AGROINDUSTRIAL WASTES: A RENEWABLE SOURCE OF MATERIALS FOR FINE-CHEMICALS AND BIOACTIVE COMPOUNDS BY GREEN CHEMISTRY METHODOLOGIES

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SUMMARY: As part of our research devoted to the utilization of renewable natural sources such as the agroindustrial wastes to get fine-chemicals and bioactive compounds, in this communication we describe some examples of recovery and reuse of phenolic compounds present in olive oil mill wastewaters. Chemical procedures with a low environmental impact have been optimized in our laboratory for a new synthesis of hydroxytyrosol and its lipophilic derivatives using tyrosol as starting material. The obtained results have been described in two our recent patents (Bernini, Mincione *et al.* 2007).

1. INTRODUCTION

Agroindustrial wastes deriving from the industrial processing of the olive oil, wine, *Citrus* fruits, wheat, rapeseed contain a considerable highly toxic phenolic component, responsible for a significant environmental problem in the production areas. Moreover, the presence of this component restricts the use of these materials as animal feed and in the biochemical processes such as the fermentation for the production of biogas. On the other hand, if selectively recovered, more phenolic compounds have a high added value being natural antioxidants useful in cosmetic, food and pharmaceutical applications.

Our research group has been experienced from some years in the selective recovery of these compounds from agroindustrial wastes by chemical and biotechnological methods in collaboration with Microbiologists and Biochemists of other universities and private industries.

The recovered more abundant phenols have been converted in new bioactive compounds and fine chemicals by green chemistry methodologies. Recently, we have published some papers about the reuse of these compounds. Flavonoids have been converted into new compounds showing antitumoral activity (Bernini, Mincione *et al.* 2003 and 2005); simple alkylated phenols into *p*-benzoquinones having selective fungicide activities (Bernini, Mincione *et al.* 2006); cinnamic acids into *p*-vinyl phenols, molecules exhibiting antioxidant properties (Bernini, Mincione *et al.* 2007). The reactions were performed in traditional and ecofriendly solvents to lower the environmental impact of the processes (Bernini, Mincione *et al.* 2007).

Recently, we have turned our attention on hydroxytyrosol (3,4-dihydroxyphenylethanol), a

molecule that can be found alone or as part of other molecules such as oleuropein (Figure 1) in olive fruits, olive leaves, virgin olive oil (Montedoro *et al.* 1992) and oil mill wastewaters (Capasso *et al.* 1999; Visioli *et al.* 1999).

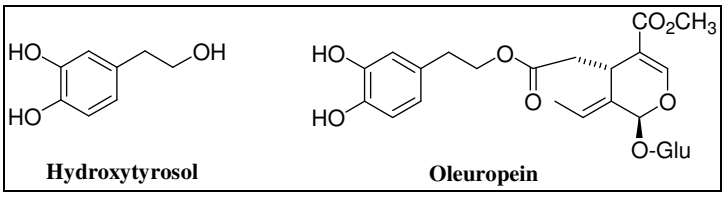


Figure 1. Chemical structures of some phenolic compounds

Hydroxytyrosol is an antioxidant of particular interest having different properties such as scavenging of free radicals, protection against oxidative DNA damage and LDL protection, prevention of platelet aggregation and inhibition of 5- and 12-lipoxygenases (Manna *et al.* 1999 and 2005). It is utilized as food additive and in many pharmaceutical and cosmetic formulations protected by international patents.

As it is commercialized only from some chemical companies at high prices, more efforts have been made to obtain it with synthetic procedures. However, no efficient and cheap procedures have been described.

Furthemore, its low solubility in hydrophobic/lipid media is a restriction to the industrial applications. Therefore, an appropriated change of the chemical structure is necessary to modify its physical properties like solubility, miscibility in oils and emulsions without to alter the antioxidant activity.

In this communication we describe a new synthesis of hydroxytyrosol and hydroxytyrosol derivatives utilizing tyrosol as starting material. As a preliminary, we report two methodologies of recovery of tyrosol and hydroxytyrosol from agroindustrial wastes.

2. RECOVERY OF TYROSOL AND HYDROXYTYROSOL FROM NATURAL SOURCES

2.1 Process for the recovery of tyrosol and hydroxytyrosol from oil mill wastewaters (OMW)

Some years ago, our research group has been engaged in a project entitled “Valorization of wastes deriving from the processing of the olives to obtain fine-chemical useful for cosmetic, pharmaceutical and dietetic applications” with a private industry based in the South of Italy (Lachifarma Srl, Lecce) and other Italian universities. A first result of this project has been the realization of a pilot-plant by Hydro Air Research for the treatment of OMW to obtain water useful for agricultural use (lower than 100 mg O_2/l of COD) and to recover polyphenolic compounds of different molecular weight.

