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Combined recovery of polysaccharides and polyphenols from *Rosa damascena* wastes

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ABSTRACT

The industrial production of rose oil generates huge amounts of waste, and its potential for integrated utilization is underexplored. A combined method for the recovery of biologically active substances from waste rose (*Rosa damascena* Mill.) biomass was investigated and proposed in the present study. It comprised preliminary ethanol treatment of the wastes aimed at the extraction of polyphenol substances and subsequent fractional extraction of polysaccharides. The results suggested that the optimal ethanol concentration for integrated recovery was 70%. Different polysaccharide fractions were extracted and investigated for the first time from the alcohol insoluble residues (AIRs) by consecutive fractional extraction with water, oxalate, diluted acid and alkali. The neutral sugar compositions of the polysaccharides suggested that they were pectic-type polysaccharides. The differential thermal analysis of the isolated polysaccharides showed that their thermal stability was in the 220–230 °C range. The overall yield of polysaccharides (25.3%) and polyphenols (4.4%) suggested that the waste rose biomass could serve as a valuable source of biologically active substances. As a result of the present study, a technological scheme for combined waste rose biomass utilization was proposed. On the basis of the technological operations and the energy assessment, calculations of the average costs of combined polyphenols and polysaccharides recovery were presented. An additional advantage of the integrated approach is that it could be further combined with other methods and applied to other essential oil plant wastes.
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1. Introduction

The Rose Valley is a region situated in Central Bulgaria, south of the Balkan Mountains. Owing to the favorable weather conditions cultivated *Rosa damascena* – one of the most famous symbols and important essential oil crops of Bulgaria (Rusanov et al., 2012), has been grown in this area for centuries. The main products from the fresh rose flowers are rose oil, concrete, absolute, and rose water, and they are widely appreciated and used in perfumery, pharmacy and the food industry. Bulgaria, Turkey and Iran are the biggest producers in the world, supplying together more than 90% of the rose oil. Distillation of around 4000 kg of fresh rose petals is necessary for the production of one kilogram of rose oil (Kovacheva et al., 2010). Due to this fact, large amounts of waste are generated, and most of the distilleries simply dump the biomass in the nearby locations. This approach, however, does not allow the recovery of the valuable biologically active substances present in the rose waste biomass, besides, biocontamination of the soil and water is possible. The main strategy for the utilization of these waste materials is an additional treatment for more complete extraction of aroma substances (Nedkov et al., 2005) taking into account the price of rose oil, water, concrete, and absolute. Other possibilities were investigated and polyphenol extracts were used for color stability improvement of strawberry beverages (Mollov et al., 2007). In previous experiments, Slavov et al. (2013, 2016) studied the possibility for obtaining water-soluble polysaccharides from

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waste rose petals. However, the analysis of the residue obtained after hot-water extraction showed that a large part of the pectic polysaccharides still remained unextracted and suggested that further investigations in this direction were needed. From a technological and ecological point of view, it is also important to combine as many methods as possible (Galanakis, 2015) for a more complete reuse of waste rose flowers, waste reduction and obtaining valuable by-products.

The aim of the present work was to explore and investigate an approach using consecutive fractional extraction for combined recovery of polyphenols and polysaccharides from waste rose (*Rosa damascena*) biomass and their characterization.

2. Materials and methods

2.1. Materials

The waste rose biomass (*Rose Damascena* Mill) was provided by EKOMAAT distillery, (Mirkovo, Sofia district, Bulgaria: 2014 harvest). The wastes were inspected for impurities, dried under vacuum at 50 °C until the water content was around 5–6% and then stored at −18 °C. The reagents used in the preparative and analytical experiments were of analytical grade and obtained from local distributors.

2.2. Methods

2.2.1. Preparative methods

2.2.1.1. Obtaining ethanolic extracts (Et) and alcohol-insoluble residues (AIR). The alcohol-insoluble residues were obtained from waste materials by treatment with 50, 70 and 95% ethanol and denoted as AIR50, AIR70 and AIR95, respectively. Briefly, 300 g dry waste rose petals were treated with 1.5 L ethanol solution for 1 h at 60 °C with constant stirring, then left for 24 h at room temperature. The mass was filtered, and the insoluble residue was washed with 0.5 L ethanol. After filtration, the ethanolic extracts were collected and the total volume was measured. The ethanolic extracts were further used for polyphenol determination and antioxidant activity analysis. The remaining alcohol-insoluble residues were dried at 50 °C under vacuum. The AIR's were further used for obtaining polysaccharides by consecutive fractional extraction.

2.2.1.2. Consecutive fractional extraction of AIR and obtaining polysaccharides. Consecutive fractional extraction of the AIRs was performed as follows:

1) **Hot-water extraction** was performed following the procedure described in Slavov et al. (2016). After extraction and filtrate reduction to 1/3 of its initial volume, the concentrated liquid containing the polysaccharides was precipitated with 3 vols of 96% ethanol (24 h at 4 °C). The mass was filtered using nylon cloth (50 μm), and then dried at 50 °C. The crude polysaccharide was re-dissolved in 250 mL H_2O and subjected to dialysis (membranes Sigma-Aldrich, D9652-100FT, Mr. cut-off 12000) for 48 h against distilled water. The retentate in the dialysis membrane was freeze dried and denoted as water-soluble polysaccharides (W).
2) **Ammonium oxalate extraction** – the residue from the hot-water extraction was treated with 1000 mL 0.05 M $(NH_4)_2C_2O_4$ at 50°C for 1 h (pH 6) with constant stirring (100 rpm); the mass was filtered through cloth (50 μm), and the residue was subjected to a second extraction with another 1000 mL 0.05 M $(NH_4)C_2O_4$ at 50°C for 1 h (pH 6) with constant stirring (100 rpm). The extract was filtered again, and both filtrates were combined and evaporated under vacuum to 1/3 of its initial volume. Then the concentrated filtrate was treated as described in 1). The retentate in the dialysis membrane was freeze dried and denoted as chelate-extracted polysaccharides (Ch).
3) **Dilute acid extraction** – the remaining residue after the chelate extraction was subjected to acid extraction with 1000 mL 0.1 M HCl at 85°C for 1 h (pH 1.2) with constant stirring (100 rpm); the mass was filtered through cloth (50 μm), and the residue was subjected to a second extraction with another 1000 mL 0.1 M HCl at 85°C for 1 h (pH 1.2) with constant stirring (100 rpm). The extract was filtered again, and both filtrates were combined and evaporated under vacuum to 1/3 of its initial volume. Then the concentrated filtrate was treated as described in 1). The retentate in the dialysis membrane was freeze dried and denoted as acid-extracted polysaccharides (Ac).
4) **Dilute alkaline extraction** – the residue from the dilute acid extraction was treated with 500 mL 0.05 M NaOH at 4°C for 17 h (pH 11.2) with constant stirring (100 rpm); the mass was filtered through cloth (50 μm), and the residue was subjected to a second extraction with another 500 mL 0.05 M NaOH at 4°C for 4 h (pH 11.2) with constant stirring (100 rpm). The extract was filtered again and the combined filtrates were precipitated with 3 vols of 96% ethanol for 24 h at 4 °C. The precipitate was filtered through cloth (50 μm) and dried (50 °C). The obtained polysaccharide was dissolved in 250 mL distilled water and dialyzed (membranes Sigma-Aldrich, D9652-100FT, Mr. cut-off 12000) for 48 h against distilled water. The retentate in the dialysis membrane was freeze dried and denoted as alkali-soluble polysaccharides (B).

