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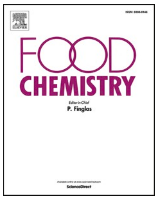

Effects of Wheat Bran-Derived Alkylresorcinols on the Physical and Oxidative Stability of Oil-in-Water Emulsions as related to pH

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ABSTRACT

Alkylresorcinols (ARs), a homolog series of phenolipids naturally occurring in many by-products, can meet the high demand of the food industry for natural antioxidants. In this study, ARs (C17–C25) were isolated from wheat bran, and their antioxidant activity was tested in oil-in-water emulsions at pH 3.5 and pH 7.0 at two concentrations (15 and 30 mg/L of emulsion) during 14 days of storage at 35 °C. Results revealed that lipid oxidation was affected by both ARs concentration and pH of the emulsion. Indeed, when ARs were added, a significant suppression of hydroperoxides and aldehydes (hexanal and nonanal) formation with a consequent prolongation of their lag phases (5-fold) was observed at pH 3.5, while at pH 7.0 the lag phases were doubled. No influence of ARs on emulsion particle size was found. The present work demonstrated how ARs could represent sustainable and innovative natural antioxidants for emulsion-based food.

1. Introduction

As a major cause of food quality deterioration, lipid oxidation is the foremost target on which researchers' efforts are focused. Lipid oxidation is governed by numerous chemical reactions triggered by factors like air, heat, and transition metals, leading to the formation of compounds such as hydroperoxides, aldehydes, and ketones that deteriorate the nutritional and sensory properties of foods and are, above all, harmful to human health. These reactions happen extensively in the presence of polyunsaturated fatty acids (PUFAs) which, on the other hand, are increasingly attracting consumers -and consequently food industries- for their benefits on human health. Besides, a vast number of foods are emulsified systems, where the control of lipid oxidation is more challenging, since the presence of water accelerates the oxidative reactions, leading to a more rapid degradation of lipids (Bravo-Díaz, 2022; Laguerre, Bily, Roller, & Birtić, 2017). The latter is especially true for oil-in-water (O/W) emulsions, where the contact area between the dispersed oil droplets and the dispersant water is very large. Indeed, the interfacial region that separates the aqueous phase from the oil one is the crucial site where oxidation reactions take place, as the prooxidants contained in the water (transition metals, photosensitizer, and enzymes) can easily interact with lipids (Bravo-Díaz, 2022). Hence, new strategies to counteract lipid oxidation are constantly being sought, including the addition of antioxidants, which quickly react with pro-oxidants through several mechanisms such as radical scavenging, transition metals chelation, or singlet oxygen quenching (Choe & Min, 2009). However, antioxidants often show weak efficacy in emulsified systems due to their poor solubility in lipids, which causes them to partition into the aqueous phase and thus away from the site of reactions (McClements & Decker, 2018). Additionally, consumer demand for natural antioxidants to replace synthetic ones is constantly growing, being often a major challenge for industries.

Wheat bran represents 10–14 % of the kernel and the main coproduct generated during the milling process. Every year 150 million tons of wheat bran are produced, most of which is used as animal feed (Chen, Mense, Brewer, & Shi, 2023). However, bran contains many nutritionally interesting phytochemicals such as β-glucans, vitamins, proteins, polyphenols, lipids, and alkylresorcinols (Cardenia et al., 2018;

Abbreviations: ARs, alkylresorcinols; AR15, alkylresorcinols added at 15 mg/L of emulsion; AR30, alkylresorcinols added at 30 mg/L of emulsion..

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Chen et al., 2023). Alkylresorcinols (ARs) are a homologous series of phenolipids naturally present in different cereals such as wheat, rye, and barley, consisting of a phenolic structure (resorcinol) and a hydrophobic chain of varying length at position 5 of the phenolic ring. In wheat bran, ARs are present (about ~0.3 %, *w/w*) with an odd chain length between 13 and 27 carbon atoms, in both saturated and unsaturated forms (Chen et al., 2023; Elder, Coupland, & Elias, 2021; Esche, Barnsteiner, Scholz, & Engel, 2012). Although the total ARs content often varies significantly, the distribution of the homologs is relatively stable within species, with a C17:0/C21:0 ratio of ~0.01 for durum wheat, ~0.1 for soft and spelt wheat, and ~1 for rye (Landberg, Marklund, Kamal-Eldin, & Åman, 2014). Since ARs are mainly present in the bran, they are also considered as biomarkers of whole grain wheat and rye foods and intake. Many beneficial biological properties have been reported for ARs, such as antioxidant, antimicrobial, anticancer, antilipidemic and neuroprotective, being also proposed as promising bioregulators of metabolic and immune processes and potential positive regulators of the gut microbiome (Zabolotneva, Shatova, Sadova, Shestopalov, & Roumiantsev, 2022). Their antioxidant capacity is due to the presence of two hydroxyl groups on the benzene ring that can donate hydrogen/electrons to radical molecules, such as lipid radicals. Thus, ARs can be suggested as natural compounds to prevent lipid oxidation in food products, especially those characterized by emulsified systems, thanks to their amphiphilic structure. While there are numerous papers on the *in vitro* antioxidant activity of ARs, to the best of our knowledge very few studies have been done on the antioxidant activity of ARs in model systems, especially as related to different pH values, and in real foods. Recently, Elder and colleagues (Elder et al., 2021) studied the antioxidant activity of individual AR homologs (chain length C17:0, C19:0, C21:0, C23:0, C25:0) extracted from rye bran in bulk oil and O/W emulsion, demonstrating that the formation of primary and secondary oxidation products was delayed in their presence. A winterized, acetonic rye bran extract of ARs was also tested in O/W emulsions at pH 7, achieving a significant increase in the lag phase of oxidation products (Elder, Coupland, & Elias, 2019). Moreover, AR individual homologs were also studied in low-moisture crackers, where they significantly extended their shelf life (Elder, Coupland, Hopfer, & Elias, 2022). However, the use of the entire fraction of ARs naturally present in plants instead of isolating them individually would be a great advantage for companies in terms of both economic and environmental sustainability. Furthermore, an additive effect of individual ARs in terms of antioxidant activity can occur when they are present together within the same system.

