Folia Biologica et Oecologica 17: 54–62 (2021)

Acta Universitatis Lodziensis

Effect of the solvent on the extraction of polyphenols from distillery stillage and on their antioxidant activity

BIOOPEN 2021 – POST-CONFERENCE ARTICLE

WIOLETA MIKUCKA, MAGDALENA ZIELIŃSKA

University of Warmia and Mazury in Olsztyn, Faculty of Geoengineering, Department of Environmental Biotechnology, Sloneczna 45G, 10-709 Olsztyn, Poland
E-mail: wioleta.mikucka@uwm.edu.pl

ABSTRACT

The increase in the costs of storage and disposal of post-production residues has resulted in the search for new directions for their recycling, which is closely related to the necessity of protecting the natural environment and promoting a circular economy. Moreover, the apparent interest shown by the food market in raw materials with high antioxidant activity implies an increasing use of by-products. The objective of the study was to determine the effect of the type and concentration of the solvent on the efficiency of extracting polyphenols from distillery stillage as well as their antioxidant activity by using several solvents: methanol:water (70:30 v/v), methanol:water (100:0 v/v), ethanol:water (70:30 v/v) or ethanol:water (100:0 v/v). The DPPH radical method was used to determine the antioxidant activity of the obtained extracts. The normalised variable (NV) and statistical measure (MS) were determined, based on which the effectiveness of the solvents was evaluated. The highest polyphenolic content and the antioxidant activity were obtained by using ethanol:water (70:30 v/v) as a solvent in the extraction of polyphenolic compounds from distillery stillage.

KEYWORDS: DPPH assay, Folin-Ciocalteu, phenolic content, methanolic extract, ethanolic extract

Introduction

One of the main trends in line with the principle of a bioeconomy is sustainable development, which bases its activities on the maximum use of resources of biological origin to protect the natural environment and reduce production costs (Stegmann *et al*. 2020). Following the principle of 'how to get more using less' is to manage and to valorise raw materials and by-products from variable production processes (Okonko *et al*. 2009). Bio-based raw materials with a high recycling potential include, for example, molasses obtained during sugar production (Fan *et al*. 2018), cereal bran (Belc *et al*. 2019) or

DOI: https://doi.org/10.18778/1730-2366.16.15

fruit and vegetable pomace (Lin *et al*. 2013, Coman *et al*. 2019).

Currently, special attention is paid to the quality and nutritional value of food. Many products have been shown to have increased susceptibility to oxidative processes that negatively affect their safety. To limit these changes, natural or synthetic substances with anti-oxidant properties are used (Lourenço *et al*. 2019). A trend has been noticed in which consumers seek to use natural antioxidants. Therefore, the search for inexpensive, efficient and available sources of compounds with antioxidant properties, mainly polyphenols, has become the subject of interest of researchers.

Distillery stillage is an example of a raw material that fits with the current above-mentioned trends. A stillage is a by-product of alcoholic fermentation. It is a valuable source of polysaccharides and volatile fatty acids, as well as natural antioxidants, namely polyphenols. However, there is still no literature data on the use of distillery stillage as a source of bioactive compounds. The stillage composition depends mainly on the processing conditions and the type and quality of the substrates, which means that it may differ among distilleries (Mohana *et al*. 2009). The most commonly used substrates for the production of alcohol are cereals, potato starch and sugar beet molasses (Smuga-Kogut 2015). Therefore, more attention should be paid to the recovery of polyphenolic compounds from by-products of the distillery industry.

