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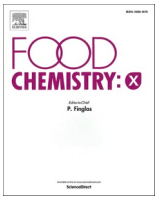

Untargeted metabolomics and conventional quality characterization of rowanberry pomace ingredients in meatballs

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ABSTRACT

In this study, a rowanberry pomace defatted with supercritical CO_2 (2%-AC), its ethanolic extract (1%-E) and extraction residue (2%-R), were tested in meatball preparation. The meatballs with 1%-E demonstrated the highest in vitro radical scavenging capacity. In the case of 1%-E the pH of meatballs was significantly lower compared to the control sample ($P = 0.0132$) on the 5-day. The lowest cooking loss was achieved when the meatballs contained mainly fibre-rich 2%-R. The UHPLC method detected 184 metabolites, including strong antioxidants, such as chlorogenic acids, 3′,4′-methylenedioxy-5,7-dimethylepicatechin, hyperin, isoquercitrin. The 1%-E was particularly effective against the development of unpleasant off-flavours caused by carbonyl compounds. Consistently, the decrease in lipid oxidation, indicated by reduced 7-dodecenal and 2,4-heptadienal contents, has been observed following the addition of rowanberry extract to meatballs. Metabolomics coupled with conventional quality evaluations provided a deeper understanding of the potential utilization and valorisation of different rowanberry pomace extracts as meat ingredients.

1. Introduction

Meat products are significantly susceptible to the decline of quality caused by oxidative processes, especially considering processing and shelf life. The loss of quality in meat, usually supported by lipid oxidation and the formation of undesired compounds, affects meat composition and processing characteristics (Munekata et al., 2020). The hydroperoxides formed while cooking meat may decompose and form volatile organic compounds such as aldehydes, alkanes, alkenes, ketones, alcohols, esters and acids (Domínguez et al., 2019). These compounds are responsible for the deterioration of the colour, texture and flavour of meat-derived products and the loss of pigments and vitamins in meat products (Domínguez et al., 2019; Aminzare et al., 2019). Grinding and heating disarrange the muscle cell configuration, deactivating antioxidative enzymes and initiating non-heme iron, increasing lipid oxidation (Gallego et al., 2015). Therefore, it is challenging food scientists to find ways to inhibit or delay these reactions by utilizing various antioxidant compounds. Whereas synthetic preservatives were mainly used in previous years, recently the emphasis has shifted to safer natural antioxidants (Domínguez et al., 2019).

Recently, food metabolomics or food omics, which aims to analyse small molecules (metabolites), such as pathogens in food systems, has

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gained attention (Fiehn, 2002). Novel analytical instruments, as well as metabolite databases, enable analysing thousands of metabolites in a single analysis and identifying novel metabolites present in food (Beale et al., 2016). The non-targeted approaches allow identifying unknown compounds relevant to food systems and identifying the biomarkers of spoilage (Rocchetti, Bernardo, et al., 2020).

In order to avoid the deteriorative impact of reactive oxygen species (ROS) on meat products, different plant-based ingredients possessing antioxidant capacity, such as the extracts obtained from fruits, vegetables, herbs, and spices, have been tested as meat ingredients (Aminzare et al., 2019). Various berries, such as blueberries, blackberries, cranberries, and grapes, have demonstrated their effectiveness as ingredients possessing antioxidant capacity for stabilizing meat products due to their high contents of antioxidant polyphenols (Lorenzo et al., 2018). Moreover, berry pomace, the solid residue of juice production (Tamkute et al., 2021) has shown good antioxidant potential. Comparing the antioxidant capacities of different berry pomace extracts, Babaoğlu et al. (Babaoğlu et al., 2022) found that the red currant pomace extract showed the highest value in the DPPH$^{\bullet}$ assay, while the water extract of chokeberry pomace exhibited the highest flavonoid and total phenolic content (TPC). In the case of incorporating the water extract of chokeberry pomace into meat products, beef patties' oxidative stability and microbiological acceptability increased during storage in the refrigerator (Babaoğlu et al., 2022). Kähkönen et al. found that 0.05 % rowanberry extract inhibited over 90% of the formation of methyl linoleate-conjugated diene hydroperoxides (Kähkönen et al., 1999). Moreover, rowanberry jam has been considered a suitable ingredient for meat dishes (Hallmann et al., 2011). Compared to the rowanberry juice or fruit samples, pomace samples have demonstrated even higher average TPCs (Sarv et al., 2021). The rowanberry pomace samples, especially originated from the hybrid cultivars (cvs), but also from some other selected rowanberry varieties possessed significant antioxidant capacity values (Sarv et al., 2021). To the best of our knowledge, only one article is available on the use of rowanberry extract in meat products; wherein methanol and ethanol extracts of the whole berries were tested in the emulsified raw pork burger patties, while pomace ingredients have not been tested in meat previously (Ganhão et al., 2010). Considering the tendency of upcycling agro-food processing by-products into higher benefit products it was of interest to test such ingredients in meat, In general, the reports on the use of processed by consecutive extractions berry pomace products in foods are rather scarce.

In terms of their antioxidant properties, it was hypothesized that the rowanberry pomace based ingredients inhibit lipid oxidation in meatballs during meat processing and/or storage. The untargeted metabolomic approach was used for evaluating the changes of metabolites in meatballs with the addition of rowanberry pomace-based ingredients possessing antioxidant capacity during their storage time period.