Hence, the aim of the present work was to evaluate the antioxidant activity of the entire ARs fraction present in wheat bran, in 1.0 % O/W emulsions. Furthermore, since the antioxidant activity of the molecules can be affected by their concentration and the pH of the environment, ARs were tested in the emulsions at two different concentrations (15 and 30 mg/L of emulsion) and pH values (3.5 and 7.0).

2. Materials and methods

2.1. Chemicals and materials

All solvents and reagents were of analytical grade. Methanol and methyl tert-butyl ether (MTBE) were purchased from VWR (Milan, Italy), *n*-hexane, diethyl ether, ethyl acetate, propan-2-ol, ethanol absolute anhydrous, isooctane, 1-butanol, and hydrochloric acid (37 %) were obtained from Carlo Erba (Milan, Italy). Anhydrous pyridine, *N*,*O*-bis(trimethylsilyl)trifluoroacetamide with trimethylchlorosilane (BSTFA:TMCS, 99:1, *v/v*), 5α-cholestan-3β-ol, 2–2′-diphenyl-1-picrylhydrazyl (DPPH), ferrozine, ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA), silicic acid (100–200 mesh, 75–150 μm), activated charcoal, Tween® 20 (polyethylene glycol sorbitan monolaurate), sodium phosphate monobasic dihydrate, sodium phosphate dibasic anhydrous, ferrous sulfate heptahydrate, barium chloride dihydrate, ammonium thiocyanate, and cumene hydroperoxide were purchased from Merk (Darmstadt, Germany). Milli-Q filter system (Millipore, Milan, Italy) was used to prepare double distilled water. N° 1 filter papers were purchased from Whatman (Maidstone, England). Solid-phase extraction (SPE) cartridges (Strata® NH_2, 55 μm, 70 Å, 1 g/6 mL; Strata® SI-1 Silica, 55 μm, 70 Å, 500 mg/3 mL) were provided by Phenomenex (Torrence, CA, USA). Wheat bran was kindly supplied by Molini Bongiovanni S.p.A (Cambiano, Italy).

2.2. Isolation of alkylresorcinols (ARs) from wheat bran

2.2.1. Lipid extraction from wheat bran

ARs were extracted from wheat bran and purified according to Esche et al. (2012), with slight modifications. Wheat bran was freeze-dried (Lio 5P, 5 Pascal, Italy) and immediately ground and sieved at 500 μm. Ten grams of the obtained bran were added with 40 mL of a *n*-hexane:chloroform solution (1:1; *v/v*) and stirred for 1 h in darkness at room temperature to extract the lipid fraction. The mixture was then filtered with a Buchner funnel with Whatman filter paper, and the solvent was removed under vacuum with a rotary evaporator (Rotavapor® R-210, Büchi, Flawil, Switzerland) at 37 °C. The obtained oil was dissolved in 10 mL of *n*-hexane.

2.2.2. Purification of ARs from lipid fraction

ARs were isolated from the lipid fraction through solid-phase extraction (SPE) following the procedure described by Esche et al. (2012), with slight modifications. Briefly, 50 mg of lipid matter extracted from wheat bran was resuspended in 1 mL of *n*-hexane and loaded on a SPE-NH_2 cartridge, previously activated with 10 mL of *n*-hexane. To remove the undesired compounds, different solvents were eluted in the following order: 10 mL *n*-hexane:diethyl ether (98:2; *v/v*), 20 mL of *n*-hexane:ethyl acetate (96:4; *v/v*), and 10 mL of *n*-hexane:ethyl acetate (5:95; *v/v*). ARs were finally eluted with 10 mL of *n*-hexane:ethyl acetate (5:95; *v/v*) followed by 5 mL of MTBE. The solvents were then removed with a rotary evaporator (Rotavapor® R-210, Büchi, Flawil, Switzerland), and residues were dissolved in 500 μL of *n*-hexane:diethyl ether (80:20; *v/v*) and loaded onto a SPE SI-1 cartridge, previously activated with 3 mL of *n*-hexane. After eluting 5 mL of *n*-hexane:diethyl ether (8:2; *v/v*) and 4 mL of *n*-hexane:diethyl ether (1:1; *v/v*) and discarding both phases, purified ARs were eluted with 3 mL of methanol. Methanol was then evaporated and the ARs dissolved in *n*-hexane: propan-2-ol (3:2; *v/v*).

2.3. Characterization of ARs

2.3.1. Identification and quantification of ARs by GC/MS and GC/FID

The isolated fraction was dried and ARs were silylated by adding 100 μL of pyridine and 200 μL of BSTFA:TMCS (99:1; *v/v*) and slowly stirring the mixture at 40 °C for 20 min in darkness. Solvents were then evaporated with nitrogen flow and 500 μL of *n*-hexane were added. After centrifugation (3 min at 3500 ×*g*), the upper phase was collected, dried, and resuspended in 100 μL of *n*-hexane.

Identification of ARs was performed in a Shimadzu QP2010 Plus GC/MS (Shimadzu, Kyoto, Japan), using a RXi-5ms fused silica capillary column (10 m, i.d. 0.1 mm, 0.1 μm film thickness; Restek, Bellefonte, PA). The AOC-5000 Pal autosampler (Shimadzu, Kyoto, Japan) was used to inject 1 μL at 345 °C with a split ratio of 1:50. Helium was used as carrier gas with a constant linear velocity of 49.9 cm/s. The oven temperature was programmed as follows: from 100 °C to 310 °C at 7 °C/min; from 310 °C to 320 °C at 1 °C/min, from 320 °C to 345 °C at 7 °C/min. The final temperature was held for 14 min. The interface and ion source temperatures were set at 230 and 200 °C, respectively. Acquisition was performed in total ion current (TIC) with a mass range of 33–600 *m/z* and a scan velocity of 1166 amu/s. The NIST08s (National Institute of Standards and Technology, Gaithersburg, USA) library was used to identify the compounds by comparing the mass spectra.

To calculate the yield of the ARs extract and its purity, 1 μL of the sample was injected in a GC/FID (GC-2010, Shimadzu, Kyoto, Japan) equipped with the same analytical column of GC/MS and under the same analytical conditions. Purity was calculated as the percentage of the sum of all ARs (both saturated and unsaturated) on the total peaks detected in the chromatogram. To quantify, 80 μL of a 5α-cholestan-3-β-ol solution (1.005 mg/mL; internal standard) were added to ARs before silylation.

