Peanut residues pre-treatment and preliminary characterization for polyphenol extraction (IFST-CAAS)

6.2.1. Analysis of peanut skin composition

Peanut skin was provided to IFST-CAAS by SJCOF peanut transformation company.
The basic composition of peanut skin was determined according to the national standard method, and the water content was determined by the rapid moisture detector (Table 11).

Table 11: Content of basic composition of peanut skin (% of residue wet weight). Values are means (n=3) ± SD.

Moisture (%)	Crude Fiber (%)	Protein (%)	Fat (%)	Sugar (%)	Ash (%)
4.54±0.04	3.64±0.00	47.28±0.70	3.40±0.06	32.45±0.50	8.78±0.02

6.2.2. Peanut skin pre-treatment and polyphenols extraction

The prepared NADES were uniform liquid at room temperature. Considering the high viscosity of NADESs, the water content of NADESs used in polyphenol extraction is 30% (w/w) (Table 12).

Table 12: Hydrogen bond acceptor (HBA)**,** hydrogen bond donor (HBD), molar ratio and abbreviation of used NADES.

Hydrogen bond acceptor (HBA)	Hydrogen bond donor (HBD)	Molar ratio (HBA: HBD)	Abbreviation
Choline chloride	Lactic acid	1:2	DES-LA
Choline chloride	Citric acid	2:1	DES-CA
Choline chloride	Urea	1:2	DES-Ure
Choline chloride	Ethylene glycol	1:2	DES-EG
Choline chloride	Glycerol	1:2	DES-Gly
Choline chloride	1,2-propanediol	1:3	DES-Pro
Choline chloride	Fructose	2:1	DES-Fru
Choline chloride	Glucose	2:1	DES-Glu

6.2.3. Effects of different solvents on peanut skin polyphenols

The eight eutectic solvents prepared have excellent performance in the extraction of peanut polyphenols. Except for fructose, the polyphenol extraction amount of these solvents is generally higher than that of traditional solvents such as water. It is worth mentioning that when the hydrogen donor is lactic acid and urea, the extraction efficiency of the corresponding NADES solvent reaches the highest level, which is 160.20 ± 1.85 mg polyols extracted per g of peanut skin (mg/g) and 160.82 ± 2.21 mg/g, respectively. When using water as a solvent, the extraction amount of polyphenols is the lowest (68.47 ± 0. 71 mg/g) (Figure 32).
The fixed factor conditions are temperature: 40℃, liquid to solid ratio: 20 mL/g, time: 10 min, ultrasound power: 250 W, water content: 30% (w/w).

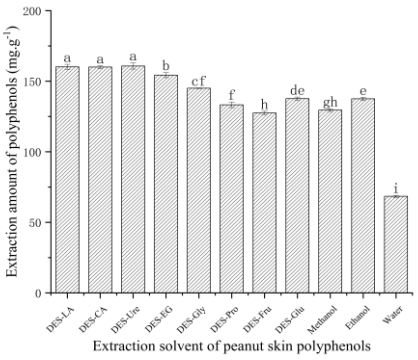


Figure 32: Extraction solvent of peanut skin polyphenols. Different superscript letters indicate significant differences at ($p < 0.05$). All values are means ($n=3$) ± SD.

Quantification of total phenolic content (TPC) after extraction was performed using Folin-Ciocalteu method[47].
As shown in Figure 32, the total phenol yield of DES-LA, DES-CA and DES-Ure was the highest, which was significantly higher than that of traditional solvents such as methanol, ethanol and water.
Considering the difficulty of solvent preparation and the influence of solvent on polyphenol activity, lactic acid was used as hydrogen donor for follow-up experiments.

6.2.4. Effect of different factors on the extraction of polyphenols

The time (5,10,15,20,25,30 min, Fig 33A), liquid-solid ratio (10,20,30,40,50 mL/ g, Fig. 33B), lactic acid / choline chloride molar ratio (3:1, 2:1, 1:1, 1:2, 1:3, Fig. 33C), ultrasonic power (200,250,300,360,400 W, Fig. 33D), temperature (30,40,50,60,70 °C, Fig. 33E), water content (10,20,30,40,50 %, Fig. 33F) were selected to replace the corresponding conventional amount in the single factor variable substitution process, and the effects of various factors on the extraction effect of polyphenols were investigated.

