NEWFEED

Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes

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Grape stem valorisation strategy: including the grape stems composition, the optimized process and the setting up of pilot plant

Deliverable number 2.1

Work Package 2	Optimization of the Valorisation and Feeding strategies
Task 2.1	Case study 1: grape stem-based ingredients for dairy sheep and cattle
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Foreword

The work described in this report was developed under the project NEWFEED: Turn Food Industry By-products into Secondary Feedstuffs via Circular-Economy Schemes (Grant Agreement number: 2013/ Call 2020 Section 1 Farming IA). If you wish any other information related to this report or the NEWFEED project please visit the project web-site (www.newfeed-prima.eu) or contact:

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Executive Summary

This deliverable provides the grape stem valorisation strategy including the grape stems composition, the optimized process, and the setting up of pilot plant. This process is included in the first case study developed in the project:

1. Use of **grape stems** from wineries for sheep and dairy cattle. This case study is led by AZTI (www.azti.es) and will be validated in Spain.

The deliverable includes

- the feedstock supply and logistics in a safe manner and a characterisation of grape stem
- Optimization of the hydrolysis process in laboratory scale to improve the nutritional and in vitro digestibility value
- The start-up pilot operation
- Optimization of the innovative and efficient process

The analysis of all the data obtained will allow the definition of the valorisation strategy and the establishment of the scale up process in WP3.

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1. Introduction

The overall objective of the project is to develop alternative feed ingredients using a circular economy approach through the conversion of food by-products into high-value secondary animal feed.

The purpose of this Deliverable is to define the grape stem valorisation strategy: including the grape stems composition, the optimized process, and the setting up of pilot plant. This process is included in the first case study developed in the project:

- ✓ The first case study will evaluate the use of grape stems from wineries as a second-generation ingredient for ruminant feed (sheep and dairy cows). This case study is led by AZTI (www.azti.es) and will be validated in Spain.

The analysis of the data obtained will allow the definition of the optimized process for the scaling up in the WP3 to ensure the successful valorisation of these by-products as alternative ingredients for animal feed.

2. Methodology

2.1 Antioxidant activity

Antioxidant activity of samples was measured using the DPPH radical scavenging activity (DRSA) method based on Brand-Williams et al. (1995) with slight modifications. DPPH (2,2-Diphenyl-1-picrylhydrazyl, D9132 Sigma Aldrich, Steinheim, Germany) in methanol (40 ppm) was prepared and 280 µl of this solution were added to 20 µl of sample solution. The mixture was incubated at room temperature in the dark for 30 min. Absorbance was measured at 515 nm. The standard comprised of water–methanol (50 % v/v) and different concentrations of Trolox (218940050, Acros Organics, New Jersey, USA). The antioxidant capacity was expressed as mg Trolox equivalent antioxidant capacity (TEAC) per litter of solvent or per g of dry matter (DM) using the calibration curve. The same samples were used as for the determination of polyphenols.

2.2 Total phenolic determination

Total phenolic content (TPC) was measured using the Folin–Ciocalteu method (Singleton 1965) with modifications. Initially, 30 µl of Folin–Ciocalteu (J/4100/08, Fischer Scientific, Loughborough, UK) solution were added to 140 µl sample, blank or standard and 140 µl of Na2CO3 7 % (w/v) (Sigma Aldrich, Steinheim, Germany). The mixture was incubated at room temperature in the dark for 1 h and the absorbance was measured at 750 nm. Gallic acid (G7384, Sigma Aldrich, Steinheim, Germany) was used as standard at a concentration range of 1.4–20 ppm and results were expressed as mg gallic acid equivalent (GAE) per litter of solvent and per g of DM sample.

2.3 Chemical analysis

The chemical analyses were carried out by the Department of Animal Nutrition of the Provincial Council of Alava (Vitoria-Gasteiz, Spain) in accordance with Royal Decree 2257/1994, of 25 November, which approves the official methods of analysis of animal feeds or foodstuffs and their raw materials (BOE, 1995).

Dry matter (DM) content was determined by oven drying at 103°C (method 6). Ash content was determined by incineration of the samples at 550°C (method 12). Nitrogen content (method 3) was determined using the macro-Kjeldahl procedure on a Kjeltec Foss (Foss, Hillerød, Denmark). Neutral detergent fibre (NDF) and acid detergent fibre (ADF) content were determined by the method of Van Soest et al. 1991. Acid detergent lignin was determined subsequent to ADF by calcination at 525 ºC. Crude fat content was determined by extraction of the sample with petroleum ether (method 4). Neutral detergent insoluble protein (NDICP) and acid detergent insoluble protein (ADICP) was determined by analyzing the NDF and ADF residues, respectively, for Kjeldahl nitrogen. The determination of Ca, P, K and Mg minerals was done by Perkin Elmer 2100DV Optical Emission Spectrophotometry (OES).

Total reducing sugars were determined by the Dinitrosalicylic acid (DNS) method adjusted to the microplate assay procedure. The DNS acid reagent was prepared by dissolving 8 g of NaOH in 100 mL of distilled water. Then, 5 g of DNS (Fischer Scientific, Loughborough, UK), 250 mL of distilled water and 150 g of potassium sodium tartrate tetrahydrate (Sigma-Aldrich, Steinheim, Germany) were added and made up to the volume (500 mL). Sample, blank or standard (25 µL), different concentrations of D-glucose (Fischer Scientific, Loughborough, UK), and 25 µL of DNS reagent were added to each well and incubated for 10 min at 100 ^{o}C. The microplate was rapidly cooled in an ice bath and 250 µL of distilled water was added to each well. Absorbance was read at 540 nm.

In vitro organic matter digestibility in the short term in vitro trial was calculated as described by Pell and Schofield (1993), where by 45 mL of a neutral detergent solution was added to each bottle and warmed at 105 °C for 1 h; then, the bottles were cooled, filtered through glass filter crucibles (Porosity 2) and washed with distilled water, ethanol and acetone. The remaining sample was dried at 100 °C overnight and then burned in a muffle furnace at 550 °C to obtain true IVOMD values.

The analysis of SCFA (acetic, propionic, butyric, isobutyric, valeric, isovaleric and caproic) of the samples of caecal contents was performed by gas chromatography using a flame ionization detector. A volume of 4 mL of diluted cecal contents mixed with one mL of a solution of 20 g/L of 4-metil-valeric acid as an internal standard, in 0.5 N HCl, was centrifuged (15,000×g for 15 min at 4◦C) to separate the liquid phase from the feed residuals. After, the liquid phase was microfiltered (premium syringe filter regenerated cellulose, 0.45 µm 4 mm, Agilent Technologies, Madrid, Spain), and 0.5 µL of liquid phase was directly injected in the

chromatograph (Agilent 6890 N, Agilent, Spain) using a capillary column (30 m× 530 um; 1-µm particle size; HP- FFAP, Agilent, Spain).

Stoichiometric methane values were estimated using equations proposed by Blümmel et

al. (1999) based on the stoichiometry of Wolin (1960).

Where:

CO_2= (C2/2) + (C3/4) + (1.5 x C4)

CH_4= C2 + (2 x C4) -CO_2

Being C2: individual concentration of acetic acid; C3: individual concentration of propionic acid and C4: individual concentration of butyric acid.

2.3.1 Calculation and statistical analysis

Fermentation kinetics were described according to the exponential model described by Krisnamoorthy et al. (1991) as:

$$Y = A(1 - e^{-c(t-L)})$$

where Y is gas production (mL/g DM) at time t, A is gas production from the insoluble fraction (mL/g DM), c is the gas production rate constant for fraction A (h−1) and L is the lag time prior to gas production (h).

The parameters A, c and L for each bottle were calculated using a non-linear regression procedure, which minimizes actual distances of data points to fitted curves by Marquardt's algorithm.

Data was subjected to analysis of variance using the mixed procedure considering the series as random effect and washing (W), hydrolysis (H) and the interaction (W x H) as fixed effects. Results are reported as least-squares means. Significant differences between treatments were declared at $P < 0.05$ using the Tukey's multiple-comparison test.

2.4 Short-Term In Vitro Batch Fermentation Trial

Samples were tested 3 in vitro series. In each series, ruminal fluid was collected from one multiparous Latxa ewe slaughtered for production purposes. Before slaughtering, ewes were fed a basal diet (80% meadow hay and 20% compound feed) for 3 weeks and had free access to fresh water and feed. Ruminal fluid was collected before the morning feeding and filtered through four layers of cheesecloth into a volumetric flask.

Then, was diluted in culture medium in a 1:4 ratio (ruminal fluid and phosphate-bicarbonate buffer, respectively) under anaerobic conditions according to Menke et al. (1979).

Approximately 500 mg of grape stem samples were weighed into 125 mL serum bottles. Each sample was incubated with 50 mL of culture medium in triplicate, the bottles were crimp sealed and incubated at a constant temperature (39°C) in the incubator for 24 hours. Gas production was released at 2, 4, 6 and 22 hours post-inoculation to avoid the pressure in the bottle headspace exceeding 48 kPa, as suggested by Theodorou et al. (1994). After 24 hours of incubation, bottles were put in the fridge for 20 min to stop fermentation for subsequent sampling for short chain fatty acids (SCFA) and in vitro organic matter digestibility (IVOMD) determination.

2.5 Long-Term in Vitro Batch Fermentation Trial

The animals, substrates and incubation procedures were the same as those described in the previous section. Approximately 1 g of each sample and 100 mL of culture medium was incubated for 96 hours at 39°C in glass bottles with an actual volume capacity of approximately 307 mL. The kinetics of gas production were recorded using the ANKOMRF gas production system (ANKOM Technology, Macedon, NY, USA). The release of the accumulated gas was done automatically (or manually in case of technical problems) through a valve attached to the module after setting the configuration parameters in the ANKOMRF software. The recording interval was set at 10 min. A threshold of 1 psi for automatic release of accumulated gases to avoid supersaturation of CO2 in the medium and a valve opening time of 500 mseg were set. Pressure values, corrected for the quantity of substrate organic matter (OM) incubated, were used to generate gas volume estimates.

2.6 Pretreatments

2.6.1 Crushing

The crushing was performed in a Comitrol® Processor Model 1700 (Urschel, Chesterton, Indiana, USA) with the objective of analysing the effect of crushing combine or not with the, washing and the hydrolysis in the nutritional composition, ruminal fermentation kinetic and in vitro digestibility of final ingredients.

2.6.2 Washing

The washing process was carried out at a 1:1.5 solid-liquid ratio for 45 minutes. the sample was filtered through a 250 µm mesh, solid samples were used for the subsequent processes and liquid samples were used for reducing sugars determination.