2.3.2. Determination of antioxidant properties of ARs

The antioxidant properties of ARs were tested in terms of radical scavenging activity (DPPH spectrophotometric assay) and metal-chelating capacity (ferrous ion-chelating spectrophotometric assay) using a BioTek Synergy HT spectrophotometric multi-detection 96-well microplate reader (BioTek Instruments, Milan, Italy). For these assays, the extract was dried with a nitrogen flush and resuspended in ethanol, and appropriate dilutions were applied. For the DPPH assay, 100 μL of DPPH solution (0.1 mM) were added to 100 μL of the sample in the 96-well microplate. After 20 min of incubation (steady-state) in darkness at 23 °C, the absorbance was read at 517 nm against a blank (Cantele et al., 2023). The inhibition percentage (I%) was calculated according to Eq. (1):

$$I\% = \left(\frac{Abs_{blk} - Abs_{sample}}{Abs_{blk}}\right) \times 100 \quad (1)$$

where $\mathrm{Abs_{blk}}$ represents the absorbance of the blank and $\mathrm{Abs_{sample}}$ represents the absorbance of the sample. The results were expressed as micromoles of Trolox equivalents per gram of sample (μmol TE/g), calculated by building a calibration curve with Trolox (25–300 μM; $y = 0.1941x + 0.0483$; $R^2 = 0.9999$).

For the ferrous-ions chelating assay, 1 mL of the extract was mixed firstly with 20 μL of ferrous chloride (2 mM) and then with 20 μL of ferrozine (6 mM). After each addition, the mixture was vigorously stirred with the vortex. A blank was also prepared, replacing the sample with ethanol. After 10 min of incubation, 200 μL was added to the 96-well microplate and the absorbance was read at 562 nm against the blank (Cantele et al., 2020). Barium chloride and ferrous sulfate were reacted to prepare fresh ferrous chloride, as ferrous ions are highly oxidizable. Eq. 1 was again used to calculate the inhibition percentage and then the result was expressed as milligrams of EDTA equivalents per gram of sample (mg EDTAE/g) by preparing a calibration curve with EDTA (0.001–0.1 mg/mL; $y = 8546.5x - 0.4859$; $R^2 = 0.9947$).

2.4. Stripping of the oil

It may be noted that the research may be hampered by the presence of more polar substances such as tocopherols, hydroperoxides, free fatty acids, phenolic acids, and carotenoids; for this reason, it is imperative to eliminate them from the oil. For example, phenolic acids, tocopherols, and carotenoids may have antioxidant properties, and even low concentration of hydroperoxides and free fatty acids may have an impact on the system's oxidative stability. In the present study, a commercial mixture of soybean and sunflower seeds oil was deprived of its polar compounds according to Boon et al. (2008), using a chromatographic column of 3.0 cm diameter and 35.0 mm length. Three layers were prepared to pack the column: 22.5 g of silicic acid (previously washed with double distilled water), 5.6 g of activated charcoal, and again 22.5 g of washed silicic acid. Silicic acid and charcoal were dissolved, respectively, in 100 mL and 70 mL of *n*-hexane. Thirty grams of oil were dissolved in 30 mL of *n*-hexane and eluted with 270 mL of the same solvent through the packed column. All the procedure was conducted under aluminum foils to avoid light and triacylglycerols were collected in an ice bath. The solvent was removed with a rotary evaporator (Rotavapor® R-210, Büchi, Flawil, Switzerland) at 37 °C and the success of the stripping process was evaluated by injecting the obtained oil into GC/FID to confirm the composition in terms of triacylglycerols and to exclude the presence of minor polar components (Cardenia et al., 2018), and by measuring the peroxide value to verify the absence of hydroperoxides (Shantha & Decker, 1994). Moreover, the total absence of tocopherols was assessed by reversed-phase high-performance liquid chromatography equipped with a photodiode array detector (RP-HPLC-PDA) (Thermo Electron Corporation, Waltham, MA, USA) according to Cantele et al., 2020. The fatty acids composition of the stripped oil was also determined (Cardenia et al., 2018) and is detailed in Table S1 of the Supplementary Material. The oil was stored at −20 °C until subsequent analyses.

2.5. Preparation of the oil-in-water (O/W) emulsions and storage conditions

According to Cardenia, Waraho, Rodriguez-Estrada, McClements, and Decker (2011), 1.0 % stripped oil, 0.1 % Tween 20 (non-ionic emulsifier) and 10 mM phosphate buffer solution at pH 3.5 and pH 7.0 were used to prepare the O/W emulsions (percentages are given for the total weight of the emulsion; *w/w*). The ARs were added to the emulsions at a concentration equal to 15 mg/L (AR15) and 30 mg/L (AR30) of emulsion. These concentrations were selected based on preliminary tests (unpublished data). To do so, the corresponding aliquot of the extract was added to a beaker and solvent was removed by flushing nitrogen; then the stripped oil was added, and the mixture was gently stirred. Emulsions were prepared according to Cantele et al. (2023). Briefly, a coarse emulsion was firstly made using an IKA T25 digital Ultra-Turrax® (IKA®-Werke GmbH & Co. KG, Staufen, Germany) for 2 min at maximum speed (20,000 rpm) and then was turned into a fine emulsion with 3 min of ultrasounds sonication at 100 W in an ice bath to maintain a temperature lower than 30 °C. Control samples without the addition of ARs were also made for each pH value. One milliliter of each emulsion was transferred into 20 mL headspace vials, sealed with PTFE/silicone septa aluminum caps, and stored at 35 °C in darkness for 14 days. For each sample and each analysis, three separate experiments ($n = 3$) were conducted.

2.6. Determination of particle size distribution and ζ-potential of the emulsions

Emulsions were diluted at a ratio of 1:50 (*v/v*) in the corresponding pH 7.0 or pH 3.5 10 mM buffer solution, and a Zetasizer Pro (Malvern Instruments, Worcestershire, UK) was used to evaluate the particle size distribution (average peak by intensity), polydispersity index (PdI), and droplet surface charge (ζ-potential). Three measurements were done for each independent experiment.

