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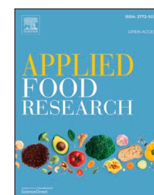

Sorbus spp. berries extraction in subcritical water: Bioactives recovery and antioxidant activity

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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

With the aim of developing a sustainable and efficient extraction of polyphenols-rich fraction from *Sorbus* spp., Microwave-Assisted Subcritical Water Extraction (MASWE) was selected as the technique of choice. Although *Sorbus* spp. berries are rich in nutraceutical compounds, they are generally underutilised. The extracts obtained contain caffeoylquinic acids (*i.e.* chlorogenic acids), flavonoids, and organic acids, all of which are active components with potential applications in innovative functional foods due to their potent antioxidant activity. To optimise the extraction protocol, different temperatures and different species of the above *genus* were studied to improve the recovery of the polyphenolic fractions and obtain products with potential high bioactivity. The best resulting sample was studied more in detail using different colorimetric assays (total content of polyphenols, flavonoids and sugars – TPC, 406.43 mgGAE/gext; TFC, 13.5 mgQE/gext; TSC, 454.1 mgGlu/gext), purification streams, and LC-MS analyses. However, the most remarkable property observed is the outstanding antioxidant activity of the extract (4.74 mmolTrolox.eq/gext). In order to obtain a refined product rich in bioactive compounds, a purification protocol was performed leading to a final TPC selectivity of about 87 % and 10.12 mmolTrolox.eq/gext. Qualitative LC-MS analyses helped to identify chlorogenic acids as predominant in the extract, along with methyl esters of sorbic acid.

1. Introduction

In recent years, a growing interest has focused on the isolation and extraction of new value-added components from plant sources, a process that involves academia as well as industry and consumers. (Galanakis, 2012) The possibility of using natural sources for the extraction of various bioactive compounds has found fertile ground in a decade where nutraceuticals and dietary supplements are experiencing a significant increase in use and production (Zhang et al., 2016). These naturally occurring compounds can be used in fortified foods with increased antioxidant activity, in dietary supplements (Nasri et al., 2014) or as food additives that help extend the shelf-life of products (Pereira et al., 2022). The use of herbal teas or the consumption of certain foods (or parts thereof) that are particularly rich in some bioactives has always existed, and recently several drugs have been derived from phytochemicals. Nowadays, scientific progress and the increased knowledge offer the possibility to improve the intake of metabolites with health-promoting effects, mainly by means of extraction and incorporation into supplements and formulations (AlAli et al., 2021). In general, natural bio-actives has shown a wide range of potential benefits, including anti-inflammatory, antioxidant, anti-aging and anti-cancer activities (Pitchaiah et al., 2017), immune-stimulant properties (Carr et al., 2017), cardiovascular protection (Affuso et al., 2010) and various other applications (Ruchi et al., 2017). The phenomenon of oxidative stress (caused by ROS - Reactive Oxygen Species) can lead to oxidative damage that negatively affects various macromolecules of our organism, such as nucleic acids, proteins, and lipids, origin of several deseases (*i.e.* cancer, tissue-damaging, aging etc.) (Juan et al., 2021). Therefore, it is important to find plant sources that are sufficiently rich in antioxidants and can provide health benefit without synthetic drugs (Bobinaitė et al., 2020). Across the plant kingdom, berry fruits are particularly rich in polyphenols, which show important activity, especially in ROS

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scavenging (Skrovankova et al., 2015).

Sorbus L., is a *genus* belonging to the Rosaceae family, commonly called rowanberries, mountain ash, or European berries. The latter is a widespread deciduous tree or bush, in particular present in the northern hemisphere, widely appreciated throughout the time for its nutritional and medicinal properties (Olszewska et al., 2012) The fruits have been traditionally used as diuretic, laxative, anti-inflammatory, cardiovascular protectant, against rheumatism and kidney issues as well as for the treatment of various gastrointestinal disorders (Shikov et al., 2014), or even as appetite-improving agent and as excellent vitamins source (Singh et al., 2021). They are edible but because of the astringent and bitter taste, food applications are limited to jelly, juice and jam, in particular in northern countries (Mrkonjić et al., 2017; Berna et al., 2012). The main components in wild rowanberries are primarily hydroxycinnamic acids (chlorogenic and neochlorogenic), and flavonols, such as quercetin and kaempferol derivatives (Gaivelyte et al., 2013; Kähkönen et al., 2001. Kylli et al., 2010) Other constituents reported in literature include triterpenes, sterols, carboxylic acids (such as sorbic acid and its esters), coumarins and cyanogenic glycosides (Sołtys et al., 2020).

To extract and isolate the bioactive compounds of rowanberries, several procedures can be applied. The most common and traditionally used are maceration, decoction, infusion, percolation, tincture, extraction with Soxhlet apparatus and hydroalcoholic extraction. In the last decades following the rising trend of highly efficient green extraction method (Chemat et al., 2020), several technologies have been experimented, such as ultrasound- (UAE) (Binello et al., 2020) and microwave-assisted extractions (MAE) (Grillo et al., 2021; Gunjević et al., 2021), subcritical water (SWE) (Aimone al et., 2023), supercritical fluids extractions (SFE) (Salgado-Ramos et al., 2023), pulsed electric fields (PEF) (Wang et al., 2023; Grillo et al., 2022), the exploitation of enzymes and many others. In recent years, all these innovative methods have been extensively used as they brought many advantages, such as shorter extraction times, lower energy consumption and the use of non-toxic solvents (mainly water), guaranteeing higher yields and cost savings. Water is the safest, cheapest and most abundant solvent and it is suitable for dissolving various polar compounds. However, its high polarity hinders the use of this solvent in cases of less polar molecules, such as polyphenols and other organic compounds. To overcome this problem, thanks to scientific innovation and technological evolution, it is possible to use water in a subcritical state, with temperatures between 100 °C and 374 °C, where physicochemical properties are progressively changing. Specifically, the polarity can be adjusted according to the necessity, by increasing temperature, allowing to reach values similar to organic solvents (*i.e.* methanol or acetonitrile (Cheng et al., 2021).

Adopting a software exploiting natural language processing (NLP), developed by Álvarez-Martínez et al. (2023), it is possible to cluster the research papers found in literature by searching "*Sorbus*" and "*Green extraction*", obtaining a world cloud that convey the most common words present in manuscripts (Fig. 1). By analysing the result, the more visible concepts are obviously *extract* and *rowanberries*, along with *phenols, flavonoids, actives* and *antioxidant.* Those keywords, which can be considered the "core" of this work, attest the direction of the research, enriched by topics which are still not involved (*i.e. green, microwaves* and others). This new and modern tool could greatly simplify the analytical procedures in literature searches and is worth mentioning and promoting in the scientific community.

