# D5.1 Initial report on characterization of Pro-Enrich raw materials and products

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# Contents



## Executive summary

WP5 relies on the materials produced by WP3. At this stage in the project, these are not finalized. However, at this moment the methodologies for measurement of target compounds have been prepared in agreement with NATAC who makes initial raw material assessments in WP2. Based on the current growing season, olive mill residues have been collected, stabilized, and stored from Slovenian olive mill partner Franka Marzi. They are now ready for measurement in WP2 and processing in WP3 and WP4. Rape seed press cake can be collected at any time during the year and citrus and tomatoes will be collected during the first quarter of 2019. With all samples collected, laboratory routines can be developed to measure target compounds.

## 1. Introduction

This is a public document reporting on the characterisation efforts of the Pro-Enrich project with respect to raw materials and products. This document serves as an informational overview of the general activities underway and is the first report of three. Any confidential details of test procedures, internal communications, and/or results will be withheld.

InnoRenew is leading WP 5, which is designed to assess the efficacy of WP3 treatments and extractions. Initial measurements of target compounds from raw material sources are being conducted by NATAC as part of WP2. It is planned that WP5 will assess the results after various preprocessing treatments or extractions have been made. At this point in the project, few treatments and extractions have been made in WP3. Therefore, this report will try to broadly describe the approach that will be taken.

**WP5: Analysis and validation**

This WP will provide in-depth analysis and characterization of the targeted compounds and generated products in the PRO-ENRICH project (as determined in WP2). Identification of valuable compounds, their abundance, and method for extraction will be determined. Performance testing of obtained target compounds will be undertaken by industry partners according to relevance in their respective business areas.

## 2. Current Status of Research

### Sample Collection

Do to the current growing season, work that has been done at this point has been with olive mill residues and rape seed press cake. Rapeseed press cake can be collected at any time throughout the year and citrus and tomatoes will be collected during the first quarter of 2019. For olive mill residues, sampling of two Slovenian olive mills was completed for this season (October and November). Sampling was conducted at the olive mills, Oljarna Krožera Franka Marzi (ProEnrich partner) and nearby cooperating olive mill, Lisjak. The following materials were sampled: olive pomace with stones, pomace without stones, stones, waste water, and olive leaves.

The two olive mills use different processing technologies, two-phase (Lisjak) and three-phase decanters (Franka Marzi). In the case of the 2-phase decanter, only pomace is obtained. The pomace from this technology has a relatively wet (~70 % water content). In the case of 3-phase decanters, the pomace is relatively dry (~10% water content) and mill water is produced as a separate phase and collected. When using 3-phase decanters, tap water was added resulting in approximately 30 % in the collected mill water.

Samples were collected every week from each mill (Figure 1). The exact date of sampling and the sample preparation for each collected sample are shown in Table 1. Approximately 2.5 liters of pomace and water were collected for each sample. From this approximately 500 g was taken to be freeze dried the next day. Both the 2 L and 500 g portions were immediately frozen at -20C. The small portion was then freeze dried the next day resulting in approximately 150 g of dry material. Olive leaves collected with some being air dried and some being freeze dried (Table 1). The stones and mill water were immediately frozen at -20 ˚C as freeze drying is not necessary. The sample preparation and the storage of olive mill waste extracts was performed according Obied et al. (2007,2008). The prepared samples are shown in Figures 1-3.

Table 1. Sample collection and preparation.

