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Original article

A by-product from virgin olive oil production (pâté) encapsulated by fluid bed coating: evaluation of the phenolic profile after shelf-life test and *in vitro* gastrointestinal digestion

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Abstract Pâté is a by-product of virgin olive oil production presenting a high phenolic content and beneficial health effects. This research aims to evaluate the storage stability of phenolic compounds of pâté and a coated formulation, and to verify the bioaccessibility of the phenolic compounds. An accelerated shelf-life test at 40 °C/75% relative humidity was carried out for 75 days. Different combinations of degradative and hydrolytic reactions impacted the results. Indeed, at the end of the storage period, 84% of phenols was recovered in pâté with free tyrosol and hydroxytyrosol showing an increase due to secoiridoids hydrolysis. The total phenolic content of coated pâté did not significantly decrease, showing a higher increment of free tyrosol and hydroxytyrosol. Simulated digestion led to the liberation of 15% of phenols by both samples. In conclusion, the coating process improved the stability of pâté phenolic compounds without affecting their bioaccessibility.

Keywords Functional food ingredient, nutraceuticals, olive oil pomace, encapsulation, hydroxytyrosol, storage stability, bioaccessibility.

Introduction

Phenolic compounds belonging to different chemical classes present in extra virgin olive oil (EVOO) have been studied largely for their health-beneficial effects. These compounds are mainly associated with tyrosol (tyr), hydroxytyrosol (OH-tyr) and their secoiridoidic precursors.

Numerous studies reported that virgin olive oil phenols provide benefits for plasma lipid levels and contrast oxidative damage (Covas *et al.*, 2006; de la Torre-Carbot *et al.*, 2010; Dinu *et al.*, 2020). On this basis, the European Food Safety Authority (EFSA) stated in 2011 that ‘Olive oil polyphenols contribute to the protection of blood lipids from oxidative stress’, allowing for this ‘health claim’ to be included in the well-known Commission Regulation (EU) No 432/2012 (EC, 2012).

Olive oil production generates large quantities of wastes such as, most importantly, olive pomace (800–850 kg t^{-1} olives). This by-product, also known as ‘alperujo’, is obtained through the use of two-phase plants and includes a combination of liquid and solid wastes such as olive pulp, olive skin, stone and water. Many studies have been aimed at reducing the environmental impact of olive pomace and/or harnessing its potential economic value (Frankel *et al.*, 2013). Indeed, this by-product is rich in important value-added compounds among which tyr and OH-tyr, present as free and bound forms, represent a prominent part (Obied *et al.*, 2005). Notably, no more than 0.5% of olive fruit phenols are transferred to EVOOs, whereas the larger part remains in the by-products (Cecchi *et al.*, 2018b). In the last few years, an innovative two-phase decanter has been developed which allows for the production of a spreadable olive pomace, named pâté, characterised by the absence of residuals of kernel and lignin. The amount of water is greater compared to olives (85–90%). In fresh pâté fat and proteins, content is about 1.5–2.25% (Cecchi *et al.*, 2018a), while in olives, the main constituent is lipids (10–25%). The protein content of the fresh pulp is relatively low, generally between 1% and 3%.

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Dietary fibre is close to 7.5% in pâté, while it varies between 1–4% in the olive fresh pulp, with the major components being cellulose, lignin and hemicellulose (Montaño *et al*., 2010).

Pâté has already been used to enrich some staple food-products (like bread, pasta and bars) proposed as valuable healthy-products and appreciated by consumers (Padalino *et al*., 2018; Cecchi *et al*., 2019; Tufariello *et al*., 2019), suggesting the use of this by-product as a functional ingredient for the development of novel food supplements. Recently, a clinical trial aimed at evaluating the effects of pâté on cardiovascular and metabolic risk factors highlighted a statistically significant reduction in plasma levels of total cholesterol, LDL cholesterol and pro-inflammatory protein MCP-1 (Dinu *et al*., 2020). In addition, pâté evaluated in a rat model was able to promote abdominal pain relief, showing a protective role related to its peculiar phenolic profile (Parisio *et al*., 2020). Although pâté phenolic compounds have been demonstrated to possess several interesting protective effects, limited data are available about the stability of these molecules in pâté (Cecchi *et al*., 2018a). Another point scarcely addressed so far is the bioaccessibility of the phenolic compounds of *Olea europaea* L. It was evaluated in different matrices like EVOO (Dinnella *et al*., 2007; Quintero-Florez *et al*., 2018; Rocchetti *et al*., 2020), table olives (D'Antuono *et al*., 2016), olive leaves (Ahmad-Qasem *et al*., 2014) and recently in pomace extracts (Ribeiro *et al*., 2020) but has not been analysed in pâté so far.

Since several phenolic compounds are acknowledged as being susceptible to high temperatures, light exposure and presence of oxygen, different strategies have been developed to tackle these problems, including which encapsulation. There are many encapsulation techniques described in the literature (e.g. emulsion, spray drying, extrusion, electrospinning, etc) and some have been successfully applied to plant polyphenols. Fluid bed coating is a widely employed encapsulation technology to increase the stability of labile molecules and, in general, to improve the functional properties of the bioactive molecules (Meiners, 2012). The coating materials can be pure materials or a mixture and can be made of sugars, gums, proteins, natural and modified polysaccharides, lipids, and synthetic polymers (Fang & Bhandari, 2010). Different approaches based on a number of binding or coating materials to process purified molecules or extracts have been developed so far but, to our knowledge, they have never been applied to complex by-products like pâté. The aim of our work is to further characterise pâté to promote its sustainable reuse as a rich source of phenols from *Olea europaea*. To achieve this goal, we studied: (i) the storage stability of each phenolic compound present in pâté (P) and in a coated pâté (CP) obtained by processing the matrix through fluid bed technology using modified starch and maltodextrin; (ii) the bioaccessibility of pâté phenolic compounds by simulated *in vitro* digestion to investigate the effects induced by the coating process on the release of specific phenols in physiologic conditions.