The production of alcohol is growing every year because this raw material is used in the chemical, pharmaceutical, cosmetic and food industries, among others (Kharayat 2012). The United States and Brazil produce 94 billion litres of ethanol annually, which is approximately 85% of the global alcohol production (Kharayat 2012). The European Union has implemented a programme obliging the use of biofuels in transport fuels (14.0% by 2025 and 19.7% by 2030) (Krzywonos *et al*. 2015). Alcohol plays a key role in the development of the global economy, but it is also a source of environmental pollution; indeed, 1 l of the produced spirit yields 9–14 l of the by-products. Distillery stillage is characterised by a high content of biodegradable organic matter (chemical oxygen demand [COD] from 15 to 176 g O_2/l) (Melamane *et al*. 2007). The stillage causes a serious ecological problem due to the high concentration of nitrogenous compounds, low pH, high temperature and dark brown colour resulting from the presence of poorly biodegradable melanoidins (Fito *et al*. 2019). Therefore, it is crucial to dispose of the stillage. Until now, the main direction in the management of the by-products of the distillery industry has been their use as fertilisers (Satyawali and Balakrishnan 2008), feed ingredients (Djukić-Vuković *et al*. 2015) and biofuel production substrates (Caruso *et al*. 2019). However, the by-products of the agri-food industry have strong antioxidant properties. Therefore, scientists are increasingly involved in research on bioactive compounds due to their important role in the prevention and treatment of the most serious diseases of civilisation, including heart disease, diabetes and cancer. In food production, they can be used as new ingredients in innovative products or as food additives (Laufenberg *et al*. 2003). The actions taken are in line with the trend of searching for natural compounds with antioxidant properties in place of synthetic antioxidants.

Considering the amounts that are generated, the high nutritional value and the potential strong antioxidant activity, it seems beneficial to use distillery by-products as a source of polyphenols or an additive to enrich the composition of certain food products. There is a lack of data on the characteristics of stillage in terms of the content of polyphenols and their antioxidant properties. Moreover, the nature and polarity of the solvent used for the recovery of these compounds are important in determining the antioxidant properties. These parameters can significantly influence the hydrogen atom transfer (HAT) or single electron (SET) mechanisms, which are crucial for measuring antioxidant properties (Perez-Jimenez and Saura-Calixto 2006). Hence, the critical point of the research seems to be the selection of an appropriate solvent for extracting polyphenols. Therefore, the objective of the study was to determine the effect of the type and concentration of the solvent on the efficiency of the extraction of polyphenols from distillery stillage and on their antioxidant activity.

Materials and methods

Materials

In this study, distillery stillage from the production of concentrated, crude ethyl alcohol from cereals (a company in north-eastern Poland) was used.

Extraction of polyphenols

The following solvents were used for the extraction: ethanol:water (100:0 v/v) (E 100%), methanol:water (100:0 v/v) (M 100%), ethanol:water (70:30 v/v) (E 70%) and methanol:water (70:30 v/v) (M 70%). To 1 g of the freeze-dried distillery stillage, 10 ml of the solvent was added. The mixtures were shaken for 45 min at room temperature (25 °C) protected light. The extraction process was carried out in triplicate.

Total polyphenolic content

The concentration of total polyphenolic compounds was determined using the colourimetric method with the Folin-Ciocalteu reagent (Singleton *et al*. 1999). This measurement is based on the reversible reduction of molybdenum(VI) present in the Folin-Ciocalteu reagent to molybdenum(V) by phenols in an alkaline medium. For the reaction, 0.25 ml of extract was mixed with 0.125 ml of Folin-Ciocalteu reagent; next, 0.5 ml of a 14% Na_2CO_3 solution was added to the flask. Distilled water was then added to adjust the volume to 10 ml. After 30 min of incubation, the absorbance was measured at 760 nm and compared with the control sample, which was prepared analogously, except using water instead of the extract. A standard curve was prepared using gallic acid (Figure 1), and the polyphenolic content is expressed as gallic acid equivalents (GAE) in mg/ml of sample.