2.6.3 Hydrolysis

The hydrolysis was performed using a Sell Symphony 7100 Bathless Dissolution Distek equipment (Distek Inc., North Brunswick, NJ, USA), controlling and monitoring temperature, time and stir speed (Figure 1). The pH of each run of the experimental design was controlled manually and adjusted with NaOH 1 M in a final volume of 500 mL.

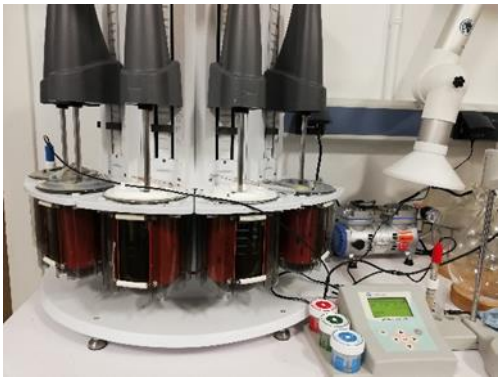

Figure 1. Sell Symphony 7100 Bathless Dissolution Distek equipment

Enzymatic hydrolysis

Enzymes were provided by Novozymes (NovozymesA/S, Bagsvaerd, Denmark). Viscozyme ® is an endo-beta-glucanase that hydrolyzes (1,3)- or (1,4)-linkages in beta-D-glucans with high mannase activity and Ultimase ® is a cellulase that hydrolyse (1,4)-beta-D-glucosidic linkages in cellulose and other beta-D-glucans. Enzyme conditions were pH 5, 55 ^{o}C, 20 h, 250 rpm, ratio 1:1 solid:water and 2 % of enzyme with respect to fibre.

The hydrolysis processes were ended by enzyme inactivation by temperature at 90 ^{o}C for 15 min. Then, the samples were centrifuged (2650 g; 15 min; ambient Tª), and two fractions were recovered: the liquid sample which corresponds to the hydrolysates and the solid fraction whose digestibility and nutritional composition for ruminant feed was further analysed. Samples were freeze-dried for further analysis.

Alkali hydrolysis

Alkali hydrolysis was carried out with NaOH, 1 % w/v, at a sample:solvent ratio of 1:1.25 (w/w). The condition selected were 3 h, 90 ^{o}C 250 rpm. In the second experimental trial, different conditions of alkali hydrolysis were conducted.

2.7 Statistical analysis

Experimental factors were considered significant when their probability (p-value) was less than 0.05 and were analysed with one-way ANOVA (analysis of variance). Normal data distribution was verified using Shapiro Wilk test and Levene test to assess the equality of variances. When equal variances were not assumed, Kruskal Wallis statistic test was used to compare samples.

3. Feedstock supply and logistics in a safe manner and a characterisation of grape stem

EU is the world-leading producer of wine with an average annual production was 167 million hectolitres (Eurostat 2019). Among the winery by-products, grape stems, pomace, and lees are the most significant ones, in which, grape stems represent 1.4-7.0 % of the initially processed raw matter.

Grape stems are rich in fibre, such as cellulose, hemicellulose and lignin and in polyphenols which are of great interest in animal nutrition as they are absorbed intestinally and bioaccumulated, confirming antioxidant and antimicrobial effects and contributing to oxidative stability. Although the benefits of using these by-products as feed ingredients have already been proven through in-vitro assays, there is still the need of gaining knowledge about other livestock species (e.g. dairy cattle and sheep) by performing not only in-vivo tests but also, analysing the potential increase that might occur in milk quality and production.

However, currently, grape stems are not valorised as highly profitable by-products and are sent for composting or discarded in open areas, causing environmental impacts.

To avoid this current treatment, the following diagram is proposed in the project (Figure 2). A recovery and stabilization process via flash drying, obtaining a grape stem ingredient, feed formulation and feeding test trials to validate the process.

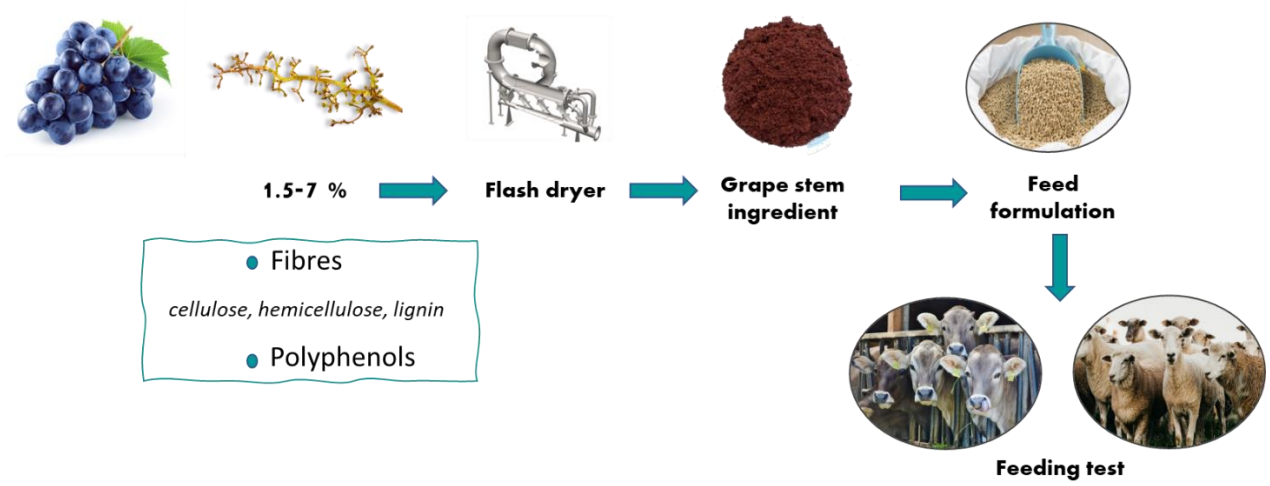

Figure 2. Propossed diagram in case study 1: use of grape stems from wineries as a second generation ingredient for ruminant feed (sheep and dairy cows).

The main activities carried out for the definition of the feedstock supply and logistic in a safe manner and characterisation were:

- Visit of several wineries with the aim of learning about the handling and operation of separating the grapes from the stems, their storage and management.

- Sampling of the grape stem (about 2,8 tons) → 13th October of 2021 during the grape harvesting period In the next figures we can see the collected grape stem in Baigorri winery previous and after the grinding process).
- Grinding of the sample (about 1 mm particle size) → 14-15th October for easy transport and drying due to the large size of the product.
- Characterisation of the ingredient.

In the next figures we can see the collected grape stem in Baigorri winery previous and after the grinding process.

- *Figure 3. Sampling of the produced grape stem in Baigorri winery.*

Figure 4. Grape stem before and after the grinding process.

The average chemical composition of grape stem-based ingredient from the Baigorri winery is shown in the Table 1. There are several reviews analysing the nutritional composition of grape stem and other winery by-products. The main conclusion is that the comparison of data is difficult due to the differences between samples related to several factors, such as, grape variety, maturity, as well as differences in extraction processes (Blackford 2021). Water is the main component of the fresh grape stem, varying from 55 to 80 % of total weight. Therefore, a stabilization process is needed to avoid the degradation process. In this study the initial moisture content of the grape stem was 70 % and, after the stabilization process by flash drying, the water content was reduced to 7.43 %. At this moisture level, the ingredient can be stored for longer periods without microbial degradation risk.

Table 1. Chemical composition of grape stem-based ingredient from Baigorri winery.

Parameters	Unit	Value
Moisture	%	7.43
Ashes	%	6.28
Energy	kJ/100 g	1480
Protein	%	4.07
Total Carbohydrates	%	81.58
Neutral detergent fibre	%	40.81
Acid detergent fibre	%	40.81
Lignin acid detergent	%	19.72
Crude fat	%	0.64
Starch	%	4.90
Total sugars (expressed in glucose)	%	19.74
Antioxidant activity (expressed as TEAC, Trolox equivalent antioxidant capacity)	mg TEAC/g DM	68,0
Polyphenols (expressed as GAE, Gallic acid equivalent)	mg GAE/g DM	64,5

Dried grape stems are characterized by low protein and starch content concomitant with a high fibre content resulting in a relatively low gross energy containing raw material. Mean values reported in the current experiment are in line with those found in the literature (Blackford 2021, Filippi 2021, Prozil 2012).

4. Optimization of the hydrolysis process in laboratory scale to improve the nutritional and in vitro digestibility value

Several pre-treatments have been used as a way to reduce fibre fraction in grape stems (Ping et al, 2011, Filippi et al, 2021). Pre-treatments such as alkali hydrolysis and acid hydrolysis are normally presented as an effective process for pre-digestion of fibre fractions, which would allow increasing the inclusion percentage of grape stems in animal diets. Alkali pre-treatments allow to reduce lignin content, while acid pre-treatments lead to a hemicellulose reduction. In addition, enzymatic hydrolysis could help with degradation of fiber fractions.

Therefore, the objective of the current study was to analyze the effect of different hydrolysis processes (enzymatic, basic and both) and washing steps on their nutrient profile and on in vitro digestibility and fermentation kinetics, with the aim of increasing their inclusion level in ruminant diets.

4.1 First experimental design

A full factorial design has been defined including, a crushing step, washing step, basic hydrolysis (NaOH, 1 % w/v, at a sample:solvent ratio of 1:1.25 w/w) and Enzymatic hydrolysis (Table 2), which were selected as the most appropriates for fibre hydrolysis based on bibliography and previous research (Filippi et al, 2021).

Both, basic and enzymatic hydrolysis were performed at laboratory-scale using a Sell Symphony 7100 Bathless Dissolution Distek equipment (Distek Inc., North Brunswick, USA), controlling and monitoring temperature, time and stir speed.

All the processes were carried out in triplicate.

Table 2. Analysed factors in the fisrt experimental design.

Grinding	Washing	Hydrolysis
Coarse (1)	Yes	Without hydrolysis
Fine (2)	No	Alkali hydrolysis
		Cellulolytic hydrolysis
		Alkali hydrolysis + Cellulolytic hydrolysis

4.1.1 Results

Washing process

Washing process was carried out with manual shaking for 45 minutes (Figure 5). Depending on the crushing, liquid samples had more or less sugars. The liquor sample obtained from the sample coarsely grinded had 22 g reducing sugars/L, while the most intense grinding led to lower sugar content in the liquor with 1.6 g reducing sugars/L. This may be due to the fact that the grinding process itself or the extra time needed for the process, may have degraded the sugars, decreasing the sugar content in the sample and therefore, in the extracted liquor.

