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Research Paper

Profiling specialized metabolites of two *Malus domestica* Borkh. varieties: *In vitro* pulp callus culture *vs* fruit peel and pulp

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A R T I C L E I N F O

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A B S T R A C T

Malus domestica Borkh. (Rosaceae) comprises different varieties of commercially widespread apples around the world and available on the market all year round. Given their economic and traditional importance, chemical profile of these fruits was thoroughly investigated defining apples as a source of different classes of phytochemicals with interesting biological properties. Enhancing the production of these bioactive molecules by *in vitro* culture techniques is of great importance for avoiding problems due to their availability, but also to express selectively some metabolites. Based on previous results showing apple pulp callus culture as good source of pentacyclic triterpenic acids, the aim of this work was to investigate the specialized metabolites produced by optimized callus cultures starting from explants of pulp fruits of two apple varieties ('Annurca' and the still unexplored 'Mela Rosa del Montefeltro') compared to those of ripe fruit pulps and peels. LC-MS/MS analyses of fruit and callus hydroalcoholic extracts allowed the identification of 72 compounds, including hydroxycinnamic acids, catechins, flavonoids, and triterpenes. The qualitative profile of peels and pulps were very similar, while differences were observed in the callus extracts. Pulps were rich in phenols including phlorizin, catechin, and procyanidins; peels contained both phenols and triterpenic acids while callus extracts were characterized only by highly produced triterpenic acids, some of which were not found in the fruits. In conclusion, this study sheds light on how cell plant culture can be considered as an alternative system for producing specialized metabolites.

1. Introduction

Malus genus (Rosaceae family) includes different varieties of commercially widespread apples around the world and available on the market all year round (Perini et al., 2014). *Malus domestica* Borkh. is the most known and cultivated species, comprising many varieties. As a member of the Pomaceae subgroup (such as pear and quince), the fruit is a false fruit; indeed, the edible part, the pulp, does not derive from the ovary of *M. domestica* flower, but from the growth of its receptacle after ovary fertilization (Maugini et al., 2006) (in this paper, however, it will be defined as a fruit). Apples are a key component of the Mediterranean diet due their flavour and nutritional value as source of sugars, pectin, minerals, and crude fibers (Shalini and Gupta, 2010; Verardo et al., 2017). They are particularly appreciated by consumers as such, but also consumed in popular dishes and traditional medicine, and for the preparation of various kinds of food products as juices, vinegar, liqueur, cider, jam, fresh apple slices, dry, and canned apple (Rachana and Gupta, 2010; Vidović et al.; 2020). Given their economic and traditional importance, chemical and biological profile of these fruits was thoroughly investigated defining apples as a source of different specialized metabolites, including flavonoids (quercetin, catechin, phlorizin) and chlorogenic acid derivatives, known for their strong antioxidant activity (Boyer and Liu, 2004; Soler et al. 2009). In addition, many triterpenic acids were also detected in apples, such as maslinic, corosolic, pomolic (Sut et al.; 2019) and annurcoic acids, the latter isolated for the first time in *M. domestica* var. 'Annurca' (D'Abrosca et al., 2006). Triterpenic acids are specialized metabolites produced by plants, useful for human and animal health, since they could act as bioactive agents against inflammation (Andre et al., 2012; Kashyap et al., 2016; Deng et al., 2021), type 2 diabetes (Ding et al., 2018), liver diseases (Liu et al., 2021), and cholesterol accumulation (Hao et al., 2020). Nowadays, many efforts are carried out for the production of plant specialized metabolites with

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healthy properties through the *in vitro* cell cultures obtained from plant organs (Mihai et al., 2010; Gawri et al., 2013; Saradha et al., 2014; Zahedzadeh et al., 2015). The *in vitro* culture of plant tissues for the bioproduction of bioactive molecules can be considered as a renewable and economically viable technique (Espinosa-Leal et al., 2018). The plant material is continuously maintained in controlled environmental conditions not depending on seasonality and it is protected by environmental pollution, as well as from the attack of microorganisms and insects. Furthermore, much less water is required for the *in vitro* culture of plant cells compared to the cultivation of plants in the field. Interestingly, this technique contributes to the conservation of plant species important for the specialized metabolites production (da Silva Santos et al., 2022). The *in vitro* callus culture, particularly significant as a starter for cell culture in the factory on an industrial scale (Lystvan et al., 2018), is usually obtained starting from explants of cotyledons, leaves, petioles, roots, nodes, and internodes of plants. The fruit pulp has rarely been used as starting material for an *in vitro* culture to obtain specialized metabolites, with only a few reported examples in the literature (Oota et al. 1983; Nawa and Ohtani, 1992; Laezza et al., 2024). Even in the case of *M. domestica*, several authors referred *in vitro* culture protocols starting from different plant organs (Teixeira da Silva et al., 2019; Dibbisa and Yusuf, 2022). In our previous work, high yields of callus production were obtained starting from explants of two Italian *M. domestica* fruit varieties: “Mela Rosa Marchigiana” (typical of the Marche region, growing 400–900 m above sea level) and ’Golden Delicious’. The results showed that these calli produced higher amount of bioactive triterpenic acids *in vitro* than *in vivo* (Verardo et al., 2017). Considering the interesting biological activities of apple specialized metabolites (Laszczyk, 2009; Ayeleso et al., 2017) and continuing our previous studies on exploring pulp-derived callus, the aim of this work was to obtain *in vitro* the callus from explants of two Italian apple varieties fruit pulp: ‘Annurca’, a fine variety typical of the Campania region, and ‘Mela Rosa del Montefeltro’, an ancient sub-variety of ‘Mela Rosa Marchigiana’ cultivated since ancient times in the North of the Marche region named Montefeltro and recently come back into vogue. As a second aim, the quali-quantitative analysis of specialized metabolites produced by both calli was performed by high-resolution Orbitrap-based electrospray ionization source mass spectrometer (HR-Orbitrap/ESI-MS) and compared with the chemical profile of peel and pulp of both apple varieties. To our knowledge, the pulp-derived callus and the chemical profile of ‘Mela Rosa del Montefeltro’ was herein performed for the first time.

2. Materials and methods

2.1. Chemicals, reagents, and apparatus

Ultra-high performance liquid chromatography (UHPLC) grade solvents (MeOH, H_2O, and HCOOH) and all analytical grade solvents were obtained from VWR (Milano, Italy). Catechin, quercetin 3-*O*-glucoside, chlorogenic acid, and euscaphic acid standards were previously isolated from other plant extracts and characterized by 1D- and 2D-NMR, and MS experiments in our laboratory. UHPLC-HR-MS was carried out with a Vanquish Flex Binary pump coupled to a Q Exactive Plus Orbitrap-based FT-MS system equipped with an electrospray ionization (ESI) source (Thermo Fisher Scientific Inc., Bremen, Germany). The separation was obtained through C-18 Kinetex® Biphenyl column (100 × 2.1 mm, 2.6 μm particle size) provided of a Security Guard™ Ultra Cartridge (Phenomenex, Bologna, Italy). Data were processed with Xcalibur 4.1 software.

2.2. Fruit samples and calli culture

The ripe fruits of *Malus domestica* var. ‘Annurca’ were purchased from large-scale retailers in October 2019, from “Mela Annurca Campana IGP Rossa del Sud” - O. P. Giaccio Frutta, Vitulazio (CE), Italy (lot number: 0511G09445A350A350), while the ripe fruits of ‘Mela Rosa del Montefeltro’ were obtained from the company “Il Sorbo Vivai”, Montefiorentino di Frontino (PU), Italy. The two apple varieties were processed to start the callus culture, as reported below, on the same day of purchase and no post-purchase conservation treatment in the laboratory was used. The ‘Annurca’ variety is a prized Italian variety of apple typical of the Campania region. The harvest of these fruits, still unripe, must begin around mid-September to prevent them from rotting and falling to the ground as they are deciduous. The ripening phase, called "redness", begins immediately with the exposure of the fruits to the sun on straw supports for 10–15 days and periodic watering with water. The ‘Mela Rosa del Montefeltro’ variety is one of the oldest Italian apples, medium sized, flattened circular in shape, with a very short stalk. The name Rosa (pink) does not derive from the colour of the skin, but from the scent, which recalls that of the rose flower. It is harvested around mid-October and stored at room temperature until the following spring.