2.2.2. Analytical methods

2.2.2.1. Total polyphenols determination. Total polyphenols were determined according to the method of Singleton and Rossi (1965) with Folin-Ciocalteu's reagent (Sigma-Aldrich, Switzerland). Gallic acid (Sigma-Aldrich, (Steinheim, Germany) was employed as calibration standard and results were expressed as gallic acid equivalents (GAE) per gram dry matter in extracts.

2.2.2.2. HPLC analysis of ethanolic extracts. High Performance Liquid Chromatography (HPLC) analyses of phenolic components was performed on an Agilent 1220 HPLC system (Agilent Technology, USA), equipped with a binary pump and UV–vis detector. A wavelength of 280 nm was used. The separation of phenolic compounds was performed using an Agilent TC-C18 column (5 μm, 4.6 mm x 250 mm) at 25 °C. The mobile phases consisted of 0.5% acetic acid (A) and 100% acetonitrile (B) at a flow rate of 0.8 mL/min. The gradient condition started with 14% B, increased linearly to 25% B between the 6th and the 30th min, then to 50% B at the 40th min. The standard compounds (gallic acid, 3,4-dihydroxy benzoic acid, chlorogenic acid, caffeic acid, *p*-coumaric acid, ferulic acid, ellagic acid, catechin, epicatechin, rutin, naringin, myricetin, quercetin, naringenin and kaempferol) were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2.2.3. Antioxidant activity assays. 1) Oxygen Radical Absorbance Capacity (ORAC) assay

was measured according to the method of Ou et al. (2001) with some modifications described in details by Číž et al. (2010). The method measures the antioxidant scavenging activity against peroxyl radical generated by thermal decomposition of 2,2′-azobis[2-methyl-propionamidine] dihydrochloride (AAPH; Sigma-Aldrich, Steinheim, Germany) at 37 °C. Fluorescein (FL; Sigma-Aldrich, Steinheim, Germany) was used as the fluorescent probe. The loss of fluorescence of FL was an indication of the extent of damage from its reaction with the peroxyl radical. The protective effect of the antioxidant was measured by assessing the area under the fluorescence decay curve (AUC) relative to that of a blank in which no antioxidant was present. Solutions of AAPH,

fluorescein and (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; Sigma-Aldrich, Steinheim, Germany) were prepared in a phosphate buffer (75 mM, pH 7.4). Dilution of the samples was also performed with the phosphate buffer. The reaction mixture (total volume 200 μL) contained FL (170 μL, final concentration 5.36×10^{-8}M), AAPH (20 μL, final concentration 51.51 mM), and sample (10 μL). The FL solution and the sample were incubated at 37 °C for 20 min directly in a microplate reader, and AAPH (dissolved in buffer at 37 °C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 μL of phosphate buffer was used instead of the extract. The antioxidant activity was expressed in micromole Trolox equivalents (μmol TE) per liter of extract. The Trolox solutions (6.25; 12.5; 25 and 50 μM) were used for defining the standard curve. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany), at excitation wavelength of 485 nm and emission wavelength of 520 nm.

2) Hydroxyl Radical Averting Capacity (HORAC) assay

The HORAC assay developed by Ou et al. (2002) measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co(II) complex, hence the protecting ability against hydroxyl radical formation. Hydrogen peroxide (Fluka, Germany) solution of 0.55 M was prepared in distilled water. A solution of 4.6 mM Co(II) was prepared as follows: 15.7 mg of $CoF_2{\cdot}4H_2O$ (Sigma-Aldrich, Steinheim, Germany) and 20 mg of picolinic acid (Sigma-Aldrich, Steinheim, Germany) were dissolved in 20 mL of distilled water. Fluorescein 170 μL (60 nM, final concentration) and 10 μL of sample were incubated at 37 °C for 10 min directly in the FLUOstar plate reader. After incubation, 10 μL H_2O_2 (27.5 mM final concentration) and 10 μL of Co(II) (230 μM final concentration) solutions were added subsequently. The initial fluorescence was measured, after which the readings were taken every minute after shaking. For the blank sample, phosphate buffer solution was used. 100, 200, 600, 800 and 1000 μM gallic acid solutions (in phosphate buffer 75 mM, pH 7.4) were used for building the standard curve. The AUC were calculated in the same way as ORAC. The results were expressed in micromole gallic acid equivalents (μmol GAE) per gram dry matter in extracts.

3) DPPH assay

The antioxidant activity of ethanolic extracts was measured by DPPH method as described by Brand-Williams et al. (1995) and modified by Dinkova et al. (2014). Briefly, 250 μL of ethanolic extract of rose waste (diluted with distilled water 1:3, v/v) were added to a 2.25 mL DPPH [2,2-diphenyl-1- picrylhydrazyl] (Merck, Germany) solution in methanol (6×10^{-5}M); the mixture was left for 15 min (kept in the dark at room temperature) so that a reaction could take place, and then the absorbance at 515 nm was read.

4) FRAP assay

The ferric reducing ability was determined according to Benzie and Strain (1996), with some modification of Dinkova et al. (2014). A mixture of 2.25 mL FRAP reagent and 250 μL ethanolic extract (diluted with distilled water 1:3, v/v) was prepared in a cuvette, and absorbance at 593 nm was measured after 4 min of reaction. The FRAP reagent consisted of 2.5 mL 2,4,6-tripyridyl-s-triazine (TPTZ; Sigma-Aldrich, Steinheim, Germany) solution (10 mM) in hydrochloric acid (40 mM), 2.5 mL of a 20 mM $FeCl_3$ water solution and 25 mL of an acetate buffer (0.3 M, pH 3.6).

2.2.2.4. Characterization of pectic polysaccharides. The neutral sugar composition of the polysaccharides was measured as alditol acetates after acid hydrolysis of the polysaccharide. In brief, the polysaccharide was hydrolyzed with 2 M trifluoroacetic acid (Sigma-Aldrich, Germany) for 3 h at 120 °C, and the monosaccharides obtained were converted to alditol acetates according to the method of Blakeney et al. (1983). The alditol acetates were analyzed by gas chromatography (6890 GC System Plus, Hewlet-Packard, Palo Alto, USA) with an SP-2380 (Supelco column) at 200 °C for 3 min, then 5 °C.min^{-1} to 250 °C; injector temperature 250 °C, detector temperature 280 °C; helium was used as carrier gas at 1 mL min^{-1}. Peak identification was based on retention times, using *myo*-inositol as internal standard.

