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Original article

Autochthonous white grape pomaces as bioactive source for functional jams

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Summary Seeds and skins from grape pomaces of Pecorello and Mantonico *cv* underwent extraction (ultrasound–assisted or maceration), in order to obtain added-value ingredients for the production of a functional pear jam. The antioxidant features of the extracts were tested by *in vitro* colorimetric assays. Among seeds, Mantonico by maceration (MSC) showed the best results, as well as Mantonico by ultrasound-assisted extraction (MBs) among skin extracts. The selected extracts were fully characterised by NMR and MS techniques, confirming the presence of many polyphenols, flavonoids and tannins among others. Pectin was then derivatised by the grafting procedure with the active molecules of MBs and MSC. The latter produced the best antioxidant polymer also without toxicity evaluated using Caco-2 cells and was used for the jam preparation. The functional pear jam showed improved antioxidant performances in comparison with its non-functional counterparts as well as its maintenance over time (15 days).

Keywords ESI-MS technique, functional food, grape pomace, jams, metabolomics NMR, pectin, polymeric conjugates, ultrasounds assisted extraction, white berries.

Introduction

Grape is one of the most consumed fruits in the world, representing with its derivatives (i.e. wine, grape juice, jams and raisins) one of the most important food crops under the economical point of view. Winemaking accounts for about 80% of the worldwide grape production, suggesting (Jelley *et al.*, 2016), that the wine supply chain is composed by the grape production and its oenological transformation followed by the packaging and trade of the obtained wines. Actually, wine industry is more complex and articulated, including distillation processes as well as by-products and wastes transformation to obtain new commodities and energy (European Regulation No. 555/2008), although there are growing new oenological applications for winemaking products. The latter aspect is of particular concern, as wine industry produces millions of tons of residues after fermentation whose storage, transformation, and/or elimination pose relevant economic and environmental problems (Beres *et al.*, 2017). Some applications have been reported for grape by-products, including the production of bioethanol from carbohydrates or the extraction of tartaric acid (Galanakis, 2012). More frequently, they are used for animal feeding, soil improver or biogas production (Lucarini *et al.*, 2018). The presence of bioactive compounds in grape waste, in particular polyphenols (Fontana *et al.*, 2013), which are well-known health-promoting agents, mainly for their antioxidant and antimicrobial properties (Xia *et al.*, 2014), highlights the possibility of its exploitation as a source of valuable molecules to be used as functional ingredients for the pharmaceutical, cosmetic and food industries (Yu & Ahmedna, 2013). Moreover, natural phenols obtained in such manner can be considered completely safe in comparison with synthetic antioxidants, widely used in the food industry but with undesirable effects on human health (Rockenbach *et al.*, 2011). Despite these advantages, there are many limitations in the practical employment of polyphenols as food industry additives. From the chemical point of view, they decompose in water or oxidised by heat and light, showing, at the same time, limited water solubility in their free form. Under the physiological point of view,

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they are rapidly metabolised and removed from the body. Besides, as most of them possess high molecular weights, their absorption can be quite low (Xia *et al*., 2014). All these factors can depress a lot their biological activities by decreasing their stability and bioavailability. Finally, many polyphenols show poor organoleptic features further diminishing their potential applications in the food field. To overcome these drawbacks, the administration of these compounds can be improved by the use of formulations in order to stabilise the phenolic structure, and at the same time enhancing water solubility and bioavailability (Parisi *et al*., 2014). In this sense, the conjugation of antioxidants to biomacromolecules, has been proved to reach the goal. This research focused on the recovery of bioactive compounds from grape wastes, seeds and skins, coming from the winemaking processes of Pecorello and Mantonico *cv*, two indigenous vine varieties cultivated in Calabria (southern Italy). Mantonico *cv* represents a historical heritage of Jonical coast that only recently raised interest from farmers and consumers. This *cv* is the autochthonous Locride's white berry. Pecorello *cv*, known locally from the end of 1800, instead is present in the Cosenza area. Suitable extraction methods have been evaluated and different *in vitro* tests confirmed the antioxidant activity suitable for food application. Then, the best extracts have been delivered as ingredients during the production of a new functional pear (*Pyrus communis)* jam, after conjugation with a polymeric matrix, namely pectin. The full chemical characterisation of the selected extracts was accomplished by NMR metabolomics and ESI-MS, two trustworthy methods able to detect the most important bioactives present in literature (Tartaglione *et al*., 2018) and responsible of antioxidant activity.

