Data article

C18 alkyl-modified silica: A suitable tool for olive biophenol green extraction

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ABSTRACT

A clean and straightforward methodology to assess the value of olive mill wastewater (OMWW) samples as well as to obtain biophenol-rich oils that can find employment in a variety of cosmetic, nutraceutical and pharmaceutical products is described. The method employs chromatographic spherical organosilica microparticles, coupled to advanced analytical techniques. OMWW samples obtained in Sicily from two different organically grown cultivars during the exceptional 2015/2016 olive oil season were analyzed, leading us to establish the conditions for optimal extraction of hydroxytyrosol and tyrosol.

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Specifications table

Subject area	Natural biomolecules green extraction and characterization.
Compounds	Tyrosol, Hydroxytyrosol, C18 alkyl-modified silica, olive mill wastewater
Data category	HPLC analysis, chromatographic separation.
Data acquisition format	HPLC chromatogram
Data type	Raw, but rationalized
Procedure	A new extraction and separation protocol for olive mill wastewater polyphenols is described as well as an HPLC analysis of the resulting extract
Data accessibility	data are with this article

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1. Rationale

Low in saturated fat, and rich in monounsaturated fatty acids from olive oil as the principal source of fat, the Mediterranean diet is a dietary pattern typical of Greece and southern Italy famous for its palatability as well as for its health benefits [1]. The diet is characterized by vegetable foods (fruit, vegetables, breads, other forms of cereals, potatoes, beans, nuts, and seeds), fresh fruit, cheese and yogurt, fish and poultry consumed in low amounts, red meat consumed and wine consumed in low to moderate amounts, normally with meals. Cumulative evidence emerging from recent epidemiological research suggests an unequivocal cardio-protective role for olive oil, and an important role in preventing several cardiovascular disease risk factors, including diabetes, metabolic syndrome and obesity and in preventing breast and digestive tract cancers [2].

Beyond monounsaturated fatty acid and even though only 2% of the total phenolic content of the milled olive fruit goes to the oil phase, the beneficial health effects of extra virgin olive oil are now firmly linked to the small amount of highly bioactive biophenols naturally present in the oil [3]. Already used as antioxidants and antimicrobials in functional foods and beverages, as well as in several cosmetic products [4], olive polyphenols will also play an important role in the treatment and prevention of inflammation, and thus of numerous free-radical mediated chronic diseases, well beyond the Mediterranean basin countries where the olive tree has been cultivated for >5000 years.

Olive biophenols are capable of scavenging free radicals and increasing shelf life of foodstuffs by retarding the process of oxidation and deterioration, thus they are suitable as a natural replacement of synthetic preservatives in food, cosmetic and pharmaceutical industries. Most biophenolics content present in the olive fruit ends in the olive mill wastewater (OMWW) ($\approx 53\%$) and in the pomace ($\approx 45\%$) [5]. OMWW originate from water naturally present in the olive fruit as well as by water added during the three-phase oil extraction process (up to 50 L of water added per 100 kg olive paste), resulting in the yearly production of 30 million m^3 of OMWW in a short period of time [6] (in Italy, between September and December), whose high phenolics content makes it recalcitrant to microbial treatment in wastewater municipal treatment plants giving place to significant extra economic costs for disposal.

OMWW is a complex matrix consisting of diverse range of hydrophilic phenolic, non-phenolic bioactive compounds and biologically inert compounds. Hence, both the recovering and purification methods play a crucial role in composition, biological activity and economic value of the corresponding extracts.

The most commonly used technique for recovery and concentration of olive phenols are based on membrane separation including micro and nanofiltration, osmotic and vacuum membrane distillation. Using these techniques, almost 80% of free low molecular weight polyphenols can be recovered with a relatively high grade of purity [7]. However, membranes do not offer selectivity for a particular biophenol species. Alternative and more selective techniques for polyphenol recovery from OMWW are the liquid-liquid extraction, often assisted by surfactants which may induce biophenol polymerization into condensed high-molecular-weight polymers with no biological activity [8]; and solid phase extraction (SPE) over resins such as Amberlite [9] or over silica [10].

We have recently developed a quick method to assess the economic value of olive mill wastewater via quantification of hydroxytyrosol and tyrosol in olive phenolic enriched extracts [11]. Now, in order to further improve the biophenol recovering process we report the comparative performance of silica and C18 alkyl-modified silica as stationary phases in the method we developed which employs also chromatographic separation.