2. Materials and methods

2.1. Plant material and extracts preparation

Sweet rowanberry cvs Likernaja (the hybrid of *S. aucuparia* × *Aronia melanocarpa*), Solnechnaja (the seedling of *S.aucuparia*) and wild rowanberry were harvested in autumn 2021 from Polli Experimental Station, (South Estonia, 58°0′44.5′′N, 25°32′16.8′′E). All fruits were immediately frozen and stored at −20 °C. The fruit was defrosted before extracting the juice by a low-speed juicer Smeg SJF01CREU (Smeg S.p. A, Guastalla, Italy). The pomace parts, which accounted for approximately 15–20% of the weight of the fresh rowanberries (Sarv et al., 2021), were freeze-dried in a VirTis Advantage Plus Benchtop Freeze Dryer Model XL-70 (SP Industries, Warminster, PA, USA) for 72 h at 30 μbar. Subsequently, three pomace samples were mixed and ground in Retsch cutting mill Retsch SM 300, (Retsch GmbH, Haan, Germany) with sieve holes diameter of 5 mm to obtain a homogenous batch. This sample was defatted by extracting with supercritical CO_2 in SCF extraction equipment Separex 5 (Champigneulles, France) for removing lipophilic substances at 40 MPa pressure, 40∘C temperature and 150 min extraction time (Tamkute et al., 2021) to obtain the 1st ingredient (AC) for meatballs. The lipophilic CO_2 extract was not used in the meat tests due to the remarkable content of polyunsaturated fatty acids in berry seeds, which may accelerate the formation of oxidation products in meatballs during storage (Venskutonis, 2020).

AC was further extracted with 1:1 (v/v) ethanol/water at solid/liquid ratio of 1:10 (w/v) using microwave-assisted extraction (MAE) for 15 min at power 300 W. After the extraction, the extract was filtered. The EtOH part of the supernatant was dried in a rotary evaporator, and the remaining water was freeze-dried in a VirTis Advantage Plus Benchtop Freeze Dryer Model XL-70 (SP Industries, Warminster, PA, USA). The dried extract was stored in a sealed package in a freezer (-20 °C) as the 2nd ingredient (E) for meatballs. The extraction residue was freeze-dried and stored as the 3rd ingredient (R) in the grip seal polythene bag at room temperature.

2.2. Preparation of meatballs

The meatballs were prepared according to the protocol of Kerner et al. (Kerner et al., 2021) with modifications. Briefly, the minced pork and salt were purchased from the local (Tartu, Estonia) butcher's shop and the food store, respectively. The components were mixed according to the recipe and the raw mixture was divided into the following portions: the control sample (88% of minced pork, 11% water, 1% salt), and the samples with ingredients AC, R and E, each with the concentrations of 1%, 2%, 3% and 5%. Six voids (Ø 4.5 cm, depth 2 cm) in the self-made moulds were filled with the raw meatball mixture. After weighing, the meatballs were cooked at 145 °C in the oven Inoxtrend E1CUA-107E (Santa Lucia di Piave, Italy) for 15 min. The cooked meatballs were cooled down to room temperature and weighed and packed into a Vision Pack Srl VP01 (Packaging Factory Holding, Lallio, Italy) under a modified atmosphere consisting of 70 % N_2 and 30% CO_2 provided by Linde GAS Limited Company (Tallinn, Estonia). To understand the effect of rowan ingredients on the physicochemical parameters of pork meatballs during cold storage, the time points 0 and 5 were chosen according to USDA Food Safety and Inspection Service https://www.fsis.usda.gov/ . Accordingly, the ideal shelf-life period of properly stored, cooked meatballs without artificial preservatives is 3–4 days in the refrigerator. Therefore, packed meatballs were stored at + 4 °C and analyzed at 0 and 5 days of storage.

2.3. Sensory evaluation

The sensory assessment of cooked meatballs was conducted by nine randomly selected trained assessors from the Estonian University of Life Sciences, Chair of Food Science and Technology, in a specially designed room with individual booths. The fresh meatballs were warmed to 55–70 °C in a microwave oven (Moulinex Micro-Chef V98, Ecully, France) and cut in half before sensory assessment. The sensory attributes for the valuation of cooked meatballs were the odour, appearance, colour, taste, juiciness, and texture. The widely used hedonic 9-point scale (Wichchukit & O'Mahony, 2015), where the points 9; 5 and 1 indicate very good, satisfying, and not satisfying assessment, respectively, was applied for sensory evaluation.

2.4. Determination of quality characteristics

The cooking loss of meatballs was calculated as the weight difference between raw and cooked but cooled to the room temperature samples in percentages. Prior to the chemical analyses, such as fat (EVS-ISO 2446:2001, Gerber method), moisture (EVS-ISO 1442:1999), protein (EVS-ISO 937:1978, Kjeldahl method), and ash content (ISO 936:1999), the meatball samples were homogenised using the Retsch GM200 laboratory homogeniser (Retsch Gmbh & Co, Haan, Germany). Seven

2Go™ pH-meter (Mettler-Toledo AG Analytical, Schwerzenbach, Switzerland) was used to determine the pH of meatball samples (5 g) homogenised with 50 mL of 0.1 M potassium chloride solution. The water activity analyser (Aqua Lab, Model Series 3 TE, Decagon Devices, Inc., Washington, DC, USA) was used to determine the water activity (aw) by achieving the equilibrium humidity of air in a tightly closed chamber. The X-Rite 964 spectrophotometer (X-Rite, Grand Rapids, MI, USA) was used for taking three replicate colour measurements of each freshly cut meatball sample from different places. The measurements were expressed numerically by CIE (International Commission on Illumination) Lab system values, where L^*, a^*, b^* mark the lightness, redness, yellowness, respectively (Mokrzycki & Tatol, 2012).

2.5. Determination of total phenolic content and in vitro radical scavenging activity

Two in vitro spectrophotometric analyses, the total phenolic content (TPC) and 2.2-diphenyl-picrylhydrazyl (DPPH•) scavenging assay, were used for preliminary screening of meat ingredients AC, R and E. The (TPC) was measured according to the method of Folin–Ciocalteu (FC) (Folin & Ciocalteu, 1927) with modifications using the gallic acid (GA) standards. The free radical scavenging capacity assay (DPPH•) with slight modifications was, according to Brand-Williams et al. (Brand-Williams et al., 1995) using Trolox as a positive control. The absorbance values of the samples during the TPC and DPPH• assays were measured at 760 nm and 515 nm, using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan). Both spectrophotometric assays were performed in four replicates using laboratory grade chemicals purchased from Sigma-Aldrich (Steinheim, Germany).

