Measurement: Food 21 (2026) 100280

Contents lists available at ScienceDirect

Measurement: Food

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

Development of bioresidue apple flour with potential application in the food industry

Chaimaa Aichouche [a], Eliana Pereira [a], Márcio Carocho [a], Maria Inês Dias [a], Ricardo C. Calhelha [a], Sandrina Heleno [a], Filipa A. Fernandes [a], Tânia C.S.P. Pires [a], Elizabete Ferreira [b], Cristina Caleja [a,*], Lillian Barros [a]

[a] CIMO, LA SusTEC, Instituto Politécnico de Bragança, Campus de Santa Apolónia, 5300-253 Bragança, Portugal
[b] Pão de Gimonde®, M Ferreira & Filhas, LDA, EN218, n° 3798, 5300-553, Gimonde, Portugal

ARTICLE INFO

Keywords:
Circular economy
Apple flour
Bioresidue
Bioactivities
Bread

ABSTRACT

Food loss and residues have attracted the attention of many sectors around the world due to negative environmental and economic effects. Several studies identify this bioresidue with potential to be explored and used as natural ingredients, benefiting environment and economy, and producing functionalized products. This study aimed at the nutritional, chemical and bioactive evaluation of flours obtained from apples discarded as bioresidue due to their low caliber or non-standard format. The nutritional evaluation of the flour highlighted an interesting protein and ash content. Three free sugars were identified in the analyzed flour, with fructose being the main sugar and five organic acids, with malic acid being the most prevalent. Regarding fatty acids, monounsaturated fatty acids predominated, with oleic and linoleic acids being the major components. Seven phenolic compounds were identified by HPLC-DAD-ESI/MS, divided into three different families: phenolic acids, flavan-3-ols, and dihydrochalcone, with phenolic acids predominating, which may justify the antioxidant and antimicrobial potential evidenced by the flour. After characterization, flour was incorporated into two types of bread and the effect of this incorporation over the shelf life was evaluated. Overall, the nutritional and chemical composition of the apple flour obtained in this study makes it a viable alternative for application in the bakery and pastry industry, thus creating alternative products and health promoters for the consumer. Additionally, the reuse of apple bioresidue will allow the recovery of waste discarded annually without added value, in addition to contributing to a positive impact on the economy and the environment.

1. Introduction

Food intake is no longer seen as just a form of hunger satiation, to be associated with the intake of nutrients and bioactive compounds, with a direct influence on consumer health and well-being [1]. In this sense, consumers are increasingly aware of their food choices and there is a greater concern with the analysis of labels, favoring products designated as healthier and more natural [2].

In this sense, there is a concern on the part of the food industry to follow new consumer trends and, in the launch of this type of products on the market [1]. The incorporation of natural ingredients with functional and bioactive properties has been tested. Nature presents several natural matrices that have been studied and tested as promising sources of natural ingredients to be used by the industry [3].

In the food industry, bioresidues are the organic, process-derived fractions (e.g., peels, pomace and seeds) generated during harvesting, sorting and manufacturing that, although often classified as waste, contain recoverable nutrients and bioactive compounds amenable to valorization through biorefinery and green-extraction strategies [4]. There is a high amount of bioresidue discarded annually by the food industry and commercialization, which has been raising a great concern not only economically but also environmentally. This type of residue has been characterized as having a very interesting nutritional and chemical composition, in addition to highlighting the presence of bioactive compounds of high interest [3]. Recent statistics showed that European food processing units might generate approximately 100 Mt of waste and by-products each year, mostly comprising the production of drinks (26 %), dairy and ice cream (21.3 %), and fruit- and vegetable-derived

* Corresponding author.
E-mail address: ccaleja@ipb.pt (C. Caleja).

https://doi.org/10.1016/j.meafoo.2026.100280
Received 3 September 2025; Received in revised form 21 January 2026; Accepted 9 February 2026
Available online 10 February 2026

products (14.8 %) [5].

Around 70 % to 80 % of the total apple produced is destined for marketing in fresh, and the apple considered to be of inferior quality is destined for the agri-food industry, for processing. Fruits that present characteristics that do not fit the classification and selection standards, such as, for example, low caliber, non-uniform colour, scars caused by insects, birds or hail, injuries resulting from cultural methodologies, such as harvesting, inadequate transport, symptoms disease or physiological problems, are rejected for marketing [6]. Initially, these fruits were intended for animal feed, but with the increase in raw material, the food industries started to process them, as a form of economic valorization [7].

The apple and its residues appear as rich sources of antioxidants that can play a role in reducing the risk of several diseases related to oxidative stress, for example, coronary heart disease, damage to the immune system, asthma, and diabetes, which has aroused great interest by the scientific community and the industry [8].

In this sense, and in order to value a by-product of the food industry, a flour was made from apples discarded as bioresidue (of the Fuji variety, as it is one of the most consumed varieties worldwide). After nutritional, chemical and bioactive characterization, this four was evaluated as a natural ingredient for application in the baking and pastery industry.We hypothesised that the incorporation of apple-derived flour would enhance the nutritional and functional profile of bakery products due to its richness in bioactive compounds. However, given the reformulation of traditional recipes, potential challenges were anticipated, particularly regarding technological and sensory attributes such as colour, texture, volume, and overall consumer acceptability of the developed products.

2. Materials and methods

2.1. Apple flour preparation

The Fuji variety apples were kindly provided by the Campotec company, based in Torres Vedras, Portugal. After reception the apples were washed with water. The apples were cut into slices and the cores and seeds were removed. The apple slices were placed to dry in the oven at 50°C for 48 hours and then crushed and reduced to flour (~20 mesh) using a lab blender (model A327R1, Moulinex, Barcelona, Spain). Subsequently, the flour was stored in a cool and dry place, protected from light, for further analysis.

2.2. Proximate composition and energy

The macronutrient composition of the apple flour was determined using official food analysis methodologies [9]. The quantification of macronutrients was done analysing the content of proteins, fats (AOAC 989.05), and carbohydrates, as well as the amounts of ash (AOAC 935.42) and moisture. The total protein content (N × 6.25) was calculated as nitrogen content by the Kjeldahl method (AOAC 991.02). The energy value was calculated according to the Regulation (EU) No 1169/2011.

2.2.1. Free sugars

A high-performance liquid chromatography (HPLC) system coupled to a refraction index (RI) detector was used to determine the soluble sugars composition, following a previously described procedure [10]. Melezitose was used as internal standard at 25 mg/mL. For the identification of the compounds, Clarity 2.4 Software DataApex 4.0 Software (Prague, Czech Republic) was used, from which the relative retention times of the sample peaks were compared with known patterns. The results were obtained by the internal pattern method and expressed in grams per 100 g of fresh weight (fw).

2.2.2. Fatty acids

Fatty acids were determined by gas chromatography with flame ionization (GC-FID) design, as previously described by Barros et al. [10]. The identification and quantification were performed by comparing the relative retention times of FAME peaks from samples with standards (standard mixture 47885-U, Sigma, St. Louis, USA) and results were recorded and processed using the Clarity software and expressed in relative percentage of each fatty acid.

2.2.3. Organic acids

Organic acids were determined using an Ultra-Fast Liquid Chromatography (UFLC, Shimadzu 20A series, Kyoto, Japan) coupled to a diode array detector.

The extraction was performed in the dark by adding 25 mL of metaphosphoric acid (4.5 %) to the sample (1.5 g), maintaining the temperature conditions of 25 °C and constant stirring (150 rpm) for 45 minutes. After extraction, the mixture was filtered through filter paper (Whatman No. 4) and subsequently through nylon filters (0.2 μm; Whatman) for analysis by UFLC (Shimadzu Corporation, Kyoto, Japan). The separation of the compounds was achieved using a C18 reverse-phase SphereClone column (Phenomenex, Torrance, California, USA) (5 μm, 250 mm × 4.6 mm i.d.) thermostated at 35°C, with elution performed using sulfuric acid (3.6 mM) at a flow rate of 0.8 mL/min, with an injection volume of 20 μL.

The detection was achieved using a DAD system, applying wavelengths of 215 nm and 245 nm (for ascorbic acid). The quantification was performed using calibration curves obtained from commercial standards (L(+)-ascorbic acid, citric acid, malic acid, oxalic acid, shikinic acid, succinic acid, fumaric acid and quinic acid), purchased by Sigma-Aldrich (St. Louis, MO, USA) and the results were expressed in g per 100 g of fresh weight (fw).

2.3. Extracts preparation

The flour sample (2 g) was submitted to maceration with 50 mL of ethanol/water (80:20, *v/v*) for 1 h at room temperature with stirring at 150 rpm. Subsequently, the mixture was filtered through filter paper (Whatman No. 4) and the process was repeated. The ethanolic fraction of the combined extracts was evaporated under reduced pressure (100 rpm, 40°C; rotary evaporator, Heidolph, Schwabach, Germany) and the aqueous phase was frozen and lyophilized (Labconco Freeze Zone 6, USA) for further analysis.

2.4. Identification and quantification of phenolic compounds

For phenolic profile analysis, the extract described was redissolved in EtOH/H_2O solution (20:80, *v/v*). The phenolic compounds composition was determined by high-performance liquid chromatography coupled with a photodiode array detection (280, 330 and 370 nm as preferred wavelengths) and a mass spectrometer with electrospray ionization (HPLC-DAD-ESI/MS). The identification of compounds was based on the comparison with commercial standards and the available literature information. The quantification was performed using the calibration curve of the most similar available standard. The results were expressed as mg/g of extract.