2. Procedure

2.1. Materials and methods

Hydroxytyrosol and tyrosol standard compounds were purchased from Extrasynthese (Genay Cedex, France). n-hexane, ethyl acetate, acetonitrile and formic acid employed both for extraction procedure and HPLC analysis were obtained from VWR (Milan, Italy). All solvents used were of analytical grade. All standards were prepared in ethyl acetate and stored at $-20\,°C$.

Fresh OMWW samples (5 L) were collected immediately after milling at two continuous three-phase olive oil mills located in western and eastern Sicily during the 2015/2016 season. The OMWW originate from two different organically-grown cultivars: Carasuola (from Sciacca) and Biancolilla (from Suvarelli). To avoid decomposition, the OMWW samples were stored at −20 °C until required for experimental use. No stabilizing agents were added.

Both OMWW samples were subjected to liquid-liquid solvent extraction prior to chromatographic separation over C18 or silica gel stationary phase and analysis. In detail, 500 mL of raw OMWW from each cultivar was centrifuged twice at 9000 rpm in 30 mL vials for 10 min (Beckman allegra X-22R centrifuge with a fix-angle rotor F0630). Centrigugation removed pulp and other suspended solids as well as of residual lipids (oil and wax). The resulting water phase was then filtered through Whatman filter paper to get rid of any residual solids.

About 350 mL of a greenish-black water phase of pH ≈ 5 was obtained after filtration. The filtrate was thus acidified to pH ≈ 2 with concentrated (2 M) HCl. The color immediately turned to reddish-black. The acidified water phase was defatted in a separatory funnel using n-hexane (3 × 25 mL). The resulting aqueous layers were extracted again with EtOAc (4 × 40 mL) in order to collect all the extractable phenolics content. The EtOAc extracts of each sample were collected together, dried over anhydrous sodium sulfate, and evaporated under vacuum (180 mbar) using a rotary evaporator (Buchi Rotovapor R-200 equipped with Vacuum Controller V-850 and Vacuum Pump V-700) at 40 °C. A yellowish-brown crude oil, of different weight depending on the cultivar, was eventually obtained.

In order to eliminate the resins from the crude, each crude extract was separately dissolved in EtOAc. Silica gel (2 g) was added to the mixture and the solvent evaporated again in a rotary evaporator. The oil adsorbed on silica was loaded on a silica gel septum (Silica gel 60 Particle size 0.063–0.200 mm, 70–230 mesh ASTM) (11 g) packed in n-hexane. The septum was eluted with hexane (100 mL) to remove any residual apolar component. After that EtOAc (100 mL) was added to the column to recover the biophenol fraction. The septum trend was followed by TLC (aluminum sheet covered by silica gel 60 F254). The eluate, for each cultivar, was evaporated in a rotary evaporator. The resulting yellowish-orange oils were named silica purified polyphenol mixes (SPPM).

The same crude samples obtained from liquid-liquid extraction were processed through a C18 reverse phase silica column (C18 carbon 17%, loading 0.73 mmol/g, 60 Å, particle size 40–63 μm). In detail, 20 g of the C18 reverse phase silica kindly donated by SilyCicle (Quebec City) was packed in 9:1 acetonitrile:acidified water (2.5% w/w formic acid). The column was eluted with the same eluent and then polarity was gradually increased up to 1:1 acetonitrile: acidified water in order to recover biophenol fractions as clean as possible. The column trend was followed again by TLC. For each cultivar, the corresponding fraction bearing tyrosol and hydroxytyrosol was pooled together, and evaporated to dryness in a rotary evaporator. The resulting light yellowish oils were called C18 purified polyphenol mixes (CPPM).

2.2. Chromatographic analysis and identification of extract components

Both extraction fractions SPPM and CPPM originating from the two cultivars were separately dissolved in 5 mL of EtOAc. 1 μL of each resulting solution was qualitatively monitored by HPLC analysis by comparison and combination of their retention times (UV detector at 280 nm) and mass spectral data with the corresponding standards. For quantitative analysis, biophenol compounds were determined at 280 nm using their respective reference compounds in different concentration.

The LC/MS chromatograph was Agilent 6130 Series Quadrupole LC/MS Systems with a G1311A Quaternary Pump, equipped with: G1329A High Performance Autosampler, G1316A Thermostated Column Compartment and G1315D Diode Array Detector (DAD). Separation was carried out using an Agilent Eclipse XBD-C18 (4.6 × 150 mm, 5 lm) column maintained at 30 °C.