In this piece of work the aim is to design a sustainable extraction protocol for rowanberries, exploiting the features of subcritical water conditions reached by means of MW irradiation. Those benign-by-design approach could pave the way towards the efficient valorisation of a fruit extremely rich in bioactives, that actually can be hardly implemented in foods applications. The outstanding antioxidant peculiarity of the recovered products, together with polyphenolic, flavonoids and sugars contents, would elucidate the high quality of the processed feedstock.

Fig. 1. World cloud obtained by NLP-assisted software, based on the research of works related to "*Sorbus*" and "Green extraction".

2. Materials and methods

2.1. Table of abbreviation

ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
BM	Benchmark
BV	Bed volume
DAD	Diode array detector
DM	Dry matrix
DPPH	2,2-diphenyl-1-picrilidraziyl
EC_{50}	Half-maximal inhibitory concentration
FC	*Folin-Ciocalteau*
GAE	Gallic acid equivalents
MASWE	Microwave-assisted subcritical water extraction
QE	Quercetin equivalents
ROS	Reactive oxygen species
S/L	Solid liquid ratio
SWE	Subcritical water extraction
TEAC	Trolox equivalents antioxidant capacity
TFC	Total Flavonoids Content
TPC	Total Polyphenols Content
TSC	Total Sugars Content
US	Ultrasound

2.2. Materials

Three different varieties of matrices were used during the study: i) *Sorbus aucuparia* L. from Balkan; ii) *Sorbus chamaemespilus* collected in Aosta Valley (Italy); iii) *Sorbus aria* (L.) Crantz. All samples were collected at the end of summer and stored in a dry and dark environment to preserve their properties.

Solvents and reagent for the colorimetric tests were purchased from Sigma Aldrich (St. Louis, MO, USA) or VWR (Radnor, Pennsylvania, USA). The acetonitrile used as HPLC eluent was CHROMASOLV® (gradient grade, for HPLC, 99.9 %) while Milli-Q H_2O was obtained using a Milli-Q Reference *A*+System (Merck Millipore, Burlington, MA, USA).

2.3. Pre-treatment

Cryo-milling was used to reduce the size of the vegetal matrix to

obtain a homogeneous starting material. The berries, after a quick rinse with distilled water, were milled with a laboratory blender (Waring Blender, HGBTWTS360, Stamford CT, USA), after freezing them with liquid nitrogen. The preventive cryogenic treatment provided a more fragile structure and protected the sample from overheating produced by mechanical stress, which could lead to thermal degradation.

2.4. Extractions

2.4.1. Conventional extractions

Hydroalcoholic extractions were selected as conventional benchmark (BM) reference. For these tests, 7 g of matrix were inserted in a round-bottom Pyrex flask with the adequate volume of $EtOH_{aq}$ 70 % solution to reach a 1:30 solid/liquid (S/L) ratio, by adapting the method used by Tian et al. (2017). The mixture was heated in an oil bath under reflux and magnetic stirring for 1 h. After the extraction the suspension was centrifuged, and vacuum filtered. The residual biomass was recovered, thoroughly washed and re-extracted with fresh solvent in the same conditions, to achieve exhaustive extraction. The recovered alcoholic fractions were evaporated under vacuum, while the remaining water was frozen with liquid nitrogen and then freeze-dried (−60 °C, 0.2 mbar, Telstar Lyotest, Azbil Telstar SL, Terrassa, Spain). The dry extract was weighed and dry yield was calculated by normalizing the result on the extracted matrix. The samples were stored at 4 °C for further analysis. The extraction of the hydroalcoholic solution at room temperature was also investigated using a maceration test where 2 g of biomass were left under magnetic stirring for 1 hour in 60 mL (S/L ratio of 1:30) of $EtOH_{aq}$ 70 %.

2.4.2. Microwave-Assisted subcritical water extractions (MASWE)

The extractions assisted by MW were carried out in a SynthWAVE multimode reactor (Milestone srl, Bergamo, Italy), following a protocol commonly used by our research group, adapted from Aimone et al. (2023). All the extractions were carried out using a nitrogen back pressure, to avoid the solvent ebullition and therefore allow to reach the subcritical conditions. At the same time N_2 preserved the oxidation-sensitive components at the high operational temperatures. Temperature control and system cool-down is provided by a chiller working at 8 °C. The samples were treated at different temperatures, ranging from 100 °C to 150 °C (heating ramp of 5 min). Extractions were performed in most of the tests using deionized water as a solvent and a solid/liquid ratio of 1:30. The biomass (10 g) was placed in a Teflon vessel (capacity 1 L) and suspended with the adequate volume of solvent. Before each extraction, the matrix was pre-treated in an US bath (40 Hz, Weber Ultrasonics, MG200TFDMF 40–80–120, Karlsbad, Germany) for 15 min. The agitation during the extraction is provided by a mechanical stirrer (550 rpm). At the end of the process the extraction mixture was separated from the residual biomass, by centrifugation at 4200 rpm for 5 min (Cence Hunan Xiangyi Laboratory Instrument Development Co., Ltd., China) and the supernatant was hot filtered under vacuum on Büchner funnel and then freeze-dried (−60 °C, 0.2 mbar, Telstar Lyotest, Azbil Telstar SL, Terrassa, Spain). The dry extract was weighed and dry yield was calculated by normalizing the result on the extracted matrix. The samples were stored at 4 °C for further analysis.

2.5. Purification

2.5.1. C18 cartridge

A Reverse Phase Sep-Pak® C18 cartridge (1 g, 2 mL bed volume, BV, Waters) was used to fractionate and purify the bioactive-rich fraction of the extract and remove the salts and sugars interferents, under vacuum, gaining inspiration from the SPE purification step carried out by Kylli et al. (2010) on *Sorbus* spp., with some adjustments. The cartridge has been conditioned with 6 mL of deionized water (corresponding to 3 BV), and then loaded with an extract-saturated solution (0.5 mL), discarding the liquid passing through the column. The system has been washed with 3 BV of deionized water, allowing the removal of the most hydrophilic fraction. Afterwards, 3 BV of MeOH have been used to elute the organic fraction from the stationary phase. Both the fractions (aqueous and organic) were dried, weighed and sored at 4 °C for further analysis.

