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Integrated utilization of grape skins from white grape pomaces

Joana A.S. Mendes[a,b,c,d], Ana M.R.B. Xavier[a,b], Dmitry V. Evtuguin[a,b,*], Luísa P.C. Lopes[c,d]

[a] CICECO, University of Aveiro, 3810-193 Aveiro, Portugal
[b] Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal
[c] Environment Department, Polytechnic Institute of Viseu, 3504-510 Viseu, Portugal
[d] CI&DETS, Polytechnic Institute of Viseu, 3504-510 Viseu, Portugal

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ABSTRACT

An approach for the integrated utilization of grape skins from white grape pomaces has been proposed. This consists of consecutive or simultaneous extraction of grape skins with neutral organic solvent and water under reflux. Organic extract is a valuable raw material for the isolation of oleanolic acid. The aqueous extract (ca 50%, w/w) is composed of essentially hexoses and suitable for the high yield (till 51%) bioethanol production at a maximum specific cell growth rate (μ_{max}) of 0.29 h^{-1}. The remained extracted grape skins are the complex of structural polysaccharides embedded into cutinous matrix. Extracted grape skins were shown to be a prospective raw material for the production of low-density boards ($d \leq 0.40$) for insulation needs. The boards produced from grape skins and bind of 8% urea–formaldehyde resin revealed reasonable tensile strength (0.4 MPa) and a low thermal conductivity (0.09–0.12 W (m K)$^{-1}$) over a wide range of temperatures (40–200 °C).

1. Introduction

The fluctuating prices, the reduction of world reserves and the adverse environmental effects of oil consumption have led to increasing demand and the development of alternative materials and energy sources that can reduce or even replace the use of fossil resources. These trends are coherent with necessities to utilize diverse agricultural residues both for economic and environmental reasons. In particular, winemaking sector produces high amounts of under-utilised by-products, which have significant economic potential being an urged challenge (Spigno et al., 2008; Ping et al., 2011; Prozil et al., 2012). This is especially important for Europe producing around 65% of wine worldwide (Commission E., 2013).

The conjunction of wastes generated in the winemaking process (grape pomaces) is constituted of skins, pulp, stalks and seeds, which account to 25–35 kg per 100 L of produced wine (Costa, 1983). Grape skins are the major component of grape pomaces contributing roughly to a half of grape pomace matter. Grape skins may be used to some extend in animal feeds, but only few other applications are known (Costa, 1983; Arvanitoyannis et al., 2006). For example, the extractives of grape skins represented by classes of organic compounds such as polyphenols and triterpenic acids can have interesting pharmaceutical and nutritional applications (Hichri et al., 2003; Ruberto et al., 2007).

Red grapes are entirely involved in fermentation and processed skins contain much less pulp and residual sugars than the skins from white grapes that are mechanically pressed to produce juice and are not subjected to ethanolic fermentation (Silva, 2003; Ruberto et al., 2007). This is an attractive feature for the eventual utilization of residual sugars of white grape skins as substrate for bioprocessing. Recently, aqueous extract of white grape skins was successfully used for the production of bacterial cellulose (Carreira et al., 2011). The eventual application of residual sugars in grape skins for the production of so-called second generation (2G) bioethanol would be another attractive application. Nowadays, bioethanol is the most recognized biofuel alternative to petrofuels and produced essentially from food stuff (so-called first generation bioethanol) (Bai et al., 2008). Last decade, 2G bioethanol has been the focus of studies using lignocellulosic materials as a carbon source (Mabee and Saddler, 2010; Naik et al., 2010; Quintero et al., 2011; Dias et al., 2012). Unlike to first generation bioethanol, 2G bioethanol derived from non-food raw materials do not compete with food production and has a higher potential for sustainable production (Naik et al., 2010; Dias et al., 2012).

The analysis of chemical composition of grape skins shows that the main structural polysaccharides are embedded into the cuticle matter and remain inaccessible to acidic hydrolysis (Mendes et al., 2013). Hence the saccharification of grape skins either by enzymatic or by acidic hydrolysis is problematic. At the same time, the cuticle matter exhibits a chemical structure sound to cork material and could be proposed for the biocomposite applications. The use of grape skins for biocomposites was never reported yet though the economic and environmental benefits of the composites involving

* Corresponding author at: CICECO, University of Aveiro, 3810-193 Aveiro, Portugal.Tel.: +351 234 401 526; fax: +351 234 370 084.
E-mail address: dmitrye@ua.pt (D.V. Evtuguin).