The molecular weights of the isolated pectic polysaccharides were determined according to Kratchanova et al. (2008) on a Waters (Milipore) system equipped with Ultrahydrogell™ 120 and Ultrahydrogell™ 500 columns (7.8 × 300 mm, Waters). Bidistilled water was used as eluent at an elution rate of 0.8 mL/min. The columns were equilibrated with Shodex pullulan (Showa DENKO, Japan) standards with molecular weights of 0.62×10^4, 1.00×10^4, 2.17×10^4, 4.88×10^4, 11.3×10^4, 20.0×10^4, 36.6×10^4, 80.5×10^4 Da.

The uronic acid content was measured by the m-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen, 1973) using 3-hydroxybiphenyl (Acros Organics, Belgium). The protein amount in the polysaccharides was determined by the Bradford method (Bradford, 1976) using AMRESCO E535-KIT (AMRESCO, Solon, Ohio, USA).

The degree of methylesterification (DM) was determined by quantification of the methanol released by alkaline de-esterification (0.5 M NaOH) for 1 h at 4 °C in the presence of divalent cations ($CuSO_4$; Merck, Germany). Reverse phase HPLC equipped with a C18 Superspher column (Merck, Germany) using 4 mM H_2SO_4 as eluent at a flow rate of 0.7 mL/min at 25 °C with refractive index detector was used for methanol determination. Isopropanol was used as internal standard, and DM was calculated as the molar ratio of methanol to 100 galacturonic acid units.

The thermo-physical properties of the pectic polysaccharide extracts were investigated by differential thermal analysis – thermogravimetric analysis (DTA-TG) with LABSY TM Sevo (Setaram, France). Before the analysis pectic extracts were dried at room temperature and 6 mbar in a desiccator with P_4O_{10} (Acros Organics, Belgium) as drying agent to 6–8% water content. The DTA-TG curves were obtained during heating of the samples (4 mg of each polysaccharide) from 10 to 320 °C, increasing the temperature by 5 °C/min. The analyses were made in air atmosphere (speed of air 20 mL/min) with corundum crucible.

2.3. Energy assessment

The energy assessment was performed as follows: The mass and energy balance of the technological operations were made and the relative mass flows for treatment of 1 kg waste material were obtained (according to the methods described in Boustead and Hancock, 1979 and Schmidt, 1997). Furthermore, having in mind the thermophysical parameters of the solvents (ethanol, water, etc.), the temperature regimens, and technical data provided by EKOMAAT distillery (Mirkovo, Sofia district, Bulgaria) the necessary energy for heating, cooling and freezing was determined. The energy assessment calculations and cost estimations were made also on the basis of the following data: average prices of energy and fuels in Bulgaria for November 2016; 1 kg waste on average is obtained from 6.62 kg rose flowers; on average, 4 kg waste rose biomass yield 1 kg polysaccharides and 176 g polyphenols (on a dry basis). Calculations were made on the basis of the assumption that 90% of the ethanol used is distilled and reused.

2.4. Statistical analysis

The analyses were run three times, and the data were given as mean values. Statistical significance was detected by analysis of variance (ANOVA, Tukey's test; value of $p < 0.05$ indicated statistical

Table 1
Yield of extracted substances and characteristics of the alcohol insoluble residues (AIR) obtained after 50%, 70% and 95% ethanol treatments of waste rose flowers.

AIR	Extracted substances, %	Yield of AIR, %	DM, %	PUC, %	Proteins, %	Ash, %
AIR50	$14.3^{a} \pm 1.1$	$85.6^{a} \pm 1.2$	$61.75^{a} \pm 0.9$	$18.65^{a} \pm 0.5$	$1.65^{a} \pm 0.2$	$2.51^{a} \pm 0.5$
AIR70	$9.5^{b} \pm 1.2$	$90.3^{b} \pm 1.1$	$62.15^{a} \pm 1.2$	$20.29^{b} \pm 0.4$	$1.84^{a} \pm 0.6$	$2.67^{a} \pm 0.9$
AIR95	$10.2^{b} \pm 1.3$	$89.6^{b} \pm 1.5$	$64.21^{a} \pm 0.8$	$21.15^{b} \pm 0.9$	$1.78^{a} \pm 0.4$	$2.56^{a} \pm 0.8$

AIR: Alcohol insoluble residues.
DM: Degree of methylesterification.
PUC: Polyuronic acid content.
[a,b]Different letters mean statistical difference (Tuckey's HSD test, $p < 0.05$).

difference). The homogeneity of variances assumption was checked by Levene's test.

3. Results and discussion

3.1. Treatment of waste with 50, 70 and 95% ethanol solutions

Treatment with ethanolic solutions of the raw plant materials is a procedure applied before the extraction of polysaccharides and aimed at the removal of low-molecular substances, such as – sugars, polyphenols, salts, etc. (Kratchanova et al., 2008). This treatment also leads to obtaining ethanolic extracts from waste rose biomass which are rich in polyphenols and could be used as antioxidant supplements and color stabilizers (Mollov et al., 2007). In order to prevent the solubilization of polysaccharides, 95% ethanol is commonly used. However, previous studies on the extractability of polyphenols found that lower ethanol concentrations (30%) were more suitable for their extraction. For this reason, an investigation on the influence of the ethanol concentration (50, 70, and 95%) on the yield of alcohol-insoluble residues and the polyuronic acid content (PUC) was performed in order to determine the ethanol concentration which would allow a combined recovery of polyphenols and polysaccharides. Concentrations lower than 50% were not considered because the polysaccharide loss was significant. The results of the experiments for obtaining alcohol-insoluble residues (AIR) are presented in Table 1.

The AIR yields after treatment with 70 and 95% ethanol did not differ significantly. The AIR50 yield was the lowest and differed statistically from AIR70 and AIR95. It could also be seen that the polyuronic acid content was the lowest for AIR50 and was significantly different from AIR70 and AIR95. These results showed that the extraction using solutions with ethanol concentrations lower than 70% led to partial solubilization of pectic-type polysaccharides. The yields of the substances extracted by ethanol confirm these observations. The dry matter content was the highest in the 50% ethanolic extract and differed statistically from the dry matter in the 70% and 95% ethanolic extracts. The other parameters, i.e. degree of methylesterification (DM), proteins and mineral content were similar for the alcohol-insoluble residues obtained after treatment with 50, 70 and 95% ethanol.

3.2. Characterization of ethanolic extracts

In order to assess the potential of rose wastes as source of polyphenols the total polyphenol content of the ethanolic extracts was investigated. Furthermore the individual phenolic compounds present in the three extracts were determined. The results for total polyphenol content (TPC) and the individual phenolic compounds are given in Table 2.

