CHEMICAL ENGINEERING TRANSACTIONS
VOL. 86, 2021
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Sauro Pierucci, Jiří Jaromír Klemeš

ISBN 978-88-95608-84-6; **ISSN** 2283-9216
DOI: 10.3303/CET2186007

Agro-industrial Wastes as Bioactive Molecules Source

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Sustainable use of renewable natural resources, through value addition, using biological life processes, is an ideal transition from an oil-based economy to bioresource economy. Addressing climate change, renewable resources such as agro-industrial wastes, currently seen as low value materials, are in fact natural sources of value-added compounds that can be used, for example, as economic sources of bioactive substances of high interest for chemical, pharmaceutical and food industries. Moreover, agro-industrial by-products such as most of organic effluents have a high organic load content and can be vaporizable through a gaseous energy carrier production (methane/hydrogen) and a digested flow for agricultural purposes, using anaerobic digestion techniques. Once adopted, biological processes of these materials may contribute to reduce the environmental pollution burden.
The present work refers to different procedures and methodologies applied to wastes valorisation that, within a biorefinery concept, had the achievement of valuable biomolecules as a core of the entire recovery line system. Several wastes/wastewaters mainly produced in the Mediterranean area were under study.

1. Introduction

Agro-industrial wastes are a growing problem in our global industrialized societies. In recent years, the decrease of the environmental impact caused by agro-industrial wastes has been the subject of growing concern. Agro-industry and in particular food industry generates large amounts of liquid, solid and gaseous effluents/wastes, which are multi-phase and multi-component.
The by-products of vegetable processing plants are regarded as low-value that represent daily tons of highly pollutant organic material, with high contents of BOD, COD and suspended solids.
Due to their high nutrient content, these wastes cause severe environmental pollution events and management problems, which could be reduced significantly if good waste management practices, comprising the valorisation/conversion of wastes, are employed. The recovery, reuse or recycling of wastes is therefore essential as these constitute the raw material for the production of different valuable final products. In addition, due to the rising costs with the treatment of solid and liquid wastes, the recovery of extracts or compounds with biological interest from agro-industrial wastes has also recently received increasing attention.
In Mediterranean countries, large amounts of toxic effluents are produced by the agro-industrial activities that have to be disposed of, often creating outbreaks of pollution.
Considering the high content of organic load, they can be valorised in terms of energy, producing biogas/methane by anaerobic digestion process, but, on the other hand, as most of them are also rich in phenolic compounds, a further valorisation may be considered regarding the previous extraction of valuable biomolecules within a biorefinery approach. Olive oil mill, cork boiling and chestnut wastes/wastewaters (La Cara et al., 2012; Marques et al., 2014; Squillaci et al., 2018; Vella et al., 2018; Maurelli et al., 2013) are some examples of agro-industrial materials that contain relevant concentrations of high-value compounds such as phenolic compounds, which are characterized by a variety of biological activities, particularly as antioxidants. The use of low-value industrial wastes to obtain phenolic compounds allows to reduce the production costs and to increase the profit margin of the first line products, in a perspective of a biorefinery concept, being

Paper Received: 12 August 2020; Revised: 28 February 2021; Accepted: 26 April 2021
Please cite this article as: Squillaci G., La Cara F., Roseiro L.B., Marques I., Morana A., 2021, Agro-industrial Wastes as Bioactive Molecules Source, Chemical Engineering Transactions, 86, 37-42 DOI:10.3303/CET2186007

possible to combine the technologies for re-use and valorise the biological materials, with a significant increase of the environmental, social and economic sustainability.
The present study presents several examples of low-value agro-industrial wastes/effluents, which can be regarded as sources for bioenergy and/or high-value compounds. Phenolic compounds and their specific biological properties (e.g. antioxidant, antibacterial, antifungal, anti-inflammatory and anti-tumour activities) together with their applications in niche markets, are issues that will be addressed in this work. Thus, the three types of effluents chosen have in common the presence of phenolic compounds in discrete / high quantities.
The interest toward phenolic compounds is well established as they greatly contribute to the benefits of human health (Roseiro et al., 2012; Tripoli et al., 2005). It is recognized that the healthy properties of phenolic compounds are connected to their antioxidant power (Balasundram et al., 2006; Wojdyło et al., 2007; El-Abbassi et al., 2012); in fact, these molecules have a positive effect on cardiovascular diseases, being able to reduce the oxidation of LDL due to their ability to scavenge superoxide radicals which are involved in the pathogenesis of atherosclerosis (Zhishen et al., 1999; Visioli et al., 2001). Phenolic molecules may reduce blood pressure and inhibit platelet aggregation (Hodgson et al., 2006; Hubbard et al., 2006), and are also considered to be responsible for the prevention of further human diseases connected to oxidative stress such as neurodegenerative syndromes and cancer (Vauzour et al., 2010; Cacciola et al., 2019). In addition to the antioxidant power, antimicrobial and anti-inflammatory effects of phenolic compounds should not be forgotten (Benevides-Bahiense et al., 2017). Nowadays, the requirement of biologically active molecules of natural origin in substitution of the mainly employed synthetic ones, and the growing attention by consumers toward more health-protective compounds, has stimulated the research of new sources for natural active molecules production (Mohd Azman et al., 2016), particularly from biowastes (Tavares et al., 2020.; da Costa-Lopes et al., 2016).