The pulps of both apple varieties were used as source of explants for *in vitro* callus induction. Before creating the pulp explants, the fruits were sterilized on the surface by washing in ethanol and then flambéed under a sterile laminar flow hood. The apples were then opened using a sterile scalpel and discs of 5 mm in diameter and about 3 mm in height were taken as explants at 0.5 cm from the peel (Verardo et al, 2017). Pulp explant cultures were first performed with ‘Annurca’ using two types of medium: Murashige and Skoog and Gamborg B5 both with the addition of 30 g/L sucrose, and supplemented with 2-isopentenyladenine (2iP, Merk D7660) 2.5 μM, plus naphthaleneacetic acid (NAA, Merk N0640) 5.0 μM, or 2.5 μM 2iP alone or 5.0 μM NAA alone. The culture medium was then brought to pH 5.8 before adding agar (0.8%), autoclaved for sterilization (120 °C, 104 kPa) for 20 min and then distributed in Petri dishes in the amount of 30 mL for each. The cultures were then incubated at 25 ± 2 °C. Callus subcultures were done every 28 days in the same culture media. The quantities of callus formed were recorded after the first two months of culture in accordance with Simoes et al. (2009) by recording the callus fresh weight (FW) and dry weight (DW) in each experimental condition. Data regarding callus biomass production were reported as the mean ± standard deviation of FW and DW in each tested culture condition. The differences between the callus biomasses produced were analysed by the statistical software MSTAT-C with the Tukey test at a 5% level of significance. The optimal culture conditions found for obtaining the callus from the ripe apple pulp were maintained to achieve material by implementing sub-cultures every 28 days in fresh culture medium. Explants of ‘Mela Rosa del Montefeltro’ ripe pulp from fruits were cultured in the same medium selected for ‘Annurca’ apple. The material was kept at -20 °C in a freezer until freeze-drying for chemical analysis.

In order to perform comparative chemical analysis between callus produced metabolites and naturally found in the fruits, peels and pulps of ‘Annurca’ and ‘Mela Rosa del Montefeltro’ apples were cut into pieces, immediately freeze-dried, and stored in airtight dark glass jars until further testing.

2.3. Extraction procedure

Freeze-dried calli, peels and pulps of both apple varieties were all extracted under the same conditions. 500 mg of each sample were subjected to ultrasound-assisted extraction (LBS2 bath, Falc Instruments s.r.l., Treviglio, Italy) with 10 mL of a EtOH:H_2O (8:2 v/v) mixture for 15 min at 20 °C (solid:liquid ratio of 1:20 g/mL). 5 mL of all obtained extracts were withdrawn, filtered using Phenex™ Teflon® (PTFE) filter membranes (0.45 μm pore size, 47 mm diameter) and 3 μL of supernatants were injected in triplicate through UHPLC-HR-Orbitrap/ESI-MS.

2.4. LC-HR-Orbitrap/ESI-MS chemical analyses of the apple extracts

2.4.1. Qualitative analysis

The metabolomic fingerprint of the hydroalcoholic extracts of calli,

Table 1
Callus biomass production after 60 days from *in vitro* culture of 'Annurca' apple ripe fruit pulp.

Media and growth regulators (μM)	Fresh weight[a] (g)	Dry weight[a] (g)
MS (Murashige and Skoog)		
5.0 NAA + 2.5 2iP	14.42 ± 0.59[a]	0.51 ± 0.05[a]
5.0 NAA	2.16 ± 0.92[b]	0.11 ± 0.03[b]
2.5 2iP	No callus	No callus
B5 (Gamborg B5)		
5.0 NAA + 2.5 2iP	7.15 ± 1.02[a]	0.27 ± 0.05[a]
5.0 NAA	2.33 ± 0.62[b]	0.11 ± 0.02[b]
2.5 2iP	No callus	No callus

[a] Data represent mean ± standard deviation; n = 9 repetition. In each column, for each culture medium, the values with the different letters are significantly different by the Tukey test at 5% of significance. NAA = naphthaleneacetic acid; 2iP = 2-isopentenyladenine.

Table 2
Callus biomass production after 60 days from *in vitro* culture of 'Mela Rosa del Montefeltro' ripe fruit pulp, in the same culture medium selected to produce callus from 'Annurca' apple pulp.

Media and growth regulators (μM)	Fresh weight a (g)	Dry weight a (g)
MS (Murashige and Skoog)		
5.0 NAA + 2.5 2iP	10.58 ± 0.29	0.24 ± 0.09

NAA = naphthaleneacetic acid; 2iP = 2-isopentenyladenine.

peels, and pulps of both apple varieties were obtained through LC-MS/MS analyses. The elution was performed using a mixture of HCOOH in H_2O 0.1% v/v (solvent A) and HCOOH in MeOH 0.1% v/v (solvent B), a linear gradient of 5 to 100% of solvent B in 26 min (flow rate 0.5 mL/min). The HR-MS spectra were acquired both in positive and negative ion mode, within a *m/z* scan range of 135–2000. The column and autosampler temperature were kept at 35 and 4 °C, respectively. The MS operated in full MS/MS scan (70,000 resolution, maximum injection time 220 ms) and data dependent mode (17,500 resolution, maximum injection time 60 ms). The ionization parameters were optimized using data as reported previously (Di Stasi et al., 2023).

2.4.2. Quantitative analysis

For the quantitative analysis of the main phenolic compounds and triterpenes, the chromatographic method was optimized for each class of compounds. In particular, the separation of phenolic compounds was performed with HCOOH in H_2O 0.1% v/v (solvent A) and HCOOH in MeOH 0.1% v/v (solvent B) (flow rate 0.5 mL/min) and a gradient as follows: 0–1.5 min, 5% B isocratic mode; 1.5–9.5 min, 5–40% B; 9.5–11.5 min, 40% B isocratic mode. The HR-MS spectra were acquired in negative ion mode (1.5 min starting acquisition), within a *m/z* scan range of 135–2000. The elution of triterpenic acids was carried out with the same mobile phase and flow rate but a different gradient: 0–1.5 min, 55% of B; 1.5–8 min, 55–65% of B; 8–14 min isocratic mode at 65% of B; 14–19 min, 65–75% of B; 19–23 min, 75–85% of B; 23–25.5 min, 85–100% of B. The HR-MS spectra were acquired in positive ion mode (1.5 min starting acquisition), within a *m/z* scan range of 420–700. The ionization parameter for both the methods were optimized as cited before. In addition, four calibration curves were constructed with quercetin 3-*O*-glucoside (concentration range of 0.05–0.000048 mg/mL), catechin (concentration range of 0.1–0.00039 mg/mL), chlorogenic acid (concentration range of 0.05–0.00019 mg/mL), and euscaphic acid (concentration range of 0.2–0.00019 mg/mL) used as external standard for the quantification of flavonoids, catechins, hydroxycinnamic acids, and triterpenes, respectively. Stock solutions of 1 mg/mL of each standard were first prepared and then the different range of concentrations was obtained in triplicate using serial dilutions. Calibration curves displayed a good linearity for all the entire concentration range with a correlation coefficient (R^2) of 0.9912 for quercetin 3-*O*-glucoside, 0.9988 for chlorogenic acid, 0.9907 for catechin, and 0.9887 for euscaphic acid. Microsoft® Office Excel was used for processing data and the amount of each compound was finally expressed as mg/g of DW ± standard deviation (SD).

