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Triterpenic and Other Lipophilic Components from Industrial Cork Byproducts

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The detailed chemical composition of the lipophilic extractives of cork and cork byproducts generated throughout industrial processing has been investigated by gas chromatography−mass spectrometry. Triterpenes (cerine, friedeline, and betulinic acid) were the major components detected. Betulinic acid is the main triterpene (11.7g/kg) identified in industrial cork powder, whereas in black condensates friedeline (95.3g/kg), betuline (13.1g/kg), and betulinic acid (12.1g/kg) are the main triperpenes. Significant fractions of α-hydroxy fatty acids (115.1g/kg) and α,ω-dicarboxylic acids (21.2g/kg) were also detected in black condensate after alkaline hydrolysis. The results demonstrate that these two industrial byproducts can be considered as promising sources of bioactive chemicals or chemical intermediates for the synthesis of polymeric materials.

KEYWORDS: ***Quercus suber*** **L. cork; cork byproducts; industrial cork powder; black condensate; lipids; triterpenes**

INTRODUCTION

Quercus suber L. is a native species of the Mediterranean region yielding a thick bark, commonly known as cork, used mainly in the production of wine stoppers and agglomerates for acoustic and thermal insulation. Portugal produces ≈185 000 tons/year of cork (2000), representing >50% of the world production (*1*).

Industrial cork processing generates substantial amounts of residues such as the so-called "cork powder", "black condensate", and "cooking wastewaters". The cooking of cork planks in boiling water is a key stage in wine stopper production, yielding cooking wastewaters as liquid effluent. Cork powder is generated mainly during the production of granulated cork for agglomerated materials. Cork residues enriched in the outer and inner surfaces of cork planks, rejected during cork stopper production, are one of the main sources of granulated cork materials. The cork powder particles have an inadequate size distribution to be used in the manufacture of agglomerates and are mainly burned to produce energy (*2*, *3*). Yearly, this byproduct represents ≈40000 tons (≈22% of the total cork production).

Black condensate is a residue of the production of black agglomerates, which involves the treatment of cork particles, without any adhesive, at temperatures in the range of 250−500 °C. During such thermal treatment of cork, vapors are formed and later condensed in autoclave pipes. Periodically, this byproduct is removed (2500 tons/year) and burned to produce energy (*2*).

In this context cork and cork byproducts can be seen as potential renewable sources of chemical commodities, particularly when the rapid depletion of fossil resources and the consequent need of sustainable development of world economy are considered. The search for new applications of cork byproducts is particularly attractive within the scope of the biorefinery concept in forest-based industries; the concomitant upgrading of all the byproducts generated during wood and forest products processing represents logical issues within this situation (*4*). Obviously, the development of new applications for cork byproducts requires a detailed knowledge of their composition, which in some aspects is still far from being completely assessed.

Natural cork is essentially composed of suberin (the main component), lignin, polysaccharides, and extractives (see, e.g., refs *5*−*13*). The relative abundance of these fractions is extremely variable, as influenced by geographical origin, quality, or even the different parts of the tree from which the cork was obtained (see, e.g., refs *5*, *11*, and *13*).

Cork extractives are mainly composed of aliphatic, phenolic, and triterpenic components. The triterpenic fraction of cork extractives (**Figure 1**) contains essentially friedeline, cerine, betuline, betulinic acid, smaller amounts of sterols such as β-sitosterol (*10*−*16*), and also a minor fraction of other friedelane and lupane type components (*17*, *18*). Although friedeline and cerine are in general identified as the main triterpenic compounds, the abundance and composition of this fraction is also highly variable (see, e.g., refs *5*, *11*, and *13*).

The abundance of some of these triterpenes in cork, together with their promising applications, directly or as precursors of bioactive components for biomedical applications (*19*−*22*), has