The data presented in Table 2 suggest that the total polyphenol content in the ethanolic extracts is very high and reaches 46.5% based on dry weight, after extraction with 70% ethanol. The chemical structure of phenolic compounds, present in the vegetative matrix could vary from single molecules (i.e. phenolic acids) to highly polymerized structures such as tannins. Therefore, mixtures of ethanol, methanol and acetone with water are most commonly used for the extraction of these compounds (Dai and Mumper, 2010; Mukhopadhyay et al., 2006). In the present study, ethanol was the preferred solvent for obtaining AIR from waste rose petals due to its low toxicity. Since the goal was the combined consecutive recovery of polyphenols and polysaccharides with a maximum yield of both substances, ethanol concentrations higher than 50% were chosen. The results in Table 2 demonstrate that ethanol-water mixtures have a better extraction efficiency compared with pure alcohol. Mixtures with water facilitate the penetration of organic solvents into the plant cells and increase polyphenols extraction (Mukhopadhyay et al., 2006). The best extraction of total polyphenols (6506.1 mg/L, respectively 464.7 mg/g dry matters extract) was achieved with 70% ethanol, whereas the use of 95% alcohol led to a 42.5% decrease in the extracted phenolic compounds. The use of 50% alcohol yielded a slightly lower polyphenols amounts (per g dry weight extract) in comparison with 70% ethanol, and the results were significantly different. A similar trend was observed for the extracted individual polyphenolic compounds. The yields of the more hydrophilic phenolic acids (neochlorogenic acid, caffeic acid, *p*-coumaric acid) with 50% and 70% alcohol were similar, whereas the yields of more hydrophobic ellagic acid and flavonoids were significantly higher when 70% ethanol was used as an extractant.

Since polyphenolic compounds present in the extracts are related to their antioxidative properties, in the subsequent experiments, the antioxidant activity of the ethanolic extracts was investigated via several methods. When assaying the antioxidant

Table 2
Content of total polyphenols and individual phenolic compounds in ethanolic extracts of waste rose flowers.

Extract	mg/g dry weight (DW) extract											
	Neochlorogenic acid	Caffeic acid	p-Coumaric acid	Ellagic acid	Quercetin	Quercetin-3-β-glucoside	Myricetin	Kaempferol	Naringin	Catechin	Epicatechin	TPC
Et50	$9.9^{a} \pm 0.6$	$2.6^{a} \pm 0.1$	$8.8^{a} \pm 1.1$	$6.1^{a} \pm 0.3$	$11.3^{a} \pm 0.6$	$48.1^{a} \pm 1.0$	$18.8^{a} \pm 0.5$	$1.2^{a} \pm 0.1$	$76.6^{a} \pm 0.9$	$37.5^{a} \pm 1.3$	$14.2^{a} \pm 0.8$	$401.9^{a} \pm 13.6$
Et70	$9.8^{a} \pm 0.5$	$2.7^{a} \pm 0.6$	$9.4^{a} \pm 0.8$	$9.4^{b} \pm 0.7$	$14.1^{b} \pm 0.5$	$63.4^{b} \pm 0.8$	$23.8^{b} \pm 0.9$	$1.7^{a} \pm 0.2$	$82.4^{b} \pm 0.7$	$69.2^{b} \pm 2.6$	$11.9^{b} \pm 0.7$	$464.7^{b} \pm 16.2$
Et95	$7.3^{b} \pm 0.3$	–	$2.4^{b} \pm 0.6$	$2.0^{c} \pm 0.3$	$10.9^{a} \pm 0.6$	$52.6^{c} \pm 1.1$	$12.8^{c} \pm 0.4$	$2.1^{a} \pm 0.9$	$64.8^{c} \pm 1.0$	$45.3^{c} \pm 2.0$	–	$311.7^{c} \pm 19.9$

Et: Ethanolic extract.
TPC: Total polyphenol content.
[a,b,c]Different letters mean statistical difference (Tuckey's HSD test, $p < 0.05$).

Table 3
Antioxidant activity of ethanolic extracts of waste rose flowers by ORAC, HORAC, DPPH and FRAP methods.

Extract	ORAC, μmol TE/g DW	HORAC, μmol GAE/g DW	DPPH, μmolTE/g DW	FRAP, μmolTE/g DW
Et50	$6523.6^{a} \pm 65$	$3034.8^{a} \pm 98$	$1058.7^{a} \pm 64$	$1281.4^{a} \pm 84$
Et70	$7762.5^{b} \pm 143$	$4515.7^{b} \pm 104$	$1448.7^{b} \pm 68$	$1548^{b} \pm 65$
Et95	$5500.85^{c} \pm 102$	$3370.7^{c} \pm 82$	$951.7^{a} \pm 87$	$1175.1^{a} \pm 75$

ORAC: Oxygen Radical Absorbance Capacity assay.
HORAC: Hydroxyl Radical Averting Capacity assay.
DPPH: 2,2-diphenyl-1- picrylhydrazyl assay.
FRAP: Ferric reducing ability of plasma assay.
Et: Ethanolic extract.
TE: Trolox equivalents.
GAE: Gallic acid equivalents.
DW: Dry weight.
[a,b,c]Different letters mean statistical difference (Tuckey's HSD test, $p < 0.05$).

activity of natural antioxidants, use of more than one antioxidant assay is recommended for a detailed understanding of the antioxidant properties of substances (Číž et al., 2010). Therefore, several assays expressing various aspects of the antioxidant action of polyphenols (ORAC, HORAC, DPPH and FRAP) were used. The methods employed, cover different aspects of the antioxidant action and give a broader view of the antioxidant potential of rose wastes. The ORAC method measures the ability of the antioxidant to scavenge peroxyl radicals via hydrogen atom transfer. These radicals are physiologically the most important ones, and the hydrogen atom transfer is the most physiologically relevant mechanism of antioxidant action. The HORAC method measures the metal-chelating activity of antioxidants under the conditions of Fenton-like reactions, hence it indicates the protecting ability of compounds against hydroxyl radical formation. The DPPH test represents the radical scavenging capacity of an antioxidant via transfer of a single electron, whereas the FRAP method is an indicator of the metal-reducing capability.

The results on the antioxidant activity (Table 3) are similar to those on the total polyphenol content, indicating that polyphenol compounds are the main contributor to the antioxidant activity of the investigated extracts. With all the methods used, the 70% ethanolic extract showed the highest antioxidant activity which was significantly different from Et50 and Et95 extracts. For example, the results on the ORAC antioxidant capacity of the Et70 extract were 10% and 39% higher, respectively, in comparison with the Et50 and Et95 extracts.

Based on the analysis of the three ethanolic extracts and on the data on obtaining AIR (Table 1), a conclusion could be made that the most suitable ethanol concentration for optimum utilization of the waste rose flowers was 70%. The higher ethanolic concentration (95%) led to obtaining AIR with slightly higher polyuronic acid content, but the extractability of polyphenolic compounds was lower. Using ethanol solution with concentrations lower than 70% led to significant losses of polysaccharides.