2.5.2. Phenol hydrolysis

To investigate the composition of the extract an acidic hydrolysis was performed to break the glycosidic bonds and to release aglycones. The adopted protocol was an adaptation from Piagnani et al. (2012): in brief, 50 mg of the extract was dissolved in a 10 mL glass tube, containing 2 mL of 50 % $EtOH_{aq}$. and the mixture was vortexed for 30 Section 2 mL of absolute EtOH were added followed by 2 mL of HCl 6 N (added dropwise). The mixture was vortexed, left for 40 min at 80 °C and then left to cooldown at room temperature. The solution was left under a nitrogen flow to remove both HCl and EtOH, while the remaining water has been removed by freeze-drying.

2.6. Analytical section

2.6.1. Total polyphenols content (TPC)

The class of polyphenols represents one of the main high-added value compounds present in this matrix. For this reason, the analysis of those components represents the core of the analytical section, performed by means of different colorimetric essays, exploiting an UV–vis (Cary 60, Agilent Technologies, Santa Clara, CA, USA) as a fast semi-quantitative method to characterize the extraction trends.

2.6.1.1. Folin-Ciocalteau (FC). The most common and widely used method to quantify polyphenols is represented by the assay which exploits the *Folin-Ciocalteau* reagent. A calibration curve is prepared with gallic acid aqueous standard solutions (with dilutions between 5 and 250 μg/mL). The resultant calibration curve is employed as a reference to quantify the samples in terms of gallic acid equivalents (GAE). For the analysis, the dried extracts were dissolved in deionized water, with concentrations ranging from 0.2 to 3 mg/mL. The experimental procedure, which is an adaptation of Singleton et al. (1999), entails the dilution of 250 μL of a freshly prepared solution of the extract with 4 mL of deionized water. In the tube are then added 500 μL of a Na_2CO_3 solution (10 % *w/v*) and 250 μL of the stock solution of *Folin-Ciocalteau* reagent (1:1 with water). The sample was kept in a dark environment and read at 725 nm, after 25 min of incubation, with a UV–vis in a plastic cuvette (1 cm). A blank was prepared using distilled water. The TPC value, determined with the calibration curve, was expressed as mgGAE/g of dry extract (selectivity) or mgGAE/g of dry matrix (yield). Analysis were performed in triplicate, reporting results as average ± the standard deviation.

2.6.2. Total flavonoids content (TFC)

Flavonoids represent a significant category of polyphenols. In order to estimate the concentration of flavonoids within the larger group of phenolic compounds, a colorimetric assay was employed. The utilized methodology is based on the protocol by Asokkumar et al. (2009), where 0.5 mL of the sample solution are mixed with 0.1 mL of 10 % (*w/v*) aluminium nitrate, 0.1 mL of potassium acetate (1 M) and 4.3 mL of 80 % ethanol. After 40 min of incubation in darkness at room temperature the absorbance was measured at 420 nm. A calibration curve (concentration ranging from 0.15 mg/mL to 0.025 mg/mL, $R^2 = 0.999$) was prepared with a standard solution of Quercetin and the results are expressed in quercetin equivalents (mgQE/g of extract). Analysis were performed in triplicate, reporting results as average ± the standard deviation.

2.6.3. Antioxidant and radical scavenging activity

The antioxidant activity of the *Sorbus* extracts is evaluated *via* radical scavenging tests, firstly with the EC_{50} radical inhibition, which describes the sample concentration required to scavenge half of the radical activity. This value has been compared with a standard solution of Trolox® expressing the results as Trolox Equivalent Antioxidant Capacity (TEAC), in µmol over gram of extract. The percentual inhibitions, calculated from UV–vis absorbances, were interpolated *via* a Probit regression (Software Bobo Least Squares ver. 0.9.1.) (Locatelli et al., 2009) for all the following protocols.

2.6.3.1. DPPH. The antioxidant activity of the extracts was evaluated following the method described by Brand-Williams et al. (1995) using the stable radical DPPH• (2,2-diphenyl-1-picrihydrazil). Compounds present in the sample cause the radical inhibition, evaluated as a dose-dependent decolouration (ten different concentrations prepared according to the sequential dilution procedure). The absorbance is monitored at 515 nm deriving the percentual inhibition used to determine EC_{50} values by fitting. For the calibration, a methanolic solution of Trolox® (EC_{50} = 7.6 µg/mL) was used. Analysis were performed in triplicate and results reported as average values (confidence interval of EC_{50} extrapolated by of the software).

2.6.3.2. ABTS·+. To evaluate the antioxidant activity of the extracts, an alternative reagent was used, based on a similar mechanism of reaction than DPPH·. The procedure from Re et al. (1999) and adapted by Aimone et al. (2023) involves the radical generation by the ABTS salt (2, 2′-azino-bis acid(3-ethyllbenzothiazolin-6-sulfonic) and a strong oxidant specie (potassium persulfate). Results are expressed by using a methanolic solution of Trolox® 15 µM (EC_{50} = 7.9 µg/mL) for the calibration. Analysis were performed in triplicate and results reported as average values (confidence interval of EC_{50} extrapolated by of the software).

2.6.4. Total sugars content (TSC)

The Total Sugars Content (TSC) contained in the samples is assessed by means of an easy and rapid colorimetric assay known as "anthrone method" (Yemm et al., 1954). The estimation of the carbohydrates content is accomplished by an initial hydrolysis to monosaccharides and their dehydration, operated by concentrated sulfuric acid, to form hydroxymethyl furfural. The anthranol, the enolic form of the reactant, binds the aldehyde group from furfural, forming a bluish-green complex. The reagent is freshly prepared dissolving 0.2 g of anthrone powder in 100 mL of concentrated 96 % sulfuric acid. 5 mL of reagent were added to 1 mL of the sample solution and incubated at 100 °C for 20 min. The mixture is cooled down to room temperature and the absorbance is registered at 620 nm. Glucose was used to prepare a calibration curve, expressing the results as mgGlu/g of extract. Analysis were performed in triplicate, reporting results as average ± the standard deviation.

2.6.5. LC-DAD-MS

Different samples were analysed by liquid chromatography equipped with a diode array detector (DAD, Water 2487 dual lambda absorbance dual detector, reading 280 and 370 nm), coupled with a quadrupole detector (Waters Micromass ZQ), both supported by MassLynx V4.1 software. The samples were dissolved in different solvents, according to their solubility, ranging from pure deionized water to MeOH solution, through different percentages mix of the two cited solvents. The system is equipped with a Synergi Hydro-RP (4 µm 80 Å, 250 × 4.6 mm, Phenomenex by Waters) and A: H_2O 2 % CH_3COOH; B: ACN 2 % CH_3COOH; as eluents; injection gradient (min,%B): 0,0; 6.50,0; 30,50; 36,100; 45,100. Samples injection volume: 20 µL with flow rate of 1 mL/min. The mass analysis was performed in ESI+ mode (Cone Voltage: 20.00 V; Capillary Voltage: 3.00 kV; Source T: 110 °C; Desolvation T: 220 °C, *m/z* range 100–1200).