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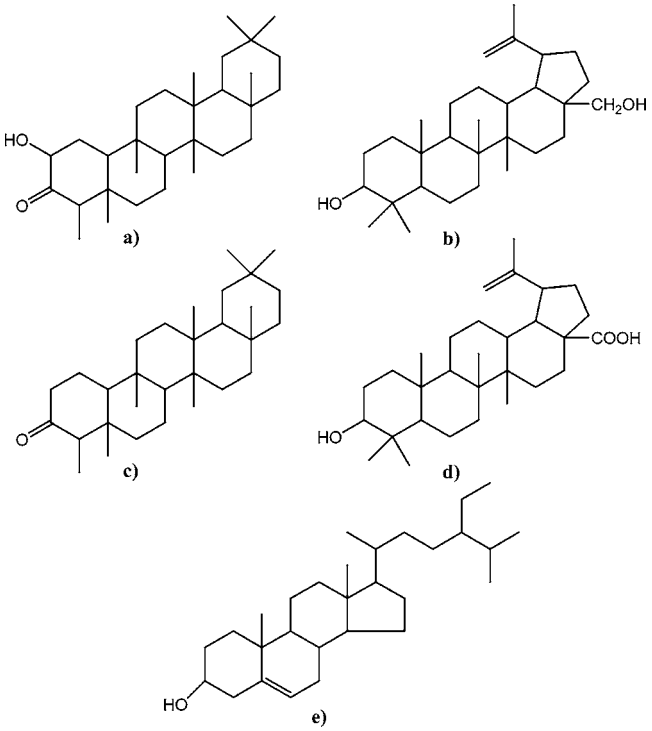

Figure 1. Major triterpenes identified in the cork samples: (**a**) cerine; (**b**) betuline; (**c**) friedeline; (**d**) betulinic acid; (**e**) β-sitosterol.

prompted our interest in studying its abundance in industrial cork byproducts.

Although there is a significant amount of work focused on cork composition (see, e.g., refs *7*−*9*), including a few studies on the composition of cork plank extractives (triterpenic and phenolic) throughout the industrial processing (*10*−*13*), there is a gap in what concerns the cork byproducts.

In the present paper a detailed study on the GC-MS analysis of the lipophilic extractives, and particularly the triterpenic fraction of cork industry byproducts, namely, industrial cork powder and black condensates, is presented. To understand the different composition of industrial cork powder, a comparative study with natural and boiled cork planks and their corresponding planar surface fractions was carried out.

MATERIALS AND METHODS

Samples. Industrial cork powder (ICP) was sampled in Corticeira Amorim mill, (Portugal, February 2005); black condensates (BCond) were sampled in Amorim Revestimentos mill (Portugal, November 2004).

Q. suber L. cork planks ("amadia" grade) were sampled from the southern part of Portugal (Herdade da Moinhola, Amorim Florestal mill, Portugal, March 2005). These included natural cork (WNC) planks after a resting period in the field and boiled cork (WBC) planks obtained after a cork cooking stage.

WNC and WBC cork planks were handily cut to isolate the corresponding inner (INC and IBC, respectively), 3−5 mm thick, and outer (ONC and OBC, respectively), 5−10 mm thick, surface fractions.

Solid samples (cork and BCond) were milled in a Retsch cross-beater mill SK1 (Haan, Germany), and the granulometric fraction of 40−60 mesh was used for analyses.

Extraction. Solid samples (≈20 g) were sequentially Soxhlet extracted with dichloromethane (DCM), methanol (MeOH), and water during 10 h for each solvent. Each sample was extracted in triplicate.

Alkaline Hydrolysis. To evaluate the presence of esterified structures in cork samples (INC, IBC, ONC, OBC, WNC, and WBC), ICP, and BCond, 20 mg samples of DCM extracts were dissolved in 0.5 M NaOH in 50% aqueous methanol and heated at 100 °C, under nitrogen atmosphere, for 1 h. The reaction mixture was cooled, acidified with 1 M HCl to pH 2, and extracted three times with DCM, and then the solvent was evaporated to dryness (*23*).

Table 1. Extractive Yields (Weight Percent) of Cork Industry Byproducts, Natural Cork, and Boiled Cork and Respective Inner and Outer Fractions[a]

	cork byproducts		natural cork			boiled cork		
solvent	ICP	BCond	WNC	INC	ONC	WBC	IBC	OBC
DCM	2.6	91.9	3.6	4.2	1.9	4.7	5.0	2.7
MeOH	1.9	5.5	1.7	2.8	1.5	2.2	3.6	1.8
water	1.4	0.3	3.7	2.1	1.4	1.1	2.6	1.1
total	5.9	97.7	9.0	9.1	4.7	8.0	11.2	5.7

[a] Each value is the average of three extractions with variation coefficients within 2.5–4.5%.