Material and methods

Pâté preparation

About 60 kg of pâté (P) from typical Tuscan cultivars (Frantoio and Moraiolo) was obtained from the processing of an olive batch of approximately 0.5 tons during the 2018 crop season (Castagneto Carducci, Livorno, Italy) immediately after the oil extraction process. P was freeze-dried at 0.4 Pa and −20 °C within 3 h of collection until reaching a constant weight (4 days, water content, 79% w/w) and stored under vacuum at room temperature. Before its use, P was finely ground using De'Longhi Braun® Household GmbH (Neu-Isenburg, Germany) Minipimer MultiQuick 5 vario MQ 30 hand blender accessory and sieved to obtain a powder with homogeneous particle size (150–300 μm).

Moisture content

The moisture content of all samples utilised in the study was assessed following the AOAC official method 993.26. Samples were weighted and let dry in a stove at 105 °C for 24 h. Then, the loss of weight was recorded, and the percentage of water content was calculated. All analyses were carried out in triplicate.

Coating process

An amount of 100 g of P was subjected to fluid bed coating with 100 mL of a mixture containing 10% w/v maltodextrin DE19 (Agrana, Austria), 10% w/v modified OSA (Octenyl succinic anhydride) starch (Capsul®, provided by Ingredion, Illinois, US) and 0.5% w/v sunflower lecithin (Cereal Docks Food, Italy) as wall materials, using a fluidised bed Mini-Glatt (Glatt, Binzen, Germany) reactor at 40 °C with an air flow of 15 $m^3 h^{-1}$. After production, the coated samples were immediately subjected to analyses.

Evaluation of the phenolic compounds

Extraction and hydrolysis

An amount of 250 mg of dried P and CP was extracted twice with 10 mL of $EtOH:H_2O$ 8:2 v/v, under magnetic stirring for 1 h, and then filtered. The obtained extract was defatted with *n*-hexane, dried under vacuum (−0.1 MPa) on a Rotavapor® R-100

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(from Büchi, Milano, Italia) and then dissolved in 5 mL of the same extractive mixture. A portion of this solution was subjected to chromatographic analysis, while another part was submitted to an acidic hydrolysis, according to the procedure validated for virgin olive oil reported in Bellumori *et al*., (2019). Briefly, 300 µL of H_2SO_4 1 M was added to 300 µL of the extract and placed in a stove at 80 °C for 2 h; then, 400 µL of distilled water was added and the solution was centrifuged and analysed by HPLC-DAD-MS.

Identification and quantification

The determination of phenolic compounds was performed before and after acid hydrolysis using an HP 1100 liquid chromatograph equipped with a DAD detector (Agilent Technologies, Palo Alto, CA, USA). The identification of phenolic compounds was carried out by an HP 1260 MSD mass spectrometer with an API/electrospray interface (Agilent Technologies); the following conditions were applied: gas temperature 350 °C, nitrogen flow rate 10.5 L min^{-1}, nebuliser pressure 35 psi (241 KPa) and capillary voltage 3500 V. The mass spectra were acquired in the m/z range 100–1000 Th in negative ion mode, setting the fragmentation energy between 80 and 120 V.

A Poroshell 120 EC-C18 column (150 × 3 mm i.d, 2.7 µm particle size, Agilent Technologies) was used to analyse the samples. The mobile phases were as follows: (A) acidified water (pH 3.2) and (B) acetonitrile; the multistep linear gradient reported by Cecchi *et al*., (2018a) was applied. The total time of analysis was 53 min, flow rate 0.4 mL min^{-1}.

A six-point calibration curve of tyrosol (Extrasynthèse, Genay, France, purity ≥ 99%) at 280 nm (R^2 = 0.999) was used to evaluate tyrosol, hydroxytyrosol and their derivatives; a six-point calibration curve of verbascoside (Sigma-Aldrich, Steinheim, Germany, purity ≥ 99%) at 280 nm (R^2 = 0.999) was used to evaluate verbascoside and its derivatives; secoiridoids at 280 nm were evaluated with the five-point calibration curve (R^2 = 0.999) of oleuropein (Extrasynthèse, purity ≥ 98%), and finally, the flavonoid content was determined using a six-point calibration curve of luteolin 7-*O*-glucoside (Extrasynthèse, purity ≥ 98%) at 280 nm (R^2 = 0.999). The total phenolic content was calculated as sum of each phenolic compound. The content of total tyrosol and hydroxytyrosol after hydrolysis was evaluated using the curve of tyrosol at 280 nm. Data were expressed as mg g^{-1} dried weight (DW) as mean ± standard deviation from triplicates; for coated P, the amount of coating material was excluded.

Stability test (Accelerated shelf-life test)

An accelerated shelf-life test of pâté (P) and coated pâté (CP) was carried out by incubation in a thermostatic chamber (Memmert, Germany) at 40 °C/ 75% relative humidity (RH) for 75 days. Twelve grams of P and CP was divided in 4 different Petri dishes each and placed in the incubator. Samples of 250 mg were collected at 0, 25, 50 and 75 days, then indicated as t_0, t_1, t_2 and t_3, and used for the different experiments.

Static simulated digestion

Static simulated digestion of P and CP, corresponding to the sample at t_0, was performed following Infogest 2.0 official protocol (Brodkorb *et al*., 2019); digested samples were taken after both gastric and intestinal steps.