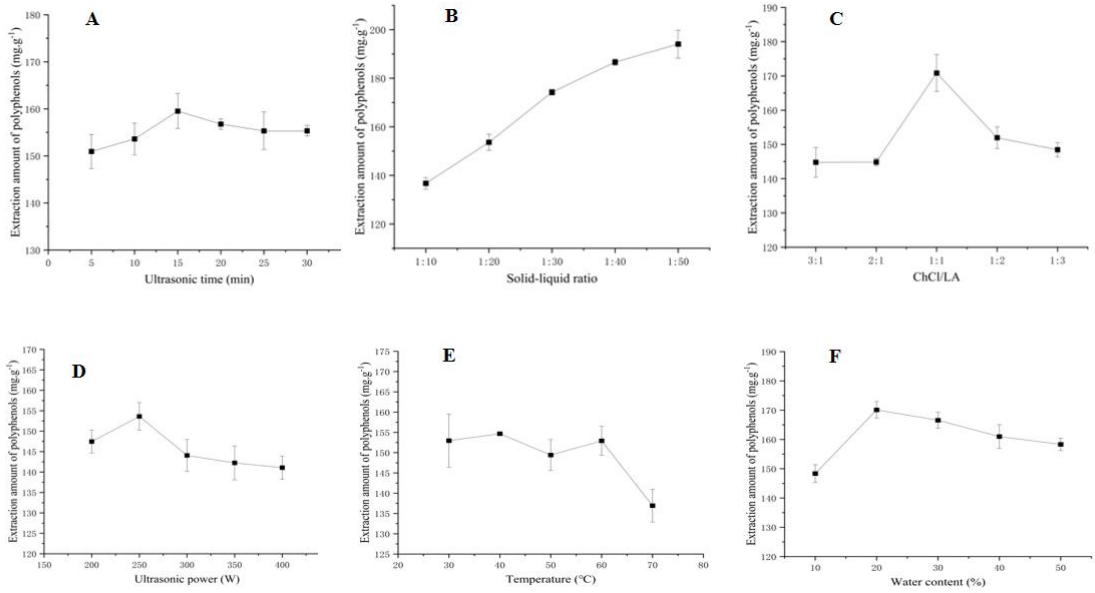


Figure 33: Results of single factor experiment on extraction of peanut skin polyphenols by NADES. All values are means ($n=3$) ± SD.

As shown in Figure 33A, within 5 ~ 15min, with the increase of ultrasonic time, the extraction amount of peanut skin polyphenols increased significantly, and then decreased with the extension of time.
It can be seen from Figure 33B that with the increase of solid-liquid ratio, the extraction amount of polyphenols from peanut skin increased significantly first and then tended to be gentle.
As shown in Figure 33C, When the molar ratio of choline chloride to lactic acid is 1:1, the yield of polyphenols is the highest, and then with the increase of the amount of lactic acid added, the yield of total polyphenols gradually decreases.
As shown in Figure 33D, when the ultrasound power was 250W, the extraction amount of peanut skin polyphenols was the highest, and then decreased with the increase of the ultrasound power.
It can be seen from Figure 33E that the extraction amount of polyphenols from peanut skin increased first and

[47] Xiu et al. (2023). Deep eutectic solvent combined with ultrasound technology: A promising integrated extraction strategy for anthocyanins and polyphenols from blueberry pomace. Food Chem., 422, 136224.

then decreased with the increase of temperature, and reached the maximum at 40 °C.
As shown in Figure 33F, with the increase of water content, the extraction amount of polyphenols from peanut skin showed a trend of increasing first and then decreasing, and reached the maximum when the water content was 20 %.

6.2.5. Orthogonal test

Considering the industrial production practice, the ratio of material to liquid was controlled as 1:10, the ultrasonic power and the molar ratio of choline chloride and lactic acid were fixed at the same time, and the optimal extraction process was determined through orthogonal experiments. The experimental factors and levels are shown in Table 13.

Table 13: Orthogonal test design and test results. All values are means (n=3) ± SD.

No.	A Temperature	B Water content	C Time	Extraction amount of polyphenols (mg/gDW)
1	1	1	1	141.790
2	2	2	2	153.697
3	3	3	3	150.839
4	1	1	2	137.989
5	2	2	3	159.735
6	3	3	1	156.057
7	1	1	3	144.477
8	2	2	1	152.523
9	3	3	2	146.863
K1	446.325	424.257	455.370	
K2	461.781	468.954	438.549	
K3	443.862	458.760	458.052	
k1	148.775	141.419	151.79	
k2	153.927	156.318	146.183	
k3	147.954	152.920	152.684	
Primary and secondary order			B>C>A	
Optimal level	A2	B2	C3	
Optimal combination			**A2B2C3**	

The results of orthogonal test showed that the optimal combination of extraction technology was $A_2B_2C_3$, that is, the extraction temperature was 40°C, the extraction time was 20 minutes, and the water content of NADES was 30%.