3. Results and discussion

3.1. Microwave-Assisted subcritical water extraction (MASWE) optimization

3.1.1. Dry yield determination

An initial MASWE screening was conducted with the aim of determining the best protocol to recover polyphenols from berries. For simplicity, the study was performed on a single variety of berries, considering the others only with the optimized extraction strategy. Temperatures of 100 °C, 125 °C, and 150 °C (30 min, S/L 1:30) were studied to evaluate different subcritical conditions. An hydroalcoholic solution was used as a benchmark under reflux conditions (BM). For all the tests, dry yields have been determined according to Section 2.4, normalizing on the extracted matrix.

As depicted in Table 1, slightly higher yields were obtained at all tested temperatures compared to conventional extraction (up to about +8 %, in the case of 150 °C, 65.42 % *vs.* 57.24 %). These results confirm the great potential of this technology and encourage to deepen the characterization of the recovered compounds.

3.1.2. Colorimetric analyses

3.1.2.1. Total polyphenols content (TPC). All the extracts were characterized with the FC method (see Section 2.6.1.1) in order to assess the total polyphenols content (see Table 2).

The TPC selectivity of the extract resulting from the extraction at lower temperature (42.51 ± 3.11 mgGAE/gext) is very low compared to the other two conditions, which in contrast have values that are almost comparable (76.33 ± 2.35 and 72.72 ± 1.55 mgGAE/gext). This result shows that increasing the subcritical conditions at higher temperatures leads to improved efficiency of polyphenol extraction. It is worth noting that, according to the literature, higher temperatures correspond to less polar extraction media (Galanakis, 2012). Considering the TPC yield (parameter that combines the extraction yield and the selectivity in polyphenols) the best result is represented by the extraction performed at 150 °C, with a net recovery of 47.27 ± 1.01 mgGAE each gram of DM extracted (see Fig. 2).

3.1.2.2. Antioxidant activity – DPPH·. Considering the antioxidant activity as a crucial feature of polyphenols, the latter has been evaluated to better characterize the extract recovered from the MASWE screening. The analysis exploited the stable radical DPPH· (see Section 2.6.3.1), expressing the results as EC_{50} and Trolox eq. (see Table 3).

As depicted by the results shown above, all the MASWE extracts show higher antioxidant capacity than the one obtained under conventional conditions. When the EC_{50} is considered, a lower value indicates a higher antioxidant capacity (the lowest is 18.8 µg/mL, 150 °C). On the other hand, if Trolox eq. are considered, a higher value indicates a better activity. The highest recorded result is 1.62 mmol/gext, for the extraction at 150 °C. The above reported results are consistent with the previous findings, pointing out as the best extraction conditions the protocol conducted at 150 °C for 30 min.

Table 1
Extraction conditions and relative dry percentage yields (%).

Temperature (°C)	Yield (%)
100	59.85
125	61.59
150	65.42
Conventional*	57.24

* Conventional extraction conditions. T: reflux.; t: 60 min × 2; solvent: $EtOH_{aq}$ 70 %.

Table 2
Total polyphenols content, FC analysis.

Temperature (°C)	TPC	
	Selectivity (mgGAE/gext)	Yield (mgGAE/gDM)
100	42.51 ± 3.11	25.42 ± 1.86
125	76.33 ± 2.35	46.56 ± 1.43
150	72.72 ± 1.55	47.27 ± 1.01
Conventional*	53.73 ± 1.81	30.95 ± 1.23

* Conventional extraction conditions. T: reflux.; t: 60 min × 2; solvent: $EtOH_{aq}$ 70 %.

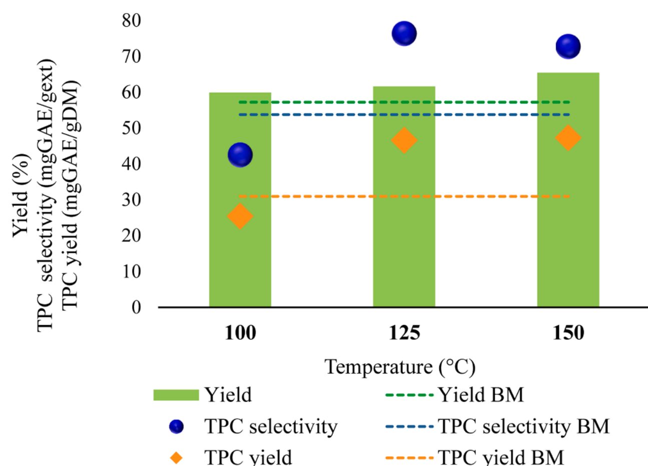

Fig. 2. Total Polyphenols Content analysis on the first set of extraction. Yield: dry yield on the matrix, expressed in %; TPC selectivity expressed in mg GAE per g of extract; TPC yield expressed in mg GAE per g of extracted DM. BM: benchmark.

Table 3
Antioxidant activity, DPPH analysis, results expressed both with EC_{50} values and Trolox equivalents.

Temperature (°C)	DPPH		
	EC_{50}		Trolox eq. (mmol/gext)
	(mg/mL)	(μg/mL)	
100	0.0370 (0.027÷0.051)	37.0	0.82
125	0.0227 (0.017÷0.030)	22.7	1.34
150	0.0188 (0.015÷0.023)	18.8	1.62
Conventional	0.039 (0.032÷0.048)	39.0	0.78

3.2. Variety screening

The first set of tests performed on *Sorbus aucuparia* defined the optimized conditions (150 °C, 30 min), that have been furtherly exploited on two other types of berries. This approach has the aim to define the most promising variety, in terms of polyphenolic content and antioxidant activity.

3.2.1. MASWE and polyphenols recovery

The results presented in Table 4 show the difference between species of rowan berries, under the same extraction conditions.

Despite the lowest dry yield between the different samples, the extract obtained from *Sorbus chamaemespilus* showed a significant polyphenols recovery. In fact, the extraction efficiency of this process resulted in a very high TPC selectivity, with 40.64 ± 0.31% *vs.* 7.27 ± 0.15 % and 5.16 ± 0.05 % (for *Sorbus aucuparia* and *aria*, respectively). Similarly, the *chamaemespilus* var. account up to 225.57 mgGAE/gDM as TPC yield, almost 5 times more than the other two. It worth notice also that the sample of *Sorbus aria* has shown the highest dry yield but the lowest TPC.