2.5. Bioactivities evaluation

2.5.1. Evaluation of antioxidant activity

Following the previously described protocols, DPPH radical-scavenging activity and reducing power were evaluated using ELX800 microplate reader (Bio-Tek Instruments, Inc., Winooski, VT, USA) at 515 and 690 nm, respectively [10]. The lipid peroxidation inhibition was evaluated by the extract capacity to inhibit the formation of thiobarbituric acid reactive substances (TBARS) using porcine (*Sus scrofa*) brain tissues as oxidizable substrates, according to the methodology

described by Barros *et al.* [10]. The results were expressed as IC_{50}, which correspond to the extract concentration responsible for inhibiting 50 % of oxidation (μg/mL). Trolox was used as a positive control.

The antihaemolytic activity of the extracts was evaluated as previously described by Lockowandt *et al.* [11]. This assay was performed using sheep blood erythrocytes and the results were expressed as IC_{50} values (μg/mL) for Δt of 60 and 120 min, which translate the extract concentration needed to protect 50 % of the erythrocyte population from the haemolytic action of 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH) for the specified time periods. Trolox was used as a positive control.

2.5.2. Evaluation of cytotoxic activity

For the cytotoxicity activity, the effect of the extract (8 mg/mL) on the growth of human tumour cell lines was evaluated by sulforhodamine B (SRB) assay to determine cell growth inhibition [12]. Four tumour cell lines were used in this assay: MCF-7 (breast carcinoma), NCI-H460 (lung carcinoma), AGS (gastric adenocarcinoma) and CaCo-2 (colon adenocarcinoma). The non-tumour cell line VERO (fibroblast-like kidney cell line from African green monkey) was also tested. Ellipticine was used as a positive control and the results were expressed as GI_{50} values (μg/mL), which correspond to the extract concentration that inhibits 50 % of cell growth.

2.5.3. Evaluation of antimicrobial activity

For evaluation of antimicrobial activity, the extracts were tested against five Gram-negative bacteria, as well as three Gram-positive bacteria. All these microorganisms were obtained from Frilabo, Porto, Portugal. The bacteria were incubated at 37°C in a suitable fresh medium for 24 hours before analysis to maintain the exponential growth phase.

The bacterial strains were clinical isolates obtained from patients hospitalized in various departments of the Hospital Center of Trás-os-Montes and Alto Douro (Vila Real, Portugal). Five Gram-negative bacteria (*Escherichia coli, Proteus mirabilis, Klebsiella pneumoniae, Pseudomonas aeruginosa*, and *Morganella morganii*) and three Gram-positive bacteria (*Enterococcus faecalis, Listeria monocytogenes*, and methicillin-resistant *Staphylococcus aureus* - MRSA) were tested.

The Determination of Minimum Inhibitory Concentration (MIC) was performed using a colorimetric assay as described by [13]. The samples were first dissolved in 5 % (v/v) dimethyl sulfoxide (DMSO) and 95 % autoclaved distilled water to reach a final stock concentration of 20 mg/mL. Then, 90 μL of this concentration was added to the first well (96-well microplate) in duplicate along with 100 μL of Tryptic Soy Broth (TSB). In the remaining wells, 90 μL of TSB were added. The samples were serially diluted to obtain concentrations ranging from 10 to 0.03125 mg/mL. Finally, 10 μL of inoculum (standardized at 1.5×10^6 Colony Forming Units (CFU)/mL) were added to all wells, ensuring the presence of 1.5×10^5 CFU. Two negative controls were prepared, one with TSB and another with the extract, while two positive controls were prepared with TSB and each inoculum, as well as another with the medium, antibiotics, and bacteria. Ampicillin and streptomycin were used for all tested bacteria, and methicillin was also used for *Staphylococcus aureus*. The microplates were incubated at 37°C for 24 hours. MIC was determined following the addition of 40 μL of 0.2 mg/mL *p*-iodonitrotetrazolium chloride (INT) and incubation at 37°C for 30 minutes. MIC was defined as the lowest concentration that inhibited visible bacterial growth, which was determined by a color change from yellow to pink if the microorganisms were viable, evaluated by visual examination. For MBC determination, 10 μL of liquid from each well that showed no color change were plated on solid medium (Blood agar with 7 % sheep blood) and incubated at 37°C for 24 hours. The MBC was defined as the lowest concentration required to kill the bacteria.

The antifungal activity was assessed as described by [14]. *Aspergillus fumigatus* (ATCC 204305) and *Aspergillus brasiliensis* (ATCC 16404) were used, and obtained from Frilabo, Porto, Portugal. The fungi were maintained on malt agar, with cultures stored at 4°C, and later transferred to fresh medium and incubated at 25°C for 72 hours. To evaluate antifungal activity, fungal spores were washed from agar plate surfaces with sterile 0.85 % saline containing 0.1 % Tween 80 (*v/v*). The spore suspension was adjusted to a concentration of approximately 1.0×10^5 in a final volume of 100 μL per well. Samples were first dissolved in 5 % (v/v) dimethyl sulfoxide (DMSO) and 95 % autoclaved distilled water to make a stock solution of 10 mg/mL. Then, 90 μL of this concentration was added to the first well of a 96-well microplate in duplicate with 100 μL of Malt Extract Broth (MEB). In the remaining wells, 90 μL of MEB were added. The samples were serially diluted to obtain concentrations ranging from 10 to 0.03125 mg/mL. MIC was determined using a serial dilution technique in the 96-well microplate. The lowest concentrations without visible growth (under a binocular microscope) were defined as MICs. Minimum fungicidal concentration (MFC) was determined by serial subcultivation of 2 μL of tested compounds dissolved in medium and inoculated for 72 hours into microplates containing 100 μL of MEB per well, followed by further incubation for 72 hours at 26°C. The lowest concentration with no visible growth was defined as MFC, indicating 99.5 % killing of the original inoculum. The commercial fungicide ketoconazole (Frilabo, Porto, Portugal) was used as a positive control.

2.6. Incorporation of apple flour as natural ingredients in bread

To evaluate the potential of apple flour, as a potential natural ingredient, it was incorporated into two types of bread (wheat bread and corn bread) usually prepared at the Pão de Gimonde bakery in Bragança (Portugal). The effects of this incorporation on different parameters throughout its shelf life were evaluated.

2.6.1. Preparation of bread

For wheat bread: 10 g of yeast were dissolved in 600 g of water and 15 g of salt were added and mixed with 900 g of wheat flour until a homogeneous dough was obtained. The dough was covered with cling film and left to rise for 30 minutes in a warm place. After molding the bread shape, it is taken to the preheated oven for 30 minutes at 200°C.

For wheat bread with apple flour the above process was followed by adding 100 grams of apple flour to the wheat flour.

For corn bread: 10 g of yeast were dissolved in 600 g of water and 15 g of salt were added and mixed with 900 g of corn flour until a homogeneous dough was obtained. The dough was covered with cling film and left to rise for 30 minutes in a warm place. After molding the bread shape, it is taken to the preheated oven for 30 minutes at 200°C. For the apple flour corn bread, the above process was followed by adding 100 grams of apple flour to the corn flour.

The samples were prepared and analysed in triplicate. Physical parameters and microbial load were evaluated in the fresh samples immediately after preparation (T0). For the remaining analyses, the samples were immediately frozen after preparation (T0) and stored at room temperature for three (T3) and five (T5) days in sealed plastic bags and protected from light with aluminum foil. After each storage period, the samples were freeze-dried and finely ground to ensure stability, homogeneity, and preservation of chemical and bioactive constituents prior to analysis.

2.6.2. Physical parameters of bread

For each bread sample, the water activity "a_w" was determined at 20°C for 5 min at the surface of each sample slice by using an activity meter instrument (AQUALAB 4TE) based on the dew-point method and with an absolute error of 0.003. The average a_w value of each slice was calculated from the a_w values estimated at its surface and at the adjacent slice surface.

The colour of the samples was measured in triplicate each group of samples, at three different points using a colorimeter (model CR-400, Konica Minolta Sensing Inc., Tokyo, Japan). The Illuminate C was used and an 8 mm diaphragm aperture was previously calibrated against

a standard white tile. CIE L^* (luminosity), a^* (green/red), b^* (blue/yellow) colour values were recorded using the data software "Spectra Magic Nx" [15].

Texture evaluation was performed using a TA.XT Plus texture analyzer with a 30 kg Stable Micro Systems load cell (Vienna Court, Godalming, UK) via a P/45 aluminum cylinder, which performed a human mouth-like lateral analysis (TPA) by pressing twice on the matrix. At pre- and post-test velocities at 3 mm/sec and the target position was set to 25 % pressure starting at 50 g of force. Expressing results by macro has been combined to reach different dimensions of texture, namely, hardness, adhesion, quaternity, cohesion, chewiness, and elasticity. The results were analyzed by Exponent software [16].

2.6.3. Determination of the proximate composition of bread

The nutritional composition (protein, fat, carbohydrates, ash, humidity, and the total energy value) of bread samples was determined according to official food analysis methodologies [9]. To determine the content of protein, fat, ash, carbohydrates and the total energy value, the procedures previously described in section 2.2. were followed.

2.6.4. Determination of the sugars and fatty acids of the bread

For the determination of free sugars and fatty acids of different bread samples, the methodologies previously described in sections 2.2.1 and 2.2.2., respectively, were followed.

2.6.5. Microbial load

The microbial load of flours (corn, wheat, apple) and different breads (wheat bread, wheat bread with apple flour, corn bread and corn bread with apple flour) were evaluated. The preparation of the samples was carried out according to the procedure described in the International Organization for Standardization [17]. In brief, the different bread samples (10 g, in triplicate) were mixed with 90 mL of peptone water and homogenized in a stomacher equipment (Star Blender, VWR, Radnor, USA) for 30 seconds at 300 units. The resulting suspensions were further diluted to obtain 3 serial dilutions (10^{-1} to 10^{-3}) and each solution was analyzed in duplicate.