Materials and Methods

Samples

Grape marc samples were provided by Le Moire srl (ctr Strivillati Motta Santa Lucia, Catanzaro, Italy) of Dr. Paolo Chirillo (Latitude: 39°05′28″N - Longitude: 16°17′35″E - Altitude: 527 m). In particular, two kind of gape pomace were used, derived by Pecorello (P) and Mantonico (M) *cv*, harvested in September and October 2017, respectively, depending on the specific maturity stage of each cultivar. All the samples were stored under vacuum at −18°C, until use. All the reagents were purchased from Merck (Italy).

Extraction procedures

The extraction of grape pomaces was carried out by using reported methods for red grapes, including seeds and skins (Nawaz *et al*., 2006; Katalinic *et al*., 2010; Carrera *et al*., 2012). Briefly, skins (B) and seeds (S) were manually separated and individually treated. According to data reported in literature, two extraction methods were employed, namely "maceration" (C) and "ultrasound-assisted" (s) (see Table S1). Each extraction was performed in triplicate and data expressed as means (±SD).

Disposable phenolic groups by Folin–Ciocalteu procedure and determination of total antioxidant activity

Amount of total phenolic equivalents in the extracts, polymers and jams was determined using Folin-Ciocalteu reagent procedure (Pan *et al*., 2007). The recorded values ranged from 0.092 (PBs) to 1.710 (MSC), as reported in Table S2. Total antioxidant activity of each extract, conjugate and jam was evaluated following a literature protocol. (Spizzirri *et al*., 2011). Each extraction was performed in triplicate and data expressed as means (±SD).

Determination of scavenging activity on DPPH and ABTS radicals

Free radical scavenging properties of extracts, conjugates and jams, were estimated towards DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2′-azinobis(3-ethylbenzothiazoline-6-sulphonic acid)) radicals (Iemma *et al*., 2010; Restuccia *et al*., 2017). Each extraction was performed in triplicate and data expressed as means (±SD).

Mass spectrometry analysis

Samples MSC and MBs re-dissolved in methanol were injected (flow rate 5 μL min^{-1}) in the electrospray source of a LCQ DECA ion trap (ThermoFinnigan, Bremen, D) and a Orbitrap Q Exactive Plus (Thermo Fisher) at resolution of 30 000 and 140 000 FWHM@m/z 200, in positive and negative ion mode. MS^n product ion spectra have been carried out inside the ion trap with helium as collision gas, and higher-energy collisional dissociation (HCD) MS/MS spectra in the Orbitrap Q Exactive Plus by using nitrogen as collision gas at collision energy 18–30% arbitrary units (Giorgetti *et al*., 2017). ChemSpider (Royal Society of Chemistry) and Phenol-Explorer 3.5 (French National Institute for Agricultural Research) have been used as auxiliary tools for the identification of the compounds.

Nuclear magnetic resonance analysis

^{1}H-NMR, ^{1}H –^{1}H TOCSY homonuclear bidimensional and ^{1}H –^{13}C HSQC heteronuclear bidimensional experiments were performed on aliquots of MBs and MSC

samples in order to assess their chemical composition. The assignment of the resonances was performed by analysing ^{1}H and ^{13}C NMR characteristics and cross-correlated signals in 2D spectra (TOCSY and HSQC spectra) and by comparison with the literature compilations (Wishart *et al.*, 2013). Quantification of the identified compounds was performed by comparison of the signal integral with the reference one, and quantities were expressed in mg of compound normalised for the aliquot weight expressed in g.

Synthesis of conjugates

The synthesis of pectin conjugates (PMSC and PMBs) has been assessed following the general procedure according to a literature method, with some modifications (Parisi *et al.*, 2010). PB (Blank pectin polymer), exploited as a control, was prepared when grafting process was carried out in the absence of the extracts.