Sugar concentration has a high effect on digestibility and ruminal fermentation kinetics' values, so, from now on and seeing that the effect of grinding is being distorted by the degradation of sugars and other components, we will not analyse the effect of this variable in the data analysis.

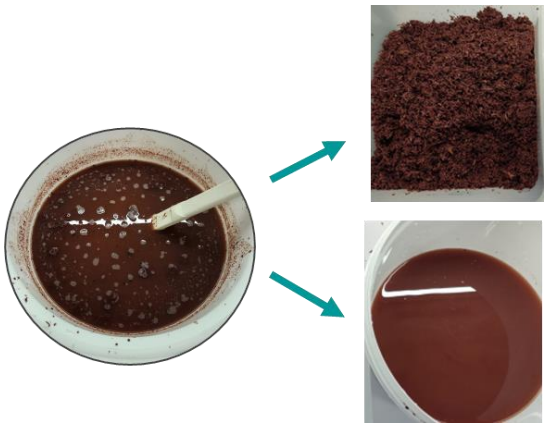

Figure 5. Washing process and the solid and liquid fractions obtained.

After the washing process, the moisture content of both raw materials increased from 73.5 % to 81.9 % in the case of grinding 1 and from 76.3 % to 84.5 % in the case of grinding 2.

When scaling up the process, the equipment that will be used, will allow to decrease the moisture content of the solid product after the washing process.

Alkali hydrolysis

Alkali hydrolysis is a highly intense process at 90 ^{o}C for 3 h at pH around 9-10. The initial pH was not adjusted, and the final pH was near to 7 in all cases, so that the sample did not have to be neutralised. Washing significantly affected the alkali hydrolysis process (Table 3). Initial and final pH were lower in samples without a previous washing step, mainly due to a reduction of sugars in the washed samples. The percentage of mass loss (%) was higher in the samples that were not washed and the reducing sugars that were released to the liquor after the hydrolysis process were also significantly higher in the samples that were not washed.

Table 3. Effect of washing process in the alkali hydrolysis of the samples.

Variables	Washing						*p*-Value
	No			Yes			
Initial pH	10.03	±	0.27	10.74	±	0.13	<0.001
Final pH	7.48	±	0.13	8.22	±	0.09	<0.001
Mass loss (%)	36.59	±	2.85	32.86	±	2.69	0.0416
Liquor reducing sugars (g/L)	60.64	±	7.80	16.86	±	1.74	<0.001

Enzymatic hydrolysis

The enzymatic process can be modified depending on the previous steps. Next table (Table 4) shows the differences in the enzymatic hydrolysis process when washing and alkali hydrolysis were applied or not.

Table 4. Effect of washing and alkali hydrolysis in enzymatic hydrolysis process (NAH:Non alakali hydrolysis; AH: Alkali hydrolysis).

Variables	No washing		Washing		*p*-value		
	NAH	AH	NAH	AH	Washing	AH	Washing*AH
Initial pH	4.18	7.38	4.1	7.59	0.1578	<0.001	0.0136
Final pH	4.59	5.63	4.61	5.08	<0.001	<0.001	<0.001
Mass loss (%)	14.81	6.99	15.73	21.31	<0.001	0.2301	<0.001
Initial reducing sugars in liquor (g/L)	24.71	23.77	9.07	6.31	<0.001	0.3737	0.6569
Final reducing sugar in liquor (g/L)	26.27	33.27	12.84	18.37	<0.001	0.0524	0.7962
Reducing sugar release in the liquor (g/L)	1.56	9.50	3.78	2.17	0.1585	<0.001	0.9153

Initial pH, final pH and mass loss during the enzymatic hydrolysis process were significantly affected by the previous washing and alkali hydrolysis processes, except in the case of initial pH that washing did not have a significant effect. The three variables were also significantly affected by the interaction of both factors.

When an interaction happens, the results must be analysed separately in groups. In this case, when washing is applied or not, a previous alkali hydrolysis increased the initial and final pH in both cases. When a washing step was not applied a previous alkali hydrolysis significantly decreased the mass loss (%). On the contrary, when a washing step was carried out, a previous alkali hydrolysis increased the mass loss.

In the case of the variables related with reducing sugars, no interactions were found between variables so they can be analysed together. Initial or final reducing sugar concentration in the liquid was significantly lower when the samples had been previously washed. A previous alkali hydrolysis did not affect the initial and final concentration of reducing sugars. However, the release of sugars was significantly affected by the previous alkali hydrolysis, when the alkali hydrolysis was applied, samples released more sugars during the enzymatic hydrolysis.

Final ingredient chemical composition

Washing (Yes/No) and Hydrolysis (Without/Alkali/Enzymatic/Alkali-Enzymatic) were selected as factors to determine their effect in the analysed variables in the final ingredient. Antioxidant capacity, total polyphenol content and reducing sugar content of final ingredients were significantly affected by the interactions between washing and hydrolysis processes, therefore, results must be analysed separately (Table 5).

Table 5. Effect of washing and hydrolysis process in the antioxidant, total polyohenols and reducing sugar content of final solid ingredient. WH:without hydrolysis; AH:ALkali hydrolysis; EH:Enzymatic hydrolysisis; AEH: Alkali-enzymatic hydrolysis; W:Washing; H: Hydrolysis. TEAC:Trolox Equivalent Antioxidant Capacity; GAE:Gallic Acid Equivalent.

Variables	No washing				Washing				*p*-Value		
	WH	AH	EH	AEH	WH	AH	EH	AEH	W	H	W*H
Antioxidant capacity (mg TEAC/g)	26.75	25.37	11.46	19.60	17.98	33.41	11.02	24.62	0.4763	<0.001	0.0019
Total polyphenols (mg GAE/g)	25.33	22.38	7.33	13.37	17.85	29.69	7.18	16.59	0.5668	<0.001	0.0047
Total reducing sugars (mg/g)	394.8	197.62	120.52	188.56	89.95	122.79	72.24	82.62	<0.001	0.0046	0.0185

When samples were not washed, alkali and alkali*enzymatic hydrolysis reduced the antioxidant capacity of samples, without differences between alkali*enzymatic and alkali hydrolysis or non-hydrolysed samples. When samples were washed, alkali hydrolysis increased the antioxidant capacity of samples compared with the other treatments and enzymatic hydrolysis reduced compared to the other treatments. No differences were found between alkali*enzymatic hydrolysis and samples without hydrolysis (Table 5).

In the case of total polyphenols in final samples, when samples were not washed, enzymatic or alkali*enzymatic hydrolysis processes decreased the content compared to only alkali or not hydrolysed samples. No differences were found between the last two. When samples were washed, otherwise, alkali hydrolysis increased final polyphenol content compared with the other analysed processes. Enzymatic hydrolysis decreased the polyphenol content, and no differences were found between no treatment and alkali*enzymatic hydrolysed samples (Table 5).

Finally, when samples were not washed, not hydrolysed samples had the highest sugar content compared to the other hydrolysis processes. No differences were found between alkali, alkali*enzymatic and enzymatic hydrolysis. When samples were washed otherwise, alkali hydrolysis led to higher sugar content in final samples compared to enzymatic and alkali*enzymatically processed samples. No differences were found between samples without hydrolysis and alkali hydrolysed samples.

The effect of the different optimization processes on grape stems chemical composition can be seen in Table 6. Both basic (11.6 vs. 5.47 %, P<0.001) and basic enzymatic (8.43 vs. 5.47 %, P=0.001) hydrolysis processes increased ash content compared to control (CTR). In addition basic hydrolysis increased the ash conent compared to basic enzymatic (11.6 vs. 8.43 %, P<0.001) or enzymatic (11.6 vs. 6.37 %, P<0.001) hydrolysis conditions, being differences between the latter significant (8.43 vs. 6.37 %, P=0.024). As for crude protein content the enzymatic hydrolysis increased the crude protein content compared to basic hydrolysis (5.88 vs. 4.81 %, P=0.042), being diffefences among all other treatments not significant.

Grape stems ash content was increased when the washing process was applied (8.60 vs. 7.35 %, P=0,011). However, as for crude protein content, diffences between washing and not washing was found not significant (5.20 vs. 5.44 %, P=0.374).

A significant interaction between washing and the enzymatic hydrolysis was found for neutral detergent fibre, acid detergent fibre, acid detergent lignin and cellulose contents. When grape stems were washed differences among hydrolysis treatments were found not significant. When grape stems were not washed, however, all hydrolysis treatments increased the neutral detergent fibre content compared to CTR. In addition both, basic enzymatic (64.2 vs. 41.6 %, P<0.001) and enzymatic treatments (55.7 vs. 41.6 %, P=0.009) increased the neutral detergent fibre content compared to basic hydrolysis. Differences between basic enzymatic and enzymatic treatments were found not significant (64.2 vs. 55.7 %, P=0.323).

When grape stems were washed both the enzymatic (56.3 vs. 43.7 %, P=0.015) and basic enzymatic hydrolysis (57.6 vs. 43.7 %, P=0.007) increased the fibre acid detergent compared to CTR, being differences among all other treatments not significant. When washing was not applied, all enzymatic hydrolysis increased the acid detergent fibre compared to CTR. In addition, the basic enzymatic hydrolysis increased the acid detergent fibre content compared to basic hydrolysis (57.6 vs. 40.6 %, P<0.001).

When grape stems were washed both enzymatic and basic enzymatic hydrolysis increased the acid detergent lignin compared to CTR or basic hydrolysis, being differences among all other treatments not significant. When grape stems were not washed all hydrolysis treatments increased the acid detergent lignin compared to CTR. In addition, the basic enzymatic hydrolysis increased the acid detergent lignin content compared to basic hydrolysis.

Whereas differences among hydrolysis treatments were found not significant for cellulose content when washing was applied, significant differences were found when washing was not applied. All of the hydrolysis treatments increased the cellulose contents compared to CTR. In addition the basic enzymatic hydrolysis increased the cellulose contents compared to the basic hydrolysis (34.0 vs. 22.5 %, P=0.038).

Table 6. Effect of different optimization processes on grape stems chemical composition.