3.3. *Consecutive fractional extractions of AIRs and physico-chemical characteristics of the extracted polysaccharides*

The data on AIR50 (Table 1) showed a decrease in the yield and polyuronic content. The AIR70 and AIR95 yields were almost similar and did not differ statistically. The highest polyuronic acid content was observed for AIR95: 21.15%. In the subsequent experiments, polysaccharide fractions were obtained from the alcohol-insoluble residues (AIR50, AIR70 and AIR95) by consecutive fractional extractions with water, 0.05 M $(NH_4)_2C_2O_4$, 0.1 M HCl and 0.05 M NaOH and their characteristics are shown in Table 4.

The overall yield of extracted polysaccharides was similar for AIR95 and AIR70 (no statistical difference was observed). The lower polysaccharides yield – of 17.54 g for AIR50 confirmed the observation (Table 1: data on the polyuronic acid content of AIR50) that pretreatment with an ethanolic solution with concentrations lower than 70% led to partial solubilization of polysaccharides. A similar trend of increase in the yield was observed for the extractability of water and chelate-soluble polysaccharides. The yields of the different types of polysaccharides showed that chelate and acid-extractable polysaccharides were the most abundant, with very close values for water-extractable polysaccharides. The overall yield also implied that waste rose flowers were a rich and promising source of polysaccharides. On the basis of the data on the uronic acid content, a conclusion could be made that they were pectic type polysaccharides. The degree of methylesterification of the water-soluble polysaccharides was above 60%. For the chelate and acid-extractable pectic polysaccharides, it could be concluded that they were middle-esterified pectic polysaccharides. Water and acid-extractable polysaccharides showed the highest total neutral sugar contents. The protein content of the pectic fractions was in the 1.1–1.3% range. The highest molecular weights were observed with water and acid-extractable polysaccharides obtained from AIR70 and AIR95, although all of them were heterogeneous. All of the oxalate-extracted polysaccharides were homogenous, and those extracted from AIR95 had the highest molecular weight. The alkali-soluble pectic polysaccharides were characterized by lower molecular weights, which was an expected result since the dilute alkali treatment could lead to partial depolymerization through β-elimination of uronic acid rich polysaccharides (Kratchanova et al., 2004). The water-soluble polysaccharide extracted from AIR70 had the highest molecular weight compared with Ch70, Ac70 and B70. The polysaccharides obtained from AIR50 were homogenous and had molecular weights by an order of magnitude of 10^4Da.

In the subsequent experiments, the pectic polysaccharides, the initial alcohol-insoluble residues and the residues after sequential extraction were hydrolyzed with 2 M trifluoroacetic acid (TFA) and their neutral sugar composition was determined (Table 5).

The most abundant neutral sugar was galactose followed by arabinose. For the water-soluble pectic polysaccharides, the ratio between galactose and arabinose was close to one which suggested presence of arabinogalactan side chains. These results confirmed the observations made by Slavov et al. (2013) in a study of the immunomodulating properties of water soluble rose pectic polysaccharides. The presence of glucose in the water-soluble polysaccharides (and not in the chelate- and acid-soluble ones) implied that this monosaccharide was present in the neutral sidechains of the macromolecules. The highest amounts of galactose in the acid-extractable polysaccharides also suggested presence of galactan-type sidechains. The lower content of arabinose in the acid-extractable pectic polysaccharides also suggested that some arabinan-type sidechains were present in the rose pectic polysaccharides since the acid conditions lead to their hydrolysis and removal during the extraction process (Kratchanova et al.,

Table 4
Characteristics of polysaccharides extracted by consecutive fractional extraction from AIR50, AIR70 and AIR95 with water (W), 0.05 M $(NH_4)_2C_2O_4$ (Ch), 0.1 M HCl (Ac) and 0.05 M NaOH (B).

Pectic fraction	Yield, g	Uronic acids, mg/g	DM, %	Neutral sugars, mg/g	Proteins, mg/g	Molecular weight, Da
W50	$4.82^{a} \pm 0.3$	$525.58^{a} \pm 6.2$	$62.3^{a} \pm 4.9$	$721.64^{a} \pm 8.1$	$24.31^{a} \pm 2.1$	3.55×10^4
Ch50	$5.58^{a} \pm 0.4$	$447.69^{a} \pm 7.8$	$35.9^{a} \pm 5.8$	$737.73^{a} \pm 12.6$	$14.15^{a} \pm 3.2$	7.18×10^4
Ac50	$6.16^{a} \pm 0.2$	$431.70^{a} \pm 3.6$	$48.1^{a} \pm 4.6$	$961.14^{a} \pm 8.6$	$23.65^{a} \pm 1.5$	1.01×10^4
B50	$0.98^{a} \pm 0.1$	$294.37^{a} \pm 8.8$	$17.8^{a} \pm 2.1$	$745.50^{a} \pm 4.8$	$30.58^{a} \pm 6.5$	8.90×10^4
Total	$17.54^{a} \pm 0.4$					
W70	$4.95^{a} \pm 0.5$	$539.74^{b} \pm 5.3$	$61.7^{a} \pm 5.4$	$886.55^{b} \pm 10.1$	$21.59^{a} \pm 1.4$	2.08×10^6 (11%) 1.63×10^5 (89%)
Ch70	$6.66^{b} \pm 0.1$	$687.21^{b} \pm 6.7$	$33.2^{a} \pm 2.4$	$757.89^{b} \pm 6.8$	$13.68^{a} \pm 3.5$	6.14×10^4
Ac70	$6.68^{a} \pm 0.4$	$462.16^{b} \pm 4.1$	$49.5^{a} \pm 3.5$	$943.86^{b} \pm 7.4$	$26.44^{a} \pm 1.5$	1.3×10^5 (41%) 2.04×10^4 (59%)
B70	$1.36^{b} \pm 0.1$	$368.32^{b} \pm 9.4$	$16.8^{a} \pm 1.8$	$715.79^{b} \pm 6.3$	$21.95^{b} \pm 2.4$	4.85×10^4 (45%) 1.96×10^3 (55%)
Total	$19.65^{b} \pm 0.5$					
W95	$5.02^{a} \pm 0.2$	$591.71^{c} \pm 4.2$	$63.5^{a} \pm 4.7$	$856.14^{c} \pm 8.2$	$33.21^{b} \pm 1.8$	1.24×10^6 (44%) 4.32×10^5 (56%)
Ch95	$6.83^{b} \pm 0.5$	$824.21^{c} \pm 10.5$	$34.8^{a} \pm 3.6$	$733.33^{a} \pm 7.8$	$11.56^{a} \pm 1.1$	6.75×10^5
Ac95	$6.78^{a} \pm 0.5$	$443.07^{c} \pm 6.1$	$51.5^{a} \pm 1.2$	$949.71^{b} \pm 4.3$	$29.24^{b} \pm 2.8$	2.25×10^6 (24%) 8.14×10^5 (27%) 3.55×10^4 (49%)
B95	$1.44^{b} \pm 0.1$	$323.73^{c} \pm 3.4$	$15.6^{a} \pm 1.1$	$802.92^{c} \pm 1.6$	$17.63^{b} \pm 3.2$	9.45×10^4
Total	$20.07^{b} \pm 0.5$					

AIR: alcohol insoluble residues.
DM: Degree of methylesterification.
Da: Dalton.
W: Water.
Ch: Chelate.
Ac: Acid.
B: Base.
[a,b,c]Different letters mean statistical difference (Tuckey's HSD test, $p < 0.05$). Comparison of the parameters was made between polysaccharides obtained with same extractants.