Pear jam preparation procedure

The preparation of jams was carried out by using fresh pears and PMSC, PB and PC (commercial pectin) as gelling agents following a modified procedure reported in literature (Igual *et al.*, 2013). Three jams were prepared labelled JMSC (jam prepared with PMSC), JPB (jam of PB) and JPC (jam of PC).

Imaging

Pectin polymers, blank and enriched with extracts were visualised to identify qualitative differences using an Olympus BX41 microscope, and the images were taken with CSV1.14 software, using a CAM XC-30 for image acquisition.

Cell viability assays

Human colorectal adenocarcinoma (Caco-2) cells were obtained from prof. Tiziano Verri (University of Salento, Italy). Cell viability was determined by using the MTT assay. The absorbance readings (570 nm) were used to determine the IC_{50} (μg mL^{-1}) using GraphPad Prism 7 Software (GraphPad Inc., San Diego, CA) (Aiello *et al.*, 2017).

Statistical analysis

The inhibitory concentration 50 (IC_{50}) was calculated by non-linear regression with the use of Prism GraphPad Prism, version 4.0 for Windows (GraphPad Software). One-way analysis of variance test (ANOVA) followed by a multicomparison Dunnett's test were applied.

Results and Discussion

Green extraction of phytochemicals

The yields of pomace samples treated by maceration were much higher than those related to ultrasound-assisted, although this latter method improves the penetration of the solvent into cellular material and leads a cleaner extraction, furnishing crude extract more soluble in the media used for *in vitro* assays (Novak *et al.*, 2008).

Evaluation of disposable phenolic groups by Folin-Ciocalteu procedure

The antioxidant potential of the extracts was influenced by cultivar characteristics, as well as the raw materials (seeds or skins) and the extraction methodologies (maceration or ultrasound-assisted). The values recorded for the different extracts showed best antioxidant activities in the extracts derived from the Mantonico variety, independently of the employed extraction technique, except for the PSs, that showed a phenolic content higher than the correspondent Mantonico sample (MSs). In general, lower amounts of phenolic compounds were detected in the skins (maximum value 0.140 meq GA/g of extract, for MBs), compared to the seed extracts. In the case of the skins extracts, the impact of the extraction technique on the phenolic content was negligible, even if the ultrasound-assisted procedure usually ensured slightly better results. On the contrary, seed extracts have proven to be richest in polyphenolic compounds, particularly when the extraction process was carried out by maceration. MSC samples (1.710 meq GA/g of extract) and PSC (0.800 meq GA/g of extract) provided the highest amounts of available phenol groups, showing values from 3 to 10 times higher than the extracts obtained from ultrasound-assisted extracted seeds.

Determination of total antioxidant activity

The results collected in Table S2 highlighted that a correlation between total polyphenol content and antioxidant capacity was not always observed. The seed extracts of both cultivars, with the exception of the MSs, returned the most significant values, emphasising the existence of a positive relationship between phenolic content and antioxidant capacity. Different behaviours were found for the peel extracts, showing a correlation only for MBs. These records confirmed that the antioxidant capacity of the natural extracts is related to total phenolic content (Novak *et al.*, 2008). However, qualitative difference of the phenolic compounds in the samples can be invoked as a

probable explanation of the poor relationship between available phenolic groups and total antioxidant activity.

Determination of scavenging effect on DPPH radical

The ability of the extracts to inhibit the DPPH radical was expressed in terms of IC_{50} (mg mL^{-1}), is shown in Table S2. A comparison of IC_{50} values shows that the skins undergoing an ultrasound-assisted extraction process lead to IC_{50} values one order of magnitude lower than the skins extracts by maceration methodology. As far as seeds are concerned, no uniformity can be found in the data, considering that the extracts characterised by lower IC_{50} value (MSs and PSC) were obtained through both extraction processes. By focusing on the sample with the lowest IC_{50} value (MSs with an IC_{50} of 0.012 mg mL^{-1}), it should highlight the absence of correspondence with the results of total antioxidant activity and available phenolic groups. This trend is typical of all the samples with the exception of PSC, confirming noteworthy antioxidant activity also against DPPH radical. These discrepancies could be justified by considering the different environment (organic and aqueous) in which the assays were performed.