2.7. Determination of primary oxidation products

Lipid hydroperoxides content of the emulsions was measured as primary oxidation markers. The procedure, described by Cardenia et al. (2011), included the addition of 0.3 mL of emulsion to 1.5 mL of isooctane:propan-2-ol (3:1; *v/v*), vortex for 30 s and centrifugation at 3400*g* for 3 min. After that, 0.2 mL of the upper phase were dissolved in 2.8 mL of a 2:1 (*v/v*) solution of methanol and butanol. Fifteen microliters of ammonium thiocyanate (30 %; *w/v*) and 15 μL of $FeCl_2$ were added to the mixture, which was vortexed and incubated at room temperature for 20 min in the dark. Absorbance was read at 510 nm. $FeCl_2$ was prepared by reacting 0.132 M $BaCl_2$ and 0.144 M $FeSO_4$. A cumene hydroperoxide standard calibration curve in the range of 10–300 μM ($y = 2.6872x + 0.0135$; $R^2 = 0.9973$) was used to perform quantification and results were expressed as millimoles of hydroperoxides per kilogram of oil (mmol/kg oil).

2.8. *Determination of secondary oxidation products*

Hexanal and nonanal content were determined as markers of the secondary oxidation reactions by headspace solid-phase microextraction gas chromatography coupled with mass spectrometry (HS-SPME-GC/MS) using a Shimadzu QP2010 Plus GC/MS (Shimadzu, Kyoto, Japan). Vials were conditioned at 40 °C for 10 min to reach the equilibrium of the volatile compounds in the headspace. A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) coated fiber (df 50/30 μm; 1 cm; Supelco, Bellefonte, PA, USA) was then exposed in the headspace of the vial for 5 min while maintaining the same temperature. To perform the chromatographic separation, the fiber was subsequently desorbed for 3 min in split mode (1:50) using helium as the carrier gas (34.8 cm/s linear velocity) in an RTX-5 fused silica capillary column (20 m, i.d. 0.10 mm, film thickness 0.10 μm; Restek, Bellefonte, PA). Column oven was programmed as 40 °C for 1 min, then up to 100 °C (5 °C/min), and up to 240 °C (30 °C/min). The final temperature was held for 1 min. Injector, transfer line, and ion source temperatures were, respectively, 260 °C, 230 °C, and 200 °C. Ions were acquired between 33 and 350 *m/z* at a scan speed of 1666 amu/s. The external standard method was used to quantify the two aldehydes with calibration curves of the corresponding pure standard (hexanal, 0.200–750 μmol/kg oil, $y = 0.5730x - 0.1349$, $R^2 = 0.9985$; nonanal, 0.146–350 μmol/kg oil, $y = 0.0408x - 0.0034$, $R^2 = 0.9945$). Results were expressed as μmol/kg oil.

2.9. *Statistical analysis*

To investigate the impact of wheat bran ARs on the physical and oxidative stabilities of the emulsions, one-way analysis of variance (one-way ANOVA) and Tukey's test was used to determine any statistical difference between the samples at a confidence level of 95 %. One-tailed Dunnett's *post hoc* test was used to calculate the hydroperoxides, hexanal, and nonanal lag phases, intended as the first point statistically different from day 0 ($p < 0.05$). IBM SPSS statistical software (version 28; IBM, Chicago, IL, USA) was used.

3. Results and discussion

3.1. *Characterization of ARs extract*

The wheat bran purified extract contained fifteen different alkylresorcinol homologs. Specifically, five ARs with a saturated chain (C17:0, C19:0, C21:0, C23:0, and C25:0) (Fig. 1A), and ten monounsaturated ARs (two positional isomers for each abovementioned homolog) (Fig. 1B). Martín-García, Gómez-Caravaca, Marconi, and Verardo (2021) found the same resorcinolic lipids composition in aleurone fractions of wheat bran. All ARs (both saturated and unsaturated) displayed characteristic ions at 268 *m/z* and 281 *m/z*, resulting from McLafferty rearrangement (Cantele, Potenziani, Bonciolini, Bertolino, & Cardenia, 2024; Martín-García et al., 2021), which were used as qualifier ions. Furthermore, molecular ions $[M^+]$ at *m/z* 492, 520, 548, 576, and 604 were also used to identify the TMS derivatives of C17:0, C19:0, C21:0, C23:0, and C25:0, respectively (details are provided in Table S2 of the Supplementary Material).

The unsaturated ARs were identified based on their retention time and also by comparing their mass spectra with those reported by the literature (Martín-García et al., 2021; Ross, Åman, Andersson, & Kamal-Eldin, 2004). Specifically, two peaks were present immediately before the peak of the corresponding saturated AR, possessing $[M^+]$-2. Consequently, molecular ions at *m/z* 490, 518, 546, 574, and 602 were used as qualifier ions for the identification of the TMS derivatives of C17:1, C19:1, C21:1, C23:1, and C25:1, respectively. Unsaturated ARs were present in small concentrations (< 10 % of the total ARs) with respect to the saturated ones. The composition of the homologs (saturated plus monounsaturated) was the following: C17, 7.5 %; C19, 41.9 %; C21, 40.8 %; C23, 7.2 %; C25, 2.6 %, as already observed by other researchers (Andersson, Kamal-Eldin, Fraś, Boros, & Åman, 2008). The ratio between C17:0 and C21:0 was 0.18, in agreement with those reported in soft wheat (Landberg et al., 2014). The extract revealed a content of ARs equal to 705.49 ± 7.51 μg/g of dry bran and a purity of 85 %. Contaminants included 2,3-dihydroxypropyl hexadecanoate, 2,3-dihydroxypropyl octadecanoate, hexadecanoic acid, and octadecanoic acid.

The antioxidant properties of ARs were assessed in terms of radical scavenging activity and ferrous ion-chelating activity as free radicals and bivalent cations such as iron and copper are the main initiators of oxidation in lipids. The purified ARs extract revealed a radical scavenging activity equal to 325.72 ± 5.50 μmol TE/g. On the other hand, the ferrous ions-chelating ability was quantified in 3.35 ± 0.11 mg EDTAE/g. The observed chelating activity was unexpected since the phenolic part of the ARs does not contain catechol or galloyl groups, but rather just a resorcinol group. The absence of two contiguous hydroxyl groups, in fact, would not allow the iron to replace their two hydrogen atoms. However, the same situation was reported by other authors for ferulic acid and its derivatives, suggesting that there are other types of interactions with iron (Sørensen et al., 2014). The direct comparison of the results with data from other studies is somewhat limited, as different