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natural fibres as fillers or reinforcing agents are of great interest (Fowler et al., 2006; Crespo et al., 2008). Natural fibres, like sugar cane bagasse, bamboo, jute, kenaf, cotton, rice husk have already been used as a biodegradable fillers (Avérous and Le Digabel, 2006; Satyanarayana et al., 2009; Faruk et al., 2012). Furthermore, the use of natural fibres derived from agro-based materials could develop and improve the economy of the specific agricultural sector.

Considering aforementioned peculiarities of chemical composition of skins from white grape, the comprehensive approach for their utilization has been proposed. This includes consecutive or simultaneous extraction of grape skins with neutral non-polar organic solvent and water under reflux. Both organic and aqueous extracts were exhaustively characterized and the aqueous extract was subjected to bioethanol production. The extractives free grape skins were examined for the production of low-density particle boards. The practical yields of bioethanol and biocomposite for insulation needs were estimated.

2. Materials and methods

2.1. Raw materials

The pomace of destemmed white grape (mixed varieties) was supplied by Quinta do Serrado (Penalva do Castelo, Portugal), a sub-holding of Tavfer group, during the 2008 vintage. The most abundant collected grape varieties were *Encruzado*, *Cerceal*, *Bical*, and *Fernão Pires*. The material was collected, dried at 40 °C and the grape skins were manually separated from the wine pomace. The grape skins were further extracted consecutively by hexane (4 h in Soxhlet) and water (1 h under reflux, solid-to-liquid ratio 1:10) or simultaneously with a mixture of hexane and water (1:1, v/v) for 4 h under reflux (solid-to-liquid ratio 1:10). After extraction by hexane–water mixture, the solid residue was separated by filtration and the liquid phase was separated to organic and aqueous phases. The solid residue had been used for preparation of biocomposites. The aqueous extract (AEX) was submitted to acid hydrolysis under reflux for 1 h (pH 1 adjusted by diluted aqueous solution of H_2SO_4). After cooling, the pH of the resulting solution was adjusted to 5.5 with NH_4OH aqueous solution. The AEX was aerated with compressed air and filtered using a 0.45 μm cellulose nitrate filter (Sartorius, sterilized). The purified AEX was used as a substrate for the production of ethanol.

2.2. Analyses

Grape skins were analyzed for ash, extractives and cellulose content. The ash content was determined by calcination of the material at 525 °C, according to the standard procedure Tappi T 211 om-93. Extractives in dichloromethane or in hexane were isolated by a Soxhlet extraction for 4 h. Cellulose content in dichloromethane extractive-free samples was determined by Kürschner–Hoffer method with relative error of 5% (Browning, 1967). Proteins in extractives-free samples were determined by treatment with 1% pepsin solution in 0.1 M HCl at 37 °C for 16 h (Pascoal Neto et al., 1996) and tannins were analyzed in extractives- and protein-free samples by the treatment with 0.1 M NaOH at 100 °C for 1 h in nitrogen atmosphere (liquid-to-solid ratio 100), followed by hot water washing until neutral reaction of filtrates as described elsewhere (Mendes et al., 2013).

The neutral monosaccharides of the grape skins, obtained after the Saeman hydrolysis were analyzed as alditol acetate derivatives (Blakeney et al., 1983; Selvendran et al., 1979) by gas chromatography (GC), using a Varian 3350 chromatograph equipped with a capillary column DB-225 J&W (30 m × 0.25 mm internal diameter; 0.15 μm film thickness). The chromatographic conditions were the following: column temperature: 220 °C; injector temperature: 220 °C; flame ionization detector (FID) temperature: 230 °C. Before injection of the samples, calibration curves for rhamnose, fucose, arabinose, xylose, mannose, galactose and glucose were obtained using high purity (99.9%) commercial products (Sigma–Aldrich Chem. Co., Madrid). The sugars analysis in AEX was carried out by HPLC according to previously published procedure (Fernandes et al., 2012). The total amounts of reducing sugars in AEX were quantified by dinitrosalicylic acid method according to a known procedure (Miller, 1959).