6.2.6. Verification test

According to the orthogonal test, three parallel experiments were carried out according to the optimal extraction process of choline chloride/lactic acid molar ratio 1: 1, material-liquid ratio 1:10, ultrasonic power 300 W, ultrasonic temperature 40 °C, ultrasonic time 20 minutes, and NADES water content 30% (Table 14).

Table 14: Validation experiments for the orthogonal optimization experiments.

No.	Extraction amount of polyphenols (mg/g DW)	Average value (mg/g DW)
1	158.031	
2	157.689	158.072±0.404
3	158.495	

6.2.7. Antioxidant activity of polyphenol extracts from peanut skin

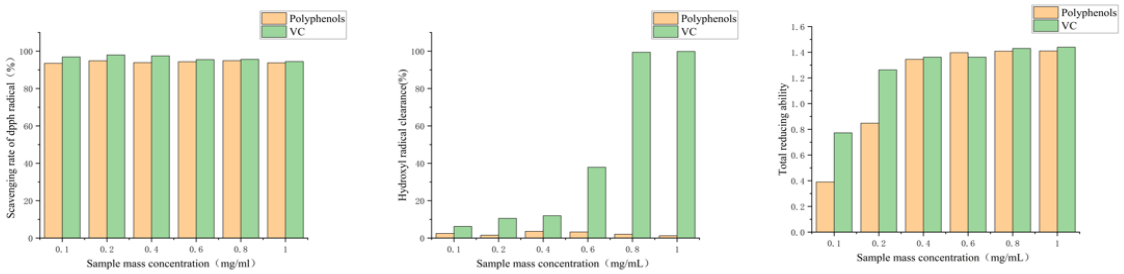


Figure 34: Different concentrations of peanut skin polyphenol and ascorbic acid (VC) solutions were prepared respectively, and VC was used as positive control. DPPH (2,2-diphenyl-1-picrylhydrazyl), hydroxyl radical scavenging experiments and total reduction ability were used to verify the antioxidant ability of red coat polyphenols.

Polyphenols from peanut skin have good DPPH (2,2-diphenyl-1-picrylhydrazyl) free radical scavenging activity (Figure 34A). The hydroxyl radical scavenging ability of red skin polyphenols was poor, a big gap with the same concentration of ascorbic acid (Figure 34B). At the concentration of 1 mg/mL, the total reducing power of peanut skin polyphenols was comparable to that of ascorbic acid (Figure 34C).

Conclusions

Based on single factor experiment and orthogonal experiment, the optimum extraction process of peanut red skin polyphenols by ultrasonic-assisted deep eutectic solvent extraction was determined. The yield of polyphenols was significantly higher than that of traditional solvents.

Peanut skin polyphenol extract was obtained by macroporous resin adsorption purification and freeze-drying. The extract had good antioxidant capacity.

Next steps

It is planned to use liquid chromatography and mass spectrometry to identify the composition of polyphenols in the extract, and to clarify the effects of different extraction processes on the extraction rate of active components and the composition of the extract.

6.3. Potato residues pre-treatment and preliminary characterization for fiber extraction (IFST-CAAS)

6.3.1. Planting area and annual production of potato in world and top 10 countries

Potato (*Solanum tuberosum* L.) is the most important food crop after wheat, maize and rice, with a global production of 374.78 million tons in 2022, and China has the largest planting area and annual production in the world [48].

6.3.2. Potato starch processing process and its by-products

In China, potatoes are mainly used for processing starch (Figure 35), which generates a large amount of waste such as residues and wastewater. According to statistics, approximately 4.5-5.5 tons of fresh residues and 10-20 tons wastewater are generated from 1 ton of starch production. Research has shown that potato residues and wastewater contained various nutritional and functional components, such as dietary fiber, protein, soluble sugars, etc. However, only small amounts of potato residues are used for animal feed, and most of them are discarded as waste directly, resulting in serious resource waste and environmental pollution.

