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Food packaging characterization, composition profiles and *in vitro* testing of micro(bio)plastics from selected petroleum- and plant-based food containers

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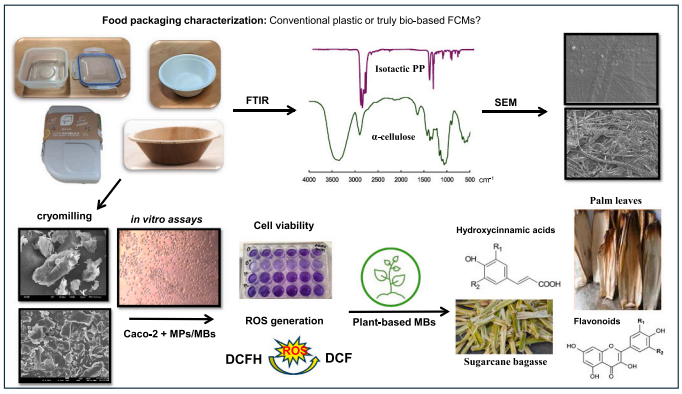
HIGHLIGHTS

- Petroleum-derived and bio-based food packaging for single and repeated use
- Food packaging items were characterized for polymer identity to detect mislabeling
- Cell viability and oxidative stress of micro(bio)plastics using *in vitro* assays
- Plant-based microbioplastics showed potential protection against oxidative stress
- Antioxidant polyphenols were found in extracts of plant-derived micro(bio) plastics

GRAPHICAL ABSTRACT



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ABSTRACT

Plastic food packaging represents a significant source of microplastics (MPs) in food and the environment, posing risks to humans and ecosystems. Replacing single-use plastics with sustainable food packaging (e.g. from renewable biomass) is essential to reduce MPs exposure, although risk assessment studies are still needed. On the other hand, food packaging often labelled as bio-based or biodegradable may contain synthetic polymers that can mislead consumers. This study investigated the composition of e-commerce food contact items (both petroleum-derived and plant-based), and the cytotoxicity of micro(bio)plastics obtained from them by cryo-milling in *in vitro* assays using Caco-2 cells. Fourier transform infrared analysis identified greenwashing and non-compliance with the EU regulatory framework in certain food packages whose main composition was polypropylene (PP)

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containing plant fiber additives. Furthermore, *in vitro* assays showed that none of the MPs tested (50–100 μm, doses 1 mg/mL) caused acute toxicity in Caco-2 cells, although some of the PP-MPs induced cellular stress (up to 25 %), while plant-based microbioplastics (MBs) reduced reactive oxygen species (ROS) levels (by 20–25 %). Particularly valuable results were derived from palm leaf (PL)-MBs where doses above 1 mg/mL induced ROS scavenging and potential cytoprotective effect in Caco-2 cells subjected to oxidative stress. This can largely be attributed to the presence of (poly)phenols (448 ± 25 μg/g) (mainly flavonols and hydroxycinnamic acids, 110 ± 7 and 297 ± 15 μg/g, respectively) in PL-MBs, which are natural antioxidants, highlighting the potential benefits of this type of active packaging for the food industry and human health.

1. Introduction

Food packaging plays a crucial role in food safety but it also contributes to environmental pollution. The demand for plastic packaging in the food industry is very high, and around 99 % of these materials are petroleum-derived [2]. For example, according to the reviewed literature, 42 % of workers in the EU use microwave-safe food containers at lunchtime (90 % of which are made of polypropylene) [16].

As part of the principles of the circular economy, conventional plastic food contact materials (FCMs) are gradually being replaced by bio-based plastics (derived wholly or partially from renewable biomass) to reduce the carbon footprint and environmental impact of FCMs derived from fossil fuels [13,18,28,30,39,45].

However, although the food packaging industry often markets bio-based plastics as “safe”, “sustainable” and “eco-friendly”, several authors have recently questioned this issue [43,47,49,52,65,7]. In fact, many bio-based materials often labelled as “natural” or “biodegradable” may actually contain natural fibers blended with synthetic polymers and undisclosed chemical additives that could migrate into food or enter the environment after disposal in landfills or domestic compost [4,47]. In addition, food packaging can also be a relevant source of food and environmental exposure to micro/nanoplastics (MNPs) [3,21,24,36,41,63,64]. For example, in Southeast Asia, some authors have estimated an annual individual intake of MPs from takeaway food packaging of approximately 195,000 MPs, one of the highest dietary intake rates in the world [21,63]. Therefore, routine use of plastic food packaging may represent a significant contribution to human exposure to MPs. Although there is ongoing debate, recent research suggests that human exposure to MNPs may pose potential health risks and may even adversely affect women during pregnancy [14,19,33,50,6,57,59].