2.6. Untargeted profiling by UHPLC-HRMS of the different meatballs

The untargeted metabolomics was used to evaluate storage time effects on prepared meatballs and the phytochemical profile of the 3 different rowanberry extracts. The time points for observation of the changes in metabolomics were selected as the day of preparation- day 0, the longest ideal period for storing homemade meatballs – day 4, and to study the possible oxidation process of packed meatballs-day 14.

Therefore, the lyophilized pork meatballs were extracted following the protocol previously reported by Pateiro et al. (Pateiro et al., 2018), with minor modifications. Briefly, one gram of each sample was extracted with 10 mL of an 80% aqueous methanol (v/v) solution (both LC-MS grade, VWR, Milan, Italy) added with 0.1% (v/v) formic acid. This mixture was subjected to an extraction system through Ultra-turrax (Ika T10, Staufen, Germany) for 5 min at room temperature. The corresponding extracts were centrifuged (Eppendorf 5810R, Hamburg, Germany) at 7800 × *g* for 15 min at 4 °C and then filtered using 0.22 μm cellulose syringe filters. Finally, the filtered samples were transferred to amber vials until instrumental analysis.

In this work, the untargeted profiling analysis was done using high-resolution mass spectrometry (HRMS) based on a Q-Exactive™ Focus Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, Waltham, MA, USA) coupled to a Vanquish ultra-high-pressure liquid chromatography (UHPLC) pump and equipped with heated electrospray ionization (HESI)-II probe (Thermo Scientific, USA). Shortly, the chromatographic separation was carried out under a gradient of acetonitrile in water (from 6% to 94% in 35 min) as mobile phase, with 0.1% formic acid as a phase modifier, using BEH C18 (2.1x100 mm, 1.7 μm) analytical column maintained at 35 °C. The injection volume was 6 μL and elution was operated with a flow rate of 200 μL/min. Full scan MS analysis was performed under the positive ionization mode and with a nominal mass resolution of 70,000 FWHM at m/z 200. The injection sequence was randomized, with three replicates for each sample. Quality control (QC) samples (prepared by pooling same aliquots of each sample) were acquired in a data-dependent (TOP N = 3) MS/MS mode, and the Top N ions were selected for fragmentation under stepped (10, 20, 40 eV) Normalized Collisional Energy. The HESI parameters were previously optimized by Rocchetti et al. (Rocchetti et al., 2021).

The raw spectral data were processed using MS-DIAL software (version 4.80) (Tsugawa et al., 2015) for post-acquisition and data filtering procedures. The MS-DIAL parameters were adapted from previously published works on LC-MS untargeted metabolomics-based analysis (Rocchetti et al., 2021). The mass features were searched in the mass range of 80–1200 m/z, having a minimum peak height of 10,000 cps. Accurate mass tolerance for peak centroiding was 0.05 Da for MS and 0.1 Da for MS/MS analysis. Retention time information was excluded from the calculation of the total identification score. The MS and MS/MS tolerance for identification was set to 0.05 Da and 0.1 Da, respectively. The identification step was based on mass accuracy, isotopic pattern (i.e., isotopic distribution, space, and abundance) and spectral matching. The total identification cut-off score was set to 50%, retaining the most common HESI + adducts. Annotation of meat metabolites was achieved against the comprehensive database known as FooDB (https://foodb.ca/). Furthermore, the software MS-Finder (Tsugawa et al., 2016) was used for in-silico fragmentation of the not annotated mass compounds, using the FooDB and Lipid Maps libraries, thus working according to a level 2 of confidence in annotation (i.e., putatively annotated compounds and structural confirmation according to spectral matching) (Salek et al., 2013). Only the compounds having an in-silico prediction score higher than 5 were retained.

2.7. Statistical and multivariate data analysis

The mean values and standard deviations (SD) of total phenolic contents (TPC) and DPPH• radical scavenging capacity (RSC) results were calculated using MS Excel 2016 and one-way analysis of the variance (ANOVA) at p value < 0.05. The statistical package R 4.2.0 was applied for statistical analysis (Minato Nakazawa, 2022) of assessors panel sensory scores and sensory score results visualization. The Linear Mixed-Effects Model (GLMM) was used to study the effects of variants, the effect of three replications and the storage period on the pH, aw and colour characteristics of the samples as well as the measurements of cooking loss, moisture, and protein and ash contents on the day 0. The Emmeans (Searle et al., 1980) package was applied for the pairwise comparison of groups and the model-assessed results were presented as least-square means.

The multivariate statistical analyses dealing with metabolomics were done using two different softwares, Mass Profiler Professional (version B.12.06; from Agilent Technologies) and SIMCA (version 16; from Umetrics, Malmo, Sweden) for data processing and normalization and supervised modelling, respectively. In this regard, both unsupervised and supervised multivariate statistics were used based on hierarchical cluster analysis (HCA), Principal Component Analysis (PCA), and orthogonal projections to latent structures discriminant analysis (OPLS-DA). The OPLS-DA models were built considering the storage time period (i.e., 0, 4, and 14 days) under investigation, also recording the model validation parameters (goodness-of-fit R^2Y) and goodness-of-prediction Q^2Y). The VIP (i.e., variables importance in projection) selection method was then used to list the most relevant meat metabolites in prediction, considering only VIP markers characterized by values higher than 1. Finally, a Fold-Change (FC) analysis was done to check the direction and the intensity of variation of the marker compounds highlighted by the VIP selection method.

3. Results and discussion

3.1. Sensory evaluation and in vitro antioxidant capacity of meatballs

Due to the harmfulness of synthetic ingredients, various natural preservatives have recently been tested to inhibit lipid oxidation and extend the shelf-life of foods (Aziz & Karboune, 2018). However, applying plant-based ingredients to food products as preservatives may

be limited due to the flavour characteristics (Dussault et al., 2014). The astringent taste of rowanberry is a major obstacle to its consumption. Therefore, it is essential to know the acceptable dose of this ingredient to achieve the sensory quality of meatballs. In our research, three rowanberry pomace powders of cvs Likernaja, Solnechnaja and wild rowanberry were pre-selected as the ones with the highest antioxidant capacity (Sarv et al., 2021). These powders were defatted and mixed to obtain the 1st ingredient (AC). The 2nd ingredient was EtOH/water microwave extract of defatted pomace (E) and the 3rd was the extraction residue (R). The TPC of these three ingredients were analyzed and the TPC value of E was almost fivefold compared to AC and 17 times higher than R (Fig. 1a).