2. Experimental setup

2.1 Feedstocks

Olive mill wastewater (OMW), the effluent generated in an olive oil campaign, was collected in an olive oil mill (three-phase continuous extraction) in Rio Maior (Portugal) and stored in the olive mill underground tanks. Chestnut manufacture process wastewater (ChW) was obtained from a company located in Avellino, Italy. Cork boiling wastewater (CBW) was obtained from a cork industry facility in Alcácer do Sal, Portugal. These substrates were stored at 4°C until use.

2.2 Wastewater parameters characterization

Total chemical oxygen demands (COD) and total nitrogen were evaluated using Spectroquant® test kits (Merck, Whitehouse Station, NJ, USA). Total and volatile solids and total and volatile suspended solids (TS, VS and TSS, VSS, respectively) were measured according to standard methods (APHA et al., 1998). Conductivity was evaluated by a conductivity meter (EcoTestr, OAKTON Instruments, Vernon Hills, IL, USA).

2.3 Total polyphenols, *ortho*-diphenols and flavonoids content

Total phenolic content was measured by the Folin-Ciocalteu method (Singleton and Rossi, 1965). Aliquots of phenolic extract, diluted to 150 µL with deionized water, were mixed with 750 µL of Folin-Ciocalteu reagent (diluted ten-folds with deionized water) and 600 µL of 7.5% (w/v) Na_2CO_3. The reaction was developed at 25 °C for 2 h in the dark, and the absorbance was read at 765 nm against a blank prepared with 150 µL of deionized water (Varian spectrophotometer, model DMS-200, Varian Analytical Instruments, Leini, Torino, Italy). The total phenolic content was estimated by a calibration curve built with increasing quantities of a standard solution of gallic acid (range 1.5–10 µg). The results were expressed as mg of GAE (Gallic Acid Equivalents)/mL.
Total *ortho*-diphenols were measured as described by (Arnow, 1937). Briefly, 100 µL of phenolic extract were diluted to 400 µL with deionized water. Then, 400 µL of 0.5 M HCl, 400 µL of 1.45 M $NaNO_2$ and 0.4 M Na_2MoO_4, and 400 µL of 1 M NaOH were added in sequence. The resulting mixture was immediately read at 500 nm using a blank made of 400 µL of deionized water. The quantification was carried out by a calibration curve obtained with increasing quantities of a standard solution of caffeic acid (range 5–50 µg). The results were expressed as mg of CAE (Caffeic Acid Equivalents)/mL.
Total flavonoid content was determined following the method of Barreira et al. (2008) with some modifications. Briefly, 250 µL of phenolic extract were mixed with 1.25 mL of deionized water and 75 µL of 5% (w/v) $NaNO_2$. After 5 min, 150 µL of 16% (w/v) $AlCl_3 \cdot 6H_2O$ were added. After 1 min, 500 µL of 1 M NaOH and 275 µL of deionized water were added, and the resulting solution was vigorously mixed. The absorbance was read at 510 nm versus a blank containing 250 µL of deionized water. The flavonoid amount was determined by a

calibration curve obtained with increasing quantities of a standard solution of catechin (range 5–75 µg). The results were expressed as mg of CE (Catechin Equivalents)/mL.

2.4 Antiradical activity

The antiradical activity was defined as the amount of antioxidant (expressed as µg of total polyphenols) necessary to decrease the initial 2,2-diphenyl-1-picrylhydrazyl (DPPH) concentration by 50% (EC_{50} = efficient concentration). The antiradical activity of the raw wastewaters was determined by a modification of the method described by von Gadow et al. (1997). Briefly, 1 mL of a 6×10^{-5} M methanolic solution of DPPH was added to 10 mL of a methanolic solution of the samples mixed and placed in 1 cm glass cuvettes. The decrease in absorbance at 515 nm was determined continuously with data acquisition at 2 s intervals with a spectrophotometer Varian Cary 50 for 16 min (until the absorbance stabilized). The inhibition percentage (IP) of the DPPH radical by the phenolic compounds of the extracts was calculated according to the formula:

$$IP = [(AC(0) - AC(t)) / AC(0)] \times 100$$

where *AC(0)* is the absorbance of the control at t = 0 min and *AC(t)* is the absorbance of the reaction solution at t =16 min (Cruz et al., 2001).