Table 5
Neutral sugar composition of pectic polysaccharides extracted with different extractants, initial AIRs and the residues after fractional extraction.

Pectic fraction (material)	Rhamnose, mg/g	Xylose, mg/g	Arabinose, mg/g	Galactose, mg/g	Glucose, mg/g	Ratio GalA/Rha
W50	$17.75^{a} \pm 2.8$	–	$102.26^{a} \pm 5.2$	$117.47^{a} \pm 4.2$	$41.08^{a} \pm 2.1$	29.6
Ch50	$10.79^{a} \pm 1.3$	–	$74.15^{a} \pm 2.8$	$97.76^{a} \pm 2.8$	–	41.5
Ac50	$35.45^{a} \pm 3.6$	–	$17.19^{a} \pm 1.0$	$273.91^{a} \pm 6.9$	–	12.1
B50	$12.53^{a} \pm 2.5$	$0.79^{a} \pm 0.1$	$2.08^{a} \pm 0.2$	$217.42^{a} \pm 5.4$	–	23.5
Initial AIR50	$8.49^{a} \pm 1.2$	$45.26^{a} \pm 3.6$	$129.35^{a} \pm 6.8$	$159.92^{a} \pm 2.4$	$21.26^{a} \pm 1.3$	
Residue50	$0.91^{a} \pm 0.1$	$31.93^{a} \pm 3.5$	$29.67^{a} \pm 2.3$	$43.27^{a} \pm 2.6$	$40.48^{a} \pm 2.0$	
W70	$26.36^{b} \pm 3.6$	–	$118.81^{b} \pm 1.4$	$145.30^{b} \pm 3.8$	$39.59^{a} \pm 3.2$	18.4
Ch70	$15.38^{b} \pm 2.8$	–	$73.58^{a} \pm 4.8$	$85.72^{b} \pm 1.5$	–	44.6
Ac70	$37.80^{ab} \pm 3.9$	–	$20.40^{a} \pm 2.5$	$301.43^{b} \pm 6.7$	–	12.2
B70	$13.2^{a} \pm 1.5$	$0.26^{b} \pm 0.1$	$0.34^{b} \pm 0.1$	$185.12^{b} \pm 2.4$	11.36 ± 1.3	27.8
Initial AIR70	$10.71^{b} \pm 0.9$	$48.69^{a} \pm 2.9$	$133.35^{a} \pm 2.5$	$168.61^{b} \pm 3.5$	$25.07^{b} \pm 2.1$	
Residue70	$0.24^{b} \pm 0.1$	$59.91^{b} \pm 4.2$	$29.63^{a} \pm 3.6$	$34.43^{b} \pm 2.1$	$44.95^{a} \pm 2.5$	
W95	$24.52^{b} \pm 1.0$	–	$119.53^{b} \pm 4.8$	$142.94^{b} \pm 5.0$	$42.36^{a} \pm 5.6$	24.1
Ch95	$13.11^{ab} \pm 1.1$	–	$74.24^{a} \pm 5.2$	$80.37^{b} \pm 2.4$	–	62.9
Ac95	$39.64^{b} \pm 2.1$	–	$11.53^{b} \pm 1.3$	$270.04^{a} \pm 3.7$	–	11.2
B95	$13.62^{a} \pm 1.5$	$0.35^{b} \pm 0.1$	$1.05^{c} \pm 0.1$	$177.53^{c} \pm 2.6$	–	23.7
Initial AIR95	$11.47^{b} \pm 0.8$	$26.18^{b} \pm 2.8$	$134^{a}.05 \pm 2.5$	$171.69^{b} \pm 3.8$	$41.11^{c} \pm 4.1$	
Residue95	$1.85^{c} \pm 0.4$	$42.68^{a} \pm 4.2$	$25.47^{a} \pm 1.4$	$47.60^{a} \pm 2.5$	$44.17^{a} \pm 1.5$	

W: Water.
Ch: Chelate.
Ac: Acid.
B: Base.
AIR: Alcohol insoluble residue.
GalA: Galacturonic acid.
Rha: Rhamnose.
[a,b,c]Different letters mean statistical difference (Tuckey's HSD test, $p < 0.05$). Comparison of the quantities of neutral sugars was made between polysaccharides obtained with same extractants.

2008). The highest ratio between galacturonic acid (GalA) and rhamnose (Rha) for the chelate-extractable pectic polysaccharides suggested that it was rich in homogalacturonans. The highest rhamnose content and the lowest GalA:Rha ratio (around 10) in the acid-extractable pectins suggested that this treatment extracted polysaccharides rich in rhamnogalacturonan I macromolecules (Ralet and Thibault, 2009). The presence of xylose only in the alkali-soluble polysaccharides implied that this treatment led to deeper degradation of the cell walls and partial extraction of hemicelluloses. An additional indication of this was the presence of glucose in the alkali-soluble fractions. Besides, the presence of significant amounts of arabinose and galactose in the residues after fractional extraction suggested that some parts of the polysaccharides still remained in the plant matrix. A possible explanation of these findings is their tight integration within the cellulose/hemicellulose