Table 4
Extraction yields and TPC analysis, with FC method.

Matrix	Yield (%)	TPC	
		Selectivity (mgGAE/gext)	Yield (mgGAE/gDM)
Sorbus aucuparia	65.42	72.72 ± 1.55	47.27 ± 1.01
Sorbus chamaemespilus	55.51	406.43 ± 3.12	225.57 ± 1.73
Sorbus aria	70.41	51.58 ± 0.52	35.83 ± 0.36

3.2.2. Antioxidant activity

Based on the results obtained in terms of polyphenols recovery, *Sorbus aria* was not considered for the determination of antioxidant activity. On the other hand, *Sorbus chamaemespilus* was selected as the most promising sample to be submitted to further characterizations. Concerning the DPPH· assay (see Section 2.6.3.1), this sample was compared to the previous data gathered for *Sorbus aucuparia* (conventional and MASWE). As reported in Fig. 3, the *chamaemespilus* extract demonstrates a marked antioxidant activity, in comparison with benchmark references.

In particular this product has an antioxidant activity (4.74 mmol-Trolox.eq/gext) which is more than 3 to 6 times higher in comparison with conventionally and MASWE extracted *aucuparia* (0.78 and 1.62 mmol/gext, respectively).

3.2.3. Characterization of the extract – colorimetric assays

As it can be appreciated from Fig. 4, the *Sorbus chamaemespilus* MASWE extract has been characterized by using different colorimetric assays, namely TFC and TSC.

As earlier explained (**see** Table 4), the TPC was investigated using the FC method (see Section 2.6.1.1) with promising results (*approx*. 40 % TPC selectivity). Further details on the composition of this fraction were investigated by the determination of flavonoids (see Section 2.6.2), with results expressed as quercetin equivalents. Indeed, the sample shows a minimal presence of this class of metabolites (13.5 ± 0.76 mgQE/gext), among other polyphenolic compounds. As for the total sugar content, the TSC of the crude extract was evaluated by the anthrone method (see Section 2.6.4), and the results showed that almost half of the extract is composed of sugars (45.41 ± 5.02 % gGlu/gext). This value cannot be

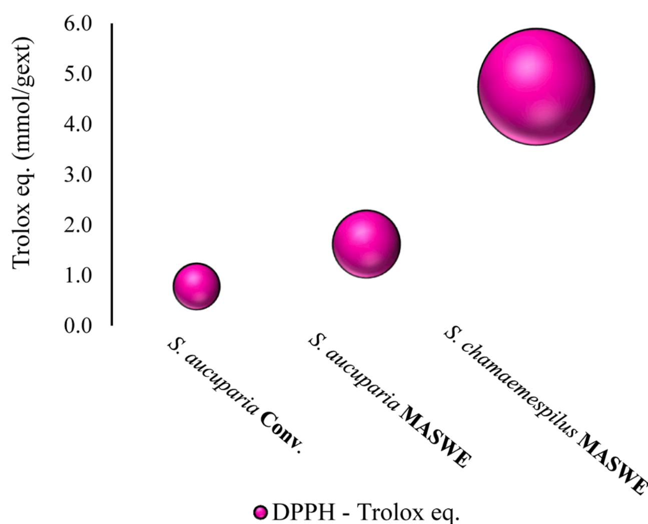

Fig. 3. Antioxidant activity determined with DPPH· radical; comparison between *Sorbus aucuparia* (conventionally extracted), *Sorbus aucuparia* MASWE (150 °C, 30 min), *Sorbus chamaemespilus* MASWE (150 °C, 30 min).

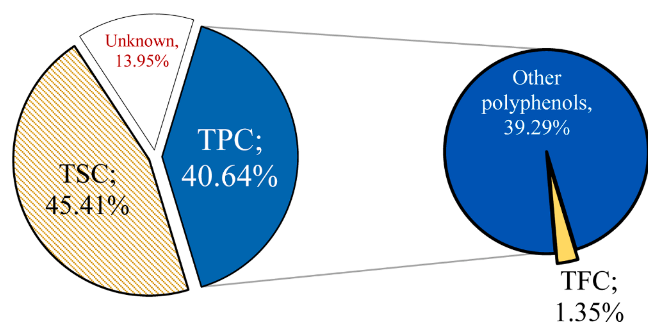

Fig. 4. Characterization of the extract (*Sorbus chamaemespilus*, MASWE 150 °C, 30 min).

directly assigned to a precise class of compounds (*i.e.* mono-, oligo- or polysaccharides), because the colorimetric assay is based on global hydrolysis and dehydration of saccharides. Based on literature data (Zlobin et al., 2012), it can be assumed that rowan berries consist of 4.2 % pectin, which could be partially responsible for the TSC value.

3.2.4. Polyphenolic fraction purification

To better understand the composition of the extract and to obtain an enriched polyphenol fraction, an SPE strategy was developed (see Section 2.5.1), in which the hydrophilic fraction, consisting mainly of sugars and salts, was separated from the rest of the extract (see Fig. 5). The organic fraction recovered from the SPE cartridge resulted in an enrichment of approx. 46 % in terms of polyphenols content (75.71 ± 0.70 % *vs*. 40.64 ± 0.31 % of gGAE/gext). Similarly, TFC content increased by an average of 35 % (1.35 ± 0.76 % *vs*. 2.10 % ± 0.81 gQE/gext) while antioxidant activity increased from 4.74 to 7.41 mmolTrolox.eq/gext, nearly doubling. Concerning the TSC, the results were higher than expected with 29.5 ± 1.98 % gGlu/gext. A possible explanation is that the detected sugars include those associated with the polyphenols (and flavonoids) in their glycosidic form. The aglycone could be formed by the strong hydrolysis during the anthrone test (see Section 2.6.4), which also releases the bound sugars. To evaluate this hypothesis, the controlled hydrolysis of the glycosides was explored (see Section 2.5.2), investigating how the glycones/aglycones ratio could affect the determination and the activity of the recovered metabolites. Therefore, according to the literature, hydrolysis was performed with HCl under reflux conditions (Piagnani et al., 2012), followed by the previously optimized fractionation of the hydrolysate on a C18 cartridge (see Section 2.5.1).