	With Washing				Without Washing					P-value		
	B	E	BE	CTR	B	E	BE	CTR	SEM	H	W	HxW
Dry matter (%)	92.4	94.1	93.8	93.4	93.2	93.7	92.1	92.6	1.07	0.767	0.434	0.681
Ash (% DM)	12.7	6.77	8.73	6.22	10.6	5.97	8.14	4.72	0.596	<0.001	0.011	0.601
Crude Protein (% DM)	5.17	6.09	5.21	5.27	4.45	5.67	5.82	4.84	0.360	0.043	0.374	0.371
Neutral Detergent Fibre (% DM)	52.9	61.9	58.6	51.1	41.6^{b}	55.7^{a}	64.2^{a}	26.4^{c}	2.28	<0.001	<0.001	<0.001
Acid Detergent Fibre (% DM)	47.3^{ab}	56.3^{a}	57.6^{a}	43.7^{b}	40.6^{b}	50.4^{ab}	61.1^{a}	24.8^{c}	2.14	<0.001	<0.001	0.002
Hemicellulose (% DM)	5.57	5.60	1.01	7.38	1.02	5.34	3.09	1.61	1.645	0.266	0.097	0.123
Celullose (% DM)	29.1	30.1	32.3	26.0	22.5^{bc}	27.1^{ab}	34.0^{a}	14.2^{c}	1.98	<0.001	0.004	0.035
Acid Detergent Lignin (% DM)	18.2^{b}	26.2^{a}	25.3^{a}	17.7^{b}	18.1^{b}	23.3^{ab}	27.2^{a}	10.6^{c}	1.40	<0.001	0.060	0.038

W: washing, H: hydrolysis, B: basic hydrolysis, E: enzymatic hydrolysis, BE: basic enzymatic hydrolysis, CTR: without hydrolysis, DM: dry matter, SEM: standard error of the mean

Organic matter digestibility and ruminal fermentation kinetics

The effect of the different optimization processes used on grape stems' in vitro digestibility and fermentation parameters are shown in Table 7. A significant interaction among washing and hydrolysis was found for all variables except for isobutyrate, isovalerate and total branch-chained short fatty acids. When grape stems were washed, basic hydrolysis improved digestibility (P<0.001) compared to control (CTR), whereas basic-enzymatic hydrolysis reduced it (P<0.001). When grape stems were not washed, none of the hydrolysis processes improved digestibility compared to CTR. Indeed, enzymatic, and basic-enzymatic hydrolysis reduced it (P<0.001), being differences between these treatments not significant.

When grape stems were washed, none of the hydrolysis processes increased total short chain fatty acid (SCFA) production compared to CTR. In fact, enzymatic (P<0.001) and basic-enzymatic (P=0.014) hydrolysis reduced total SCFA production compared to CTR, being differences between the latter not significant (P=0.605). In addition, non-significant differences were found between CTR and basic hydrolysis (P=0.995) and between basic hydrolysis and basic-enzymatic hydrolysis (P=0.102). When grape stems were not washed, enzymatic (P<0.001) or basic- enzymatic (P<0.001) hydrolysis reduced SCFA production compared to CTR or compared to basic hydrolysis. Differences between enzymatic and basic-enzymatic hydrolysis (P=0.989), or between CTR and basic hydrolysis (P=0.068) were not found significant.

Regarding individual molar proportions of SCFA, when grape stems were washed, non-significant differences were found in acetate (C2) molar proportions among the different treatments. However, when grape stems were not washed, the basic-enzymatic hydrolysis increased C2 molar proportions compared to CTR (P=0.037), being differences among all other treatment non-significant. Similarly, when grape stems were washed, non-significant differences were observed among the different treatments for butyrate (C4) molar proportions, but when grape stems were not washed all the hydrolysis processes reduced C4 molar proportions compared to CTR, being differences among them non-significant. When grape stems were washed, the basic hydrolysis reduced valerate (C5) molar proportions compared to CTR (P=0.036), with differences among all other treatments being not significant. However, when grape stems were not washed all the hydrolysis processes reduced C5 molar proportions compared to CTR, being differences among them non-significant. Regarding the acetate to propionate (C2:C3) ratio or the acetate plus butyrate to propionate(C2+C4:C3) ratio, when grape stems were washed none of the hydrolysis processes affected these two ratios compared to CTR. However, the basic-enzymatic hydrolysis reduced both the C2:C3 (P=0.031) and C2+C4:C3 (P=0.033) ratios compared to basic hydrolysis. When grape stems were not washed, all the hydrolysis processes resulted in higher C2:C3 and C2+C4:C3 ratios compared to CTR, with differences among them being non-significant.

Washing increased isobutyrate (0.682 vs. 0.585 mol/100 mol, P=0.001), isovalerate (1.03 vs. 0.782 mol/100 mol, P=0.001) and total branched-chain short fatty acid (1.71 vs. 1.37 mol/100 mol, P=0.001) molar proportions compared to not applying the washing step.

Basic-enzymatic hydrolysis (0.738 vs. 0.537 mol/100 mol, P<0.001) and enzymatic hydrolysis (0.671 vs. 0.537 mol/100 mol, P=0.007) increased isobutyrate molar proportions compared to CTR. Similarly, basic-enzymatic hydrolysis increased isobutyrate molar proportions compared to basic hydrolysis (0.738 vs. 0.587 mol/100 mol, P=0.004). Non-significant differences were found among all other treatments.

Basic-enzymatic hydrolysis (1.10 vs. 0.730 mol/100 mol, P<0.001) and enzymatic hydrolysis (1.00 vs. 0.730 mol/100 mol, P=0.007) increased isovalerate molar proportions compared to CTR. Similarly, basic-enzymatic hydrolysis (1.10 vs. 0.785 mol/100 mol, P<0.001) and enzymatic hydrolysis (1.00 vs. 0.785 mol/100 mol, P=0.007) increased isovalerate molar proportions compared to basic hydrolysis. Non-significant differences were found among all other treatments.

Finally, basic-enzymatic hydrolysis (1.84 vs. 1.27 mol/100 mol, P<0.001) and enzymatic hydrolysis (1.67 vs. 1.27 mol/100 mol, P=0.007) increased branched-chain short fatty acid molar proportions compared to CTR. Similarly, basic-enzymatic hydrolysis (1.84 vs. 1.37 mol/100 mol, P<0.001) and enzymatic hydrolysis (1.67 vs. 1.37 mol/100 mol, P=0.003) increased branched-chain short fatty acid molar proportions compared to basic hydrolysis. Non-significant differences were found among all other treatments.

The effect of the different optimization processes used on grape stems' in vitro gas production parameters are shown in Table 8. A significant interaction among washing and hydrolysis was found for all variables. When grape stems were washed basic hydrolysis increased potential gas production compared to CTR (P=0.022) and enzymatic hydrolysis (P=0.004), being differences among all other treatments not significant. Regarding the lag phase, differences among treatments were found not significant. Differences in gas production rate among experimental treatments also were not significant.

When grape stems were washed, however, the basic-enzymatic hydrolysis significantly reduced gas production rate compared with CTR (P=0,019), being differences among all other treatments not significant. When grape stems were not washed none of the treatments increased potential gas production compared to CTR. Indeed, enzymatic, and basic-enzymatic hydrolysis reduced it (P<0.001), being differences among all other treatments not significant. The basic enzymatic treatment reduced the lag phase compared to CTR (P<0.001) and the basic treatment (P=0.004) with differences among all other treatments being not significant.

Table 7. Effect of different optimization processes on grape stems in vitro digestibility and fermentation parameters.

	With Washing				Without Washing					P-value		
	B	E	BE	CTR	B	E	BE	CTR	SEM	H	W	HxW
IVOMD (%)	48.6^{a}	27.5^{c}	35.0^{bc}	37.6^{b}	57.2^{a}	36.7^{b}	39.9^{b}	61.6^{a}	1.34	<0.001	<0.001	<0.001
SCFA (mmol/100 mL)	4.17^{ab}	2.81^{c}	3.34^{bc}	4.39^{a}	5.13^{a}	3.78^{b}	3.50^{b}	6.00^{a}	0.241	<0.001	<0.001	0.017
Individual SCFA proportions (mmol/100 mmol)												
C2	73.2	70.5	67.4	71.2	69.9^{ab}	69.7^{ab}	71.8^{a}	64.3^{b}	1.44	0.083	0.103	0.007
C3	17.4	19.3	21.8	18.5	20.3	20.4	18.8	24.1	1.30	0.287	0.083	0.020
C4	7.06	7.51	7.54	7.80	7.67^{b}	7.55^{b}	6.87^{b}	9.25^{a}	0.338	<0.001	0.046	0.001
IsoC4	0.624	0.705	0.791	0.609	0.551	0.637	0.685	0.466	0.0307	<0.001	0.001	0.767
C5	0.816^{b}	0.894^{ab}	0.851^{ab}	0.987^{a}	0.881^{b}	0.853^{b}	0.781^{b}	1.21^{a}	0.0547	<0.001	0.113	0.001
IsoC5	0.863	1.14	1.23	0.869	0.707	0.857	0.973	0.590	0.0506	<0.001	<0.001	0.517
BCSFA	1.49	1.85	2.02	1.48	1.26	1.49	1.66	1.06	0.0772	<0.001	<0.001	0.678
C2:C3	4.20^{a}	3.66^{ab}	3.54^{b}	3.85^{ab}	3.44^{a}	3.42^{a}	3.81^{a}	2.69^{b}	0.1405	0.002	<0.001	<0.001
(C2+C4):C3	4.61^{a}	4.05^{ab}	3.92^{b}	4.27^{ab}	3.82^{a}	3.79^{a}	4.20^{a}	3.08^{b}	0.151	0.005	<0.001	0.001

W: washing, H: hydrolysis, B: basic hydrolysis, E: enzymatic hydrolysis, BE: basic enzymatic hydrolysis, CTR: without hydrolysis, IVOMD: in vitro organic matter digestibility, SFCA: short chain fatty acid, C2: acetate, C3: propionionate, C4: butyrate, C5: valerate, BCSFA: branched-chain short fatty acid, SEM: standard error of the mean

Table 8. Effect of different optimization processes on grape stems in vitro gas production parameters.

	With Washing				Without Washing					P-value		
	B	E	BE	CTR	B	E	BE	CTR	SEM	H	W	HxW
A (mL/ g OM)	138^{a}	109^{b}	130^{ab}	113^{b}	146^{ab}	128^{b}	125^{b}	163^{a}	20.2	<0.001	<0.001	<0.001
c	0.096	0.135	0.112	0.122	0.125^{ab}	0.117^{ab}	0.110^{b}	0.170^{a}	0.0913	0.010	0.103	0.026
L (h)	-2.31	-2.92	-2.42	-2.87	-1.32^{b}	-2.24^{ab}	-4.28^{a}	-0.713^{b}	0.554	0.007	0.158	0.002

W: washing, H: hydrolysis, B: basic hydrolysis, E: enzymatic hydrolysis, BE: basic enzymatic hydrolysis, CTR: without hydrolysis, A: potential gas production, c: gas production rate, L: lag time, SEM: standard error of the mean

4.1.2 Conclusions

1. Applying a washing step prior to the hydrolysis process affects the results obtained by the hydrolysis itself.
2. When grape stems were washed, the basic hydrolysis improves the nutritional value of grape stems by increasing *in vitro* digestibility and potential gas production but at the expense of a less efficient fermentation process.
3. When grape stems were washed, the basic hydrolysis compared to control does not reduce the fibre content but improves the nutritional value of grape stems by increasing *in vitro* digestibility and potential gas production at the expense of a less efficient fermentation process.
4. When grape stems were not washed, none of the hydrolysis processes tested improve the nutritional value of grape stems.
5. Alkali hydrolysis was selected for further optimization through a Box-Behnken design. The washing step was removed from the study as the alkali hydrolysis should work as a washing itself.