Determination of scavenging effect on ABTS radical

The scavenging capacity of the extracts against the ABTS radical was expressed in terms of IC_{50} (mg mL^{-1}), as shown in Table S2. The analysis of the IC_{50} values displayed that the antioxidant capacity of the peel extracts has been at least two orders of magnitude higher that the extracts obtained from the seeds, while the extraction technique seems to have a limited influence on the molecules that specifically interact with the ABTS radical. MSC and MSs returned the lowest IC_{50} values (0.0017 and 0.0019 mg mL^{-1} respectively), partially confirming the data observed in the evaluation of scavenging activity against DPPH radical. A comparison of the collected data with the literature analyses appears very problematic due to the lack of information about the antioxidant activity of the extracts of the cultivars investigated in this study. Furthermore, a qualitative-quantitative comparison should take into account that many factors, including weather conditions, fruit ripening, soil and place of growth, largely affected antioxidants' distribution. An interesting study evaluating seven grape Apulian cultivars, showed differences in the total phenolic content of red and white grape skins (Giovanelli & Brenna, 2007). The obtained results supported the idea that the phenolic content of different grapes mainly depended on the different variety and not on the colour of the skins (Baiano *et al.*, 2009).

Molecular fingerprints of the extracts

After comparison of the data obtained from *in vitro* studies, the extracts MSC and MBs were selected and further analysed by ESI-MS. The mass spectra of the selected extracts (MSC and MBs), in terms of antioxidant properties, evidenced significant differences in metabolic profiles (Figure S1). Putative attributions of compounds, based on high resolution and tandem MS ESI (+) analysis of MBs (see Tables S1–S4 in SI), identified several sugar derivatives, including mono- and disaccharides and also several polyphenols such as hydroxyferulic acid, formononetin, (iso)sakuranetin (never recorded elsewhere), 76 results of probable flavonoids, coumaroyl-1,5-quinolactone, feruloyl tartaric acid and the coumarin glycoside esculin (MS: 363.0684, in wine) (Pantelić *et al.*, 2016). ESI (−) analysis also showed typical wine acids such as malic acid and tartaric acid, as well as glycosides and methylated glycosides. MS analyses of the seeds revealed many antioxidant compounds compared to those in the skins. Although in ESI (+) the identified compounds were only mono-, di- and tri- saccharides; ESI (−) analysis identified several antioxidant compounds such as catechin, leucofisetinidin, luteoforol, several tannins and other molecules with high m/z values (Tables S3–S6).

NMR characterisation of the extracts

The resonance assignment of ^{1}H NMR spectra of MBs and MSC was performed by the analysis of ^{2}D NMR experiments (TOCSY, HSQC) as reported in Figures S2–S7, and by comparing the observed chemical shifts with ones from literature data. From the analysis of NMR spectra, several molecule classes, including aminoacids, organic acids, carbohydrates and miscellaneous were identified and quantified (Table 1). Of particular interest is the presence of phenols such as Gallic acid, Salicylic acid, p-Coumaric acid, Catechin, Quercetin-3-O-Galactoside and Pyrogallol. The presence of molecules such as 2,3-butanediol, ethanol and methanol could be ascribed to fermentation processes.

Synthesis of antioxidant polymers

Pectin is a natural polymer commonly used for food applications, and considering its biodegradability and biocompatibility in physiological environments, it was chosen as polymer backbone to be functionalised with the antioxidant molecules contained in the extracts. In this context, the choice of the extract able to ensure the better performance plays a crucial role. The detailed analyses of the data concerning the antioxidant properties of the extracts highlight as MSC and MBs displayed the better characteristics in term of available phenolic groups and total antioxidant

Table 1 Quantitative analysis of MSC and MBs by ^{1}H NMR. The SD is 3% of the measured amount