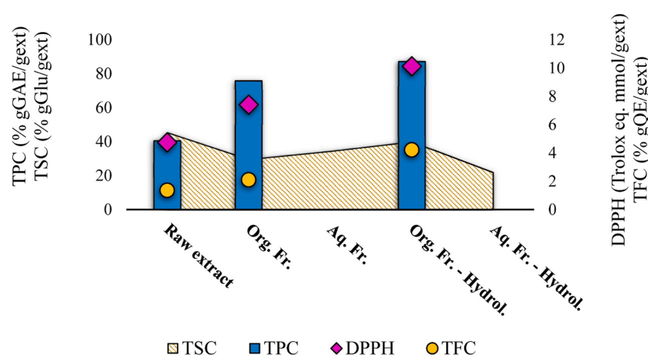

Fig. 5. Analysis of the extract purification, according to different stages and fractions: Raw extract: no purification; Org. Fr.: Organic fraction obtained from the cartridge separation; Aq. Fr.: Aqueous fraction obtained from the cartridge separation; Org. Fr. - Hydrol.: Organic fraction obtained from the cartridge separation performed on the hydrolysed extract; Aq. Fr. - Hydrol.: Aqueous fraction obtained from the cartridge separation performed on the hydrolysed extract. Reported assays: TSC Total Sugars Content, expressed on the primary axis; TPC Total Polyphenols Content, expressed on the primary axis; DPPH antioxidant activity, expressed on the secondary axis; TFC Total Flavonoids Content, expressed on the secondary axis.

The results presented in Fig. 5 attests that the hydrolysis achieved the desired purpose. The TPC selectivity of the collected organic fraction dramatically increase of more than 86 % respect to the raw extract (75.71 % *vs.* 40.64 %). This same fraction, after the hydrolysis, results furtherly enhanced of more than 15 %, from 75.71± 0.70 % to 87.26 ± 2.20 %. In summary, merging the purification strategy with the hydrolysis protocol it is possible to achieve a 2-fold TPC selectivity increase. The results presented above have been additionally confirmed by the proportional increase of the antioxidant activity. The DPPH· value has reached 10.12 mmolTrolox.eq/gext, more than the doubled, with respect to the 4.74 mmolTrolox.eq/gext of the raw extract itself and an increasing of 27 % if the results of the two organic fractions are compared. Following the achievement of this result, the alternative radical ABTS·+ (see Section 2.6.3.2) was employed to comprehensively analyse the antioxidant feature of the *Sorbus chamaemespilus* extract. In Fig. 6 is reported the comparison between the two determinations, where the antioxidant power is equally expressed as Trolox eq.

The increasing of the activity is recognized by both the protocols, but the discrepancies between DPPH· and ABTS·+ results are likely due to different reactivities of the antioxidant compounds. Consequently, it is necessary to assess which method may be more suitable (*i.e.* sensitive). In literature, it is possible to find different references about antioxidant power of *Sorbus* spp. berries. The several numbers of *Sorbus* existing species represent a barrier when attempting to make comparisons between results, within disparate samples and adopted protocols (Sarv et al., 2021; Mikulic-Petkovsek et al., 2017; Olszewska et al., 2012). In this context, considering the values given by the two protocols, the more feasible method seems to be ABTS·+, because of the better discrimination range. Indeed, for an extract characterized by a low antioxidant activity the returned value of Trolox eq. is lower than the one given by DPPH·, on the contrary, with a high antioxidant activity it gives higher results. Thus, the slope of the trend ensures a better discrimination between small variations of antioxidants. Furthermore, the increasing trend of antioxidant capacity achieved through purification goes hand in hand with the increase in polyphenol content. The spheres in Fig. 6 were represented using the as area the FC values and at a glance, it is easy to verify how the increase in Trolox eq. is proportionally correlated with the increase in dimension. Hence, this evidences that the antioxidant power is most likely primarily attributed to the class of polyphenols.

3.3. LC-DAD-MS analysis

To have a deeper characterization of the extracts, several samples

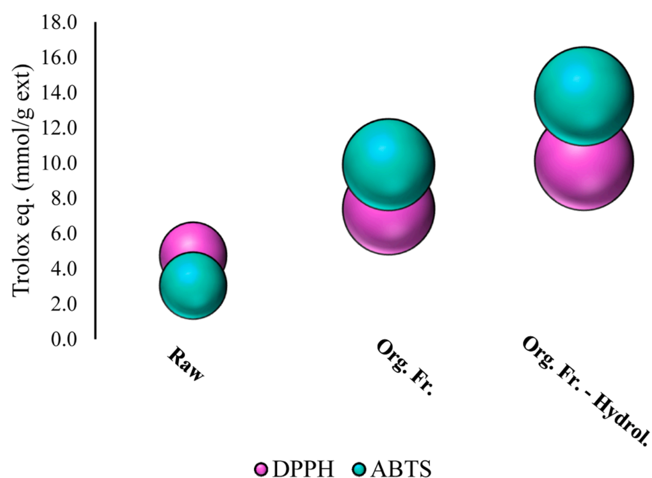

Fig. 6. Increasing trend of the antioxidant activity obtained through the purifications of the extract.

have been analysed by using LC-DAD-MS (see Section 2.6.5). Chromatograms of the fractionated organic phase after C18 purification, have been reported hereafter. As already known in literature, the predominant compounds present in rowanberries extract are chlorogenic acids (Kylli et al., 2010) and different class of flavonoids (Hukkanen et al., 2006). Within the analysis performed on the enriched organic fraction, it is possible to notice the presence of several compounds, in particular chlorogenic acids, quercetin derivatives, sorbic acid and its methyl ester. As it can be depicted in Fig. 7, two predominant peaks (Rt 17.02 min and 19.47 min) are ascribable to chlorogenic and neochlorogenic acid: a definitive discrimination between the two isomers could be achieved by comparing the retention times of standard solution.

Several signals can be detected (see Fig. 8) after searching for quercetin mass. By investigating the rutin mass ($m/z + 1$: 611.52) signal, two peaks are displayed. One peak at 22.15 min is ascribable to a different molecule (non-quercetin-like) with m/z 610, while the signal at 23.65 min is likely due to rutin. This assumption is supported by the coexistence at the same Rt of a signal of $m/z + 1$: 465.10, resulting from the cleavage of the bond between the two moieties composing rutinose, forming isoquercetin (quercetin-3-*O*-glucoside, m/z 464.10), according to literature data (Sawada et al., 2012). The same mass can be also detected at a different Rt (24.29 min), assignable to a native molecule, occurrent in the extract. Those two signals (Rt: 23.65 min and 24.29 min) can be clearly identified also on the chromatogram of the quercetin mass research ($m/z + 1$: 303.24), supporting the abovementioned identifications, since quercetin represent a core constituent of both rutin and quercetin-3-*O*-glucoside. Conversely, a peak at 29.81 min, not composed by additional fragmentations, can be attributed directly to quercetin (m/z 302.24).