3. Results

3.1. Callus induction

The explants of 'Annurca' and 'Mela Rosa del Montefeltro' apple fruit pulp formed with friable callus on the upper face and along the cutting surface in both MS and B5 culture media containing NAA (5.0 μM) plus 2iP (2.5 μM) or NAA (5.0 μM) alone. The two-culture media containing 2iP (2.5 μM) alone didn't induce callus formation, and the explants died after about 10 days of culture after turning dark in colour. The combination of MS medium with 5.0 μM NAA and 2.5 μM 2iP induced the highest biomass production of callus from 'Annurca' apple ripe pulp explants (Table 1). These plant growth combinations were maintained, and subcultures were grown every 28 days to obtain a sufficient amount of plant material for extractions and analysis *in vitro*. The callus obtained in MS and B5 media containing NAA alone (5.0 μM) grew with difficulty and could not be used for biomass production since its growth was too slow and an insufficient amount of cellular material was produced. In the same culture medium selected for the 'Annurca' apple pulp explants, the 'Mela Rosa del Montefeltro' ones also reacted and started the production of white and crumbly callus (Table 2), which was subcultured and treated in the same way of the 'Annurca' callus.

3.2. Chemical fingerprint of apples fruits and calli extracts

The metabolomic fingerprint of peel, pulp, and callus extracts of both apple varieties was obtain through UHPLC-HR-Orbitrap/ESI-MS analysis, registered in both positive and negative ion mode. Metabolites were tentatively identified by comparison of full mass spectra, fragmentation patterns, and measured accurate mass with data reported in literature, considering a mass error <5 ppm on the experimental molecular formula. A total of 72 compounds were found, including hydroxycinnamic acids, catechins, flavonoids, and triterpenes (Table 3). As shown in Fig. 1, the qualitative chemical profiles of peels and pulps of both apple varieties were very similar sharing most of the metabolites, while differences were observed in both callus extracts. Phenolic compounds were detected only in the fruit extracts at low retention times (4–12 min), while triterpenes were represented in all chromatograms at high retention times (17–23 min). Among phenols, 47 compounds (1–47) were tentatively identified in peels and pulps and classified as flavonoids, hydroxycinnamic acids, and (epi)catechin derivatives. According to previous studies (Sommella et al., 2015), flavonoids were glycosides of isorhamnetin, kaempferol, quercetin, and phlorizin, while hydroxycinnamic acids were identified as ferulic, coumaric, and quinic acids derivatives (Cioni et al., 2023). In addition, catechin (1) and epicatechin (11) were detected in both *Malus* varieties, together with 23 procyanidins mostly annotated as dimers, trimers, and oligomers (Karonen et al., 2004; Navarro-Hoyos et al., 2021). A cyanidin hexoside (8), an anthocyanin, was detected only in 'Annurca' peel as expected due its red colour.

The callus cultures obtained from the pulp of both apple varieties displayed a limited number of phenolic compounds, in contrast with their prevalence in both peels and pulps. On the other hand, as previously reported by Verardo et al. 2017, a higher number of triterpenic acids were detected in calli than in peels and pulps. In detail, a total of twenty-five triterpenes were tentatively identified in positive ion mode, with three of them (49, 54, and 55) detected only in calli and two of them (63 and 66) detected only in peels. The most represented compounds were hydroxylated ursolic acid derivatives, while only two compounds (49 and 62) were annotated as hydroxylated derivatives of oleanolic acid. Among ursolic acid derivatives, compounds 51, 52, 53,

Table 3
Mass spectra data (positive and negative ion mode) of compounds detected and tentatively identified in peel, pulp, and callus extracts of *Malus domestica* var. 'Annurca' (MANN) and var. 'Mela Rosa del Montefeltro' (MRM). Some peaks remained unidentified.