Total aerobic mesophiles (ISO 4833-2:2013): in duplicate using the pour plate technique. Specifically, 1 mL of each dilution the sample was placed in a Petri dish and 15 mL of Plate Count Agar (PCA, Liofilchem Co., Roseto degli Abruzzi, Italy), were added and the plates were then homogenized. After solidification of the medium, the plates were incubated in an inverted position at 30 °C for 72 h. Colony counts were performed on plates containing between 15 and 300 colonies (Limit of Quantification (LOQ) = 1 log (Colony Forming Units) CFU per g).

Coliforms (ISO 4832:2006): were quantified following the methodology describe for total aerobic mesophiles, using the Violet Red Bile Lactose Agar (VRBLA, Liofilchem Co., Roseto degli Abruzzi, Italy). The plates were incubated at 30 °C for 48 h, in reversed position. Colony counts were performed in plates that had between 10 and 150 colonies (Limit of Quantification (LOQ) = 1 log (Colony Forming Units) CFU per g).

Yeasts and Molds (ISO 21527-2:2008): were quantified in duplicate using the spread plate technique. Concretely, 0.2 mL of the suspensions were pipetted onto a plate containing 15 mL of the Dichloron Glycerin Selective Agar (DG18, Liofilchem Co., Roseto degli Abruzzi, Italy) and were spread .The plates were incubated in the upright position at 25 °C for 5 days, and the counting was performed on plates with less than 150 colonies (Limit of Quantification (LOQ) = 1.7 log (Colony Forming Units) CFU per g). Yeasts were counted after 3 days and molds were counted after 5 days of incubation.

Bacillus cereus (ISO 7932:2004): were quantified following the methodology describe for yeasts and molds, using Bacillus cereus Agar Base (PEMBA, Liofilchem Co., Roseto degli Abruzzi, Italy. The plates were incubated at 30 °C for 24–48 h, in reversed position. Counting was performed in the plates having between 10 and 150 colonies (Limit of Quantification (LOQ) = 1.7 log (Colony Forming Units) CFU per g).

2.7. Statistical analysis

Throughout the whole document, all data was expressed as mean ± standard deviation. Furthermore, to better understand the effect of the addition of the apple flour and storage time, for all analyses the samples were analyzed through a two-way ANOVA with type III sums of squares using the SPSS Software, version 25. This multivariate general linear model treats the two factors, incorporation (I) and storage time (ST) as independent, thus allowing the effect of each one to be analyzed independently, providing more insight on their contribution towards the outcome. If a significant interaction (<0.05) was recorded among the two factors (I × ST), these were evaluated simultaneously, and some general conclusions and tendencies were extracted from the estimated marginal means (EMM), when possible. If there was no significant interaction (>0.05), each factor was evaluated independently using a simple Student's T test (for I) or a Tukey's multiple comparison test (ST) when the means were homoscedastic, and a Tamhane's T2 for non-homoscedastic samples. Homoscedasticity was evaluated using a Levene's test. All analyses were carried out using a significance level of 0.05. A Principal Components Analysis (PCA) was performed with the control and apple incorporated breads. This included a categorical principal components analysis, with an oblimin rotation using a Kaiser normalization, and was also performed in the SPSS software. In terms of the principal component analysis (PCA), it was performed for all parameters of the analyzed breads, both the control and apple incorporated bread, for all storage times. The use of this statistical tool allows to understand how each component (analysis performed) correlates to the different storage times and flour (wheat, corn and apple), through visual perception. For the microbiological analysis of flours, differences among flour types for each microorganism were assessed separately using one-way analysis of variance (ANOVA). When significant differences were detected, mean comparisons were performed using Tukey's multiple comparison test. Homoscedasticity was evaluated using Levene's test.

3. Results and discussion

3.1. Nutritional and chemical characterization of apple flour

The results of the nutritional composition of apple flour obtained from Fuji apple residues are shown in Table 1.

After drying, a large reduction in moisture content was achieved. According to the food composition table of the National Institute of Health Dr. Ricardo Jorge, wheat flour (type 55) (flour traditionally used by the bakery and pastry industry) has a moisture content of 13.40 %. Therefore, the apple flour obtained in this study has a lower moisture content than wheat flour (type 55). In this way, since the prepared apple flour presented a lower moisture content value than the wheat flour (type 55) which is an essential parameter also in the product preservation.

Table 1 reveals that apple flour has a fat content of 0.061±0.003 g/100g. A study by Serra et al. [18] reported a higher value in Fuji apple flour with a value of 0.54 g/100g). In turn, a study developed by Coelho and Wosiacki [19] showed a lipid value of 1.31 g/100g in apple pomace flour.

Considering Table 1, it was found that the ash content in apple flour is 1.5±0.1 g/100g. According to the food composition table of the National Institute of Health Dr. Ricardo Jorge, 100 g of dried apple contains approximately 1.4 g of ash, and therefore, the result obtained is relatively close to what is mentioned in the literature. This value agrees with a study carried out previously by Coelho and Wosiacki [19] in which he described an ash content of 1.4 % in apple pomace. The same ash value in apple flour was recorded by Zlatanovic et al [20]. In turn, Serra et al. [18] in a comparative study of 5 varieties of apple pomace flour described ash values ranging between 1.2 and 1.9 (g/100g). According to the National Institute of Health food composition table Dr. Ricardo Jorge, wheat flour (type 55) contains an ash content of 0.50 g per 100 g

Table 1
Nutritional and chemical composition of apple flour.

Nutritional Composition	
Moisture (%)	6.5±0.1
Fat (g/100g)	0.061±0.003
Ash (g/100g)	1.5±0.1
Proteins (g/100g)	3.959±0.002
Carbohydrates (g/100g)	97.5±0.1
Energy (Kcal/100g)	394.5±0.2
Free sugars	**Amount (g/100g)**
Fructose	8.9±0.5
Glucose	6.9±0.4
Sucrose	1.18±0.04
Total	17±1
Organic acid	**Amount (mg/g)**
Oxalic acid	2.7±0.2
Quinic acid	5.1±0.7
Malic acid	20±1
Citric acid	4±1
Fumaric acid	0.10±0.01
Total	32±3
Compound name	**Amount (%)**
C10:0	0.35±0.01
C11:0	0.64±0.03
C12:0	0.51±0.03
C15:0	0.32±0.02
C15:1	0.95±0.04
C16:0	19±1
C16:1	0.77±0.02
C17:0	0.45±0.02
C18:0	5.3±0.2
C18:1n9	38.8±0.1
C18:2n6	27±1
C18:3n3	1.64±0.03
C20:1	3.9±0.1
C22:0	0.63±0.03
SFA	27±1
MUFA	44.5±0.1
PUFA	28±1

Results are presented as mean ± standard deviation. Caproic acid (C6:0); Caprylic acid (C8:0); Capric acid (C10:0); Undecylic acid (C11:0); Lauric Acid (C12:0); Pentadecylic acid (C15:0); Palmitic acid (C16:0); Palmitoleic acid (C16:1); Margaric Acid (C17:0); Stearic acid (C18:0); Oleic acid (C18:1n9); Linoleic acid (C18:2n6); Linolenic acid (C18:3n3); Eicosenoic acid (C20:1); Behenic Acid (C22:0); SFA-Saturated Fatty Acids; MUFA- Monounsaturated Fatty Acids; PUFA-Polyunsaturated fatty acids.

of product. From this analysis, it can be concluded that apple flour has a higher content than wheat flour.

Regarding the protein content (Table 1), the apple flour analyzed in this study presented 3.959±0.002 g/100g of protein. These values appear in agreement with some studies found in the literature that nutritionally evaluate apple pomace flours. Zlatanovic et al [20] present a protein content that varies between 3.2 and 5.8 g/100g in apple pomace flours. Similarly, Coelho and Wosiacki [19] described a protein content of 3.35 g/100g in apple pomace flour. In the study developed by Sato [21], apple pomace from eleven cultivars were compared and the protein content values ranged from 3.75 to 4.65 g 100/g.

Fructose, glucose and sucrose were the free sugars found in the apple flour obtained in this study (Table 1). According to the values the sugar with the highest content in the flour was fructose in an amount of 17±1 g/100g.

The sugar content in apples can vary depending on different conditions, which may explain the differences found in the different studies presented in the literature. Apples exposed to sunlight contain a higher percentage of sugar compared to fruits in the shade. Likewise, sugar content can also be affected by harvest periods [22]. In the study developed by Coelho and Wosiacki [19], apple pomace flour had higher total sugar content (35.11 g/100g), with 22.31 g/100g of glucose being highlighted. When comparing apple pomace flours, Zlatanovic et al [20] showed glucose values that ranged between 9 and 18 (g/100g), fructose between 21 and 35 (g/100g) and sucrose between 5.6 and 9.7 (g/100g). /100g).

Malic acid (Table 1) emerged as the major organic acid in apple flour. This organic acid emerged predominating along with citric acid in Fuji apples [23]. In a comparative study between different apple species, malic acid content ranged between 1.72 and 2729 mg/g [24]. Likewise, Pires, et al [13] highlighted the majority presence of malic acid followed by quinic and oxalic acids (1.36, 0.15, 0.101 mg/100 g, respectively) in "Bravo de Esmolfe" apples.

The individual composition of fatty acids as well as the content of saturated, monounsaturated and polyunsaturated fatty acids is shown in Table 1. The results show an outstanding amount of unsaturated fatty acids, due to the high contribution of oleic acid.