In vitro studies with human cell lines have shown that the degree of toxicity of petroleum-derived microplastics (MPs) varies depending on the cell type, particle size, concentration, polymer type and additives, among others [11,17,22,25,26,35,53,54,60,62]. To our knowledge, little is known about the toxicity of microbioplastics (MBs); only a few studies focusing on specific natural biodegradable polymers, such as polylactic acid (PLA) have been published [43,61]. These investigations conclude that PLA-MBs trigger oxidative stress through excessive production of reactive oxygen species (ROS). Furthermore, since biodegradability enhances the release of microparticles [49], MBs can enter the human food chain and compromise the safety of bio-derived FCMs.

In the present study, we investigated the nature and chemical composition of selected food contact items from the e-commerce market (both petroleum-derived and plant-based), using attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) and scanning electron microscopy (SEM) to assess their compliance with the recent EU regulatory framework on “greenwashing”. Following the characterization of the FCMs and based on these results, this study aimed to evaluate the cytotoxicity of cryomilled micro(bio)plastics derived from them using *in vitro* assays with Caco-2 cells (mimicking human intestinal epithelial cells) and endpoints based on cell viability and ROS production. Finally, the correlation between the observed *in vitro* test results (in particular ROS levels) and the presence of natural antioxidant compounds in MBs obtained from plant fiber-based FCMs, has been investigated using liquid chromatography-quadrupole-time-of-flight mass spectrometry (LC-QTOF-MS).

2. Materials and methods

2.1. Reagents and materials

Crystal Violet (CV), 2’,7’-dichlorofluorescein diacetate (DCFH-DA) and tert-butyl hydroperoxide solution (t-BOOH) (70 % in water) were purchased from Sigma-Aldrich. Stock solutions of DCFH-DA (10 mM in DMSO) and CV (0.2 % in H_2O/MeOH 99:1) were prepared and stored until use at −80 ºC and 4 ºC, respectively. Folin-Ciocalteu reagent, sodium carbonate, phosphate-buffered saline (PBS), sodium dodecyl sulphate (SDS), acetone, formic acid, hydrochloric acid (37 %), HPLC grade methanol and acetonitrile were supplied by Panreac. LC-MS grade acetonitrile was supplied by Scharlab S.L. Gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, kaempherol, kaempherol-3-*O*-glucoside, quercetin, quercetin-3-*O*-glucoside, rutin, caffeic acid, ferulic acid, p-coumaric acid, 5-*O*-caffeoylquinic acid, (+)-catechin, epicatechin and procyanidin B1 were provided by Sigma-Aldrich. Ultrapure water used in all experiments was obtained from a Milli-Q purification system (Merck, Millipore).

The polypropylene MPs reference material (63–100 μm) was kindly provided by the EU Joint Research Center (JRC) (Geel, Belgium).

2.2. Food packaging samples

The selected FCMs include reusable polypropylene (PP) lunchboxes containing triclosan, an antibacterial agent (Lock & Store™); disposable bowls made of plant fibers (Häago™) used in the catering industry; a bio-based lunchbox (Ajaa™) and a coffee cup and lunchbox containing plant fibers (Harko™) (Table 1 and Figure S1, Supplementary Material). All items were purchased through online sales retail platforms.

2.3. Preparation of MPs and MBs

The FCMs were mechanically ground into thin strips and then cryomilled using a laboratory cryogenic ball-mill made of stainless steel (Retsch, Germany). At the end of the process, the particles were dry-sieved using stainless steel mesh filters and size fractions between 50 and 100 μm were selected for *in vitro* testing. No additional sterilization or washing steps were applied after sieving to avoid leaching of additives and bioactive compounds [25].