| Number | Sample label | Type of sample | Olive mill | Sampling date | Information about the sample | freezing | freeze drying | air drying |
|---|---|---|---|---|---|---|---|---|
| 1 | vz. 1 | pomace without stones | 2 phase (Lisjak) | 14.10.2018 | 80% 'Leccino', 20 % other, probably 'Maurino' | x | x | |
| 2 | vz. 2 | pomace without stones (70%) | 3 phase (Marzi) | 14.10.2018 | 50% 'Istrska belica', 50% 'Leccino' | x | x | |
| 3 | vz. 2 | mill water | 3 phase (Marzi) | 14.10.2018 | 50% 'Istrska belica', 50% 'Leccino' | x | | |
| 4 | vz. 3 | pomace with stones | 2 phase (Lisjak) | 21.10.2018 | 'Leccino' | x | x | |
| 5 | vz. 4 | pomace without stones (70%) | 3 phase (Marzi) | 21.10.2018 | 'Leccino' | x | x | |
| 6 | vz. 4 | mill water | 3 phase (Marzi) | 21.10.2018 | 'Leccino' | x | | |
| 7 | vz. 5 | pomace without stones | 2 phase (Lisjak) | 28.10.2018 | Mixed varities | x | x | |
| 8 | vz. 6 | pomace without stones (70%) | 3 phase (Marzi) | 28.10.2018 | Mixed varities | x | x | |
| 9 | vz. 6 | mill water | 3 phase (Marzi) | 28.10.2018 | Mixed varities | x | | |
| 10 | vz. 6 | leaves | 3 phase (Marzi) | 28.10.2018 | Mixed varities | | x | x |
| 11 | vz. 6 | stones | 3 phase (Marzi) | 28.10.2018 | Mixed varities | x | | |
| 12 | vz. 7 | pomace without stones | 2 phase (Lisjak) | 4.11.2018 | 'Istrska belica' | x | x | |
| 13 | vz. 8 | pomace without stones (70%) | 3 phase (Marzi) | 4.11.2018 | Mixed varities | x | x | |
| 14 | vz. 8 | mill water | 3 phase (Marzi) | 4.11.2018 | Mixed varities | x | | |
| 15 | vz. 8 | leaves | 3 phase (Marzi) | 4.11.2018 | Mixed varities | | x | x |
| 16 | vz. 8 | stones | 3 phase (Marzi) | 4.11.2018 | Mixed varities | x | | |
| 17 | vz. 9 | pomace without stones | 2 phase (Lisjak) | 11.11.2018 | 'Leccino' | x | x | |
| 18 | vz. 10 | pomace without stones (70%) | 3 phase (Marzi) | 11.11.2018 | 'Istrska belica' | x | x | |
| 19 | vz. 10 | mill water | 3 phase (Marzi) | 11.11.2018 | 'Istrska belica' | x | | |
| 20 | vz. 10 | leaves | 3 phase (Marzi) | 11.11.2018 | 'Istrska belica' | | x | x |
| 21 | vz. 10 | stones | 3 phase (Marzi) | 11.11.2018 | 'Istrska belica' | x | | |

Figure 1. Samples of pomace (left) and waste water (right).

Figure 2. Freeze-dried pomace

Figure 3. Air dried olive leaves collected for measurements.

Figure 4. Olive mill water.

Figure 5. Olive stones.

## Measurement methodologies

WP2 leader NATAC and WP5 leader InnoRenew have coordinated efforts to ensure the same methodologies are used and that the measured values are comparable. Methodologies for analysis of olive residues, tomatoes, and citrus have been prepared at this time, with rapeseed analysis to follow. The following methodologies are planned to be used for measurement of target compounds from Pro-Enrich agricultural residues.

*Determination of phenolic compounds in pomace and mill water*

After sampling and initial freezing, pomace from two-phase and three-face decanters was lyophilized and crushed to form powder and then stored until the analysis at -20˚ C. The mill water was frozen and stored until analysis at -20˚ C. This is the current state of the project. However, we plan to use the following methodology for upcoming measurements:

Standards:

-2-(4-hydroxyphenyl) ethanol (tyrosol), Purity > 97%

-2-(3,4-Dihydroxyphenyl)ethanol (3-Hydroxytyrosol), Purity ≥ 98.0%

- Verbascoside, Purity ≥ 99%
- 3',4',5,7-Tetrahydroxyflavone (luteolin), Purity ≥98%
- 4',5,7-Trihydroxyflavone (apigenin), Purity ≥99%

Reagents:

- methanoic acid, 98-100% (V / v) or less
- methanol for HPLC
- acetonitrile for HPLC
- HPLC water
- n-hexane, analytical purity
- Hydrochloric acid

Phenolic compounds in olive pomace and mill water, such as hydrocystyrosol glucoside, hydroxytriol, tyrosol, verbascoside, lutelline glucoside, lutelline, and apigenin will be extracted using 60% (w/w) aqueous methanol solution with the addition of hydrochloric acid (Obied et al., 2005). Samples are then to be analysed using reverse phase high-performance liquid chromatography (HPLC), according to COI/T.20/Doc No 29 (2018). Detection will be at 280 nm, with the exception of the flavonoids luteolin and apigenin, which are detected at 340 nm. Calibration curves for tyrosol (mass fraction from 30 to 800 mg/kg; y = 0.0811a) will be constructed using standard compounds. Total phenolic compounds will be determined according to the IOC publication and quantified using the response factor for tyrosol. The term "total phenolic compounds" refers to the "biophenolic minor polar compounds" determined according to the IOC method.