SEC analyses of AEX, dissolved in ultra pure water with 0.1 M $NaNO_3$ and 0.02% NaN_3 to a concentration of 0.4–0.5% (4–5 mg mL^{-1}), were performed on a PL-GPC 110 system (Polymer Laboratories Ldt., UK) equipped with refraction index (RI) detector using two Plaquagel-OH MIXED 8 μm columns (300 mm × 7.5 mm). The temperature of the injector and the columns was kept constant at 36 °C. The eluent, ultra pure water containing of 0.1 M $NaNO_3$ and 0.02% NaN_3, was pumped at a flow rate of 0.9 mL min^{-1}. The SEC columns were calibrated using pullulan standards (Polymer Laboratories, UK).

About 10 mg of extractives soluble in dichloromethane or in hexane were trimethylsilylated (TMS) and analyzed by gas chromatography coupled with mass detector (GC–MS). Briefly, the sample was dissolved in 0.5 mL of pyridine containing tetracosane as internal standard and silylated by adding 250 μL of bis(trimethylsilyl)trifluoroacetamide and 50 μL of trimethylchlorosilane. The mixture was heated to 70 °C for 40 min and the derivatized products analyzed by GC–MS. Each sample was injected twice and the results were averaged. GC–MS analysis was carried out on a Trace Gas Chromatograph 2000 series coupled with a Finnigan Trace MS mass detector, employing a DB-1J & W capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness), using helium as carrier gas (35 cm/s). The chromatographic conditions were as follows: initial temperature – 80 °C for 5 min; temperature rate – 4 °C/min; final temperature – 285 °C for 10 min; injector temperature – 290 °C; transfer line temperature – 290 °C; split ratio – 1:75. Compounds were identified as TMS derivatives by comparing their mass spectra with those in GC–MS spectral library or with data from the literature and in some cases, by injection of standards.

2.3. Fermentation assays

Saccharomyces cerevisiae 4072 was provided by the Portuguese Yeast Culture Collection on agar tubes. The culture was transferred to yeast medium (YM) agar plates, containing 20 g of agar, 10 g of glucose, 3 g of yeast extract and 3 g of malt extract per litre of distilled water and maintained at 4 °C, after grown at 28 °C ± 0.5 °C during 3 days. The inocula for the fermentations were prepared with one colony of the yeast strain reactivated in YM liquid medium and incubated at 28 °C during 15 h.

Fermentations of 250 ml working volume were carried out with 70% (v/v) of ennobled AEX, 10% (v/v) of supplementary medium (SM1 or SM2) and 20% of inoculum in Erlenmeyer flasks of 500 mL incubated at 180 rpm and 28 °C in an orbital incubator.

SM1 was used for *fermentation 1*, contained 2.5 g L^{-1} yeast extract, 2.0 g L^{-1} $(NH_4)_2HPO_4$ and 1.0 g L^{-1} $(NH_4)_2SO_4$ and $MgSO_4 \cdot 7H_2O$ and SM2 for *fermentation 2*, containing the same supplement except yeast extract that was not added. Sterile samples were taken during time for analysing biomass sugars and ethanol concentrations.

Biomass was determined by monitoring of optical density at 620 nm (OD^{620}) and using a calibration curve of OD^{620} versus biomass dry weight. Sugars and ethanol concentrations were analyzed by high-pressure liquid chromatography (HPLC) as described previously (Xavier et al., 2010).

2.4. Biocomposites preparation and mechanical characterization

Biocomposites were prepared using hexane and water extractive free grape skins and 8% (w/w) of urea–formaldehyde resin with 1% of ammonium sulphate added as catalyst, using a hydraulic press with electrically heated plates (Carver). The samples were compression-moulded into panels and the density of the board was controlled by a mould with fixed volume. The target density of the panels was ca 350–400 kg/m^3 with dimensions of 10 cm × 10 cm × 0.8 cm. The pre-formed mixture of grape skins and resin was pre-heated at 210 °C during 2 min, pre-pressed for 1 min and, after the pressure relief for 30 s to liberate the vapours, pressed again under the same conditions for 3 min. After pressing, panels were removed from the hot press and cooled at room temperature.