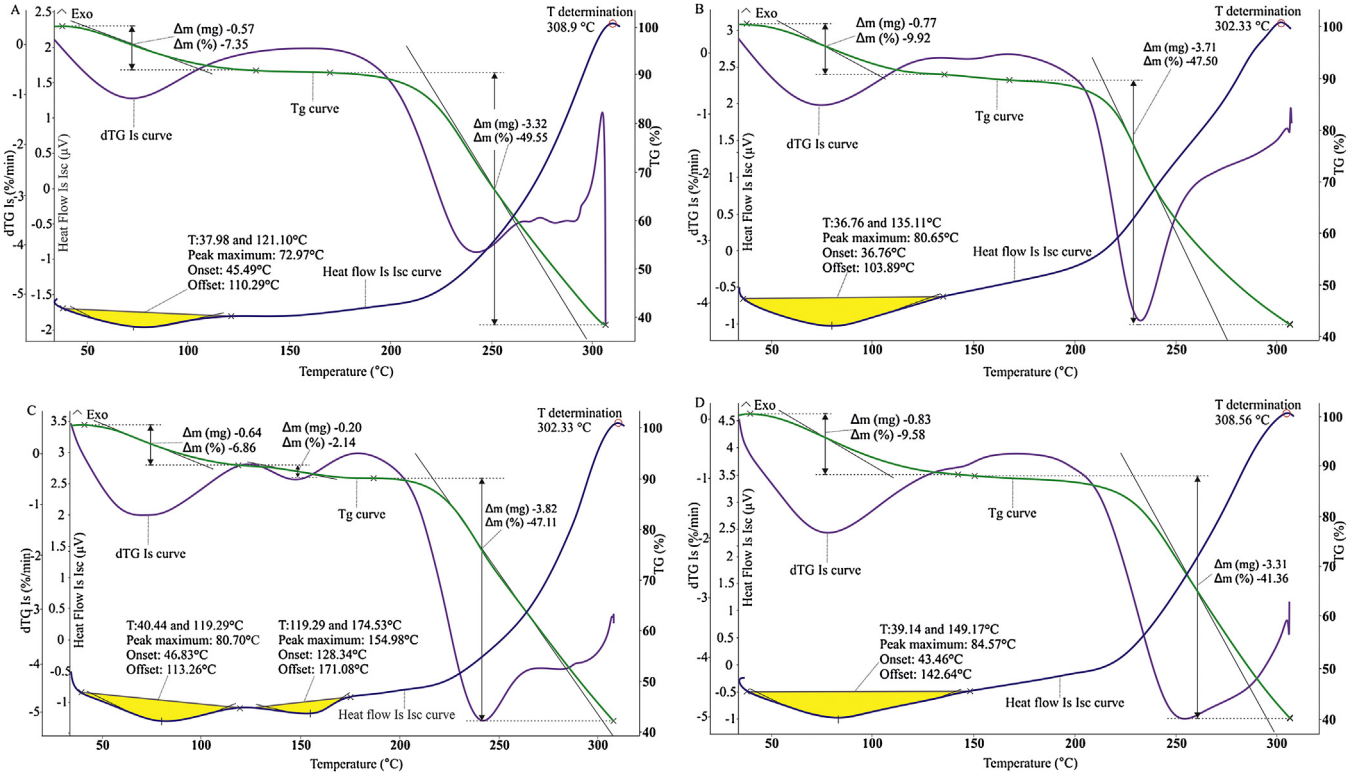


Fig. 1. DTA-TG thermograms of pectic polysaccharides extracted with different extractants from AIR70 by consecutive fractional extraction (A – water-soluble; B – chelate-soluble; C – acid-soluble; D – alkali-soluble pectic polysaccharides). TG – the curve of termogravimetry analysis; dTG Is – the curve of changes with time of the first derivative of the termogravimetry analysis; Heat flow Is Isc – the differential thermal analysis curve, measured with temperature and mass changes of the sample.

matrix, since large amounts of xylose and glucose were also found in the residues.

3.4. Analysis of polysaccharide properties by DTA-TG

The differential thermal-thermogravimetric analysis (DTA-TG) of polysaccharides gives information about changes in their physical and chemical properties (thermal stability, glass transition temperature, sublimation, processes of evaporation of capillary bonded water, degradation, dehydration and oxidation of the macromolecules) upon heating. This technique is very useful for the study of polymeric materials (thermoplastics, thermosets, elastomers, composites, plastic films, fibers, polysaccharides used for coatings, etc.) and the information derived could be used for corroborating predicted material structures, for comparing properties of natural biopolymers, or as a chemical analysis tool. In this connection, it was of interest to investigate the physical and chemical properties of the isolated polysaccharides by DTA-TG. Having in mind the optimal valorization conditions and the results on the influence of ethanol concentration on the polyphenols and polysaccharides yield, only the pectic fractions obtained from AIR70 were analyzed (Fig. 1). The polymeric structures (in this case, polysaccharides) can lose part of their mass via three major ways: release of low-molecular adsorbed substances, chemical reactions and decomposition. The presence of endothermic peaks at the beginning of heating in the 38–145 °C range (Fig. 1, Heat flow Is Isc curve) for all of the investigated polysaccharides suggested processes of evaporation of the adsorbed or capillary bound water molecules. Such endothermic peaks in the range of 40–150 °C were common for hydrophilic colloids (polysaccharides) and were found by Appelqvist et al. (1993), Einhorn-Stoll et al. (2007) and Godeck et al. (2001). This information was confirmed from the dTG Is curves which represent changes with time of the first derivatives of the termogravimetry curve and express the absolute mass changes of the samples. For the acid-extractable pectic polysaccharide, a second endothermic peak appeared immediately after the first one (from 120 until 175 °C). In this case, apart from the water molecule evaporation, some chemical reactions or initial processes of degradation of the macromolecules probably started to take place. This observation is valuable because it confirms the different type and structures, hence the different properties of the pectic polysaccharides present in the vegetative matrix and extracted by different extractants. A similar results about significant differences in the thermal behavior of laboratory-extracted and available commercial pectins and even between commercial pectins supplied by different producers (Einhorn-Stoll et al., 2014) was also observed. This, however, complicated to a certain extent the investigation of the properties of complex biopolymers but nevertheless, the information obtained by DTA-TG analysis is important for the examination of their structures and properties.

The TG curve in Fig. 1 (and dTG Is curve) leads to the conclusion that an inflection point (glass-transition temperature) exists for the four investigated pectic polysaccharides at which the polymeric (pectic) macromolecule changes the flexibility of its segments and hence its viscoelastic properties. In our previous X-ray diffraction investigations (Slavov et al., 2016) on pectic polysaccharides isolated from different plant wastes, the polysaccharides were found to have relatively low crystallinity levels. This glass-rubber transition is much more pronounced with lower initial degrees of crystallinity of the macromolecules. The glass-transition temperature of the investigated pectic polysaccharides was in the 70–75 °C range and the change in weight was 6–9% of the initial mass. This was confirmed from the dT/dt (dTG Is) curve which peaks match the endothermic peaks observed in the DTA curve (Heat flow Is Isc curve) and the area of the endothermic peak is proportional to the mass changes. The advantages of expressing the results with the

