

Enzyme-Assisted Extraction and Ultrafiltration of Value-Added Compounds from Sour Cherry Wine Pomace

María C. Roda-Serrat*, Cecilie Lundsryd, Simone Rasmussen, Rime B. El-Houri, Peter B. Lund, Knud V. Christensen

Department of Chemical Engineering, Biotechnology and Environmental Technology, University of Southern Denmark. Campusvej 55, 5230 Odense M, Denmark
mcs@kbm.sdu.dk

The production of sour cherry wine results in large amounts of biowaste known as press cake or pomace. This pomace contains among others the fruit skin and flesh that have been previously reported to be a rich source of anthocyanins and phenolic acids. These components can be extracted and formulated to be used as natural pigments, nutraceuticals or cosmeceuticals. In the present work, sour cherry wine pomace has been extracted in aqueous buffers and ultrafiltered using ceramic membranes. Two enzyme formulations based on pectinases and cellulases have been added to the extraction media, and their influence on the extraction yield in batch mode, ultrafiltration performance and product degradation has been assessed. Sour cherry wine pomace has been found to contain relatively low amounts of anthocyanins ($48.6 \pm 0.9 \text{ mg kg}^{-1}$) and phenolic acids ($82.1 \pm 2.9 \text{ mg kg}^{-1}$) as compared to biowaste from other sour cherry food products. The use of enzyme formulations based on cellulase and pectinase was not shown to improve the total amount of anthocyanin and phenolic acids extracted. However, an increase in ultrafiltration permeate flux was observed when the enzymes were used.

1. Introduction

In a world with finite resources, the concept of circular economy is gaining in popularity as opposed to traditional consume-dispose linear economy. This framework aims at extending product lifetimes as well as recycling the available resources in a closed-loop whenever possible (Bocken et al. 2017). Waste valorisation does not only reduce the waste management costs, but also provides a potential revenue in the form of new products and resources (Vea et al. 2018).

In the case of the agricultural and food industries, the biowaste produced has a great potential for valorisation as it is typically a source for fine chemicals, nutrients, materials and fuels. Current biowaste management typically involves the use as animal feed, composting, landfilling and incineration (Lin et al. 2013). However, these techniques are not always feasible and can in some cases be limited by regulatory issues. In the recent decades more focus has been given to bioenergy carrier production in the form of biogas, biodiesel, bioalcohol etc. (Bhatia et al. 2018). Following the emerging concept of biorefinery, a more integrated approach to waste management aims at the recovery of multiple valuable sources following a bio-cascading principle. This entails a valorization hierarchy from high to low value, that covers all the range from recovery of fine chemicals to production of energy. This integrated approach does not only diversify but also maximize the output from the biowaste valorisation process (Tonini et al. 2013).

Sour cherry (*Prunus Cerasus L.*) is seldomly consumed fresh due to its astringent taste. For this reason, it is seen as an industrial crop that is generally processed into juice, wine, jam or marmalade. During processing, large amounts of biowaste containing seeds, fruit skin and flesh are produced. This biowaste, also known as pomace or press-residue, has at present no industrial use.

However, sour cherry pomace has been reported to contain several types of bioactive secondary metabolites. On the one hand, the kernel on its seeds contain oils rich in polyunsaturated fatty acids, tocopherols, β -carotene, and phenolic compounds that can be extracted in organic solvents or in supercritical conditions

(Yılmaz and Gökmen, 2013). The skins and flesh are on the other hand rich in phenolic compounds such as anthocyanins and hydroxycinnamic acids (Yılmaz et al. 2018).

The highest extraction yields for anthocyanins are obtained by using ethanol (Pinelo et al. 2005), acidified water-ethanol mixtures (Jusoh et al., 2018) or acidified methanol (Vagiri and Jensen, 2017). However, the biorefinery industry prefers to avoid the use of large amounts of organic solvents, when possible. The main reason for this is to comply with a more sustainable strategy and to avoid the presence of trace amounts of solvents in the final products.

