

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

Article

Supercritical CO₂ Extraction of Antioxidant Bioactive Components from Apple Peels and Their Modulation of Complex I Activity in Isolated Mitochondria

Antonella Aresta^a; Pietro Cotugno^b; Ciro Leonardo Pierri^c; Lucia Trisolini^a; Carlo Zambonin^a

^a Department of Biosciences, Biotechnology and Environment, University "Aldo Moro", Via Orabona 4, Bari, 70125, Italy

^b Department of Chemistry, University "Aldo Moro", Via Orabona 4, Bari, 70125, Italy

^c Department of Pharmacy, Pharmaceutical Sciences, University of Bari "Aldo Moro", Via Orabona 4, Bari, 70125, Italy

Correspondence: Dr Antonella Maria Aresta, Department of Biosciences, Biotechnology and Environment, University "Aldo Moro", Via Orabona, 4, 70126-Bari (Italy), Tel./Fax: +39.080.5442021

Abstract: This study aimed to extract bioactive compounds from apple (*Malus domestica*) peel waste using supercritical CO₂ extraction. The bioactive fractions from apple peels from the three Italian cultivars were extracted applying a temperature of 60 °C and a pressure of 250 bar for 15 min. 20% ethanol was used as a co-solvent. The extraction process was carried out at a flow rate of 2 mL/min and their total polyphenol (TP), anthocyanin (TA), ascorbic acid (AA), and antioxidant activity contents (TAC) were measured. The extracts from the Stark cultivar had the highest levels of polyphenols, anthocyanins, and ascorbic acid, while the Royal Gala cultivar had the highest total antioxidant activity. Chromatographic analyses were also performed to characterize polyphenols. The extracts were then tested for their effect on the mitochondrial NADH-ubiquinone oxidoreductase (Complex I) activity on mitochondria isolated from human embryonic kidney cells (HEK239) cells, with the Stark extracts showing the most positive response in terms of NADH oxidation. These findings highlight the potential of apple peel waste as a source of functional phytocompounds and suggest the Stark cultivar extracts may have pharmacological applications. This study supports the circular bioeconomy by promoting the use of waste products as a valuable resource.

Keywords: SFE; UHPLC; apple peels; bioactive compounds; in vitro activity

Introduction

In the last decade, scientific research has focused on new and more advantageous methods for the extraction of bioactive molecules, such as polyphenolic compounds and vitamins, from food waste [1-5]. The main reasons for the growing interest in these matrices are in their properties, namely they provide the food industry with an efficient and environmentally friendly solution for waste disposal and can represent a rich and economical source of useful molecules, if properly treated.

Apple (*Malus domestica*) is a type of fruit available all year round, typical of temperate zones, although widespread throughout the world for economic and cultural reasons [7-9]. In 2020, according to the Food and Agriculture Organization report, world apple production was estimated at ~86 million tons, i.e.~28% less than that of bananas, with 120 million tons, but ~8% and 10% more than grapes and oranges, respectively. Among the leading producing countries, China ranks with a production of ~40.5 million tons. Approximately 18% of global production is transformed into drinks, syrups, jams, purees, etc., generating large quantities of by-products whose disposal can constitute an environmental problem and add additional costs to food producers [9,10]. However, due to the high content of natural compounds important for human health, apple waste is an important resource, which can be used to obtain products of greater economic and nutritional value [10-16].

Apple peel is a rich source of dietary fiber [15,17], minerals [17], flavonoids [15,18] and phenolic acids [15,18]. Polyphenols and dietary fiber exert a positive influence on the prevention of various diseases and have a positive effect on human health [2,16,19,20]. The distribution of polyphenols, molecules important to balance oxidative stress and inflammatory processes [2,18,19], varies between the peel and its flesh, being the first richer in flavonoids, such as quercetin glycosides and cyanidin glycosides [2,12].

Nutritionists have detected that the apple is also an essential source of vitamins, such as C, B1, B2, B3, and A [17,21,22]. Various reports indicate that, like the polyphenols, the content of vitamin C is higher (1.5- to 3.3-fold greater), in the peel compared to its flesh. Vitamin C is a powerful antioxidant with a dietary reference intake of 25-90 mg/day. Its content in the peel ranged from 99.2 ± 10.0 to 300.9 ± 10.8 mg/100g [21,22].

Therefore, given the potentially high value of apple waste and the need to ensure a healthy environment for a sustainable economy, the development of analytical methods capable of valorizing industrial by-products in an environmentally sustainable way is in high demand. In fact, new methods are currently available in the literature for the best use of this source [1,2,5,10,12,16]. Previously, conventional extraction techniques (maceration and Soxhlet) were the only ones used in the extraction of phytocompounds from apple. The extracts were obtained by treating fresh or dried (oven-dried or freeze-dried) peels with solvents of different polarity, such as aqueous acetone or ethanol solutions. Although acetone proved to be more suitable than the oven-dried/ethanol combination for the extraction of polyphenols from apple peels, the latter procedure was preferred because it was more suitable for food uses [16]. Unfortunately, conventional methods are not environmentally friendly and easily applicable to large-scale samples, such as industrial ones.

Recently, green extraction technologies are preferred to conventional methods for transforming apple waste into high value-added products [1,2,10].

Supercritical fluid extraction (SFE) constitutes a more cost-effective alternative to conventional procedures because it is simple, eco-friendly and can be applied to systems on different scales. In fact, it can be used in the laboratory for analytical (from few hundred milligrams to a few grams of sample) or for preparative purposes (a few hundred grams of sample), but also to treat quantities of raw material in the order of kilograms (pilot systems) up to tons (industrial sector) [2,16].

In this work, carbon dioxide (CO₂) was used as supercritical fluid with ethanol as modifier, for the simultaneously extraction of the bioactive molecules from apple peel. The waste apple peels were obtained from three among of the most common cultivars in Italy from organic (Royal Gala and Stark) and non-organic farming (Gala).

Each SC-CO₂ extract was characterized in terms of total phenolic content, flavonoid, anthocyanin, vitamin C and antioxidant activity. In addition, the antioxidant activity of each extract was assayed in *in vitro* tests on mitochondria from the human embryonic kidney cell line (HEK293) [23] to estimate NADH-ubiquinone oxidoreductase (Complex I) activity in presence of the investigated extracts.

Several mitochondrial diseases but also some cancers [24], and more in general mitochondrial dysfunction associated to ageing, may depend on Complex I impairment, since cataracts, diabetic mellitus, short stature, neuromuscular degeneration, cardiomyopathy, sensorineural hearing loss, and kidney failure, etc. [25-26], thus, it becomes crucial to find small molecules able to stimulate Complex I activity and/or mitochondrial function.