Bending strength f_m (Eq. (1)) was assessed according to NP EN 310:2000 norm on a testing machine Servosis ME-405/5 using 10 cm × 5 cm × 0.8 cm plates:

$$f_m = \frac{3F_{max}l}{2bt^2} \quad (1)$$

where F_{max} is the load to rupture (N), l is the distance between two support points (mm), and b and t are the width and thickness of tested plates (mm), respectively.

The water absorption (WA) and thickness swelling (TS) were determined after soaking of boards in water (25 °C, 24 h) adapting to ASTM D 1037-93. The thermal conductivity of the samples was measured on a thermal conductance tester (C-Matic Thermal Conductance Tester, TCHM-DV), according to ASTM C-518-91 and ASTM E 1530-93 standard procedures, using 0.8 cm thick circular plates (Ø = 5 cm).

3. Results and discussion

3.1. Grape skins characterization

The general chemical composition of skins from white grape pomace (mixed varieties) is presented in Table 1. Major constituents were inorganic compounds (18.3%), cellulose (12.5%) and water-soluble compounds (48.0%). The compounds soluble in dichloromethane and in hexane accounted to 2.4% and 1.3%, respectively. The content of proteins and tannins were ca 7 and 3%, respectively. Hence, by misbalance, the content of other constituents (structural hemicelluloses, cuticle matter and waxes) was about 9%. It may be concluded that the amount of water-soluble compounds in white grape skins was roughly twice than that reported in red grape skins, whereas the amount of structural polysaccharides (cellulose and hemicelluloses) was substantially lower (almost half) (Mendes et al., 2013). In addition, white grape skins contained almost half amounts of proteins and tannins of the red grape skins (Table 1).

Table 1
Chemical composition of grape skins.

Components	Rel. abundance, % dry sample
Ash	18.3
Extractives	
Dichloromethane	2.4
Hexane	1.3
Water	48.0
Cellulose (*Kürschner–Hoffer*)	12.5
Proteins	6.7
Tannins	3.4
Composition of neutral sugars	
Rhamnose	1.2
Fucose	0.4
Arabinose	3.1
Xylose	7.2
Mannose	4.2
Galactose	33.1
Glucose	50.8

The analysis of neutral sugars in grape skins was carried out after their previous extraction with dichloromethane (Table 1). Among hemicelluloses, arabinogalactan was suggested to be the most abundant by analogy with polyoses composition from cell wall materials of white grape skins reported previously (Lecas and Brillouet, 1994).

3.2. Characterization of extractives

Grape skins were subjected to consecutive extraction by hexane and water and the corresponding extracts analyzed by a set of chromatographic and mass spectrometry techniques. Thus, hexane extractives were analyzed by GC–MS according to previously developed methodology (Mendes et al., 2013). Total Ion Chromatogram (TIC) and identified compounds are presented in Supplementary data (Fig. A and Table A). Four main classes of extractives were clearly distinguished: fatty acids, higher alcohols, sterols and terpens/terpenoids (Fig. 1). Moreover, fatty acids and triterpenoids were predominant. Octacosanoic (0.46 mg g^{-1} of grape skins), hexadecanoic (0.37 mg g^{-1} of grape skins) and 9,12-octadecadienoic acid (0.38 mg g^{-1} of grape skins) were the most abundant among detected fatty acids. The most abundant sterol was β-sitosterol (0.47 mg g^{-1} of grape skins), whereas the most abundant terpenoid in hexane extract was oleanolic acid with 1.09 mg per gram of grape skins.

The use of dichloromethane instead of hexane in extraction of grape skins allowed not only to increase the extractives yield but also to change the proportions among different classes of extractives (Fig. 1). Thus, the relative proportion of triterpenoids increased significantly, especially the oleanolic acid (11.1 mg g^{-1} of grape skins).

Oleanolic acid, a related compound to betulinic acid, is known as a precursor for various drugs (antiviral activity, including anti-VIH, anti-stress, anti-inflammatory, anti-tumoral, etc.) and may be considered as a promising added value product (Hichri et al., 2003; Liu, 2005; Pollier and Goossens, 2012). According to preliminary results obtained on the purification by crystallization from hot dichloromethane solution, a purity of oleanolic acid of 85% was achieved what is a promising value. The isolation of this compound in its pure form could be applied for the valorization of extractives from grape skins.