2.4. Caco-2 cell cultures

Human colon adenocarcinoma Caco-2 cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM-F12, Biowhittaker), supplemented with 50 mg/L each of penicillin, gentamicin and streptomycin (Sigma-Aldrich) and 10 % fetal bovine serum (FBS) (Biowhittaker) (hereafter DMEM-FBS+). Caco-2 cells were maintained at 37 ºC in a humidified 5 % CO_2 incubator.

2.5. Cell treatments

Caco-2 cells were seeded in 24-well microtiter plates (5 ×104 cells/

Table 1
Description of the food containers.

Food package type (Brand name)	**Acronym**	**e-commerce market**	**Plastic category**	**Material information from manufacturer/ supplier**
Lunchbox (Lock & Store™)	PPTCS	USA	Petroleum-based, non-biodegradable	Reusable Microwave safe Polypropylene Antibacterial
Bowl (Häago™)	PL	EU	Plant-based, biodegradable	100 % natural (palm leaf) Disposable/ Compostable Microwave and freezer safe Hot/cold food
Bowl (Häago™)	SCB	EU	Plant-based, biodegradable	100 % natural (sugarcane bagasse) Disposable/ Compostable Microwave and freezer safe Hot/cold food
Lunchbox (Ajaa™)	bio-PE	EU	Bio-based, non-biodegradable	100 % natural (sugarcane juice/ minerals) Renewable materials Reusable
Coffee mug	PPBF	China	Biocomposite, non-biodegradable	Bamboo fiber Reusable Eco-friendly
Lunchbox (Harko™)	PPWS	China	Biocomposite, non-biodegradable	Wheat straw Reusable Eco-friendly Microwave safe

well). This cell line is widely used in biochemical and nutritional research as a model of human colonocytes in cell culture, since it retains its morphology and most of its functions during culture [42]. After 24 h, the DMEM-FBS+ (500 µL) medium was replaced with suspensions of MPs/MBs in DMEM (1 mg/mL of MPs/MBs of particle sizes ranging from 50 to 100 µm), previously homogenized by sonication for 1 min and subsequently vortexed to ensure uniform dispersion of MPs/MBs in the culture medium. A reference material consisting of pristine-PP MPs (63–100 µm) was also used for comparison purposes. Each sample (6 replicates/plate) was analyzed in at least three independent experiments. Caco-2 cells in DMEM or t-BOOH (200 µM in DMEM) solution (6 replicates/plate) were used as negative and positive controls, respectively. After 24 h, MPs/MBs were removed, and cell viability and ROS generation were determined as follows:

2.6. Cell viability

Cell viability was investigated by staining attached cells with the CV dye, which binds to proteins and DNA of living cells and is therefore an indirect method to quantify cell death [15]. Briefly, after removing the MPs/MBs suspensions, cells were washed with PBS, then 0.2 % CV stock solution (see 2.1) was added, and the cells were incubated for 30 min at room temperature. After incubation, the wells were washed twice with water. A 1 % SDS solution was then added to lyse attached cells, and absorbance at 560 nm was measured with a microplate reader. Absorbance readings were normalized to those of negative controls (cells exposed to medium without MPs/MBs) to calculate the percentage of live cells.

2.7. ROS generation

Intracellular ROS production was quantified using a fluorometric assay based on the oxidation of reduced dichlorofluorescein (DCFH-DA) (a non-fluorescent compound) which is converted in the presence of ROS to highly fluorescent dichlorofluorescein (DCF) [8].

Briefly, after completion of the incubation with MPs/MBs, Caco-2 cells were incubated with 5 µM DCFH-DA (see 2.1) for 30 min at 37 ºC. Residual DCFH-DA dye was removed with PBS and then DMEM was added. Fluorescence was measured at excitation and emission wavelengths of 485/530 nm, respectively, using a microplate reader (Bio-Tek, USA). Absolute fluorescence signals were normalized to those of negative controls (unexposed cells in DMEM) and corrected for the number of live cells (CV assay, see 2.6) to calculate the percentage of ROS.

2.8. Fourier transform infrared spectroscopy (FTIR) analyses

The basic composition of the food packages included in this work was identified by FTIR in attenuated total reflection (ATR) mode using a Nicolet iS50 ATR-FTIR spectrophotometer (Thermo Fisher Scientific, USA). Spectra were recorded in the range of 500–4000 cm^{-1} with a resolution of 4 cm^{-1} and an average of 64 scans. Polymer identification was performed by comparing the spectral correspondence of the recorded spectra with the reference spectrum of isotactic polypropylene, polyethylene, and cellulose.