In this context, the aim of this preliminary work is to quantify the antioxidant fractions present in the investigated apple peel extracts and to assay Complex I activity in presence of the investigated extracts for estimating their abilities in stimulating the oxidation of NADH and mitochondrial function for possible future pharmacological applications.

2. Materials and Methods

2.1. Apple collection and apple peel preparation

Apples from Stark, Gala, and Royal Gala cultivar were acquired by local suppliers in the period August-September 2021.

The fruits were washed with tap water and thin peels were obtained from each cultivar and then cut into small pieces, put on Petri plates, weighed, and immediately covered with paper to avoid light exposure. Samples were frozen at -20 °C for 24 hours and finally freeze-dried for 48 hours in a ScanVac cool safe lyophilizer supplied by *Chemie* (BA, Italy), operating at a temperature of -50 °C and a pressure of 0.5 torr. Freeze-dried peel samples were stored into a dryer at room temperature in the dark, until the time of use.

2.2. Supercritical fluid extraction

SC-CO₂ extraction was then carried out according to Aresta et al [4], with modifications. The SC-CO₂ system was a Spe-ed SFE from *Applied Separations* (Allentown, PA, USA). One gram of each freeze-dried peel sample was mixed with a small quantity of a dispersing material, Ottawa sand (O₂Si, 50-70 mesh particle size, *Applaied Separation*), to facilitate the contact between the supercritical fluid and the sample particles. The mixture was placed into a 10 mL extraction cell, which was sealed under pressure before being introduced into the oven at 60 °C.

Carbon dioxide (supercritical fluid grade, SCF) from *Rivoira* (Milan, Italy) was used as extraction solvent with 20 % ethanol (liquid chromatographic grade, LC; *Sigma-Aldrich*, Milan, Italy) as co-solvent. Before each extraction, the manual purging of the co-solvent pump was necessary both to ensure the correct flow at the same stage and to prevent the pump from undergoing a pressure stroke during extraction. Then, a static period of 3 minutes was applied before starting the extraction by activating the co-solvent pump. Subsequently, the dynamic extraction process was carried out at 60 °C and 250 bar for 15 minutes, with carbon monoxide and ethanol (0.4 mL/min) at a total flow rate of 2 mL/min. The extracts were collected in amber vials using the heated micrometric valve, set at 70 °C. At the end of each extraction cycle, an ethanol cleaning cycle was carried out using a stainless-steel by-pass instead of the jar, for 3 minutes, to remove all residues.

The extract sample was then filtered through a syringe filter device with a 25 mm diameter and 0.45 µm PTFE (polytetrafluoroethylene) membrane (*Sigma-Aldrich*), before to be subjected to analytical analysis.

2.3. Total Antioxidant capacity (TAC) Assay

The assay was performed according to slightly modified previous literature protocols [27]. An aliquot (10 µL) of SC-CO₂ extract (S) was transferred into a test tube containing 0.99 mL of 0.1 mM 2,2-diphenyl-1-picrylhydrazyl (DPPH; *Sigma Aldrich*), or ethanol (EtOH). The solutions were kept in the dark for 1 h at room temperature. The absorbances were read at 517 nm against EtOH using a UV/Visible spectrophotometer (UV-1650 PC; *Shimadzu*, Kyoto, Japan). The radical scavenging activity (RSA) was expressed as the percentage of the DPPH consumed, which was calculated using the following formula:

$$\%RSA = 100 - [(AS - AEtOH) \div ADPPH] \times 100 \quad (1)$$

where AS is the absorbance of the extract with DPPH, AEtOH is the absorbance of the extract without DPPH, and ADPPH is the absorbance of the DPPH solution. The calibration curve was prepared with standard solutions of (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox; *Sigma Aldrich*) in the range 1-100 mM. The TAC was expressed as Trolox equivalent antioxidant capacity (TEAC) (mmole of Trolox /100 g of f.d.m) [28].

2.4. Quantification of antioxidant compounds

2.4.1. Total phenols (TPs) determination

The TPs content was evaluated by using 4-benzoylamino-2,5-dimethoxybenzenediazonium chloride hemi [zinc chloride] salt, namely Fast Blue BB diazonium salt (FB BB D; *Sigma Aldrich*), according to Medina et al. protocol [28]. Briefly, 0.1 mL of 0.1 % (w/v) FB BB D reagent in 0.1 % ethanol was added at 1 mL of SC-CO₂ extract previously diluted 1:10 in water. Samples were accurately mixed and 0.1 mL of 20 % (w/v) Na₂CO₃ in 1 M NaOH was added to each test tube. The absorbances of samples were measured at 420 nm at 0 and 30 min and the difference between two measurements was used for the estimation. Gallic acid (GA; *Sigma Aldrich*) was used as the standard compound (calibration curve in the range 0.02-20 µg/mL) for quantification of TPs, expressed as mg of GA/100 g of freeze-dried material (fdm). Each measurement was performed in triplicate.

2.4.2. Total flavonoids (TFs) determination

The content of TFs was carried out following the method proposed by Zhishen et al. [29], with a few modifications. Briefly, 50 µL of SC-CO₂ extract was dried in a test tube and 0.5 mL of distilled water (suitable for HPLC; *Sigma-Aldrich*) was added to the residue. After accurate mixing, 37.5 mL of 5% (w/v) NaNO₂ (*Sigma-Aldrich*) was added to each test tube, which was then kept for 5 min at room temperature. Subsequently, 37.5 mL of 10 % (w/v) AlCl₃ (*Sigma-Aldrich*) was added. After 6 min, 250 mL of 1 M NaOH was added, mixed and then each tube received other 300 mL of distilled water. The absorbance at 510 nm of the mixture was finally measured, against a blank sample obtained in the same mode. GA was used as the standard compound for quantification of TFs, expressed as mg of GA/100 g of fdm. Each measurement was performed in triplicate.

2.4.3. Total anthocyanins (TAs) determination

The monomeric anthocyanin content of the SC-CO₂ extract from apple peel was measured using a spectrophotometric pH differential protocol [30]. An aliquot (0.1mL) of apple peel extract was mixed thoroughly with 0.9 mL of 0.025 M KCl pH 1 buffer. The absorbance of the mixture was measured at 515 and 700 nm against a distilled water blank. Another aliquot was combined similarly with 25 mM sodium acetate buffer pH 4.5, and the absorbance of the solution was measured at the same wavelengths. Finally, the differences between (A_{515nm} - A_{700nm}) pH 1 and (A_{515nm} - A_{700nm}) pH 4.5 were used for the determination of total anthocyanins. Cyanidin-3-glucoside chloride (CG, Reference Substance; *Sigma-Aldrich*) was used as the standard compound for quantification of anthocyanin content in each apple peel extract. Total anthocyanins were expressed as mg of CG/100 g of fdm. Each measurement was performed in triplicate.