Sample Derivatization. Prior to GC-MS analysis, ≈20 mg of each sample was trimethylsilylated as described before (*23*): the residue was dissolved in pyridine (250 μL), and components containing hydroxyl and carboxyl groups were converted to their TMS ethers and esters, respectively, by adding bis(trimethylsilyl)trifluoroacetamide (250 μL) and trimethylchlorosilane (50 μL). After the mixture had stood at 70 °C for 30 min, the TMS derivatives were analyzed by GC-MS.

GC-MS Analysis. GC-MS analyses were performed using a Trace GC 2000 gas chromatograph, connected to a mass selective detector, Finnigan Trace MS, using helium as carrier gas (35 cm s^{-1}) and equipped with a DB-1 J&W capillary column (30 m × 0.32 mm i.d., 0.25 μm film thickness). The chromatographic conditions were as follows (*23*): isothermal at 80 °C for 5 min, ramped from 80 to 285 °C (4 °C min^{-1}), and then isothermal at 285 °C for 15 min; injector temperature, 250 °C; transfer line temperature, 285 °C; split ratio, equal to 1:50. The MS was operated in the electron impact mode with an electron impact energy of 70 eV and collected data at a rate of 1 scan s^{-1} over a range of *m*/*z* 33−800. The ion source was maintained at 200 °C.

For quantitative analysis GC-MS was calibrated with pure reference compounds representative of the major classes of components present in the extracts (stigmastanol, oleanolic acid, betulinic acid, palmitic acid, behenic acid, and ellagic acid), relative to the internal standard, *n*-tetracosane. The respective multiplication factors needed to obtain correct quantification of the peak areas were calculated as an average of six GC-MS runs.

Quantitative results were obtained as the average of four concordant injections. Identification of chromatographic peaks was based on the equipment's spectral library and also on comparison with previously published data, reference compounds, ion fragmentation patterns, and/or retention times according to the Results and Discussion.

Chemicals. Palmitic acid (99% purity), behenic acid (99% purity), stigmastanol (97% purity), betulinic acid (90% purity), *n*-tetracosane (99% purity), oleanolic acid (97% purity), and ellagic acid (97% purity) were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain).

RESULTS AND DISCUSSION

Cork and Cork Byproduct Extractives Content. The extraction yields of the studied samples are shown in **Table 1**. The yields found for ICP are significantly lower that those observed for cork samples (WNC and WBC), whereas these latter ones are within the typical values found for "amadia" grade cork (see, e.g., ref *5* and *7*). To understand this observation (and also the differences in chemical composition discussed below) and considering that ICP is enriched in the outer and inner surface fractions of cork planks, rejected during cork stopper manufacture, the extraction yields and composition of these surface fractions were analyzed. As can be seen from **Table 1**, the extraction yields of the outer surface fraction of cork (either

Table 2. Major Components (Milligrams per Kilogram) of Industrial Cork Powder (ICP) and Cork in the DCM Extract and Total (DCM and MeOH)[a]

	ICP		WNC		WBC		INC		ONC		IBC		OBC	
	DCM	total	DCM	total	DCM	total	DCM	total	DCM	total	DCM	total	DCM	total
betulinic acid	9860	11719	2196	2234	1177	1230	5037	5203	1571	1621	1457	1496	1884	1936
cerine	1886	2060	4635	4648	6083	6144	7328	7542	2468	2703	7809	7942	1919	1938
friedeline	1792	2009	2308	2323	2684	2745	3554	3829	1100	1370	4592	4959	1732	1778
betuline	764	875	324	335	331	343	446	481	464	492	417	435	358	371
β-sitosterol	254	254	539	539	778	778	648	648	247	247	916	916	244	244
ursolic acid		104	130	130	tr	17	tr	25	tr	35	tr	tr	tr	20
lupeol		60		71	tr	67	tr	64	tr	0	tr	43	tr	20
total triterpenes	14555	17081	10132	10289	11053	11323	17013	17791	5850	6468	15192	15791	6136	6307
ellagic acid		1347		1222		1513		2784		1211		5852		1110
total	15065	19254	13658	15065	12358	14212	18278	19685	6298	8173	16054	22587	7146	8441

[a] Each value is the average of four injections with variation coefficients within 1.1–5.0%.