Sequential stages of filtration, microfiltration, ultrafiltration, nanofiltration and reverse osmosis, schematized in Figure 1, allow to recover, after chromatographic purification and concentration, 1g/l of hydroxytyrosol and 0.6 g/l of tyrosol with purity higher than 98%. Tyrosol have been utilized as starting material to prepare hydroxytyrosol by oxidative pathway. These compounds have been also used for the formulation of preparations for topical use to oppose melanogenesis process and to induce derma de-couloring and de-pigmenting (Villanova *et al.* 2006).

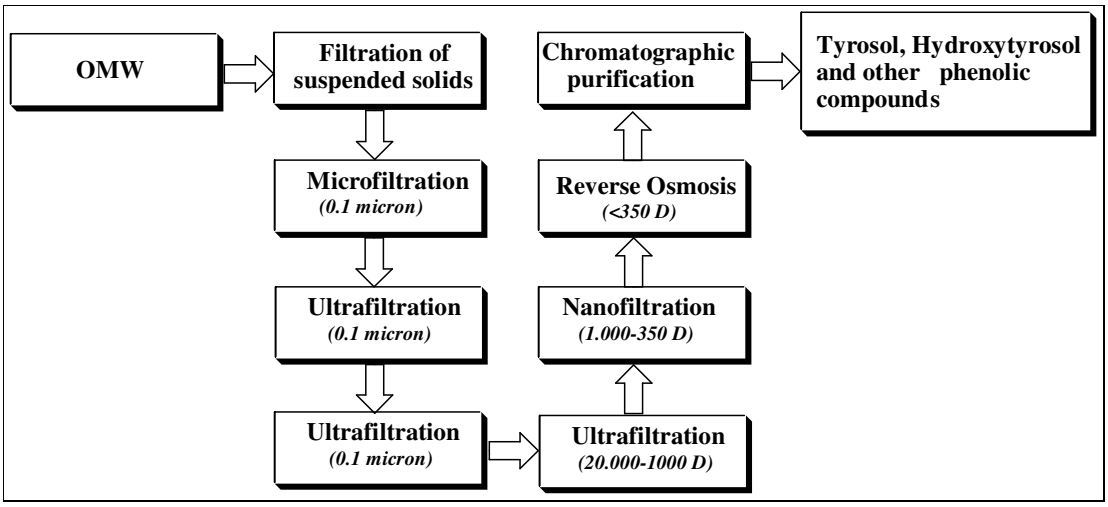
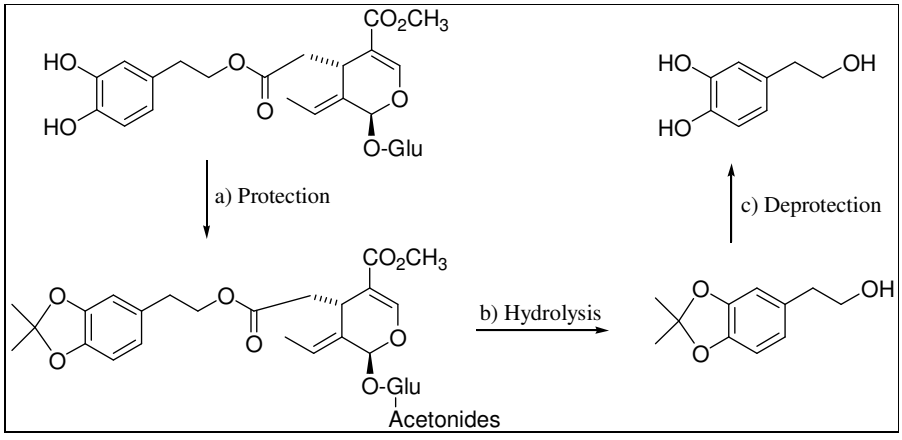


Figure 1. Recovery of phenolic compounds from OMW

2.2 Obtaining hydroxytyrosol from freshly extracted oleuropein

Oleuropein is a secondary metabolite present in considerable amounts in all parts of olive trees (6-9% w/w in dry leaves) and it is responsible for the bitter taste of unprocessed olives. In oleuropein the hydroxytyrosol moiety is esterified by elenolic acid. During the milling process for olive oil production the released endogenenous β-glycosidases hydrolyze the ester bond present in oleuropein and afford hydroxytyrosol.