2.4.4. Ascorbic Acid (AA) determination

The quantification of AA was performed according to Kschonsek et al. [18]. Briefly, an aliquot (0.2 mL) of SC-CO₂ extracts, previously diluted 1:10 with ultra-pure water, was mixed with 0.3 mL of trichloroacetic acid (0.31 M; *Sigma Aldrich*) and centrifuged (5 min, 17,000 g). Then, 0.3 mL of supernatant was mixed with 0.1 mL of DNP reagent [one volume of thiourea solution (0.83 M in distilled water), one volume of copper sulphate solution (24 mM in distilled water) and 20 volumes of 2,4-dinitrophenylhydrazine solution (0.11 M in 4.5 M sulfuric acid)]. All reagents were provided by *Sigma Aldrich*. The mixture was heated in a thermomixer for 1 h at 60 °C. The samples were cooled in an ice bath for 5 min. Then, 0.4 mL of sulfuric acid (9 M; *Sigma Aldrich*) was added and mixed. The samples were kept in the dark for 20 min. Finally, each sample was transferred into a semi-micro cuvette and measured at 520 nm. Each measurement was performed in triplicate. In the same way, a calibration line was obtained with six standard solutions of AA (Reference Standard; *Sigma Aldrich*) covering the concentration range of 0.06 to 6 mg/mL.

2.5. UHPLC-DAD Analysis

Chromatographic analyses were performed using an UHPLC system (LC20ADXR; *Shimadzu*, Milan, Italy) equipped with a binary pump (LC20ADXR; *Shimadzu*), an auto sampler (SIL20ACXR; *Shimadzu*) and a UV-diode array detector (PDA-1; *Shimadzu*) with a flow cell (10 mm, 1/1600, steel, 2.4 mL, LWL). The chromatographic column was an Accucore XLC18 (150x4.6 mm, 4 μ m; *Thermo Scientific*, Milan, Italy) equipped with an Accucore XLC18 precolumn (10x4 mm, 4 μ m; *Thermo Scientific*).

The mobile phase used was (A) 0.1% formic acid in water, (B) acetonitrile (*Sigma Aldrich*) with a flow rate of 1.2 mL/min. The gradient elution program was as follows: B (8%) (2 min), 8% B to 15% B (3 min), 15% B to 25% B (5 min), 25% B to 35% (5 min), 35% B to 50% B (5 min), B (50%) (15 min), and re-equilibration of the column, (5 min). The total chromatographic run time was 45 min. Injection volume was 20 μ L.

Spectra were acquired in the range of 220–500 nm, while meticulous composite chromatograms were obtained using the Max Plot software of the PDA-1 detector, which automatically selects and stores the maximum absorbances for each peak, thus providing the maximum signal for all compounds detected. The phenolic compounds were identified by comparing their retention times, UV-visible with those obtained from standard compounds (10 μ g/mL; all *Sigma-Aldrich*), when available. Otherwise, compounds were tentatively identified comparing the obtained information with available data reported in the literature.

An enzymatic deconjugation was also performed as follows: 0.1 mL of enzymatic solution for glycosides hydrolysis [obtained by dissolving 10 mg of β -Glucuronidase from bovine liver (type B-1, \geq 1,000,000 units/g solid; *Sigma Aldrich*) in 13 mL of 0.1 M acetate buffer, pH 5.0] was added to 0.05 mL of SC-CO₂ extract and the resulting mixture was immediately incubated overnight (i.e. approximately 17 hours) at 37 °C. Before being subjected to chromatographic analysis, the mixture was filtered through a syringe filter device with 13 mm diameter (0.45 μ m PTFE; *Sigma-Aldrich*).

The experiments were performed in triplicate.

2.6. Evaluating biological activity

2.6.1. Cell cultures

HEK293 cells were obtained from the American Type Culture Collection (<https://www.atcc.org/products/crl-1573>). For the proposed analyses, HEK293 were maintained in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with sodium pyruvate and stable glutamine (*Euroclone*, Pero (MI), Italy), supplemented with 10 % (v/v) fetal bovine serum (FBS; *Euroclone ECS0180L*, Pero, Milan, Italy), and 1 % (v/v) penicillin-streptomycin (*Euroclone*, ECM0010, Pero, Milan, Italy). The cells were cultured in a humidified atmosphere with 5 % CO₂ and 37 °C.

2.6.2. Mitochondrial isolation

Cells were grown in T75 flasks. At confluence cells were detached by trypsin and centrifuged at 125 g for 5 min, the supernatant was discarded, and the pelleted cells were resuspended in Ringer NaCl buffer (135 mM NaCl, 20 mM HEPES, 0.8 mM MgSO₄, 3 mM KCl, 1.8 mM CaCl₂, 11 mM D-glucose, pH = 7.5) according to Palacino et al [31]. Afterwards cells were centrifuged at 125 g for 5 min and the obtained pellet was suspended in 2 mL of A buffer (sucrose 320 mM, Tris-HCl 5 mM, EGTA 2 mM, pH = 7.4), and homogenized with a glass-Teflon grinder kept in ice. Thus, the homogenate underwent a centrifugation step at 4 °C for 6 min at 2000 g to remove nuclei and tissue particles, while the supernatant was collected for a further centrifugation at 4 °C for 15 min at 12,000 g to pellet mitochondria. The entire isolation procedure of subcellular fractions was carried out according to Palacino et al. [31]. The mitochondria amount was determined in according to Bradford methods [32].

2.4.3. Complex I activity measurements

Rotenone-sensitive NADH (β -Nicotinamide adenine dinucleotide) ubiquinone oxidoreductase (Complex I) activity was measured spectrophotometrically following the decrease of NADH absorbance at 340 nm as described by Spinazzi et al. [33], with some modifications. It was used a reaction mix containing 25 μ g sample mitochondria, TNS buffer 3 % (w/v), 50 mM potassium phosphate buffer pH 7.5, 600 mM KCN (*Sigma Aldrich*), and 90 mM NADH (\geq 97% pure, grade HPLC; *Sigma Aldrich*). The mitochondria were incubated for 2 min with apple peel extracts diluted 1:100, 1:1000 or 1:10000 in H₂O or with only ethanol used as a control (at the same dilutions in water) before starting the reaction. The reaction was started by adding 30 mM Deciubiquinone (\geq 97% pure, grade HPLC; *Sigma Aldrich*). NADH (β -Nicotinamide adenine dinucleotide) ubiquinone oxidoreductase (Complex I) specific activity was checked by adding an excess of Rotenone 100 μ M. In parallel, as a further control test, it was prepared a cuvette containing the same quantity of extract (ethanol) dilutions and the mitochondrial sample, in presence of 100 μ M Rotenone (\geq 95% pure; *Sigma Aldrich*), which is a Complex I selective inhibitor.