A variety of plant cell wall degrading enzymes have been reported to facilitate the release of intracellular products into the extraction media. Typically used formulations include cellulases, hemicellulases and pectinases, as reviewed by Puri et al. (2012). The resulting extracts require purification and concentration before they can be reintroduced in the market. In this regard, membrane filtration offers a palette of processes that can be tailored to the specific biowaste processing depending on its particularities (Castro-Muñoz et al., 2016).

2. Materials and Methods

In the present study, anthocyanins and phenolic acids were recovered from sour cherry wine pomace. The experimental design for the study is shown in Figure 1. The extraction was performed using acidified water and assisted by two enzyme formulations, namely Celluclast[®] 1.5L and Fructozym[®] Flash-C, that are based on cellulases and pectinases, respectively. Ultrafiltration with ceramic membranes was performed to produce a particle-free extract containing the anthocyanins and phenolic acids. The possible degradation of the products by the enzymes was assessed by an enzyme side activity test performed on ultrafiltration permeate.

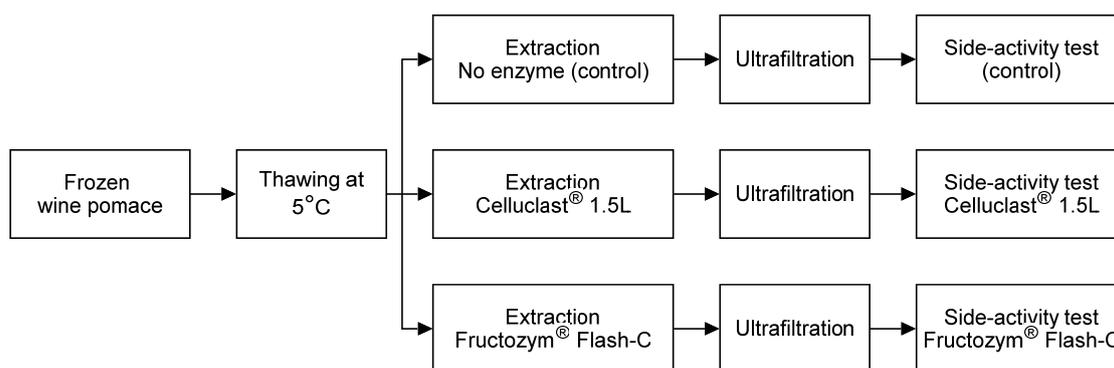


Figure 1: Flow diagram of the experiments performed in this study

2.1 Plant material, reagents and membrane

Sour cherry wine pomace from the variety *Prunus Cerasus L.* was kindly provided by Frederiksdal Kirsebærvin (Harpelunde, Denmark) on November 2017. The pomace was received in a big batch and stored in small buckets at -18 °C until processing. The individual buckets were thawed at 5 °C prior to extraction.

All aqueous extraction media were prepared using 50 mM citric acid in demineralised water. The enzyme preparation Celluclast[®] 1.5L was purchased from Sigma Aldrich (Brøndby, Denmark) and Fructozym[®] Flash-C was kindly provided by Erbslöh Geisenheim AG (Geisenheim, Germany).

Citric acid monohydrate, trifluoroacetic acid and sodium hydroxide were purchased from Sigma Aldrich (Brøndby, Denmark) whereas HPLC grade acetonitrile was purchased from VWR (Søborg, Denmark). The pure standards of cyanidin-3-glucoside and neochlorogenic acid were purchased from Extrasynthese (Genay Cedex, France) and Sigma Aldrich (Brøndby, Denmark), respectively.

The alkaline membrane cleaning product RO Chlodan was purchased from Novadan A/S (Kolding, Denmark)

2.2 Methodology for batch extractions

The batch extractions were performed in 1 L jacketed reactors equipped with overhead stirring. The temperature in the reactors was controlled by a thermostated water bath.