Test with the 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical

The antioxidant activity was measured using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) method according to Moure *et al*. (2001). Two hundred microlitres of the extract was added to 2 ml of DPPH solution at a concentration of 0.033518 g/250 ml of methanol. The method is based on determining the degree of reduction of the DPPH radical by antioxidants contained in the sample. The decrease in absorbance was measured at 515 nm for 16 min. The antioxidant activity was calculated according to the formula:

$$\text{Antioxidant activity (\%)} = \frac{(A_0 - A_t)}{A_0} \times 100$$

where A_0 is the initial absorbance of the DPPH solution and A_t is the absorbance of the DPPH solution after 16 min.

Statistical analysis

To determine the efficiency of the solvents used for the extraction of polyphenolic compounds from the distillery stillage, the unitisation of the variables was used (Guzik *et al*. 2005). Using this method, variables ranging from 0 to 1 were obtained. The STATISTICA 13.1 software (StatSoft) was employed for statistical analysis. The following formula was used for the calculations:

$$NV = (X_J - X^{min}_J)/(X^{max}_J - X^{min}_J),$$

where NV is the normalised variable, X_J is the average value of the variable, X^{max}_J is the maximum value of the variable, X^{min}_J is the minimum value of the variable and J is the type of solvent.

A statistical measure (M_S) was used to determine the ability of solvents to extract effectively bioactive compounds from the distillery stillage. It was calculated according to the following equation:

$$M_S = 1/p \sum N_V,$$

where M_S is the statistical measure, p is the number of variables and N_V is the normalised variable.

Results

The total polyphenolic content was determined by the Folin-Ciocalteu method and is reported as GAE equivalents by reference to the standard curve (Figure 1). Table 1 shows the total polyphenolic content in the distillery stillage and their antioxidant activity. These values depended on the solvent used.

The highest polyphenolic content was in the extracts obtained with the use of E 70% (0.134 ± 0.009 mg GAE/ml) and M 70% (0.121 ± 0.011 mg GAE/ml). The lowest polyphenolic content was obtained when using solvents without the addition of water: 0.064 ± 0.021 mg GAE/ml and 0.078 ± 0.018 mg GAE/ml, respectively, for M 100% and E 100%.

All extracts had strong DPPH radical scavenging activity; the scavenging activity after 16 min of incubation ranged from 64% to 88% of the initial radical concentration (Table 1). Figure 2 shows the DPPH radical scavenging activity over time. Among the solvents used, the

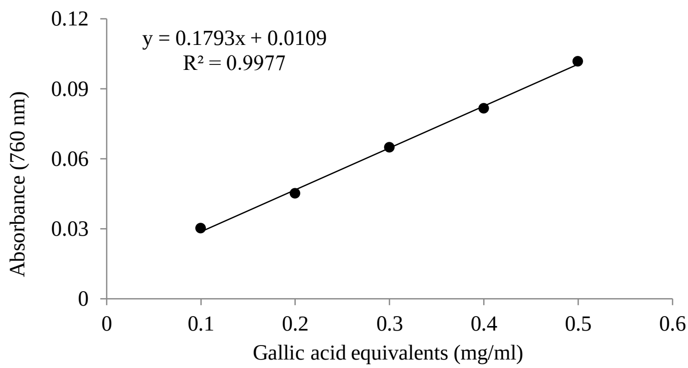

Figure 1. Standard curve for the determination of total polyphenolic compounds in extracts.

Table 1. Total polyphenolic content in distillery stillage and their antioxidant activity.

Solvent	Total polyphenolic content (mg GAE/ml)	Antioxidant activity (%)
E 70%	0.134 ± 0.009	88 ± 0.010
E 100%	0.078 ± 0.018	70 ± 0.021
M 70%	0.121 ± 0.011	78 ± 0.013
M 100%	0.064 ± 0.021	64 ± 0.030

mixtures with water were characterised by higher polarity, and the obtained extracts showed the highest content of extracted polyphenolic compounds and high antioxidant activity. The highest antioxidant activity was in the E 70% (88% ± 0.010%) and M 70% (78% ± 0.013%) extracts. In the case of the M 100% and E 100% extracts, the DPPH radical scavenging activity ranged from 64% ± 0.030% to 70% ± 0.021% after 16 min of incubation.