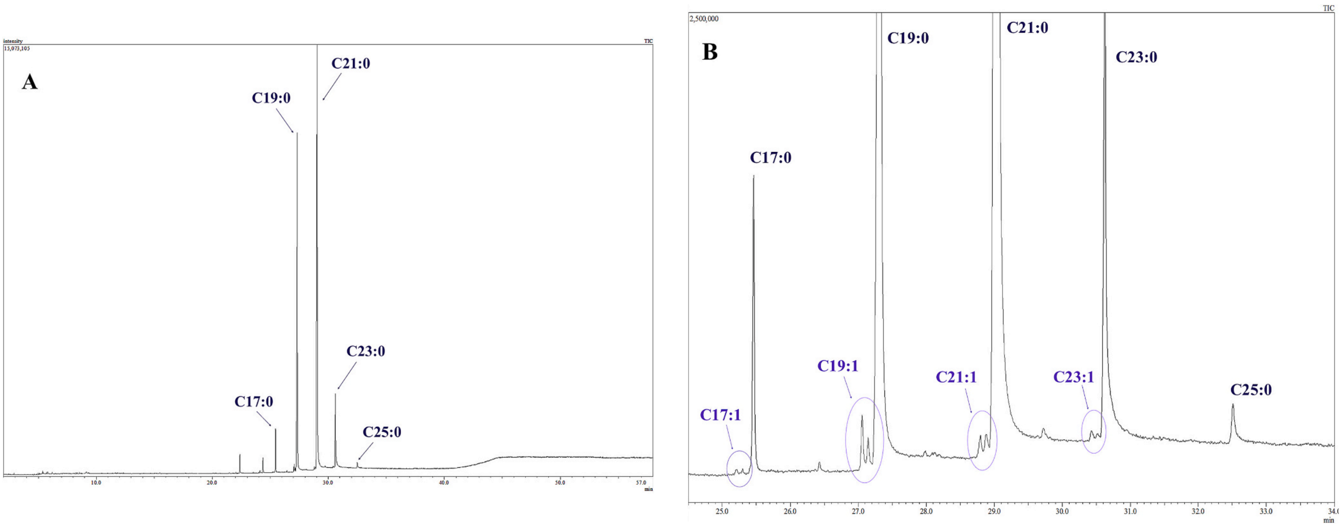

Fig. 1. GC/MS chromatogram of the wheat bran extract, saturated AR homologs (A panel) and monounsaturated AR homologs (B panel) are reported. The monounsaturated ARs of C25 are present at trace levels and just detectable in single ion monitoring (SIM).

operating conditions significantly impact this type of *in vitro* analysis. However, it is worth mentioning here that the antioxidant activity of alkylresorcinols has been observed in numerous studies and across different systems. For instance, Elder et al. (2019) demonstrated good antioxidant activity in oil-in-water emulsions of an acetonic rye bran extract containing alkylresorcinols, due to the ability to scavenge oxygen radicals. Two years later, the same authors proved the radical scavenging properties of synthetic alkylresorcinols homologs (individually and in mixtures) in both O/W emulsions and bulk oil (Elder et al., 2021). Their efficacy has also been confirmed in low-moisture crackers (Elder et al., 2022), and in a minced-meat model (Cantele et al., 2024). Additionally, Hładyszowski, Zubik, and Kozubek (1998) reported in their study that increasing the alkyl chain (from 1 to 15 carbon atoms) on the resorcinol ring, the Fe^{2+}-induced peroxidation was reduced in liposomes. In this case, the high affinity with the membrane and thus the ease in penetrating it, stopping the propagation of oxidation, are likely to be responsible for the activity of the tested compounds, as demonstrated by Durand et al. (2017).

However, *in vitro* assays for antioxidant activity do not reflect the behavior of the same molecules in more complex systems like food. In fact, the reactions that take place between molecules depend not only on their structures but also on the physical-chemical phenomena occurring in their surroundings. Therefore, before hypothesizing the use of new antioxidants by the food industry, their behavior in different environments should be studied to predict their efficacy in different matrices. For this reason, the ARs purified from wheat bran have been tested in O/W emulsions at two different pH values.

3.2. *Influence of ARs on the physical stability of the emulsions*

Droplets dimension was investigated to monitor the physical instability processes that can occur during the storage of emulsified systems. These phenomena include flocculation, coalescence, and creaming, and all involve, albeit in different ways, the aggregation of oil particles leading to the separation of the discontinuous phase from the continuous ones and thus loss of stability.

Results of particle sizes are reported in Fig. 2 and detailed in Tables S3 and S4 of Supplementary Material. In general, slightly larger particle sizes were noted in the neutral environment than in the acidic one. In fact, at day 0 a range of 254.9–290.4 nm was measured for the emulsions at pH 7.0, while at pH 3.5 dimensions were between 204.5 and 218.5 nm. The data from the literature on how pH affects the particle size of oil droplets in O/W emulsions prepared with Tween 20 is unclear. For instance, Cardenia et al. (2011) noticed smaller particles when the emulsions were prepared with a buffer solution at pH 7 (170–188 nm) compared to pH 3.5 (260–328 nm). On the other hand, both Jiao et al. (2022) and Teo et al. (2016) reported in their work that pH has no effect on the dimension of the particles of the emulsions, obtaining consistent results ranging from pH 2 to pH 10. Further studies are required to better understand this aspect. Under the conditions tested, ARs had no effect on the size of the oil droplets, either in relation to their concentration or the pH of the system as no significant differences with respect to the control were observed throughout the experiments ($p > 0.05$). Thus, ARs probably did not contribute to reducing the interfacial tension of the droplets. Nevertheless, sufficiently small particles were obtained to ensure kinetically stable emulsions, as no physical instability occurred during the 14 days of storage, evidenced by the constant particle size ($p > 0.05$) and unchanged visual appearance.

Along with the particle size of the oil droplets, the electrical charge displayed on the surface (ζ-potential) is also necessary to be monitored because it can determine the fate of an emulsion in terms of its physical stability. High values of charge, whether positive or negative, ensure strong repulsion between particles, thus averting their aggregation. Conversely, a weak charge, close to neutrality (0 mV), may not guarantee electrostatic repulsion leading to instability. In addition, ζ-potential can also affect the oxidative stability of emulsions by changing the rate of lipid oxidation (Bayram, Laze, & Decker, 2023; Cardenia et al., 2011).