Identification and quantification of phenolic compounds was obtained using a G6120B Single Quadrupole LC/MS system equipped with an electrospray ionization source (ESI). For target compound analysis, a flow injection analysis (FIA) was carried out to determine the fragmentor setting to improve the compound response. The potential chosen was 200 V. ESI work conditions were: capillary voltage 5000 V, gas flow rate 13 L/m, gas temperature 300 °C and nebulizer pressure 60 Psi. To obtain the best sensitivity, the quadrupole was used in SIM mode. Optimum separation was achieved with a binary

Table 1

Amounts of hydroxytyrosol and tyrosol in the SPPM and CPPM extracts.

Entry	Hidroxytyrosol (mg/L)	Tyrosol (mg/L)
Biancolilla SPPM	65.90 ± 3.29	16.04 ± 0.81
Biancolilla CPPM	63.34 ± 3.16	18.25 ± 0.91
Cerasuola SPPM	125.43 ± 6.27	29.85 ± 1.49
Cerasuola CPPM	127.81 ± 6.37	28.12 ± 1.41

mobile phase gradient at a flow rate 0.5 mL/min. The mobile phase consisted of a binary solvent system using (A) water/formic acid pH 3.1, and (B) acetonitrile which were previously degassed. The gradient elution program was as follows: 0–15 min, 10–60% B; 15–20 min, 60–10% B (namely starting at t = 0 min with 10% solvent B and 90% A, and gradually growing during 15 min to B = 60%). After 15 min (when the analysis is over) the B relative amount (%) rapidly decreases during the subsequent 5 min so as to return the column in the original condition, ready for the successive separation and analytical run.

3. Data, value and validation

Despite the substantial differences between silica gel and C18 organosilica gel in hydrophilic/lipophilic balance, mesh number, granulometry and elution solvents, both selectivity and retention capacity of the two silica-based materials were fully comparable. Indeed, both SPPM and CPPM showed a similar and superimposable HPLC profile in which tyrosol and hydroxytyrosol appear to be the major components and in the same quantity (Table 1).

In practical and commercial applications, octadecyl-silica will be the best option for separation rather than normal phase, due to several reasons. First, the hydrophobic stationary phase works well for retention of most organic analytes, so that water can be used as a mobile phase in conjunction with less polar solvents such as MeCN and MeOH, but also ecofriendly biosolvents such as ethyl lactate [12]. Reverse phase chromatography enables also to use pH selectivity to improve separation.

Normal phase silica, on the other hand, requires the use of toxic *n*-hexane or of costly biosolvents such as *d*-limonene [13]. Volatile hexane quickly evaporates locally contaminating air. In chronical exposure it can cause central nervous system effects such as vertigo dizziness and confusion. The US National Institute for Occupational Safety and Health has set a recommended exposure limit for hexane isomers of 100 ppm (350 mg/m^3) over an 8 h workday [14], and those levels are very difficult to respect assuming an industrial process. Moreover, *n*-hexane is highly flammable, and storing great quantity is not recommended.

4. Conclusion

Two different chromatographic separations were attempted with the aim of remove hydrophilic resins and "poor" biophenols obtaining enriched mixes (SPPM and CPPM) rich in valued biophenols (mainly tyrosol and hydroxytyrosol) that were compared via quantification of tyrosol and hydroxytyrosol using an analytical procedure lately optimized [11].

Deeb and co-workers in Jordan successfully used normal phase silica to separate the biophenols in OMWW prior to thorough NMR and MW analyses. The list of solvents used to elute the biophenols (n-Hexane, ethyl acetate, methanol, chloroform, dichloromethane, ammonia, benzene, and pyridine) [10] gives the scope of the potential health and environmental impact.

In 2007 researchers in Italy first suggested a method the use of a C18-solid phase cartridge and EtOAc as eluent for fractionation of the phenolic raw extract from OMWW [15]. The fractionated extracts were then analyzed using low biophenol specific HPLC. Now, the use of a silica reverse phase supplied by a leading manufacturer of chromatographic spherical organosilica microparticles, coupled to advanced analytical techniques that led us to establish the conditions for optimal extraction of hydroxytyrosol and tyrosol, establish a clean and straightforward methodology to assess the value of

OMWW samples and, for companies interested, also to obtain biophenol-rich extract that can find employment in a variety of cosmetic, nutraceutical and pharmaceutical products.

Acknowledgements

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