The minced pork (moisture 67.43%, protein 18.49%, fat 13.85%, and ash 0.96%) was mixed with 11% water and 1% salt as well as the rowanberry pomace-based ingredient. The concentrations from 1% to 5% of ingredients were sensory evaluated and the best of each group were selected for further tests. The meatballs without any ingredients were evaluated in every test as a reference.

The panellists gave the highest average score for the colour (7.8) of the samples with 3%-AC, followed by the juiciness of the samples with 1%-R and the control, both with an average score of 7.5. In addition, the samples with 1%-R achieved the best score of 7.5 for the taste. The odour was most acceptable in the case of 1%-R. The samples with more than 1%-E scored<5 for taste and were not acceptable for further use. Both AC and R with concentrations 1–3% got the average scores of sensory attributes more than 5; therefore, 2%-AC and 2%-R as well as 1%-E were chosen for further experiments.

The in vitro antioxidant capacity of lyophilized meatballs was tested, using their ability to scavenge stable diphenyl-picrylhydrazyl radical DPPH• assay. Compared to the meatballs without any ingredients (control), the meatballs with ingredients had remarkably higher DPPH• assay values: with the addition of 1%-E, 2%-AC, and 2%-R, the antioxidant potential of meatballs was more than 15-, 10- and 5- fold higher, respectively (Fig. 1b). These results can be crucial in slowing oxidation and deleterious processes occurring during meat processing and/or storage.

3.2. Proximate composition and cooking losses of meatballs

Adding plant-based fibres to meat products allows producers to supply the food with better texture or moisture holding capacity and reduce the content of animal-based proteins and saturated fatty acids (Paglarini et al., 2022). In the current study, the plant-based ingredients accounted just for 1–2%; therefore, the moisture, ash and protein contents were not affected remarkably.

However, the fat content in meatballs decreased by adding the plant-based ingredients, especially the fibre-rich AC and R. In addition, as indicated by ANOVA (Table 1), the ingredient R affected the juiciness of meatballs by keeping more than 2% higher moisture content compared to the control sample and by reducing the cooking loss more than 13%. These results agree with the previous study, where a remarkable decrease in cooking loss was achieved with 3% sugarcane fibre addition to meatballs (Mena et al., 2020). In contrast to fibre-rich ingredients, the lyophilized extract E increased the cooking losses significantly.

Table 1
Proximate composition of cooked pork meatballs and cooking losses.

Sample	Moisture (g/100 g)	Protein (g/100 g)	Fat (g/100 g)	Ash (g/100 g)	Cooking loss (%)
Control	57.90 ± 2.33[a]	20.74 ± 0.35[a]	21.05 ± 0.86[b]	2.04 ± 0.276[a]	23.33 ± 2.05[ab]
AC (2%)	58.48 ± 4.03[a]	20.59 ± 0.24[a]	15.20 ± 4.54[a]	2.00 ± 0.045[a]	24.27 ± 2.42[ab]
E (1%)	57.62 ± 2.13[a]	20.64 ± 1.22[a]	19.33 ± 1.38[bc]	1.85 ± 0.027[a]	26.23 ± 4.97[a]
R (2%)	59.31 ± 1.83[a]	19.40 ± 1.25[b]	17.00 ± 0.30[ac]	1.94 ± 0.109[a]	20.17 ± 3.66[b]

[a, b, c] Different letters in columns indicate significant differences between least square means ($p < 0.05$) by Tukey's multiple comparison's post hoc test. Control—meatballs without ingredients, AC (2%) —meatballs with 2% of defatted with supercritical CO_2 rowanberry pomace, E—meatballs with 1% of EtOH/water extract of AC, R—meatballs with 2% of extraction residue.

3.3. Determination of physicochemical parameters

The main quality characteristics, which play an important role in defining consumers' preferences, are the juiciness, colour, freshness and tenderness of meat products. These quality characteristics are affected by the physicochemical parameters, such as pH, water activity (aw) and colour (Tamkute et al., 2021). The decrease in pH can lead to an unacceptable taste in food but also provide an inhibitory effect against spoilage or pathogenic microorganisms (Barcenilla et al., 2022).

In current study, after 5 days of storage at 4 °C, the pH was significantly lower in the meatballs with 1%-E compared to the control sample ($P = 0.0132$) (Fig. 2 a). The pH reduction in meatballs can be explained by the higher concentration of chlorogenic acids present in E, compared to the fibre-rich ingredients AC and R (Sarv et al., 2021). However, in the case of the samples with AC and R, the pH remained stable during the 5-days of storage, due to some content of chlorogenic acid, while the pH of the control sample increased. Tamkute et al. found that the pH of cooked ham samples with chokeberry extract remained constant during a prolonged (36 days) storage period at 4 °C, while the pH of control sample increased (Tamkute et al., 2021). The "easily perishable" meat products aw greater than 0.95 and pH greater than 5.2 must be stored at or < 5 °C (Halagarda & Wójciak, 2022). The measured aw values of the meatballs in this study ranged within 0.974–0.987 (Fig. 2 b), while the highest reduction (1.3%) in aw values were achieved when the fibre-rich 2%-R was added to meatball pastry. Such a small decrease in aw doesn't have any significant influence on the self-life and storage conditions of