2.9. Scanning electron microscopy (SEM) analyses

SEM images of the surface of intact food packages and the MPs/MBs were obtained using a JEOL JSM-6335F scanning electron microscope (Tokyo, Japan), equipped with an energy dispersive X-ray analyzer (EDX). The surface of small pieces of the food packaging was coated with a thin conductive layer of gold before being observed under a microscope. For the MPs/MBs, sample preparation consisted of pouring a few mg onto a coverslip and coating it with a gold layer. An acceleration voltage of 5–20 kV was used with an effective distance of 8–15 mm. SEM images were obtained at different magnifications from 80 to 20,000 X.

2.10. Extraction of (poly)phenolic compounds from MBs and determination of Total Phenolic Content (TPC)

The extraction of (poly)phenols was performed following the procedure described by [37]. Briefly, 125 mg of MBs (from plant-derived Häago™ bowls, Table 1) were mixed with 4 mL of a methanol/water (50:50, *v/v*) solvent mixture (M1) for 1 h by shaking, and then centrifuged at 1250 x g for 10 min. The supernatant was collected and the MBs were re-extracted by shaking with 4 mL of the acetone/water (70:30, *v/v*) solvent mixture (M2) for 1 h and then, centrifuged under the same conditions. Both supernatants were combined and diluted to 10 mL with the M1 +M2 (50:50, *v/v*) solvent mixture. The extracts were evaporated to dryness under a gentle nitrogen stream. Finally, 250 µL of the 1 % formic acid/acetonitrile (90:10, *v/v*) solvent mixture was added, and the extracts were filtered through 0.45 µm cellulose syringe filters.

The TPC was determined by the Folin-Ciocalteu assay [48]. First, a standard calibration curve of gallic acid was prepared over the concentration range of 10–400 mg/L in a 1 % aqueous formic acid/-acetonitrile (90:10, *v/v*) solution. Then, 25 µL of the sample extracts, standards, or the solvent mixture was mixed with 25 µL of the Folin-Ciocalteu reagent and allowed to react for 3–8 min at room temperature. Subsequently, 500 µL of sodium carbonate (75 g/L) solution and 750 µL of water were added, and the mixture was stirred in the dark at room temperature for 1 h. Absorbance was then measured in 96-well microtiter plates using a microplate reader set to 750 nm. Interpolation from the standard calibration curve yielded the TPC value, expressed as µg of gallic acid equivalents (GAE) per g of MBs. All extracts were measured in triplicate.

2.11. Identification and quantitation of (poly)phenolic compounds by LC-QTOF-MS analysis

(Poly)phenolic compounds in PL and SCB-MBs extracts were tentatively identified and quantified by targeted LC-MS/MS analysis performed on an Agilent 1200 series LC instrument (Agilent Technologies, USA) coupled to an Agilent G6530A quadrupole-time-of-flight (QTOF) mass spectrometer with a JetStream electrospray (ESI) ionization source according to the procedure described in Mateos et al. [37]. The separation was performed using a Luna C_{18} analytical column (50 ×2 mm, 5 μm) protected by a guard column of the same stationary phase (both from Phenomenex, USA) and a gradient of water (eluent A) and acetonitrile (eluent B), both acidified with 0.1 % formic acid at a flow rate of 0.4 mL/min. The gradient elution started with 5 % eluent B and increased linearly to 30 % B in 15 min, then to 50 % B in 5 min, and finally to 90 % B in a another 5 min, where it remained isocratic for 4 min; finally returned to initial conditions (5 % B) in 1 min and was held for 7 min for column reconditioning. The injection volume was set at 5 μL. The QTOF analyzer was operated in negative ion mode within the range 100–970 *m/z* with the following parameters: capillary voltage, 3.5 kV; collision energy 150 V; drying gas temperature, 325°C; drying gas flow, 10 L/min; pressure of the nebulizer gas, 25 psi. The Masshunter Data Acquisition B.04.01 and Masshunter Qualitative/Quantitative Analysis B.07.00 software (Agilent Technologies, USA) were used for system control and data analysis, respectively. The identification of (poly)phenolic compounds in MBs extracts was performed by comparing retention times, mass accuracy and MS/MS fragments in negative mode with their standards, if available, or by comparing the characteristics of their mass spectra with those reported in mass spectral databases (namely, MassBank and PubChem databases) or the literature.