The enzyme dosage of 200 mL ton⁻¹, the pH of 3.0 and the temperature of 50 °C were selected based on the manufacturers recommendations. The extraction media of 50 mM citric acid in water and the pomace-to-

solvent ratio of 1 to 5 were selected after preliminary screening (data not shown), and were kept constant throughout the experiments.

2.3 Test of enzyme side activities

Three samples of 80 g of ultrafiltration permeate were kept in a water bath at 50 °C for 24 hours. The solutions were kept in the dark by wrapping with tin foil, and the pH was adjusted to 3.0 when needed. Fructozym[®] Flash-C and Celluclast[®] 1.5L were added to two of the samples in the same dose used for the filtration experiments, while the third one was kept as a control sample. Small aliquots were taken for analysis at different time intervals.

2.4 Methodology for ultrafiltration

The ultrafiltration experiments were performed using 3 L feed in a 5 L jacketed reactor with overhead stirrer and external temperature control. The enzymes were added to the reaction mixture and the reaction was carried out for 1 hour. Afterwards, the feed was passed through a sieve to remove the cherry stones, and then fed back to the reactor. At this point the ultrafiltration step started by pumping the feed through the membrane module in cross-flow mode. The ultrafiltration membrane element used was a tubular single-channel Al₂O₃/ZrO₂/TiO₂ membrane from Atech Innovations GmbH (Gladbeck, Germany) with molecular weight cut off of 25 kDa (membrane reference number UF25kDZ 267451). The membrane length was 500 mm, and the effective membrane area was 0.011 m². The trans-membrane pressure was adjusted manually via a needle valve at the retentate exit from the module. The pressure was monitored at the inlet and outlet of the module, and the trans-membrane pressure was calculated as the average between these two. The retentate flow was measured by a flow meter, and the permeate flux was monitored by a scale at 15 s time intervals. Based on preliminary studies (data not shown), the cross-flow velocity was kept at 3 m s⁻¹ and the trans-membrane pressure at 0.5 bar. The cleaning protocol of the membrane consisted of initial rinsing with clean water followed by recirculation of a 1.5 % solution of the alkaline cleaning agent RO Chlodan. A final rinsing step with clean water was performed before the pure water flux was measured.

2.5 Sample analysis

The dry matter content of the pomace was determined as the weight difference upon drying ~2 g of sample at 105 °C in a hot air oven overnight.

For the samples containing particles (extraction mixture and retentate), 10 mL aliquots were centrifuged for 15 minutes at 5 °C and 4000 rpm in a Thermo Scientific Sorvall ST 16R Centrifuge (Hvidovre, Denmark). The supernatant was then collected and filtered through a 0.22 µm syringe filter. The permeate samples were directly microfiltered by a syringe filter.

The quantification of anthocyanins and phenolic acids was done by high performance liquid chromatography (HPLC) on an Agilent Technologies 1200 Series HPLC system controlled by Open Lab CDS software (Version 1.9.0). The column was a Luna[®] Omega (3 µm PS C18 100 Å, LC Column, 150x3.0 mm, Phenomenex) equipped with a guard column (SecurityGuard[™] ULTRA, Phenomenex) and thermostated at a temperature of 40 °C. The mobile phase consisted of 0.05 % trifluoroacetic acid in water (solvent A) and 0.05 % trifluoroacetic acid in acetonitrile (solvent B). The binary solvent gradient was as follows: 0 – 15 min, 8 – 16 % B; 15 – 25 min; 16 – 30 % B; 25 – 28 min; 30 – 90 % B; 28 – 31 min, 90 % B; 31 – 35 min, 90 – 8 % B; 35 – 36 min, 8 % B. The solvent flow was set at 0.5 mL min⁻¹ and the sample injection volume was 20 µL. Chromatograms were recorded at 320 nm and 520 nm for detection of phenolic acids and anthocyanins, respectively.

Quantification was performed using calibration curves based on solutions of pure external standards of cyanidin-3-glucoside and neochlorogenic acid ($R^2 > 0.999$). For simplification, the anthocyanin content is therefore expressed as mg cyanidin-3-glucoside equivalents, and phenolic acid content as mg neochlorogenic acid equivalents.