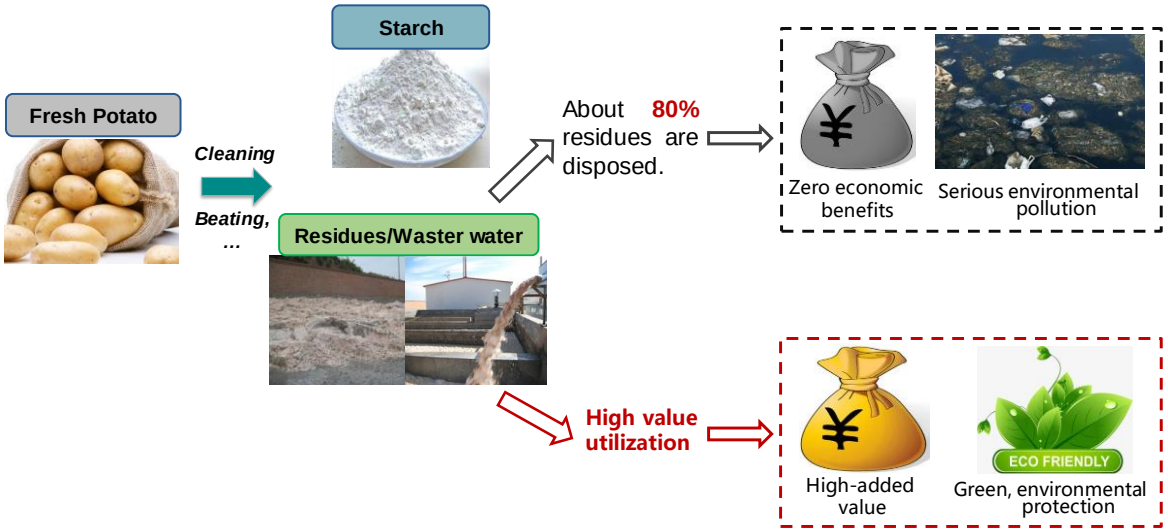


Figure 35: Current status of potato starch processing.

6.3.3. Proximate composition of potato residues and its dietary fibers

Potato residues were obtained from potato starch processing industry (Inner Mongolia Autonomous Region, China).

Starch and dietary fiber was the main composition in potato residues (Table 15, Figure 36). Further, the content of cellulose, pectin, hemicellulose, and lignin in potato residues was about 21.51%, 10.86%, 5.59%, and 2.79%, respectively.

The extraction processes of potato dietary fiber were optimized using thermostable α- amylase, the extraction rate and purity of potato dietary fiber was 85.18% and 92.78%, respectively.

[48] http://www.fao.org/faostat/en/#data/QCL

Table 15: The proximate composition of potato starch processing residues and dietary fibers (g/100 gDW). The data are mean (n=3) ± (SD).

Composition	Potato residues	Potato dietary fiber
Starch	54.96±2.86	1.19±0.36
Ash	1.34±0.01	1.56±0.09
Protein	6.35±0.05	3.52±0.18
Fat	0.32±0.02	0.27±0.04
Total dietary fiber	34.40±0.33	92.78±0.57
Insoluble dietary fiber	26.71±0.73	73.18±0.32
Soluble dietary fiber	7.69±1.79	19.60±1.53

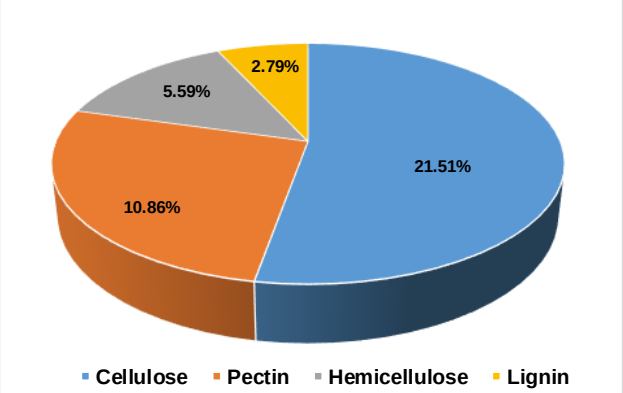


Figure 36: The content of cellulose, pectin, hemicellulose, and lignin in potato starch processing residues (g/100 gDW).

6.3.4. Monosaccharides and uronic acids of potato dietary fibers

Six monosaccharides and 2 uronic acids were detected in potato dietary fiber (Table 16). The total dietary fiber and insoluble dietray fiber mainly contained glucose, soluble dietary fiber mainly contained galactose and galacturonic acid (Table 16).

Table 16: Monosaccharides and uronic acids content of total, insoluble, and soluble dietary fibers. Letters in the same row represent significant difference using Duncan's method (g/100 gDW).