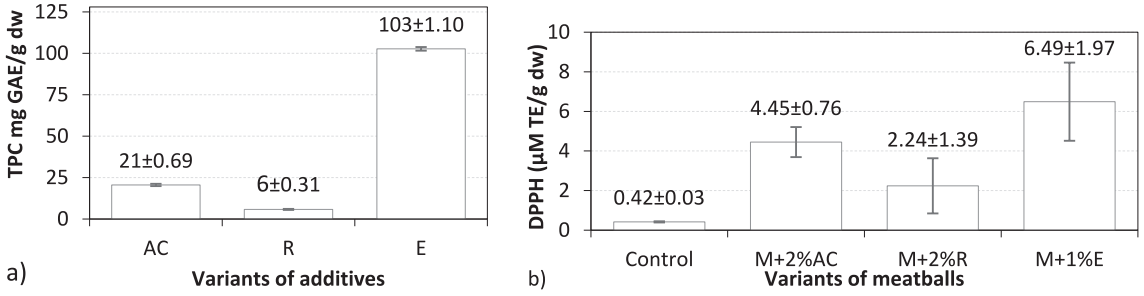

Fig. 1. The total phenolic content (TPC) of rowanberry pomace-based ingredients for meat a): Control—without ingredients, AC— defatted with supercritical CO_2 rowanberry pomace, E— EtOH/water extract of AC, R—extraction residue; and b) antioxidant capacity (DPPH) of meatballs with and without ingredients: Control—without ingredients, M + 2 %AC— meatballs with 2% of defatted with supercritical CO_2 rowanberry pomace, M + 1 %E— meatballs with 1% of EtOH/water extract of AC, M + 2 %R— meatballs with 2% of extraction residue.

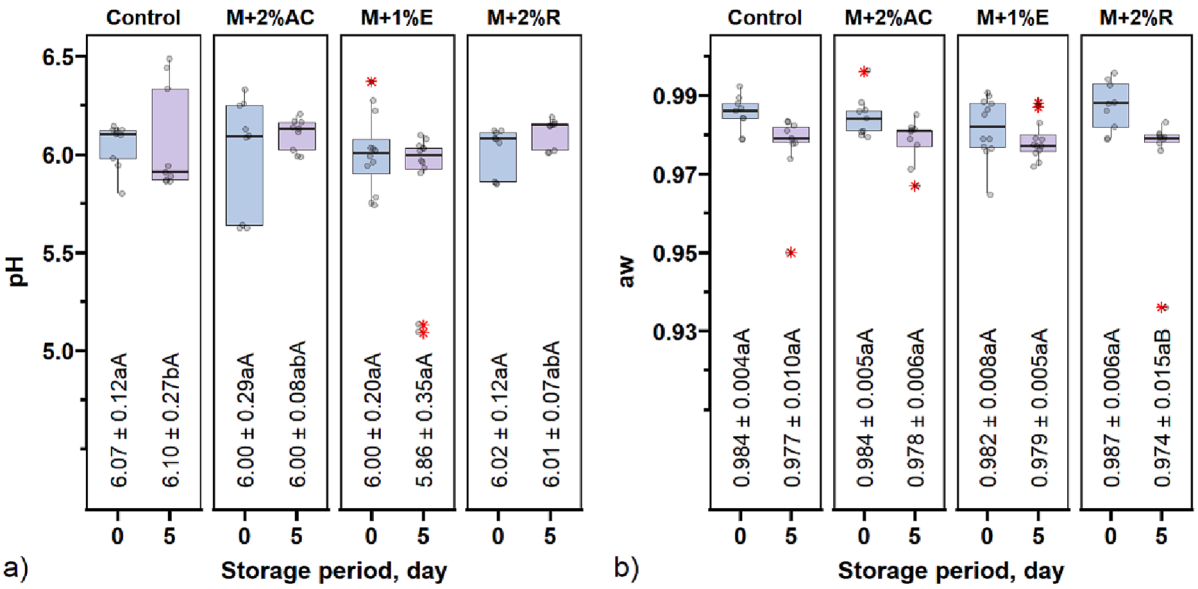

Fig. 2. Physicochemical parameters of meatballs (pH and aw) on the day of preparation and after five days of storage Actual values are presented with grey dots; outliers are denoted with a red asterisk; mean values are presented as least-square means ± standard deviations; different lowercase letters indicate the statistical difference ($p < 0.05$) between the variants within the same day; different capital letters indicate the statistical difference ($p < 0.05$) between the days within the same variant). Control—without ingredients, M + 2 %AC— meatballs with 2% of defatted with supercritical CO_2 rowanberry pomace, M + 1 %E— meatballs with 1% of EtOH/water extract of AC, M + 2 %R— meatballs with 2% of extraction residue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

meatballs. The other ingredients (2%-AC and 1%-E) caused an even lower reduction of aw, 0.7 and 0.3%, respectively. Similarly, in the previous study (Tamkute et al., 2021) there was only a marginal effect on aw of pork products in the case of the cranberry pomace ethanol extract addition.

The state and content of myoglobin, storage temperature, pH, and packaging affect the colour of raw and cooked meatballs (Tamkute et al., 2021). The colour is also the first parameter that indicates the possible microbial or oxidative spoilage of meat products. The discolouration of red meat could occur due to the oxidation of the iron atoms in red oxy-haemoglobin (Peiretti et al., 2020). In the current study, all the rowanberry pomace-based ingredients decreased the lightness (L^*) of meatballs, likely due to the high content of anthocyanins in rowanberries (Sarv et al., 2021) (Fig. 3a). This kind of darkening of meat products has been previously mentioned for chokeberries, blueberries, grapes and blackcurrants (Peiretti et al., 2020; Tamkute et al., 2021). In the current case, the ingredients increased the redness (a^*) up to 48% (Fig. 3 b). The ingredients with higher amounts of bioactive components, such as 1%-E and 2%-AC, decreased the yellowness (b^*) of meatballs by 1.87% and 0.42%, respectively, but 2%-R increased b^* value by 0.51%, compared to the control, during 5 days of storage (Fig. 3 c).