The composition of the simulated digestion fluids was the same described in the protocol. The fluids were prepared 1.25× concentrated, allowing the addition of enzyme solutions and $CaCl_2$, that is, 0.75 mM in simulated salivary fluids (SSF), 0.075 mM in simulated gastric fluids (SGF) and 0.3 mM in simulated intestinal fluids (SIF), just before the experiments. The experiments were performed in a shaking thermoblock, at 37 °C. Two tubes were filled with 250 mg of P and other 2 tubes with 250 mg of CP. For the oral phase, the samples were reconstituted with 250 µL of distilled water in order to reach the correct consistency of the bolus, as suggested by the protocol, and SSF was added in ratio reconstituted sample: SSF of 1:1 w/v. Human salivary α-amylase (A1031, Sigma-Aldrich, Milan, Italy) was previously added to SSF to reach 75 U mL^{-1} concentration in the final mixture. After two minutes of salivary digestion, the samples were mixed with the SGF in ratio 1:1 and the pH was adjusted to 3.0 using a 5 M HCl solution. Pepsin from porcine gastric mucosa (P6887, Sigma-Aldrich) was added to obtain 2000 U mL^{-1} activity, and the mixture was incubated for 60 min. The gastric digestion was stopped by adding SIF in a final ratio of 1:1 v/v and bile salts (C1254, Sigma-Aldrich) at 10 mM final concentration, adjusting the pH to 7.0 with a 5 M NaOH solution. One tube containing P and one containing CP were immediately centrifuged (4500 ***g***, SX4250 rotor; Allegra x-22R, Beckman Coulter, USA) for 10 min at 10 °C, and the supernatants were snap-frozen with liquid nitrogen. Pancreatin 4x USP (P1750, Sigma-Aldrich) was added to the other 2 tubes to obtain 100 U mL^{-1} of trypsin activity. The mixture was incubated for 120 min in the thermoblock. The samples were then centrifuged and the supernatants frozen with liquid nitrogen. All the samples were finally lyophilised. The experiment was performed in duplicate (*n* = 2).

The recovery, expressed as the percentage of phenolic compounds released from P and CP after the

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gastrointestinal digestion, was calculated as previously reported by the following:

Recovery (%) = Cd/Cu × 100.

where Cd is the phenolic compound concentration in gastric or intestinal phase fraction and Cu is the initial polyphenols concentration in undigested P or CP. The recovery (%) after the intestinal phase is defined as the bioaccessible fraction of phenolic compound.

Antioxidant capacity (AOC) of the phenolic extracts

The samples that underwent to the accelerated shelf-life test and static simulated digestion were tested by ABTS test. The assay was assessed based on the method of Thaipong *et al.*, (2006), with some modifications. The working solution was obtained mixing 7.4 mM of ABTS$^{\cdot +}$ solution with 2.6 mM potassium persulphate solution, in equal quantities, and allowing them to react for 12 h at room temperature in the dark. Absorbance of the solution was adjusted to 0.75 ± 0.02 units at 734 nm using methanol. The assay was performed on a 96-well microplate (Sarstedt, Nümbrecht, Germany), in which 20 μL of standard or sample was mixed with 200 μL of ABTS$^{\cdot +}$ solution and incubated for 10 min. The decrease of absorbance was monitored at 734 nm after 10 min using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Trolox (Sigma-Aldrich) served as a standard and the results were expressed in μM Trolox equivalents (TE) g^{-1} dry weight. The experiments were performed in triplicate.

Statistical analysis

Analyses were performed in triplicate, and the results expressed as mean values ± standard deviation. The Pearson coefficient was computed to test possible correlations. Statistical significance was evaluated by the analysis of variance and *F*-test ($p \leq 0.05$) using Microsoft Excel statistical software. Fisher's LSD test was applied to compare the mean values using the software DSAASTAT v. 1.1.3.

Results and discussion

Phenolic profile of pâté and coated pâté

The chromatographic profiles of pâté (P) and coated pâté (CP) extracts were found to be very similar, assuring that the coating did not cause any change on the phenolic content (Fig. 1). The phenolic compounds detected in P and CP by HPLC-DAD-MS were those typically present in virgin olive oil and in olive fruit (Lozano-Sanchez *et al.*, 2017; Cecchi *et al.*, 2018a). More than 20 compounds were detected. In particular, simple phenols such as OH-tyr and tyr, verbascoside and its hydroxylated isomers (compounds 8 and 9), luteolin as the main flavonoid, and several secoiridoidic compounds were identified.

As reported in previous studies (Lozano-Sanchez *et al.*, 2017; Cecchi *et al.*, 2018a), P contains a significantly higher percentage of phenolic compounds in comparison to other by-products like olive pomace and olive-mill wastewaters. In the present study, the phenolic content was calculated by HPLC-DAD as the sum of the individual compounds. At the same time, an acidic hydrolysis was applied to simplify the analytical determination of the total content of OH-tyr and tyr. This allows evaluation of both the free forms and those linked to the secoiridoidic nucleus. Table 1 reports the amount of the main phenolic compounds present in P and CP at t_0, before and after hydrolysis.

The P extract contained 19.95 ± 0.34 mg g^{-1} dry weight (DW) of total phenolic compounds before hydrolysis and 14.55 ± 0.23 mg g^{-1} of OH-tyr and tyr (free and bound forms) after hydrolysis. Similar values were recorded for CP (20.87 ± 0.53 and 15.34 ± 0.72 mg g^{-1} DW) before and after hydrolysis, respectively, and not significant differences were observed between the two extracts (Table 1). As observed in our previous study (Cecchi *et al.*, 2018a), the ratio OH-tyr:tyr is about 9:1 after hydrolysis. According to the EFSA health claim for EVOO (EC, 2012), 5 mg of total OH-tyr and its derivatives (necessary to comply with the claim) could be reached by less than approximately 0.4 g of dry P.

Stability of phenolic compounds over time

It is well known that phenolic compounds, despite their potential health benefits to humans, are very sensitive to heat and light and can undergo oxidation or degradation processes, drastically reducing their effectiveness (Parisi *et al.*, 2014). Although the degradation of virgin olive oil phenolic compounds was assessed in several studies under different storage or accelerated heating conditions (Daskalaki *et al.*, 2009; Li *et al.*, 2014; Krichene *et al.*, 2015), no data are available on the stability of the same phenols in P. One of the objectives of this study was to determine the stability over time under standardised conditions of phenolic compounds in P and to evaluate whether a protective coating based on OSA modified starch and maltodextrin could improve the stability of these molecules. Indeed, these materials already proved to increase the stability of some phenolic compounds in other food by-products (Ballesteros *et al.*, 2017; Zanoni *et al.*, 2020). The shelf-life and effect of the coating process were examined by incubating P and CP in a thermostatic chamber for 75 days, under controlled conditions of temperature and humidity (40 °C and 75%