Monosaccharides and uronic acids	Total dietary fiber	Insoluble dietary fiber	Soluble dietary fiber
Rhamnose	3.93±2.07b	1.94±1.92c	7.58±7.76a
Arabinose	7.14±3.34a	7.82±8.71a	4.83±4.7b
Xylose	0.64±0.34a	0.74±0.78a	0.07±0.01b
Mannose	0.12±0.10a	0.11±0.45a	0.16±0.05a
Galactose	33.97±3.27b	17.54±19.32c	58.59±68.79a
Glucose	9.76±6.19a	40.24±9.89a	2.36±15.33b

Galacturonic Acid	4.34±10.02ab	2.86±3.24b	5.69±4.87a
Glucuronic Acid	0.16±0.04ab	0.12±0.59b	0.24±0.22a

Conclusions

Potato residues are rich in starch and dietary fiber, and the potato dietary fiber can be increased from 34.40 g/100 g to 92.78 g/100g after the starch is removed by thermostable α-amylase. Compared to total and insoluble dietary fiber, soluble dietary fiber consists of larger amount of galactose.

Next steps

(1) The high hydrostatic pressure assisted enzymatic modification method will be used to improve the content of soluble dietary fiber, the parameters for high hydrostatic pressure, reaction time, and cellulase addition will be optimized.

(2) IC, NMR, FTIR, etc., can be applied to clarify the chemical composition, structural characteristics, and biological activities such as hypoglycemic and blood lipids-lowering of modified potato dietary fiber.

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

6.4. Apple pomace pre-treatment and preliminary characterization for polyphenol extraction (IFST-CAAS)

6.4.1. Comparison of apple pomace from concentrated juice (FC) and not concentrated juice (NFC) dried with different drying methods

The wet apple pomace, both from concentrated apple juice (FC) and from not concentrated apple juice (NFC), were provided to IFST-CAAS by SDIC apple juice production company.
The residues were dried with different drying methods, which are hot air drying 60°C (HAD 60), heat pump drying 60°C (HPD 60), and freeze drying (FD), with the industrial dried pomace (ID) as the control. As shown in Figure 37, the industrial dried sample has shown loose granular form, while the samples dried with other drying techniques have shown larger granules. The color of FC apple pomace appeared slightly darken than that of NFC apple pomace, suggesting that FC pomace undergone more pronounced browning during the juice production.

6.4.2. Moisture content of apple pomace from concentrated apple juice (FC) and from not cencentrated apple juice (NFC)

As shown in Table 17, the initial moisture content (MC, wt) of FC apple pomace was 77.53±0.87%, slightly higher than that of NFC apple pomace (76.54±0.61%). The final moisture content of industrial dried apple pomace was 17.83±0.10%. For the other samples dried in the lab with different drying techniques, the moisture content of the apple pomace was all below 1%.

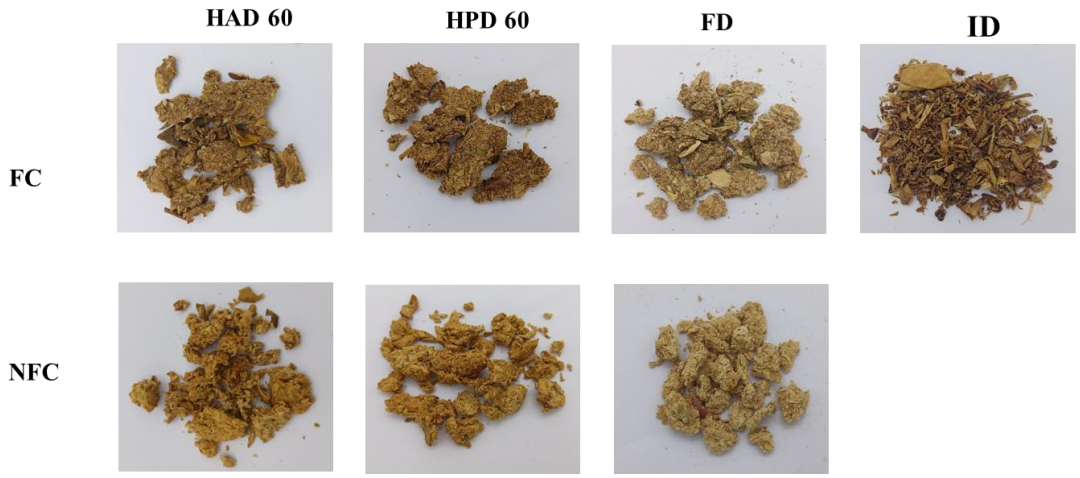


Figure 37: Comparison of the morphology of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) under different drying methods. HAD 60: hot air drying 60°C; HPD 60: heat pump drying 60°C; FD: vacuum freeze-drying; ID: industry drying.

