Meat Science 200 (2023) 109160

Contents lists available at ScienceDirect

Meat Science

journal homepage: www.elsevier.com/locate/meatsci

Evaluation of different blackcurrant seed ingredients in meatballs by using conventional quality assessment and untargeted metabolomics

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ARTICLE INFO

Keywords:
Ribes nigrum L. seeds
Pork meatballs
Antioxidants
Meat quality
Metabolomics

ABSTRACT

Blackcurrants are sources of phenolic compounds, such as anthocyanins, possessing strong antioxidant, antimicrobial and antifungal activity. Therefore, the addition of different blackcurrant pomace ingredients may affect the overall meat quality. The actual chemical profile and bioactivities of blackcurrant pomace ingredients may strongly depend on its preparation; for instance, in our study the highest values of the in vitro antioxidant capacity were determined for blackcurrant seeds after supercritical CO_2 extraction. Starting from these background conditions, in this work, we evaluated the ability of three different concentrations (namely 1, 3, and 5% *w*/w) of blackcurrant (BC) seeds following EtOH/water extraction (BC-AE), before supercritical fluid CO_2 extraction (BC-RS), and after supercritical fluid CO_2 extraction (BC-ASC) to affect different quality parameters of pork meatballs. These latter were stored considering three different time-points, namely 1, 3 and 6 days at 4 °C packed under modified atmosphere (i.e., 70% N_2 and 30% CO_2). Untargeted metabolomics allowed to identify several lipid and protein-related oxidation products involved in redox reactions, such as 13-L-hydroperoxylinoleic acid, (12*S*,13*S*)-epoxylinolenic acid, 9,10-epoxyoctadecenoic acid, glutathione, glutathione disulfide, L-carnosine, L-ascorbic acid, and tocotrienols. Besides, multivariate statistics applied on the metabolomics dataset confirmed that the chemical profile of meatballs was an exclusive combination of both BC inclusion levels and type of BC-ingredients considered. Our findings showed that the higher the concentration of BC seed ingredients in meatballs, the lower the cooking loss and the higher the fibre content. Also, all the ingredients significantly affected the colour parameters.

1. Introduction

Meat is an important part of a human diet as a source of high biological value proteins. Health claim for meat, as a product contributing to the improvement of iron absorption, has been approved by the EFSA according to Commission Regulation (EU) No 1018/2013 of 23 October 2013 amending Regulation (EU) No 432/2012 establishing a list of permitted health claims made on foods other than those referring to the reduction of disease risk and to children's development and health. On the other hand, IARC (International Agency for Research on Cancer) included processed and red meat into 1 and 2A categories of cancer causing substances, respectively (IARC, 2018). In addition, meat is an expensive source of proteins, and its production is related to a heavy environmental impact (Djekic, 2015; Gonzalez, Marquès, Nadal, and Domingo, 2020). Therefore, there is a clear tendency of developing meat products by partial or full substitution of meat by plant origin-based and

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https://doi.org/10.1016/j.meatsci.2023.109160
Received 8 December 2022; Received in revised form 6 March 2023; Accepted 6 March 2023
Available online 10 March 2023

non-conventional ingredients as a source of cheaper proteins (Rocchetti et al., 2023). In addition, plant origin ingredients containing antioxidant phytochemicals are being tested in meat products as the inhibitors of oxidation and formation of carcinogenic compounds in processed meat. Finally, upcycling of agri-food processing side-streams (by-products and waste) for the recovery of high nutritional value substances and development of health beneficial ingredients for various applications, including meat products, has become one of the most important trends in food science and technology. Regarding safety and stability, many meat products are microbiologically sensitive foods, while oxidation of their lipids and proteins are among the main chemical changes, which affect meat sensory quality and nutritional value. The rationale of the present study is closely linked to all above-mentioned aspects, and it will be shortly explained.

Minced meat products, which are particularly sensitive to the microbiological and oxidative processes during storage, were selected for our study. Several studies (Akcan, Estévez, and Serdaroğlu, 2017; Anton et al., 2019; Djordjević et al., 2018; Vasilev et al., 2017) have confirmed that meat patties, meatballs, which are popular products in Europe, oxidize more rapidly compared to fresh meat. Lipid oxidation reduces the product quality by affecting negatively colour, nutritional value, causing off-flavors (rancidity), forming toxic compounds and increasing health risks (Anton et al., 2019; Vargas-Ramella et al., 2021). Therefore, plant origin ingredients with antimicrobial and antioxidant potential have been tested in various meat products (Babaoğlu, Unal, Dilek, Poçan, and Karakaya, 2022; Jurčaga et al., 2021; Lorenzo et al., 2018).

Fruit processing by products such as juice press-cake (pomace) contain large fraction of valuable nutrients, while the majority of berry (small fruit) pomaces, including black currants (BC) (Basegmez et al., 2017), are exceptionally rich in polyphenolic antioxidants. Therefore, small fruits are of a particular interest for upcycling into higher value substances. During last few years an efficient green consecutive extraction platform with the increasing polarity solvents has been proposed and tested for the valorization of berry pomace of various small fruits (Bobinaitė et al., 2020; Kitrytė et al., 2020; Kitrytė et al., 2020; Kitryte, Laurinavičiene, Syrpas, Pukalskas, and Venskutonis, 2020; Tamkutė, Liepuoniūtė, Pukalskienė, and Venskutonis, 2020). High value berry pomace extracts were tested in meat products and were shown to slow-down oxidation, inhibit the growth of pathogenic microorganisms (Tamkutė, Vaicekauskaitė, Gil, Carballido, and Venskutonis, 2021), increase antioxidative potential and reduce the proliferation of cancer cells (Tamkutė et al., 2022). However, after each extraction step together with soluble extracts high amounts of insoluble residues are obtained. The studies on testing such residues are rather scarce, while their nutritional composition may be of interest for various foods. For instance, after supercritical carbon dioxide ($scCO_2$) extraction the main fraction of antioxidant polyphenolics remain in the residue, and therefore it may be recognized as antioxidant dietary fibre (Varnaitė et al., 2022), while after each extraction the proportion of proteins is increasing. In addition, considering the complexity of berry pomaces consisting of seeds, exocarp and endocarp residues with different composition mechanical pre-fractionation may increase the diversity of the ingredients obtained as it was demonstrated for sea-buckthorn (Kitrytė et al., 2017). In general, the seeds accumulate higher amounts of oil and protein, while the skins are richer in polyphenolics. Consequently, separation of different anatomical parts before solvent extraction may provide some extra value for the recovered fractions of upcycling.

Consequently, our study by testing solid fractions of BC pomace biorefining in meat products applies an innovative concept. For this purpose, BC pomace, as well as its residues after extraction with $scCO_2$ (defatting), ethanol and water were added to meat products at different concentrations. Blackcurrant (*Ribes nigrum* L.) is a native to central and northern Europe species, yielding dark purple fruits, which are particularly rich in vitamin C and anthocyanins. To the best of our knowledge the concept of using BC pomace solid residues after consecutive extraction steps have not been reported previously neither in meat nor in any other food formulation. Besides commonly used methods for the evaluation of product quality, non-targeted metabolomics was used to screen the effect of BC ingredients on the composition of small molecules, known as metabolites. This approach is gaining popularity and is being used to assess the quality and chemical changes in foodstuffs (Rocchetti, Gallo, Nocetti, Lucini, and Masoero, 2020), however, less information is available on the use of untargeted metabolomics for assessing the oxidation processes in meat products, while this approach may help to find and identify biomarkers of spoilage.

2. Materials and methods

2.1. Chemicals and reagents

Sulfuric acid (H_2SO_4), boric acid (H_3BO_3) and hydrogen chloride (HCl; 0.561 M) originated from Sigma-Aldrich Chemie (Steinheim, Germany). Sodium hydroxide (NaOH, 50%) was purchased from Ingle AS (Ingliste, Estonia), and Kjeltabs (FOSS Analytical A/S, Denmark) tablet was from Oridor Eesti OÜ (Tartu, Estonia). Ethanol (95% and 78%) was from Estonian Spirit OÜ (Tallinn, Estonia). Heat-stable α-amylase, amyloglucosidase, protease, analytical grade Celite and MES/TRIS buffer were from Megazyme Ltd. (Wicklow, Ireland). Acetone was from Honeywell International Inc. (Charlotte, NC, USA). The solvents used for UHPLC-HRMS analysis, namely water, acetonitrile, and formic acid (LC-MS grade) were purchased from VWR (Milan, Italy).

2.2. Preparation and evaluation of blackcurrant seeds ingredients

2.2.1. Extraction procedures

Dried and mechanically separated from the skins, BC pomace seeds were from the last harvest from one batch to ensure the freshness of the additive and avoid the changes in chemical composition that may appear during the long time storage, donated by the Cooperative Ribes LT (Biržai, Lithuania). The flowchart of ingredient preparation is presented in Supplementary fig. 1. The seeds were milled using 0.5 mm sieve and extracted with $scCO_2$ at the previously optimized parameters (Basegmez et al., 2017). This processing step gave lipophilic extract and defatted residue, further abbreviated as BC-ASC. Lipophilic extract as a product containing highly unsaturated oil has not been used in further experiments. Six portions of BC-ASC, 100 g each were mixed with 300 mL 96% ethanol in a 500 mL flask and extracted in a platform universal shaker PSU20 (Biosan, Riga, Latvia) for 2 h at 155 rpm. The solid residues were re-extracted with 200 mL 96% ethanol. The extracts were filtered in a Buchner funnel Whatman filter paper no.1 (Whatman International Ltd., Maidstone, U.K.), combined and evaporated in a rotary vacuum evaporator Rotavapor R-210 (Büchi Labortechnik, Flawil, Switzerland) at 40 °C. The yields from seeds and skins were 2.70 ± 0.13% and 3.46 ± 0.21%, respectively. The residue after ethanol extraction was dried at 40 °C and 6 its portions, 80 g each, were extracted with 400 mL of hot water in a shaker for 2 h. The residue was re-extracted 2 times with 200 and 150 mL of hot water. The extracts were decanted, combined, centrifuged in a refrigerated centrifuge Velocity 18R (Dynamica Scientific Ltd., Livingston, UK) at −18 °C during 20 min and freeze-dried in a Maxi Dry Lyo (Jonan Nordic A/S, Aelleröd, Denmark). The yields from the seeds and skins were 7.22 ± 0.23% and 3.85 ± 0.19%, respectively. Thus, the third ingredient, the residue after all extractions (BC-AE), was obtained for testing in meat products. Ethanol and water extracts are a good source of polyphenolic antioxidants, mainly anthocyanins, and may be used in formulation of health beneficial ingredients. However, they were excluded from further experiments because the main aim of this study was to test in meat the effects of raw seeds and their processing by-products after different steps of extraction.