3.4. *Untargeted chemical profiling of meatballs added with rowanberry ingredients during storage*

The untargeted UHPLC-Orbitrap analysis on the different meatballs allowed the putative annotation of 402 compounds according to their

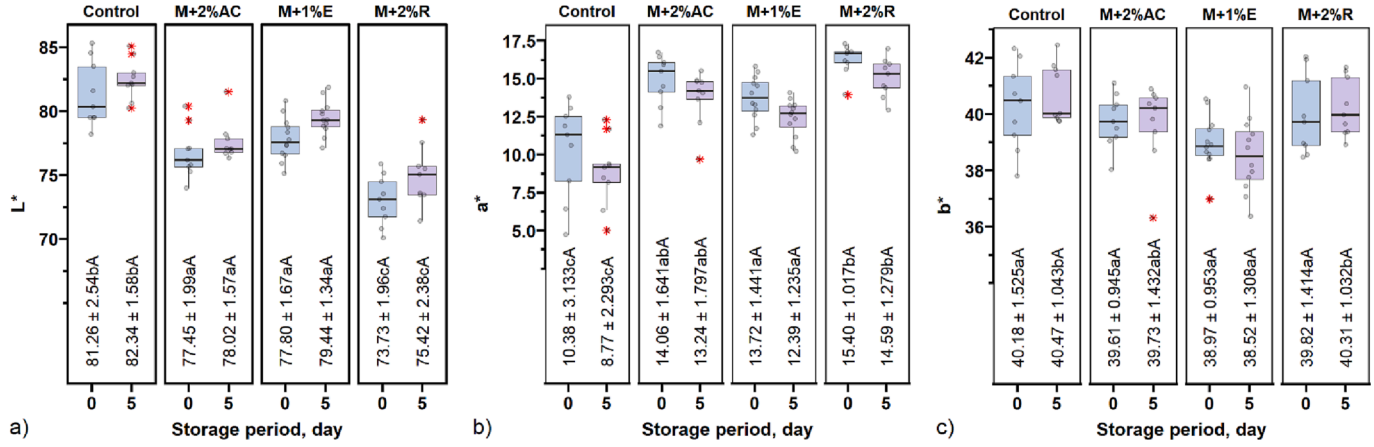

Fig. 3. Colour characteristics of meatballs (L*, a*, b*) on the day of preparation and after five days of storage Actual values are presented with grey dots; outliers are denoted with a red asterisk; mean values are presented as least-square means ± standard deviations; different lowercase letters indicate the statistical difference ($p < 0.05$) between the variants within the same day; different capital letters indicate the statistical difference ($p < 0.05$) between the days within the same variant. Control—without ingredients, M + 2 %AC— meatballs with 2% of defatted with supercritical CO_2 rowanberry pomace, M + 1 %E— meatballs with 1% of EtOH/water extract of AC, M + 2 %R— meatballs with 2% of extraction residue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

individual abundance values and composite mass spectra (MSMS). The number of chemical features annotated by untargeted metabolomics reflects the overall complexity of the meat matrix under investigation. A detailed list of all compounds annotated, with the corresponding mass spectra, isotopic profile, and identification-related information, is provided as **supplementary material**.

Afterwards, a multivariate statistical approach based on both unsupervised and supervised methods was used to group samples according to their similarity in the measured mass features. Firstly, the unsupervised hierarchical cluster analysis (HCA) was used to naïvely group samples according to intrinsic similarities in their chemical profile, and the corresponding heat-map (based on the Fold-Change, FC, and variations of each annotated compound) is reported in Fig. 4. The HCA consisted of two main groups: the first cluster hierarchically included the control samples at the different time-points of storage time (i.e., 0, 4, and 14 days), whilst the second cluster showed all the meatballs prepared with the different pomace ingredients (i.e., 2%-AC, 1%-E, and 2%-R). The heat-map highlighted the potential effect of rowanberry pomace on modifying the meat metabolomic profile. Similar separation trends were obtained by inspecting the unsupervised PCA score plot (**supplementary material**), highlighting a clear separation between the control samples and the meatballs with added ingredients along the first principal component (PC1). Looking at the unsupervised statistical findings, the impact of storage time was particularly evident when considering 2%-AC and 1%-E added samples. Therefore, to confirm the results highlighted by unsupervised multivariate methods, a supervised