In the course of a scientific collaboration with Prof. A. Gambacorta (University of "Roma Tre"), a new acetonide of the freshly extracted oleuropein has been synthesized. After hydrolysis, a new hydroxytyrosol derivative has been obtained and after deprotection, hydroxytyrosol have been recovered in good yield (Scheme 1).



Scheme 1. Obtaining hydroxytyrosol from oleuropein

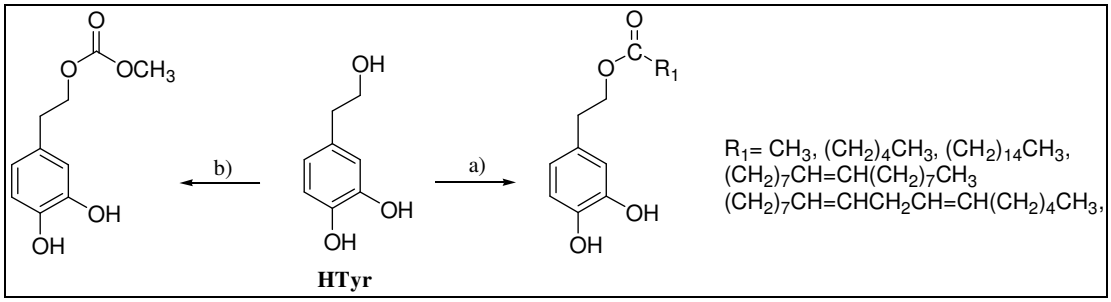
3. CHEMICAL ECOFRIENDLY PROCEDURES TO CONVERT TYROSOL INTO HYDROXYTYROSOL AND LIPOPHILIC HYDROXYTYROSOL DERIVATIVES

3.1 Selective esterification of tyrosol and hydroxytyrosol

Many literature data indicate that the potent antioxidant activity of hydroxytyrosol is due to the presence of the *ortho*-diphenolic function.

The chemoselective esterification of hydroxytyrosol on the alcoholic function is a strategy to modify its solubility and miscibility in oils and emulsions. Acyl chlorides have been utilized to get lipophilic hydroxytyrosol (Scheme 2, path a).

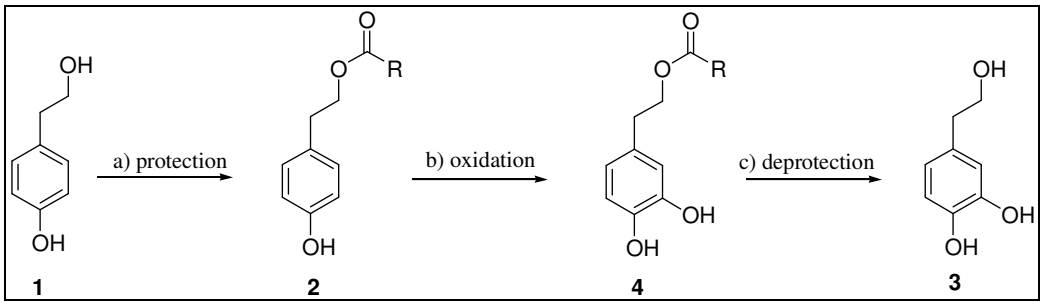
Furthemore, a new carboxymethylated hydroxytyrosol has been prepared by chemoselective carboxymethylation with dimethyl carbonate, a reagent with a low environmental impact (Scheme 2, path b). This lipophilic derivative hydroxytyrosol has been tested as antioxidant and it showed an activity similar to hydroxytyrosol.



Scheme 2. Selective esterification of hydroxytyrosol HTyr

3.2 Conversion of tyrosol into hydroxytyrosol by chemical methodologies

A new and cheap synthetic procedure has been pointed out in our laboratory. Tyrosol is the starting material. It has been selectively derivatized on the alcoholic function as previously reported, them oxidized to hydroxytyrosol derivatives. The following hydrolysis permitted to obtain hydroxytyrosol in good yields (Bernini, Mincione *et al.* 2007) and in a short time.