Other components characteristic of rowanberries can be detected (see Fig. 9): both sorbic acid (Rt 20.65 min) and methyl sorbate (Rt 24.99 min) can be found by searching the relative masses.

4. Conclusions

In this work, a MASWE protocol was optimised as a green and sustainable strategy for the recovery of antioxidant components from different species of rowanberries. Main investigation of experimental conditions was performed on one species and then the achieved results transferred to the others (150 °C for 30 min). The analysis of all the samples showed a higher concentration of polyphenols (40.64 % gGAE/gext) and antioxidant metabolites (4.74 mmolTrolox eq/gext) for *Sorbus chamaemespilus* compared to the other two varieties. Further remarkable achievements were obtained by using multiple purification steps. A C18 cartridge was exploited to separate different fractions according to their hydrophilicity, yielding an organic fraction with a higher polyphenol content (+46 %) and a doubled antioxidant activity. To improve the enrichment of bioactives, the same purification protocol was repeated after an acid hydrolysis, obtaining a final product reaching 87 % TPC selectivity and an enhanced antioxidant activity (10.12 mmolTrolox.eq/gext). A preliminary LC-DAD-MS analysis confirmed the presence of several bioactive compounds, such as chloro- and neochlorogenic acid, sorbic acid and quercetin derivatives, molecules that can be used in several fields, such as the formulation of food supplements, preservatives, and fortifications or in the production of cosmeceuticals. The presented work demonstrates the feasibility and the exploitability of MASWE as a sustainable and efficient process aimed to recover high value-added products derived from a natural and unexploited source such as *Sorbus* spp. berries.

Ethical statement - Studies in humans and animals

The work by Aimone et al., entitled "*Sorbus spp* berries extraction in

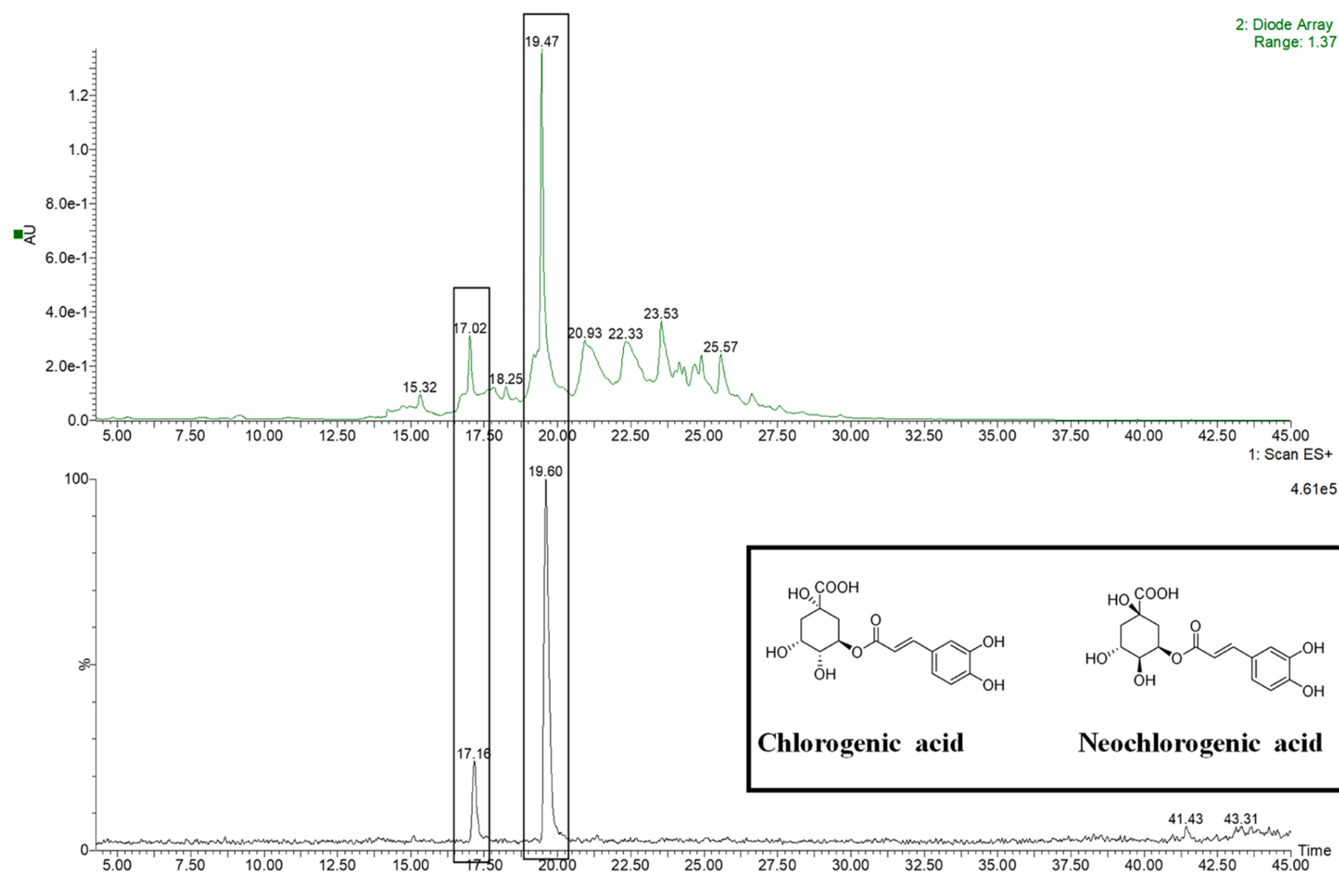

Fig. 7. LC-DAD-MS analysis: DAD spectra and mass research for chlorogenic and neochlorogenic acids ($m/z + 1$: 355.31).