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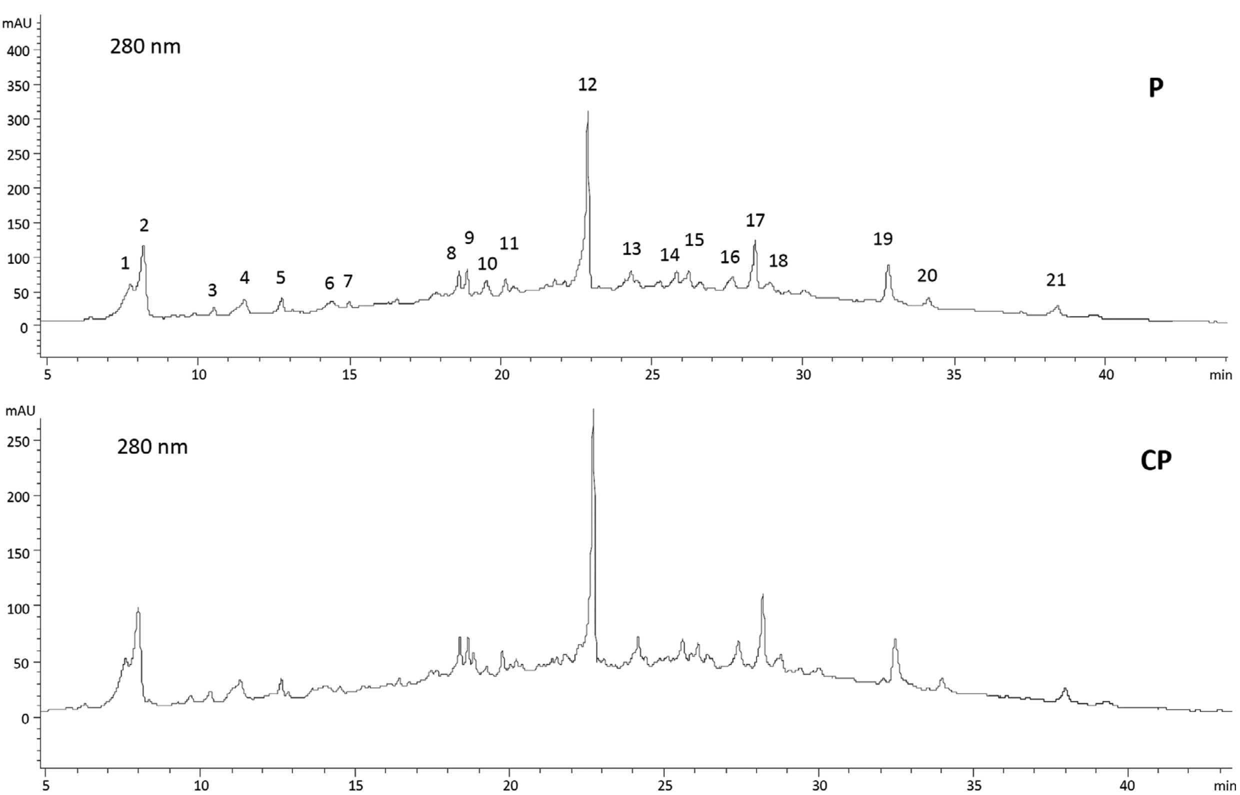

Figure 1 HPLC-DAD chromatograms detected at 280 nm of pâté (P) and coated pâté (CP) extracts. 1, OH-tyrosol glucoside; 2, OH-tyrosol; 3, tyrosyl glucoside; 4, tyrosol; 5-7, unknown; 8, β-OH-acteoside 1; 9, β-OH-acteoside 2; 10–11, unknown; 12, verbascoside; 13-18, secoiridoids; 19, luteolin; 20-21, secoiridoids.

RH). These conditions are those typically used to accelerate degradative reactions in order to assess the stability of food ingredients and pharmaceuticals over a shorter time interval.

Figure 2 reports the total phenolic content of P and CP extracts determined at 0, 25, 50 and 75 days of storage, before and after acidic hydrolysis. The P extract showed a significant decrease in total phenolic content after 25 days, with 87.9% of the total phenols recovered. Only a further slight degradation was observed for the rest of the incubation time, with a decrease of about only 4% detected after 75 days. This trend was confirmed also after hydrolysis considering the sum of the total tyr and OH-tyr content (free and bound forms), which decreased to 80.1% at t_1. With regard to the P extract, at the end of the stability test the total recoveries of phenols evaluated before and after the hydrolytic procedure were 83.8% and 75.2%, respectively.

Interestingly, the CP extract did not show a significant decrease in the total phenolic content during the incubation time, indicating that the carbohydrate wall materials protected phenols from degradation, as already documented in previous studies (Ballesteros *et al.*, 2017; Zanoni *et al.*, 2020). After hydrolysis, a significant decrease of about 10% was observed after 25 days of storage; the total content of OH-tyr and tyr remained stable for the rest of the stability test.

During the stability test, the antioxidant capacity (AOC) of P and CP extracts was determined by ABTS assay (Fig. 3). AOC values at time zero were 378 ± 17 and 425 ± 35 μmols Trolox g^{-1} DW for P and CP, respectively. These results are in the same range of values reported by Martínez-Patiño *et al.*, (2019) on olive pomace and determined by ABTS test. According to the trend observed for the phenolic content reported in Fig. 2, AOC showed a decrease of about 40% for P and 50% for CP passing from t_0 to t_1, followed by a plateau in which a further significant degradation is visible only for the CP sample from t_2 to t_3. Differently from that depicted in Fig. 2, no significant differences were observed among P and CP extracts at each of the selected times. This result suggests that the products derived from degradation of the original compounds could not be detected by the applied analytical methods, but may still contribute to the AOC

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Table 1 Content of the main phenolic compounds present in pâté (P) and coated pâté (CP) extracts at different times of stability test, before and after hydrolysis