2.5 HPLC analysis of phenolic compounds

Aliquots of 0.1 mL of each sample were diluted in 5 mL of acid methanol (70:29:1; methanol:water:HCl) and incubated at 37 °C for 30 min in a rotary shaker. The suspension was centrifuged for 15 min at 3500 rpm and the supernatant was recovered and used for polyphenols assay.
HPLC/UV analysis of single phenolic compounds was performed utilizing a 250 × 4.6 mm (5 µm) C18 Hypersil column (Thermo Electron Corporation, Bellefonte, PA, USA) used with a Securityguard precolumn (Phenomenex, United Kingdom) with a C18 cartridge in combination with a Thermo-Finnigan Surveyor HPLC system (solvent degasser, quaternary pump, thermostatically controlled column oven set at 25 °C a photodiode array detector set to collect overall data from 200-600 nm, and selected wavelengths of 230, 254 and 280 nm).
Peak identifications were confirmed from retention times, UV spectroscopic data, and direct comparison to pure standards. The solvent flow rate was 0,9 mL/min and the mobile phase was a four-step linear solvent gradient system (0–30 min, 10% B; 30–35 min, 55% B; 35–40 min, 100% B; 40-45 min, 100% B) using 2% acetic acid in water as solvent A and 0.5% acetic acid in 50% acetonitrile as solvent B. Identification of phenolic compounds in the extracts was performed by HPLC-UV, comparing the relative retention times and UV spectra with those of standard solutions (Nazzaro et al., 2011)

2.6 Statistical analysis

All tests were performed in triplicate and expressed as mean ± Standard Deviation (SD) calculated by Microsoft Excel 2013. Statistical analysis was carried out by GraphPad Prism (version 5). Significant differences were determined by two-way analysis of variance (ANOVA) with Bonferroni post-tests. Mean values were considered not significantly different at $p \geq 0.05$.

3. Results and Discussion

Three case studies were chosen from agro-industrial residues of the Mediterranean area: the vegetation waters of the oil mill, the cork boiling waters and the processing waters of chestnuts.
The three chosen feedstocks, collected in production plants in Italy and Portugal, were analysed to evaluate their main chemical-physical characteristics, which are shown in Table 1:

Table 1: Characterization of CBW, ChW and OMW

Parameter	CBW	ChW	OMW
pH	5.8 ± 0.0	4.54 ± 0.0	4.96 ± 0.0
COD (kg m^{-3})	6.5 ± 0.1	5.60 ± 0.04	54.3 ± 2.3
TS (kg m^{-3})	5.13 ± 0.08	3.36 ± 0.14	28.2 ± 0.4
VS (kg m^{-3})	4.05 ± 0.04	3.00 ± 0.14	15.8 ± 3.2
TSS (kg m^{-3})	0.58 ± 0.11	2.69 ± 0.37	3.18 ± 0.07
VSS (kg m^{-3})	0.15 ± 0.07	2.58 ± 0.42	0.53 ± 0.0
Conductivity (mS cm^{-1})	1.5 ± 0.1	1.08 ± 0.06	11.7 ± 0.6
Total Nitrogen (kg m^{-3})	0.04 ± 0.00	0.175 ± 0.004	0.56 ± 0.02

From the feedstocks characterization (Table 1), the three substrates showed similar pH values that are in the acid range and whose maximum value is below 6.0. Concerning the organic load, OMW is a concentrated effluent that holds high organic matter content, represented by the highest concentrations in solids and where the COD content is greater than 50 kg m^{-3}. In contrast, the other two (CBW and ChW) are very diluted substrates with around COD concentrations of 9 times lower than OMW.
Another relevant aspect that distinguishes OMW from the other two feedstocks, concerns the conductivity parameter. Distinct values of about 11 mS cm^{-1} and 1.1-1.5 mS cm^{-1} were registered in OMW and CBW-ChW, respectively.
Table 2 shows the amount of the phenolic compounds present in the wastes object of the present study.