The apple flour had fourteen fatty acids in its composition, highlighting the oleic (C18:1n9), linoleic (C18:2n6) and palmitic (C16:0) acids as the major fatty acids in a percentage of 38.8 ± 0.1 %, 27 ± 1 % and 19 ± 1 %, respectively. This predominance is responsible for the prevalence of unsaturated fatty acids in relation to saturated fatty acids in this flour. These results agree with the data presented in a comparative study of eight apple cultivars [23] where linoleic acid appears as the majority. In a study developed by Pires et al. [13] analysing the acid composition of "Bravo de Esmolfe" apples, palmitic and linoleic acids predominated with 28.94 % and 15.8 %, respectively.

3.2. *Phenolic composition of apple flour*

The chromatographic responses (retention time, wavelengths of maximum absorption, and mass spectral data) and tentative identification of the phenolic compounds present in the apple flour extract are described in Table 2. The corresponding quantification is also present in Table 2. Seven phenolic compounds were found, divided into three different families of compounds: three phenolic acids (caffeic acid and caffeoylquinic acid derivatives), two flavan-3-ols ((epi) catechin derivatives), and two dihydrochalcone (phloretin O-glycosides).

The phenolic profile of apple fruits has been extensively studied by other authors [25–28,13,29,30] However, as far as the authors knowledge, this is the first study of the phenolic profile of apple flour. Overall, the profile found is coherent with the previously published ones, apart from the fact that the number of phenolic compounds is very low. This could be due to processing steps regarding the flour preparation that can lead to the degradation of the compounds.

Regarding their amounts, peak 1, tentatively identified as caffeic acid hexoside, was the major peak found, with 0.71±0.01 mg/g extract. Consequently, the total phenolic acids group presented the major concentration, 1.217±0.001 mg/g extract, which is in coherence with the results presented by Arraibi et al. [25] in hydroethanolic and aqueous extracts of Spanish and Belgian apple pomace. In turn, the flavan-3-ol group was the second main group found, which is consistent with the results presented by Arraibi et al. [25]. Finally, the dihydrochalcone group, represented by phloretin O-glycosides, despite being present, was found in trace amounts in the studied sample. Again, this can be due to the processing steps needed for flour preparation. Nonetheless, the presence of this type of compounds is always very important due to pharmacological properties (such as antioxidant, anti-inflammatory, antibacterial and antiviral properties) of these compounds [31], which add value to the matrix sample in which they are found.

The amount of total phenolic content present in apple flour was 1.4119±0.0004 mg/g extract, with the most representative compounds being phenolic acids. Ćetković et al. [32] in a comparative study of different apple pomace shows a total phenolic content that varies from 0.692 and 1.474 (mg/g). Also, Feng et al. [33] showed in their study in apple pulp from Malus domestica Borkh a value of total phenolic compounds ranging between 0.096 and 0.416 (mg/g). The phenolic composition identified in the apple flour in this study is lower than that presented in the study by Pires et al. [13], which highlighted the presence of 15 phenolic compounds in 'Bravo de Esmolfe' apples. Despite

Table 2
Retention time (Tr), wavelengths of maximum absorption in the UV-Vis region (λ_{max}), attempt to identify and quantify phenolic compounds in apple flour extract (mean ± SD).

Peak	Rt (min)	λmax (nm)	[M-H]⁻ (*m/z*)	MS² (*m/z*)	Tentative identification	mg/g extract
1	4,87	364	341	179 (100),161 (34),135(5)	Caffeic acid hexoside	0.71 ±0.01
2	6,66	322	353	191(12),179 (1),173 (100),161 (1),135(2)	4-*O*-caffeoylquinic acid	0.29 ±0.01
3	7.24	327	353	191 (100),179 (6),173 (2),161 (1),135(1)	5-*O*-caffeoylquinic acid	0.212 ±0.004
4	8,58	281	577	451(24),425 (100),407 (21),289(12)	B-type (epi) catechin dimer	0.118 ±0.003
5	11.39	280	865	739(74),713 (44),695 (100),577 (64),575 (37),425 (10),407 (9),289 (8),287(7)	B-type (epi) catechin trimer	0.077 ±0.002
6	18,08	285	567	273(100)	Phloretin-2-*O*-xyloglucoside	tr
7	22.89	285	435	273(100)	Phlorizin (phloretin-2-*O*-glucoside)	tr
					TPA	1.217 ±0.001
					TF3O	0.195 ±0.001
					TdhC	tr
					TPC	1.4119 ±0.0004

TFA - Total phenolic acids; TF3O – Total Flavan-3-ol; TdhC - Total dihydrochalcone; TPC - total phenolic compounds; tr - traces. Calibration curves: caffeic acid (y = 388345x + 406369, R^2 = 0.9939, peak 1), chlorogenic acid (y = 168823x - 161172, R^2 = 0.9945, peak 2 and 3), catechin (y = 84950x - 23200, R^2 = 0.9968, peak 4 and 5) and isoliquiritigenin (y = 42820x + 184902, R^2 = 0.9964, peak 6 and 7)

this difference, in both studies 5-O-caffeoylquinic acid was the main compound.

3.3. Bioactive properties of apple flour

The antioxidant activity was evaluated through four in vitro assays: DPPH radical scavenging activity, reducing power, thiobarbituric acid reactive substances (TBARS) inhibition, and oxidative hemolysis inhibition (OxHLIA). The results in Table 3 are displayed as EC_{50}/IC_{50} values (representing the sample concentration that provides 50 % antioxidant activity). Considering that the lower the EC_{50}/IC_{50} value, the greater the antioxidant activity [34], it appears that apple flour showed greater antioxidant activity than trolox in the reducing power test and similar values for the DPPH and TBARS assays. The presence of phenolic compounds in numerous vegetables, fruits, and natural plants is thought to be responsible for their antioxidant properties. Polyphenols' radical-scavenging capacity is determined by their molecular structure and the availability of phenolic hydrogens [35].

In the literature, several studies have reported the excellent antioxidant activity of several species of apples and by-products. Hamauzu et al. [36] and Luo et al. [37] have reported lower EC_{50} values when using the DPPH scavenging activity methodology in Malus domestica (EC_{50}= 8.4 mg/100 mL and 0.26 mg/mL, respectively).

The antibacterial activity of hydroethanolic extracts obtained from apple flour was tested against a panel of eight food bacteria (Enterobacter cloacae, Escherichia coli, Pseudomonas aeruginosa, Salmonella enterica, Yersinia enterocolitica, Bacillus cereus, Listeria monocytogenes e Staphylococcus aureus) and eight clinical bacterial strains (Escherichia coli, Klebsiella pneumoniae, Morganella morganii, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis, Listeria monocytogenes e MRSA). Table 3 presents the results obtained for each extract in minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC). The food bacteria most sensitive to apple flour were Yersinia enterocolitica and Staphylococcus aureus with lower MIC values (0.6 mg/mL). In turn, the most sensitive clinical bacteria were Escherichia coli and MRSA with MICs of 1.25 mg/mL. However, apple flour was not bactericidal for any of the bacteria tested up to the maximum concentration tested (10 mg/mL).

The antifungal activity of the hydroethanolic extracts obtained from apple flour was tested against a panel of two fungi (*Aspergillus brasiliensis* and *Aspergillus fumigatus),* and the results expressed in minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (MFC), being presented in Table 3. The apple flour showed the ability to inhibit the growth of both tested fungi (MIC and MFC of 10 mg/mL).

Several studies have reported promising antimicrobial activity of different apple varieties and by-products, namely apple pomace. Luo et al. [37] and Pires et al. [13] highlight the excellent antimicrobial activity of apples of the Fuji and "Bravo de Esmolfe" varieties, respectively. Fuji apples had lower MIC values than "Bravo de Esmolfe" and our apple flour, since the strains used in the present study and in the study by Pires et al. [13] were obtained from clinical isolates with multi-resistant profiles.

The activity of apple flour on the growth of the four human tumor cell lines (MCF-7, NCI-H460, AGS and CaCo) and for the primary culture of non-tumor cells (VERO) is presented in Table 3 and are expressed in values of the concentration of extract responsible for inhibiting cell proliferation by 50 % (GI_{50}), values in μg/mL. The results showed that apple flour only showed positive results (GI_{50} <400 μg/mL) demonstrating the ability to inhibit the growth of the NCI-H460 (lung cancer) cell line. The results also allow to verify that the apple flour extract did not express cytotoxicity against non-tumor cell culture, VERO (GI_{50} > 400 μg/mL).

3.4. Alternative breads

After characterizing the apple flour, four types of bread were made: Traditional wheat bread, wheat bread with apple flour, traditional corn bread, corn bread with apple flour (Table 4).

Considering the results and discussion, the following tables are presented with two levels, the top referring to the case of the corn bread and the bottom to the wheat bread. As detailed in the statistics section of the materials and methods, the tables are represented in such a way that the values of the incorporation (I) are represented as means of each storage time (ST) including both incorporation times. This type of representation allows the aforementioned independent analysis of each factor individually and thus, the standard deviations should not be regarded as an accuracy of an individual analysis as it includes the variation of the non-fixed factor (I or ST). If a significant interaction among these two factors is detected (I × ST $p<0.05$), no multiple comparisons can be carried out, meaning that both factors, I and ST contributed to the changes in the breads, hindering concrete conclusions, although general tendencies can sometimes be concluded from the Estimated Marginal Means (EMM) plots. Inversely, if this value is higher than 0.05, each factor was classified individually using either Tukey's or Tamhane T2 tests depending on the homoscedasticity of the distribution, for ST, and a Student's T test for I.

Table 3
Antioxidant, antimicrobial and antiproliferative activity of the hydroethanolic extract of apple flour (mean ± SD).