P	t_0	t_1	t_2	t_3
OH-tyr glu	1.83 ± 0.01 a	2.50 ± 0.10 c	2.14 ± 0.04 b	2.54 ± 0.07 d
OH-tyr	2.61 ± 0.07 a	3.84 ± 0.08 b	4.49 ± 0.15 d	4.16 ± 0.04 c
Tyr glu	0.19 ± 0.01 c	0.13 ± 0.01 b	0.12 ± 0.01 ab	0.10 ± 0.01 a
Tyr	0.69 ± 0.03 a	0.85 ± 0.03 b	0.89 ± 0.01 b	1.03 ± 0.02 c
OH- acteoside 1	0.32 ± 0.01 a	0.36 ± 0.01 a	0.54 ± 0.06 b	0.31 ± 0.01 a
OH- acteoside 2	0.28 ± 0.01 a	0.39 ± 0.01 c	0.29 ± 0.01 b	0.38 ± 0.01 c
Verbascoside	2.44 ± 0.06 c	1.76 ± 0.02 b	1.68 ± 0.04 b	1.51 ± 0.01 a
Luteolin	0.70 ± 0.02 c	0.56 ± 0.00 b	0.51 ± 0.01 a	0.51 ± 0.02 a
Secoiridoids	9.87 ± 0.13 c	6.2 ± 0.02 b	6.39 ± 0.16 b	5.26 ± 0.05 a
Total phenols	19.95 ± 0.34 c	17.53 ± 0.01 b	18.03 ± 0.32 b	16.73 ± 0.15 a
After hydrolysis	t_0	t_1	t_2	t_3
OH-tyr	13.08 ± 0.22 b	10.09 ± 0.54 a	10.05 ± 0.04 a	9.49 ± 0.16 a
Tyr	1.47 ± 0.01 a	1.57 ± 0.07 a	1.50 ± 0.01 a	1.46 ± 0.03 a
Sum of tyr + OH-tyr	14.55 ± 0.23 b	11.66 ± 0.47 a	11.54 ± 0.03 a	10.95 ± 0.13 a
CP	t_0	t_1	t_2	t_3
OH-tyr glu	1.74 ± 0.01 a	1.95 ± 0.02 a	2.73 ± 0.16 b	3.36 ± 0.12 c
OH-tyr	2.81 ± 0.02 a	5.33 ± 0.04 bc	5.46 ± 0.05 c	5.30 ± 0.08 b
Tyr glu	0.19 ± 0.02 ab	0.27 ± 0.06 b	0.15 ± 0.01 a	0.10 ± 0.01 a
Tyr	0.80 ± 0.02 a	0.99 ± 0.01 b	1.06 ± 0.03 c	1.46 ± 0.01 d
OH-acteoside 1	0.39 ± 0.02 b	0.57 ± 0.01 c	0.60 ± 0.03 c	0.28 ± 0.01 a
OH-acteoside 2	0.31 ± 0.02 a	0.48 ± 0.01 c	0.38 ± 0.01 b	0.41 ± 0.02 b
Verbascoside	2.25 ± 0.08 b	2.15 ± 0.01 b	1.95 ± 0.05 a	1.86 ± 0.03 a
Luteolin	0.71 ± 0.01 b	0.54 ± 0.01 a	0.53 ± 0.00 a	0.54 ± 0.00 a
Secoiridoids	10.57 ± 0.33 d	7.84 ± 0.24 c	7.13 ± 0.12 b	6.28 ± 0.10 a
Total phenols	20.87 ± 0.53 a	21.27 ± 0.03 a	20.89 ± 0.48 a	20.76 ± 0.35 a
After hydrolysis	t_0	t_1	t_2	t_3
OH-tyr	13.82 ± 0.66 b	12.16 ± 0.01 a	11.53 ± 0.43 a	11.67 ± 0.13 a
Tyr	1.52 ± 0.05 a	1.63 ± 0.05 a	1.72 ± 0.19 a	1.74 ± 0.02 a
Sum of tyr + OH-tyr	15.34 ± 0.72 b	13.79 ± 0.05 a	13.25 ± 0.62 a	13.41 ± 0.11 a

Data are expressed as mg g^{-1} dried weight (DW) as mean ± standard deviation of three independent analyses. Different letters in the same line indicate significant differences ($p \leq 0.05$) for each compound over time. OH-tyr, hydroxytyrosol; Tyr, tyrosol; glu, glucoside.

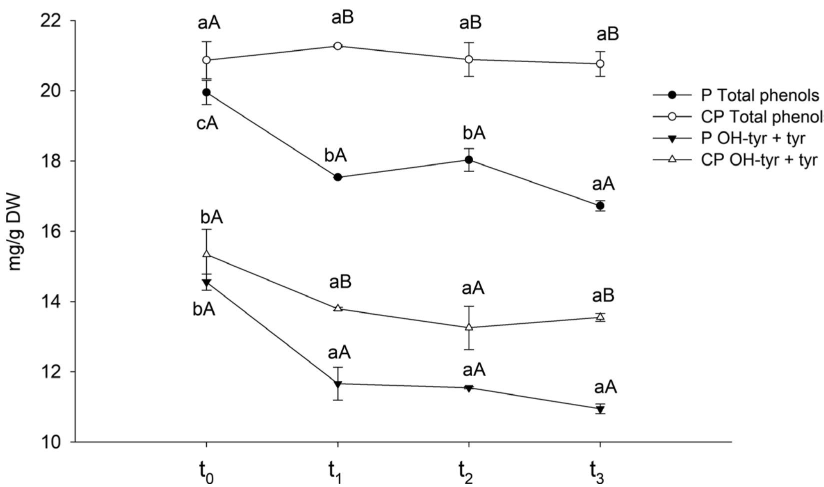

Figure 2 Phenolic content in pâté (P) and coated pâté (CP) extracts evaluated before and after acidic hydrolysis, at different times of stability test. Data are expressed as mg g^{-1} dried weight (DW) as mean ± standard deviation from triplicates. The lowercase letters indicate significant differences ($p \leq 0.05$) over time; the uppercase letters indicate significant differences between P and CP for the same time of stability test. OH-tyr hydroxytyrosol; tyr, tyrosol.