3. Results and Discussion

3.1. Supercritical fluid extraction

The parameters applied during supercritical fluid extraction (SFE) were found in the literature [1-4,34]. Having a low polarity, ethanol was selected as extraction co-solvent to help carbon dioxide for vitamin C extraction, while a percentage of 20% was chosen to favor the extraction of polyphenols. Besides, a mild temperature (60°C) was selected to avoid thermal degradation of the compounds and preserve the antioxidant properties of the extracts [4,34]. Given these observations, the samples were prepared according to the protocol optimized by Aresta et al. for the determination of polyphenols and vitamins in winemaking by-products by extraction with SC-CO₂ [4].

3.2. Quantification of antioxidant compounds

The specific spectrophotometric assays for the determination of the total antioxidant capacity and the quantification of the antioxidant compounds present in the extracts of Stark, Gala and Gala Royal apple peel obtained by SFE (2.2 paragraph), have been selected from the literature [28] considering specific features, such as sensitivity, specificity, and ease of execution. TPs were detected using the FB BB D reagent which, unlike the more common Folin-Ciocalteu, is not sensitive to reducing sugars which can be naturally present in extracts and determine overestimates with Folin-Ciocalteu [28].

Table 1 shows the average values of the complete set of determinations (TAC, TPs, TFs, TAs and AA) performed on each apple peel SC-CO₂ extract. The data obtained were in good agreement with the existing literature for all the analytes except for ascorbic acid, which was found in all extracts at concentration levels lower than expected [17,18].

Table 1. TAC, TPs, TFs, TAs and AA apple peel SC-CO₂ extracts content.

Cultivar	TAC mmole Trolox /100 g (n=3)	TPs mg GA/100 g (n=3)	TFs mg GA/100 g (n=3)	TAs mg CG/100 g (n=3)	AA mg/100 g (n=3)
Stark	5.74 \pm 0.07	379 \pm 68	120 \pm 2	7.0 \pm 0.3	43.75 \pm 6.05
Gala	2.20 \pm 0.04	152 \pm 25	90 \pm 1	5.0 \pm 0.3	9.02 \pm 1.65
Royal Gala	5.95 \pm 0.89	136 \pm 14	115 \pm 2	4.8 \pm 0.1	25.59 \pm 1.96

T-test showed some statistically significant differences between the extracts of the three cultivars in the five assays (p $<$ 0.05). Stark extracts were higher in TP, TA, and AA than the other two. Instead, it showed no significant differences in comparison with Royal Gala for TAC. Gala and Royal Gala were similar in TP and TA. On the other hand, vitamin C contents, were very different between the

three extracts. In particular, the Gala apples were characterized by lower levels and lower antioxidant activity. Then, phytochemicals can contribute in different ways to the antioxidant activity of the extracts, and the poor correlation between the estimated amounts of the analytes and the antioxidant power of the samples.

3.3. UHPLC-DAD Analysis

Chromatographic analyses confirmed the existence of differences between samples deriving from apple waste of different origin. Figure 1 shows chromatograms relevant to the three SC-CO₂ extracts (Stark, Gala and Royal Gala)

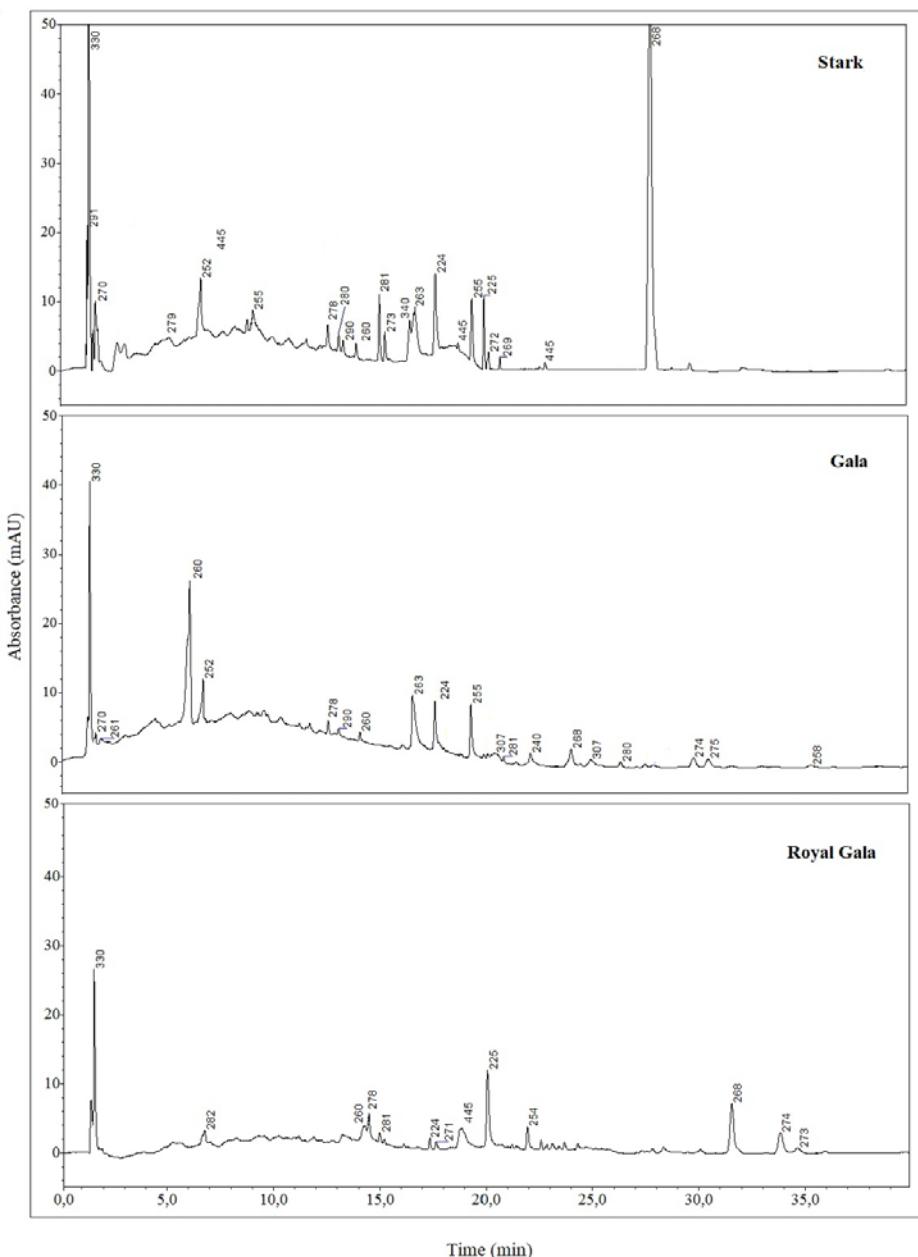


Figure 1. LC-UV-DAD chromatograms (max plots) of SC-CO₂ extract from the apple peel of Stark, Gala and Royal Gala cultivar. The numbers refer to the λ_{max} detected for each peak. Chromatographic and acquisition conditions are described in section 2.5.