Quantification was achieved by preparing external calibration curves with the following standards (gallic acid, protocatechuic acid, 4-hydroxybenzoic acid, 5-*O*-caffeoylquinic acid, caffeic acid, ferulic acid, *p*-coumaric acid, kaempherol, kaempherol-3-*O*-glucoside, quercetin, quercetin-3-*O*-glucoside, rutin, catechin, epicatechin, procyanidin B1) at five different concentration levels from 0.01 to 25 μM. The limits of detection and quantification ranged from 0.003 to 0.008 μM and from 0.005 to 0.01 μM, respectively. The inter- and intra-day precision (as the coefficient of variation, ranging from 3.6 % to 7.5 %) was considered acceptable and allowed the quantification of (poly)phenolic compounds (quantified as equivalents of the respective parent molecules when available or of the most chemically related compound).

2.12. Statistical analysis of in vitro assay data

Results are expressed as the mean ± standard deviation (SD). Statistical analysis was performed using SPSS version 29.0. Prior to statistical analysis, data homogeneity of variance was tested using Levene's text. For multiple comparisons, one-way analysis of variance (ANOVA) was used to assess significant differences between the mean values of the different biological parameters in the control and treatment groups. A p value < 0.05 was considered to indicate statistical significance. In addition, hierarchical cluster analysis was performed using Statgraphics. Distance was represented as the squared Euclidean distance, and clustering was calculated according to Ward's method.

3. Results and discussion

3.1. Characterization of the FCMs

This work focuses on food packaging purchased exclusively through e-commerce, as it has become an essential part of global retail. The European Parliament recently approved a Directive ([10]/825) that prohibits greenwashing claims, such as "environmentally friendly", "biodegradable", and "natural", without official proof. The phenomenon of greenwashing is relatively common in the e-commerce market, as platforms have not yet established robust regulatory and control mechanisms [58]. Therefore, the first part of the work consisted of evaluating the composition of the food packaging materials to assess their compliance with the new EU Directive.

3.1.1. FTIR spectra

The identity of the Lock & Store™ polypropylene lunchboxes (Table 1, Figure S1 Supplementary Material) was confirmed in previous work [36]. Fig. 1 shows the spectra of the other FCMs included in this study. The FTIR spectra of the plant fiber-based Häago™ bowls (Fig. 1a) match the reference spectrum of α-cellulose, the main component of plant fibers. The broad band observed at 3370 cm^{-1} and the high intensity band at 1031 cm^{-1} are common characteristic bands of cellulose corresponding to the -OH stretching vibration and the asymmetric stretching of C-O-C, respectively [40]. On the other hand, the FTIR spectrum of the Ajaa™ lunchbox (Fig. 1b) shows characteristic absorption bands at 2918 cm^{-1} and 2848 cm^{-1}, corresponding to asymmetric and symmetric stretching vibrations -CH_2; bending -CH_2 at 1471 cm^{-1} confirming that it is polyethylene (PE). Moreover, the spectrum shows an additional band at 1018 cm^{-1}, which matches the reference spectrum of a clay mineral (hydrated magnesium silicate $Mg_3Si_4O_{10}$ x H_2O, talc) commonly used as a reinforcing inorganic filler to improve the overall performance of plastic FCMs [46]. Although the FTIR spectrum cannot differentiate whether PE comes from renewable or petroleum resources, according to the information provided by the supplier it is a renewable material (100 % natural) and can therefore be classified as bio-PE. While bio-PE is produced from agricultural resources (e.g. sugarcane), it is non-biodegradable, and manufacturers must include this information on the packaging label, as terms such as renewable, or "natural" can cause considerable confusion among consumers.

Regarding the coffee mug and the Harko™ lunchbox, the information on the sales website only mentions that they are made of "eco-friendly" bamboo fiber and wheat straw, respectively; however, their FTIR spectra (Fig. 1c) closely match those of isotactic PP. Indeed, the characteristic absorption peaks at 2950, 2916 and 2870 and 2835 cm^{-1} are attributed to stretching vibrations of -CH_3 and -CH_2, while the peaks at 1455 and 1375 cm^{-1} indicate the presence of bending vibrations of -CH_2 and -CH_3 of PP, respectively. Furthermore, two weak bands are observed around 3370 cm^{-1} (-OH groups) and 1640 cm^{-1} that could correspond to those of the reference spectrum of α-cellulose. In conclusion, these food containers are a clear example of greenwashing, as they are made primarily of non-biodegradable petroleum-derived plastics (PP) and contain an unspecified percentage of plant fibers (bamboo or wheat straw) added as additives/fillers.