To summarize, the following BC ingredients were produced: BC seeds

before supercritical CO_2 extraction (BC-RS), after $scCO_2$ extraction (BC-ASC), and after EtOH/water extraction (BC-AE).

2.2.2. Evaluation of BC seed ingredients

Proximate composition of BC-RS, BC-ASC and BC-AE was determined from ground samples in four parallel replicates using standard methods: moisture (LST EN ISO 665:2020), protein (LST EN ISO 8968-1:2014, LST EN 12135:2001 and LST ISO 937:2000), fat (LST EN ISO 659:2000 (ISO 659:2009)), ash (ISO 749:1977), and crude fibre (LST EN ISO 6865:2001 (ISO 6865:2000)) content.

The TPC (Total Phenolic Content) was measured with Folin-Ciocalteu's reagent as originally described by Singleton, Orthofer, and Lamuela-Raventós (1999) and adapted to the BC solids by using QUENCHER approach. Briefly, 10 mg of sample or cellulose (blank) were mixed with 150 μL of distilled H_2O, 750 μL of Folin-Ciocalteu's reagent, and 600 μL of Na_2CO_3 solution, vortexed for 15 s, shaken at 250 rpm for 2 h in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 760 nm. Gallic acid ($C_6H_2(OH)_3CO_2H$) solutions (150 μL) at various concentrations (0–80 μg/mL) were used for calibration.

$ABTS^{\bullet+}$ (2,2′-azino-bis(3-ethylbenzo-thiazoline-6-sulfonic acid) decolorisation assay was performed according to Re et al. (1999), which is based on the reaction of a radical cation with antioxidant resulting in colour change. Pomace or cellulose (blank) were mixed with 25 μL of MeOH and 1500 μL of working $ABTS^{\bullet+}$ solution, vortexed for 15 s, shaken at 250 rpm for 2 h in the dark, centrifuged (4500 rpm, 5 min) and the absorbance of optically clear supernatant was measured at 734 nm. Trolox solutions (25 μL) at various concentrations (0–1500 μmol/L MeOH) were used for calibration.

ORAC (Oxygen Radical Absorbance Capacity) method was performed as described by Prior et al. (2003) and Davalos, Comez-Gordovez, and Bartolome (2004) by using fluorescein as a fluorescent probe. The reaction was carried out in 75 mM phosphate buffer (pH 7.4). BC solids or cellulose (blank) were mixed with 150 μL of PBS solution (75 mmol/L) and 900 μL of fluorescein solution (14 μmol/L PBS), vortexed for 15 s, shaken at 250 rpm for 60 min in the dark, and centrifuged (4500 rpm, 5 min). Optically clear supernatant (175 μL) was transferred to the 96-well black opaque microplates, pre-incubated for 15 min at 37 °C, followed by rapid addition of 25 μL of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH) solution (240 mmol/L) as a peroxyl radical generator using a multichannel pipette. The fluorescence was recorded every cycle (1 min × 1.1), total of 90–140 cycles. Further experimental and data handling were the same as reported for extract analysis. Trolox solutions (150 μL) at various concentrations (0–500 μmol/L PBS) were used for calibration.

2.3. Preparation of pork meatballs

The preparation of pork meatballs from three different meat batches and their packaging was followed by the previously reported protocol Kerner, Jõudu, Tänavots, and Venskutonis (2021) with some modifications. Minced pork meat (moisture 65.9, protein 18.2, fat 15.1 and ash 0.93%) was purchased from a local commercial abattoir (Tartu, Estonia). Three batches of pork meatballs were formulated to contain 88% of minced pork meat, 10% of tap water and 1% of salt (control sample) and tested samples contained 1, 3, and 5% of different BC seed ingredients. The mixture of meatballs was prepared manually using tap water and salt, and the meatballs were shaped with a weight of about 30 g, a diameter of 4.5 and 2 cm of height by using a self-made form. The assessors carried out the preliminary sensory evaluation of meatballs with different concentrations of BC seed ingredients to test product acceptance. In general, the assessors positively evaluated the taste and smell of the meatballs and noted dark colour of the products with BC seed ingredients. Three meatballs were used for analysis (pH, water activity a_w and colour measurements) for each storage time. The meatballs were cooked in a pre-heated oven Inoxtrend E1 CUA-107E (Treviso, Italy) at 145 °C × 15 min, and then cooled down to a room temperature. Packaging was carried out under modified atmosphere conditions consisting of 70% N_2 and 30% CO_2 (Linde GAS AS, Tallinn, Estonia) with a Vision Pack S.r.l. VP01 (Packaging Factory Holding, Lallio, Bergamo, Italy) and stored in cooled conditions at 4 °C. Analyses were conducted at 1, 3, and 6 days of storage, with the exception of metabolomics that was used to evaluate the optimum inclusion levels (%) by looking at specific marker compounds in the prepared meatballs.

2.4. Measurement of chemical composition, fibre content and cooking loss of meatballs

For the chemical composition analyses the meatball samples (at least 200 g) were ground and homogenized in a laboratory homogenizer Retsch GM200 (Retsch Gmbh & Co, Haan, Germany). The cooked meatballs were analysed for moisture (EVS-ISO 1442:1999), protein (EVS-ISO 937:1978, Kjeldahl method), fat (EVS-ISO 2446:2001, Gerber method), and ash content (ISO 936:1999). Carbohydrates were calculated from fat, protein, moisture, and ash content. Samples were randomly selected and cooked across all samples and batches within three months.

Dietary fibre (total, soluble and insoluble) in pork meatballs was measured using Megazyme Dietary Fibre Assay Kit (K-TDFR-100A) (Megazyme Ltd., Wicklow, Ireland) by following the AOAC 991.43 method. Cooking loss was measured by weighing the meatballs before heat treatment and after cooling the cooked meatballs down to a room temperature. The cooking loss was expressed in %.

2.5. Measurement of pH, a_w and colour parameters

pH was measured by a Seven 2Go™ pH-meter (Mettler-Toledo AG Analytical, Schwerzenbach, Switzerland), calibrated with pH 4 and 7 buffer solutions. 5 g of sample was homogenized with 50 mL of 0.1 M potassium chloride solution in Retsch GM200 (ISO 2917:1999) laboratory homogenizer. Water activity was measured in a water activity analyzer (Aqua Lab, Model Series 3 TE, Decagon Devices Inc., Washington, DC, USA). For the colour measurements, meatballs were cut into halves after opening the package and three replicate measurements were recorded by a spectrophotometer X-Rite 964 (X-Rite, Grand Rapids, MI, USA) on the internal area of the freshly cut surface in different places to obtain the average value. The results were expressed by CIE (International Commission on Illumination) Lab system values (D65 and observer angle of 10°), namely L^*—lightness, a^*—redness, b^*—yellowness. The total colour difference (ΔE_{Lab}) between the control and test samples was calculated by using the following formula (Eq. 1):

$$\Delta E_{Lab} = \sqrt{\left(L_0^* - L_1^*\right)^2 + \left(a_0^* - a_1^*\right)^2 + \left(b_0^* - b_1^*\right)^2}, \quad (1)$$

where ΔE_{Lab} is the total colour difference between the control and test samples; L_0^*, a_0^*, and b_0^* are the means of the colour parameters determined for the control samples; and L_1^*, a_1^*, and b_1^* are the means of the colour parameters determined for the test samples.

In the interpretation of the results, the following was assumed:

- when $0 < \Delta$ELab < 1—the observer does not notice the difference;
- when $1 < \Delta$ELab < 2—only an experienced observer may notice the difference;
- when $2 < \Delta$ELab < 3.5—an unexperienced observer also notices the difference;
- when $3.5 < \Delta$ELab < 5—a clear difference in colour is noticed and;
- when $5 < \Delta$ELab—an observer notices two different colours (Mokrzycki and Tatol, 2011).

2.6. Untargeted profiling based on UHPLC-HRMS

Regarding the extraction process for the metabolomics analysis, pork

meatballs were thawed at room temperature and then extracted following the protocol previously reported by Pateiro et al. (2018), with minor modifications. Briefly, one gram of each sample was extracted with 10 mL of 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 based on the utilization of an 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 was obtained through a 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 (2.1 × 100 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 with a positive ionization mode and a nominal mass resolution of 70,000 FWHM at *m/z* 200. The injection sequence was randomized, with three replicates for each sample. Pooled quality control (QC) samples (prepared by pooling same aliquots of each sample) acquisition was achieved 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 (Rocchetti et al., 2020).

The raw spectral data were processed using the software MS-DIAL (Version 4.80) (Tsugawa et al., 2016) 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; Rocchetti, Michelini, Pizzamiglio, Masoero, and Lucini, 2021). Features were searched in the mass range 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 score cut off was set to 50%, retaining the most common HESI+ adducts. Annotation of meat metabolites was achieved against the comprehensive database, known as FooDB (http://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, Steinbeck, Viant, Goodacre, and Dunn, 2013). Only the compounds having an in silico prediction score higher than 5 were retained.