Some polyphenols were identified by comparing both the chromatographic retention times and the UV-visible spectra taken at the apex of each peak with those obtained from standard compounds analyzed under the same conditions.

Table 2 lists the standards that have been subjected to chromatographic analysis, their retention times (RT, min), the λ_{max} recorded by the detector and the presence or absence in the considered cultivar.

Table 2. Retention times of standard compounds, λ_{max} detected, and presence in SC-CO₂ extracted.

RT (min)	Compound	λ_{max} (nm)	Presence in SC-CO ₂ extracted
2.0±0.1	Gallic acid	270	Stark and Gala
5.0±0.1	(+)-Catechin	279	Stark
5.6±0.1	Vanillic acid	260	Gala
9.1±0.1	Rutin	255	Stark
12.4±0.1	trans-Resveratrol	307	
13.8±0.1	Quercetin	370	
14.3±0.2	Cinnamic acid	278	Royal Gala

Rutin, is a glycoside of the flavonol quercetin, in which the aglycone is bound to the disaccharide α -L-rhamnopyranosyl-(1→6)- β -D-glucopyranose. It is found in a wide variety of plants and apple peel and is believed to be a rich source of flavonoids [7,35]. However, it was unexpectedly detected only in Stark extracts.

Then, all the SC-CO₂ extracts were subjected to enzymatic hydrolysis, to obtain the respective aglycones. The glycosylated deconjugates were then subjected to LC analysis in order to check their presence. Figure 2 shows, for instance, an LC chromatogram of a deconjugate Stark sample. Both the retention time and the UV-visible spectrum of the peak eluting at 13.9 minutes were found clearly comparable to those of the standard of quercetin analysed using the same chromatographic conditions. On the contrary, analysis of the deconjugates related to the other two cultivars confirmed the absence of the flavonol.

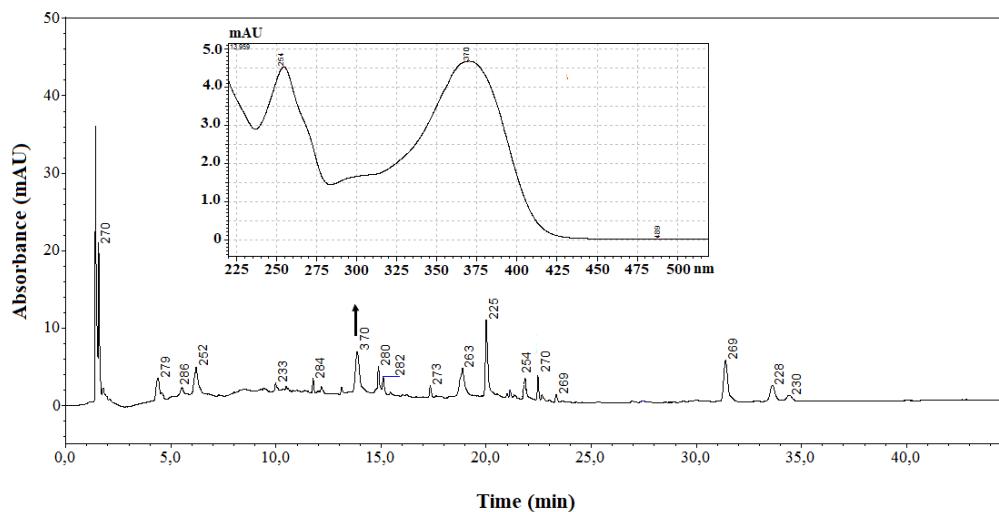


Figure 2. LC-UV-DAD chromatograms (max plots) of enzymatically hydrolyzed SC-CO₂ extract from the apple peel of Stark. The numbers refer to the λ_{max} detected for each peak. The figure shows the spectrum of the compound at 13.9 minutes, coincident with quercetin. Chromatographic and acquisition conditions are described in section 2.5.

The careful inspection of retention times and spectra and the provisional identification, also comparing the obtained information with the literature data showed the probable presence of catechins (λ_{max} 280 nm), caffeic acid (238 nm), cyanidins (278 nm), chlorogenic acid (324 nm), isoflavones (260 nm), retinoid (340 nm) and carotenoids (445 nm) [36].

In the chromatograms relative to the SC-CO₂ extracts of Stark samples, it is evident the presence of a very abundant peak at the retention time of 31.3 min, whose UV-visible spectrum is reported in

figure 3. Interestingly, this spectrum is shared with other peaks of the same chromatogram, namely the one with RT to 18.9, 22.5 and 23.4 minutes. Comparing the chromatographic data relevant to this extract with those of the other cultivar analysed, only one peak in the chromatogram relevant to the Gala Royal extract showed a peak eluting at 18.9 min, also characterized by the same UV-visible spectrum. It is worth noting that Gala Royal and Stark cultivars are both from organic farming.

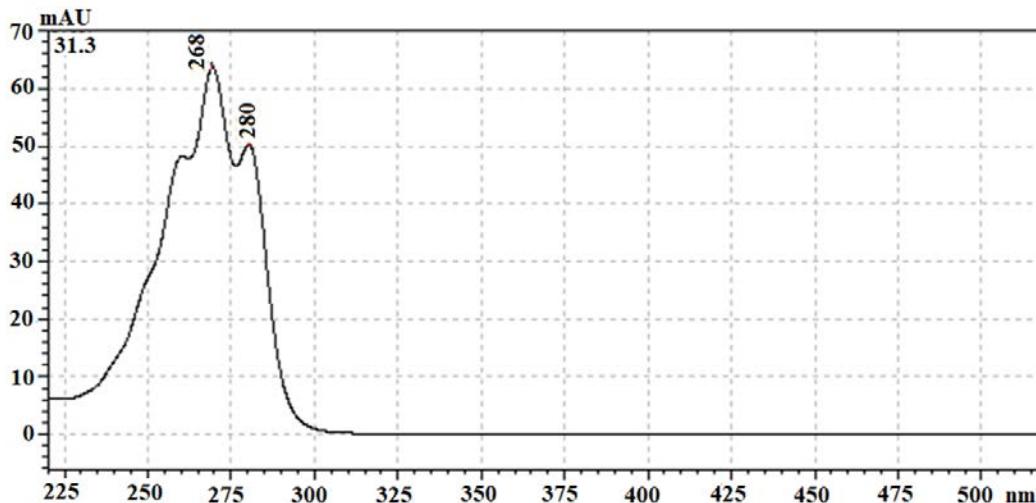


Figure 3. UV-visible spectrum of unknown compounds at 18.9, 22.5, 23.4 and 31.3 min. Chromatographic and acquisition conditions are described in section 2.5.