2.6 Data analysis and presentation

All experiments were performed in triplicate and the results are presented as mean value ± standard deviation. Statistical significance was assessed by means of one-way ANOVA with a significance level of $P < 0.05$. The error bars in the figures show the standard deviation.

Unless otherwise stated, all results are given relative to the fresh weight of the sample.

3. Results and discussion

3.1 Characterization of the pomace

The initial characterization of the pomace revealed that the batches of starting material were heterogeneous on their amount of moisture. This was confirmed by the measured dry matter content of 47 ± 10 % wt. This

variation was attributed to the settlement of the liquid fraction of the wet pomace at the bottom of the container in which it was delivered. As a result, mixing and homogenization of the different buckets was performed to avoid deviation in the results due to sample inhomogeneity.

The total fresh weight of the pomace was approximately distributed as follows: 65 % stones, 31 % flesh, and 4 % sticks and stems. It is to be noted that the flesh, which is the source for anthocyanins and phenolic acids, accounts for less than a third of the total fresh weight of the pomace.

3.2 Yield of extraction of the pomace

Figure 2 shows the evolution of the extraction yields with time for aqueous extractions in batch mode with and without enzymes. In all cases the extraction of anthocyanins was complete after 6 hours. Whereas in the case of phenolic acids, the yield had not become completely constant after 12 hours of extraction.

The highest yields reported were in fact for the extraction without enzymes; with yields of $48.6 \pm 0.9 \text{ mg kg}^{-1}$ for anthocyanins and $82.1 \pm 2.9 \text{ mg kg}^{-1}$ for phenolic acids.

Yilmaz et al. (2015) performed extractions of de-stoned pomace from sour cherry juice using water-ethanol solutions at different temperatures and reached anthocyanin yields up to $410 \pm 2 \text{ mg kg}^{-1}$. This yield is much higher than the one reported in this study. However, this result is expected when the pomace comes from fruits that have only been cold pressed, a process much gentler. It seems feasible that the ethanol formed during fermentation aids the extraction of anthocyanins and phenolic acids into the wine, and therefore a smaller fraction remains in the pomace. The quantity of the components of interest in biowaste depends to a large extent on the processing conditions of the primary food product. Vagiri and Jensen (2017) reported that maceration, higher temperatures, and the use of enzymes during juice production resulted in a larger anthocyanin content in the juice, and subsequent lower content in the pomace.

The use of the enzyme Celluclast[®] 1.5L seemed to decrease the extraction yield of anthocyanins, whereas Fructozym[®] Flash-C decreased the yield of both anthocyanins and phenolic acids. These observations could be explained by inhomogeneity of the samples, or by degradation of some of the products by the enzymes tested. This hypothesis was further investigated using the enzyme side activity assays described in section 3.3 Effect of enzymes side activity on enzymes and phenolic acids.

