Valorization of wine industry waste as a source of antioxidants for an enriched animal diet
(Valorificareareziduurilorindustrieivinicoleprinnutriţiaanimalăîmbogăţităînantioxidanţi)

Synthetic scientific report 2013-2016

Phase 2013

Objectiv 1.1 Water extraction of grape pomace (ET), polyphenols composition and antioxidant activity evaluation.

Activity 1.1.1 Water extraction of grape pomace (ET) and total polyphenol content

The grape pomace resulted from red wine fermentation was collected from ValeaCalugareasca winery, dried and provided for this project by Mr.Stroie Constantin the general manager of DionisAgrifood company.The grape pomace was subsequently grinded in view of extraction. After the hot water extraction, the total polyphenols (TP) were determined by the Folin-Ciocalteu method. The results were expressed as mg gallic acids equivalents (GAE)/L, and for the ET extract the total polyphenol quantity was **422.91±13.27 mg GAE/Lof ET extract.**

Activity 1.1.2 Analysis byUV-Vis spectroscopy and high performance liquid chromatography coupled with mass spectroscopy (LC-MS)of the polyphenol composition of ET

The spectrum of the grape pomace water extract (**ET**) shows a absorption maximum at λ =270 nm. This indicates the presence of **flavan-3-ol, catechinandprocianidin**type compounds.

For the LC-DAD-MS analysis, 500 µl of ET were filtered through a SPARTAN 13, 0.45 µm (Whatman) filter before the chromatographic separation.
The analysis was carried on in the “electrospray positive ion” (ESI+) mode, on aAQA (Thermoquest/ Finnigan) mass spectrometer, coupled to a P4000LC (Finnigan) pump and a UV6000LP diode array detector (Finnigan). The separation was done on aM-1021290001 Chromolith performance RP-18ET column.
According to the UV-Vis and mass spectra, in the composition of the ET extract there were identified derivatives of **phenolic, protocatechuic, caffeic, gallicand vanillic acids**. These derivatives of phenolic acids are formed with one of the B procyanidins (which is an

epicatechindimmer). Cathechin and epicatechin were also identified, being among the important components of this extract.

Activity 1.1.3 Antioxidant activity determination for ET: FRAP and DPPH tests, inhibition of soy pure lipoxygenase-1

Determination of total antioxidant activity as ferric reducing power - FRAP

The **FRAP**value of the TE extract is **13,85±1,47**.

Determination of antiradical antioxidant activity by DPPH test

The antiradical activity of the extract is measured asTrolox equivalents, and is calculated following an equation obtained from a calibration curve.

The antiradical activity was calculated for 5 concentrations: 0,42 mg GAE/ml extract, 0,28 mg GAE/ml extract, 0,21 mg GAE/ml extract, 0,14 mg GAE/ml extract, 0,007 mg GAE/ml extract. By graphical representation of the antiradical activity according to the concentration, the **EC_{50}**index was calculated as being **1.56 mg GAE/ml extract**.

Determination of the pure soy lipoxygenase-1 inhibition (sLOX) in the presence of ET extract

Kinetics of thelipoxygenase reaction and determination of the sLOXinhibition in the presence of ET extract

The enzymatic activity was measured using a Specord 250 (Analytic Jena) spectrophotometer at 234 nm and 25°C. The sLOXactivity is determined by the increase in absorbance at 234- by the formation of reaction products- after the addition of linoleic acid in borate buffer along with the enzyme.

The ET extract does not show the inhibition of lipoxygenase.

By achieving the entire project objective for the year 2013, the ET grape pomace extract was obtained, which was analysed by high performance liquid chromatography (HPLC) coupled with mass spectrometry.Thereby, it was underlined that the ET contains oligomers formed from phenolic acids and B procyanidin (epicatechin dimer).ET shows antiradical

and ferric reduction antioxidant activity, but does not show anti-lipoxygenase activity.

Phase 2014

Objective 1. *In vitro* and *in vivo* experimental evaluation of ET effect on swine

Activity1.1. Carrying out the ET treatments on porcine intestinal epithelial cells (IPEC-1): spectrophotometric evaluation of polyphenols in the culture media after the ET treatment of the cells and the spectrophotometric evaluation of polyphenols in the intracellular matrix after the ET treatment.

IPEC-1 cells, a line of porcine intestinal epithelial cells, seeded at $2x10^5$ cell/ml were allowed to adhere for 24h and then treated with grape pomace extract (ET) in concentrations of 250 ng/ml, 500 ng/ml and 1000 ng/ml.The cell cytotoxicity was measured by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] assay, thus the concentrations were chosen in terms of not being toxic and lethal for the cells. The treatment lasted for a short incubation period of 3h and for a long incubation period of 24h. The absorbance was measured at 570nm using a microplate reader (TECAN SUNRISE, Austria) and the absorbance of the background at 650nm was subtracted.