4.2 Second experimental design

With the aim of optimizing the hydrolysis parameters for the selected alkali hydrolysis, a Box-Behnken design (BBD) was carried out. The BBD was composed of three factors, two levels and centre points. The selected factors for the alkali hydrolysis process were solid concentration (%), hydrolysis time (h) and temperature (°C).

shows the low (−1), centre (0) and high levels (+1) of each factor (Table 9).

The range for each variable of the experimental design was established considering the scalability of the process regarding processing costs.

Box–Behnken designs are experimental designs for response surface methodology defined by the following characteristics:

- Each factor, or independent variable, is placed at one of three equally spaced values, usually coded as −1, 0, +1. (At least three levels are needed for the following goal.)
- For instance, the Box–Behnken design for 3 factors involves three blocks, in each of which 2 factors are varied through the 4 possible combinations of high and low. It is necessary to include centre points as well (in which all factors are at their central values).

Table 9. Three factors involved in teh Box-Benken design, and their low and high values.

Factors	Units	Low	Centre	High
A:Temperature	^{o}C	60	75	90
B:Time	h	1	2	3
C:Solids	%	33	36.5	40

Table 10 shows the 15 runs carried out in the design. The pH and the stirring were maintained controlled (pH 9 and 250 rpm). The centre points allow the estimation of pure error and the system performance at any experimental point within the studied range (Ahmad et al, 2020). The hydrolysis was executed in random order to avoid bias.

Some controls were added to the trials:

- Control without any treatment (no washing-no extra crushing-no hydrolysis).
- Control with washing step.
- Control with extra crushing.
- Control with extra crushing and washing step.

Table 10. Runs and coditons of the second experimental design.

Runs	Solids (%)	Temperature (^{o}C)	Time (h)
1	36.5	75	2
2	36.5	75	2
3	33	90	2
4	33	75	1
5	36.5	60	3
6	33	60	2
7	36.5	90	1
8	36.5	60	1
9	33	75	3
10	40	60	2
11	36.5	90	3
12	40	75	3
13	40	90	2
14	40	75	1
15	36.5	75	2

Results

Parameter Optimization via Box–Behnken Design (BBD)

The experimental and predicted responses for each variable—Sugars, antioxidant capacity, polyphenols, digestibility, protein, ash, ADF, Lignin, NDF and total VFA (volatile fatty acids) of each hydrolysis condition included in the experimental design, are shown in Table 11.

The experimental responses were fitted to a polynomial model to assess the effect of variables on the response. The insignificant coefficients for the full quadratic model and their significance levels were analysed. Since there were multiple insignificant terms, the model was reduced by removing them using a p-value of 0.05 as the cut-off.

Table 11 shows the obtained and predicted values for the response variables.

Table 11. Box–Behnken experimental design, obtained and predicted values for the response variables.

Run	Sugars (mg/g DM)		Polyphenols (mg GAE/g DM)		Antioxidant Activity (g TEAC)		Organic Matter Digestibility (%)		Protein (%)		Ash (%)		ADF (%)		Lignin (%)		NDF %		Total VFA (mmol/100 mL)	
	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred	Obs	Pred
1	215	207	24,0	22,2	31,1	24,3	33,0	33,0	7,1	6,6	12,1	12,1	49,2	50,6	39,5	37,5	50,5	51,7	4,9	4,8
2	204	207	21,8	22,2	23,2	24,3	38,9	33,0	6,4	6,6	10,5	12,1	44,6	50,6	36,7	37,5	46,2	51,7	5,1	4,8
3	204	210	31,7	28,5	37,4	32,7	43,0	41,9	6,5	6,3	13,7	13,3	47,6	46,7	34,8	31,9	47,9	46,1	5,4	5,4
4	194	205	15,6	15,7	19,7	21,6	36,0	36,5	6,8	6,9	10,0	9,2	51,1	51,5	36,0	36,7	53,5	53,6	5,2	5,2
5	123	140	22,5	19,5	21,0	18,2	28,5	27,9	5,6	5,6	12,1	10,8	52,4	52,0	27,2	25,1	53,2	51,5	4,7	4,7
6	205	179	24,4	22,4	19,4	17,2	34,7	33,9	6,4	6,3	10,1	12,4	47,6	46,9	27,4	28,5	48,1	46,4	5,3	5,3
7	214	198	25,6	28,6	24,4	27,3	33,0	33,5	6,3	6,3	11,3	12,6	51,1	51,5	34,2	36,2	51,8	53,4	5,1	5,1
8	197	212	19,1	20,9	21,2	21,5	31,9	32,0	6,4	6,3	9,3	7,8	50,3	50,6	27,8	25,8	50,1	51,6	5,0	5,0
9	118	127	16,8	21,8	16,0	21,0	34,4	35,7	5,7	5,8	12,7	11,7	52,5	53,7	26,2	27,1	53,9	57,2	5,0	5,0
10	230	224	14,4	17,6	19,3	24,0	29,0	30,2	6,3	6,5	10,3	10,8	44,4	45,2	26,9	29,7	44,6	46,4	5,0	5,0
11	123	108	33,8	32,1	37,0	36,7	34,7	34,5	5,7	5,7	16,0	17,5	56,6	56,3	30,1	32,0	57,1	55,6	4,9	4,9
12	126	115	16,2	16,0	30,0	28,1	28,0	27,4	6,6	6,5	15,6	16,4	56,0	55,7	41,6	40,8	58,0	57,8	4,7	4,7
13	120	146	30,0	31,9	30,7	32,8	29,6	30,4	6,6	6,6	23,8	21,5	49,8	50,5	44,9	43,7	50,7	52,4	5,2	5,2
14	209	200	25,1	20,1	26,4	21,4	31,0	29,7	6,7	6,7	9,9	11,0	52,9	51,7	37,0	36,1	62,6	59,3	5,0	5,0
15	201	207	20,9	22,2	18,6	24,3	27,1	33,0	6,2	6,6	13,8	12,1	58,0	50,6	36,2	37,5	58,5	51,7	4,6	4,8

Results were expressed as a second-order polynomial equation, as shown in Equation (2):

$$Yij = A0 + \sum AiXi + \sum AiiXi2 + \sum AijXiXj + \varepsilon \quad (1)$$

where Yij is the response function (Sugars, polyphenols, antioxidant activity, organic matter digestibility, protein, ash, ADF, NDF, lignin and total VFA), A0 is the regression coefficient for the intercept, Ai is the coefficient of the linear term, Aii is the coefficient for the quadratic term, Aij is the coefficient of the interaction term, and ε is the error.

Table 12 shows the ANOVA significance levels of each variable and Table 13 regression model coefficients.

One of the linear terms (temperature) was highly significant for the studied polyphenols, antioxidant activity, sugars, ash and lignin content responses. In the case of reducing sugar content, time was also statistically significant. Solid concentration in the reaction was a significant term in the organic matter digestibility and lignin content responses. In the case of time linear term, it affected significantly to the protein content. Among the interactions, reducing sugar content was affected by the interaction between temperature and time and the quadratic term of time. In addition, polyphenol content of final ingredient was also affected by the quadratic term of temperature. Finally, lignin concentration in the final ingredients was affected by the quadratic term of temperature (Table 12). No significant effects were found for ADF (%), NDF (%) and Total VFA (mmol/100 mL).

The fitted model goodness was assessed by the coefficient of determination (R2) and the R2 adjusted, which are shown in Table 13. The highest values for R2 and adjusted R2 are for the sugar content (R2 87.96 and Adj. R2 66,27), Lignin (R2 90,92 and Adj. R2 74,59). The high value of R2 for the models indicates there was a good correlation between the experimental and predicted response values (Table 11). On the contrary, in the case of the prediction of ADF, NDF and total VFA the obtained R2 and the R2 adjusted were much lower, revealing a worse relation between the experimental and predicted values. However, the generated models were significant for all the variables studied.

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Table 12. ANOVA significance levels.

	p-Value									
	Sugars (mg/g DM)	**Polyphenols (mg GAE/g DM)**	**Antioxidant Activity (mg TEAC/g DM)**	**Organic Matter Digestibility (%)**	**Protein (%)**	**Ash (%)**	**ADF (%)**	**NDF (%)**	**Lignin (%)**	**Total VFA (mmol/100ml)**
A:Temperature	0,0465	0,0122	0,0446	0,2044	0,7302	0,0184	0,4476	0,4413	0,0091	0,3498
B:Time	0,0041	0,4667	0,5311	0,6009	0,0314	0,0647	0,3775	0,7798	0,2948	0,0873
C:Solids	0,2297	0,6028	0,4824	0,041	0,352	0,1095	0,7459	0,4078	0,0269	0,1125
AA	0,1969	0,028	0,4666	0,8635	0,1071	0,3608	0,4485	0,1865	0,0289	0,1215
AB	0,3668	0,2708	0,3699	0,5468	0,7834	0,7094	0,7211	0,8325	0,5839	0,873
AC	0,018	0,1245	0,6308	0,3729	0,8304	0,092	0,5713	0,5496	0,1345	0,7904
BB	0,0118	0,1593	0,7702	0,5298	0,1472	0,3761	0,1544	0,0971	0,1121	0,3787
BC	0,702	0,0884	0,5989	0,8594	0,1902	0,5609	0,848	0,6314	0,0613	0,8521
CC	0,1271	0,1411	0,9451	0,7515	0,296	0,4132	0,5846	0,993	0,6734	0,0727

Significant terms are in red (P < 0.05).