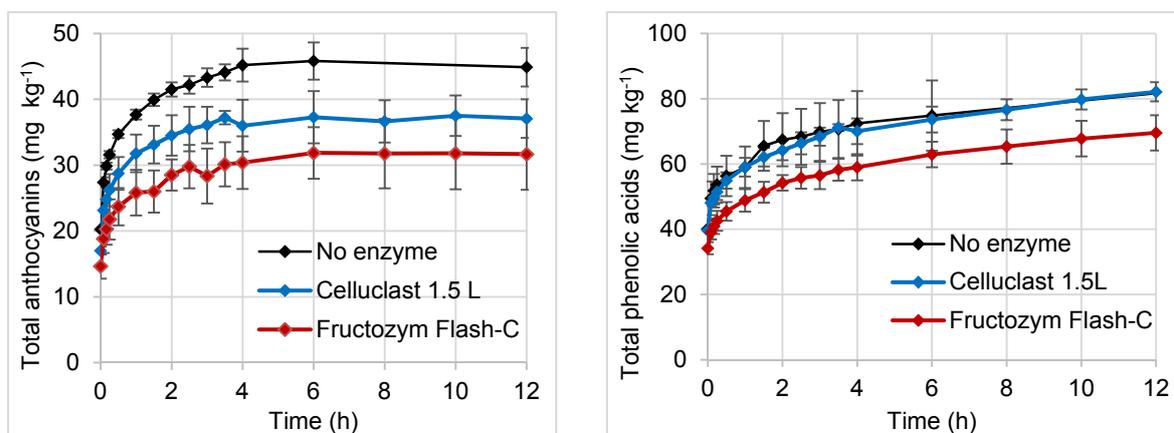


Figure 2: Yield of anthocyanins (a) and phenolic acids (b) observed upon aqueous extraction using a 50 mM citric acid solution at 50 °C for 12 hours.

3.3 Effect of enzymes side activity on anthocyanins and phenolic acids

Stability tests in presence of enzymes were performed to confirm that no flavonoid glycosidase activity was present in the commercial enzyme preparations. Figure 3 shows that both groups of components show good stability at the conditions tested (enzyme dosage 200 mL ton^{-1} , 50 °C, pH 3.0, dark, 24 h). The concentration of phenolic acids stays sharp at 100 % whereas that of anthocyanins seem to increase by between 5 to 10 %. This observation may be caused by depolymerization of polymeric anthocyanidins during the incubation time to form anthocyanin monomers. Since no degradation was observed, the differences in the batch extraction yields could be attributed to different initial contents in the pomace and/or pH variations within the experiments. Lee and Wroslstad (2004) also reported little effect of enzymatic treatment on the extraction of blueberry pomace, therefore suggesting that the enzymatic effect will depend on the composition of each biowaste matrix, and that extraction techniques should be tailored for each specific case.

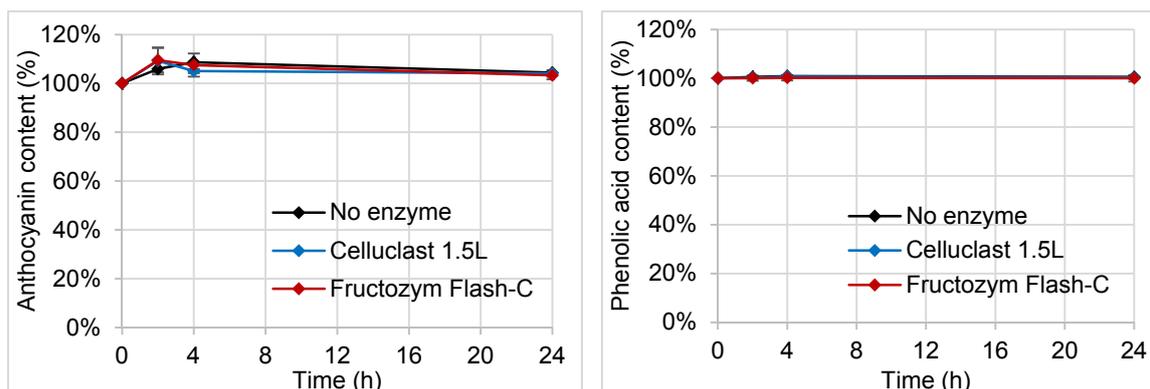


Figure 3: Normalized content of anthocyanins (a) and phenolic acids (b) observed upon the enzyme degradation essays performed at 50 °C, pH 3.0 and in the dark.

3.4 Effect of enzyme addition on ultrafiltration

As shown in Figure 4, the addition of enzymes to the extraction media resulted in an increase in the permeate flux during filtration. Initial flux decay due to membrane fouling was observed, however a stationary flux was achieved in most cases and was maintained for filtration times up to one hour.

In their review, Echavarría et al. (2011) report different membrane applications in the juice industry in which plant cell hydrolysing enzymes are used for different applications such as clarification, to increase of the yield in the press and to ease cross-flow filtration efficiency. Larger permeate fluxes in the presence of enzymes have been attributed to a decreased viscosity of the juice, and a reduction of particle size in the feed, which prevents fouling of the membrane by large particles. At the same time, the enzymes may also degrade the fouling layer and act as a self-cleaning step during operation.