ONC or OBC) are significantly lower than those found for whole cork and similar to those reported for ICP (although, as will be described below, the compositions of these two fractions are significantly different). The lower extraction yields of the outer surface fraction of cork can be due to the high environmental exposure of these outer surface fractions, which, certainly, promotes degradation and removal of cork components from the outer surface. On the other hand, the inner cork fractions (INC and IBC) tend to have slightly higher extraction yields than the corresponding cork samples, indicating a higher concentration of extractives in the inner surface of cork.

It is also worth mentioning that DCM and MeOH yields for boiled cork (and fractions) are apparently higher than those of its natural cork counterparts; this is due to the loss of water-soluble extractives during the boiling stage.

Finally, the DCM extraction yield of black condensate (BCond) is ≈92%, and the overall extraction yield is ≈98%. The high extraction yields found are consistent with the volatile nature of this fraction.

Chemical Composition of Cork and Cork Byproduct Lipophilic Extractives. The chemical composition of cork and cork byproduct extracts is presented in **Table 2** (cork, cork fractions, and ICP) and **Table 3** (black condensates).

From a qualitative point of view, the compositions of all cork, cork fractions, and ICP extracts are quite similar and mainly made up of triterpenic compounds, followed by smaller amounts of fatty acids, aliphatic alcohols, and phenolics. In addition to these families, considerable amounts of long-chain ω-hydroxy- and α,ω-dicarboxylic acids and phenolics in esterified forms were identified in BCond extractives.

Cerine, friedeline, and betulinic acid (**Figure 1**) are the dominant components identified. Smaller amounts of other triterpenoids such as betuline, β-sitosterol, ursolic acid, and lupeol were also identified. Triterpenic compounds were identified by systematic interpretation of their mass spectra and also by their elution order.

For friedeline, the most important fragments are found at *m*/*z* 426, corresponding to $[M]^+$, at *m*/*z* 411 $[M - CH_3]$, at *m*/*z* 341 associated with ring A cleavage, at *m*/*z* 302 and 273 involving the fragmentation of ring D, and, finally, at *m*/*z* 205 arising from the fragment containing A and B rings (*16*, *24*, *25*). The mass spectra of cerine TMS derivative is also characterized by the typical fragments of saturated friedelane skeletons (*24*): at *m*/*z* 341, 302, 273, and 205; additionally, the molecular ion at *m*/*z* 514 and the fragment at *m*/*z* 73 are also observed as prominent peaks. The TMS derivative of betulinic acid presents a fragmentation pattern typical of a saturated lupane skeleton, involving ring C cleavage (*24*, *26*), with an important fragment at *m*/*z* 189 and others at *m*/*z* 279, 292, and 320. The molecular ion at *m*/*z* 600 and fragments resulting from the loss of methyl and the isopropyl units, respectively, at *m*/*z* 585 and 557, are also observed. The identification of betulinic acid was further confirmed by injecting a reference sample. Betuline shows fragmentation patterns similar to those described for betulinic acid, and its identification was also confirmed by comparison with published data (*26*).

The identification of the β-sitosterol TMS derivative was based on both the characteristic fragmentation ions and the literature data (*27*, *28*). The molecular ion at *m*/*z* 486 $[M^+]$ and the related peaks at *m*/*z* 471 $[M - 15]$, *m*/*z* 396 $[M - 90]$, corresponding to the 1,2-elimination of the trimethylsilanol group, and *m*/*z* 381 $[M - 90 - 15]$ are observed. The mass spectrum of β-sitosterol also shows characteristic fragments of 3-hydroxy-Δ^5-sterols at *m*/*z* 129 and at *m*/*z* 357 $[M - 129]$, derived from the cleavage of ring A through C3−C4 and C1−C10 bonds (*29*).

All of the identified triterpenes have been previously reported as cork components (*13*, *15*, *16*).

The quantitative analysis of the extracts revealed that, regardless of the extraction time, the extraction of triterpenes with DCM was incomplete, particularly in the case of ICP samples, in which significant amounts of these components were still detected in the MeOH extract. Therefore, **Table 2** presents the abundances of the studied components in DCM and the total abundance (DCM plus MeOH). Although all studied cork and ICP DCM extracts contain the same major components, they differ significantly in the relative abundance of individual compounds, especially in betulinic acid. In fact, whereas in the studied natural (WNC) and boiled cork (WBC) samples cerine (1.9−7.9 g/kg) is generally the major component, followed by friedeline (1.4−5.0 g/kg) and betulinic acid (1.2−5.2 g/kg), in the case of ICP, betulinic acid is the major component (11.7 g/kg), representing >68% of the triterpenic fraction.