Table 13. Regression model coefficients

	Coefficients									
	Sugars (mg/g DM)	**Polyphenols (mg GAE/g DM)**	**Antioxidant Activity (mg TEAC/g DM)**	**Organic Matter Digestibility (%)**	**Protein (%)**	**Ash (%)**	**ADF (%)**	**NDF (%)**	**Lignin (%)**	**Total VFA (mmol/100ml)**
Intercept	-2483,14	-34,4082	-8,18664	47,1055	24,4754	271,223	-55,9088	13,951	134,312	33,9907
A:Temperature	23,5746	-4,51287	-0,634163	1,0584	0,183444	-2,41068	0,307623	1,54454	1,69341	-0,130325
B:Time	137,89	28,0502	-29,0878	2,2275	-1,89405	-3,26107	-22,9474	-10,04	-22,2405	0,332083
C:Solids	96,4958	10,2141	3,26473	-2,2152	-1,27446	-10,0326	6,04575	-1,23189	-9,02532	-1,31427
AA	-0,0326037	0,0217333	0,0117537	0,00165	-0,00140741	0,00551111	-0,00847963	-0,0174722	-0,0208796	0,000768519
AB	-0,285167	0,0806667	0,211833	0,0848333	0,003	0,0311667	0,0561667	0,0366667	-0,058	0,001
AC	-0,517619	0,0391905	-0,0314286	-0,0367143	0,000666667	0,0469524	0,0257143	0,0301429	0,0505238	0,00047619
BB	-35,0433	-1,835	-1,03542	-1,38375	-0,276667	-1,1975	3,89208	5,23625	-2,98042	-0,0895833
BC	-0,466429	-0,719286	0,517143	-0,105	0,0671429	0,210714	0,128571	-0,36	1,02143	-0,005
CC	-0,794762	-0,161837	-0,0198299	0,0560204	0,0153741	0,0897959	-0,110646	0,00193878	0,0564966	0,0171769
R^2	87,9553	77,9792	67,6387	70,5625	80,5962	85,2816	56,236	65,1399	90,9248	79,1878
Adj R^2	66,2748	38,3418	9,3884	17,5751	45,6693	58,7885	0	2,39182	74,5894	41,7257

Response surface plots were analysed in the responses with significant factors. Figure 6-Figure 7 and Figure 8 show the response surface plots of the significant variables. In the case of reducing sugars, higher sugar content in the final ingredient was obtained when temperature and time were lower. On the contrary, polyphenol and antioxidant components were in a significantly higher concentration when temperature and time were higher. Same effect is shown in the surface plot of ash concentration (Figure 7). In protein concentration variable, otherwise, longer time led to lower protein content in the final ingredient.

In the case of lignin concentration, higher temperature and solids led to higher lignin concentration, on the contrary, maintaining lower solids, the lignin concentration of the final ingredient was lower, regardless of the hydrolysis temperature. Finally, digestibility value was highly affected by the solids, obtaining higher values at lower solid conditions in the hydrolysis.

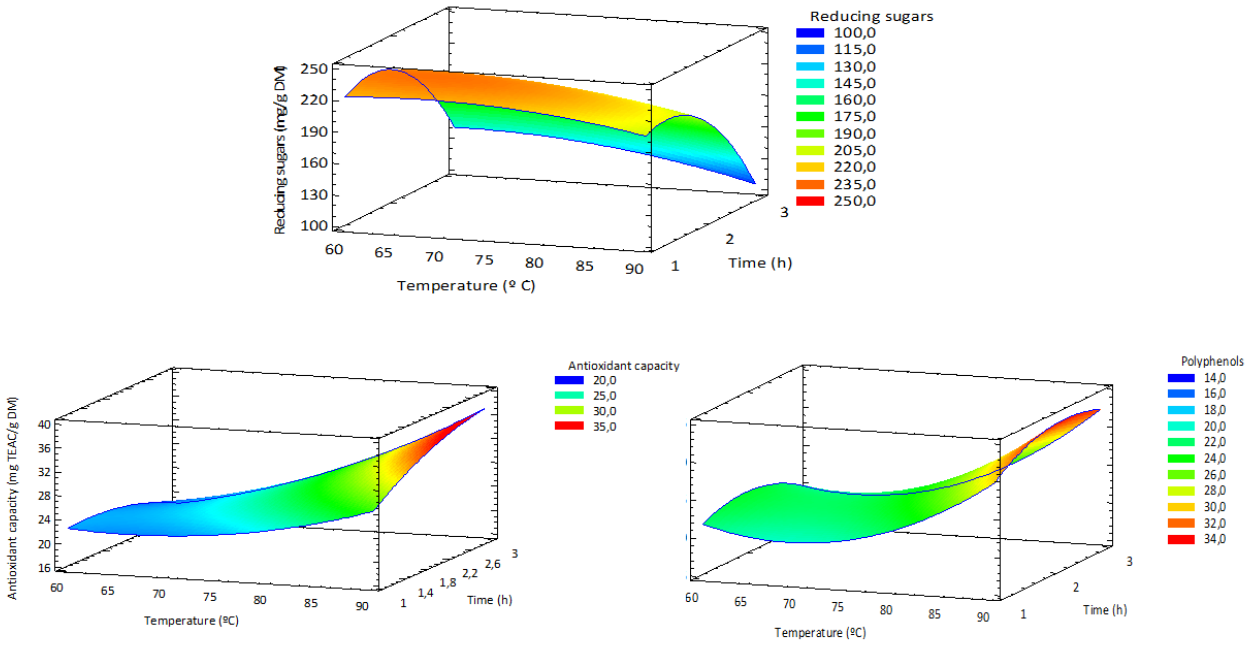

Figure 6. Response surface plots of reducing sugars, antioxidant activty and polyohenols in the final samples.

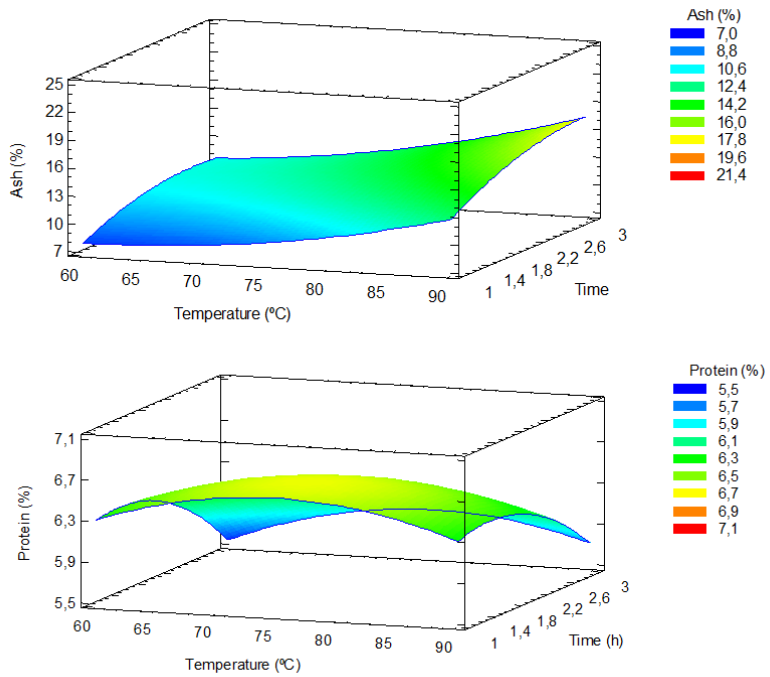

Figure 7. Response surface plots of Ash and protein concentration of final ingredients.

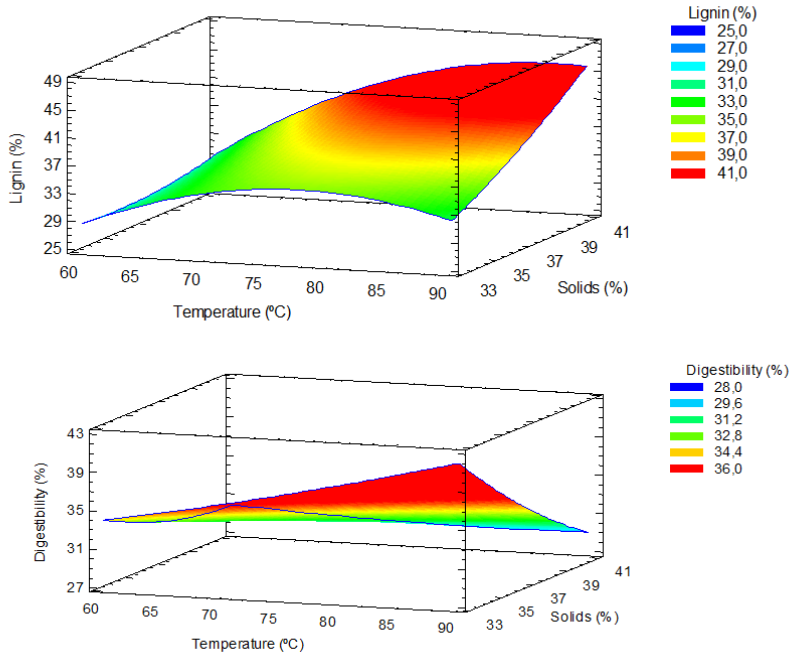

Figure 8. Response surface plots of digestibility value and lignin concentration of the final ingredients.

One of the main objectives of the hydrolysis process is to improve the digestibility value of the final ingredients. Therefore, digestibility parameter has been selected as the response variable to select the best conditions of the hydrolysis. The selected optimum value for digestibility is of 41,98% (Table 14). To obtain this value the statistical program has selected the next conditions.

Table 14. Optimun hydrolysis conditions for digestibility increase.

Factor	Selected conditions
Temperature (ºC)	89,9997
Time (h)	2,3119
Solids (%)	33,0

These conditions have been selected for the WP3 scale-up of the hydrolysis process.

Next table (Table 15) shows the values of the volatile fatty acid and methane production in the different runs carried out.

Table 15. Volatile fatty acid production (mmol/100mL) in the different ruminal fermentation of hydrolysed samples.

Run	TOTAL VFA	acetic	butiric	isobutiric	isovaleric	propionic	valeric	c2c3	c2c4c3	BCVFA	CH4
1	4,87	71,33	7,53	0,26	0,29	20,18	0,50	3,55	3,93	0,55	0,84
2	5,11	70,56	7,57	0,24	0,25	20,45	0,47	3,50	3,88	0,49	0,90
3	5,39	71,84	7,33	0,26	0,27	19,90	0,46	3,62	3,99	0,53	0,88
4	5,23	70,71	7,84	0,25	0,25	20,44	0,49	3,48	3,86	0,50	0,91
5	4,74	71,40	7,83	0,26	0,26	19,38	0,48	3,72	4,13	0,51	0,82
6	5,27	69,96	8,07	0,24	0,22	21,06	0,51	3,34	3,72	0,46	0,89
7	5,09	70,65	7,96	0,26	0,25	20,22	0,49	3,52	3,91	0,51	0,90
8	5,03	70,45	8,18	0,25	0,24	20,58	0,49	3,43	3,83	0,48	0,84
9	4,99	71,75	7,70	0,25	0,24	19,42	0,47	3,71	4,11	0,49	0,86
10	5,02	69,54	8,39	0,25	0,20	21,39	0,50	3,25	3,65	0,45	0,86
11	4,86	72,05	7,50	0,28	0,28	18,87	0,49	3,86	4,25	0,56	0,86
12	4,67	72,03	7,38	0,15	0,16	19,56	0,46	3,71	4,09	0,32	0,79
13	5,24	72,84	7,03	0,24	0,14	19,57	0,43	3,72	4,08	0,39	0,92
14	4,98	70,69	7,66	0,22	0,12	21,17	0,47	3,34	3,70	0,34	0,83
15	4,56	71,01	7,67	0,22	0,15	19,94	0,47	3,61	3,99	0,37	0,81

Regarding the control samples, next table shows the results of composition, digestibility and total volatile fatty acid production (Table 16).