Table 17: Moisture content (MC, wt) of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC). HAD 60: hot air drying 60°C; HPD 60: heat pump drying 60°C; FD: vacuum freeze-drying; ID: industry drying. All the experiments were conducted in triplicates and expressed as the mean ± SD.

	FC (wt %)	NFC (wt %)
Initial MC	77.53±0.87%	76.54±0.61%
ID	17.83±0.10%	
HAD	0.007±0.01%	0.011±0.01%
HPD	0.004±0.01%	0.006±0.01%
FD	0.002±0.01%	0.007±0.01%

6.4.3. The color of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) with different drying methods

For FC samples, industry dried and vacuum freeze-dried sample exhibited higher brightness and redness values. Hot air dried and heat pump dried samples had comparable colorimetric parameters. Compared to FC samples, NFC samples exhibited greater brightness and redness values (Figure 38).

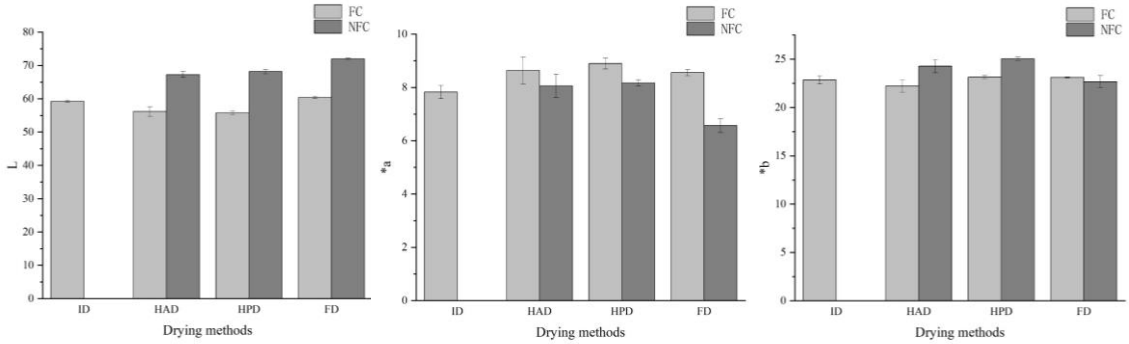


Figure 38: Color analysis of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) with different drying methods. HAD 60: hot air drying 60°C; HPD 60: heat pump drying 60°C; FD: vacuum freeze-drying; ID: industry drying. Bars represent the mean (n=3) ± SD.

6.4.4. The total polyphenols content of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) with different drying methods

NFC apple pomace exhibited higher total polyphenol content (Figure 39) presumably due to the antioxidants added during the fruit juice processing, protecting the polyphenols from degradation. Besides, NFC apple pomace was more susceptible to the drying process. Vacuum freeze-drying proved to be the most effective method for preserving total polyphenols in NFC samples. FC apple pomace processed under industry drying also demonstrated relatively high total polyphenol content, possibly for immediate drying after apple juice extraction, minimizing phenol degradation during the subsequent storage. The other FC apple pomace, transported under freezing conditions prior to laboratory drying, might have undergone phenol loss during these processes.

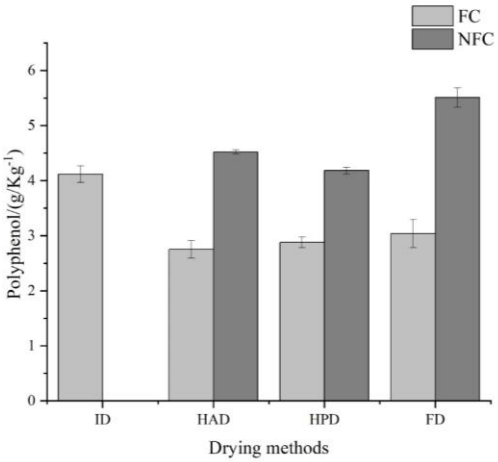


Figure 39: Polyphenol content of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) with different drying methods. HAD 60: hot air drying 60°C; HPD 60: heat pump drying 60°C; FD: vacuum freeze-drying; ID: industry drying. Bars represent mean (n=3) ± SD.

6.4.5. The total flavonoids content of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) with different drying methods

The flavonoid content in industrial dried apple pomace was the highest, indicating that immediate drying was most beneficial for the preservation of flavonoid compounds (Figure 40). Flavonoids can be degraded during the freezing, storage, and the subsequent drying processes. However, the flavonoid content in FC apple pomace was not significantly affected by the drying method. In contrast, the content of flavonoids in NFC apple pomace was greatly affected by drying method. The total flavonoid content was highest in NFC apple pomace samples processed using vacuum freeze-drying.