Peak[a]	t_R	Compound	Formula	[M–H]⁻	[M+H]⁺	Product ion[b,c]	Error (ppm)[b]	Extracts[d]
Flavonoids								
10	5.9	Kaempferol/Luteolin hexoside	$C_{21}H_{20}O_{11}$	447.0937	449.1076	285.04	+ 0.91	A
33	8.7	3-Hydroxyphloretin 2-*O*-xylosylglucoside	$C_{26}H_{32}O_{15}$	583.1676	585.1813	289.07, 271.06	+ 1.30	A, B, D
36	9.4	Quercetin 3-*O*-glucopyranoside	$C_{21}H_{20}O_{12}$	463.0886	465.1026	300.03	+ 0.86	A, B, D, E
37	9.4	Rutin	$C_{27}H_{30}O_{16}$	609.1469	611.1600	301.03, 300.03, 271.02	+ 1.30	A
38	9.9	Phloretin pentosylhexoside (isomer I)	$C_{26}H_{32}O_{14}$	567.1725	569.1865	273.07, 167.03	+ 1.00	A, B, D, E
39	10.1	Quercetin pentoside (isomer I)	$C_{20}H_{18}O_{11}$	433.0780	435.0923	300.03	+ 0.85	A, B, D, E
40	10.1	Phloretin pentosylhexoside (isomer II)	$C_{26}H_{32}O_{14}$	567.1725	569.1865	273.07, 167.03	+ 1.00	A, B, D, E
41	10.4	Phlorizin	$C_{21}H_{24}O_{10}$	481.1355, ([M+HCOO]⁻)	437.1442	273.07, 167.03	+ 0.85	A, B, D, E
42	10.5	Quercetin deoxyhexoside	$C_{21}H_{20}O_{11}$	447.0936	449.1064	300.03, 255.03	+ 0.73	A, B, D, E
43	10.5	Quercetin pentoside (isomer II)	$C_{20}H_{18}O_{11}$	433.0780	435.0909	300.03	+ 0.91	A, B, D, E
44	11.0	Isorhamnetin hexoside	$C_{22}H_{22}O_{12}$	477.1042	479.1185	315.05, 300.03, 285.04	+ 0.73	A, D
45	11.4	Kaempferol/Luteolin pentoside	$C_{20}H_{18}O_{10}$	417.0828	419.0974	285.04, 284.04, 255.03	+ 0.19	A, D
46	12.0	Kaempferol/Luteolin acetyl hexoside	$C_{23}H_{22}O_{12}$	489.1044	491.1193	285.04, 284.04, 255.03	+ 1.12	A
47	12.0	Isorhamnetin pentoside	$C_{21}H_{20}O_{11}$	447.0937	449.1079	314.04; 299.02; 271.02	+ 0.91	A
Hydroxycinnamic acids								
3	4.8	Dihydrocoumaroyl hexoside	$C_{15}H_{20}O_{8}$	327.1088	329.0051	165.05; 147.04	+ 0.79	A, B, D, E
4	4.8	*p*-Coumaric acid hexoside (isomer I)	$C_{15}H_{18}O_{8}$	325.0926	327.0081	187.04; 163.04; 145.03	– 0.89	A, B, D, E
5	4.8	Chlorogenic acid (isomer I)[e]	$C_{16}H_{18}O_{9}$	353.0879	355.1023	191.05, 173.04	+ 0.25	A-F
7	5.4	*p*-Coumaric acid hexoside (isomer II)	$C_{15}H_{18}O_{8}$	325.0926	327.0081	187.04; 163.04; 145.03	– 0.89	A, B, D, E
13	6.5	Coumaroylquinic acid (isomer I)	$C_{16}H_{18}O_{8}$	337.0932	339.1074	191.05, 173.04; 163.04	+ 0.92	A, B, D, E
14	6.5	Chlorogenic acid (isomer II)	$C_{16}H_{18}O_{9}$	353.0879	355.1023	191.05, 173.04	+ 0.25	A-F
18	6.9	Ferulic acid hexoside	$C_{16}H_{20}O_{9}$	355.1038	357.1182	193.05, 175.04	+ 0.96	A, B, D, E
25	7.5	Coumaroylquinic acid (isomer II)	$C_{16}H_{18}O_{8}$	337.0932	339.1074	191.05, 173.04; 163.04	+ 0.92	A, B, D, E
Anthocyanins								
8	5.4	Cyanidin hexoside	$C_{21}H_{21}O_{11}$		449.10730, [M]⁺	287.05, 241.05, 187.04	+ 0.53	A
Catechins and Procyanidins								
1	4.1	Catechin[e]	$C_{15}H_{14}O_{6}$	289.0718	291.0862	245.08, 109.03	+ 0.14	A, B, D, E
2	4.1	Procyanidin dimer (isomer I)	$C_{30}H_{26}O_{12}$	577.1353	579.1498	559.12, 451.10, 425.09, 289.07; 125.02	+0.26	A, B, D, E
6	4.9	Procyanidin tetramer (isomer I)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]²⁻)	578.1430 ([M + H]²⁺)	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
9	5.4	Procyanidin trimer (isomer I)	$C_{45}H_{38}O_{18}$	865.1995	867.2131	577.13, 407.07, 289.07, 287.06, 125.02	+ 1.11	A, B, D, E
11	5.9	Epicatechin	$C_{15}H_{14}O_{6}$	289.0718	291.0862	245.08, 109.03	+ 0.14	A, B, D, E
12	5.9	Procyanidin dimer (isomer II)	$C_{30}H_{26}O_{12}$	577.1353	579.1496	559.12, 451.10, 425.09, 289.07; 125.02	+ 0.26	A, B, D, E
15	6.5	Procyanidin trimer (isomer II)	$C_{45}H_{38}O_{18}$	865.1995	867.2131	577.13, 407.07, 289.07, 287.06, 125.02	+ 1.11	A, B, D, E
16	6.5	Procyanidin pentamer (isomer I)	$C_{75}H_{62}O_{30}$	720.1595 ([M – H]²⁻)	722.1738 ([M + H]²⁺)	575.12, 451.10, 425.09, 289.07, 125.02	+ 0.68	A, B, D, E
17	6.5	Procyanidin tetramer (isomer II)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]²⁻)	578.1430 ([M + H]²⁺)	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
19	6.9	Procyanidin tetramer (isomer III)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]²⁻)	578.1430 ([M + H]²⁺)	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
20	6.9	Procyanidin hexamer (isomer I)	$C_{90}H_{74}O_{36}$	864.1923 ([M – H]²⁻)	866.2050 ([M + H]²⁺)	577.13, 575.13, 425.08, 407.07, 287.05	+ 1.84	A, B, D, E
21	6.9	Procyanidin pentamer (isomer II)	$C_{75}H_{62}O_{30}$	720.1595 ([M – H]²⁻)	722.1738 ([M + H]²⁺)	575.12, 451.10, 425.09, 289.07, 125.02	+ 0.68	A, B, D, E
22	7.2	Procyanidin trimer (isomer III)	$C_{45}H_{38}O_{18}$	865.1995	867.2131	577.13, 407.07, 289.07, 287.06, 125.02	+ 1.11	A, B, D, E
23	7.2	Procyanidin tetramer (isomer IV)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]²⁻)	578.1430	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
24	7.2	Procyanidin pentamer (isomer III)	$C_{75}H_{62}O_{30}$	720.1595 ([M – H]²⁻)	722.1738 ([M + H]²⁺)	575.12, 451.10, 425.09, 289.07, 125.02	+ 0.68	A, B, D, E
26	7.5	Procyanidin trimer (isomer IV)	$C_{45}H_{38}O_{18}$	865.1995	867.2131	577.13, 407.07, 289.07, 287.06, 125.02	+ 1.11	A, B, D, E
27	7.5	Procyanidin tetramer (isomer V)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]²⁻)	578.1430 ([M + H]²⁺)	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
28	7.9	Procyanidin pentamer (isomer IV)	$C_{75}H_{62}O_{30}$	720.1595 ([M – H]²⁻)	722.1738 ([M + H]²⁺)	575.12, 451.10, 425.09, 289.07, 125.02	+ 0.68	A, B, D, E
29	7.9	Procyanidin tetramer (isomer VI)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]²⁻)	578.1430 ([M + H]²⁺)	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
30	7.9	Procyanidin hexamer (isomer II)	$C_{90}H_{74}O_{36}$	864.1923 ([M – H]²⁻)	866.2050 ([M + H]²⁺)	577.13, 575.13, 425.08, 407.07, 287.05	+ 1.84	A, B, D, E
31	8.7	Procyanidin hexamer (isomer III)	$C_{90}H_{74}O_{36}$	864.1923 ([M – H]²⁻)	866.2050 ([M + H]²⁺)	577.13, 575.13, 425.08, 407.07, 287.05	+ 1.84	A, B, D, E
32	8.7	Procyanidin pentamer (isomer V)	$C_{75}H_{62}O_{30}$	720.1595 ([M – H]²⁻)	722.1738 ([M + H]²⁺)	575.12, 451.10, 425.09, 289.07, 125.02	+ 0.68	A, B, D, E
34	9.0	Procyanidin hexamer (isomer IV)	$C_{90}H_{74}O_{36}$	864.1923 ([M – H]²⁻)	866.2050 ([M–H]²⁺)	577.13, 575.13, 425.08, 407.07, 287.05	+ 1.84	B, D, E

(*continued on next page*)

Table 3 (*continued*)