The spectra of these unknown compounds are similar to those of some lipophilic compounds. Carotenoids are pigments synthesized by all plants, many bacteria, and some fungi, widespread in nature, with more than 700 species, very important for human health. Some carotenoids are precursors of vitamin A, which exerts multiple functions: during embryonic development, the immune response and in the visual cycle [37].

3.3. Evaluating biological activity

To examine the effect of each apple peel extract on mitochondria, the NADH oxidoreductase activity was measured on mitochondria isolated from HEK293 cells in presence of different dilutions of the investigated extracts.

Firstly, it was noticed that the complex I activity measured from mitochondria extracted from the cultured selected cells after the addition of the three apple peel extracts at 1:100 dilution was unchanged with respect the ethanol control. It was instead observed an increase of complex I activity in presence of the Stark apple peel extract at 1:1000 and above all at 1:10000 dilution, with respect to the ethanol control at the same dilution, whereas the Complex I activity in presence of Gala and Royal Gala apple peel extracts was unchanged (Figure 4).

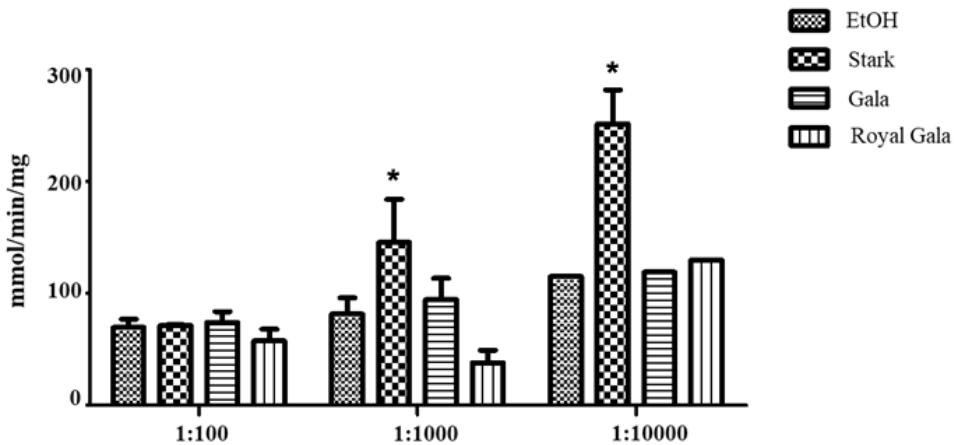


Figure 4. NADH ubiquinone oxidoreductase activity in HEK cells mitochondria incubated with SC-CO₂ extract diluted from Stark, Gala and Royal Gala waste. Values represent mean rates (nmol/min/mg) \pm SEM obtained from at least four independent experiments. * $p < 0.05$, nonparametric Wilcoxon test between mitochondria administered with 1:100, or 1:1000, or 1:10000 and nontreated mitochondria in the corresponding ethanol (EtOH). Dilution in water (1:100, 1:1000, or 1:10000).

While at the employed dilutions, it appears that ethanol presence does not affect mitochondrial function, coherently with Tempio et al. [38], and that SC-CO₂ extracts from the apple peel of Stark cultivar at the investigated increasing dilution are able to stimulate Complex I activity. It is observed that Stark extracts are richer in bioactive molecules than the other two extracts. Above all, it is observed that Stark extracts contains almost twice the concentration of AA, TPs and TAs compared to the other two investigated extracts.

While it is observed that the most concentrated Stark extracts are relatively well tolerated by mitochondria (comparable to the other investigated extracts), more diluted Stark extracts produce a significant stimulation of Complex I activity. The latter observation can be ascribed either to the minimum concentration of bioactive molecules able to produce a positive effect without causing any toxicity, or to the fact that the specific combination of AA, TPs and TAs in Stark extracts at the assayed increasing dilutions is able to exert a more efficient protective effect on mitochondria, compared to the other two extracts. Indeed, it is known that an excess of antioxidant molecules, while may counteract ROS formation into mitochondria, can alter the endogenous ROS signaling between the cytosol and mitochondria, and can even result in toxicity [39,40]. Indeed, it is known that apple peel are enriched in vitamins (i.e., vitamin E and vitamin K [41-42]) that can be structurally related to ubiquinone that can be able to target either mitochondrial proteins of the inner membrane or other FAD/NADH/ubiquinone dependent dehydrogenases widely expressed in other cell-compartment [39].

More in general, our speculations about the protective role of the tested extracts on mitochondrial function are coherent with Carrasco-Pozo et al. hypothesis [43], about a possible protective role for dietary polyphenols on mitochondria. The authors also showed that quercetin, resveratrol and rutin protected Caco-2 cells against indomethacin-induced mitochondrial dysfunction. About this, the hypothesized protective role was related to the ability of some antioxidant molecules to enter cells and accumulate in mitochondria. In addition, a structural similarity between quercetin and ubiquinone has been proposed by authors, probably responsible for the protective effect [43]. Similarly, Skemiene et al. [44], proposed that anthocyanins can behave as electron acceptors in complex I, resulting in increased ATP production in pathological conditions, such as after ischemia.

Based on the experimental results obtained, coherent with analogous findings described in literature, it is evident that Stark apple peel extracts appear to be a potentially advantageous dietary supplement for human health. For this reason, soon, it will be the subject of further studies aimed at determining, at a qualitative and quantitative level, the individual polyphenols concentration. The

role of each compound, in terms of antioxidant capacity and, above all, in terms of protective activity on mitochondria in presence of pro-apoptotic molecules will be explored by performing mitochondrial respiration assays on mitochondria extracted from different cell-lines.

Conclusions

In this work it has been shown that bioactive molecules can be extracted simultaneously from apple peels by means of SFEs. In particular, the waste apple peels, obtained from the Italian cultivar from organic farming (Stark) seems the most promising due to the highest content of total polyphenols, anthocyanins and vitamin C, whose ratio appears to be the more balanced for preserving or stimulating mitochondrial function without inducing any toxicity.