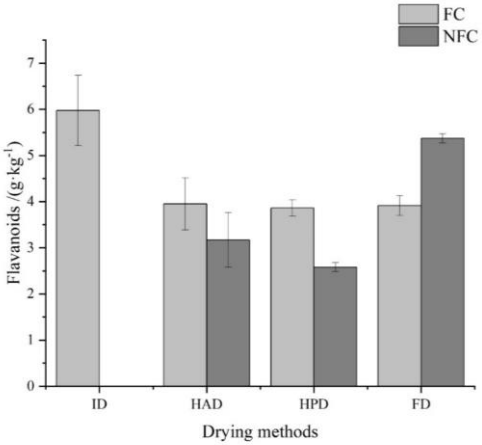


Figure 40: Flavonoids content of apple pomace from concentrated apple juice (FC) and from not concentrated apple juice (NFC) with different drying methods. HAD 60: hot air drying 60°C; HPD 60: heat pump drying 60°C; FD: vacuum freeze-drying; ID: industry drying. Bars represent mean (n=3) ± SD.

Conclusions

Compared to FC, NFC apple pomace exhibits superior color, higher total phenol content, and higher total flavonoid content. Immediate drying in the factory is most conducive to the retention of nutrients. Among

the three laboratory drying methods, vacuum freeze-drying resulted in the highest total phenol and total flavonoid content in apple pomace.

7. Conclusions

The work performed in WP2 (EU, WP2 Tasks 2.1 and 2.2; CN WP2 Tasks 2.1-2.4) during the first 18 months of EU-AgriLoop and the first 8 months of CN-AgriLoop, brought at the development of several green pre-treatments and extractions processes for targeted and highly demanded molecules (i.e., proteins, polyphenols, carotenoids, cutin, fibers) from a wide range of agro-residues, specifically in Europe tomato pomace, grape pomace, brewery spent grain, and in China peanut cake meal, peanut skin, apple pomace and potato waste.

- **Protein** extraction by enzymatic hydrolysis using proteases such as bromelain, papain and trypsin (free and immobilized) and by microbial treatments, revealed that tomato seeds and brewery spent grains (from WP3) should be considered as a priority for protein recovery, in Europe. In China, proteins were extracted from peanut meal and the alkaline/acid precipitation process was that yielding the highest content of proteins, while the most promising properties (water holding/water binding, gel property) were found for alcohol precipitated proteins. Precise characterization of proteins/peptides (SDS-PAGE gel analyses, and LC-MS/MS sequencing) from different residues and the techno-functional and bioactivity properties of the peptide/protein extracts are actually under evaluation. Selected fractions will be sent to WP4 (Task 4.1) for the production of functional materials.

- **Cutin** in the form of polymer and monomers mixture, was extracted from tomato peels. An effort was made to use a more sustainable process by replacing organic solvents (DMSO) with ethanol (for precipitation) and to substantially shorten the reaction time. Very high yield (80% w/w) and high purify final fractions were obtained. Cutin depolymerization by means of different alkaline media was evaluated to assess the impact of the process on purity and monomer/oligomer structures. The fingerprint of cutin polymers and oligomers was revealed by NMR. The bactericidal action of the cutin oligomer mixture showed to be effective in killing up to 97% of *S. aureus* (Gram + bacteria). Next tests will be extended also on *E. coli* (Gram – bacteria). Some cutin fractions have already been delivered to WP4 for material testing.

- **Polyphenols** were extracted from tomato residues and grape pomace at the European side, and from peanut skins at the Chinese side. In the case of tomato and peanut skin, , choline chloride-based eutectic solvents (NADES) were used, and NADES extraction showed better yields compared to traditional organic solvents. UPLC-UV-MS was applied to identify and quantify the main phenolic compounds in tomato skin, namely quercetin and naringenin. The extraction of polyphenols from apple pomace by NADES, and the precise identification of polyphenols from peanut skins, are currently underway. Similarly, the extraction of carotenoids from tomato peels has been carried out by NADES at European level, and lycopene and B-carotene have been identified as the main components .- **Dietary fibres** were extracted from potato waste with a high yield of 85% and characterised for their composition. Insoluble and soluble dietary fibres from potato waste were respectively richer in glucose and galactose.

Further optimization of the extraction processes is in progress in order to obtain higher purity and more targeted techno-functional characteristics of the extracted molecules or more sustainable and scalable extraction processes.