Peak[a]	t_R	Compound	Formula	[M–H]⁻	[M+H]⁺	Product ion[b,c]	Error (ppm)[b]	Extracts[d]
35	9.0	Procyanidin tetramer (isomer VII)	$C_{60}H_{50}O_{24}$	576.1275 ([M – H]$^{2-}$)	578.1430 ([M + H]$^{2+}$)	575.12, 451.10, 289.07, 125.02	+ 0.31	A, B, D, E
Triterpenes								
48	17.4	2,25-Epoxy-2α,3β,19α-trihydroxy-urs-12-en-28-oic acid Cuneataol	$C_{30}H_{46}O_6$	501.3227	503.3364	485.32, 467.31, 457.34, 439.32, 421.31, 403.30	– 0.43	A, C, D, F
49	17.8	2β,3β,16α-Trihydroxyolean-12-en-23,28-dioic acid Zanhic acid	$C_{30}H_{46}O_7$	517.3174	519.3317	501.32, 455.31, 437.30, 409.31, 391.30	+ 0.13	C, F
50	18.1	3,25-Epoxy-2β,3α,19α-trihydroxy-urs-12-en-28-oic acid Pomaceic acid	$C_{30}H_{46}O_6$	501.3225	503.3364	485.32, 467.31, 457.34, 439.32, 421.31, 403.30	– 0.43	A, C, D, F
51	18.2	Trihydroxy-urs-12-en-28-oic acid Euscaphic acid[e]	$C_{30}H_{48}O_5$	487.3433	489.3571	471.34, 453.33, 435.32, 425.32, 407.33	– 0.72	A, C, D, F
52	18.4	Trihydroxy-urs-12-en-28-oic acid Arjunic acid	$C_{30}H_{48}O_5$	487.3433	489.3571	471.34, 453.33, 435.32, 425.32 407.33, 205.16	– 0.72	A-F
53	18.6	Trihydroxy-urs-12-en-28-oic acid Tormentic acid	$C_{30}H_{48}O_5$	487.3433	489.3571	471.34, 453.33, 435.32, 425.32, 407.33, 201.16	– 0.72	A-F
54	19.1	Carboxyursolic acid	$C_{30}H_{44}O_6$	499.3070	501.3208	483.31, 465.30, 455.32, 437.30, 419.30	+ 1.00	C, F
55	19.4	3β,19α-Dihydroxy-2-oxours-12-en-28-oic acid Pirolonic acid	$C_{30}H_{46}O_5$	485.3277	487.3416	469.33, 451.32, 441.34, 423.33, 405.31	– 0.41	C, F
56	19.8	1α,19α-Dihydroxy-3-oxours-12-en-28-oic acid Annurcoic acid	$C_{30}H_{46}O_5$	485.3276	487.3414	469.33, 451.32, 441.34, 423.33, 405.31	– 0.82	A-F
57	20.1	3β,19α-Dihydroxy-urs-12-en-28-oic acid Pomolic acid	$C_{30}H_{48}O_4$	471.3482	473.3626	455.35, 437.34, 409.35, 391.34, 191.18	+ 0.13	A-F
58	20.3	Trihydroxy-urs-12-en-28-oic acid Epitormentic acid	$C_{30}H_{48}O_5$	487.3435	489.3571	471.34, 453.33, 425.34, 201.16	– 0.72	A-F
59	20.7	3-Oxo-dihydroxy-urs-12-en-28-oic acid	$C_{30}H_{46}O_5$	485.3277	487.3414	469.33, 451.32, 441.34, 423.33, 405.31	– 0.82	A-F
60	20.7	Dihydroxy-urs-12-en-28-oic acid (isomer I)	$C_{30}H_{48}O_4$	471.3482	473.3626	455.35, 437.34, 427,36, 409.35, 391.33, 205.16	+ 0.13	A-F
61	20.8	3β-*p*-Coumaroyloxy-dihydroxyurs-12-en-28-oic acid (isomer I)	$C_{39}H_{54}O_7$	633.3807	635.3936	453.33	– 0.99	A-F
62	21.1	2α,3β-Dihydroxy-olean-12-en-28-oic acid Maslinic acid	$C_{30}H_{48}O_4$	471.3482	473.3626	455.35, 437.34, 427.36, 409.35, 391.33, 205.16	+ 0.13	A-F
63	21.1	3β-*p*-Coumaroyloxy-dihydroxyurs-12-en-28-oic acid (isomer II)	$C_{39}H_{54}O_7$	633.3807	635.3936	453.33	– 0.99	A
64	21.3	2α,3β-Dihydroxy-urs-12-en-28-oic acid Corosolic acid	$C_{30}H_{48}O_4$	471.3482	473.3626	455.35, 437.34, 427.36, 409.35, 391.33, 205.16	+0.13	A-F
65	21.6	Dihydroxy-urs-12-en-28-oic acid (isomer II)	$C_{30}H_{48}O_4$	471.3486	473.3626	427.36, 409.35	+ 0.13	A-F
66	21.8	3β-*p*-Coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid (isomer I)	$C_{39}H_{54}O_6$	617.3853	619.3985	437.34; 147.04	+ 0.14	A; B
67	21.8	Oxo-hydroxy-urs-12-en-28-oic acid (isomer I)	$C_{30}H_{46}O_4$	469.3326	471.3471	453.34, 435.32, 425.34, 407.33	+ 0.45	A-F
68	22.2	Oxo-hydroxy-urs-12-en-28-oic acid (isomer II)	$C_{30}H_{46}O_4$	469.3324	471.3471	453.34, 435.32, 425.34, 407.33, 201.16	+ 0.45	A-F
69	22.4	Ursolic/Oleanolic acid	$C_{30}H_{48}O_3$	455.3533	457.3676	439.36, 411.36, 393.35	– 0.04	A, C, D, F
70	22.8	Oxo-urs-12-en-28-oic acid (isomer I)	$C_{30}H_{46}O_3$	453.3381	455.3519	437.34, 409.35, 313.25, 189.16	– 0.15	A, C, D, F
71	22.8	3β-*p*-Coumaroyloxy-2α-hydroxy-urs-12-en-28-oic acid (isomer II)	$C_{39}H_{54}O_6$	617.3856	619.3985	437.34; 147.04	+ 0.14	A, C, D, F
72	23.3	Oxo-urs-12-en-28-oic acid (isomer II)	$C_{30}H_{46}O_3$	453.3381	455.3519	437.34, 409.35, 313.25	– 0.15	A, C, D, F

[a] Peaks are listed by their elution order in each compound class
[b] Product ions and mass error of compounds 1–47 are referred to the [M – H]⁻ parent ion, while for compounds 48–78 to the [M + H]⁺ parent ion
[c] Base ion peaks are shown in bold
[d] A = MANN peel, B = MANN pulp, C = MANN callus, D = MRM peel, E = MRM pulp, F = MRM callus
[e] Confirmed by injection of a pure standard.

and 58 were a group of isomeric trihydroxy ursolic acid with a [M + H]⁺ at *m/z* 489.3571, showing the loss of two water molecules and a HCOOH during the fragmentation process (Hu et al., 2020). In particular, compound 51 showed product ions at *m/z* 471.34 ([M + H – H_2O]⁺, –18 u), 453.33 ([M + H – 2H_2O]⁺, –36 u), 435.32 ([M + H – 3H_2O]⁺, –54 u), 425.32 ([M + H – H_2O – HCOOH]⁺, –64 u), and 407.33 ([M + H – 2H_2O – HCOOH]⁺, –82 u), leading to identify the molecule as euscaphic acid, confirmed by literature data (Zhang et al., 2022) and the injection of a pure standard. Compounds 48 and 50 were epoxytrihydroxy ursolic acids isomers, displaying both a [M + H]⁺ at *m/z* 503.3364 and product ions at *m/z* 485.32 ([M + H – 18]⁺), 467.31 ([M + H – 36]⁺), 457.34 ([M + H – 46]⁺), 439.32 ([M + H – 64]⁺), 421.31 ([M + H – 82]⁺), and 403.30 ([M + H – 100]⁺), due to the loss of three H_2O and a HCOOH molecules. According to the literature data (Waldbauer et al., 2016; Sut et al., 2019) and elution order, the structure of cuneataol and pomaceic acid was assigned to 48 and 50, respectively. Compounds 55 and 56 were isomeric oxodihydroxy ursolic acid derivatives with a molecular protonated ion at *m/z* 487.3414. In detail, compound 56 was annotated as annurcoic acid, displaying a base ion peak at *m/z* 423.33 in the MS^2 and other minor product ions at *m/z* 469.33 ([M + H – 18]⁺), 451.32 ([M + H – 36]⁺), 441.34 ([M + H – 46]⁺), and 405.31 ([M + H – 82]⁺), generated by the loss of a carboxyl and two hydroxyl groups from the parent ion. Similarly, 55 showed the same fragmentation pattern of 56, but a base ion peak at *m/z* 441.34 allowed its characterization as pirolonic acid (Waldbauer et al., 2016). Compounds 57, 62, and 64 were a group of isomeric dihydroxy ursolic acid with a [M + H]⁺ at *m/z* 473.3626, while differences were seen in their fragmentation pathway. In particular, compound 57 showed a base ion peak at *m/z* 191.18 and product ions at *m/z* 455.35 ([M + H – 18]⁺), 437.34 ([M + H – 36]⁺), 427.36 ([M + H – 46]⁺), and 409.35 ([M + H – 64]⁺), due to the neutral

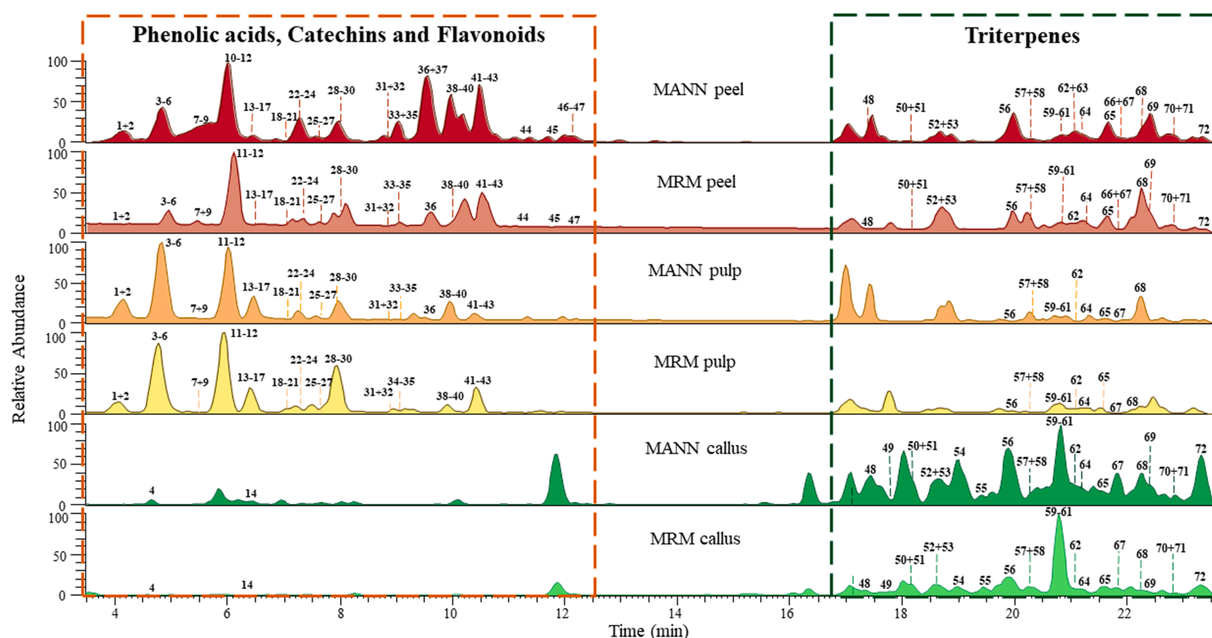

Fig. 1. Comparison of qualitative profiles of peel, pulp, and callus extracts of both apple varieties, obtained in positive ion mode. Each number corresponds to the compound listed in Table 3. MANN = *Malus domestica* var. 'Annurca'; MRM = *Malus domestica* var. 'Mela Rosa del Montefeltro'. Not numbered peaks remained unidentified.