	Molecule	MBs amonut (mg/g)	MSC amount (mg/g)
Aminoacids	Leucine	0.46	2.18
	Valine	0.61	1.99
	Isoleucine	0.33	1.46
	Threonine	0.30	2.12
	Alanine	0.50	1.77
	Proline	6.31	/
	Phenylalanine	0.40	/
Organic Acids	Lactic acid	0.70	2.11
	Sorbic acid	2.37	/
	Acetic acid	0.29	/
	Acetamide	0.34	/
	Quinic acid	13.44	/
	Succinic acid	0.93	3.45
	Malic acid	42.32	32.71
	Tartaric acid	6.99	/
	Shikimic acid	0.79	/
	p-Coumaric acid	0.50	6.87
	Gallic acid	0.24	/
	Salicylic acid	0.22	/
	Formic acid	0.15	0.34
Carbohydrates	Glucose	490.27	301.57
	Rhamnose	8.11	0.07
	Sucrose	4.29	87.82
	Raffinose	4.02	64.53
Miscellaneous	2,3-Butanediol	0.12	0.87
	Ethanol	2.49	6.51
	Methanol	0.37	3.54
	Glycosidated flavonoids (equivalents of Quercetin-3-glucoside)	5.58	76.23
	Catechin	0.91	34.30
	Pyrogallol	0.30	/
	Hydroxymethylfurfural	6.14	/
	Nicotinamide	0.14	0.40

capacity. In this sense, higher values of disposable phenolic equivalent ensure a relevant amount of antioxidant substrate in the grafting reaction. The synthetic strategy involved a biocompatible and water-soluble system based on ascorbic acid/H_2O_2 redox pair as radical initiators. The reaction between hydroxyl radicals and sensible residues in the side chains of pectin allows the activation of the polysaccharide towards radical reactions, promoting the insertion of antioxidant molecules. Literature data suggest that ortho- and para-positions relative to the hydroxyl group are the preferred target on the phenolic ring of the antioxidant molecule (Kobayashi & Higashimura, 2003). This synthetic strategy permitted to synthesise two conjugates, labelled PMSC and PMB_S, by pectin derivatisation with the active molecules in MSC and MBs, respectively. Unreacted species were removed by dialysis process (MWCO: 12–14 000 Da). Finally, lyophilisation process gained a porous material extensively characterised by antioxidant tests. To confirm the antioxidant properties of conjugates a control polymer (PB) was synthesised in the same conditions of the conjugates but without extract. In Fig. 1 are reported the images of PB, PMBs and PMSC, analysed at electronic microscope, to emphasise the qualitative characteristics.

Antioxidant properties of the conjugate polymers

Antioxidant properties of PMBs and PMSC were investigated performing the same assays employed for the extracts. The results concerning the polymers did not exactly follow the same trend of the correspondent extracts. Specifically, PMSC contains a lower amount of available phenolic groups (about 2 times) than PMBs, probably due to the poor reactivity during the grafting reaction of the phenolic compounds contained in MSC. However, the polyphenols present in MSC to the polymeric chain of pectin, ensured the best antioxidant and scavenging activities, both in aqueous and organic environment, clearly indicating in PMSC the most performing polymer. In particular, PMSC showed a total antioxidant activity of 0.241 meq CT/g of polymer, a value about 1.5 times higher than PMBs. The IC_{50} values showed as the scavenging activity of PMSC in an organic environment was of the same order of magnitude, but almost seven times greater than PMBs. This trend appeared more evident in aqueous environment and the inhibition profiles towards the ABTS radical appeared very different. In this case, the IC_{50} appeared to be optimal for PMSC, while at its maximum concentration, PMBs failed to reach 50% ABTS radical inhibition. Finally, PB did not show any interference giving negative results to all the used assays. The collected data clearly indicated PMSC as the best candidate able to guarantee the preparation of the functional jam.

Jams preparation and antioxidant properties

PMSC was proposed for the production of jam with improved antioxidant properties. The fruit chosen was pear because it contains low amount of antioxidant molecules (0.27–0.41 mg of phenols per grams of pulp), in comparison with other fruits (Reiland & Slavin, 2015). Specifically, in this work three jams were prepared, using PMSC, PB, PC. After 45 days of storage, the antioxidant properties of the obtained jams were investigated over time after the application of the extraction procedure described in the experimental section. Taking into account the average time of consumption of a jam (approximatively 2 weeks