The analysis of water-soluble extractives by SEC (Fig. 2) revealed that about 90% of dissolved compounds were of low molecular weight (<450 Da, fraction 4) and nearly 10% were removed from skins in polymeric form (fractions 1–3). Grape skins contain notable amounts of pectins and glucomannans soluble in water (Lecas and Brillouet, 1994; Prozil et al., 2012) and this presence in extract of

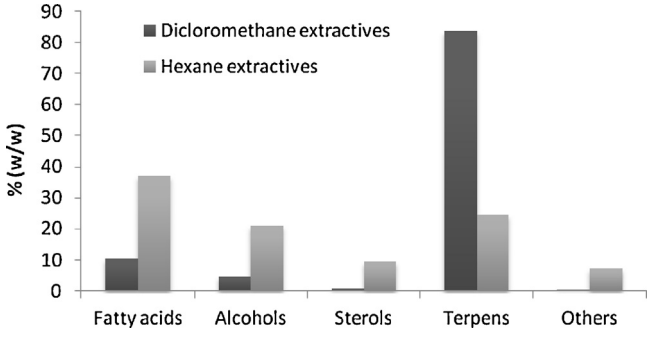

Fig. 1. Major families of extractive compounds identified in dichloromethane and hexane extracts from grape skins.

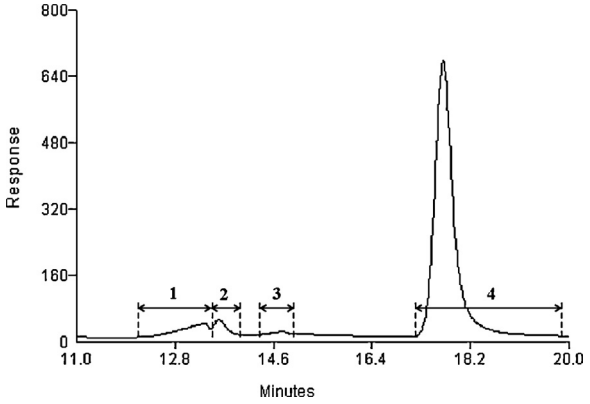

Fig. 2. SEC elution curve of aqueous extract from grape skins.

high molecular weight polysaccharides (40–500 kDa) is not surprising. The eventual contribution of proteins to water extract is plausible because proteins contribute to ca 7 wt.% of grape skins (Table 1). HPLC analysis of aqueous extract showed a set of free monomeric sugars, essentially hexoses: (galactose (46.2%), glucose (43.4%), fructose (5.5%), arabinose (3.5), mannose (1.1%) and xylose (0.3%). Hence the aqueous extract of white grape skins is a valuable hexose-containing substrate for the yeast fermentation into ethanol. The potential of aqueous extracts for the ethanol production was examined using *S. cerevisiae.*

Interestingly, the simultaneous extraction of grape skins by water–hexane (1:1, v/v) mixture under reflux followed by separation of aqueous and organic phases revealed very similar yields of sugars and extractives as was detected after consecutive extraction by hexane and water. Therefore, the extraction of grape skins can be carried out in one step with mixture of water and hexane. However, in this case, the aqueous phase should be liberated from the partially dissolved hexane by stripping in order to recover the organic solvent and to permit the subsequent biochemical processing of sugar's extract.

3.3. Fermentation assays

Fermentation assays were made by *S. cerevisiae* 4072 using the aqueous extract from white grape skins that was previously submitted to a brief acidic hydrolysis after adjusting pH to 1 with H_2SO_4. With this weak hydrolysis oligo-sugars were converted in monosaccharides, the suitable sugars for microbial fermentations. Before starting fermentations, in order to provide good conditions for alcoholic fermentation, pH was adjusted to 5.5 and a medium supplementation was made by salts with or without yeast extract. Yeast extract is a source of nitrogen, vitamins and enzymatic cofactors usually used with sugars in order to accelerate metabolic reactions.