The results of droplet surface charge are depicted in Fig. 3 and detailed in Tables S5 and S6 of Supplementary Material. Since day 0, all the emulsions displayed negative ζ-potential values at both pH levels. Although the Tween 20 used in this work is frequently regarded as a non-ionic emulsifier, observing a negative droplet surface charge was not unexpected since this phenomenon has previously been described by other researchers (Cardenia et al., 2011; Cui, Cho, McClements, Decker, & Park, 2016). Either free fatty acids in the system or contaminants in the Tween 20 could be the cause of this negative surface charge, or even the preferential adsorption by the oil droplets of OH^- ions (rather than H_3O^+) from water (Hur, Decker, & McClements, 2009). The pH led a different trend in the surface charge of the emulsions. At pH 7.0, the droplets charge at day 0 was −12.33 ± 1.39 mV (control), −10.28 ± 0.96 mV (AR15), and − 8.79 ± 0.63 mV (AR30), which dramatically decreased during the experiment, reaching at day 14 values equal to −50.62 ± 3.57 mV (control), −54.03 ± 0.88 mV (AR15), and − 54.76 ± 1.62 (AR30). Moreover, no significant differences ($p > 0.05$) were noted between the control and AR15 or AR30 during the experiment. On the other side, at pH 3.5 the surface charge was still negative but closer to 0 mV and, although a decrease in values was again observed during the experiment, this was not as drastic as in the case of the neutral environment. Indeed, the droplet surface charge of the control went from −2.15 ± 0.51 (day 0) to −5.88 ± 0.07 (day 14) mV, while for AR15 from −2.35 ± 0.25 (day 0) to −5.55 ± 0.55 (day 14) mV, and that of AR30 from −2.21 ± 0.25 mV (day 0) to −4.43 ± 0.37 mV (day 14). No differences were noted among the samples except on the last day, where AR30 showed a lower negative charge value than the control and AR15 ($p < 0.05$). This difference in the electrical charge noted on day 14 could

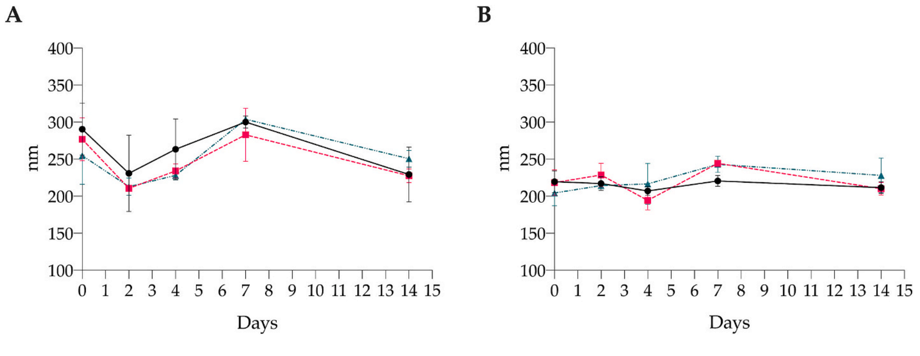

Fig. 2. Particle size of the O/W emulsions during 14 days of storage at pH 7.0 (**A**) and pH 3.5 (**B**): (●) control, (■) AR15, (▲) AR30. Each point represents the mean ± standard deviation of three independent replicates ($n = 3$). Results of ANOVA with Tukey's *post hoc* test are reported; different letters indicate significantly different means ($p < 0.05$) among the samples within the same day; where no letters are displayed, no significant differences were observed ($p > 0.05$).

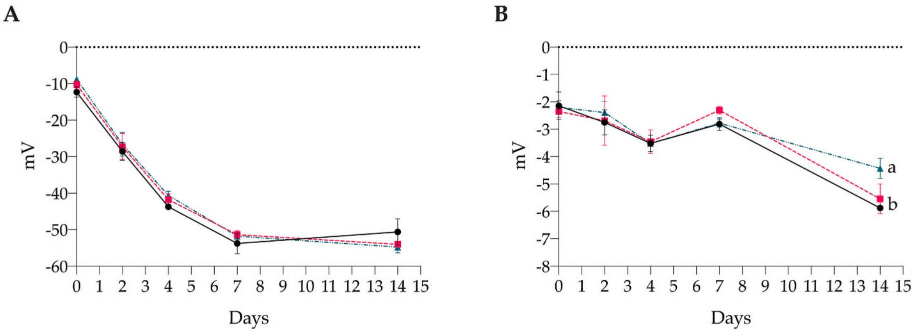

Fig. 3. Droplet surface charge of the O/W emulsions during 14 days of storage at pH 7.0 (**A**) and pH 3.5 (**B**): (●) control, (■) AR15, (▲) AR30. Each point represents the mean ± standard deviation of three independent replicates (n = 3). Results of ANOVA with Tukey's *post hoc* test are reported; different letters indicate significantly different means ($p < 0.05$) among the samples within the same day; where no letters are displayed, no significant differences were observed ($p > 0.05$).

be due to the different rate of lipid oxidation. In fact, certain compounds generated from the oxidation of the lipids, such as fatty acids, can expose at the interface (where they migrate) negative charges if the operating pH value is greater than their pK_a. This aspect will be discussed in detail in Section 3.3. On the other hand, we cannot exclude the possibility that contaminants from the emulsifier used, as well as its degradation products, may have contributed to the reduction of the surface negative charge during the experiment. In fact, the presence of peroxides in Tween 20 and their influence on emulsion systems have already been reported (Villeneuve et al., 2021).

One of the reasons why no differences in particle size or surface charge were observed could be due to the lipophilicity of the isolated alkylresorcinols. In fact, the latter possess an alkyl chain long enough to make them too hydrophobic to partition at the oil-water interface (Berton-Carabin & Villeneuve, 2023). More likely, however, these molecules were not present in the system at concentrations sufficient to influence the surface charge of the oil droplets.

On the other hand, the results of the polydispersity index (PdI) were in the range of 0.41–0.46 and 0.36–0.45 at pH 7 and 3.5, respectively (detailed in Tables S7 and S8 of the Supplementary Material) confirming their suitability for the dynamic light scattering analysis.

3.3. *Influence of ARs on the oxidative stability of the emulsions*

Emulsions were formulated with ARs extracted from wheat bran and stored at 35 °C for two weeks in the absence of light while measuring primary and secondary oxidation products, *i.e.*, hydroperoxides, and hexanal and nonanal, respectively. The formation of these products was influenced by both the pH of the emulsion and the concentration of the phenolipids (Figs. 4, 5, 6). Results are also detailed in Tables S9–S14 of the Supplementary Material.