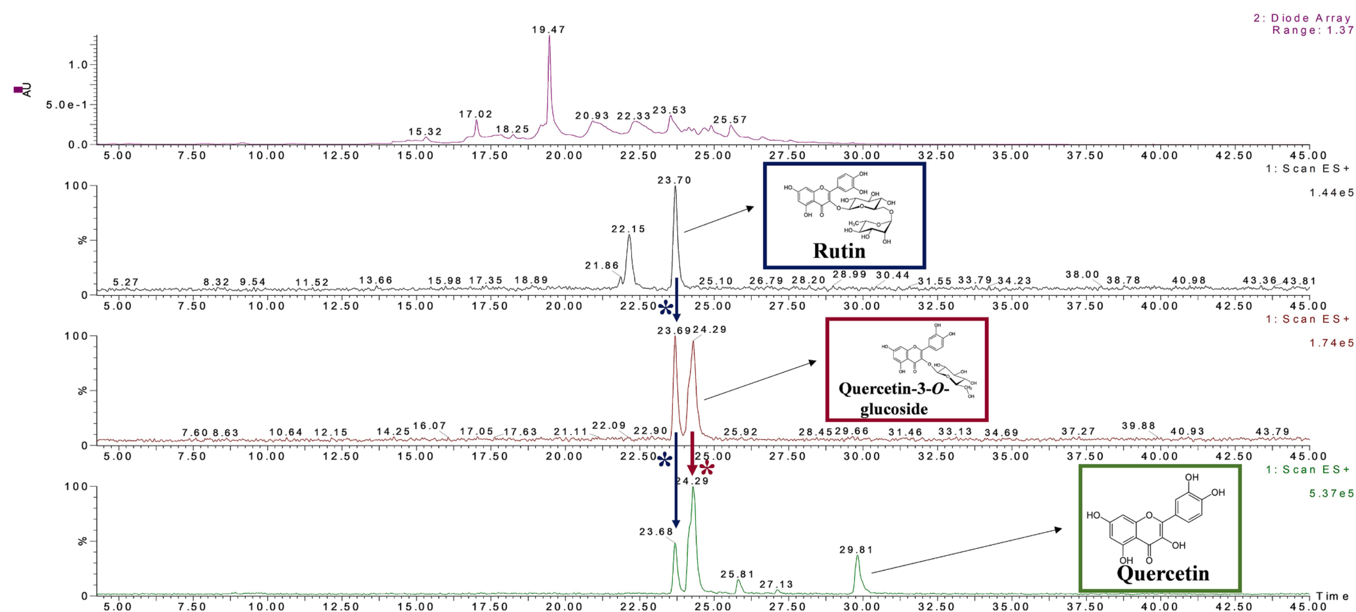

Fig. 8. LC-DAD-MS analysis of quercetin-derivatives: DAD spectra and mass research for rutin (*m/z* + 1: 611.52), quercetin-3-*O*-glucoside (*m/z* + 1: 465.10) and quercetin (*m/z* + 1: 303.24); * indicate the mass fragmentation peaks.

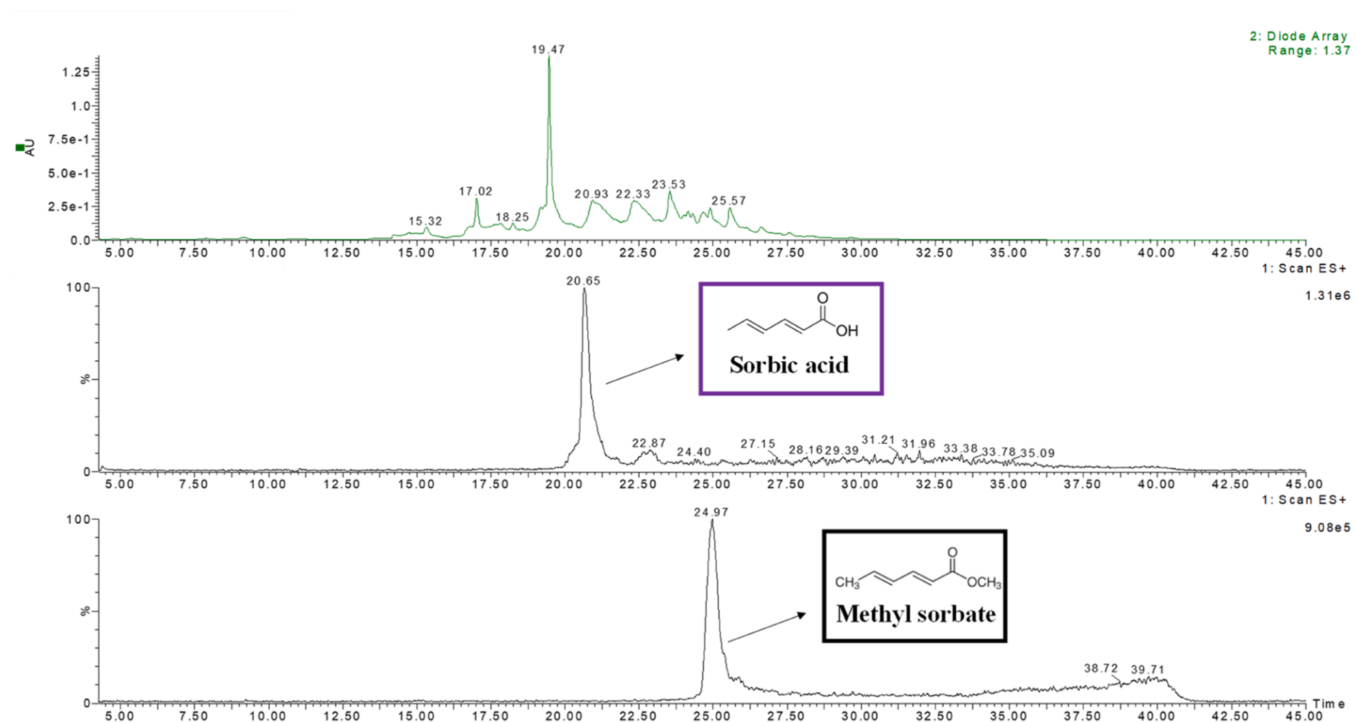

Fig. 9. LC-DAD-MS analysis: DAD spectra and mass research for sorbic acid (*m/z* + 1: 113.12) and methyl sorbate (*m/z* + 1: 127.15).

subcritical water: bioactives recovery and antioxidant activity", did not involved any studies in humans and animals.

CRediT authorship contribution statement

Clelia Aimone: Data curation, Investigation, Writing – original draft. **Emanuela Calcio Gaudino:** Conceptualization, Data curation, Validation. **Mladen Brncic:** Conceptualization, Formal analysis, Validation. **Francisco J. Barba:** Conceptualization, Formal analysis, Validation. **Giorgio Grillo:** Conceptualization, Methodology, Supervision, Writing – review & editing. **Giancarlo Cravotto:** Conceptualization, Supervision, Writing – review & editing.

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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