From **Table 2** it can be observed that whole cork samples (WNC and WBC) together with the inner surface fractions (INC and IBN) show higher amounts of triterpenes than the outer surface (ONC and OBC), which is in agreement with the extraction yields presented in **Table 1**. On the other hand, cooked samples (WBC, IBC, and OBC), when compared with the corresponding natural fractions (WNC, INC, and ONC), tend to show comparable amounts of triterpenes but, again, it should be mentioned that, if corrected for the loss of water-soluble extractives, an effective decrease in the amount of extractives would be generally observed.

Table 3. Composition of the DCM Extractives of the Black Condensate (Grams of Compound per Kilogram of Dry BCond) before (BH) and after (AH) Alkaline Hydrolysis[a]

compound	BH	AH
aliphatic alcohols	8.08	94.78
C(18:0)		0.80
C(20:0)	0.09	3.25
C(22:0)	2.78	30.56
C(24:0)	4.18	41.55
C(25:0)		3.45
C(26:0)	1.04	15.18
fatty acids	7.31	88.45
C(9:0)		1.61
C(16:0)	0.07	1.85
C18:0)		0.79
C18:1)		2.69
C(18:2)		1.10
C(20:0)	tr	1.86
C(22:0)	2.50	25.55
C(23:0)		1.61
C(24:0)	4.74	39.34
C(26:0)		12.04
ω-hydroxy fatty acids		115.10
C(16:0)		1.63
C(18:1)		6.69
C(20:0)		2.37
C(22:0)		63.94
C(24:0)		35.99
C(26:0)		3.69
α,ω-alkanedioic acids		21.19
C(16:0)		2.38
C(18:0)		3.70
C(18:1)		6.75
C(20:0)		1.10
C(22:0)		7.25
phenolic compounds	2.89	28.74
ferulic acid		10.96
vanillic acid	0.11	0.73
3-vanillylpropanol	0.07	3.81
vanillylpropanoic acid		5.36
benzoic acid		3.48
cathecol	1.16	1.23
unidentified	0.24	3.16
triterpenes	110.23	185.63
friedeline	79.46	95.29
unidentified friedeline derivative	0.96	2.00
unidentified friedeline derivative	8.15	6.73
betuline	1.32	13.13
betulinic acid	3.88	12.08
ergostene		8.49
hydroxy-Δ^{12}-dehydrolupen-3-one	3.10	17.23
β-sitosterol	8.96	20.93
stigmastan-3,4-diene	2.56	2.93
unidentified	1.20	6.81
others/unidentified	35.39	79.57
total	163.90	612.66

[a] Each value is the average of four injections with variation coefficients within 1.1–5.0%.

The amounts of triterpenic compounds found for ICP (≈17 g/kg) and OBC (≈6 g/kg) show that, regardless of the similar extraction yields, these fractions are not directly related, as was stated above. Although the abundances of most triterpenes (cerine, friedeline, and betuline) are comparable, the high concentration of betulinic acid in ICP cannot be related to the composition of OBC or directly to any other cork fraction. The significant differences between the composition of ICP and cork must be due to the variability of cork composition and, consequently, of ICP referred to above.

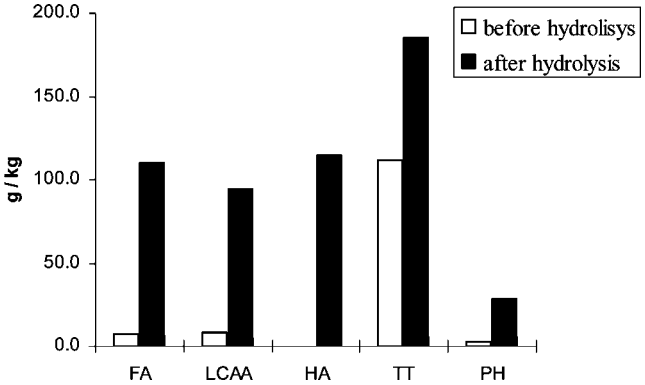

Figure 2. Contents of the major families of compounds identified by GC-MS in the DCM extract of the BCond before and after hydrolysis: fatty acids (FA); long-chain aliphatic alcohols (LCAA); hydroxyacids (HA); triterpenes (TT); phenols (PH).