In general, the addition of ARs to the emulsions resulted in a significant delay in the lag phase of both hydroperoxides and aldehydes formation compared to the emulsions without phenolipids (controls). This behavior could be ascribed to the radical scavenging and metal chelating activities of ARs observed in the *in vitro* tests. However, the ARs were more effective under the acidic conditions rather than under the neutral one. In fact, the lag phase of hydroperoxides at pH 7.0 was prolonged from 4.5 h (control) to 1 day when AR15 and AR30 were added, whereas that of hexanal was extended from 1 day to 2 days. For nonanal, the lag phase remained the same (3 days), but its formation was delayed by 1 day. In fact, in the control nonanal was found after one day, while in the emulsions with AR15 and AR30 it was found only from the second day of storage. At pH 3.5, the lag phase of hydroperoxides was extended from 1 to 4 and 5 days for AR15 and AR30, respectively. Compared to the 3-day lag phase shown by the control, the hexanal lag phase was equal to 5 days for AR15 and 8 days for AR30. Moreover, the addition of the alkylresorcinols in the acid emulsions led to a significant delay in the formation of this aldehyde, being detected only on day 4 (AR15) and day 7 (AR30), compared to the control where it was observed immediately after 2 days. The same scenario was observed for nonanal, which first appeared on day 2 in the control and then increased significantly on day 6 (lag phase 5 days), while in AR15 and AR30 it developed similarly to hexanal after 4 and 7 days, respectively, both with a lag phase of 8 days.

Therefore, the acidic pH favored better oxidative stability of the emulsions, as evidenced by the longer lag phases. This has already been found in other studies (Bayram et al., 2023; Cantele et al., 2023). A first explanation might be found in the charge of the emulsifier, as it can have a major impact on the oxidative state of the system. In fact, although the oil droplets exhibited a negative surface charge either at pH 7.0 or pH 3.5, this was higher under neutral conditions. Strong negative charges accelerate lipid oxidation since they attract more easily the cations responsible for initiating the chain reactions. Among these, iron is one of the most crucial due to its strong prooxidant activity and high solubility and reactivity (Bayram et al., 2023). Transition metals pose a very serious problem for food shelf life because, due to their ubiquitous nature, they are easily found in food, coming from numerous sources such

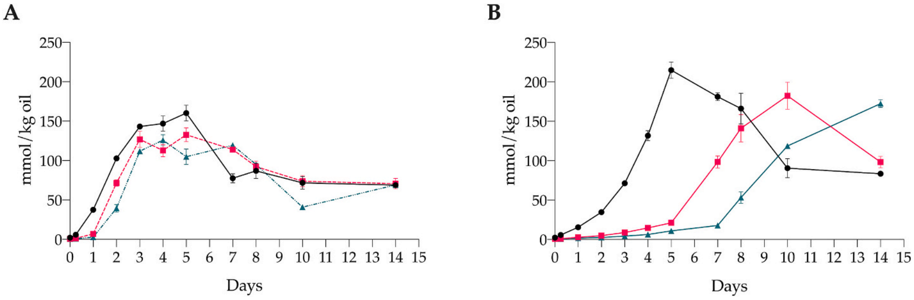

Fig. 4. Hydroperoxides content in the O/W emulsions during 14 days of storage at pH 7.0 (**A**) and pH 3.5 (**B**): (●) control, (■) AR15, (▲) AR30. Each point represents the mean ± standard deviation of three independent replicates (n = 3). Lag phases were calculated through Dunnett's *post hoc* test.

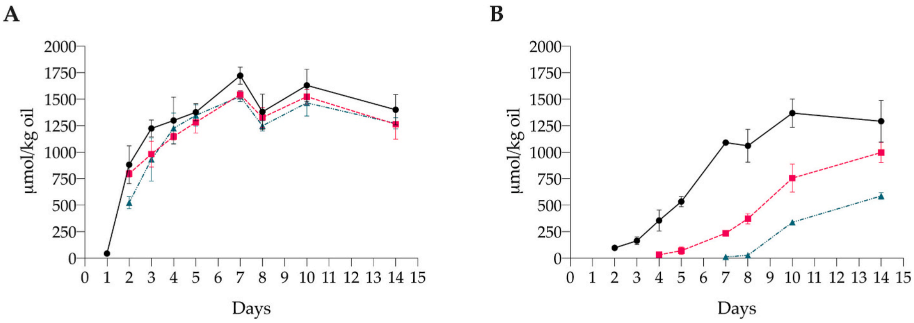

Fig. 5. Hexanal content in the O/W emulsions during 14 days of storage at pH 7.0 (**A**) and pH 3.5 (**B**): (●) control, (■) AR15, (▲) AR30. Each point represents the mean ± standard deviation of three independent replicates (n = 3). Lag phases were calculated through Dunnett's *post hoc* test.

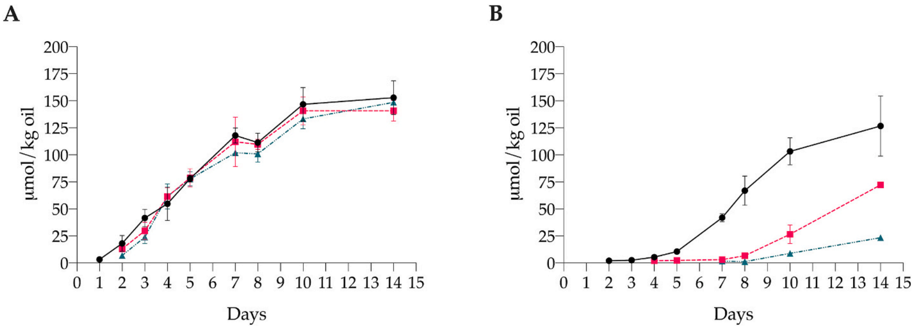

Fig. 6. Nonanal content in the O/W emulsions during 14 days of storage at pH 7.0 (**A**) and pH 3.5 (**B**): (●) control, (■) AR15, (▲) AR30. Each point represents the mean ± standard deviation of three independent replicates (n = 3). Lag phases were calculated through Dunnett's *post hoc* test.

as food ingredients, water, food processing equipment and packaging. (Bayram et al., 2023; Waraho, Cardenia, Rodriguez-Estrada, McClements, & Decker, 2009). They greatly speed up lipid oxidation by promoting the breakdown of lipid hydroperoxides into highly reactive alkoxyl and peroxyl radicals that can then spread oxidation by easily abstracting a hydrogen from fatty acids (Waraho et al., 2009). Thus, researchers are always pursuing antioxidants, especially natural ones, that can reduce the catalytic effect of metal ions preventing lipid oxidation and blocking its propagation by quenching free radicals.