2.7. Statistical analysis

Statistical analyses were performed with the statistical package R 4.2.0. (R Core Team, 2019). The effects of variants, storage period, and their interaction and the random effect of three batches (experimental replications) within three months on the samples' pH, colour characteristics, a_w, were studied by the Linear Mixed-Effects Model (GLMM). The Emmeans (Lenth, 2022) and multcomp (Hothorn, Bretz, and Westfall, 2008) packages were used to carry out the pairwise comparison of the groups. Tukey's multiple comparison post hoc test was used to determine the groups' least square mean differences at the significance level of $\alpha = 0.05$. The effects of variants and four batches on the sample moisture, protein, and ash content as well as on the cooking loss were measured only on day 1 by GLMM. All model-assessed results are presented as least-square means.

The multivariate statistical analyses to elaborate the metabolomics-based dataset were done using two different softwares, namely Mass Profiler Professional (Version B.12.06; from Agilent Technologies) and SIMCA (Version 16; from Umetrics, Malmo, Sweden) for data processing and normalization (Rocchetti, Michelini, et al., 2021). The multivariate data analysis was based on different approaches, namely unsupervised hierarchical cluster analysis (HCA), unsupervised principal component analysis (PCA), and supervised orthogonal projections to latent structures discriminant analysis (OPLS-DA). The OPLS-DA models were built considering the % of inclusion of the different functional ingredients (i.e., 0, 1, 3, and 5%) under investigation, also recording the model validation parameters (goodness-of-fit R^2Y together with goodness-of-prediction Q^2Y). Confidence limits of 95% and 99% were used to check for the presence of outliers (suspect and strong outliers, respectively, according to Hotelling's T2 approach), while cross-validation (CV-ANOVA, $p < 0.01$) and permutation testing (number of random permutations = 200) were used to validate and exclude overfitting, respectively. 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.

Three replicate determinations were performed and standard deviations calculated in case of characterisation of proximate composition and antioxidant capacity values of the BC ingredients. The values are presented as means with standard deviations.

3. Results and discussion

3.1. Characterisation of BC ingredients

3.1.1. Antioxidant capacity characteristics

The in vitro antioxidant capacity indicators measured for BC ingredients used in our study are listed in Table 1. All values were significantly higher in the $scCO_2$ extraction residue than in the raw dried seeds. It may be explained by the removal of oil and other lipophilic compounds leading to the increased proportion of polyphenolic antioxidants and better availability of antioxidative active groups for the electron/hydrogen atom transfer (SET/HAT) in the determination reaction system. Lu and Yeap Foo (2003) reported that phenolic composition of BC seed residue after extraction of lipophilic compounds with $scCO_2$ was similar to that of the whole berries. Further re-extraction of the residue with polar solvents EtOH and water decreased all values; however, TPC and $ABTS^{\bullet+}$ remained still quite high. ORAC of the final residue was 5.7-fold lower than that of the $scCO_2$ extraction residue. These results may be explained by the differences in the assays; TPC and $ABTS^{\bullet+}$ are based on a SET, while ORAC measures the ability to inactivate peroxy-radicals by donating hydrogen. It may be assumed that EtOH/water extraction efficiently removed hydrogen donating

Table 1
Total phenolic content and in vitro antioxidant activity of blackcurrant seeds and their residues after extractions ($n = 30$).

Sample	TPC, mg GA/g	ABTS, TE mg Trolox/g	ORAC, TE mg Trolox/g
BC-RS	42.07 ± 0.53	74.12 ± 0.99	13.35 ± 0.52
BC-ASC	62.09 ± 1.16	141.31 ± 3.00	15.62 ± 0.46
BC-AE	31.54 ± 0.87	109.63 ± 1.60	2.73 ± 0.05

Abbreviations: BC-RS – blackcurrant seeds before CO_2 extraction, BC-ASC – blackcurrant seeds after supercritical fluid CO_2 extraction and BC-AE – blackcurrant seeds after EtOH/water extraction. TPC = total phenolic content; ORAC = oxygen radical absorbance capacity.

polyphenolic antioxidants, while the antioxidants and or the functional groups participating in the SET mechanisms may be both extracted and formed or become available during and after extraction, respectively.

3.1.2. Proximate composition

The composition of BC ingredients is presented in Table 2. There are no remarkable differences in proximate composition; however, some increase in protein and dietary fibre content in the extraction residues may be observed. It may be explained by the removal of soluble in $scCO_2$, EtOH and water substances during each step of extraction and therefore the residues contain slightly larger fractions of insoluble proteins and fibre. In general, extraction residues become the ingredients with higher content of proteins than meat.

3.2. Proximate chemical composition, fibre content and cooking losses of pork meatballs

The addition of BC seed ingredients affected the chemical composition and cooking loss of the pork meatballs (Table 3). Almost all the samples had higher protein content compared to control sample; on the other hand, fat content in all samples decreased. Ash content was not remarkably affected.

Reduction of cooking losses is an important parameter for meat industries. The lowest cooking loss was in the meatballs with BC-ASC5, which also had high protein and fibre content. From the results we can see, that in all samples with the addition of 5% of BC seed additives, the cooking loss was lower compared to control and other samples with lower concentrations. The results about cooking loss are in line with Mena et al. (2020), reporting that incorporation of 3% of sugarcane fibre increased the cooking yield. Also, our findings agreed with Choi et al. (2012), dealing with the addition of increasing concentration of *Laminaria japonica* powder (i.e., 1, 3 and 5%), and reporting an increase in the fibre content and a corresponding decrease of cooking loss. The highest cooking loss was in BC-RS1, followed by BC-AE1 (27.30 and 27.05%, respectively). In general, it was noted that the higher the concentration of BC seed ingredients in meatballs, the lower the cooking loss.

The interest in dietary fibres (DF) to be added into food products has gained popularity due to their important role for human health; in addition, DF has an effect on some technological properties, e.g. cooking loss, yield and prevention of lipid oxidation (Zinina et al., 2019). Table 3 represents the DF results in pork meatballs with blackcurrant seed ingredients.

From the current data, it is evident that the highest inclusion levels of the functional ingredients affected the fibre content in pork meatballs. In particular, the higher the concentration, the higher fibre content, which also confirms one of the hypotheses, i.e., the percentage dietary fibre increases after CO_2 extraction. In samples BC-ASC, the DF content is higher compared to other samples.

3.3. Effect of blackcurrant seed ingredients on the pH and water activity (a_w) of pork meatballs

The effect of the BC seed ingredients on the pH values for 6 days storage of pork meatballs is described on Fig. 1. pH is an important quality parameter of meat and its products (Barrón-Ayala et al., 2020), also affecting colour intensity and water holding capacity; the decrease in pH can lead to an unacceptable taste. According to statistical analysis, pH values were affected by the added BC seed ingredients at different concentrations. The lowest pH after 3 and 6 days of storage was in control sample, below 6.0, which tend to cause oxidation (Barrón-Ayala et al., 2020). In case of other samples with blackcurrant, the pH values remained the same. The similar result was found by Tamkutė et al. (2021), who added chokeberry pomace extracts into pork burgers and the pH values with extracts slightly increased and afterwards remained the same, while the pH of control sample decreased. Besides, Hautrive, Piccolo, Rodrigues, Campagnol, and Kubota (2019) and Sánchez-Zapata et al. (2010) noted the highest pH values in meat samples added with fibre rich additives, likely due to the chemical characteristics of each fibre tested.

Water activity (a_w) is a crucial factor in food indicating the ratio of water available for microorganisms to grow, also affecting other quality characteristics of meat products. The a_w-values (Fig. 2) ranged during the 6 days within 0.978–0.984 and were quite stable within the samples and storage days. Similar results reported Tamkutė, Gil, Carballido, Pukalskienė, and Venskutonis (2019); Tamkutė, Vaicekauskaitė, Gil, et al. (2021) in case of adding chokeberry and cranberry pomace ethanol extracts; for instance, the a_w in cooked ham with chokeberry extract was 0.978–0.983 during the whole period of storage. In our study slight decrease in a_w-values during storage was observed for the samples BC-RS1, BC-RS3, and BC-RS5.

Table 2
Proximate chemical composition of blackcurrant seed extracts ($n = 3$).

Sample	Moisture, %	Protein, %	Dietary fibre, %	Minerals, %
BC-RS	5.83 ± 0.14	20.17 ± 0.04	17.28 ± 0.18	3.24 ± 0.03
BC-ASC	7.86 ± 0.20	21.15 ± 0.33	17.40 ± 0.32	3.68 ± 0.05
BC-AE	7.45 ± 0.15	22.53 ± 0.31	19.74 ± 0.12	2.44 ± 0.005

Abbreviations: BC-RS – blackcurrant seeds before CO_2 extraction, BC-ASC – blackcurrant seeds after supercritical fluid CO_2 extraction and BC-AE – blackcurrant seeds after EtOH/water extraction.

3.4. Effect of additives on colour parameters of pork meatballs

Colour is an important quality parameter, besides that purchasing and acceptability decisions by consumers are made by the colour of the product. So far as the effect of endogenous BC pigments is unavoidable in case of using their ingredients in meat products, in our study colour parameters were measured both for the products without BC ingredients ('true' or typical colour) and with them. Therefore, our objective was to measure the effects of BC ingredient on the colour of the final products. The changes of L^*, a^* and b^* values of pork meatballs with BC seed ingredients during chilled storage are presented on Figs. 3, 4 and 5. Colour characteristics of meatballs show significant differences. The lightest sample during the storage period was control. The same effect was noticed by Jurčaga et al. (2021) by using blackcurrant and Kamchatka honeysuckle extracts in frankfurters. Obvious was the tendency, the higher the BC seed ingredients concentration, the darker the sample (lower L^*-value). Reduction of lightness was also noticed by Jia, Kong, Liu, Diao, and Xia (2012), Nowak, Czyzowska, Efenberger, and Krala (2016) and Anton et al. (2019).