In the present study, the use of plant cell wall degrading enzymes has shown improved efficiency of the filtration process as compared to the experiments without enzymes. This approach can be further investigated with other biowaste streams richer in high value products in order to increase the process yields.

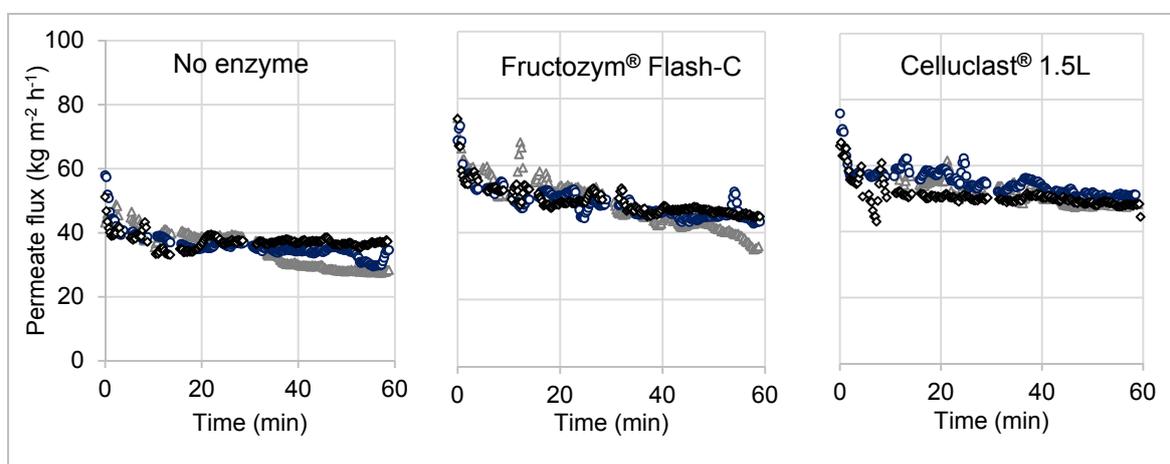


Figure 4: Permeate flux measured during ultrafiltration without enzyme (left), using Fructozym® Flash-C (center), and Celluclast® 1.5L (right) with a 20 kDa ceramic membrane at 0.5 bar, 50 °C and 3 m s⁻¹. Different symbols and colors stand for experimental repetitions.

4. Conclusions

Following the biorefinery concept, biowaste from sour cherry wine production has been investigated as a possible source of anthocyanins and phenolic acids. Aqueous extractions have been performed in mildly acidic water with and with the help of the plant cell lysing enzyme formulations Fructozym® Flash-C and Celluclast® 1.5L.

Based on the observations from this study, the addition of enzymes did not show any consistent differences in the extraction yield of either phenolic acids or anthocyanins. The enzymes did not show degradation of either of the products at the operation conditions (dosage 200 mL ton⁻¹, pH 3.0, 50 °C, darkness, 24 h). Nevertheless, both enzymes caused an increase in permeate flux during ultrafiltration with ceramic membranes. The overall yields reported were very low: 48.6 ± 0.9 mg kg⁻¹ for anthocyanins and 82.1 ± 2.9 mg kg⁻¹ for phenolic acids, which definitely limits the potential of sour cherry wine biowaste as a source of high value products.

Other sour cherry biowaste streams may need to be considered in the future, such as sour cherry juice pomace, juice sediment or even the cherry stones. In principle, different biowaste streams based on fruits and berries can be processed by extraction and purification with the method suggested in this research work, with slight modifications such as the type of enzyme, the type of membrane, and/or the operating parameters.

Acknowledgments

This research work was funded by the European Regional Development Fund as part of the Interreg North Sea Region project 38-2-4-17 BIOCAS, circular BIOMass CAScade to 100%, to whom the authors would like to express their gratitude.