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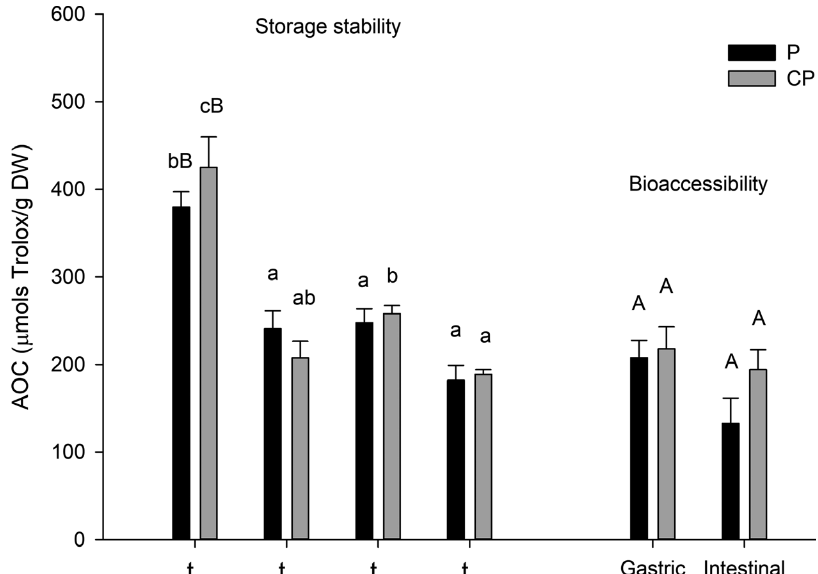

Figure 3 ABTS results of P and CP extracts at different times of stability test and after *in vitro* gastrointestinal digestion. Data are expressed as μmols Trolox equivalent g^{-1} (mean ± standard deviation) from triplicates. The lowercase letters indicate significant differences ($p \leq 0.05$) over time during the stability test; the uppercase letters indicate the differences between the t_0 sample and the digested samples.

of the extracts. Indeed, it is acknowledged that phenolic compounds subjected to oxidation may undergo chemical modifications enhancing their radical scavenging capacity as described by Nicoli *et al.*, (2000) and Di Mattia *et al.*, (2009). Such compounds may have preferentially originated in P samples due to the greater degradation of the phenols, and thus, have contributed to a higher extent to AOC in comparison to CP. In this sense, even though P samples showed lower concentrations of phenols, the final AOC turned out to be similar.

As shown in Table 1, the phenolic profile of P extract varied over time and a significant increase was observed for the content of OH-tyr glucoside, OH-tyr and tyr, which ranged from 1.83 to 2.54 mg g^{-1} DW, 2.61–4.16 mg g^{-1} DW and 0.69 to 1.03 mg g^{-1} DW, registering an increase from t_0 to t_3 of 39%, 59% and 49%, respectively. On the other hand, verbascoside, luteolin and the total secoiridoids contents showed a significant reduction over the 75 days of storage: 38%, 27% and 47%, respectively. The loss of verbascoside and secoiridoids was associated with the increase of free OH-tyr and tyr, the main products deriving from the spontaneous hydrolysis occurring during the storage of P, as already observed under not standardised conditions (Cecchi *et al.*, 2018a). Interestingly, the analysis of P after hydrolysis indicates that total OH-tyr was subjected to a net degradation (23%) from t_0 to t_1, while a significant decrease was not observed until t_3. At the same time, tyr content was not affected until 75 days of storage. This could possibly be due to the lower reactivity of tyr in comparison to the ortho-diphenol OH-tyr in terms of scavenging capacity, which leads to a higher degradation of OH-tyr over time (Benavente-Garcia *et al.*, 2000). The same trend was observed for the CP extract. A significantly high increase was recorded for the content of OH-tyr glu (93%), OH-tyr (89%) and tyr (82%) from t_0 to t_3, suggesting that the coating favours the spontaneous hydrolysis of the precursors of these phenols in the sample. At the same time, in CP, verbascoside, luteolin and the total secoiridoids contents showed a reduction accounting for 17%, 24% and 41%, respectively. Interestingly, the reduction of verbascoside and secoiridoids was lower in comparison to that observed for P, while the opposite was expected to explain the high increase of OH-tyr glu, OH-tyr and tyr.

Analysis of the hydrolysed extracts highlights a lower degradation of total OH-tyr (16% from t_0 to t_3) while tyr, similarly to P, did not significantly vary.

These results might be due to a combination of different phenomena running at different rates. On one side, the hydrolysis of secoiridoidic compounds produced a net increase of the free forms of tyr and OH-tyr, but on the other, the decrease of the total OH-tyr observed after acid hydrolysis indicates that a degradation of this molecule occurred in both P and CP samples, even if with less intensity in CP. These findings confirm that the coating process contributed to the stability of OH-tyr over time.

Bioaccessibility of phenolic compounds by simulated *in vitro* digestion

The bioaccessibility of P and CP phenolic compounds was determined using a widely accepted *in vitro* gastrointestinal digestion procedure (Minekus *et al.*, 2014) which provides a useful alternative to animal and

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Table 2 Quantification of the phenolic content in pâté (P) and coated pâté (CP) in the gastric and intestinal phases before and after acidic hydrolysis