Table 2: Total polyphenols, ortho-diphenols and flavonoids content

Compounds	CBW	ChW	OMW
Total phenols	1.20 ± 0.02 (mg GAE/mL)	0.060 ± 0.002 (mg GAE/mL)	1.32 ± 0.09 (mg GAE/ mL)
Ortho-diphenols	0.70 ± 0.09 (mg CAE/ mL)	0.048 ± 0.003 (mg CAE/mL)	0.89 ± 0.05 (mg CAE/ mL)
Flavonoids	0.26 ± 0.02 (mg CE/ mL)	0.012 ± 0.001 (mg CE/mL)	0.56 ± 0.12 (mg CE/ mL)
Antiradical Activity (EC_{50})	7.20 ± 0.11	8.50 ± 0.14	8.53 ± 0.45

GAE, CAE and CE = Gallic acid, Caffeic acid and Catechin Equivalents; EC_{50} = efficient concentration

Ortho -diphenols are not usually measured in natural extracts/wastes but here it was decided to estimate their amount because such type of molecules is known for their strong antioxidant power. In fact, the presence of the hydroxyl group in *ortho* position (catechol structure) increases the antioxidant activity of the compounds through the stabilization of the phenoxyl radical (Natella et al., 1999).
OMW was the waste with the highest content of total phenolic compounds, *ortho*-diphenols and flavonoids per mL of sample, while ChW contained the lowest amount among of the three wastewaters considered. Nevertheless, the antioxidant power, expressed as antiradical activity (EC50) were similar for the three feedstocks.
The molecular profile of the phenolic compounds presented in CBW, ChW, and OMW is shown in Table 3:

Table 3: Phenolic compounds detected by HPLC analysis

Phenolic compounds (μg/mL)	CBW	ChW	OMW
Gallic acid	19.5 ± 3.7	3.21 ± 0.16	15.4 ± 1.8
Catechin	0.6 ± 0.05	1.50 ± 0.22	18.7 ± 0.9
Protocatechuic acid	8.5 ± 1.0	2.64 ± 0.29	1.05 ± 0.09
Caffeic acid	2.14 ± 0.15	0.59 ± 0.04	11.5 ± 0.91
Vanillic acid	2.0 ± 0.19	tr	0.85 ± 0.1
Ellagic acid	96.5 ± 11.5	9.32 ± 0.28	2.3 ± 0.42
p-coumaric acid	4.1 ± 0.26	5.23 ± 0.15	1.9 ± 0.18
Ferulic acid	6.5 ± 0.78	7.89 ± 0.32	23.5 ± 1.3
o-coumaric acid	3.8 ± 0.29	tr	1.1 ± 0.3
Trans-cinnamic acid	3.7 ± 0.37	1.06 ± 0.26	0.95 ± 0.08
Rutin	nd	15.9 ± 1.26	2.52 ± 0.26
Quercetin	nd	nd	6.2 ± 0.46
Oleuropein	nd	nd	1075 ± 0.76
Hydroxytyrosol	nd	nd	64.2 ± 2.6
Tyrosol	nd	nd	40.5 ± 1.6

tr, trace – nd, not detected

Ellagic acid was most abundant in CBW and it was also the main represented phenolic compound in this waste. Recently, ellagic acid has generated high interest thanks to its properties and possible applications as antioxidant, anticancer and antimicrobial agent. It was capable of preventing wrinkles generated by UV light exposure, making it a valuable ingredient in a number of cosmetic formulations (Bae et al., 2010).

Moreover, ellagic acid showed antimicrobial activity towards human pathogens. According to Ghudhaib and co-workers, it was more effective than the antibiotics streptomycin and gentamycin, chosen as comparison, in inhibiting growth on agar plates of some pathogenic bacteria as *Klebsiella pneumonia*, *Staphylococcus epidermatis*, and *Salmonella typhi* (Ghudhaib et al., 2010).
Rutin was the most abundant phenolic compound in ChW. It was also detected in OMW, but in less amount, and totally absent in CBW. The flavonol quercetin was only detected in OMW. Its properties are common to the majority of phenolic compounds. It is an antioxidant, anti-apoptotic and anti-inflammatory agent and its applications are essentially in the therapeutic field (Alrawaiq et al., 2014)
Hydroxytyrosol, tyrosol and oleuropein were only present in OMW according to the fact that these compounds are characteristics of olive and olive oil. Furthermore, they were also the most abundant phenolic compounds among those detected in this waste (Belaqziz et al., 2017).
A quantitative assessment on potential for recovery of the phenolic compounds and flavonoid, as well as from the data shown in the present study, can be also performed analysing the large existing literature data (Arshadi et al., 2016; Chaves et al., 2020).

4. Conclusions

The results of the study carried out on the three case studies (CBW, ChW and OMW) showed that their phenolic composition can be very different both from a qualitative and quantitative point of view. However, even if the OMW seem to be, as demonstrated by a large literature on the subject, the most promising effluent quantitatively in terms of phenolic compounds content, qualitatively, also the other two feedstocks are interesting sources of molecules with high biological activity. In particular, CBW has a high content of ellagic, gallic, protocatechuic and *o*-coumaric acid, while ChW contains also rutin, in addition to the aforementioned acids. Finally, these case studies results demonstrated that it is possible to use the residues of these particular agro-industrial processes to obtain molecules with high added value for the food, pharmaceutical and cosmetic industries, prior to further conversion in energy, under a bio-refinery and circular economy perspectives.

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