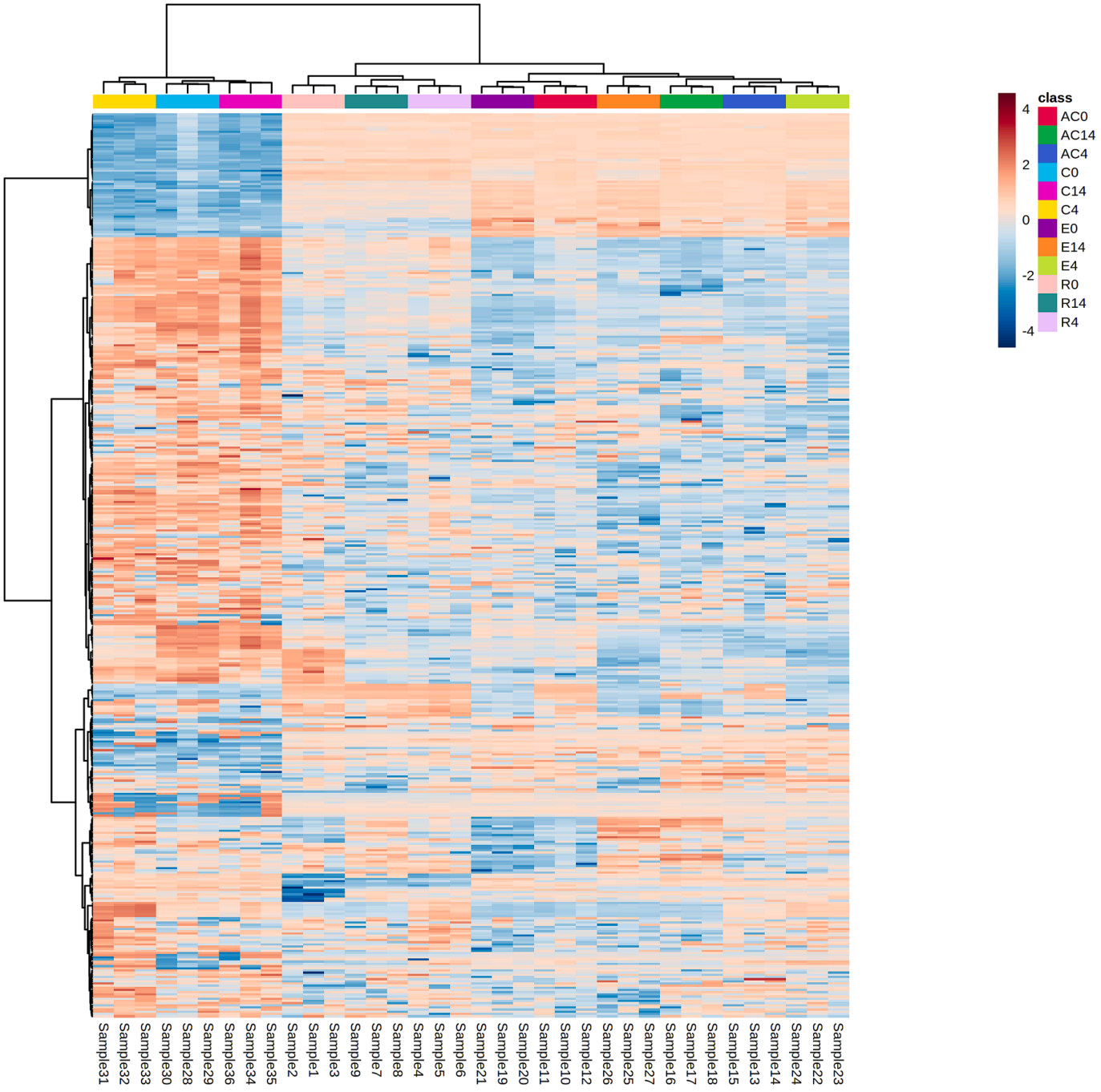

Fig. 4. Unsupervised hierarchical cluster analysis (HCA) considering the chemical profile of the different meatballs prepared with rowanberry functional ingredients (AC, E, and R) *vs* the control (C), at the different storage time-points (i.e., 0, 4, and 14 days).

orthogonal projection to latent structures discriminant analysis (OPLS-DA) was used to investigate the impact of storage time on the chemical profile of meatball samples. As shown from the OPLS-DA score plots reported in (**supplementary material**), each prediction model showed a clear separation trend between the different time points of storage time, recording more than acceptable goodness of fitting ($R^2Y > 0.9$) and prediction ability (Q^2 greater than 0.5) values. These prediction models confirmed the effectiveness of the OPLS-DA in predicting the major changes in the chemical composition of meatballs during storage at 4 °C. As the next step, we evaluated the changes in meat metabolites considering the last time point (i.e., 14 days), being more informative about the potential impact of oxidative processes on meat components and considering the protective role exerted by rowanberry pomace ingredients. Therefore, a new OPLS-DA model was built using only the meatball samples at 14 days of storage. As highlighted from the OPLS-DA score plot (Fig. 5), the control sample (C14) was separated from the added-samples (R14, E14, and AC14) along the orthogonal latent vector. Instead, meatballs with added rowanberry ingredients showed some differences in their chemical profile, with 2%-R and 1%-E samples found very close to each other. In contrast, a more characteristic chemical profile characterized the 2%-AC sample. Overall, the OPLS-DA model built consisted in excellent cross-validation and goodness parameters, with R^2X (cum) = 0.719, R^2Y (cum) = 0.993, and Q^2Y (prediction ability) = 0.919. Afterwards, the variable importance in projection (VIP) approach was used to select the most discriminant metabolites of the OPLS-DA model built. This approach revealed 184 discriminant metabolites having a VIP score higher than 1 (i.e., high prediction ability). These marker compounds are reported in **Table S1** (**supplementary material**), grouped in chemical classes provided by the comprehensive database FooDB. Additionally, we evaluated the Log Fold- Change (FC) variations between the three different treatments with the control. Looking at the discriminant markers reported in Table S1 (**supplementary material**), we found mainly terpenoids (52 compounds), amino acids (26 compounds), fatty acid derivatives (including esters, acids, and alcohols), polyphenols (16 compounds) and other compounds (e.g., aldehydes and ketones). Interestingly, fatty acid derivatives, aldehydes, and ketones were found to be up accumulated in the control sample compared to added-meatballs Table S1 (**supplementary material**). Overall, 1%-E was the most active rowanberry ingredient against the accumulation of aldehydes and ketones, recording cumulative LogFC values of 7.16 and 7.27, respectively, compared with the control (C) at 14 days of storage time. Regarding lipid oxidation phenomena, carbonyl compounds are described as a major by-product potentially affecting meat quality because of the off-flavours development due to the volatile fraction. Looking at our findings (**supplementary material**), the five discriminant aldehydic compounds were characterized by high LogFC values, such as 7-Dodecenal and 2,4-Heptadienal. This latter has already been detected as a marker associated with oxidative processes on meat components and its overall up-accumulation outlined a possible protective effect of the rowanberry pomace on lipid oxidation (Rocchetti, Lorenzo, et al., 2020). In this scenario, unsaturated lipids are chemically unstable and easily affected by degradation (Falowo et al., 2014). Our results revealed that linoleic acid derivatives showed an overall down-accumulation in the control sample (C), thus indicating a potentially higher lipidic peroxidation that was preserved by the addition of rowanberry pomace ingredients. However, looking at single marker compound, alpha-linolenic acid (i.e., one of the most involved in lipid peroxidation) was up-accumulated after 14 days only in meatballs added with 2%-R and 1%-E, while it showed a slight decrease when compared with the control (supplementary material). Another interesting result was related to the overall changes of glycerophospholipids in the three different pairwise comparisons under investigation Table S1 (**supplementary material**). In our experimental conditions, these compounds were characterized by an overall up-accumulation in the control sample, and the markers showing the higher values were phosphatidylethanolamine (PE) derivatives, such as PE (14:1(9Z)/14:1(9Z)), PE (14:0/14:0), PE (14:0/14:1(9Z)). Additionally, an up-accumulation of 25-Hydroxycholesterol was registered Table S1 (**supplementary material**); it is a steroid derivative showing high LogFC score values for each comparison under investigation (on average: 3.05). Its presence has been previously detected in two typical Italian pork products and is likely correlated to lipid and cholesterol oxidative processes (Novelli et al., 1997).