Redness significantly decreased in control sample (from 10.14 to 7.77) during 6 days storage period. The addition of BC seed additives caused the increase in redness compared to control sample, which may be explained by the anthocyanin content, but after 6 days of storage in most of the samples a^*-values decreased. The same results for lightness and redness values were observed by Ganhão, Estévez, Armenteros, and Morcuende (2013), Jia et al. (2012) and Chung, Choi, Yu, and Choi (2018). Lorenzo, Sineiro, Amado, and Franco (2014) highlights that if the redness values range between 4.6 and 10.8, the colour is perceived as brown in pork meat, so meatballs with blackcurrant seeds after EtOH/water extraction (BC-AE) and control sample, look brown during the 6 days of storage. For the control sample it represents an expected trend, as it is only heat treated.

The highest score for yellowness was in control samples, which remained stable during 6 days of storage. Discolouration has found in many researches with plant based ingredients (Anton et al., 2019; Re et al., 1999) and can be explained by browning reactions between lipid oxidation products and amines in meat. The lowest b^* values were in meatballs with 5% of blackcurrant seeds after EtOH/water extraction and 5% of blackcurrant seeds after supercritical fluid CO_2 extraction.

Table 3
Proximate chemical composition and cooking losses of pork meatballs. Values are least square means ± standard error.

Sample	Moisture (%)	Protein (g/100 g)	Fat (g/100 g)	Ash (g/100 g)	Carbohydrates (g/100 g)	Fibre content (%)	Cooking loss (%)
Control	53.27 ± 1.79^{ab}	21.32 ± 0.47^{bc}	18.98 ± 0.60^{c}	2.06 ± 0.046^{a}	0.29 ± 0.000^{a}	0.00 ± 0.00^{f}	26.79 ± 1.59^{a}
BC-RS1	53.82 ± 2.95^{ab}	21.77 ± 0.57^{ab}	18.73 ± 1.09^{bc}	2.13 ± 0.011^{a}	0.29 ± 0.000^{a}	0.181 ± 0.004^{a}	27.30 ± 1.31^{a}
BC-RS3	53.80 ± 3.64^{ab}	21.71 ± 0.19^{abc}	16.73 ± 1.29^{abc}	2.03 ± 0.030^{a}	1.60 ± 0.179^{b}	1.104 ± 0.028^{b}	23.96 ± 0.81^{ab}
BC-RS5	54.57 ± 2.12^{a}	20.29 ± 0.57^{c}	17.17 ± 0.43^{abc}	1.96 ± 0.019^{a}	2.90 ± 0.089^{cd}	1.608 ± 0.020^{c}	20.48 ± 2.22^{b}
BC-ASC1	51.67 ± 3.67^{b}	22.88 ± 0.41^{a}	15.97 ± 1.21^{ab}	2.00 ± 0.022^{a}	0.45 ± 0.069^{a}	0.206 ± 0.009^{a}	25.23 ± 1.74^{ab}
BC-ASC3	54.80 ± 4.05^{a}	21.87 ± 0.46^{ab}	15.06 ± 1.45^{ad}	1.94 ± 0.086^{a}	2.30 ± 0.179^{bd}	1.327 ± 0.016^{d}	22.82 ± 1.83^{ab}
BC-ASC5	52.75 ± 0.03^{ab}	22.85 ± 0.75^{a}	12.54 ± 0.98^{d}	1.98 ± 0.007^{a}	3.60 ± 0.000^{c}	2.128 ± 0.016^{e}	19.77 ± 2.18^{b}
BC-AE1	53.47 ± 3.91^{ab}	22.32 ± 0.6^{ab}	16.88 ± 1.12^{abc}	2.05 ± 0.053^{a}	0.3 ± 0.047^{a}	0.191 ± 0.004^{a}	27.05 ± 1.86^{a}
BC-AE3	54.71 ± 2.56^{a}	21.85 ± 0.35^{ab}	16.36 ± 0.67^{abc}	1.92 ± 0.087^{a}	1.55 ± 0.470^{b}	1.074 ± 0.047^{b}	24.45 ± 1.56^{ab}
BC-AE5	55.75 ± 2.91^{a}	21.56 ± 0.46^{abc}	14.54 ± 1.23^{ad}	1.48 ± 0.128^{b}	3.35 ± 0.112^{c}	1.585 ± 0.034^{c}	20.64 ± 1.10^{b}

a, b, c, d — Different letters in columns indicate significant differences between means ($P < 0.05$) by Tukey's multiple comparison's post hoc test. Control—without additives, BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – blackcurrant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%.

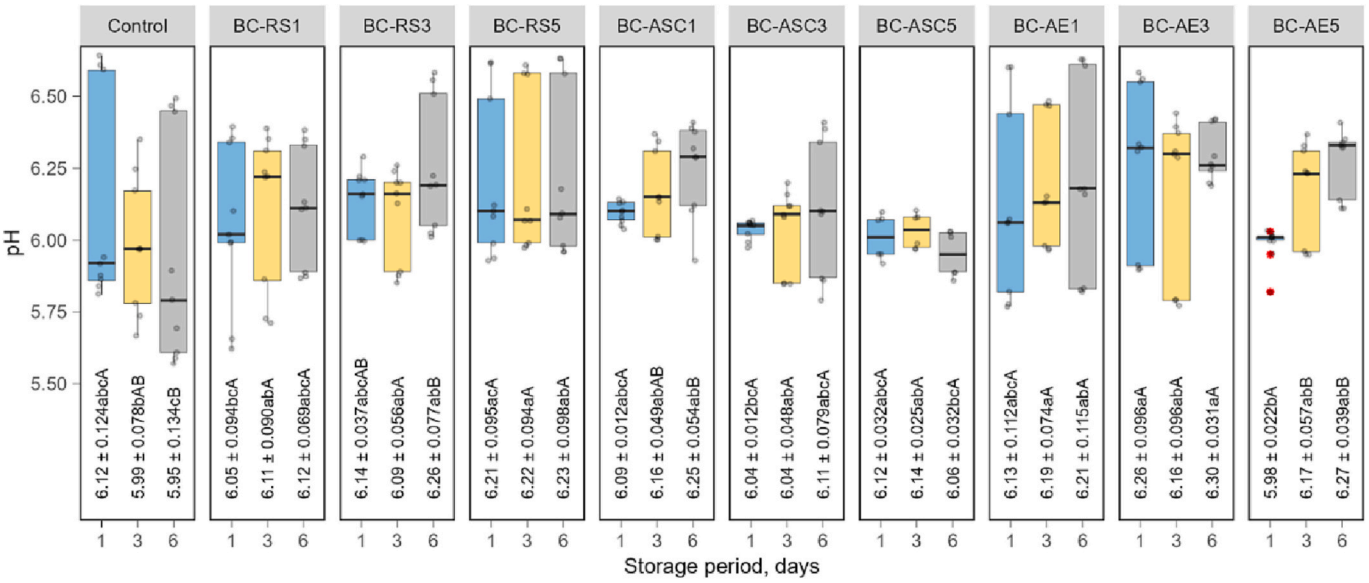

Fig. 1. Effect of BC seed ingredients and storage period (days) on the pH-value of cooked pork meatballs. Values are least square means ± standard error. The actual values are presented with grey dots, outliers with red asterisk. Control—without additives, BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – blackcurrant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Fig. 6 shows that the addition of different BC seed ingredients affected the total colour difference (ΔE_{Lab}) between control and samples with additives. ΔE as a standard measurement quantifies the difference between two colours. It is evident that clear difference in colour is noticed ($\Delta E_{Lab} > 3.5$), especially in meatballs with the addition of BC-AE and BC-ASC, a bit less in samples with BC-RS. Some decrease in ΔE_{Lab} in samples BC-RS can be seen on day 3, but still a clear difference is noticed. In case of BC-AE5, the ΔE_{Lab} was highest after 6 days of storage, while some decrease was noticed in sample BC-ASC5 compared with the 1 day of storage.