Finally, the analyses of the MeOH extracts (**Table 2**) also allowed the detection of small amounts of ursolic acid and lupeol in several samples and, more remarkably, considerable amounts of ellagic acid (2.0−5.0 g/kg).

Apart from the major components reported above, small amounts of fatty acids (≈330 mg/kg) and aliphatic alcohols (≈346 mg/kg) were also identified in the studied DCM extracts. The most abundant fatty acids identified were docosanoic acid and tetracosanoic acid, followed by hexadecanoic acid, linoleic acid, and stearic acid. The main aliphatic alcohols identified were hexadecanol, octadecanol, docosanol, and tetracosanol.

The analysis of the DCM and MeOH extracts after alkaline hydrolysis did not reveal any substantial increase in the amounts of identified compounds.

Chemical Composition of the Black Condensate Lipophilic Extractives. The GC-MS analysis of BCond revealed that it is mainly composed of triterpenes (11.0%) followed by small amounts of aliphatic alcohols, fatty acids, and phenolic compounds, accounting for ≈16% of the mass of the black condensate (**Table 3**).

Considering that BCond is a fraction of cork volatile enough to be released and condensed during high-temperature processing of cork to produce black agglomerates, the identification of only 16.4% of the BCond mass was unexpectedly low. To investigate the presence of a fraction of esterified lipophilic components, the DCM extract was submitted to alkaline hydrolysis before GC-MS analysis. The results obtained (**Table 3**) demonstrate a considerable increase in the amounts of detected compounds (accounting for ≈61% of the mass of dry BCond).

With alkaline hydrolysis, the amount of triterpenes increased to 18.6%, (**Table 3**; **Figure 2**). Friedeline is the most abundant compound (9.5%), followed by smaller amounts of other triterpenes such as betuline, betulinic acid, and β-sitosterol. The increase in the amount of triterpenic structures after hydrolysis is an indication that such components are present in esterified forms in BCond. Considering that cork and ICP extract analyses after alkaline hydrolysis (results not shown) have not confirmed the presence of esterified triterpenic structures, their presence in BCond extracts must result from condensation reactions with fatty acids during the thermal treatment of cork granulates.

However, the increase in the amount of detected compounds (**Table 3**; **Figure 2**) is mainly due to the increase in the amounts of aliphatic alcohols (from 0.8 to 9.5%), fatty acids (from 0.7 to 8.9%), and phenolic compounds (from 0.3 to 2.8%), whereas ω-hydroxy fatty acids, α,ω-alkanedioic acids, absent before

hydrolysis were detected in considerable amounts after hydrolysis (11.5 and 2.1%, respectively). These are suberin-derived components (see, e.g., refs *7*, *8*, and *30*), the presence of which in BCond might result from the cleavage of more labile ester functionalities of suberin macromolecular structure during thermal treatment, releasing oligomeric ester type structures not volatile enough to be detected by GC-MS analysis; during alkaline hydrolysis full depolymerization occurs, allowing the detection of the reported fractions.

In conclusion, the compositions of the triterpenic fractions of cork and cork byproducts are similar from a qualitative point of view; however, from a quantitative point of view, a high variability was found: whereas in natural and boiled cork samples cerine and friedeline are the major compounds, followed by betulinic acid, the latter is the major component of cork powder (ICP), whereas friedeline is the major triterpene of black condensates (BCond).

Industrial cork byproducts can be considered as abundant sources of triterpenic compounds (around 17 and 185 g/kg, respectively, for ICP and BCond) and particularly of betulinic acid and friedeline, which are known to have promising applications, directly or as precursors of bioactive components for biomedical applications (*19*−*22*).

Black condensates can also be considered as a source of other aliphatic components and particularly of ω-hydroxy fatty acids and α,ω-dicarboxylic acids, promising building blocks in the synthesis of polymeric materials from renewable sources (*31*−*35*). Although this was not considered in the present paper, such hydroxy fatty acids may also be easily obtained from cork powder suberin depolymerization (see, e.g., refs *6* and *7*), thus constituting another alternative to upgrade this cork byproduct.

The results obtained demonstrate that ICP and BCond can be valuable resources of chemicals; the development of methodologies to isolate and adequately purify those compounds/fractions, instead of simply burning the residues, will be a relavant contribution to the valorization of cork as a renewable resource.

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