In the intracellular matrix, after 24h of incubation of the IPEC cells with ET, the best absorption of the extract polyphenols is recorded for the highest administered dose (1000ng/ml). The absorption maximum, λ max, in this case was 287.1, higher than the one of the ET extract. The spectrum of the grape pomace water extract (ET) shows an absorption maximum at λ =270 nm. This bathochromic increase shows a slight oxidation of the molecules in the cells.

An interesting fact is that at the lowest administered dose, an intense absorption at λ max = 295.6 nm was noticed in the extracellular medium. This result could be motivated by the oxidation reactions that possibly occur between the polyphenols in the ET and the components of the extracellular medium (proteins, other polyphenols). A general observation can bemade in the case of all measurements, which is the oxidation of the ET polyphenols both in the cells and the extracellular medium.This oxidation gives absorption maximums between λ max =276 nm and λ max=627.0. This extended modulation of the oxidation can be explained by inter- and intra-molecular reactions determined by the oxidative potential of the polyphenols.For λ max with values between 393.3 and 487.5, oxidation compounds were identified - *o*-quinones.

Activity 1.2. Carrying out the *in vivo*experiment 1 on weaned piglets (10-30 kg)

In the frame of the *in vivo* experiment, even though the project activities stipulate only the study of whole grape pomace from which the ET extract was obtained and analyzed, two types of grape pomace were used; with (GPS+) and without (GPS-) seeds, incorporated in two diets (D1/GPS+ si D2/GPS-). The percentage in which the grape pomace was supplied in the pigs' diet was 3%.

18 crossbred starter piglets, were divided into 3 experimental groups (6 piglets/group) and assigned to one of the 3 treatments: control (normal diet for weaned pig-C); diet with integral GP (diet 1-D1); diet with GP without seeds (diet 2-D2) for 42 days. At the end of this period, bloodand organ samples were collected from all three groups and kept at -80^{0}C for further analysis.

Activity 1.3. Determination of immunologic and biochemical parameters

Determination of biochemical parameters.

The biochemical parameters were determined using an automatic BS-130 Chemistry analyzerand processed according to the manufacturer's instructions. The determined biochemical parameters were: glucose, total cholesterol, triglycerides, phosphorus, calcium, magnesium, iron, total protein, albumin, bilirubin, urea, creatinine, alkaline phosphatase (ALKP), aspartate aminotransferase (TGO/AST), alanine transaminase (TGP/ALT), gamma - glutamyltranspeptidase (GGT) and lactate dehydrogenase (LDH).

Statistical analysis.Experimental data were analyzed with the program Stat View 5.0, performing one-way analysis of variance (ANOVA), followed by a Fisher PSLD test. The *p* values lower than 0.05 were considered significant.

Following the results of the biochemical parameters analysis we can say that dietary supplementation with 3% GPs did not negatively influenced the general health status of piglets.All parameters were within the normal limitsbut the levels of glucose, calcium and magnesium in plasma were increased (results statistically significant).

Determination of immunologic parameters. Determination of concentrations of immunoglobulin subclasses (IgG, IgA, IgM)

The total concentration of immunoglobulin subclasses was measured by ELISA (Bethyl). The serum samples were diluted 1/80000, 1/4000 and 1/8000 in saline Tris buffer for the detection of IgG, IgA and IgM, respectively, following the producers recommendations.Dilutions of certain serum samples with known concentrations were used as standards and the results were expressed as mg of immunoglobulin subclass /mL.

GP diets modulated also the humoral immune response. GPS- induced the increasing of the synthesis of nonspecific immunoglobulin M and G (IgM, IgG) with key role in the generation of the first and long lasting immunity, while GPS+ had a contrary effect.

By achieving the full objective and the proposed activities for the year 2014, it has been demonstrated that polyphenols in the grape pomace extract are absorbed by the cells in the oxidized form. The oxidation of these compounds, mediated by *o*-quinones, was observed both in the extracellular medium and the intracellular matrix.

The *in vivo* experiment on weaned piglets that received a diet enriched with 3% grape pomace, indicates the fact that this residue can be incorporated in the feed of piglets, the biochemical and immunological analyses proving that the health state of the animals is good, as well as in the case of the control lot that received the classical diet.

Phase 2015

Objective 1. Evaluation by the*in vivo*experimental studies of the ET administration effect on pig