3.5. *Changes of the chemical profile of meatballs as a function of different inclusion levels of BC, AE, and ASC ingredients*

The untargeted metabolomics analysis of the different meatballs allowed to putatively annotate 749 metabolites. A detailed list containing the compounds identified, with the corresponding abundances and composite mass spectra (both MS1-isotopic and MSMS), and other information, is provided in supplementary material (Supplementary Table 1). Overall, the enrichment analysis built considering the annotated meat metabolites (reporting only metabolite sets containing at least 2 entries) revealed a great abundance of amino acids and peptides (65 compounds), followed by flavonoids (43 compounds), fatty acids and conjugates (27 compounds), prenol lipids (22 compounds), and fatty esters (20 compounds). Among the compounds annotated, we found several primary and secondary oxidation markers of both lipids and proteins involved in redox reactions, such as 13-L-hydroperoxylinoleic acid, (12*S*,13*S*)-epoxylinolenic acid, 9,10-epoxyoctadecenoic acid, glutathione, glutathione disulfide, L-carnosine, L-ascorbic acid, and others (Supplementary Table 1). The metabolomics dataset also revealed a marked distribution of phenolic compounds, likely deriving from BC seeds. Accordingly, the most represented class of polyphenols were flavonoids, with a great abundance of anthocyanins (such as glycosylated forms of cyanidin, petunidin, and delphinidin), flavones, flavonols, and other compounds. Other annotated classes were represented by phenolic acids (e.g., gallic, chlorogenic, and sinapic acids), stilbenes, lignans, and tyrosol-derivatives. It is known from scientific literature that BC seeds are a potential source of bioactive phenolic compounds (Basegmez et al., 2017); accordingly, Lu and Yeap Foo (2003) showed that BC seed residues from oil extraction were characterized by an array of polyphenols dominated by anthocyanins (mainly rutinosides and glucosides of delphinidin and cyanidin), and flavanols (such as glycosidic forms of myricetin, quercetin, and kaempferol). The BC seeds have attracted much interest also for their high content in nutritionally

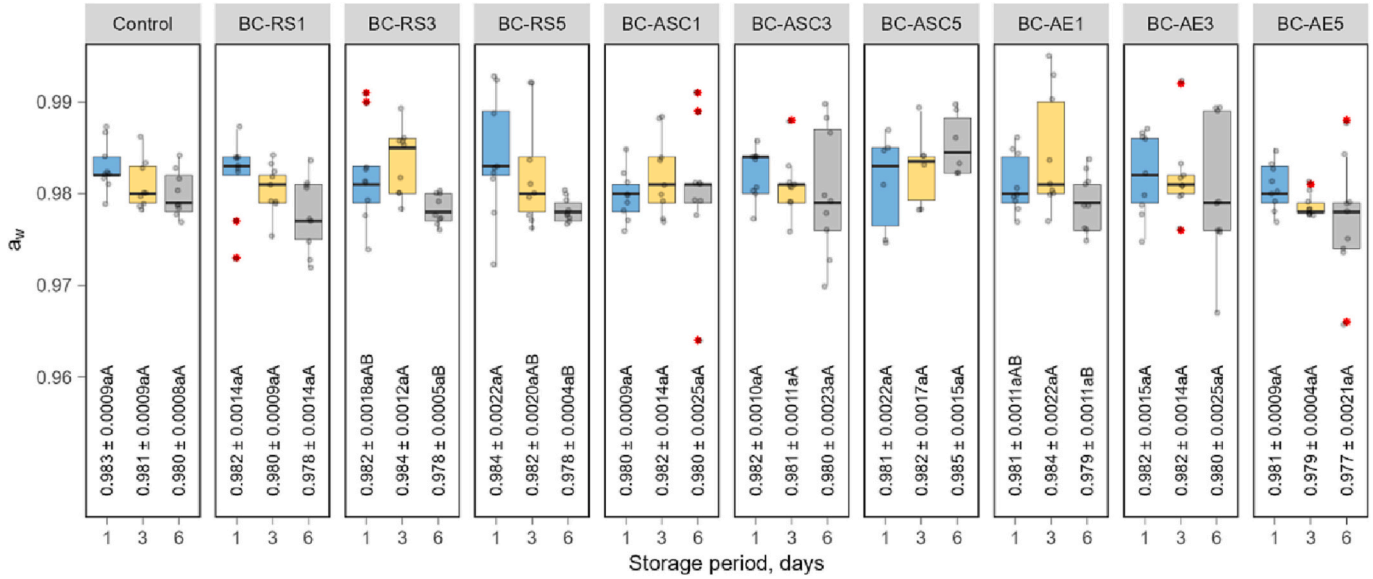

Fig. 2. Effect of BC seed ingredients and storage period (days) on the a_w-value of cooked pork meatballs. Values are least square means ± standard error. The actual values are presented with grey dots, outliers with red asterisk. Control—without additives, BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – blackcurrant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

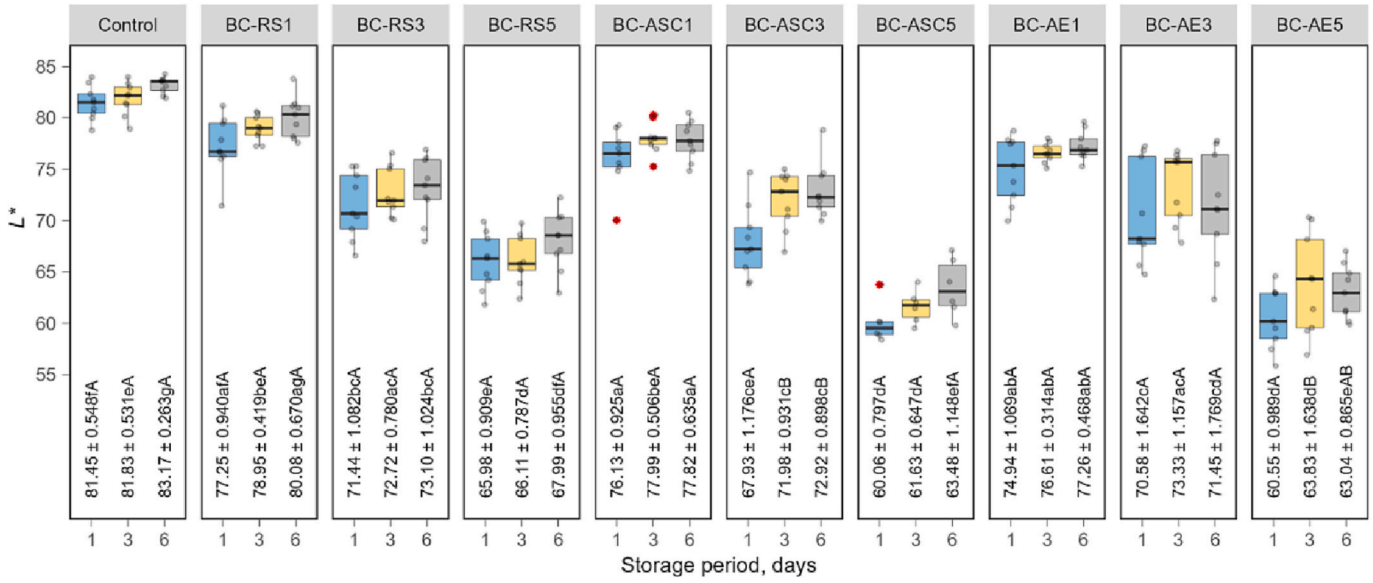

Fig. 3. Effect of BC seed ingredients and storage period (days) on the L^* (lightness) value of cooked pork meatballs. Values are least square means ± standard error. The actual values are presented with grey dots, outliers with red asterisk. Control—without additives, BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – blackcurrant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

desirable polyunsaturated fatty acids, such as γ-linolenic acid, α-linolenic acid, and steridonic acid. Under our experimental conditions (untargeted metabolomics), we annotated the only α-linolenic acid and several epoxy- and hydroxy-derivatives likely associated with oxidation processes (Supplementary Table 1).

As the next step, considering the complexity of the experimental dataset of compounds, multivariate statistical analyses (based on both unsupervised and supervised tools) were used to depict the impact of the different BC-functional ingredients on the metabolite profile of the formulated meatballs. The unsupervised hierarchical cluster analysis (Fig. 7) clearly separated the control sample vs the meatballs formulated with different inclusion levels of BC, AE, and ASC ingredients.

Overall, BC-RS1 and BC-ASC1 were included in the same sub-cluster, while meatballs prepared with BC-AE1, BC-AE3, and BC-AE5 were found to possess a similar chemical profile. Interestingly, an additional sub-cluster consisted in meatballs prepared on one side with BC-RS3 and BC-RS5 and on the other side by samples added with BC-ASC3 and ASC5 (Fig. 7). Taken together, the unsupervised statistics revealed that the most effective treatments able to provide significant differences in the chemical profile of meatballs were those based on the addition of high levels (3–5%) of blackcurrant seeds before and after supercritical fluid CO_2 extractions.

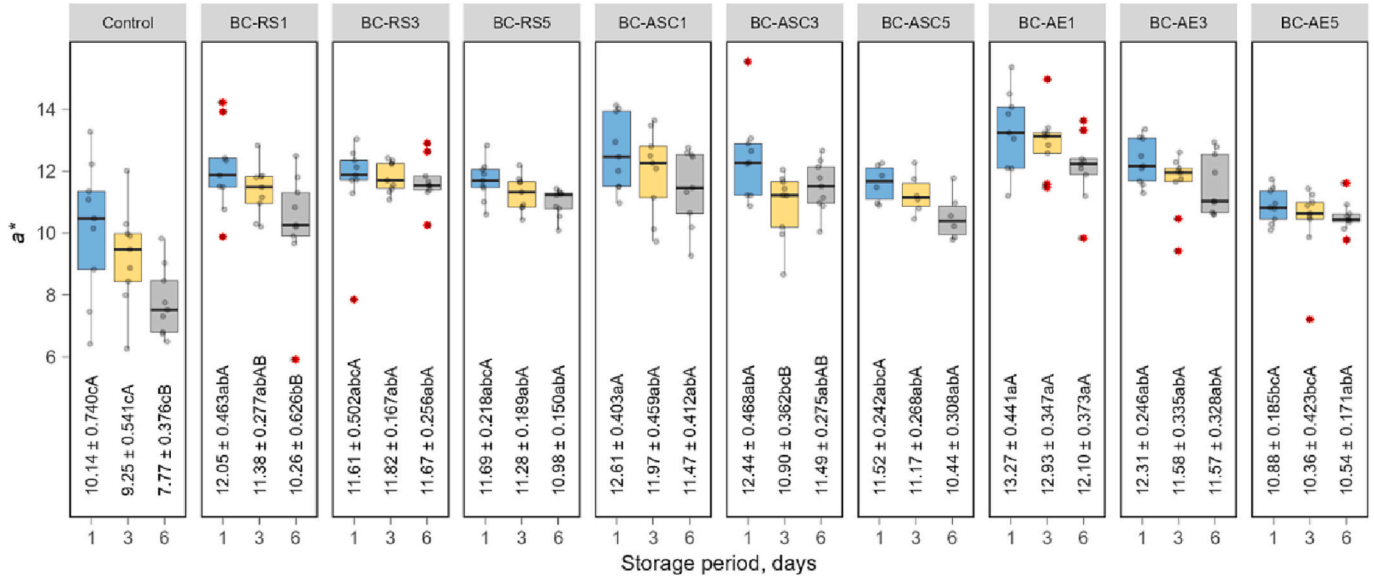

Fig. 4. Effect of BC seed ingredients and storage period (days) on the a^* (redness) value of cooked pork meatballs. Values are least square means ± standard error. The actual values are presented with grey dots, outliers with red asterisk. Control—without additives, BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – black-currant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