References

- Bhatia, S.K., Joo, H.-S., Yang, Y.-H., 2018, Biowaste-to-bioenergy using biological methods – A mini-review, *Energy Conversion and Management*, 177, 640-660.
- Bocken, N.M.P., Olivetti, E.A., Cullen, J.M., Potting, J., Lifset, R., 2017, Taking the Circularity to the Next Level: A Special Issue on the Circular Economy, *Journal of Industrial Ecology*, 21, 476-482.
- Castro-Muñoz, R., Yáñez-Fernández, J., Fila, V., 2016, Phenolic compounds recovered from agro-food by-products using membrane technologies: An overview, *Food Chemistry*, 213, 753-762.
- Echavarría, A.P., Torras, C., Pagán, J., Ibarz, A., 2011, Fruit Juice Processing and Membrane Technology Application, *Food Engineering Reviews*, 3, 136-158.
- Jusoh, Y.M.M., Idris, A.A., Khairudin, N., Zaidel, D.N.A., Hashim, Z., Mahmood, N.A.N., Zakaria, Z.Y., Muhammad, I.I., 2018, Effect of Solvent pH, Microwave Power and Extraction Time on Microwave-Assisted Extraction of *Hibiscus rosa-sinensis*, *Chemical Engineering Transactions*, 63, 541-546.
- Lee, J., Wrolstad, R.E., 2004, Extraction of anthocyanins and polyphenolics from Blueberry Processing Waste, *Journal of Food Science*, 69, 564-573.
- Lin, C.S.K., Pfaltzgraff, L.A., Herrero-Davila, L., Mubofu, S.A., Clark, J.H., Koutinas, A.A., Kopsahelis, N., Stamatielatu, K., Dickson, F., Thankappan, S., Mohamed, Z., Brocklesby, R., Luque, R., 2013, Food waste as a valuable resource for the production of chemicals, materials and fuels. Current situation and global perspective, *Energy and Environmental Science*, 6, 426-464.
- Pinelo, M., Rubilar, M., Jerez, M., Sineiro, J., Núñez, M.J., 2005, Effect of Solvent, Temperature, and Solvent-to-Solid Ratio on the Total Phenolic Content and Antiradical Activity of Extracts from Different Components of Grape Pomace, *Journal of Agricultural and Food Chemistry*, 53, 2111-2117.
- Puri, M., Sharma, D., Barrow, C.J., 2012, Enzyme-assisted extraction of bioactives from plants, *Trends in Biotechnology*, 30, 37-44.
- Tonini, D., Martinez-Sanchez, V., Astrup, T.F., 2013, Material Resources, Energy, and Nutrient Recovery from Waste: Are Waste Refineries the Solution for the Future?, *Environmental Science and Technology*, 47, 8962-8969.
- Vagiri, M., Jensen, M., 2017, Influence of juice processing factors on quality of black chokeberry pomace as a future resource for colour extraction, *Food Chemistry*, 217, 409-417.
- Vea, E.B., Romeo, D., Thomsen, M., 2018, Biowaste valorisation in a future circular bioeconomy, *Procedia CIRP*, 69, 591-596.
- Yılmaz, C., Gökmen, V., 2013, Compositional characteristics of sour cherry kernel and its oil as influenced by different extraction and roasting conditions, *Industrial Crops and Products*, 49, 130-135.
- Yılmaz, F.M., Karaaslan, M., Vardin, H., 2015, Optimization of extraction parameters on the isolation of phenolic compounds from sour cherry (*Prunus cerasus* L.) pomace, *Journal of Food Science and Technology*, 52, 2851-2859.
- Yılmaz, F.M., Görgüç, A., Karaaslan, M., Vardin, H., Bilek, S.E., Uygun, Ö., Bircan, C., 2018, Sour Cherry By-products: Compositions, Functional Properties and Recovery Potentials – A Review, *Critical Reviews in Food Science and Nutrition*, DOI: 10.1080/10408398.2018.1496901.