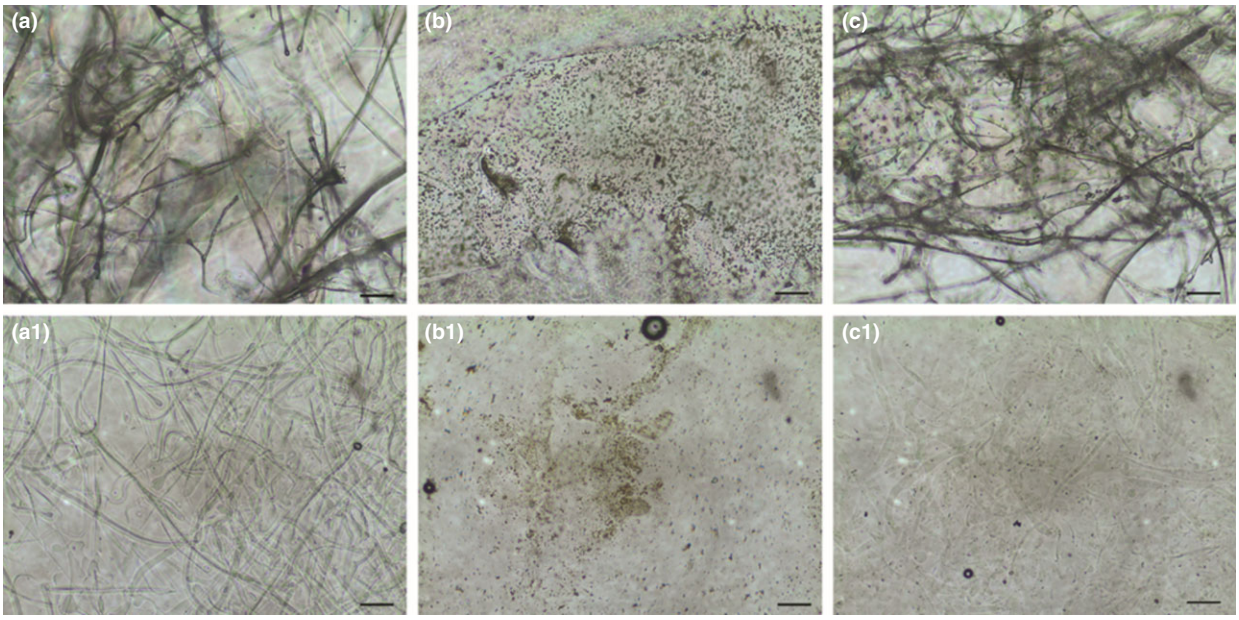

Figure 1 Optical mycroscope analyses of the pectin polymers. The named a, b, c images are Blank, MBs and MSC, while a1, b1 and c1 are the same ones but solubilised in glycerol. [Colour figure can be viewed at wileyonlinelibrary.com]

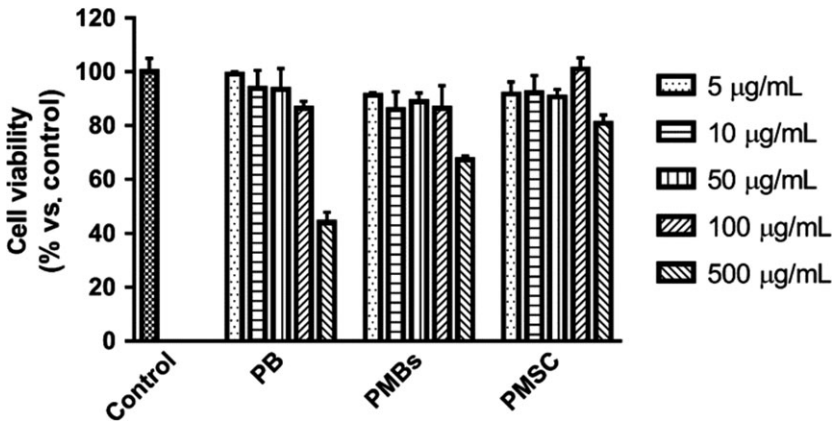

Figure 2 Viability assay in Caco-2 cell line.