Antioxidant activity								
	DPPH scavenging activity (EC_{50}, mg/mL)		**Reducing power (EC_{50}, mg/mL)**	**TBARS (EC_{50}, mg/mL)**		**OxHLIA (IC_{50}, µg/mL)**		
						Δ*t* 60 min		Δ*t* 120 min
Apple flour	4.7±0.2		2.44±0.04	0.64±0.02		232±8		566±10
Trolox	4.2±0.1		4.1±0.1	0.54±0.8		21.8±0.3		44±1
Antimicrobial activity								
	Apple flour		**Positive Control Streptomicin 1mg/mL**	**Methicilin 1mg/mL**			**Ampicillin 20mg/mL**	
Antibacterial Activity	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Food Bacteria								
Gram-negative bacteria								
Enterobacter Cloacae	>10	>10	0.007	0.007	n.t.	n.t	0.15	0.15
Escherichia coli	10	>10	0.01	0.01	n.t.	n.t.	0.15	0.15
Pseudomonas aeruginosa	>10	>10	0.06	0.06	n.t.	n.t.	0.63	0.63
Salmonella enterica	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Yersinia enterocolitica	0.6	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Gram-positive bacteria								
Bacillus cereus	>10	>10	0.007	0.007	n.t.	n.t.	n.t.	n.t.
Listeria monocytogenes	2.5	>10	0.007	0.007	n.t.	n.t.	0.15	0.15
Staphylococcus aureus	0.6	>10	0.007	0.007	0.007	0.007	0.15	0.15
Clinical bacteria								
	Apple Flour		**Positive control Ampicillin (20mg/mL)**	**Imipenem (1mg/mL)**			**Vancomycin (1mg/mL)**	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Gram-negative bacteria								
Escherichia coli	1.25	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
Klebsiella pneumoniae	>10	>10	10	>10	<0.0078	<0.0078	n.t.	n.t.
Morganella morganii	2.5	>10	>10	>10	<0.0078	<0.0078	n.t.	n.t.
Proteus mirabilis	10	>10	<015	<0.15	<0.0078	<0.0078	n.t.	n.t.
Pseudomonas aeruginosa	>10	>10	>10	>10	0.5	1	n.t.	n.t.
Gram-positive bacteria								
Enterococcus faecalis	2.5	>10	<0.15	<0.15	n.t.	n.t.	<0.0078	<0.0078
Listeria monocytogenes	>10	>10	<0.15	<0.15	<0.0078	<0.0078	n.t.	n.t.
MRSA	1.25	>10	<0.15	<0.15	n.t.	n.t.	0.25	0.5
			Ketaconazole 1mg/mL					
Antifungal Activity	MIC	MFC		MIC		MFC		
Aspergillus brasiliensis	10	10		0.06		0.125		
Aspergillus fumigatus	10	10		0.5		1		
Antiproliferative activity								
				Apple flour (µM)			**Ellipticine (µM)**	
Tumoral cell lines		AGS		>400			1.23±0.03	
		CaCo-2		>400			1.21±0.02	
		MCF_7		>400			1.02 ± 0.02	
		NCI-H460		53±5			1.01±0.01	
Non-tumoral culture		VERO		>400			0.6±0.1	

MIC: Minimum inhibitory concentration; MBC: Minimum bactericidal concentration; MFC: Minimum fungicidal concentration. GI_{50} values correspond to the concentration that causes 50 % inhibition of cell proliferation; AGS - human gastric adenocarcinoma; CaCo-2 - human colon adenocarcinoma MCF-7 - human breast adenocarcinoma; NCI-H460 - human lung carcinoma.n.t. – non detecterd.

3.5. Principal component analysis

Fig. 1 shows the biplot of the PCA. In terms of the PCA, it was performed using SPSS, using the principal component method, based on eigenvalues greater than 5 (according to the scree plot), applying a direct oblimin rotation with a Kaiser normalization. In terms of the variance, the first two dimensions accounted for 71.3 % (dimension 1 accounted for 51.2 % and dimension 2 for 20.0 %). Fig. 1 shows the biplot, in which the objects are well clustered while the loadings are scattered throughout. When the objects and loadings are close, a high correlation is implicit. Thus, in terms of clustering, the wheat and apple flours were clustered on the right side, including all three storage times, 0, 3 and 5 days (highlighted in orange), while the wheat flour was clustered near the origin of the chart. Here, two clusters were formed, namely T0 and T3 wheat flour was clustered together (black highlight) and wheat flour stored for 5 days was clustered above, highlighted in red. In terms of the corn and apple flours, they were clustered closer to each other, and thus show lower differences among each other. Specifically, the flour with corn and apple was clustered together (highlighted in purple) at the top of the chart, while the corn flour at T0 and T5 were clustered near the middle section (highlighted in blue), while corn flour at three days was clustered between the two other clusters (highlighted in green). In terms of correlation between the objects and loadings, it is clear that wheat (red and black cluster) highly correlates with SFA and C16:0 (not shown in the Fig.), as well as b^* (yellow-blueness) of both the top and bottom of the breads, as well as two dimensions of texture, springiness and cohesiveness. Wheat and apple flour (orange cluster) highly correlate with MUFA and a^* (red-greeness) of the middle and inferior part of the bread, as well as carbohydrates and the Kcal. It is also correlated, although to a lower extent to fat, ash and total soluble sugars. Corn and apple flours (purple cluster) highly correlate to the three texture dimensions of hardness, chewiness and gumminess, as well as fructose, meaning these values define this cluster. Corn flour after three days somewhat correlates with these values, but also with glucose and b^* (red-greeness) of the middle of the breads. Finally, corn flour at T0 and T5 (blue cluster) are highly

Table 4
Different samples of bread prepared (Author authorship).

	Traditional wheat bread	Wheat bread with apple flour	Traditional corn bread	Corn bread with apple flour
External appearance				
Interior appearance				

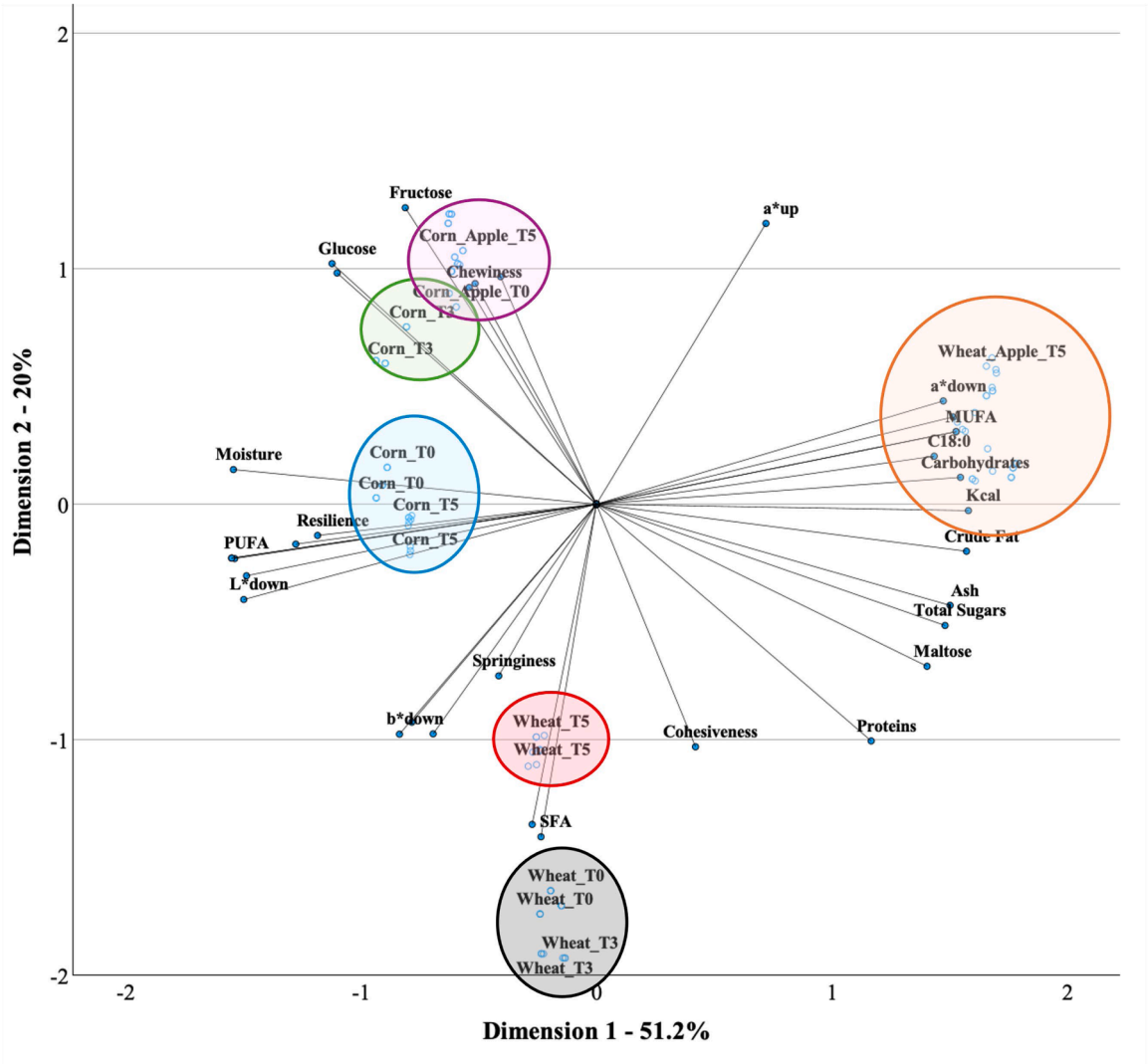

Fig. 1. Biplot of objects (flours and storage times) and component loadings (assays).

correlated with resilience (texture dimension), C18:3, PUFA and *L** (lightness).