Phenolic compound	Sample	t_0	Gastric phase	Intestinal phase
OH-tyr glu	P	1.83 ± 0.01 b	0.45 ± 0.06 (25%) a	0.31 ± 0.01 (17%) a
	CP	1.74 ± 0.00 a	0.37 ± 0.03 (21%) a	0.30 ± 0.00 (17%) a
OH-tyr	P	2.61 ± 0.07 a	0.50 ± 0.12 (19%) a	0.32 ± 0.04 (12%) a
	CP	2.81 ± 0.02 a	0.39 ± 0.02 (14%) a	0.26 ± 0.01 (9%) a
Tyr glu	P	0.19 ± 0.01 a	0.11 ± 0.00 (60%) a	0.08 ± 0.00 (41%) a
	CP	0.19 ± 0.02 a	0.12 ± 0.00 (63%) a	0.07 ± 0.01 (38%) a
Tyr	P	0.69 ± 0.03 a	0.23 ± 0.00 (34%) a	0.16 ± 0.00 (24%) a
	CP	0.80 ± 0.02 b	0.24 ± 0.01 (29%) a	0.16 ± 0.02 (20%) a
OH-acteoside 1	P	0.32 ± 0.00 a	0.11 ± 0.01 (35%) a	0.07 ± 0.00 (21%) a
	CP	0.39 ± 0.02 b	0.16 ± 0.01 (41%) b	0.09 ± 0.00 (23%) b
OH-acteoside 2	P	0.28 ± 0.00 a	0.10 ± 0.01 (37%) a	0.07 ± 0.01 (26%) a
	CP	0.31 ± 0.02 a	0.09 ± 0.01 (29%) a	0.09 ± 0.00 (31%) a
Verbascoside	P	2.44 ± 0.06 a	0.19 ± 0.07 (8%) a	0.07 ± 0.00 (3%) a
	CP	2.25 ± 0.08 a	0.15 ± 0.02 (7%) a	0.06 ± 0.00 (3%) a
Luteolin	P	0.70 ± 0.02 a	0.06 ± 0.00 (8%) a	n.d.
	CP	0.71 ± 0.01 a	0.06 ± 0.00 (8%) a	n.d.
Secoiridoids	P	9.87 ± 0.13 a	2.27 ± 0.17 (23%) a	1.67 ± 0.04 (17%) a
	CP	10.57 ± 0.33 a	2.01 ± 0.12 (19%) a	1.59 ± 0.02 (15%) a
Total phenols	P	19.95 ± 0.34 a	4.40 ± 0.40 (22%) a	2.95 ± 0.08 (15%) a
	CP	20.87 ± 0.53 a	4.03 ± 0.24 (19%) a	2.83 ± 0.02 (14%) a
After hydrolysis				
Phenolic compound	**Sample**	t_0	**Gastric phase**	**Intestinal phase**
OH-tyr	P	13.08 ± 0.22 a	2.49 ± 0.40 (19%) a	1.41 ± 0.09 (11%) a
	CP	13.82 ± 0.66 a	2.09 ± 0.02 (15%) a	1.38 ± 0.01 (10%) a
Tyr	P	1.47 ± 0.01 a	0.52 ± 0.01 (36%) a	0.35 ± 0.01 (24%) a
	CP	1.52 ± 0.05 a	0.50 ± 0.00 (33%) a	0.41 ± 0.02 (27%) b
Sum of tyr + OH-tyr	P	14.55 ± 0.23 a	3.02 ± 0.39 (21%) a	1.76 ± 0.08 (12%) a
	CP	15.34 ± 0.72 a	2.59 ± 0.02 (17%) a	1.79 ± 0.00 (12%) a

Data are expressed as mg g^{-1} dried weight (DW) as mean ± standard deviation from three independent analyses. The recovery (%) value for each digestion phase is reported in round brackets. Different letters indicate significant differences ($p \leq 0.05$) between P and CP. OH-tyr, hydroxytyrosol; Tyr, tyrosol; glu, glucoside; n.d., non-detectable.

human models. In this model, the chemical composition of digestive fluids, pH and residence time simulates the human physiological conditions of the gastrointestinal tract. This kind of approach is used in many fields of food and nutritional science as human trials are often expensive, require considerable resources and may be ethically questionable (Alminger *et al*., 2014).

Table 2 shows the concentrations of the main phenolic compounds in P and CP before and after the simulated digestion, and the recovery percentages for both the gastric and intestinal phases.

The P sample showed a release in the gastric phase of about 22% of the total phenols, similarly to that observed for the CP (19%); no significant differences were detected between the two samples. After the intestinal phase, the recovery percentages were lower, with a bioaccessibility of 15% for P and 14% for CP, with no significant differences observed between the two samples. The same trend was observed for the AOC (Fig. 3) that showed a decrease of 45% for P and 49% for CP after the gastric phase; a further decrease of 20% for P and 6% for CP was noted after the intestinal phase. A high correlation was observed (>0.97) between these data and the content of phenolic compounds determined before and after the acidic hydrolysis. No significant differences were found between the P and CP in terms of AOC, meaning that the coating process did not affect the antioxidant capacity of the phenolic compounds subjected to digestion.

Regarding the individual phenols, hydroxytyrosol and its glucoside were the most abundant compounds found in both the gastric and intestinal fractions. The recovery of this compound in the gastric phase was 19% and 14% for P and CP, respectively, and lower values of 12% and 9% were recorded for the intestinal phase. Notably, tyrosol glucoside showed the highest

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recovery in the gastric phase, that is, about 60% both for P and CP, while a bioaccessibility of about 40% was registered after the intestinal phase. On the contrary, luteolin showed the lowest recovery at 8% after the gastric phase and non-detectable after the intestinal phase. A similar profile was observed for verbascoside which showed a 3% bioaccessibility after the intestinal phase. These results are partially in accordance with previous data in the literature. The recent work of Ribeiro *et al.*, (2020) dealt with evaluation of the stability of phenolic compounds and other nutrients from a freeze-dried fraction obtained by the centrifugation of olive pomace. The recovery (%) profile after gastrointestinal digestion showed a decrease for all phenolic compounds moving from the gastric to the intestinal phase, with verbascoside being non-detectable after digestion. Also OH-tyr showed scarce stability to the intestinal environment, with a recovery lower than 1%. This result is not in agreement with our data which showed a release of OH-tyr of about 10%. On the contrary, D'Antuono *et al.*, (2016) applied a different digestion protocol in a study on table olives cv *Bella di Cerignola* and reported a higher *in vitro* bioaccessibility of the total phenols, with OH-tyr and tyr as the most recovered molecules (i.e. 86% and 99%) and luteolin as the least (7%).

Rocchetti *et al.*, (2020), applying the same protocol as that used in our study, found only traces of luteolin after the digestion and observed a relatively low bioaccessibility for most of the phenols in five commercial EVOOs. Nevertheless, relatively high values of tyrosol equivalents were recorded during the pancreatic phase (on average, 66%), perhaps as a consequence of the hydrolysis of some precursors, like the oleuropein aglycone. Also in the study of Dinnella *et al.*, (2007) the recovery of tyr among 10 EVOO samples subjected to simulated digestion was very high (>90%).