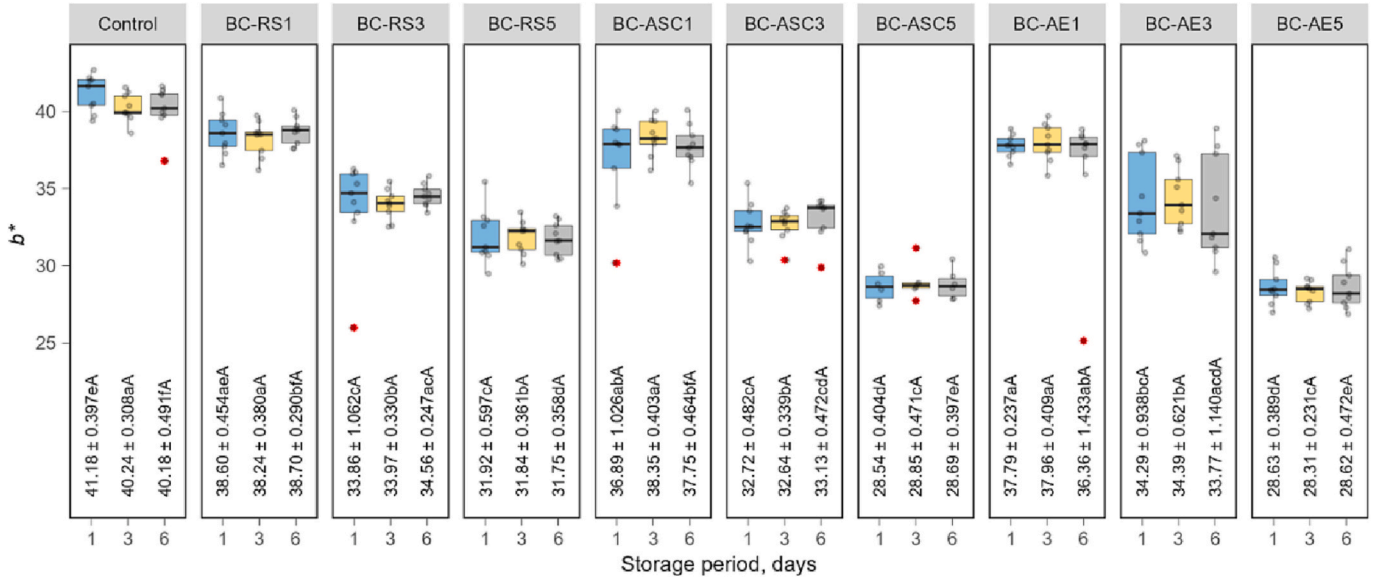

Fig. 5. Effect of BC seed ingredients and storage period (days) on the b^* (yellowness) value of cooked pork meatballs. Values are least square means ± standard error. The actual values are presented with grey dots, outliers with red asterisk. Control—without additives, BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – black-currant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Starting from the unsupervised findings, the following supervised statistical tool based on OPLS-DA was used to better understand what class of compounds was likely involved in driving the differences observed when considering the formulated pork meatballs. A first discriminant model based on the different inclusion levels as a class membership criterion is shown as Supplementary fig. 2. The supervised prediction model was characterized by excellent goodness-related parameters, being the R^2Y (goodness of fitting) = 0.976 and the Q^2Y (goodness of prediction) = 0.788. The orthogonal projections of the different meatball samples based on the chemical profile revealed also that the most specific chemical profile could be obtained when using the maximum inclusion level (i.e., 5%), while lower inclusion levels (1 and 3%) finally determined few chemical differences. On the other hand, clear differences were observed again by comparing the functional meatballs with the control sample, thus confirming the unsupervised findings (Fig. 7). Thereafter, three additional OPLS-DA prediction models were built to separately evaluate the inclusion levels of the functional BC-ingredients under investigation, namely 1% (Fig. 8A), 3% (Fig. 8B), and 5% (Fig. 8C).

As can be observed from Fig. 8, each inclusion level of BC-ingredients

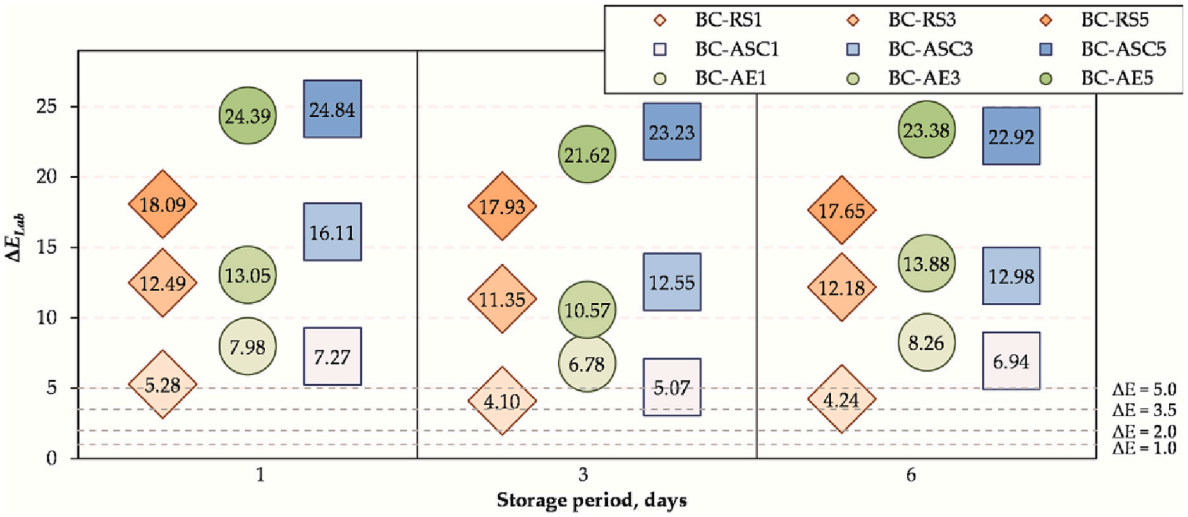

Fig. 6. The total colour difference (ΔE_{Lab}) between control and test samples during the storage. $0 < \Delta E_{Lab} < 1$—the observer does not notice a difference, $1 < \Delta E_{Lab} < 2$—only an experienced observer may notice the difference, $2 < \Delta E_{Lab} < 3.5$—an unexperienced observer also notices the difference, $3.5 < \Delta E_{Lab} < 5$—a clear difference in colour is noticed, and $5 < \Delta E_{Lab}$—an observer notices two different colours. BC-RS – blackcurrant seeds before CO_2 extraction at concentrations 1, 3 and 5%, BC-ASC – black-currant seeds after supercritical fluid CO_2 extraction at concentrations 1, 3 and 5% and BC-AE – blackcurrant seeds after EtOH/water extraction at concentrations 1, 3 and 5%.