In terms of the discriminating power of the different analyses, the principal PCA revealed that Dimension 1 (51.2 % of the explained variance) was primarily driven by compositional parameters such as proteins, crude fat, total sugars, carbohydrates, energy (kcal), and

specific fatty acids (C18:0 and MUFA), which strongly discriminated the wheat and apple flours positioned on the positive side of the axis. Conversely, moisture, PUFA, resilience, and color attributes (L^* and b^*) contributed to the negative side of Dimension 1, differentiating the corn-based samples, which exhibited lighter composition and distinct textural characteristics. Dimension 2 (20 % of the variance) was mainly explained by simple sugars (glucose and fructose) and a* values, which were associated with corn and apple formulations and reflect the contribution of apple-derived sugars and color. On the negative side of Dimension 2, SFA and additional color parameters were linked to the wheat formulations without apple. Overall, the PCA demonstrates a clear separation of samples driven primarily by compositional richness, fatty acid profile, and sugar-related attributes. The findings in this work were similar to the ones of Valková et al. [38], in which the supplementation of apple pomace powder in wheat bread resulted in an increase of total carbohydrates, ash and antioxidant polyphenols. Also, Liang et al., [39], who supplemented biscuits with apple pomace saw a change in the texture profile.

3.6. Microbial load

3.6.1. Individual flours

Fig. 2 shows the microbial load of the different flours. Wheat flour is the product with the greatest diversity of microorganisms, with total aerobic mesophiles (4.2 ± 0.2 $\log_{10}$ CFU/g), coliforms (4.1 ± 0.2 $\log_{10}$ CFU/g), yeasts (2.1 ± 0.2 $\log_{10}$ CFU/g), and molds (2.73 ± 0.05 $\log_{10}$ CFU/g), detected in concentrations above the minimum determination limits. This was followed by apple flour, in which total aerobic mesophiles (2.5 ± 0.2 $\log_{10}$ CFU/g) and molds (2.9 ± 0.2 $\log_{10}$ CFU/g), were detected. Only the total aerobic mesophiles (3.3 ± 0.2 $\log_{10}$ CFU/g), grew in corn flour. Statistical analysis confirmed that the microbial loads differed significantly among flours depending on the microorganism analyzed ($p < 0.05$). Total aerobic mesophiles differed significantly among all flours. Coliforms and yeasts were detected exclusively in wheat flour, which presented significantly higher counts compared to corn and apple flours. Regarding molds, wheat and apple flours showed similar microbial loads, both significantly higher than those observed in corn flour. *Bacillus cereus* was not detected in any of the flours analyzed. The development of these microorganisms is influenced by different factors, such as the availability of nutrients, the level of water activity, moisture, temperature and their remarkable resistance to acidic pH values [40]. In this context, apple flour has a relevant concentration of organic acids (32 ± 3 mg/g), which contributes to a pH suitable for mold growth. In addition, the presence of nutrients such as proteins and sugars favor the development of these microorganisms. These results are consistent with those of Ito et al. (20217) in the study on the effect of radiation on apple pomace flour, in which the presence of mold colonies was observed in the non-irradiated sample. However, the authors did not present the value of their quantification but claimed that it was below the legally permissible limit (<10^2 CFU/g). The results are not directly comparable as they are the same matrix, but different samples and the results were expressed in different units. The presence of microorganisms in flours used in the production of processed foods is generally not of particular concern because these products are subjected to high heat treatments or preservation processes such as pasteurization and sterilization that eliminate or significantly reduce the microbial load.

3.6.2. Breads

Table 5 shows the microbial load of the different breads. No growth of *B. cereus* or yeast was detected in either type of bread. Regarding the growth of total aerobic mesophiles, wheat flour breads (control and with incorporation of apple flour) were found to have a greater number of colonies forming units, which was expected as wheat flour (Fig. 2) also had higher levels of total aerobic mesophylls compared to corn flour. It was also found that in wheat bread there was a significant decrease in the growth of total aerobic mesophylls from T0 to T3 and a significant increase from T3 to T5, while the incorporation with apple at 10 % was able to decrease the counting in these microorganisms.

In each row, for the storage time different letters mean significant statistical differences, both with an overall significance value of 0.05. The presented standard deviations were calculated from results obtained under different operational conditions. Therefore, these values should not be regarded as a measure of precision, rather as the range of the recorded values.

Still, higher percentages of apple flour should increase this inhibition of growth. In corn bread, significant interaction was found between the factors studied, but some trends can be observed in the plots of estimated marginal means (EMM) (Fig. 3).

At T0 both samples showed contamination with these microbes, although at T3 they reduced to 0 for the apple incorporated bread, and finally no contamination was found in both breads at T5. This could be due to the lack of favorable environment for these organisms in bread. As far as the growth of coliforms is concerned, no contamination by these microorganisms was detected in the corn bread, as expected. In contrast, the control sample of the wheat bread showed coliforms, while

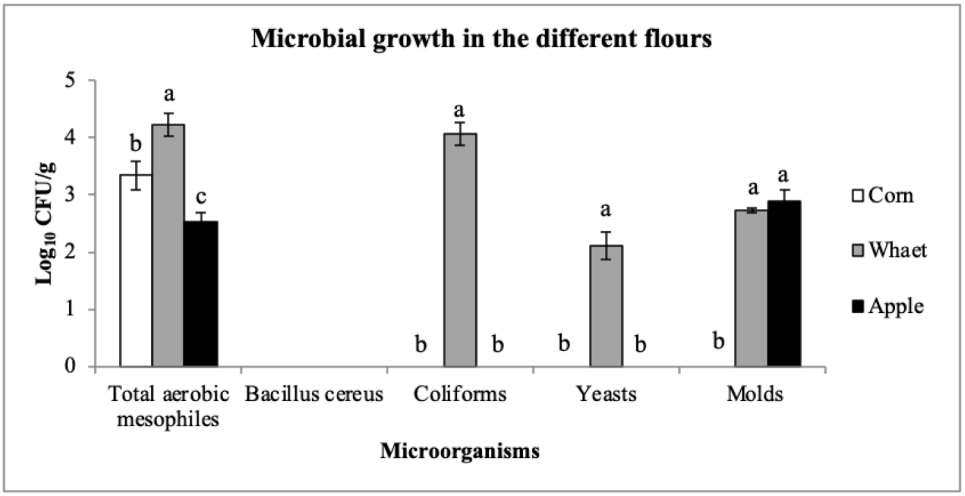

Fig. 2. Microbial growth in different flours expressed as Log_{10} CFU/g. Values are presented as mean ± standard deviation ($n = 3$). For each microorganism, differences among flours were evaluated separately using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Different letters indicate statistically significant differences among flours within the same microorganism ($p < 0.05$). *Bacillus cereus* was not detected in any of the samples.

Table 5
Microbial load of the two bread types, expressed in $\log_{10}$ CFU/g.

Corn bread						
		Total aerobic mesophiles	***Bacillus cereus***	**Coliforms**	**Yeasts**	**Molds**
Incorporation (I)	Control	1±1	-	-	-	1±1
	Apple	0.7±1	-	-	-	1±1
p-value (n=9)	Student T test	<0.001	-	-	-	0.422
Storage Time (ST)	T0	2.2±0.2	-	-	-	0±0
	T3	1±1	-	-	-	0±0
	T5	0±0	-	-	-	2.6±0.1
p-value (n=18)	Tukey's HSD test	<0.001	-	-	-	<0.001
I × ST (n=27)	*p*-value	<0.001	-	-	-	0.52
Wheat bread						
Incorporation (I)	Control	2±1	-	0.4±0.7	-	1±1
	Apple	2.4±0.3	-	0±0	-	0±0
p-value (n=9)	Student T test	0.544	-	<0.001	-	<0.001
Storage Time (ST)	T0	2.3 ± 0.3^{b}	-	1±1	-	0±0
	T3	2.0 ± 0.1^{a}	-	0±0	-	0±0
	T5	2.8 ± 0.4^{b}	-	0±0	-	1±1
p-value (n=18)	Tukey's HSD test	0.002	-	<0.001	-	<0.001
I × ST (n=27)	*p*-value	0.065	-	<0.001	-	<0.001

the wheat bread with 10 % apple flour was not contaminated with these microorganisms. These results indicate that the antioxidant compounds contained in the apple flour may have an inhibitory effect on the growth of coliforms.

A significant interaction was found for both breads in terms of mould growth, thus, some tendencies were extracted from the EMM plots (Fig. 3 (A)).

Regarding molds growth in corn bread, no significant interaction was found between the factors studied, verifying that storage time is the factor that significantly influences fungal growth in this type of bread. In wheat bread, significant interaction was found between the factors, though certain patterns can be observed in EMM plots (Fig. 3 (A)). Molds in wheat bread samples were only detected in control samples at time T5, suggesting that the antioxidant compounds present in apple flour may have inhibited the development of these contaminants.

As far as could be ascertained, there are no published studies investigating microbial growth in corn breads with added apple flour or in wheat breads with added apple flour. This work therefore represents a pioneering contribution to this field of research.

4. Conclusions

Reducing food waste and losses is part of the drivers of the circular economy, which can contribute to fighting pollution and boosting the economy. In this line, it is considered that the process of converting fruit by-products into flour is an economical and viable method for valorization and incorporation into a variety of food products and is a potential source of natural food ingredients. The bakery and pastry industry has been looking for new health-enhancing alternative ingredients as an alternative to traditional ingredients that can recreate new alternative products that can be offered to consumers with some dietary restrictions.

In this sense, this study demonstrated that the valorization of apple by-products from the agri-food industry, through their conversion into flour, constitutes a viable and sustainable strategy for obtaining a value-added food ingredient with application in baking. Apple flour revealed a relevant nutritional and bioactive composition, standing out for its content of phenolic compounds and the observed antioxidant and antimicrobial activity.