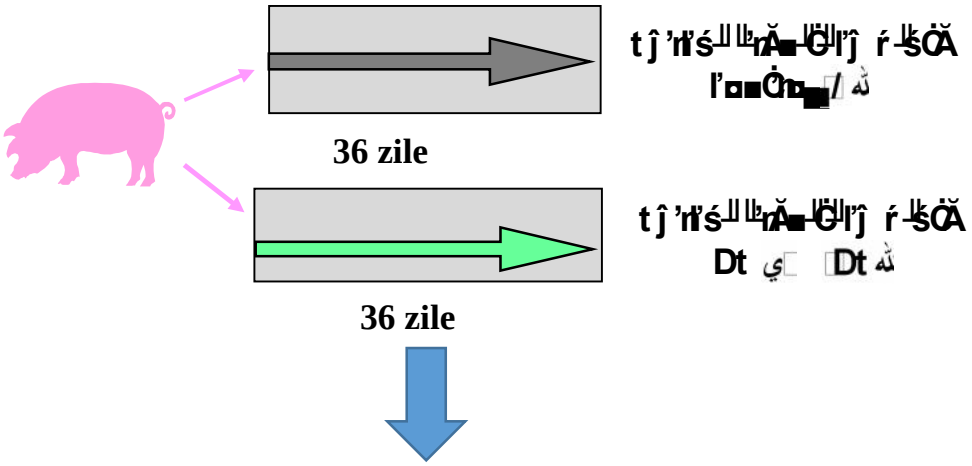
Activity 1.1. Analysis of the polyphenols' absorption in internal organs of pigs collected in the *in vivo* experiment 1

The organs' tissue samples were kept at -80C and meanwhile frozen were milled in liquid nitrogen. For the polyphenols' absorption evaluation the samples were extracted with methanol, sonicated, centrifugated and the supernatant collected and kept at -20 C until the spectra were measured.

By UV-Vis spectroscopy the spectra were determined for each collected organ (brain, heart, duodenum, colon, liver, kidneys, lymph nodes, spleen)andmuscle*Longissimus dorsi*, in total more than 200 spectra. To analyze the spectra from the software program the function "Substraction" was used for substracting the control spectrum from the treatment one, and the „Overlay" function for overlying the resulted spectra from substraction.

Activity 1.2. Carrying out the *in vivo* experiment 2 on weaned piglets (10-30 kg):
- *in vivo*administrationof ET
-Samples' collection

For this experiment, a total number of 20 crossbred TOPIG hybrid [(Landrace × Large White) × (Duroc × Pietrain)] pigs with an average body weight of 10.70 ± 0.8 kg were allocated to two experimental groups (10 pigs/group). The animals individually identified by ear tag were housed in pens and fed with experimental diets for 30 days using basal diet (control group) and basal diet with 5% grape pomace (D/GP group).At the end of the experiment (36 days) animals were slaughtered and samples from the following organs were collected: liver, spleen, duodenum, colon, kidney,*Longissimus dorsi* muscle, mesenteric lymph nodes,heart and brain. The samples were kept at -80^0C until the analysis(spectra determination).The frozen organ samples were milled in liquid nitrogen.The protocol of the experiment is presented below.



In vivo experiment2on weaned piglets

Activity 1.3. Analysis of polyphenols' oxidation in organ's and *Longissimus dorsi* muscle's samples

The analysis of polyphenols' oxidation was done like in the case of absorption (Activity 1.1/ 2015) by UV-Vis spectroscopy.The sample preparation, spectra determination and their analysis was done like in the case of absorption (Activity 1.1/ 2015)

The wavelengths at which absorptionmaximawere recorded for all organs analyzed indicate that polyphenols were metabolized and were thus absorbed in the internal organs of piglets - λmax between 274.9 and 298 nm. Grape pomace aqueous extract (ET) spectrumshows an absorption maximum at λ = 270 nm. This bathochromic change shows a slight oxidation of polyphenols after ingestion. In terms of oxidation λmaxbetween 315 nm and 588 nm can be justified by an extended modulation of oxidation by inter and intramolecular reactions (proteins, other antioxidants) due to the polyphenols'oxidative potential

By achieving the full objective and the proposed activities for the year 2015 it has been demonstratedthat the polyphenols in piglets feed in which grape pomace was included in

proportions of 3% and 5%, are metabolized and then absorbed in the organs studied (brain, heart, duodenum, colon, liver, kidney, mesenteric glands, spleen) and *Longissimus dorsi*musclein oxidized form.

Phase 2016

Objective 1.Correlation of the parameters determined *in vitro*and *in vivo*regarding the absorption and oxidation of polyphenols from ET.

Activity 1.1. Analysis and correlation of *in vitro*and*in vivo*results

By achieving the full objective and the proposed activities for the year 2016 by determining the UV-Vis spectral fingerprints, it has been shown that the intestinal epithelial cells IPEC-1 may be an*in vitro* model for the qualitativeabsorption of polyphenols in the duodenum, the mesenteric lymph nodes, spleen and kidney in pigs. Because in terms of digestion and nutrient absorption, the pig is a model for human nutrition, we can extrapolate the aboveresult and at the level of intestinal digestion and absorption of polyphenols in humans. This conclusion is important to demonstrate the correlation that exists between the *in vitro* and *in vivo* studied models regarding the qualitative absorption of dietary polyphenols at the cellular and tissues' level. Thus in this case the cost and time to perform the experiments *in vivo* on pigscan be reduced, using the *in vitro* method of testing on intestinal epithelial cells IPEC-1.