Table 16. Effect of grinding and washing process in the nutritional composition, digetsibility and total volatile fatty acid prodcution.

Grinding	Washing	Sugars (mg/g)	Polyphenols (mg GAE/mg)	Antioxidant activity (mg TEAC/g)	Digestibility (%)	Ash (%)	Protein (%)	ADF (%)	Lignin (%)	NDF (%)	Total VFA (mmol/100mL)	CH4 (mmol)
No	No	199	27,8	35,0	28,2	10,2	5,7	36,0	21,3	46,0	4,85	0,79
No	Yes	117	31,3	33,3	22,5	7,3	6,2	47,4	22,9	51,5	4,29	0,79
Yes	No	234	38,0	34,2	30,3	9,1	5,5	40,6	24,2	45,0	5,32	0,86
Yes	Yes	121	30,6	35,3	21,9	7,4	5,7	51,0	22,4	53,2	5,45	0,79

Comparing these results with the obtained in the hydrolysed samples of the second experimental design (Table 11), in general there is an increase in the digestibility of some of the hydrolysed samples (until 43 %), similar or lower values in Total volatile fatty acid production, and an increase in the lignin concentration. There is also an increase of the ADF and NDF concentration in samples when the hydrolysis was carried out.

Washing decreases sugar concentration in samples and increases the ADF and NDF concentration, leading to a decrease in digestibility.

5. Start-up pilot operation

In the start-up pilot operation, the main objective was to ensure that proposed equipment and bioprocess will successfully operate at pilot scale in WP3, including corrective and adjustment actions. The pilot plant is in RIERA NADEU company, in Granollers (Spain).

5.1 Objective

The objectives of the initial tests were:

- Drying the product to a final moisture content of 10 %.
- Drying the entire sample of 1.5 tonnes of product.
- Observe the behaviour of the product and critical points such as the feeding system and once inside the drying chamber.

5.2 Methods

The correct operation of the installation, in order to obtain a correct final product, is based on the variation of the parameters that condition the humidity, temperature and granulometry of the product coming out of the RINA-JET turbo-dryer (Figure 9). These parameters are modified depending on the humidity, temperature and granulometry measured during the tests. In the specific case that concerns us, the following parameters have been varied:

- Feed rate
- Inlet air temperature
- Outgoing air temperature
- Air flow rate
- Inlet product humidity
- Inlet product particle size

The system introduces, by means of a header fan, hot air or inert gas at low pressure to a direct or indirect heating system, depending on the product or application, into the Drying Chamber, the RINA JET itself, creating a disintegration and circulation effect. The product to be dried is introduced in a controlled (dosed)

way through a spray nozzle (when dealing with suspensions and solutions by means of a pump) or through the upper part by means of a duct, Venturi, auger, alveolar dosing valve or conveyor belt (when drying wet solids). RINA-JET dryers allow drying products whatever their physical presentation.

In the drying chamber, the product is disaggregated producing two effects: a vacuum as a consequence of the speed that the particles acquire and an increase of the contact or transfer surface, which causes an intense drying action with a minimum heating of the solid.

Particle classification is produced by the centrifugal effect in the toroidal recirculation section and by suction control, with the most humid and/or agglomerated particles returning to the chamber, while the disintegrated and dry particles are directed to the air-dust separation elements (Cyclone and Automatic Filter).

The RINA-JET thermal drying system is designed to maintain the process parameters by automatic adjustments of the feed rate, ensuring a constant evaporation for each temperature level.

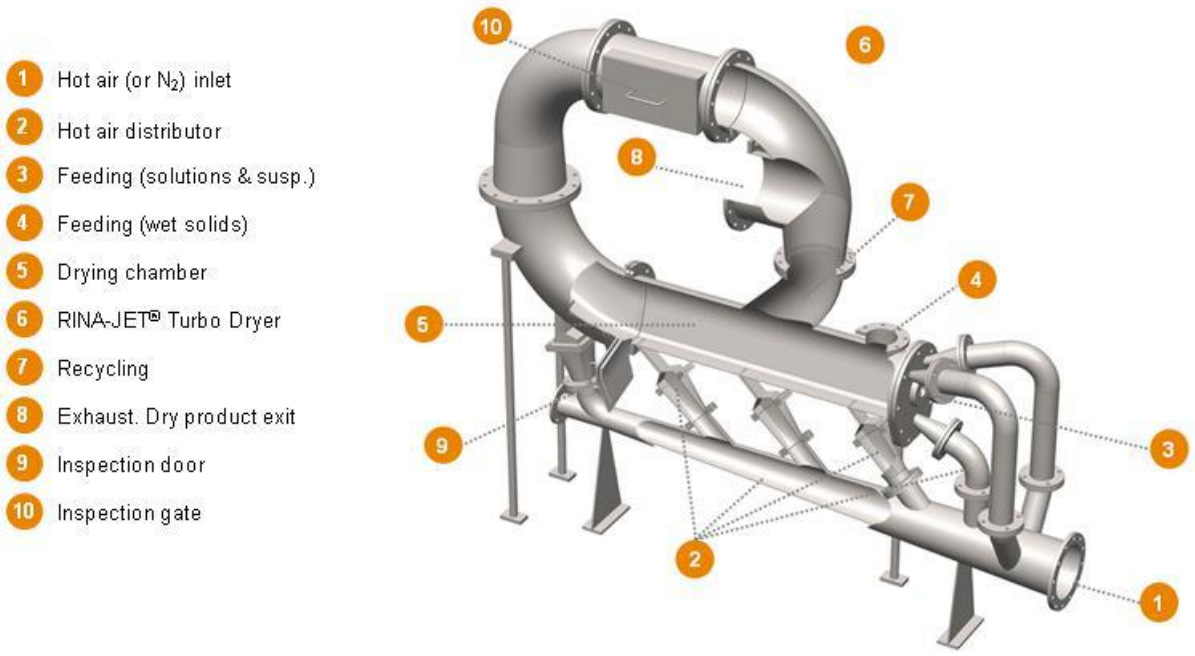

Figure 9. Diagram of the RINA-JET turbo-dryer.

5.3 Results

In the next diagram (Figure 10), the grape stem recollected in October 2021 was stabilized by drying in a flash dryer. 1760 Kg of fresh grape stem were dried and a total of 259 Kg of dried ingredient were obtained.

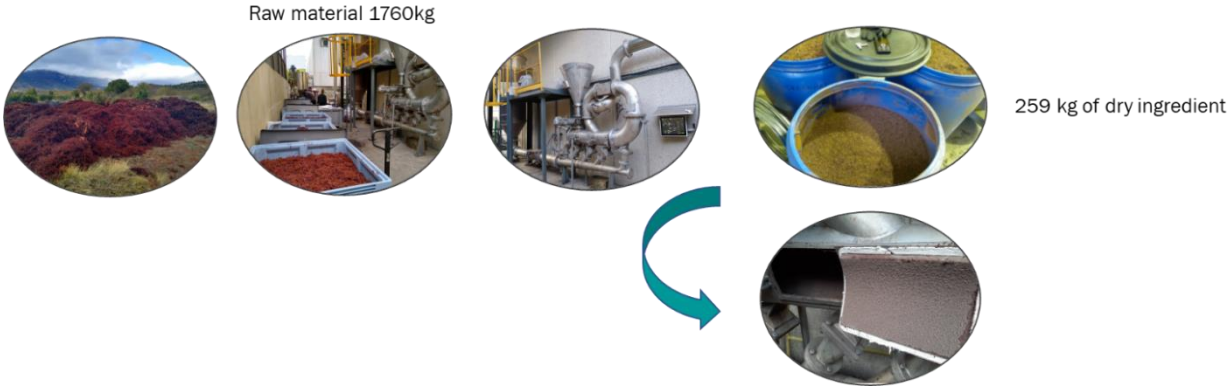

Figure 10. The drying process developed at RIERA NADEU.

The results obtained in the tests were positive as the final product reached the required moisture standard, apparently with good properties after the flash drying process.

Due to the "stops" in the tests, mainly because the high sugar content of the sample which make it to stick to the dryer chamber walls, it was not possible to have an exact measurement of the feeding rate, but only an approximate value. In addition, several stops were made as the product started to burn when it was stuck to the walls.

5.4 Conclusions and comments

Although the test was concluded with satisfactory results, points to be taken into account when designing a future industrial installation for the application in question were detected:

- The feeding system must be the indicated for this type of product, helping it to disintegrate.
- Some type of alternative pre-drying could be applied, to help correct dosage and at the same time reduce the sugars that make the product sticky.
- Possibility of a coating inside the drying chamber to alleviate/improve the possible adherence of the sugars mentioned.

Even with this series of modifications, further tests should be carried out, as the drying process cannot be guaranteed in a continuous way due to the nature of the product, therefore, a washing process of the sample before its drying was proposed in order to eliminate the free sugars present in the sample and improve the drying efficiency. This washing process has been taken into account in the experimental designs of the hydrolysis optimization process in laboratory scale to improve the nutritional and in vitro digestibility value.

5.5 Definition of the washing process

Taking into account the drying problems associated with the sugars present in the sample, AZTI started defining an efficient and scalable way to develop a pre-drying washing process. For this purpose, two lab scale trials were carried out for the scaling up of the washing step in WP3 with a “Pulp Wash” technology

provider, where the main objective was to analyze different parameters to later work in the soluble solid's recovery, from the wine grape stems to avoid the sugars problem adhering to the dryer surface. Once the sugars are recovered, the liquor can be used as fruit sugars, after concentration, or to ferment and produce bioethanol. The solid part free of sugars, would be used as feed for ruminants after a drying process.

5.5.1 First trial

In the first trial (Figure 11) the objective was to know the recovery efficiency of these initial sugars from the grape stem, by using a counter current, one step washing process, and evaluate the use of pulp wash Technology for this purpose.