The phenolic compounds will be further identified and quantified using an ultrahigh-pressure liquid chromatography system (UHPLC), interfaced with a qTOF mass spectrometer (UHPLC-ESI-QTOF / MS). UHPLC is equipped with a Poroshell 120 column (EC-C18; 2.7 µm; 3.0 × 50 mm). The following elution gradient will be used: water/ formic acid (99.05: 0.5, v/v) (A) and acetonitrile/ methanol (50: 50, v/v) (B); 0 → 3.96 min, 96% A; 3.96→ 4.45 min, 50% A; 4.45 → 5.94 min, 40% A; 5.94 → 7.12 min, 0% A (Miklavčič Višnjevec et al., 2018). Well-known phenolic compounds such as hydroxytirosol, tyrosol, verbascoside, luteolin and apigenin can be quantified as well.

**HPLC conditions:**

Column: Poroshell 120 column (EC-C18; 2.7 µm; 3.0 × 50 mm).
Mobile phase: A: water/ formic acid (99.05: 0.5, v/v), B: Acetonitrile/Methanol (50: 50, v/v)
Wavelengh: 280 nm, 340 nm
Injection volume: 10 µL
Run time: 10 min
Temperature: 20ºC

*Determination of phenolic compounds in olive leaves*

Standards:

-2-(4-hydroxyphenyl) ethanol (tyrosol), Purity: > 97%

-2-(3,4-Dihydroxyphenyl)ethanol (3-Hydroxytyrosol), Purity: ≥ 98.0%

- verbascoside, Purity: ≥ 99%

- oleuropein, Purity ≥ 98.0%

Reagents:

- methanoic acid, 98-100% (v / v)

- methanol for HPLC

- ethanol for HPLC

- acetonitrile for HPLC

- n-hexane, analytical purity

- HPLC water

Olive leaves will be milled to form a powder and then lyophilized.

Phenolic compounds are then extracted by means of ethanol/water solution (70: 30, v/v) and quantified by high-performance binary gradient liquid chromatography (HPLC) equipped with DAD with measurement wavelength at 280 nm. The procedure is described in more detail elsewhere (Butinar *et. al.*, 2012; Miklavčič Višnjevec *et al*., 2016; Bučar-Miklavčič *et al.*, 2016).

**HPLC conditions:**

Column: Phenomenex Synergi 4 µm Hydro-RP 80 Å (250 x 4.6 mm i.d.) column (Torrance, CA, USA) or likewise

Mobile phase: A: water/ formic acid (99.05: 0.5, v/v), B: Acetonitrile/Methanol (50: 50, v/v)

Wavelengh: 280 nm

Injection volume: 10 µL

Run time: 90 min

Temperature: 20ºC

*Crude protein mass balance from rapeseed press cake*

**Analytical method:**

Dumas method

**Objective:**

To obtain a quantitative measure of the degree of protein solubilization from various raw materials. As a measure of crude protein, the amount of nitrogen x 6.25 is used.

**Materials:**

- Dry raw material (rapeseed or other source)
- Aqueous extract from raw material
- Protein analyzer (Rapid N-Cube, Elementar)

**Method:**

The amount of nitrogen is determined for both the dry raw material and the aqueous protein extract using the Protein analyzer.

**Results:**

The degree of solublization is determined as the amount of nitrogen in aqueous extract divided by the amount of nitrogen in the raw material.

*Protein composition of aqueous rapeseed extract*

**Analytical method:**

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE)

**Objective**:

To evaluate the protein composition of aqueous extracts from rapeseeds and other raw materials.