loss of water molecules and a HCOOH. On the other hand, compounds 62 and 64 showed the same fragmentation pattern of 57, but a base ion peak at *m/z* 205.16. According to the comparison with their fragmentations and elution time with literature data (Sut et al., 2018), the structure of pomolic acid was attributed to 57, while 62 and 64 were tentatively identified as maslinic and corosolic acid, respectively. The sequential loss of a coumaroyloxy group and a water molecule $[M + H - 164 - 18]^+$ in the MS^2 spectra was characteristic of compounds 61, 63, 66, and 71. In detail, compounds 61 and 63 were tentatively assigned as 3*β*-*p*-coumaroyloxy-dihydroxyurs-12-en-28-oic acid isomers, displaying both a $[M + H]^+$ at *m/z* 635.3936 and a product ion at *m/z* 453.33 ($[M + H - 164 - 18]^+$). Similarly, a molecular protonated ion at *m/z* 619.3935 and a product ion at *m/z* 437.34 ($[M + H - 164 - 18]^+$) allowed the identification of compounds 66 and 71 as 3*β*-*p*-coumaroyloxy-2*α*-hydroxy-urs-12-en-28-oic acid isomers (McGhie et al., 2012). Compounds 67 and 68 were isomeric oxohydroxy ursolic acids with a $[M + H]^+$ at *m/z* 471.3471 and product ions at *m/z* 453.34 ($[M + H - 18]^+$), 435.32 ($[M + H - 36]^+$), 425.34 ($[M + H - 46]^+$), and 407.33 ($[M + H - 64]^+$), suggesting the loss of a water molecule and a HCOOH during the fragmentation process. According to McGhie et al. (2012), the structure of oxo-hydroxy-urs-12-en-28-oic acid was assigned to the two isomers 67 and 68. The structure of zanhic acid was assigned to compound 49 by considering a $[M + H]^+$ at *m/z* 519.3317 and product ions at *m/z* 501.32 ($[M + H - 18]^+$), 455.31 ($[M + H - 64]^+$), 437.30 ($[M + H - 82]^+$),

Table 4
Amount (mg/g DW ± SD) of main triterpenes identified in *Malus domestica* var. 'Annurca' (MANN) and var. 'Mela Rosa del Montefeltro' (MRM) peels and calli.

Peak	Compound	PEEL		CALLUS	
		MANN	MRM	MANN	MRM
	mg/g DW± SD				
48	Cuneataol	–	–	0.162 ± 0.02	0.0876 ± 0.03
49	Zanhic acid	–	–	0.0851 ± 0.02	0.0675 ± 0.03
50	Pomaceic acid	–	–	1.06 ± 0.005	0.971 ± 0.008
51–53	Euscaphic acid/Arjunic acid/Tormentic acid	0.0714 ± 0.02	0.430 ± 0.007	2.69 ± 0.2	3.12 ± 0.5
54	Carboxyursolic acid	–	–	0.544 ± 0.01	0.325 ± 0.03
55	Pirolonic acid	–	–	0.211 ± 0.02	0.209 ± 0.02
56	Annurcoic acid	2.18 ± 0.3	0.858 ± 0.1	12.6 ± 0.4	10.1 ± 0.4
57	Pomolic acid	0.132 ± 0.04	0.112 ± 0.003	0.425 ± 0.03	0.357 ± 0.01
58	Epitormentic acid	–	–	0.0693 ± 0.02	0.118 ± 0.03
59	Oxo-dihydroxy-urs-12-en-28-oic acid	–	–	0.243 ± 0.002	0.223 ± 0.004
61	3*β*-*p*-Coumaroyloxy-dihydroxyurs-12-en-28-oic acid (isomer I)	0.150 ± 0.01	–	–	–
62	Maslinic acid	0.110 ± 0.03	0.120 ± 0.002	1.58 ± 0.4	1.54 ± 0.5
63	3*β*-*p*-Coumaroyloxy-dihydroxyurs-12-en-28-oic acid (isomer II)	0.112 ± 0.01	–	–	–
64	Corosolic acid	0.239 ± 0.05	0.465 ± 0.1	2.63 ± 0.2	1.98 ± 0.5
66	3*β*-*p*-Coumaroyloxy-2*α*-hydroxy-urs-12-en-28-oic acid (isomer I)	0.126 ± 0.02	0.0285 ± 0.003	–	–
67	Oxo-hydroxy-urs-12-en-28-oic acid (isomer I)	0.0865 ± 0.01	0.0448 ± 0.01	0.346 ± 0.01	0.184 ± 0.02
68	Oxo-hydroxy-urs-12-en-28-oic acid (isomer II)	0.0995 ± 0.01	0.140 ± 0.003	0.136 ± 0.01	0.574 ± 0.02
69	Ursolic acid/Oleanolic acid	0.835 ± 0.03	0.244 ± 0.003	0.634 ± 0.03	0.490 ± 0.02
70	Oxo-urs-12-en-28-oic acid (isomer I)	0.0235 ± 0.003	0.00196± 0.007	0.211 ± 0.01	0.156 ± 0.02
71	3*β*-*p*-Coumaroyloxy-2*α*-hydroxy-urs-12-en-28-oic acid (isomer II)	0.661 ± 0.4	0.183 ± 0.004	–	–
72	Oxo-urs-12-en-28-oic acid (isomer I)	0.0722 ± 0.003	0.0392 ± 0.006	0.799 ± 0.01	0.458 ± 0.006
Total		4.90 ± 0.9	2.67 ± 0.2	24.4 ± 1	20.9 ± 2