Scheme 3. Synthesis of hydroxytyrosol **3**

3. EXPERIMENTAL STUDY

3.1 Experimental procedure

3.1.1 Synthesis of lipophilic hydroxytyrosol derivatives.

Hydroxytyrosol (1.0 mmol) was solubilized in dimethyl carbonate (5 ml) as solvent at room temperature and the acyl chloride was added (1.2 mmol). After 24 h, the mixture was extracted with ethyl acetate. After chromatographic purifications, the products were characterized by NMR.

3.1.2 Synthesis of carboxymethylated hydroxytyrosol.

A mixture of hydroxytyrosol (1.0 mmol), DBU (1.2 mmol) and DMC (8.0 mL) was heated to reflux (T=90°C). The reaction was monitored by thin layer chromatography (TLC) and by gas-mass analysis (GC-MS). After the disappearance of the substrate, the work-up of the reaction was achieved. The reaction mixture was cooled to room temperature and DMC was evaporated under vacuum as an azeotropic mixture with methanol (DMC/CH_3OH=1/3) boiling at 64°C. The residue was solubilized in ethyl acetate and washed with a solution of HCl 1N. The organic extracts were treated with saturated solution and dried over Na_2SO_4, filtered and concentrated under vacuum. Purification of crude mixture by chromatography on silica gel using hexane/ethyl acetate (4/1) as eluent give exclusively the methyl carbonate 2, characterized by spectroscopic analysis (yield 98%, Table 1). When the reaction was performed in the presence of sulphuric acid (20%), a similar experimental procedure was followed.

3.1.3 Synthesis of carboxymethylated hydroxytyrosol.

The experimental procedures are under patents (Bernini, Mincione *et al.* 2007).

3.1.4 Antioxidant activity of hydroxytyrosol and carboxymethylated hydroxytyrosol.

The antioxidant activities were determined using DPPH as free radical. For each antioxidant different concentrations were tested (expressed as the number of moles of antioxidant/mol DPPH). Aliquotes of compounds solution in methanol was added to a 2.8 mL of $6x10^{-5}$M methanolic DPPH solution, to achieve different concentrations expressed as the number of moles of compounds /mole of DPPH. The decrease in absorbance was determined using a HP 8453 diode array spectrophotometer at 516 nm (ε_{516} 10357 ± 162 $M^{-1}cm^{-1}$) at 25°C for different ranges of time until the reaction reached a plateau. For each antioxidant concentrations tested, the reaction kinetics were plotted. From these graphs the percentage of DPPH remaining at the steady state was determined and corrected respect of a control DPPH solution without compounds. Percentage of DPPH remaining values were transferred into another graph showing the residual DPPH at the steady state as a function of molar ratio of antioxidant to DPPH and from which the antioxidant activity expressed as the amount of antioxidant necessary to decrease the initial DPPH concentration by 50% (Efficient Concentration, EC_{50}, mol/L antioxidant/mol/L DPPH) was extrapolated.

All spectrophotometric data were acquired using a HP 8453 diode array spectrophotometer. Disposable cuvettes (1 cm x 1 cm x 3 cm) from Hellma.

4. RESULTS AND DISCUSSION

Commercial acyl chlorides have been utilized to obtain the corresponding hydroxytyrosol derivatives. Reactions proceeded in good conversions and yields without catalysts (77-89%).

Of particular interest is the new carboxymethylated hydroxytyrosol, obtained by a selective carboxymethylation of hydroxytyrosol with dimethyl carbonate (DMC), an ecofriendly chemical used as solvent as well as reagent in alternative to hazardous and toxic carboxymethylating agent.

The antioxidant activity of this compound has been determined by the DPPH reduction method by plotting the remaining percentage of DPPH as a function of the molar ratios of compound over DPPH. An EC_{50} (Efficient Concentration) of 0.11±0.02 (mmol compound/mmol DPPH) has been determined, comparable to that of the hydroxytyrosol.

A new synthesis of hydroxytyrosol has been optimized in our laboratory (Bernini, Mincione et al. 2007). Only three steps are necessary to obtain the final product with purity higher than 98%. The procedure is simple and cheap and the starting material is the natural abundant tyrosol.

5. CONCLUSIONS

In this communication we described some examples of utilization of tyrosol present in olive oil mill waste waters. New eco-friendly procedures for the chemoselective protection of the alcoholic chain of hydroxytyrosol have been described to obtain lipophilic derivatives useful for industrial applications.

A new and efficient synthesis of hydroxytyrosol has been optimized in our laboratory by an innovative methodology.

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