Regarding those discriminant terpenoids (including monoterpenoids, diterpenoids, triterpenoids, tetraterpenoids, and sesquiterpenoids) and phenolic compounds (mainly flavonoids and phenolic

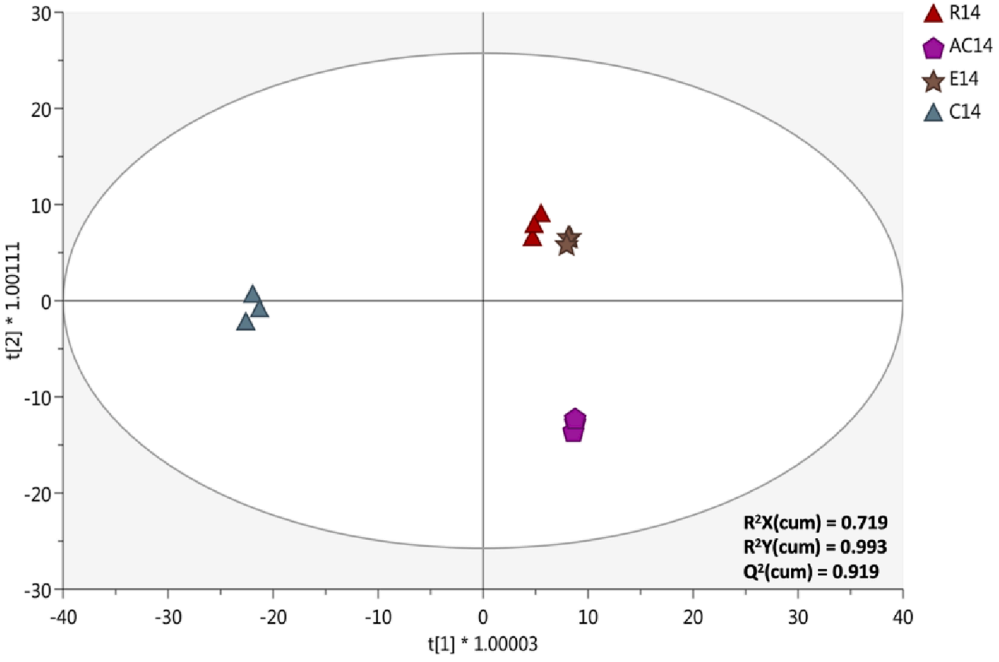

Fig. 5. Supervised orthogonal projection to latent structures discriminant analysis (OPLS-DA) considering the chemical profile of the different meatballs prepared with rowanberry functional ingredients (AC, E, and R) *vs* the control (C), at 14 days of the storage time period.

acids), most of the compounds were down accumulated in the control sample (C) and then clearly associated with the addition of rowanberry pomace ingredients in meatballs. Also, these secondary metabolites were characterized by similar LogFC values for each comparison against the control, thus showing a similar impact of the pomace ingredients during the storage time period (14 days). Several triterpenoid compounds of interest, such as 3-*trans*-*p*-coumaroylrotundic acid (previously detected as a biomarker of blueberry) (Das et al., 2022) and glycyrrhetinic acid (widely studied for its inflammatory properties) (Ming et al., 2013), have been identified following the addition of rowanberry ingredients. However, the main important phenolic compounds associated with adding rowanberry ingredients in meatballs were chlorogenic acid (average LogFC *vs* C = 13.98) and isoquercitrin (average LogFC *vs* C = 12.30).The distribution of these compounds in rowanberries is not novel, as well as their antioxidant activities against free radicals have been previously reported (Sarv et al., 2021). Therefore, the strong up-accumulation of these compounds at the end of storage time in meatballs added with pomace ingredients proved our hypothesis on greater protection against lipid oxidation phenomena.

4. Conclusions

In this work, natural ingredients possessing antioxidant capacity obtained by rowanberry pomace valorisation have been used as potential ingredients for meat products for preventing oxidation processes. By the day 5th, the meatballs with ingredients 2%-AC and 1%-E containing a higher amount of bioactives had decreased the yellowness (b^*) of meatballs. In the case of 1%-E, the pH of meatballs decreased, presumably due to the high concentration of chlorogenic acids, while in the case of 2%-R and 2%-AC, the pH remained stable, and the pH of the control sample increased. The increase in pH may indicate microbiological spoilage via the release of NH_3.

The PCA and OPLS-DA models were used to evaluate the chemical profiling of meatballs on days 0, 4 and 14. The results demonstrated the impact of storage time on changes in meatballs' chemical composition, especially in the case of 2%-AC and 1%-E added-samples with higher amounts of polyphenols.

In total, 184 discriminant metabolites were detected, consisting of 52 terpenoids, 26 amino acids, 16 polyphenols, aldehydes, and ketones, in addition to fatty acid derivatives. The most effective rowanberry ingredient against the development of unpleasant flavours caused by carbonyl compounds was 1%-E, at day-14 in the storage-time test. In addition, by the day-14 the concentration of linoleic acid derivatives had decreased only in the control sample (C).

Overall, these findings suggest the suitability of rowanberry pomace extract as a potential ingredient possessing antioxidant capacity for food products. In addition, the untargeted metabolomics can be used for assessing meat quality as well as evaluating the impact of antioxidants from rowanberry on the modifications of pork meatballs composition during their longer storage time. This approach allows finding possible correlations between natural by-products added to meat and its deterioration (mainly considering lipid peroxidation phenomena). Therefore, the approach gave the ideas for future studies, where the heating-induced effects on antioxidants must be considered. Moreover, for establishing the overall effects of various plant-based ingredients on the shelf-life of meat products, the microbiological characteristics should be determined in the future.

CRediT authorship contribution statement

Viive Sarv: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Kristi Kerner:** Formal analysis, Methodology. **Petras Rimantas Venskutonis:** Methodology, Supervision. **Gabriele Rocchetti:** Formal analysis, Methodology, Writing – original draft. **Pier Paolo Becchi:** Formal analysis, Methodology. **Luigi Lucini:** Methodology, Supervision. **Alo Tänavots:** Visualization. **Rajeev Bhat:** Resources, Supervision.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2023.100761.

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