The results of fermentation 1(*ferm 1*) with yeast extract and 43 g L^{-1} of sugars can be compared with results from fermentation 2 (*ferm 2*), without yeast extract and with 45 g L^{-1} of sugars, in Fig. 3. This shows a typical time coarse for a batch process with a short lag phase followed by a long exponential phase and the beginning of the stationary phase. In both cases monosaccharides were successfully converted to ethanol in 12–15 h and the remaining content (ca 3 g L^{-1}) that could not be consumed certainly was xylose, a pentose that *S. cerevisiae* is not able to metabolize. The addition of yeast extract as micronutrients supplement (*ferm 1*) led to a higher ethanol production (22.0 g ethanol L^{-1}) when compared to *ferm 2* without addition of yeast extract (Table 2). Maximum specific growth rate (μ_{max}) in *ferm 1* was 0.29 h^{-1} lower than in *ferm 2* (0.40 h^{-1}) meaning that growth was more significant in this

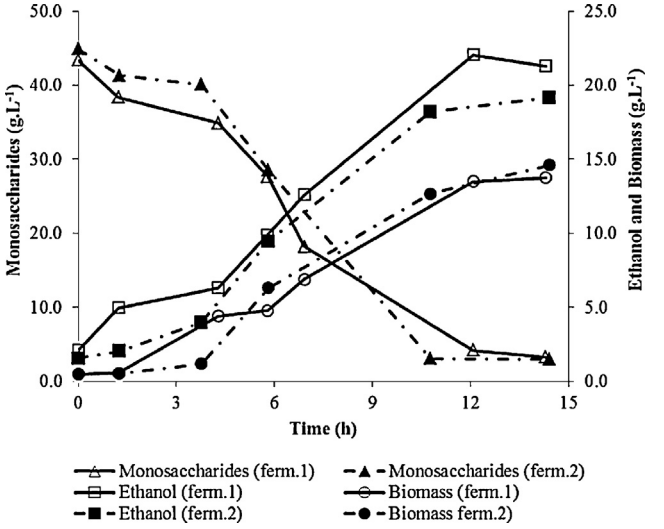

Fig. 3. Time course of aqueous extract from grape skins alcoholic fermentation into ethanol. Medium supplementation was prepared with salts and yeast extract (Ferm 1) or with salts and without yeast extract (Ferm 2).

Table 2
Results of fermentation of aqueous extracts from grape skins.

Parameters	Fermentation 1	Fermentation 2
Initial substrate (g L^{-1})	43.4	44.9
Final substrate (g L^{-1})	3.3	3.0
Initial biomass (g L^{-1})	0.48	0.48
Final biomass (g L^{-1})	13.8	14.6
Ethanol concentration, max (g L^{-1})	22.0	19.1
Max. specific growth rate, μ_{max} (h^{-1})	0.29	0.40
Uptake rate, r_s (g L^{-1} h^{-1})	2.81	2.91
Biomass grow rate, Q_p (g L^{-1} h^{-1})	1.34	1.22
Yield of biomass, $Y_{X/S}$ (g g^{-1})	0.33	0.34
Yield of ethanol, $Y_{P/S}$ (g g^{-1})	0.51	0.42

ferm 2. Alcoholic fermentation metabolism was more developed in *ferm 1* and respiration growth metabolism was more developed in *ferm 2*. Ethanol productivity (1.34 g ethanol L^{-1} h^{-1}) and yield (0.51 g ethanol per gram of sugar) were higher in *ferm 1* in relation to *ferm 2* where ethanol productivity was 1.22 g ethanol L^{-1} h^{-1} and yield was 0.42 g ethanol per gram of sugar (Table 2). In *ferm 1* the theoretical yield for ethanol production was attained, hence it may be concluded that the addition of yeast extract to AEX allowed the shift of metabolic pathways from respiration/cell growth to the fermentative one. These results show that there is a slight advantage in adding yeast extract to the fermentation medium but even without yeast extract addition good results were obtained for ethanol production, since some micronutrients could come to this aqueous extract directly from grape skins. Taking into account the obtained ethanol yield and the sugars fermentation efficiency (0.95), the estimated production per 1 tonne of absolutely dry grape skins could attain nearly 245 kg or 310 L of ethanol.