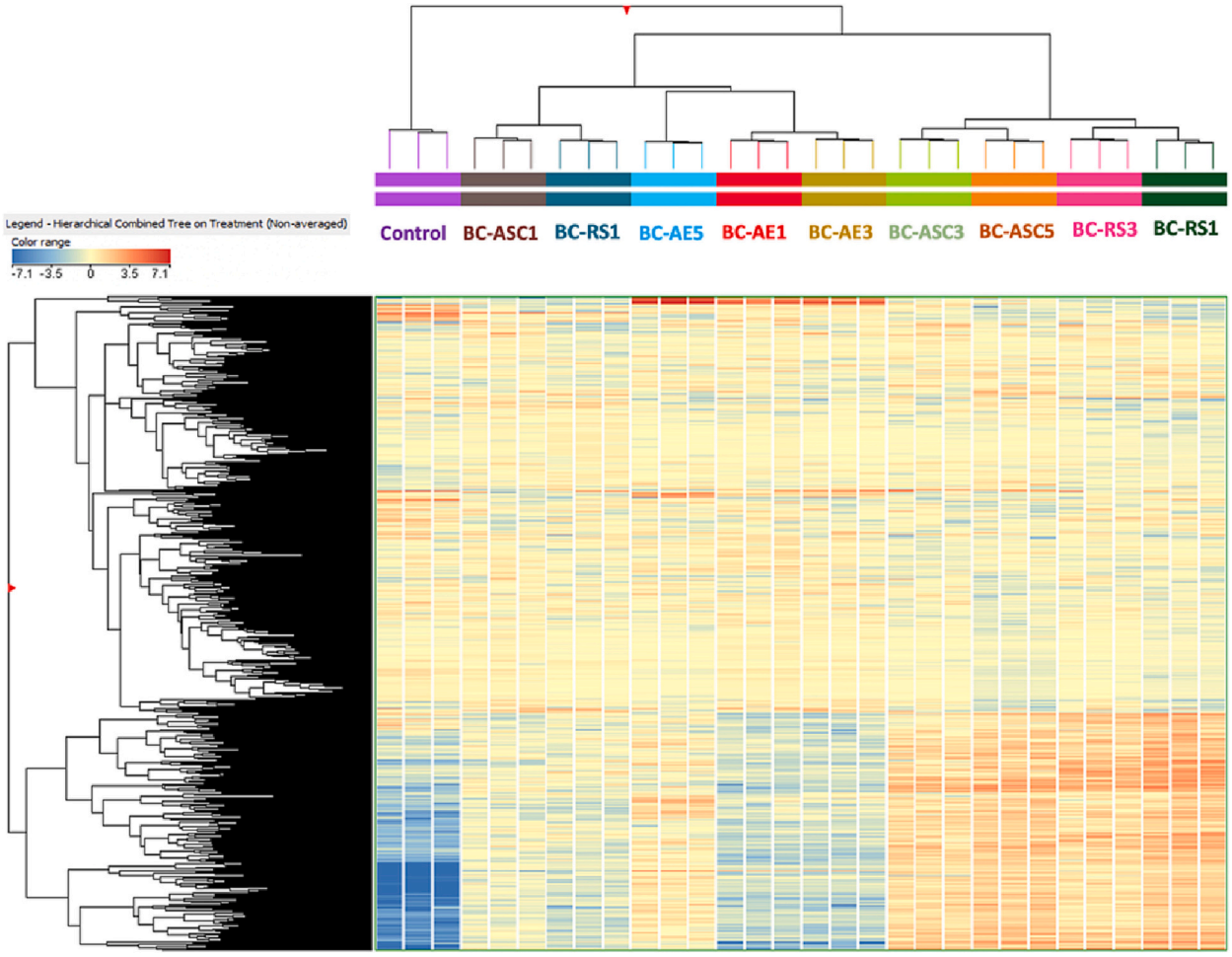

Fig. 7. Unsupervised hierarchical cluster analysis built considering the compounds annotated by untargeted metabolomics in the different meatballs, according to the different inclusion levels of BC-based ingredients (i.e., BC-ASC, BC-AE, and BC-RS).

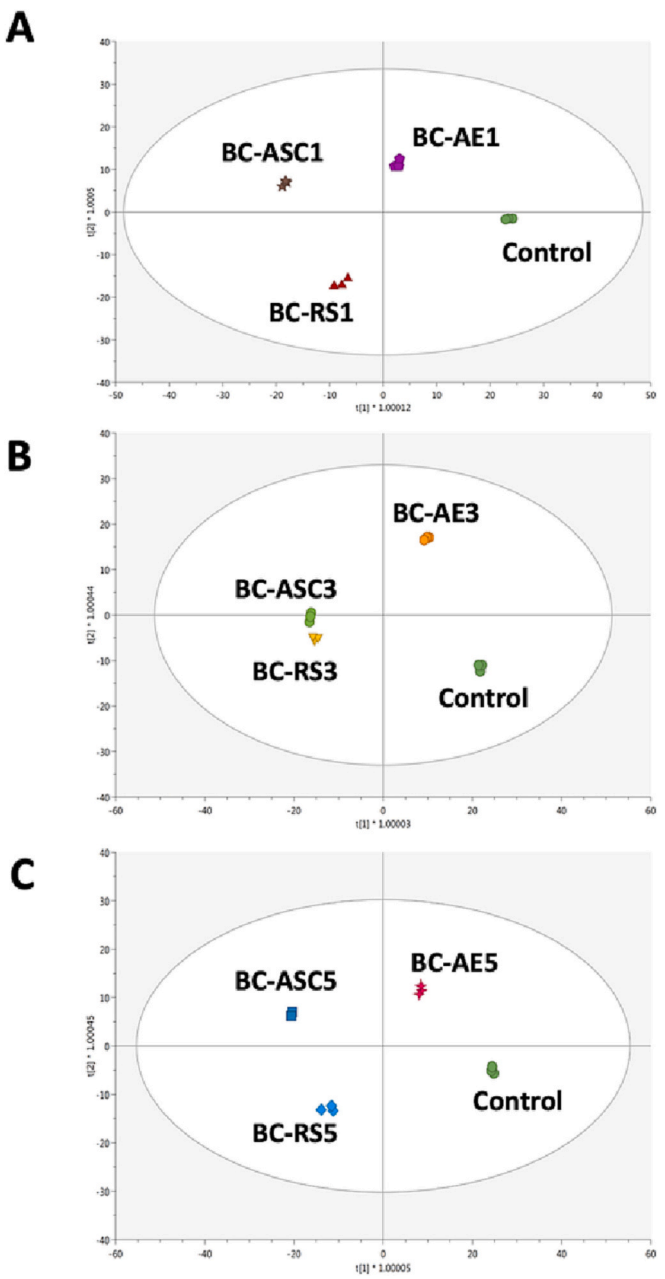

Fig. 8. OPLS-DA models considering the different inclusion levels of BC-functional ingredients (i.e., BC-RS, BC-ASC, and BC-AE) in the pork meatballs. [A] = 1% inclusion; [B] = 3% inclusion; [C] = 5% inclusion.

provided a different chemical profile of pork meatballs when compared with the control sample (Fig. 8A, B, and C). Overall, most of the discriminant variation was correlated with the utilization of BC-ASC and BC-BC ingredients. This trend was not surprising; in fact, defatted BC seed product (BC-ASC) is the residue after CO_2 extraction, therefore, it is very similar from a chemical point of view to the initial material (BC-RS) but containing a small amount of residual lipids. Accordingly, the orthogonal latent vector of the OPLS-DA prediction model separated the BC-AE sample from the others, and this was true for each inclusion level tested (i.e., 1, 3, and 5% *w*/w) under investigation. The discriminant marker compounds of each OPLS-DA model inspected (Fig. 8) were then extrapolated using a VIP selection method and considering those having a discriminant score > 1.

Looking at each OPLS-DA model separately, that one built considering the 1% inclusion levels resulted in 316 discriminant metabolites, with the highest VIP score recorded for the sesquiterpenoid pyrocurzerenone (1.49; Supplementary Table 1), followed by the monocyclic diterpene cembrene (1.44; Supplementary Table 1). Among the VIP markers we also found glutathione disulfide, showing a VIP score = 1.33. This compound was always down-accumulated in the BC-added pork meatballs when compared with the control, recording a maximum down-accumulation value in pork meatballs prepared with 1% of BC-RS (Log Fold-Change = −1.35), thus suggesting a potential role of BC-functional ingredients against redox impairment. Regarding polyphenols, the VIP selection method identified 35 discriminant compounds, with maximum VIP scores recorded for five compounds, namely hesperetin (1.28, a marker of BC-RS samples), dihydroresveratrol (1.25, a marker of BC-AE samples), myricetin (1.17, a marker of BC-RS samples), luteolin 7-galactoside (1.17, showing similar LogFC variations for each treatment vs the control), and cyanidin-3-*O*-alpha-arabinopyranoside (1.16, a marker of BC-ASC samples). Interestingly, we found other three discriminant anthocyanins resulting from the inclusion of 1%-BC functional ingredients, namely delphinidin 3-rutinoside, petunidin-3-*O*-beta-glucopyranoside, and delphinidin-3-*O*-beta-glucopyranoside. The maximum increase of discriminant anthocyanins in the BC-added pork meatballs was detected in samples added with 1% of BC-ASC when compared with BC-RS and BC-AE pork meatballs. Interestingly, the Fold-Change analysis on the discriminant compounds revealed that the flavan-3-ol ampelopsin (VIP score = 1.10) was the best marker of the 1% inclusion level, independently from the extraction technique used to obtain the BC-functional ingredients, showing a maximum LogFC value in BC-ASC pork meatballs (i.e., 11.86) when compared with the control samples. Regarding the lipid compounds related to oxidation processes, we found a marked increase of 13-L-hydroperoxylinoleic acid in each BC-added pork meatball sample when compared with the control. Lipid oxidation is known to result in the formation of several by-products potentially affecting human health. Accordingly, the autoxidation of linoleic acid, the most abundant PUFA in mammalian tissues, potentially produces two monohydroperoxides, namely 13-hydroperoxylinoleic acid and 9-hydroperoxylinoleic acid (Onyango, 2016). Besides, among the VIP discriminant compounds, we also detected an increase of some oxo-derivatives of hydroperoxides of fatty acids, such as 9-oxohexadecanoic acid (VIP score = 1.08; maximum LogFC value following the addition of BC-ASC, being 4.55) and (E)-10-oxo-8-decenoic acid (VIP score = 1.18; maximum LogFC value following the addition of BC-RS, being 1.37). Another marker of lipid peroxidation known as (11*R*,12*S*,13*S*)-Epoxy-hydroxyoctadeca-cis-9-cis-15-dien-1-oic acid (VIP score = 1.04) was found to decrease only in pork meatballs added with 1% of BC-AE (LogFC = − 0.46). According to literature, the straight-chain volatiles are derived by oxidation of unsaturated fatty acids (mainly linoleic and α-linolenic acids) which produces a range of flavor-active volatile aldehydes, ketones, alcohols, and alkyl furans. The volatile aldehydic products and alkyl furans contribute to the development of off flavors in lipid-rich foods, while both volatile and non-volatile aldehydes lower the nutritional value of food by reacting with essential nutrients such as lysine and thiamine (Wanjala, Onyango, Abuga, Onyango, and Makayoto, 2021). In our experimental conditions, the 1% inclusion level of the different BC ingredients determined an overall and significant down-accumulation of α-linolenic acid (VIP score = 1.11), with an average LogFC value = −2.84), whilst an overall increase of several alkyl furans, likely related to oxidation processes, was detected (Supplementary Table 1). Accordingly, the metabolite ethyl 2-furanacrylate (derived from 2-ethylfuran) showed a marked increase in BC-added pork meatballs and this was true mainly when considering the addition of 1% BC-AE1 (LogFC = 2.58).