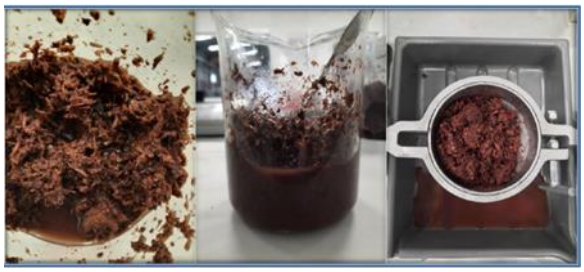

Figure 11. Washing process in the first trial.

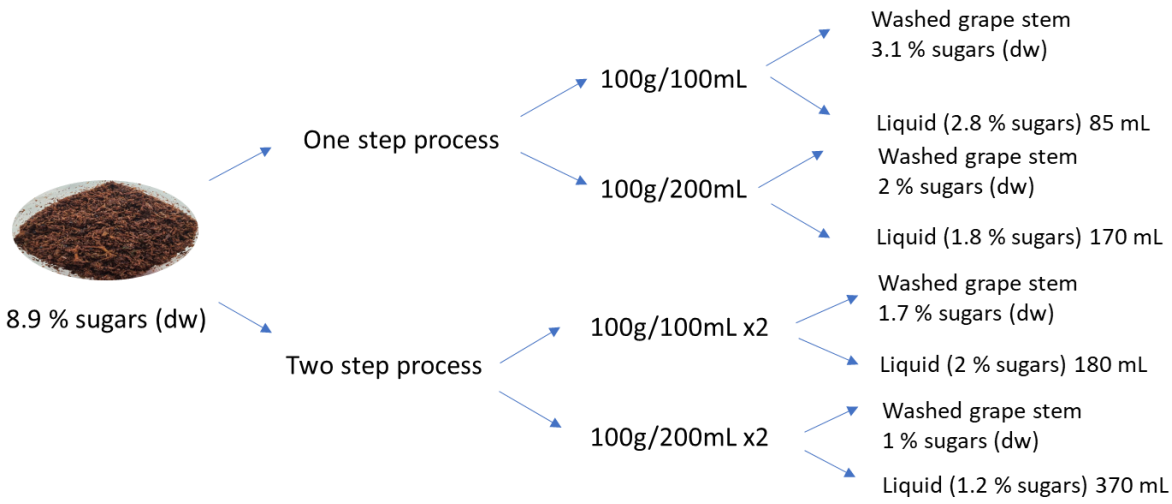

Figure 12. Experimental results of the differents washing processes developed in the first trial.

In the Figure 12, 2 processes were carried out. A one step process using two solid:liquid ratios 1:1 and 1:2, and a two-step process also with two solid:liquid ratios 1:1 and 1:2. Increasing the solid liquid ratio increased the sugar extraction of the final solid sample, but the liquor had less sugar concentration.

When the two-step process was performed, the lowest sugar content was found in the washed grape stem (1-1.7 % sugars), but the liquor sugar concentration is also lower (1.2-2 % sugar).

The initial tests showed a good sugars recovery efficiency from the initial raw material. As conclusion:

- The obtained liquor had very good characteristics to be concentrated and used in the food sector: Low pulp content.
- It was well clarified. Not presence of pectin that could complicate the concentration process.
- The liquor had a high sugar concentration.

5.5.2 Second trial

In the second trial the objective was to know the recovery efficiency of these initial sugars from the grape stem, but at this time, improving the efficiency of liquor sugar recovery through mechanical agitation and increasing the time of agitation.

100 gr of grape stem and 200 ml of water were mixed and were shaken for 3 minutes. Initial Brix of the sample was 8,9. The following table shows the obtained results (Table 17):

Table 17. Results of the second washing trial in the technology provider laboratory.

Tests		Obtained waste weight (g)	Obtained liquor volume (mL)	Brix (after washing)	Liquor Brix	Mass recovery (%)	Acid (%)	Solids (%)
1.1	100 g/ 200 mL	129	165	2,4	5,0	65,11	0,58	8
1.2		135	160	2,3	4,9	65,01	0,57	6
1.3		137	160	2,3	4,9	64,49	0,63	7

The main conclusions were:

- Mechanical agitation and longer time showed a slight improvement on sugar recovery.
- The grape stem integrity was not affected by the mechanical agitation.
- The obtained liquor from this test was sent to AZTI in order to analyze the product and the viability of the bio-ethanol transformation and other applications.

The solid samples obtained were sent to RIERA NADEU so that they could analyse the possibility of drying once the cleaning and elimination of sugars is carried out. They concluded that the sample could be able to pass directly to the dryer with a improve of the efficiency in the drying process.

In the case of the liquor, a sample was sent to NTUA for the assessment of its applicability as a substrate to produce bioethanol, due to their extensive experience in the production of bioethanol from by-products.

In NTUA they performed the fermentation tests (18h, 30 ºC, *Saccharomyces Cerevisiae*) to the samples. They concluded that all the glucose was consumed to ethanol and almost 70% of the fructose. Thus a mean fermentation yield of 81% was achieved.

In addition, we have also analysed the sugar profile of the liquor samples to better define the future market applications (Table 18).

Table 18. Sugar profile of the washed grape satem liquor.

Samples	Fructose (%)	Glucose (%)	Total sugars (%)
1	1,02	1,32	2,35
2	1,01	1,27	2,28

5.5.3 Scale up of the washing process

Before scaling up in WP3, an industrial test was performed in the technology provider facilities (JBT –Spain) for the verification of the pulp washing technology in high quantities.

1,5 tons of grape stems were collected and crushed using Urschel Comitrol® technology to reduce the particle size and guaranty the homogenization of the samples.

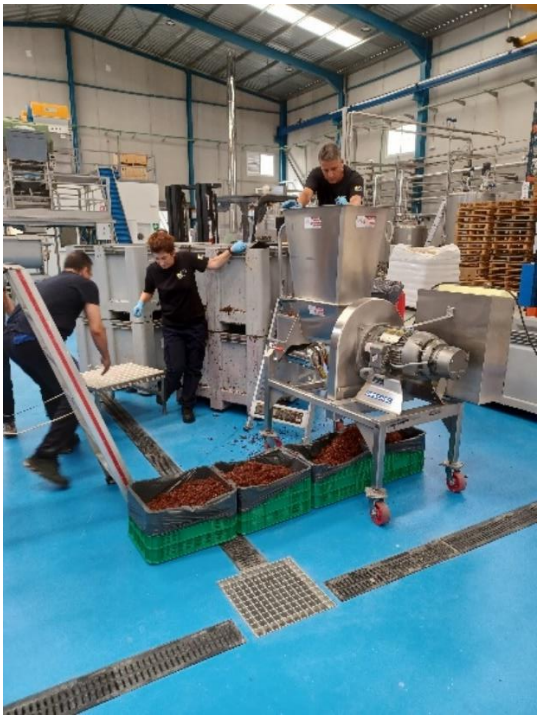

Figure 13. Image of Urschel Comitrol® technology.

Once crushed, grape stems were washed using JBT technology (Figure 14) to obtain two different raw materials:

1. Semi-solid rich in fibre (80 % moisture)
2. Liquor rich in sugars (5° brix)

Figure 14. Image of JBT technology.

The obtained ratios of the intermediate and final products are shown below:

100 kg of grape stems (75 % moisture)

+

100 Kg of water

↓

100 kg de washed grape stems (80 % moisture) → Animal feeding

+

68,5 Kg of liquor (5° Brix) → Bioethanol

After crushing and washing, washed grape stems were sent to RIERA NADEU for the drying process (Figure 15). The drying process was successfully conducted and without problems associated to the sugars as happened before removing sugars. So, the washing step will be a pre-drying process in the WP3 trials.

Figure 15. Image of RIERA NADEU technology.

6. Optimization of the innovative and efficient process

One of the main objectives of the case study 1 in the WP2, is to optimize the enzymatic and chemical hydrolysis processes in laboratory scale to improve the nutritional and in vitro digestibility value of the grape stem-based ingredient.

To carry out a hydrolysis process, water or another solvent must be added to the solid to be digested. Once the hydrolysis process is finished, it is necessary to optimize the drying process, which must combine a mechanical dewatering and a thermal drying to stabilize the hydrolysed ingredients.

In the next diagram (Figure 16) AZTI and RIERA have stablished the steps needed for the drying of the hydrolysed grape stem.

Figure 16. The steps stablished for the drying of the hydorlysed grape stem. From left to right: hydrolysis tank; centrifugation (outside and inside view); flash drying of the solid sample.

Centrifugation is a mechanical method of separating immiscible liquids, or solids and liquids by the application of centrifugal force. Centrifuges are instruments that allow samples to be subjected to intense forces that produce the separation in a short time of particles that have a higher density than that of the surrounding medium. The RINA 200 Series models are vertical axis centrifuges with automatic discharge

dedicated to solid-liquid separation by high-speed filtration. The centrifugation process was carried out at 1100 rpm with a 150 µm mesh.

The whole process was well conducted, and results obtained indicated that the centrifugation and subsequent drying process using the RINA-JET Turbo-Dryer with toroidal configuration is suitable for the required process and could be scalable in WP3.

7. General conclusions

Grape stems are rich in fibre and low in energy, which makes it difficult to include them in percentages higher than 5 % of total feed. However, it is a product rich in bioactive compounds such as polyphenols, that have an antioxidant and antimicrobial effect, among others, which can be beneficial for ruminal digestion.

In order to favour its inclusion in ruminant diets, a protocol has been established for collection and subsequent washing, which will be scaled up in the near future, to reduce the sugar content of the ingredient. This will facilitate drying and improving the efficiency of the process. In this way, we will obtain a liquor rich in sugars, which can be used in the food sector and/or for bioethanol production, and an established ingredient for animal feeding.

These preliminary tests have also allowed us to design the drying process after the hydrolysis processes of the grape stem, where we will proceed with mechanical drying by centrifugation and subsequent drying by flash drying.

When grape stems were washed, the basic hydrolysis improves the nutritional value of grape stems by increasing *in vitro* digestibility and potential gas production but at the expense of a less efficient fermentation process. When grape stems were not washed, none of the hydrolysis processes tested improve the nutritional value of grape stems. However, Alkali hydrolysis was selected for further optimization through a Box-Behnken design. The washing step was removed from the study as the alkali hydrolysis should work as a washing itself. In the alkali hydrolysis process the optimum conditions selected by the second experimental design for digestibility improvement are 90 ºC, 2,3 h of hydrolysis at 33%. With these conditions a final digestibility of 41 % is obtained. Hydrolysed and non-hydrolysed samples will be validated in WP3 feeding trials.

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