**Materials:**

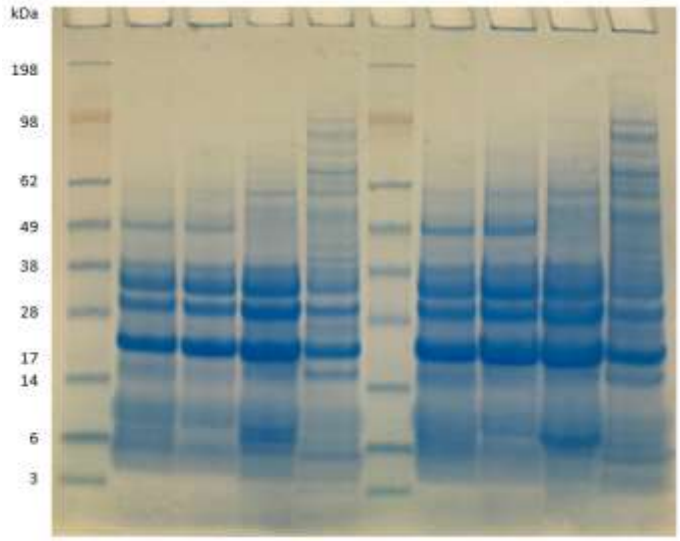
- 4-12% acrylamide gels (Genscript)
- NuPAGE MES SDS running buffer (Thermo Fisher Scientific)
- NuPAGE LDS sample buffer x4 (Thermo Fisher Scientific)
- Protein standard (SeeBlue Plus2 pre-stained protein standard)
- Dithiothritol (DTT) reducing agent
- Protein extract of interest
- Coomassie stain (InstantBlue, Expedeon)

**Method:**

Mix protein extract DTT (10 mM final conc.) and LDS sample buffer (1x final conc.) and heat for 10 minutes. Samples are then loaded on 4-12% acrylamide gels and voltage is aaplied. Proteins are visualized using Coomassie stain.

**Results:**

A typical result is shown below (rapeseed extract):



*Determination of hesperidin and other flavonoids in citrus products by HPLC-DAD*

**Standards:**

Hesperidin, Purity: 96,8%

Reagents:

Phosphoric acid, HPLC grade

Water, HPLC grade

Acetonitrile, HPLC grade

Dimethylsulfoxide (DMSO), HPLC grade

The determination of hesperidin and other flavonoids in citrus products will be performed according to the internal NATAC protocol.

Approximately 5 g of the dry-milled sample (less than 15% humidity) is placed in a 500 mL beaker. 200 mL of dimethylsulfoxide is added and agitated at room temperature for 1 hour. The sample solution is then filtered and the liquid is transferred to a 250 ml volumetric flask. The solid was rinsed with 40 mL dimethylsulfoxide, transferred to the volumetric flask and the same solvent is refilled to the mark. The solution was filtered through 0.45 µm membrane filter with a syringe and a vial for HPLC.

**Chromatographic conditions**

Column: Symmetry C18 5 µm; 250 x 4,6 mm (or similar)
Mobile phases: Phase A: 0.04% Phosphoric acid/Water (v/v), Phase B: Acetonitrile
Wavelength: 280 nm
Temperature: 35ºC
Flow: 1 ml/min
Injection Volume: 10 µL

*Determination of total carotenoids in citrus and tomato waste material by HPLC-DAD*

**Standards:**

according to the carotenoids present in the sample

Reagents:

- Methanol, HPLC grade
- Hexane, HPLC grade
- Isopropanol, HPLC grade

The determination of total carotenoids in citrus and tomato waste material will be performed according to the internal NATAC protocol.

Approximately 10 g of a dry-milled sample (less than 15% humidity) is placed in a 500 mL beaker. 200 mL of methanol is added and agitated at 40ºC protected from light and air for 1 hour. The liquid is then filtered and transferred to a 250 ml volumetric flask. The solid is rinsed with 40ml of methanol, transferred to the volumetric flask, and then refilled with the same solvent to the mark.

The solution is then filtered through 0.45 µm membrane filter with a syringe and a vial for HPLC measurement is then prepared.

**Chromatographic conditions**

Column: Symmetry C18 5 µm; 250 x 4,6 mm (or similar)
Mobile phases: Phase A: Methanol, Phase B: Isopropanol:Hexane (1:3)
Wavelength: 447 nm
Temperature: 25ºC
Flow: 0.5 ml/min
Injection Volume: 5 µL

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## 3. Conclusions

1) Olive mill waste streams have been sampled from the Franka Marzi mill and neighbouring Lisjak mill in Slovenia. These materials have been stabilized and stored for measurements in the upcoming months.
2) Methodologies have been prepared and selected in cooperation between the InnoRenew CoE and NATAC. NATAC will be analysing target compounds present within raw materials in WP2 and InnoRenew will use the same procedures for measuring target compounds in fractions produced from techniques used in WP3 and WP4.