after the opening of the jar), colorimetric tests were performed just after opening ($t = 0$ days) and then after a week and 15 days before opening. The results concerning the content of total phenolic groups, total antioxidant activity and scavenging properties in hydrophilic and lipophilic environments were displayed in Figure S8. At zero time, the analysis of the available phenolic groups, expressed as mg GA/g of jam, confirmed a considerable increase of this parameter, as consequence of PMSC use as gelling agent, respect to JPB and JPC, shows similar values to each other. In addition, JMSC had a higher total antioxidant activity and a better scavenging capacity, both towards the ABTS and DPPH radicals. In particular, in aqueous environment, JMSC showed to be very effective. Figure S8 showed the trend of over the 15 days of phenolic groups. In particular, JMSC sample underwent a slight decrease (2.5% after 7 days and 3.9% after fifteen) while, the jam prepared using PC showed a decrease three times higher after 7 days and almost one order of magnitude after 15 days. This finding clearly indicated that the presence of PMSC not only increased the total content of available phenolic groups, but allowed to maintain their concentration at high level over time. Analysing the total antioxidant activity, the same trend was

observed, with a decrease, after 15 days, of 5.3% for the JMSC matrix, which becomes, in the same period of time equal to 10.8% for JPC and JPB. However, the best results were recorded when the scavenging activity was monitored over time and the inhibition was particularly effective in aqueous environment against the ABTS species, compared to organic one. The data concerning IC_{50} extrapolated by the inhibition profiles towards the ABTS radical, showed that the enriched jam appeared more effective than the control matrices (JPC and JPB). The IC_{50} value recorded for JMSC was found to be about three times lower than JPC at $t = 0$ and four times after 15 days.

Cell viability

The confirmed stability over time of pear-jams, prompted us to further investigate if the polymers used as ingredients, were able to reduce the oxidative stress *in vivo*. In this sense before starting a pilot translational study, evaluation of viability of cells is suitable. In particular, the use of Caco-2 cell lines is a useful device because these cells are able to mimic the behaviour in the human gastro-intestinal tract. The viability was calculated at 5, 10, 50, 100 and 500 μg/mL to estimate if these polymers are able to induce toxic effects (Fig. 2). Any toxic effect was observed also at the highest dose of 500 μg/mL, demonstrating a safe use of these polymers for the development of functional food.

Conclusion

In this paper, wine-making wastes were used as suitable sources of bioactive components. In particular, the extracts were obtained by using pomaces of autochthonous Calabrian white grapes, Pecorello and Mantonico *cv*. Extractions were carried out in green conditions by using only hydro-alcoholic mixtures. Mass spectrometry and NMR analyses revealed the presence of several compounds including polyphenols and sugar derivatives, which confer to the extracts suitable antioxidant properties, making these an attractive source for the development of functional foods. In this context, a pectin polymer was developed to enrich pear jam. This jam demonstrated good antioxidant properties and a suitable use as food was demonstrated by using Caco-2 cell line.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Extraction conditions and yields of pomace samples treated by maceration and ultrasound-assisted extraction. (SD±3%).

Table S2. Antioxidant activity of extracts from skins and seeds of Mantonico and Pecorello *cv* and pectin-antioxidant conjugates.

Table S3. ESI (−) data with putative identification of most significant ions in MBs extract.

Table S4. ESI (+) data with putative identification of most significant ions in MBs.

Table S5. ESI (−) data with putative identification of most significant ions in MSC.

Table S6. ESI (+) data with putative identification of most significant ions in MSC.

Table S7. ^{1}H NMR resonance assignment of MBs and MSC.

Figure S1. ESI (+) and ESI (−) fingerprints of the extracts.

Figure S2. ^{1}H-NMR spectrum of MBs.

Figure S3. ^{1}H-NMR spectrum of MSC.

Figure S4. ^{1}H-^{1}H TOCSY NMR spectrum of MBs.

Figure S5. ^{1}H-^{1}H TOCSY NMR spectrum of MSC.

Figure S6. ^{1}H-^{13}C TOCSY NMR spectrum of MBs.

Figure S7. ^{1}H-^{13}C TOCSY NMR spectrum of MSC.

Figure S8. Available Phenolic group (a), total antioxidant activity (b), scavenging activity against DPPH (c) and ABTS (d) radicals of JMSC, JPB e JPC as a function of time.