DW: fresh weight; SD: standard deviation

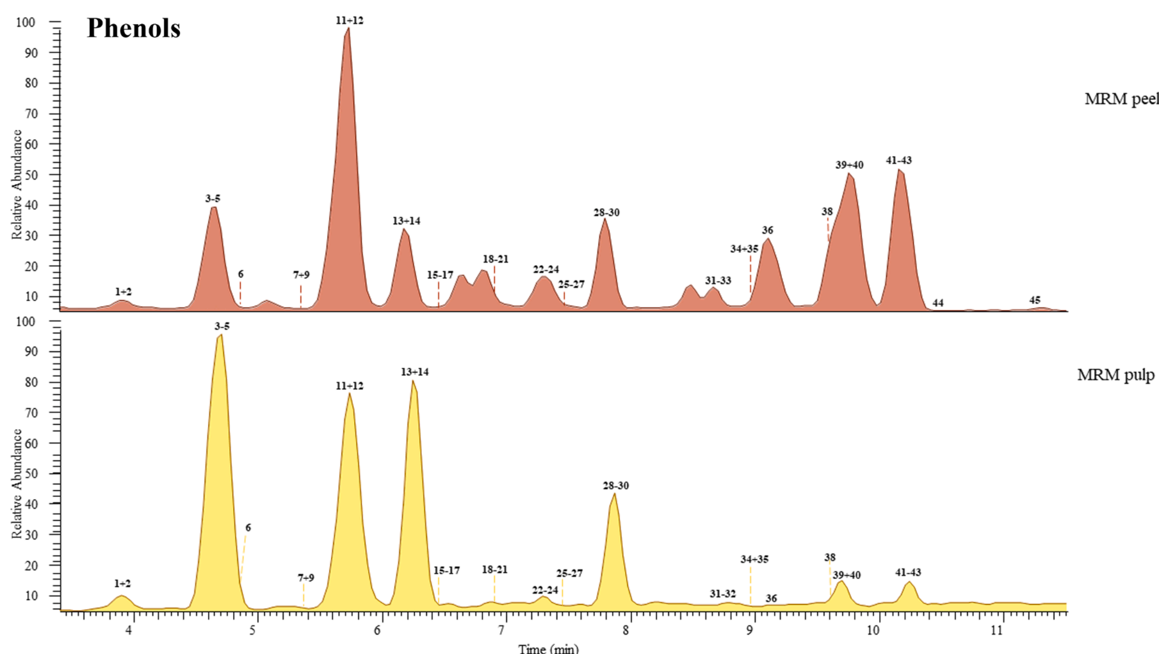

Fig. 2. Phenol profiles of peel and pulp extracts (negative ion mode) of *Malus domestica* var. 'Mela Rosa del Montefeltro' (MRM). Compound numbers correspond to those listed in Table 3.

409.31 ($[M + H – 110]^+$), and 391.30 ($[M + H – 128]^+$), indicating the presence of two carboxylic and two hydroxy groups (Maisto et al., 2023). Finally, the other detected compounds were tentatively identified as carboxyursolic acid (54, $[M + H]^+$ at m/z 501.3208) and oxo-urs-12-en-28-oic acid isomers (70 and 72, $[M + H]^+$ at m/z 455.3519).

3.3. Triterpene amount in peel and callus extracts

LC-MS quantitative analysis showed the main differences and similarities between the three apples parts in the triterpenic acid content. In detail, these compounds were in a larger amount in calli than peels, while in pulps their quantity is under the limit of quantification. As shown in Table 4, callus of 'Annurca' showed a total triterpenic acid content of 24.4 ± 1 mg/g DW, which was five times greater than that observed in the corresponding apple peels (4.90 ± 0.9 mg/g DW). Similarly, calli of 'Mela Rosa del Montefeltro' showed an eightfold increase in triterpene content (20.9 ± 2 mg/g DW) when compared to the peels (2.67 ± 0.2 mg/g DW). Among the quantified compounds, annurcoic acid (56) was the main component in both peels and calli, showing a significant raising *in vitro* cell cultures (12.6 ± 0.4 and 10.1 ± 0.4 mg/g DW, in 'Annurca' and 'Mela Rosa del Montefeltro', respectively). Among other triterpenes, epitormentic acid (58) was observed to be the least abundant in 'Annurca' callus (0.0693 ± 0.02 mg/g DW), while zanhic acid (49) was the least represented in 'Mela Rosa del Montefeltro' callus (0.0675 ± 0.03 mg/g DW), and 3-oxo-urs-12-en-28-oic acid (70) displayed lowest abundance in both peels (0.0235 ± 0.003 and 0.00196± 0.007 mg/g DW in 'Annurca' and 'Mela Rosa del Montefeltro', respectively).

3.4. Phenol amount in pulp and peel extracts

Given the lack of existing studies on *M. domestica* var. 'Mela Rosa del Montefeltro', LC-MS conditions were optimized (Fig. 2) and quantitative analysis of the phenolic compounds was carried out and compared to 'Annurca' fruit. In detail, peels and pulps appeared rich in these compounds, whereas in calli extracts their quantity was below the limit of quantification. As shown in Table 5, peels of 'Annurca' showed a total phenolic content of 5.46 ± 0.7 mg/g of DW, which was higher than that observed in the corresponding apple pulps. On the other hand, the phenolic content was found to be comparable in both pulp and peel of 'Mela Rosa del Montefeltro'. Furthermore, in both apple varieties, peels exhibited a greater amount of flavonoids compared to the pulp, hydroxycinnamic acids were more abundant in the pulps than in the peels, while the catechin content was similar between peels and pulps. Among the quantified flavonoids, quercetin derivatives and phloretin pentosylhexoside were the most abundant in peels and pulps of both apple varieties. In particular, quercetin 3-*O*-glucoside (36) was the most plenty in 'Annurca' peel (1.12 ± 0.1 mg/g) DW, but the least abundant in 'Mela Rosa del Montefeltro' pulp (0.00225 ± 0.0002 mg/g DW). Phloretin pentosylhexoside isomers (38 and 40) were three times higher in peel than in pulp of 'Annurca', whereas their content was observed to be two times higher in peels than in pulp of 'Mela Rosa del Montefeltro'. In addition, chlorogenic acid (5 and 13 isomers) was the most abundant hydroxycinnamic acid in peels and pulps of both apple varieties, while *p*-coumaric acid hexoside isomer II (7) was the least abundant. Finally, catechin (1) and procyanidin dimer isomer I (2) were observed to be in higher amount in pulp than in peel of the corresponding apple, in contrast with epicatechin (11) and procyanidin dimer isomer II (12), which were higher in peels than in pulps.

4. Discussion

Apples contain a large range of specialized metabolites, including phenolic compounds and triterpenes (Butkevičiūtė et al., 2022). Apple consumption has been linked to a lower risk of cardiovascular diseases, cholesterol and lipid oxidation, and cancers (Lin et al., 2022), suggesting a role of apple chemical components in health benefits. Furthermore, different studies reported the beneficial effects of these fruits on cardiovascular system, acting as modulator of inflammation, blood pressure, and diabetes (Weichselbaum et al., 2010; Guo et al., 2017; Zielinska et al., 2019). However, a multitude of factors, ranging from the type of apple and soil contamination to the timing of harvest, geographical location, and storage conditions, influence the chemical composition of apples (Kschonsek et al., 2018). Even though these factors were minimized in this study due to the use of two apple varieties produced in small defined geographical regions and without any storage before processing for callus culture, the effect of season remains a

Table 5
Amount (mg/g DW ± SD) of main phenolic compounds detected in 'Annurca' (MANN) and 'Mela Rosa del Montefeltro' (MRM) peels and pulps.