3.1.2. SEM micrographs

The morphology of the food packaging samples was also examined using SEM-EDX (Figure S2, Supplementary Material). Analysis of the Lock & Store™ lunchbox (Figure S2a) shows a fairly smooth surface, and the elemental composition corresponds to C. In contrast, the disposable bowls made from palm leaves (Figure S2b) and sugarcane bagasse (Figure S2c) exhibit a rough and complex fibrous structure, respectively. The EDX spectra show the presence of C and O from cellulose, as well as other minerals naturally present in plant biomass (e.g. palm leaves, Figure S2b), while the spectrum of the sugarcane bagasse bowl (Figure S2c) only shows C and O, as most of the minerals are lost during the extraction of juice from sugarcane stalks.

The Ajaa™ lunchbox, made of bio-PE (Figure S2d), features a rough surface and an elemental composition of C and small amounts of O, Si and Mg from inorganic reinforcing fillers.

SEM micrographs of the coffee mug (Figure S2e) and the Harko™ lunchbox (Figure S2f) show significant differences: the coffee mug has a smooth surface with the presence of protrusions, while the lunchbox appears rougher and more porous.

Elemental analysis confirmed that the predominant element is C

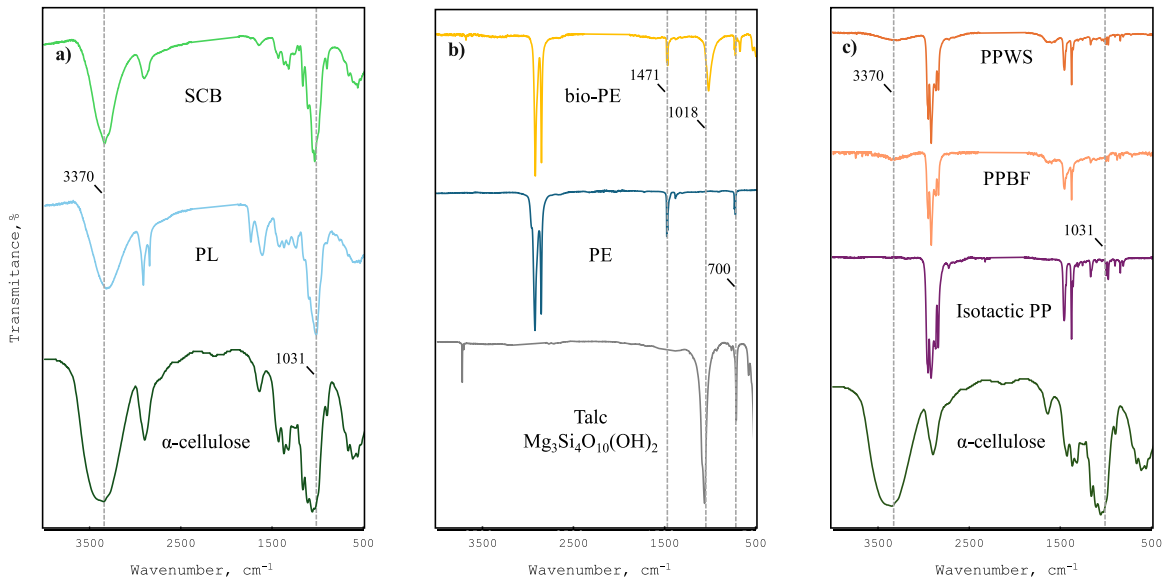

Fig. 1. FTIR fingerprints of a) Häago™ palm leaf (PL) and sugarcane bagasse (SCB) plant fiber bowls; b) Ajaa™ lunchbox; c) coffee cup and Harko™ lunchbox and comparison with the reference spectra of α-cellulose, polyethylene and isotactic polypropylene.

from PP. However, SEM images at higher magnifications (5000–20,000 x) reveal several white spots (insets e and f) whose elemental composition corresponds mainly to C, O and other elements (Ca, K, Na, S, Cl), which could be attributed to plant fibers (bamboo fiber and wheat straw) added as fillers in very low quantities.