The statistical measures were calculated using the unitisation of the variables. Table 2 shows the efficiency of the solvents that were used to determine the total polyphenolic content and their antioxidant activity in the stillage extracts.

The E 70% extract had the highest value of the statistical measure (0.90 ± 0.131) and the M 100% extract had the lowest value (0.22 ± 0.282). These data are correlated with the total polyphenolic content and the antioxidant activity obtained in this study. The highest total polyphenolic content and antioxidant activity were obtained by using E 70% as the solvent in the extraction of polyphenolic compounds from distillery stillage.

Discussion

Polyphenolic compounds are secondary metabolites of plants, differing in terms of chemical properties, structures and molecular weights (Wilska-Jeszka 2007). These compounds are generally soluble in water (Shahidi and Naczk 2011). Due to their proven beneficial effects on the human body, they are of interest to medicine, science and food producers (Pandey and Rizvi 2009). The development of an efficient procedure for polyphenolic compound extraction from different sources is a challenge due to the complex matrix, the structural diversity of phenolic compounds and

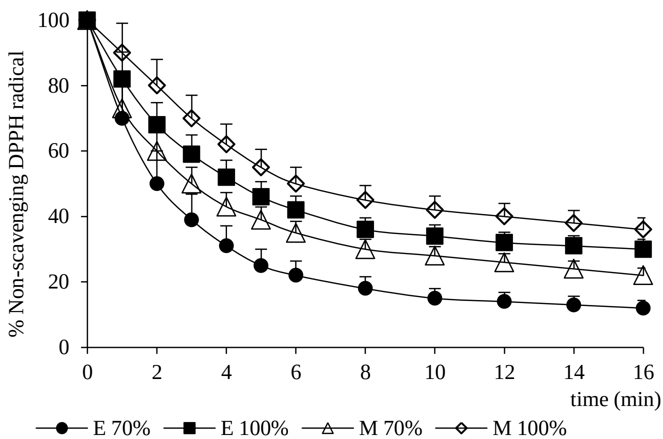

Figure 2. DPPH radical scavenging activity by distillery stillage extracts.

Table 2. Assessment of the effectiveness of the solvents used.

Solvent	N_{V1}	N_{V2}	M_S
E 70%	0.81	0.99	0.90 ± 0.131
E 100%	0.30	0.34	0.33 ± 0.032
M 70%	0.80	0.92	0.86 ± 0.081
M 100%	0.24	0.20	0.22 ± 0.282

their interaction with other cellular components. For this study, distillery stillage (a by-product of crude ethyl alcohol production) was selected as the source of polyphenols. The efficiency of polyphenol extraction mainly depends on the solvent that is used (Azmir *et al*. 2013). Organic solvents (extractants) – such as acetone, ethyl acetate, methanol, ethanol and propanol, or mixtures thereof – are used due to their high selectivity for polar compounds (Araujo *et al*. 2015). From the most commonly used solvents, ethanol is more polar than methanol, extracting preferentially more lipophilic and structurally complex phenolic compounds; moreover, ethanol is less toxic than methanol (Araujo *et al*. 2015). Therefore, this study investigated the polyphenolic content recovered from distillery stillage with two extraction solvents (ethanol, methanol) applied as pure solvents or mixtures with water (E 70% and M 70%).