The incorporation of apple flour into wheat and corn breads proved to be technologically adequate when applied at a percentage of 10 %, previously defined based on preliminary tests, in order to guarantee the maintenance of the structural, visual and sensory characteristics traditionally appreciated by consumers. At this concentration, it was possible to develop an enriched product without compromising the texture, color or nutritional composition of the bread, simultaneously guaranteeing an improvement in its functional profile and a reduction in the microbial load.

Thus, the results obtained confirm that the objective of the study was achieved, highlighting the potential of apple flour as an alternative functional ingredient in the baking industry. Looking to the future, it will be relevant to explore different percentages of apple flour incorporation, as well as to further evaluate the rheological properties of the dough and conduct sensory validation with consumers, to assess the product's acceptability and support its eventual application and commercial exploitation.

Funding

This work was arried out under Agenda VIIAFOOD - Plataforma de Valorização, Industrialização e Inovação Comercial para o Agro-Alimentar (n.° C644929456-00000040), a project supported under the PRR (www.recuperarportugal.gov.pt), and financed by the European Union/Next Generation EU.

CRediT authorship contribution statement

Chaimaa Aichouche: Writing – original draft, Formal analysis. **Eliana Pereira:** Writing – review & editing, Visualization, Validation, Methodology, Formal analysis, Data curation. **Márcio Carocho:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation. **Maria Inês Dias:** Writing – review & editing, Validation, Software, Methodology, Formal analysis, Data curation. **Ricardo C. Calhelha:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Sandrina Heleno:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation. **Filipa A. Fernandes:** Writing – original draft, Methodology, Formal analysis, Data curation. **Tânia C.S.P. Pires:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation. **Elizabete Ferreira:** Validation, Methodology, Investigation. **Cristina Caleja:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lillian Barros:** Writing – review & editing, Validation, Supervision, Resources, Project administration, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

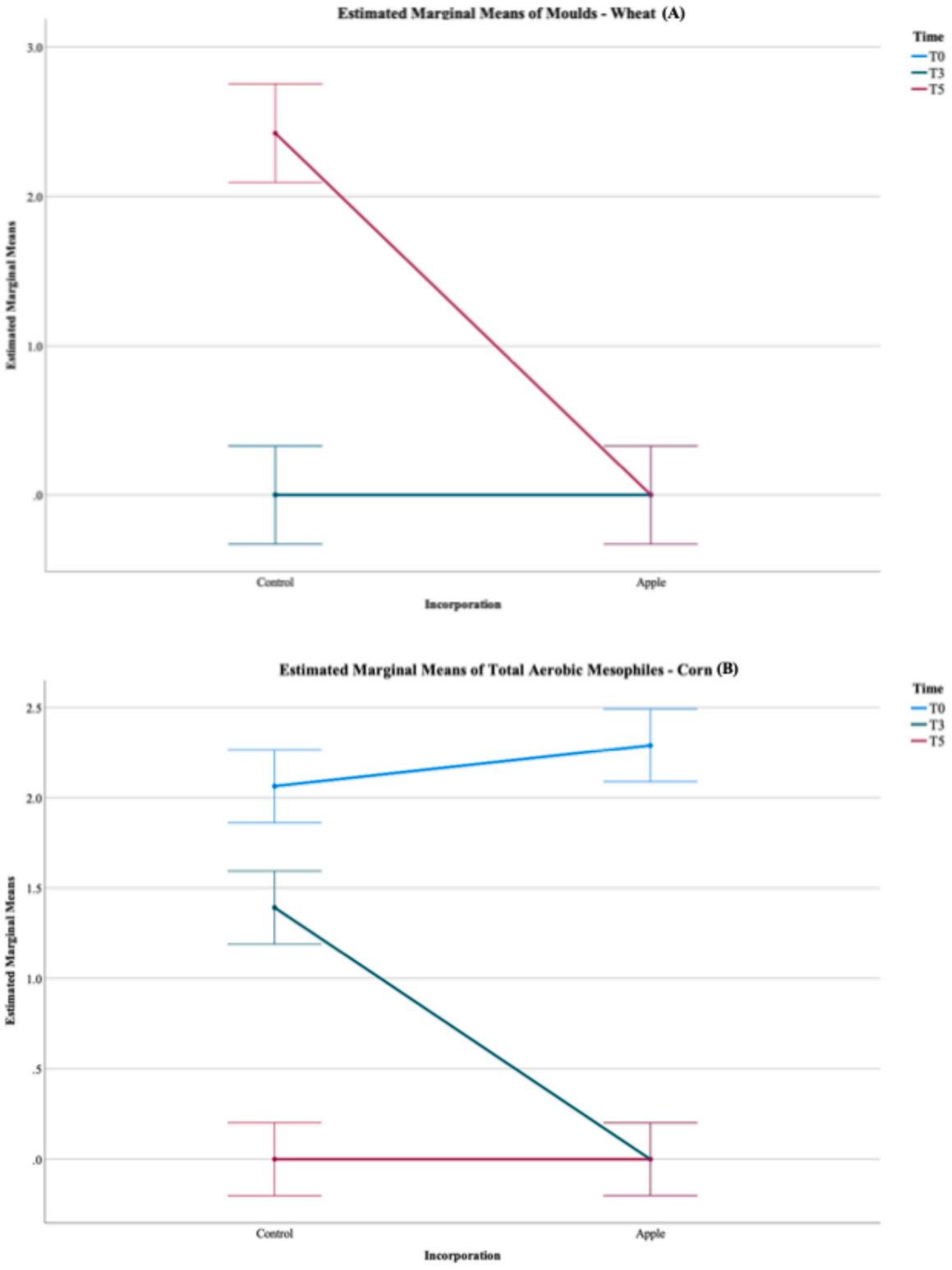

Fig. 3. EMM plots of moulds (A) in wheat sample, and total aerobic mesophiles (B) in corn sample.

the work reported in this paper.

Acknowledgements

This work was supported by national funds through FCT/MCTES (PIDDAC): CIMO, UID/00690/2025 (10.54499/UID/00690/2025) and UID/PRR/00690/2025 (10.54499/UID/PRR/00690/2025); and SusTEC, LA/P/0007/2020 (https://doi.org/10.54499/LA/P/0007/2020). C. Caleja is thankful for her contract through the project VIIA-FOOD (PRR | Aviso n.°02/C05 -i01/2022; n.° de agenda: C644929456 -00000040). The authors are grateful to FEDER Cooperación Interreg VI A Espanha – Portugal (POCTEP) 2021-2027 for financial support through the project TRANSCoLAB PLUS 0112_TRANSCOLAB_PLUS_2_P. The authors also thank the National funding by FCT- Foundation for

Science and Technology, through the institutional scientific employment program-contract with L. Barros (CEEC-INST, https://doi.org/10.54499/CEECINST/00107/2021/CP2793/CT0002).

References

[1] D.B. Kovačević, D. Brdar, P. Fabečić, F.J. Barba, J.M. Lorenzo, P. Putnik, Strategies to achieve a healthy and balanced diet: fruits and vegetables as a natural source of bioactive compounds, Agri-Food Ind. Strateg. Healthy Diets Sustain. 2 (2020) 51–88.

[2] R.H. Liu, Dietary bioactive compounds and their health implications, J. Food Sci. 78 (2013) A18–A25.

[3] P.C.C. Lima, B.S. Sousa, A.T. Santini, D.C. Olivera, Aproveitamento agroindustrial de resíduos provenientes do abacaxi 'pérola' minimamente processado, 2, Universidade Federal de Viçosa, UFV. Instituto Federal de Educação, Ciência e Tecnologia do Sul de Minas Gerais. Holos, 2017, pp. 122–136.

[4] Y. Jaouhari, F. Travaglia, L. Giovannelli, A. Picco, E. Oz, F. Oz, M. Bordiga, From industrial food waste to bioactive ingredients: A review on the sustainable management and transformation of plant-derived food waste, Foods 12 (11) (2023) 2183, https://doi.org/10.3390/foods12112183.

[5] M. Maric, A.N. Grassino, Z. Zhu, F.J. Barba, M. Brncic, S. Rimac, An overview of the traditional and innovative approaches for pectin extraction from plant food wastes and by-products: ultrasound-, microwaves-, and enzyme-assisted extraction, Trends Food Sci. Technol. 76 (2018) 28–37.

[6] Codex Standard for Apples. (2010). Codex Stan 299-2010. Disponível em: http://www.codexalimentarius.net. In).

[7] C. Paganini, A. Nogueira, F. Denardi, G. Wosiacki, Análise da aptidão industrial de seis cultivares de maçãs, considerando suas avaliações físico-químicas (Dados da Safra 2001/2002), Ciênc. Agrotecnologia 28 (2004) 1336–1343.

[8] M. Zhang, G. Zhang, Y. You, C. Yang, P. Li, F. Ma, Effects of relative air humidity on the phenolic compounds contents and coloration in the 'Fuji' apple (*Malus domestica* Borkh.) peel, Sci. Hortic. 201 (2016) 18–23.

[9] AOAC (Association of Official Agricultural Chemists) (2016). In W. Horwitz, & G. Latimer (Eds.). The official methods of analysis of AOAC international (20th ed.). Gaithersburg, MD, USA: AOAC International.

[10] L. Barros, C. Pereira, I.C.F.R. Ferreira, Optimized analysis of organic acids in edible mushrooms from Portugal by Ultra fast liquid chromatography and photodiode array detection, Food Anal. Methods 6 (2013) 309–316.