Acknowledgments: The authors are very grateful to thank Dr. Nicoletta De Vietro for assistance in the first preparation of the draft.

Funding: This research received no external funding

Author Contributions: Conceptualization, A.A. and C.Z.; Methodology, A.A., P.C., C.L.P.; Validation, A.A., P.C. and C.L.P.; Investigation, A.A., P.C., L.T.; Resources, A.A.; Data Curation, A.A.; Writing – Original Draft Preparation, A.A. and C.L.P.; Writing – Review & Editing, A.A.; Supervision, C.Z.; Funding Acquisition, A.A. and C.Z.

Institutional Review Board Statement: “Not applicable”

Informed Consent Statement: “Not applicable”

Conflicts of Interest: The authors declare no conflict of interest.

References:

1. Cai, H.L.; You, S.Y.; Xu, Z.Y.; Li, Z.M.; Guo, J.J.; Ren, Z.Y.; Fu, C.L. Novel extraction methods and potential applications of polyphenols in fruit waste: a review. *Journal of Food Measurement and Characterization* 2021, 15(4), 3250-3261.
2. Putra, N.R.; Rizkiyah, D.N.; Abdul Aziz, A.H.; Che Yunus, M.A.; Veza, I.; Harny, I.; Tirta, A. Waste to Wealth of Apple Pomace Valorization by Past and Current Extraction Processes: A Review. *Sustainability* 2023, 15, 830.
3. Vetralla, M.; Ferrentino, G.; Zambon, A.; Spilimbergo, S. A Study about the Effects of Supercritical Carbon Dioxide Drying on Apple Pieces. *International Journal of Food Engineering* 2018, 4 (3), 186-190.
4. Aresta, A.; Cotugno, P.; De Vietro, N.; Massari, F.; Zambonin, C. Determination of Polyphenols and Vitamins in Wine-Making by-Products by Supercritical Fluid Extraction (SFE). *Analytical Letters* 2020, 53 (16), 2585–2595.
5. Garofulic, I.E.; Zoric, Z.; Pedisic, S.; Brncic, M.; Dragovic-Uzelac, V. UPLC-MS₂ profiling of blackthorn flower polyphenols Isolated by ultrasound-assisted extraction. *J. of Food Science* 2018, 83(11), 2782-2789.
6. Ma, G.L.; Chen, Y.T. Polyphenol supplementation benefits human health via gut microbiota: A systematic review via meta-analysis, *J. of Functional Food* 2020, 66, Article Number103829-11
7. Chen, Z.; Yu, L.; Liu, W.; Zhang, J.; Wang, N.; Chen, X. Research progress of fruit color development in apple (*Malus domestica* Borkh.). *Plant Physiol Biochem*. 2021, 162, 267–279.
8. Vasylieva, N.; James, H. Production and trade patterns in the world apple market. *Innovative Marketing* 2021, 17(1), 16-25.
9. Fotirić Akšić, M.; Dabić Zagorac, D.; Gašić, U.; Tosti, T.; Natić, M.; Meland, M. Analysis of apple fruit (*Malus × domestica* Borkh.) quality attributes obtained from organic and integrated production systems. *Sustainability* 2022, 14(9), 5300-18.

10. Rabetafika, H.N.; Bchir, B.; Blecker, C.; Richel, A. Fractionation of apple by-products as source of new ingredients: current situation and perspectives. *Trends in Food Science and Technology* 2014, 40, 99-114.
11. Rupasinghe, V.H.P.; Wang, L.; Huber, M.G.; Pitts, L.N. Effect of baking on dietary fibre and phenolics of muffins incorporated with apple skin powder. *Food Chemistry* 2008, 107, 1217-437.
12. Shehzadi, K.; Rubab, Q.; Asad, L.; Ishfaq, M.; Shafique, B.; Nawaz Ranjha, M. M. A.; Mahmood, S.; Mueen-Ud-Din, G.; Javaid, T.; Sabtai, B.; Farooq, R. A Critical Review on Presence of Polyphenols in Commercial Varieties of Apple Peel, their Extraction and Health Benefits. *J Bio Sci & Res* 2020, 6(2), 1-8.
13. Hidalgo, A.; Brandolini, A.; Čanadanovic-Brunet, J.; Ćetkovic, G.; Tumbas-Šaponjac, V. Microencapsulates and extracts from red beetroot pomace modify antioxidant capacity, heat damage and colour of pseudocereals-enriched einkorn water biscuits. *Food Chemistry* 2018, 268, 40-48.
14. Mendoza-Wilson, A.M.; Castro-Arredondo, I.S.; Espinosa-Plascencia, A.; Robles-Burgueño, M.R.; Balandrán-Quintana, R.R.; Bermúdez-Almada, M.C. Chemical composition and antioxidant-prooxidant potential of a polyphenolic extract and a proanthocyanidin-rich fraction of apple skin. *Heliyon* 2016, 2, 00073-21.
15. Henríquez, C.; Speisky, H.; Chiffelle, I.; Valenzuela, T.; Araya, M.; Simpson, R.; Almonacid, S. Development of an ingredient containing apple peel, as a source of polyphenols and dietary fiber. *Journal of Food Science* 2010, 75, 172-181.
16. Massini, L.; Rico, D.; Martin-Diana, A.B.; Barry-Ryan, C. Valorisation of apple peels. *European Journal of Food Research & Review* 2013, 3(1), 1-15.
17. Lecerf, J.-M.; Périquet, A.; Carlin, F.; Lanckriet, S.; Paris, N.; Robaglia, C.; Gleizer, B.; Belzunces, L.; Cravedi, J.-P.; Calvarin, J. Comparison of pesticide residue and specific nutrient levels in peeled and unpeeled apples. *J Sci Food Agric* 2023, 103, 496-505.
18. Kschonsek, J.; Wolfram, T.; Stöckl, A.; Böhm, V. Polyphenolic compounds analysis of old and new apple cultivars and contribution of polyphenolic profile to the in vitro antioxidant capacity. *Antioxidants (Basel)* 2018, 7(1), 20-34.
19. Hussain, T.; Tan, B.; Yin, Y.; Blachier, F.; Tossou, M.C.; Rahu, N. Oxidative stress and inflammation: what polyphenols can do for us?. *Oxid Med Cell Longev*. 2016, 2016 number: 7432797-9.
20. Dahl, W.J.; Stewart, M.L. Position of the Academy of Nutrition and Dietetics: health implications of dietary fiber. *Journal of the Academy of Nutrition and Dietetics* 2015, 115(11), 1861-1870.
21. Vicki Brower, V. An Apple a Day May Be Safer Than Vitamins, *JNCI: Journal of the National Cancer Institute* 2008, 100, 770-772.
22. Lemmens, E.; Alos, E.; Rymenants, M.; De Storme, N.; Keulemans, W. Dynamics of ascorbic acid content in apple (*Malus x domestica*) during fruit development and storage. *Plant Physiology and Biochemistry* 2020, 151, 47-51.
23. Hu, J.W.; Han, J.Z.; Li, H.R.; Zhang, X.; Liu, L.L.; Chen, F.; Zeng, B. Human Embryonic Kidney Cells: A Vehicle for Biopharmaceutical Manufacturing, Structural Biology, and Electrophysiology, *Cell Tissues Organs* 2018, 205(1), 1-8.
24. Punzi, G.; Porcelli, V.; Ruggiu, M.; Hossain, M.F.; Menga, A.; Scarcia, P.; Castegna, A.; Gorgoglione, R.; Pierri, C.L.; Laera, L.; Lasorsa, F.M.; Paradies, E.; Pisano, I.; Marobbio, C. M. T.; Lamantea, E.; Ghezzi, D.; Tiranti, V.; Giannattasio, S.; Donati, M.A.; Guerrini, R.; Palmieri, L.; Palmieri, F.; De Grassi, A. SLC25A10 biallelic mutations in intractable epileptic encephalopathy with complex I deficiency. *Human Molecular Genetics* 2018, 27, 499-504.