The results reported by Ahmad-Qasem *et al.*, (2014) reveal that the digestion process significantly ($p \leq 0.05$) affected the composition and the total phenolic content of some olive leaf extracts; particularly, verbascoside and oleuropein showed a moderate resistance to gastric digestion, but their concentration during the intestinal phase dramatically decreased. In addition, the scarce bioaccessibility found for luteolin was partly explained by its poor solubility in acidic conditions.

In our study, both tyrosol and hydroxytyrosol showed a lower bioaccessibility compared to their glycosylated derivatives (Table 2). This could be related to the higher solubility of the glycosylated phenols in the aqueous solutions, such as simulated fluids. On the contrary, Ribeiro *et al.*, (2020), by analysing the same molecules, did not find significant differences among aglycones and glycosides.

Our results show that verbascoside displayed a poor bioaccessibility (3%), while its hydroxylated derivatives, that is, β-OH-acteosides 1 and 2, were characterised by higher recoveries, of 21% and 26% in P and 23% and 31% in CP, respectively. This could be due to the higher polarity of β-OH-acteosides in comparison to verbascoside, facilitating the release of the former in the simulated fluids, even though we cannot exclude different degradation kinetics as an explanation of the markedly lower recovery of verbascoside.

Our data showed lower recoveries of tyr and OH-tyr in the bioaccessible phases compared to those reported by other authors (Dinnella *et al.*, 2007; D'Antuono *et al.*, 2016; Rocchetti *et al.*, 2020). In particular, Rocchetti *et al.*, (2020) observed average bioaccessibility values for tyr of 53.2% and 65.2%, for gastric and intestinal phases, respectively. Moreover, as mentioned above, the authors reported a decline in the concentration of secoiridoids (oleuropein derivatives) during the digestion process, with an increase of OH-tyr concentration. Other authors also observed an increased recovery of OH-tyr and tyr due to the hydrolysis of secoiridoid derivatives after the digestion process in several virgin olive oils cultivars (Quintero-Florez *et al.*, 2018). Our results before and after hydrolysis (Table 2) did not confirm this trend with both total secoiridoids and OH-tyr content decreasing during the digestion.

The higher recovery values of tyr experienced by Rocchetti *et al.*, (2020) using the same digestion protocol can be reasonably explained by the fact that the authors analysed oil samples, for which partition of the phenolic compounds into the physiologic fluids is facilitated due to the liquid vehicle (oil) and a partial hydrophilic nature of these molecules. On the contrary, the release of phenolic compounds from a more complex matrix like P would be hampered by the interactions of the molecules with other constituents like proteins and fibres present in considerable amounts. The olive cultivar could also represent another reason for variability, as highlighted by Quintero-Flórez *et al.*, (2018) who found large differences in the bioaccessibility of phenolic compounds (from 8% to 36%) between different virgin olive oil varieties.

Overall, the lower recovery of OH-tyr compared to tyr can be explained taking into consideration its intrinsic fragility under physiologic digestion conditions. Pereira-Caro *et al.*, (2012) showed a loss of 20% for OH-tyr incubated under pancreatic conditions while gastric digestion did not bring about any modification. A possible reason for the decreased recovery of phenols moving from the gastric to the intestinal phase is that the conditions of the latter (neutral pH, presence of bile salts) could promote structural modifications, via oxidation and polymerisation, which may reduce their bioaccessibility (Yoshino *et al.*, 1999). In

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addition, olive phenolic compounds, in particular secoiridoids, can bind proteins with different affinities, and this contributes to explaining the low bioaccessibility measured. This result might suggest that these unrecovered phenols can pass into the colon and, as previously observed by the Simulator of Human Intestinal Microbial Ecosystem (SHIME® system), can positively interact with the human microbiota increasing *Lactobacillaceae* and *Bifidobacteriaceae* (Giuliani *et al*., 2019).

Finally, another possible explanation for the low recovery of phenolic compounds could be the digestion protocol used. Indeed, the simulated fluids-to-solid ratio in the Infogest Protocol applied here is lower when compared to other protocols (Dinnella *et al*., 2007; Ahmad-Qasem *et al*., 2014; D'Antuono *et al*., 2016). This parameter can limit the dissolution of the molecules, especially if not completely soluble in buffered solutions. Thus, the achievement of a generally lower recovery of secoiridoids and free phenols was not unexpected.

Conclusions

Our data showed that the phenolic compounds of pâté resulted partially affected by the accelerated stress conditions. The coating process proved to be an effective approach for the improvement of the storage stability of these compounds under standard conditions. Differently from previous studies on virgin olive oil or leaf extracts, a lower release of pâté phenolic compounds was observed during simulated digestion, even though the phenolic profiles of these matrices are very different from pâté. Interestingly, the protective coating did not reduce the bioaccessibility of pâté phenols. Despite the low bioaccessibility of the phenols in pâté, recent studies have shown significant positive effects after the intake of pâté in animal models and in humans. In conclusion, dry pâté represents a convenient, stable and easy to manipulate matrix which can be proposed as new functional ingredient/supplement, and less than half gram of dry pâté can cover the amount of total hydroxytyrosol and tyrosol needed to comply with the EFSA claim applied to EVOO. Further efforts in the evaluation of other coating materials could help to improve the bioaccessibility of the dry pâté phenolic compounds.

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Conflicts of interest

There are no conflicts of interest to declare.

Author contribution

Maria Bellumori: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Writing-original draft (equal). **Laura De Marchi:** Data curation (equal); Formal analysis (equal); Investigation (equal). **Federica Mainente:** Data curation (equal); Formal analysis (equal). **Francesca Zanoni:** Data curation (equal); Formal analysis (equal). **Lorenzo CECCHI:** Data curation (equal); Formal analysis (equal); Writing-original draft (equal). **Marzia Innocenti:** Conceptualization (equal); Writing-review & editing (equal). **Nadia Mulinacci:** Conceptualization (equal); Resources (equal); Supervision (equal); Writing-review & editing (equal). **Gianni Zoccatelli:** Conceptualization (equal); Funding acquisition (equal); Project administration (equal); Resources (equal); Supervision (equal); Writing-review & editing (equal).

Ethical approval

Ethics approval was not required for this research.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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