3.4. Biocomposites from grape skins

Extractives-free grape skins (EFGS) have a rough yield of ca 50% and composed of structural polysaccharides (cellulose and hemicelluloses) embedded into the cuticle matter. Due to certain similarity of cuticle matter to cork material, the former was examined in biocomposite for insulation needs. If the necessity of grape skins extraction with organic solvent is optional, their extraction with water prior to produce insulation boards is highly desirable to fulfil the requirements related to moister and biological resistance of final biocomposites.

Table 3
Properties of low density boards produced from extracted grape skins.

Raw material	Density (kg m^{-3})	Bending strength (MPa)	WA (%)	TS (%)	Thermoconductivity, W (m K)$^{-1}$ [a]
Extracted grape skins	380	0.4	85.2	17.4	0.094

[a] 45 °C.

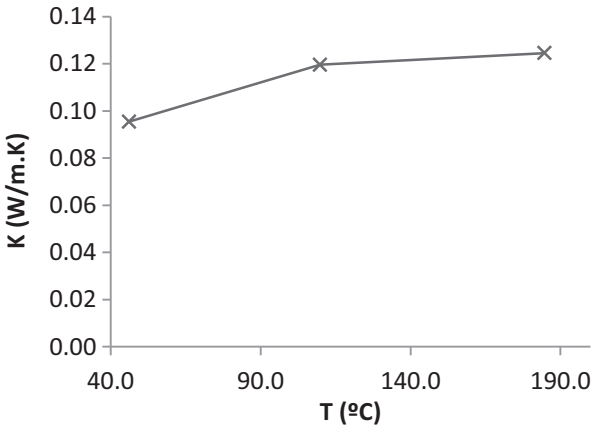

Fig. 4. Thermal conductivity of low density boards produced from grape skins.

The boards of low density (ca 0.38 g cm^{-3}) were prepared from EFGS by hot pressing in mould using an industrial urea–formaldehyde (UF) resin as a binder. The load of UF resin was 8% of based on dry board weight. Any hydrophobic additives were used. The produced boards were evaluated for mechanical resistance (bending strength), dimensional stability (water absorption (WA) and thickness swelling (TS) under soaking in water) and insulation capacity (thermal conductivity). In general, bending strength of obtained boards was moderate, when compared to that reported for low density particleboards from agricultural residues (ca 1 MPa) with the same resin (Heslop, 1997), but revealed rather good dimensional stability under humidification, e.g., the TS was 17.4% and WA of 85.2% (Table 3). For WA and TS indices, boards produced from grape skins were better than reported for boards from other agricultural residues such as kenaf core (Xu et al., 2004) or wheat straw (Heslop, 1997) and for tall wheatgrass (Zheng et al., 2007). The last fact could be explained by the presence of relatively high amounts of hydrophobic cuticle matter in grape skins (Mendes et al., 2013). Worth noting that the mechanical properties of insulation boards can be enforced, if necessary, by their laminating with appropriate material (e.g., veneer, high pressure laminates, etc.).

Boards prepared from extracted grape skins were examined for thermal conductivity in a wide range of temperatures (40–200 °C) (Fig. 4). The thermal conductivity was of 0.09 W (m K)$^{-1}$ at 40 °C, which is in the range of those reported for the commercial insulating materials, such as cork and polymeric foams, that usually vary between 0.04 and 0.16 W (m K)$^{-1}$ (Sequeira et al., 2009). In addition, thermal conductivity did not deteriorate at temperatures as higher as 100 °C, which is an important characteristic for insulation materials. Taking into attention the yield of grape skins after the extraction with water (ca 50%) and the density of the insulation boards (ca 0.4), one tonne of absolutely dry grape skins can yield about 1.25 m^3 of thermal insulation boards.

4. Conclusions

The comprehensive utilization of grape skins from white grape pomaces by either simultaneous or consecutive extraction with hexane and water has been proposed. The organic extract contained high amounts of valuable oleanolic acid. Almost half of grape skins matters are water-soluble hexoses easily fermentable into bioethanol with a high fermentation rate. The extracted grape skins were revealed to be a prospective raw material for the production of low density boards suitable for insulating needs. Roughly up to 11 kg of oleanolic acid, 250 L of ethanol and 1.25 m^3 of low density boards can be produced from one tonne of grape skins.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.indcrop.2013.05.003.

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