		PEEL		PULPS	
Peak	Compound	MANN	MRM	MANN	MRM
Flavonoids					
10	Kaempferol/ Luteolin hexoside	0.0951 ± 0.003	–	–	–
35	3-Hydroxyphloretin 2-*O*-xylosylglucoside	0.0452 ± 0.05	0.00401 ± 0.001	0.00379 ± 0.0002	–
36	Quercetin 3-*O*-glucopyranoside	1.12 ± 0.1	0.235 ± 0.04	0.0247 ± 0.002	0.00225 ± 0.0002
37	Rutin	0.0337 ± 0.002	–	–	–
38	Phloretin pentosylhexoside (isomer I)	0.969 ± 0.1	0.121 ± 0.06	0.256 ± 0.1	0.0636 ± 0.01
39	Quercetin pentoside (isomer I)	0.477 ± 0.1	0.639 ± 0.03	0.0107 ± 0.0003	0.0524 ± 0.003
40	Phloretin pentosylhexoside (isomer II)	0.0256 ± 0.002	0.00539 ± 0.001	0.00815 ± 0.001	0.00302 ± 0.0003
41	Phlorizin	0.121 ± 0.09	0.0743 ± 0.02	0.00962 ± 0.0004	0.0328 ± 0.003
42	Quercetin deoxyhexoside	0.258 ± 0.1	0.357 ± 0.02	0.0212 ± 0.001	0.00792 ± 0.002
43	Quercetin pentoside (isomer II)	0.0524 ± 0.003	0.0131 ± 0.002	–	–
44	Isorhamnetin hexoside	0.0487 ± 0.003	0.00494 ± 0.001	–	0.00492 ± 0.001
46	Kaempferol/ Luteolin pentoside	0.00831 ± 0.0003	0.00307 ± 0.001	–	–
Hydroxycinnamic acids					
4	*p*-Coumaric acid hexoside (isomer I)	0.00937 ± 0.001	0.00209 ± 0.001	0.0129 ± 0.0006	0.0119 ± 0.0007
5+14	Chlorogenic acid	1.07 ± 0.07	0.559 ± 0.01	1.88 ± 0.03	1.45 ± 0.01
7	*p*-Coumaric acid hexoside (isomer II)	0.0420 ± 0.0001	0.0531 ± 0.01	0.0131 ± 0.001	–
13	Coumaroylquinic acid (isomer I)	0.0318 ± 0.001	0.0166 ± 0.002	0.0560 ± 0.001	0.0259 ± 0.001
18	Ferulic acid hexoside	0.0222 ± 0.02	0.0373 ± 0.01	0.0377 ± 0.0005	0.0123 ± 0.0004
25	Coumaroylquinic acid isomer (isomer II)	0.0466 ± 0.001	0.0244 ± 0.003	0.0431 ± 0.001	0.0219 ± 0.002
Catechins and Procyanidins					
1	Catechin	0.153 ± 0.01	0.0509 ± 0.002	0.197 ± 0.01	0.0769 ± 0.01
2	Procyanidin dimer (isomer I)	0.137 ± 0.01	0.0525 ± 0.003	0.198 ± 0.01	0.0700 ± 0.01
9	Procyanidin trimer (isomer I)	0.0155 ± 0.01	–	0.0270 ± 0.01	–
11	Epicatechin	0.100 ± 0.02	0.0843 ± 0.003	0.0621 ± 0.02	0.0554 ± 0.01
12	Procyanidin dimer (isomer II)	0.392 ± 0.003	0.441 ± 0.07	0.350 ± 0.01	0.359 ± 0.004
15	Procyanidin trimer (isomer II)	0.0108 ± 0.01	0.0103 ± 0.01	0.0109 ± 0.01	0.00639 ± 0.01
17	Procyanidin tetramer (isomer II)	0.0191 ± 0.01	0.0171 ± 0.01	0.0273 ± 0.01	0.0203 ± 0.01
19	Procyanidin tetramer (isomer III)	0.0187 ± 0.01	0.0257 ± 0.01	0.0214 ± 0.01	0.0204 ± 0.01
22	Procyanidin trimer (isomer III)	0.0988 ± 0.01	0.134 ± 0.01	0.0759 ± 0.01	0.0892 ± 0.01
23	Procyanidin tetramer (isomer IV)	0.0236 ± 0.01	0.0359 ± 0.0004	0.0248 ± 0.01	0.0166 ± 0.01
28	Procyanidin pentamer (isomer IV)	0.0197 ± 0.01	0.0417 ± 0.003	0.0271 ± 0.01	0.0338 ± 0.01
30	Procyanidin hexamer (isomer II)	–	0.0166 ± 0.01	0.0171 ± 0.01	0.0182 ± 0.01
	Total flavonoids	3.25 ± 0.6	1.46 ± 0.3	0.334 ± 0.1	0.167 ± 0.02
	Total hydroxycinnamic acids	1.22 ± 0.09	0.692 ± 0.04	2.04 ± 0.03	1.52 ± 0.01
	Total catechins and procyanidins	0.988 ± 0.1	0.910 ± 0.1	1.04 ± 0.1	0.766 ± 0.1
	Total phenols	5.46 ± 0.7	3.06 ± 0.4	3.41 ± 0.2	2.45 ± 0.1

DW: dry weight; SD: standard deviation

critical point. For this reason, different strategies were developed to produce plant bioactive compounds which are typically found in lower concentrations in fresh fruits or other plant parts avoiding the season and plant reproductive cycle restrictions (Urquiza-López et al., 2021). In this context, cell culture technology has become a promising alternative technique to produce bioactive compounds using as starting material functional food. Based on promising results emerged in our previous work in which the callus was obtained from starting apple pulp (Verardo et al., 2017), herein the callus was inducted starting from pulp explants of two different Italian apple varieties, optimising the experimental conditions of culture medium. Interestingly, the presence of cytokinin (2iP in this case) was found to be essential to obtain durable callus culture from fruit pulp. Accordingly, previous works showed that the presence of cytokinin 6-benzyladenine (BA) induced durable production of callus from fruit pulp (Verardo et al., 2017; Verardo et al., 2019; De Bellis et al., 2022; Laezza et al., 2024). In this experiment, the use of 2iP instead of BA showed that this growth regulator could also be used to obtain stable and long-lasting callus production from apple pulp explants without inducing a dramatic quali/quantitative variation of the phytocomplex composition, compared to previous research (Verardo et al., 2017; Verardo et al., 2019; De Bellis et al., 2022). In a very recent published work (Laezza et al., 2024) yeast extract was used as elicitor to obtain callus cultures starting from the 'Annurca' apple pulp. Interestingly, elicitation improved phenol production, increasing procyanidin B2, epicatechin, and chlorogenic acid levels, whereas the calli obtained in our experiments demonstrated a selective accumulation in the triterpenic acids according to previous studies (Veraldo et al., 2017), while phenols were detected only in traces even though the used pulp starting material was found to be rich in phenolic derivatives. Another noteworthy result was the amount of recovered triterpenic acids, resulting greatly higher in callus cultures compared to that found in the peels and detected only in traces in both the pulps. Although 'Mela Rosa del Montefeltro' peel contained a lesser amount of triterpenic acids than 'Annurca' peel, the callus cultures of both pulps were shown to be comparable sources of these metabolites, being 'Mela Rosa del Montefeltro' callus a very efficient *in vitro* system for selectively producing apple bioactive metabolites (8 times higher than peels). Furthermore, several triterpenic acids not found in peels (*e.g.* 48–50, 54, 55, 58, and 59) were detected in callus cultures even if in a low amount. On the contrary, compounds 61, 63, 66, and 71 were not found in the callus extracts. These findings highlighted how different experimental conditions and crucial components could improve the *in vitro* culture response not only in terms of biomass and durability, but also in terms of classes of bioactive metabolite production when applied to the same plant species, even among plant varieties. Accordingly, Veraldo et al. (2017) reported a higher triterpenic acids production in callus culture from 'Mela Rosa Marchigiana' pulp variety than that of 'Golden Delicious' (~ 53 *vs* 35 mg/g DW) and both contents were much higher than those found in the fruit pulp. As emerged from the whole chemical study, the poorly known 'Mela Rosa del Montefeltro' fruit was richer in phenolic compounds even if in a lower amount than 'Annurca' apple. The valorization of ancient and neglected plant varieties is, therefore, actually relevant as starting material for developing new strategies to improve bioactive substance

production, as well as resource of nutraceuticals themselves. To our knowledge, this is the second report about the possibility of producing specialized metabolites through the *in vitro* cell cultures starting from explants of 'Annurca' apple pulp, but since plant cell culture can be considered an alternative system for the production of specialized metabolites useful for health, the development of different protocols could be of interest for a possible application of this technique on a large scale.

5. Conclusion

The results obtained in this study represented a step forward towards the use of fruit pulp as potential explant for *in vitro* cell culture. The use of this technique with apple pulps was useful for bioactive specialized metabolites production regardless external factors such as environmental conditions, soil, seasonal growth period or geographical location. Furthermore, the studied calli pulp was proved as an alternative source of triterpenic acids, some of which produced in higher amount *in vitro* than *in vivo*. Thus, this study sheds light on how cell plant culture can be considered as an alternative system for producing specialized metabolites starting from natural functional foods.

CRediT authorship contribution statement

Maria Vitiello: Writing – original draft, Visualization, Investigation, Formal analysis, Data curation. **Alessandra Braca:** Writing – review & editing, Supervision, Resources, Project administration, Methodology. **Marinella De Leo:** Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation. **Daniele Fraternale:** Writing – review & editing, Writing – original draft, Resources, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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