[11] L. Lockowandt, J. Pinela, C.L. Roriz, C. Pereira, R.M.V. Abreu, R.C. Calhelha, M. J. Alves, L. Barros, M. Bredol, I.C.F.R Ferreira, Chemical features and bioactivities of cornflower (*Centaurea cyanus* L.) capitula: the blue flowers and the unexplored non-edible part, Ind. Crops Prod. 128 (2019) 496–503.

[12] R.M.V. Abreu, I.C.F.R. Ferreira, R.C. Calhelha, R.T. Lima, M.H. Vasconcelos, F. Adega, R. Chaves, M.J.R.P. Queiroz, Anti-hepatocellular carcinoma activity using human HepG2 cells and hepatotoxicity of 6-substituted methyl 3-amino-thieno [3,2-b] pyridine-2-carboxylate derivatives: *In vitro* evaluation, cell cycle analysis and QSAR studies, Eur. J. Med. Chem. 46 (2011) 5800–5806.

[13] T. Pires, M.I. Dias, L. Barros, M.J. Alves, M. Oliveira, C. Santos-Buelga, I. Ferreira, Antioxidant and antimicrobial properties of dried Portuguese apple variety (Malus domestica Borkh. cv Bravo de Esmolfe), Food Chem. 240 (2018) 701–706, https://doi.org/10.1016/j.foodchem.2017.08.010.

[14] S.A. Heleno, I.C.F.R. Ferreira, A.P. Esteves, A. Ćirić, J. Glamočlija, A. Martins, M. Soković, M.J.R.P. Queiroz, Antimicrobial and demelanizing activity of Ganoderma lucidum extract, p-hydroxybenzoic and cinnamic acids and their synthetic acetylated glucuronide methyl esters, Food Chem. Toxicol. 58 (2013) 95–100.

[15] Â. Fernandes, A.L. Antonio, J.C.M. Barreira, M.B.P.P. Oliveira, A. Martins, I.C.F. R Ferreira, Effects of gamma irradiation on physical parameters of Lactarius deliciosus wild edible mushrooms, Postharvest Biol. Technol. 74 (2012) 79–84, https://doi.org/10.1016/j.postharvbio.2012.06.019.

[16] M. Carocho, P. Morales, M. Ciudad-Mulero, V. Fernández-Ruiz, E. Ferreira, S. Heleno, P. Rodrigues, L. Barros, I. Ferreira, Comparison of different bread types: chemical and physical parameters, Food Chem. 310 (2020) 125954, https://doi.org/10.1016/j.foodchem.2019.125954.

[17] International Organization for Standardization. (2003). ISO 15213: 2003 - microbiology of food and animal feeding stuffs horizontal method for the enumeration of sulfite-reducing bacteria growing under anaerobic conditions. https://www.iso.org/standard/26852.html.

[18] A.C.S. Serra, N.D.S.V. de Souza, D.A.B. de Oliveira, L.B. da Silva, L.M.G.E. da Silva, Development, bromatological and sensory analysis of biscuit prepared on apple flour (Malus communis), Res. Soc. Dev. 10 (15) (2021) e320101521413-e320101521413.

[19] L.M. Coelho, G. Wosiacki, Avaliação sensorial de produtos panificados com adição de farinha de bagaço de maçã, Ciênc. Tecnol. Aliment. 30 (3) (2010) 582–588.

[20] S. Zlatanovic, S. Ostojić, D. Micić, S. Rankov, M. Dodevska, P. Vukosavljević, S. Gorjanović, Thermal behaviour and degradation kinetics of apple pomace flours, Thermochim. Acta 673 (2019) 17–25.

[21] M.F. Sato, R.G. Vieira, D.M. Zardo, L.D. Falcão, A. Nogueira, G. Wosiacki, Apple pomace from eleven cultivars: an approach to identify sources of bioactive compounds, Acta Sci.. Agron. 32 (1) (2010) 29–35, https://doi.org/10.4025/actasciagron.v32i1.3176.

[22] T. Nilsson, K.H. Gustavsson, Postharvest physiology of "Aroma'' apples in relation to position on the tree, Postharvest Biol. Technol. 43 (2007) 36–46.

[23] J. Wu, H. Gao, L. Zhao, X. Liao, F. Chen, Z. Wang, X. Hu, Chemical compositional characterization of some apple cultivars, Food Chem. 103 (1) (2007) 88–93, https://doi.org/10.1016/j.foodchem.2006.07.030.

[24] B. Ma, Y. Yuan, M. Gao, C. Li, C. Ogutu, M. Li, F. Ma, Determination of predominant organic acid components in malus species: correlation with apple domestication, Metabolites 8 (4) (2018) 74, https://doi.org/10.3390/metabo8040074.

[25] A.A. Arraibi, Á. Liberal, M.I. Dias, M.J. Alves, I.C.F.R. Ferreira, L. Barros, J.C. M. Barreira, Chemical and bioactive characterization of spanish and belgian apple pomace for its potential use as a novel dermocosmetic formulation, Foods 10 (8) (2021) 1949, https://doi.org/10.3390/foods10081949.

[26] X. Cao, C. Wang, H. Pei, B. Sun, Separation and identification of polyphenols in apple pomace by high-speed counter-current chromatography and high-performance liquid chromatography coupled with mass spectrometry, J. Chromatogr. A 1216 (19) (2009) 4268–4274, https://doi.org/10.1016/j.chroma.2009.01.046.

[27] D. De Paepe, K. Servaes, B. Noten, L. Diels, M. De Loose, B. Van Droogenbroeck, S. Voorspoels, An improved mass spectrometric method for identification and quantification of phenolic compounds in apple fruits, Food Chem. 136 (2) (2013) 368–375, https://doi.org/10.1016/j.foodchem.2012.08.062.

[28] L. Montero, M. Herrero, E. Ibáñez, A. Cifuentes, Profiling of phenolic compounds from different apple varieties using comprehensive two-dimensional liquid chromatography, J. Chromatogr. A 1313 (2013) 275–283, https://doi.org/10.1016/j.chroma.2013.06.015.

[29] F. Sánchez-Rabaneda, O. Jáuregui, R.M. Lamuela-Raventós, F. Viladomat, J. Bastida, C. Codina, Qualitative analysis of phenolic compounds in apple pomace using liquid chromatography coupled to mass spectrometry in tandem mode, Rapid Commun. Mass Spectrom. 18 (5) (2004) 553–563, https://doi.org/10.1002/rcm.1370.

[30] A. Wojdyło, J. Oszmiański, P. Laskowski, Polyphenolic compounds and antioxidant activity of new and old apple varieties, J. Agric. Food Chem. 56 (15) (2008) 6520–6530, https://doi.org/10.1021/jf800510j.

[31] M. Stompor, D. Broda, A. Bajek-Bil, Dihydrochalcones: methods of acquisition and pharmacological properties - A first systematic review, Molecules (2019), https://doi.org/10.3390/molecules24244468.

[32] G. Ćetković, J. Čanadanović-Brunet, S. Djilas, S. Savatović, A. Mandić, V. Tumbas, Assessment of polyphenolic content and in vitro antiradical characteristics of apple pomace, Food Chem. 109 (2) (2008) 340–347, https://doi.org/10.1016/j.foodchem.2007.12.046.

[33] S. Feng, J. Yi, X. Li, X. Wu, Y. Zhao, Y. Ma, J. Bi, Systematic review of phenolic compounds in apple fruits: compositions, distribution, absorption, metabolism, and processing stability, J. Agric. Food chem. 69 (1) (2021) 7–27, https://doi.org/10.1021/acs.jafc.0c05481.

[34] K.A. Arbos, R.J.S.D. Freitas, S.C. Stertz, M.F. Dornas, Atividade antioxidante e teor de fenólicos totais em hortaliças orgânicas e convencionais, Ciênc. Tecnol. Aliment. 30 (2) (2010) 501–506.

[35] B. Birasuren, N.Y. Kim, H.L. Jeon, M.R. Kim, Evaluation of the antioxidant capacity and phenolic content of agriophyllum pungens seed extracts from Mongolia, Prev. Nutr. Food Sci. 18 (3) (2013) 188–195, https://doi.org/10.3746/pnf.2013.18.3.188.

[36] Y. Hamauzu, H. Yasui, T. Inno, C. Kume, M. Omanyuda, Phenolic profile, antioxidant property, and anti-influenza viral activity of Chinese quince (Pseudocydonia sinensisSchneid.), quince (Cydonia oblongaMill.), and apple (Malus domesticaMill.) fruits, J. Agric. Food Chem. 53 (4) (2005) 928–934, https://doi.org/10.1021/jf0494635.

[37] J. Luo, P. Zhang, S. Li, N.P. Shah, Antioxidant, antibacterial, and antiproliferative activities of free and bound phenolics from peel and flesh of fuji apple, J. Food Sci. 81 (7) (2016) M1735–M1742.

[38] V. Valková, H. Duranová, M. Havrlentová, E. Ivanisová, Ján Mezey, Z. Tóthová, L. Gabríny, M. Kacaniová, Selected physico-chemical, nutritional, antioxidant and sensory properties of wheat bread supplemented with apple pomace powder as a by-products from juice production, Plants 11 (9) (2022) 1256, https://doi.org/10.3390/plants11091256.

[39] X. Liang, L. Feng, J. Ran, J. Sun, X. Chen, Z. Jiao, B. Liu, L. Jiao, Influence of adding steam-exploded apple pomace on wheat flour characteristics and biscuit quality, J. Food Sci. Technol. 57 (8) (2020) 3031–3039, https://doi.org/10.1007/s13197-020-04336-2.

[40] B. Zaffora, L. Coisne, C. Gérard, Survival models to estimate time to visible mold growth on new paper-based food-contact materials under varying environmental conditions, LWT – Food Sci. Technol. 193 (2024) 115767, https://doi.org/10.1016/j.lwt.2024.115767.