25. Wallace, D.C. Mitochondrial DNA in aging and disease. *Sci Am.* 1997, 277:40–7.
26. Di Mauro, S; Schon, E.A. Mitochondrial DNA mutations in human disease. *Am J Med Genet.* 2001, 106, 18–26.
27. Al-Duais M.; Müller, L.; Böhm, V.; Jetschke, G. Antioxidant capacity and total phenolics of cyphostemma digitatum before and after processing: Use of different assays. *Eur. Food. Res. Technol.* 2009, 228, 813–821.
28. Medina, B. Simple and rapid method for the analysis of phenolic compounds in beverages and grains. *Journal of Agricultural and Food Chemistry* 2011, 59 (5), 1565–71.
29. Zhishen, J.; Mengcheng, T.; Jianming, W. The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. *Food Chem.* 64 1999, 555–559.
30. Lee, J.; Durst, R.W.; Wrolstad, R.E. Determination of total monomeric anthocyanin pigment content of fruit juices, beverages, natural colorants, and wines by the pH differential method: collaborative study. *J AOAC Int.* 2005, 88(5), 1269–1278.
31. Palacino, J.J.; Sagi, D.; Goldberg, M.S.; Krauss, S.; Motz, C.; Wacker, M.; Klose, J.; Shen, J. Mitochondrial Dysfunction and Oxidative Damage in parkin-deficient Mice. *The Journal of Biological Chemistry* 2004, 279, 18614–18622.
32. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976, 72, 248–254.
33. Spinazzi, M.; Casarin, A.; Pertegato, V.; Salviati, L.; Angelini, C. Assessment of mitochondrial respiratory chain enzymatic activities on tissues and cultured cells. *Nat Protoc.* 2012, 31, 1235–46.
34. Da Porto, C.; Natolino, A. Supercritical fluid extraction of polyphenols from grape seed (*Vitis vinifera*): Study on process variables and kinetics. *The Journal of Supercritical Fluids* 2017, 130, 239–245.
35. Pandey, J.; Bastola, T.; Tripathi, J.; Tripathi, M.; Kumar R.; Rokaya, R.K.; Dhakal Bhawana, R.; Ravin, D.C.; Bhandari, R.; Poudel, A. Estimation of Total Quercetin and Rutin Content in *Malus domestica* of Nepalese Origin by HPLC Method and Determination of Their Antioxidative Activity. *Journal of Food Quality* 2020, Article ID 8853426. (13p)
36. Malapert, A.; Reboul, E.; Loonis, M.; Dangles, O.; Tomao, V. Direct and Rapid Profiling of Biophenols in Olive Pomace by UHPLC-DAD-MS. *Food Anal. Methods* 2018, 11, 1001–1010.
37. Shao, X.; Bai, N.; He, K.; Ho, C.-T.; Yang, C.S.; Sang, S. Apple Polyphenols, Phloretin and Phloridzin: New Trapping Agents of Reactive Dicarbonyl Species. *Chem. Res. Toxicol.* 2008, 21, 10, 2042–2050.
38. Tempio, A.; Niso, M.; Laera, L.; Trisolini, L.; Favia, M.; Ciranna, L.; Marzulli, D.; Petrosillo, G.; Pierri, C.L.; Lacivita, E.; Leopoldo, M. Mitochondrial Membranes of Human SH-SY5Y Neuroblastoma Cells Express Serotonin 5-HT7 Receptor. *Int. J. Mol. Sci.* 2020, 21, 9629.
39. Tragni, V.; Primiano, G.; Tummolo, A.; Cafferati Beltrame, L.; La Piana, G.; Sgobba, M.N.; Cavalluzzi, M.M.; Paterno, G.; Gorgoglione, R.; Volpicella, M.; Guerra, L.; Marzulli, D.; Servidei, S.; De Grassi, A.; Petrosillo, G.; Lentini, G.; Pierri, C.L. Personalized Medicine in Mitochondrial Health and Disease: Molecular Basis of Therapeutic Approaches Based on Nutritional Supplements and Their Analogs. *Molecules* 2022, 27, 3494.
40. Gęgotek, A.; Skrzypkiewska, E. Antioxidative and Anti-Inflammatory Activity of Ascorbic Acid. *Antioxidants* 2022 11, 1993.
41. Chun, J.; Lee, J.; Ye, L.; Exler, J.; Eitenmiller, R.R. Tocopherol and tocotrienol contents of raw and processed fruits and vegetables in the United States diet. *Ournal of Food Composition and Analysis* 2006, 19, 196–204.

42. Dismore, M.L; Haytowitz, D.B; Gebhardt, S.E.; Peterson, J.W.; Booth, S.L. Vitamin K content of nuts and fruits in the US diet. *Research and Professional Brief* 2003, 103, 1650-1652.
43. Carrasco-Pozo, C.; Mizgier, M.L.; Speisky, H.; Gotteland, M. Differential protective effects of quercetin, resveratrol, rutin and epigallocatechin gallate against mitochondrial dysfunction induced by indomethacin in Caco-2 cells. *Chemico-Biological Interactions* 2012, 195, 199-205.
44. Skemeiene, K.; Liobikas, J; Borutaite, V. Anthocyanins as substrates for mitochondrial complex I - protective effect against heart ischemic injury. *FEBS J.* 2015, 282(5), 963-71.