A few bottlenecks were identified, in particular microbial contamination of tomato pomace raw materials. This problem was overcome by the development of a drying process by the producer, which took advantage of the heat generated by internal biogas production.. In addition grinding processes reduced bacterial contamination, and ionic liquid extraction of cutin eliminated it. On the other hand, no evidence of harmful contamination with hazardous metals has been established on tomato pomace. These analyses will be fed into WP1 (T1.3), focused on mycotoxin and chemical contamination.

The initial scarce availability of some feedstock was also resolved by contacting additional producers.

8. It should be mentioned that, for this interim deliverable, we have deliberately chosen to separate the European and Chinese activities into two distinct sections, although common target molecules and extraction methods are used in some cases. This is mainly due to the delay in start-up, with the European partners starting 10 months before the Chinese partners. Collaborations and exchanges between the two side partners will enable EU and CN activities to be integrated into joint sections, in future deliverables. Data Management Plan follow-up

N°	Dataset name	Open Data	Closed Data	Means of dissemination	Maximum delay before access	Data set access
1	WP2.1_2.2_UNIBO-BIGEA_AT	Data on extraction of proteins from tomato, grape and BSG feedstocks		Publications, conferences, Webinars	Once published	Not yet available
2	WP2.1_FHNW_AH/PC	Data on extraction of proteins and peptides from tomato feedstocks		Publications, conferences, Webinars	Once published	Not yet available
3	WP2.1_UNIBO-DICAM_NR	Data on extraction of compounds from tomato feedstocks by means of microbial strains		Publications, conferences, Webinars	Once published	Not yet available
4	WP2.1_2.2_ITQB_CSP	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.		Publications, conferences, Webinars	Once published	Not yet available
5	WP2.1_INRAE_CA	Raw material pretreatment and extraction: Data on amount of enriched fractions after dry fractionation. Data on extraction yield (mg/g) of polyphenols and carotenoids after NADES extraction.		Publications, conferences, Webinars	Once published	Not yet available
6	WP2.1_4.2_TOMA_TB	Data on humidity %, microbiological charge, lactic acid bacteria, dry matter, proteins, total fat, total fibre, total sugars, ash, lycopene of tomato pomace feedstocks supplied to partners.		Publications, conferences, Webinars	Once published	Not yet available
7	WP2.1_2.2_INRAE_BIA_BB	Data on extraction of cutin monomers. Spectroscopic analyses (FTIR) of different raw data and the extraction fraction of cutin monomer. extraction yields for each raw material; Chemical data		Publications, conferences, Webinars	Once published	Not yet available

		composition (GC MS) and size distribution of the cutin monomer extract.				
8	WP2.1_IFST-CAAS_HH	Basic composition of peanut meal	Extraction technology,properties of functional components	publications, conferences	Once published	Not published
9	WP2.2_IFST-CAAS_AMS	Basic composition of peanut skin	Extraction technology,properties of functional components	publications, conferences	Once published	Not published
10	WP2.3_IFST-CAAS_MMM	Basic composition of potato residue	Extraction technology,properties of functional components	publications, conferences	Once published	Not published
11	WP2.4_IFST-CAAS_XJ	Basic composition of apple pomace	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	WP2.1_2.2_UNIBO-BIGEA_AT	UNIBO	Annalisa Tassoni	no	no	No (after publishing)
2	WP2.1_FHNW_AH/PC	FHNW	Aurélie Hoeffel/Philippe Corvini	no	no	No (after publishing)
3	WP2.1_UNIBO-DICAM_NR	UNIBO	Noura Raddadi	no	no	No (after publishing)
4	WP2.1_ITQB_CSP	ITQB	Cristina Silva Pereira	no	no	No (after publishing)
5	WP2.1_INRAE_CA	INRAE_IATE	Chahinez Aouf	no	no	No (after publishing)
6	WP2.1_4.2_TOMA_TB	TOMAPAINT	Tommaso Barbieri	no	no	No (after publishing)
7	WP2.1_2.2_INRAE_BIA_BB	INRAE_BIA	Benedicte Bakan	no	no	No (after publishing)
8	WP2.1_IFST-CAAS_HH	IFST-CAAS	Hui Hu	Require data transfer contract with european partners for closed data	No	Yes
9	WP2.2_IFST-CAAS_AMS	IFST-CAAS	Aimin Shi	Require data transfer contract with european partners for closed data	No	Yes
10	WP2.3_IFST-CAAS_MMM	IFST-CAAS	Mengmei Ma	Require data transfer contract with european partners for closed data	No	Yes
11	WP2.4_IFST-CAAS_XJ	IFST-CAAS	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.