In the present work, as the experiment proceeded and lipid oxidation progressed, the negative surface charge increased, and this was particularly intense at pH 7.0. The increase in negative charge during oxidation has already been observed several times and is due to the partitioning of oxidation products, such as free fatty acids, at the interface. Here, if their pK_a value is lower than the pH of the system, some of their functional groups could be deprotonated leading to the increase of the negative charge of the droplets and thus attracting more cationic metals by diffusing the oxidation reactions (Waraho et al., 2009). Conversely, if the pH is lower than the pK_a of these products, the groups will be protonated, and the surface charge will remain steadier. The second reason why emulsions at pH 3.5 were more oxidatively stable is related to the antioxidant activity of the ARs. In fact, from the results of the *in vitro* tests, already discussed in Section 3.1, these phenolipids seem to be able to act both as metal chelators and free radical quenchers. At pH 7.0, although their efficacy was almost negligible, the ARs still slightly counteracted lipid oxidation. However, the ARs showed their actual efficacy at pH 3.5. Jovanovic, Steenken, Hara, and Simic (1996) reported how the redox potential of antioxidants changes as a function of the pH value of the system in which they are located. Since the ability to quench free radicals by donating an electron or a hydrogen atom depends on the redox potential of the molecule, the antiradical activity is therefore also dependent on the pH of the surrounding environment. The different antioxidant activity of molecules depending on the working pH has already been observed many times. In our previous work (Cantele et al., 2023) the antioxidant activity of two synthetic alkylferulates (C4 and C8) was significantly higher in emulsions at pH 3.5 than in those at pH 7.0. Bayram et al. (2023) reached the same conclusions where antioxidants such as α-tocopherol, myricetin, and taxifolin resulted in greater oxidative stability of O/W emulsions at acidic pH than at neutral ones. Huang, Frankel, Schwarz, and German (1996) found that the highest stability of O/W emulsions was obtained when α-tocopherol was added at pH 3.0, while the lowest when α-tocopherol was added at pH 7.0. However, the improved oxidative stability obtained at pH 3.5 could also be due to a different partitioning of ARs in the system and/or slower degradation of ARs. In fact, it has already been observed that by decreasing the pH, the antioxidants may change position by reaching the sites of oxidation reactions, and the rate of their depletion may be significantly decreased (Berton-Carabin & Villeneuve, 2023; Kiokias & Oreopoulou, 2022). In general, since antioxidants possess ionizable groups, the pH of the surrounding system (aqueous phase in O/W emulsions) greatly impacts their polarity (McClements & Decker, 2000). Indeed, when the antioxidant is ionized, its affinity for the aqueous phase increases, while the non-ionized form results less polar and thus with lower affinity for the aqueous phase (McClements & Decker, 2000; Schwarz, Frankel, & German, 1996). However, the overall polarity of the antioxidant determines whether it predominantly remains in the aqueous phase or partitions into the oil phase in both its ionized and nonionized forms. For example, gallic acid, a highly polar antioxidant, remains mostly in the aqueous phase at both pH 7 and pH 3, while Trolox, a less polar antioxidant, shifts from 90 % in the aqueous phase at pH 7 (acid group fully ionized) to only 30 % at pH 3, preferring the oil phase as the acid group is only partially ionized (McClements & Decker, 2000). Moreover, the charge present at the interface given by the emulsifier, which is modulated also by the pH of the aqueous phase, can attract or repulse the antioxidants based on their charge as well (Schwarz et al., 1996).

4. Conclusions

In this study, we examined the effect on the physical and oxidative stability of 1 % oil-in-water emulsions of ARs isolated from wheat bran as related to their concentration and the pH of the system. Indeed, considering their amphipathic nature that would allow them to position themselves near the sites of oxidation reactions, ARs could represent good candidates for increasing the shelf life of emulsion-based foods. However, it is also well known how both the concentration and the characteristics of the system in which they work are crucial for the molecules to perform their antioxidant activities. The pH value was the main key to the success of these antioxidants in counteracting lipid oxidation in the emulsions. Under neutral conditions, the ARs showed poor efficacy, while at pH 3.5 they were found to greatly extend the lag phases of both hydroperoxides and aldehydes. Moreover, by increasing their concentration in the system, the antioxidant effect was enhanced. Their efficacy appeared to be related to their ability to chelate transition metals but more importantly to scavenge free radicals, reactions that in this case could therefore be favored by low pH. ARs, on the other hand, appear not to significantly affect the physical characteristics of emulsions. Therefore, this study provides preliminary insights for hypothesizing the use of alkylresorcinols, extracted from a by-product generated in large quantities worldwide, as natural antioxidants in emulsion-based foods, without likely affecting their physical characteristics. Based on these results, it will be possible to carry on further studies in more complex systems, undoubtedly necessary to contemplate the actual and practical use of these compounds in the food sector. Indeed, food matrices constitute particularly complex multicomponent systems, where numerous factors influence the behavior of each component and their interactions, all of which need to be considered and investigated in detail. Another crucial aspect to be taken into account will be the extraction process and the associated production costs. In fact, further research will be necessary to develop an efficient, cost-competitive and green process able to produce food-grade ARs. If these goals are achieved, the valorization of wheat bran as a source of food-grade ARs will increase, contributing to a reduction in the environmental impact of the agri-food industry.

CRediT authorship contribution statement

Carolina Cantele: Writing – original draft, Visualization, Investigation, Formal analysis, Writing – review & editing. **Ambra Bonciolini:** Investigation. **Andrea Mario Rossi:** Resources. **Marta Bertolino:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization. **Vladimiro Cardenia:** Writing – review & editing, Supervision, Resources, Project administration, 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 the work reported in this paper.

Data availability

No data was used for the research described in the article.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.141659.

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