Regarding the 3% inclusion level, the supervised OPLS-DA model revealed 325 discriminant metabolites showing a VIP score > 1 (Supplementary Table 1). A Venn diagram was used to evaluate the common and exclusive discriminant marker compounds as a function of the different inclusion levels. The comparison between 1 vs 3%-added samples showed 233 common discriminant compounds, whilst 42 and

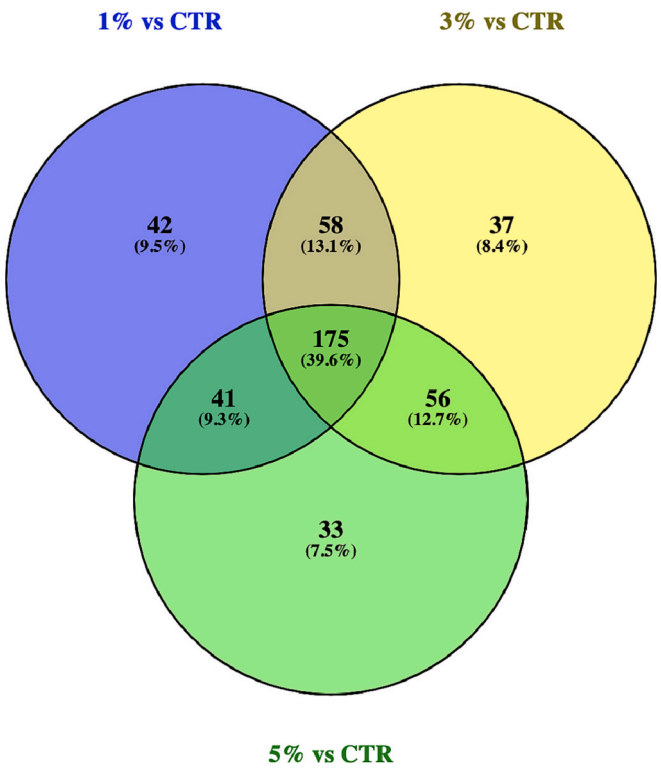

Fig. 9. Venn Diagram built considering the VIP discriminant metabolites of each OPLS-DA prediction model built considering the different inclusion levels of BC-ingredients under investigation (i.e., 1, 3, and 5%).

37 were found as exclusive discriminant markers (Fig. 9).

Among the 37 exclusive markers we found some phenolics, such as isorhamnetin (maximum increase following the addition of BC-ASC, LogFC = 8.96) and 5,7-dihydroxy-4′-methoxy-8-methylflavanone (maximum increase following the addition of BC-RS, LogFC = 7.91). Additionally, some peptides (such as Pro-Ile, Leuc-Asp, and Glu-Phe) exclusively discriminated the 3% inclusion level of BC ingredients when compared with the control sample (Fig. 8B). Among the marker compounds of the redox imbalance, we found again the glutathione disulfide (VIP score = 1.40). Overall, the variation of this compound for the 3% inclusion level was lower when compared with the 1%-added samples (being, on average, −0.38 vs − 0.78), thus potentially suggesting a lower efficiency of these ingredients to counteract oxidative stress at this inclusion level (3%). This trend could be related to the presence of phenolic compounds in the BC-added pork meatballs. Accordingly, plant-derived antioxidant polyphenols (such as phenolic acids and different sub-classes of flavonoids) have been described to possess both pro-oxidative and antioxidative properties, mainly depending on factors such as their metal-reducing potential, chelating behaviour, pH, and solubility characteristics (Babich, Schuck, Weisburg, and Zuckerbraun, 2011). Looking at marker compounds of lipid oxidation, the most effective ingredient in reducing the number of oxidation-related derivatives was represented by 3% BC-AE. The addition of this latter to pork meatballs was found to reduce the 13(*S*)-hydroperoxylinolenic acid (LogFC = −1.01) and (12*S*,13*S*)-EOD;(12*S*,13*S*)-epoxylinolenic acid (LogFC = −0.87). Regarding the aromatic profile of the product, the development of a certain flavor of cooked meat is related to volatile compounds (VOCs) generated during heating, because of Maillard reaction, lipid oxidation, interactions between Maillard reaction products and lipid oxidation products, and thermal degradation of thiamine (Kosowska, Majcher, and Fortuna, 2017). Thiamine is an important vitamin that naturally occurs in pork meat (ranging from 0.8 to 1.1 mg/100 g). The thermal degradation of this compound results in the appearance of transient and final VOCs likely affecting odour development, such as thiazoles, thiophenes and furans. In our experimental conditions, thiamine (VIP score = 1.11) was down-accumulated following the 3% of each BC-ingredients, with a corresponding increase of 2,5-dimethyl-3-(methyldithio)furan (previously reported in literature to possess a meaty flavor) (Kosowska et al., 2017). Finally, as far as is concerned the 5% inclusion of BC-ingredients in the pork meatballs is concerned, the Venn Diagram (Fig. 9) showed only 33 exclusive VIP discriminant compounds, whilst a total of 231 discriminant compounds were in common between 3 and 5% inclusion levels, thus suggesting a similar impact of higher % of inclusion on the metabolomic profile of cooked pork meatballs.

Looking at some discriminant compounds (Supplementary Table 1), we found several medium-chain aldehydes related to the flavor (Sohail et al., 2022), such as 2,5-undecadienal (VIP score = 1.08), nona-2,4,6-trienal (VIP score = 1.11), and (2E,4Z,7Z)-2,4,7-decatrienal (VIP score = 1.01) and showing controversial trends when considering their LogFC variations vs the control samples. Trienals may be the products of oxidation of linolenic acids due to the three double bonds. BC contains both, γ-linolenic acid, α-linolenic acids, and even after $scCO_2$-E some amounts remain in the residues. It is also important to highlight another important reaction, i.e., the Maillard reaction, able to affect both flavor and colour of the cooked meat product. The Maillard reaction is varied with meat composition (e.g., amino acids and glucose) and the heating conditions, including temperature, pH, water activity, etc., during meat cooking (Liu et al., 2020), which consequently causes different meat flavor. Different from the lipid degradation, the Maillard reaction mainly generates the volatile sulfur-containing compounds, nitrogen-containing heterocyclic compounds, and oxygen-containing heterocyclic compounds (Sun et al., 2022). Among these compounds, we found, among the others, 4-methyl-5-thiazoleethanol (VIP score = 1.10), methoxypyrazine (VIP score = 1.05), 2-ethyl-5-methylpyrazine (VIP score = 1.12), and several furan derivatives (Supplementary material). Overall, similar trends when compared to the previous inclusion levels were observed for the lipid peroxidation markers, with a great and proportional increase of 13-L-hydroperoxylinoleic acid in the BC-added pork meatballs and recording a maximum increase (LogFC = 4.97) for the BC-ASC added sample. Therefore, our untargeted metabolomic findings suggested that both the % of inclusion (i.e., 1, 3, and 5%) and the extraction process of the BC matrix (i.e., RS, AE, and ASC) represent critical parameters to be carefully evaluated to improve the final quality of the cooked pork meat products. However, as suggested in scientific literature (Pogorzelska-Nowicka, Atanasov, Horbańczuk, and Wierzbicka, 2018), the enrichment with bioactive ingredients from plant sources (e.g., by-products and/or waste) still represents a very powerful way to produce healthier meat products, and further studies on the valorization of these BC ingredients appear of great interest.

4. Conclusions

Blackcurrant seed ingredients as potential antioxidants and fibre-rich materials were tested in pork meatballs at the concentrations of 1, 3 and 5%. Ingredients had significant effect on the meatballs colour, causing the reduction of lightness and increase in redness compared to control sample, pH values were affected by the added BC seed ingredients at different concentrations, whereas water activity values remained the same within the samples and 6 storage days. The lowest cooking loss was observed in all samples with the highest extract concentration (BC-RS5–20.48 ± 4.44, BC-ASC5–19.77 ± 4.37 and BC-AE5–20.64 ± 2.19%), compared to the control sample (26.79 ± 3.18%). Fibre content of meatballs increased as the concentration of BC ingredients increased. Untargeted metabolomics results revealed some specific effects of different BC ingredients, namely raw (BC-RS), CO_2-defatted (BC-ASC) and extracted by polar extracts (BC-AE) seeds. Blackcurrant seeds after supercritical fluid CO_2 extraction (full defatting) and the absence of easily oxidising PUFA indicate the most effective ingredient in reducing the number of oxidation-related derivatives. Consequently, our study

proved that the untargeted metabolomics approach can be used as a powerful tool to evaluate the impact of different BC-fractions on those typical oxidative reactions of the meatballs during storage. Taken together, our findings demonstrated that BC seed ingredients may be considered as a promising antioxidants and fibre-rich materials in pork meat products which may increase their nutritional quality related to health benefits.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meatsci.2023.109160.

CRediT authorship contribution statement

Kristi Kerner: Conceptualization, Formal analysis, Investigation, Methodology, Writing – original draft. **Rita Kazernavičiūtė:** Formal analysis, Methodology. **Ivi Jõudu:** Conceptualization, Methodology, Resources, Project administration, Funding acquisition. **Gabriele Rocchetti:** Conceptualization, Formal analysis, Methodology, Investigation, Writing – original draft. **Luigi Lucini:** Writing – review & editing, Supervision. **Alo Tänavots:** Visualization, Writing – review & editing. **Shehzad Hussain:** Methodology, Formal analysis. **Petras Rimantas Venskutonis:** Conceptualization, Methodology, Writing – review & editing, Resources, Supervision.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgments

This study was supported by the European Union's Horizon 2020 research and innovation program project ERA Chair for Food (By-) Products Valorisation Technologies of Estonian University of Life Sciences—VALORTECH (grant agreement No 810630). Gabriele Rocchetti acknowledges the project PON REACT EU DM 1062/21 (57-G-999-13) funded by the Ministero dell'Università e della Ricerca (MUR).

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