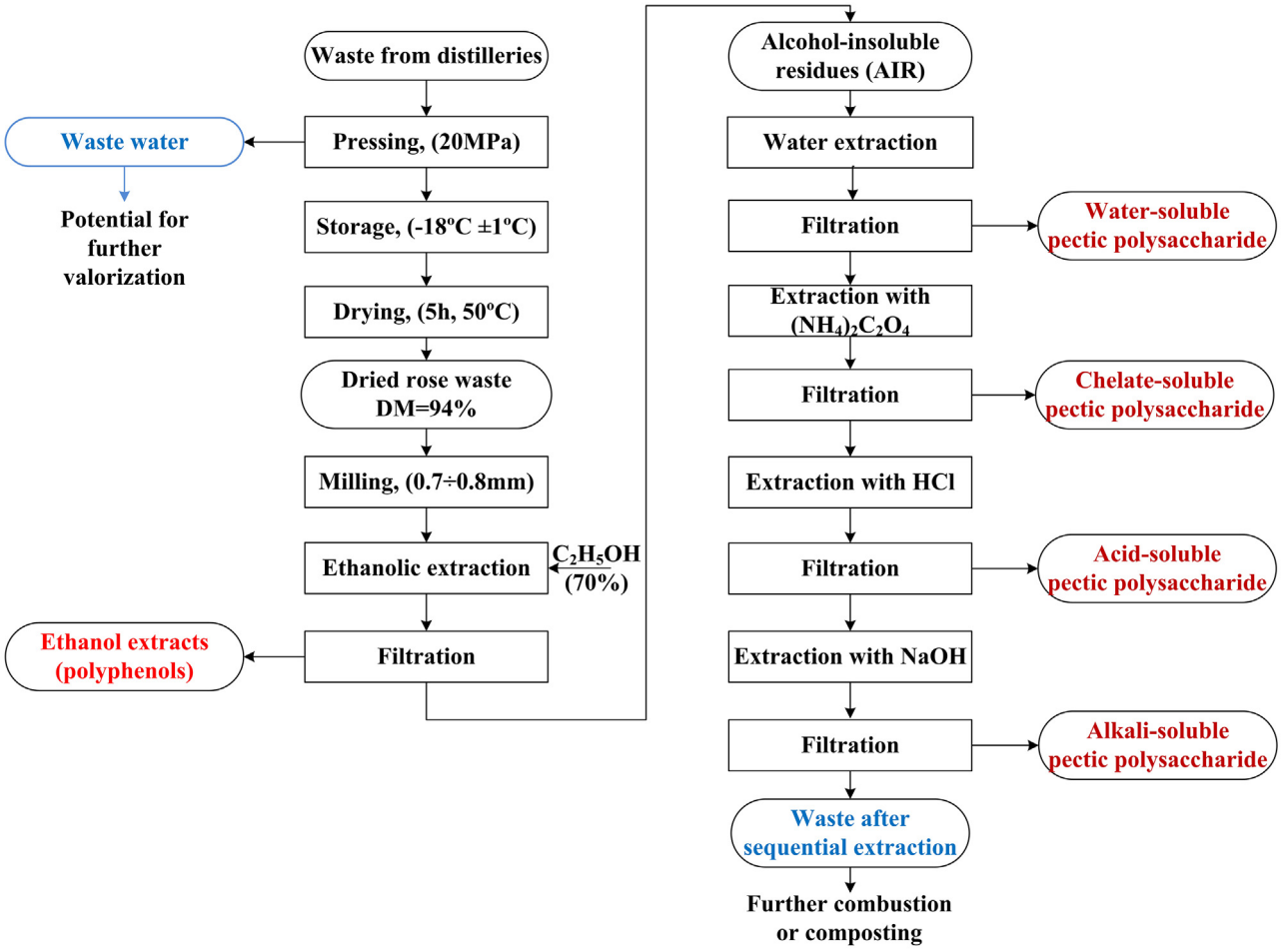


Fig. 2. Technological scheme for combined recovery of polyphenols and polysaccharides from waste rose biomass.

dTG Is curve simultaneously with the heat flow curve was that the dTG Is curve presents more accurately the processes of mass loss during heating of the samples.

While investigating the thermal stability of different pectic polysaccharides, Appelqvist et al. (1993), Einhorn-Stoll et al. (2007) and Einhorn-Stoll and Kunzek (2009) found that it was in the 220–240°C range. Data from the DTA-TG curves in the present study suggested that significant degradation of polysaccharide macromolecules started to take place around 220–230°C which was in agreement with previous studies on similar polysaccharides.

3.5. *Combined recovery of polyphenols and polysaccharides from waste rose biomass*

Several main approaches aiming at valorization of rose flower wastes have been described in the literature. The most commonly applied ones are the extraction of aroma substances, usually after enzymatic or chemical hydrolysis (Stefanov, 2016); the recovery of biologically active substances – flavonoids, polyphenols, polysaccharides, etc. (Mollov et al., 2007; Shikov et al., 2012, 2008; Slavov et al., 2016, 2013); the bio-sorption of pollutants (Iqbal et al., 2013; Rabbani et al., 2016); composting (Onursal and Ekinci, 2015; Tosun et al., 2008); and biogas production (Akgül et al., 2014). Based on

Table 6
Energy assessment and calculations of the average costs of combined recovery of polyphenols and polysaccharides from waste rose biomass.

Processes	For treatment of 1 kg waste (dry matter)[a]		For production of 1 kg polysaccharides (dry matter)[b]	
	Value	Energy costs, euro/kg[c]	Value	Energy costs, euro/kg[c]
Heating (120 °C), kJ/kg	66 862	1.22	267 448	4.88
Cooling (20 °C), kJ/kg	32 438	0.26	129 752	1.04
Freezing (−25 °C), kJ/kg	2780.4	0.05	11 121.6	0.20
Electricity, kWh/kg	9.23	0.75	36.9	3.0
Total, euro/kg	–	2.28[d]	–	9.12[d]

[a] 1 kg waste on average was obtained from 6.62 kg rose flowers.
[b] On average 4 kg waste rose biomass yielded 1 kg polysaccharides and 176 g polyphenols (on a dry basis).
[c] According Bulgarian energy market prices (November 2016).
[d] Calculations were made on the basis of assumption that 90% of ethanol used was distillated and reused.

the experimental data obtained in the present study, a technological scheme (Fig. 2) for combined recovery of biologically active substances from waste rose biomass was proposed. The approach includes an extraction of polyphenols with 70% ethanol (4.4% overall yield) and consecutive fractional extraction of polysaccharides (25.3% overall yield).

Based on the technological processes, the treatments of the waste rose biomass and average prices of energy and fuels in Bulgaria (for November 2016), calculations (according to Boustead and Hancock, 1979 and Schmidt, 1997) on the necessary energy and average cost of the operations were made and presented in Table 6.

According to the calculations treatment of 1 kg waste rose biomass would cost approximately 2.28 euro, and for the production of 1 kg polysaccharides and 176 g polyphenols – 9.12 euro. Further investigations on the potential for re-use of the waste waters (which remained after steam distillation of the fresh rose flowers) and the wastes after the consecutive fractional extraction of polysaccharides are needed and could also be included in the technological scheme for a more complete utilization of wastes from the rose oil industry.

4. Conclusions

The main advantage of the method employed consists in the combination of ethanolic pretreatment and consecutive fractional extraction of pectic polysaccharides. This approach allow for two valuable by-products to be simultaneously obtained as final products. The best results were achieved in polyphenol extraction with 70% ethanol. This pretreatment extracted the highest amounts of phenolic substances (4.4% overall yield) with pronounced antioxidant activity, while preserving the polysaccharides extracted further by consecutive fractional extraction (25.3% overall yield). The different types of pectic polysaccharides present in the waste rose biomass were isolated and characterized for the first time. As a result of the experiments, a technological scheme and energy assessment of the combined recovery of polyphenols and polysaccharides were proposed. Another advantage of this integrated approach is that it could further be combined with other methods and thereby achieve more complete utilization of the rose biomass and lower the discarded wastes.

Conflict of interest

The authors declare no conflict of interests.

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