In the present study, it was confirmed that ethanol was a more effective solvent than methanol in recovering polyphenolic compounds from the distillery stillage. The use of E 70% resulted in a total polyphenolic content of 0.134 mg GAE/ml, while the use of M 70% gave a total polyphenolic content of 0.121 mg GAE/ml. Librán *et al*. (2013) identified the most effective conditions (treatment time and ethanol concentration) for the extraction of phenolic compounds from grape marc and determined the polyphenolic content and the antioxidant activity of the extracts. The highest recovery of phenolic compounds (3.12 mg GAE/g grape pomace) was recorded after a 2 h extraction in a 75% liquid mixture of ethanol. Extending and shortening the extraction time did not increase the extraction efficiency. The highest extraction efficiency was obtained using a 75% ethanol solution because the higher concentration of ethanol led to a reduction in the antioxidant and organoleptic properties of phenolic compounds. Ryznar-Luty *et al*. (2009) noticed that the polyphenolic content in distillery residues depends on many factors, including the quality of the raw material tested as well as the place of origin and the type of raw materials used for alcoholic fermentation. The authors also noted that during transport, distillery residues are exposed to environmental factors such as temperature, humidity and environmental pollution, which also affect their polyphenolic content.

In the case of the analysed extracts obtained from the stillage, solvents of different polarity were used. There were significantly higher polyphenolic content and antioxidant activity in extracts obtained using a mixture of alcohol and water than with 100% solvents. When analysing the influence of the solvents on a group of polyphenolic compounds, there was a correlation between higher solvent polarity and higher antioxidant activity. These results were obtained with different amounts of water in the solvent, a finding that shows variability of the antioxidant potential in different environments. Specifically, hydrophilic antioxidants are more effective in a non-polar system, while lipophilic antioxidants show a better effect in a polar solution (Porter *et al*. 1989). The activity of phenolic acids increases significantly if they contain two ortho hydroxyl groups in the molecule. Such compounds with high antioxidant activity include, for example, ferulic acid, and the presence of the third hydroxyl group causes a further increase in antioxidant activity, as is the case for gallic acid, among other molecules (Rosicka-Kaczmarek 2004).

The strong reduction potential of the stillage extracts was verified in the DPPH radical scavenging assay. The ability to scavenge free radicals depends on the content of substances with antioxidant properties. The lower polyphenolic content in methanol compared with ethanol extracts was reflected in a reduction in DPPH radical scavenging activity by 10% (mixture of methanol and water) and 6% (pure methanol). Emmons and Peterson (1999) reported that the activity of polyphenolic compounds to reduce the DPPH radical depends on the location and the number of hydroxyl (–OH) and methoxy (–OCH3) groups. Peterson *et al*. (2001) showed that the decrease in polyphenolic content in oat grain causes a decrease in the DPPH radical reduction activity. The authors found a significant correlation between the polyphenolic content and the ability to eliminate the DPPH radical.

The antioxidant activity of phenolic compounds, apart from their primary activity to break free radical reactions, is also based on secondary mechanisms such as the activity to scavenge radicals, to chelate heavy metal ions and to decompose the formed peroxides (Moure *et al*. 2001). Therefore, to characterise fully the raw materials in terms of their antioxidant properties, it is important to use various methods. The tested ethanol extracts from the distillery stillage showed a relatively high content of polyphenolic compounds and their antioxidant activity, determined by the reduction of the DPPH radical.

Conclusions

The extracts from the distillery stillage were characterised by a diverse content of polyphenolic compounds and antioxidant activity resulting from the use of various solvents for extraction. However, the ethanol extracts had a higher polyphenolic content than the methanol extracts. The E 70% extract had the highest polyphenolic content and antioxidant activity. In addition, the polyphenolic content correlated with the antioxidant activity. There was higher polyphenolic content and antioxidant activity in extracts obtained with 70% solvent solutions compared with 100% solvent solutions, a factor that is related to the change in the solvent polarity. The results indicate a potential use of by-products from the distillery industry as a source of natural bioactive compounds. The observed antioxidant activity can be attributed both to the mechanisms exerted by phenolic compounds and to the synergistic effect of various phytocompounds. However, further research in this area should be carried out to determine the profile of phenolic compounds and the spectrum of their activity.

Acknowledgements

Wioleta Mikucka is a recipient of a scholarship from the Programme Interdisciplinary Doctoral Studies in Bioeconomy (POWR.03.02.00-00-I034/16-00), which is funded by the European Social Fund.

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