The use of plant fibers as additives in plastic FCMs has been banned in the EU since 2021. However, composite products made of conventional plastic and plant fibers are still marketed in China [49] which is the source market for both items (Table 1).

Based on these findings, government regulators should establish more controls in the e-commerce market and audit product information provided by manufacturers to ensure the authenticity of product descriptions and compliance with legislation.

3.2. *"In vitro" assays*

Once the actual composition of the food packages was determined, the cytotoxicity of the MPs/MBs obtained from them (Fig. 2) was investigated.

Some studies have estimated a global human consumption of MPs of 0.1–5 g per week [44]. However, assessing the actual dietary intake of MPs is a current scientific challenge [35], due to the lack of standardized analytical methodologies and suitable reference materials for their reliable detection and quantification [9]. Furthermore, dietary intake of MPs can vary considerably depending on several factors, including lifestyle, diet pattern and geographic location, among others.

In this work, the doses selected for *in vitro* testing were 1 mg/mL of MPs/MBs. Although these doses could be considered relatively high, they are similar to those reported in other studies with different human cellular models [17,35], since the goal of *in vitro* testing is to understand the mechanisms of toxicity at the cellular level in the most adverse scenarios (e.g. long-term MPs accumulation in the intestinal mucosa).

3.2.1. *Cell viability and ROS generation*

Previous *in vitro* experiments in Caco-2 cells have already shown that MPs size has an important impact on their cellular uptake and toxicity [53,55]; therefore, we have not addressed this aspect.

On the other hand, irregularly-shaped MPs cause more harmful physical effects on cells than spherical MPs [33,55], which poses a higher risk. In this sense, unlike most studies, which have used commercially manufactured synthetic spherical particles, this study employed MPs/MBs and a PP-MPs reference material (50–100 μm) obtained by cryomilling to better replicate MPs present in the environment and human tissues [25].

Therefore, rather than focusing on the size or shape of MPs/MBs, the study aimed to evaluate differences in toxicity of various types of MPs/MBs due to their composition and physicochemical characteristics (e.g. lipophilicity and hydrophilicity), which may influence the leaching of additives or bioactive compounds.

As shown in Fig. 3a, none of the MPs derived from PP-FCMs (e.g. pristine-PP, PPTCS, PPWS, PPBF) or bio-PE caused significant changes in cell viability. However, MBs obtained from plant-derived FCMs (e.g. PL and SCB) significantly increased cell viability (by 30–35 %) compared to the control.

Furthermore, the measured intracellular ROS (Fig. 3b) clearly show two opposing patterns: some PP-MPs significantly increased ROS levels by up to 25 %, while cells exposed to MBs from plant-based FCMs showed a significant reduction in ROS generation (by 20–25 %) compared to the control cells. In other cases, ROS levels (bio-PE, PPWS) were either not statistiscally different from controls or were intermediate (PPBF) between those of controls and the highest ROS levels observed.

In summary, it could be suggested that none of the MPs tested damaged cellular integrity during the incubation period. On the contrary, some PP-MPs induced cellular stress, while plant-derived MBs reduced ROS generation.

Furthermore, a hierarchical cluster analysis was used to identify similarities and differences. As shown in Fig. 4, the dendrogram classified MPs/MBs into three main clusters, where the length of the vertical lines in the dendrogram measures the separation between the merged clusters. Cluster 1 corresponds to MBs from plant-derived FCMs with a non-cytotoxic effect and lower ROS levels than the control group. Cluster 2 includes bio-PE and PP-MPs containing bamboo or wheat straw fibers, with non-cytotoxic effect and ROS levels comparable to those of the control group. Cluster 3 corresponds mainly to PP-MPs (pristine and with triclosan), without cytotoxic effect, but which trigger moderate oxidative stress in Caco-2 cells.

Overall, none of the PP-MPs induced an acute toxic effect, as no significant differences in cell viability were observed in Caco-2 cells after 24 h exposure. These results are consistent with those reported by other authors using lower or similar concentrations of MPs [17,25]. However, PPTCS MPs induced moderate oxidative stress in the same

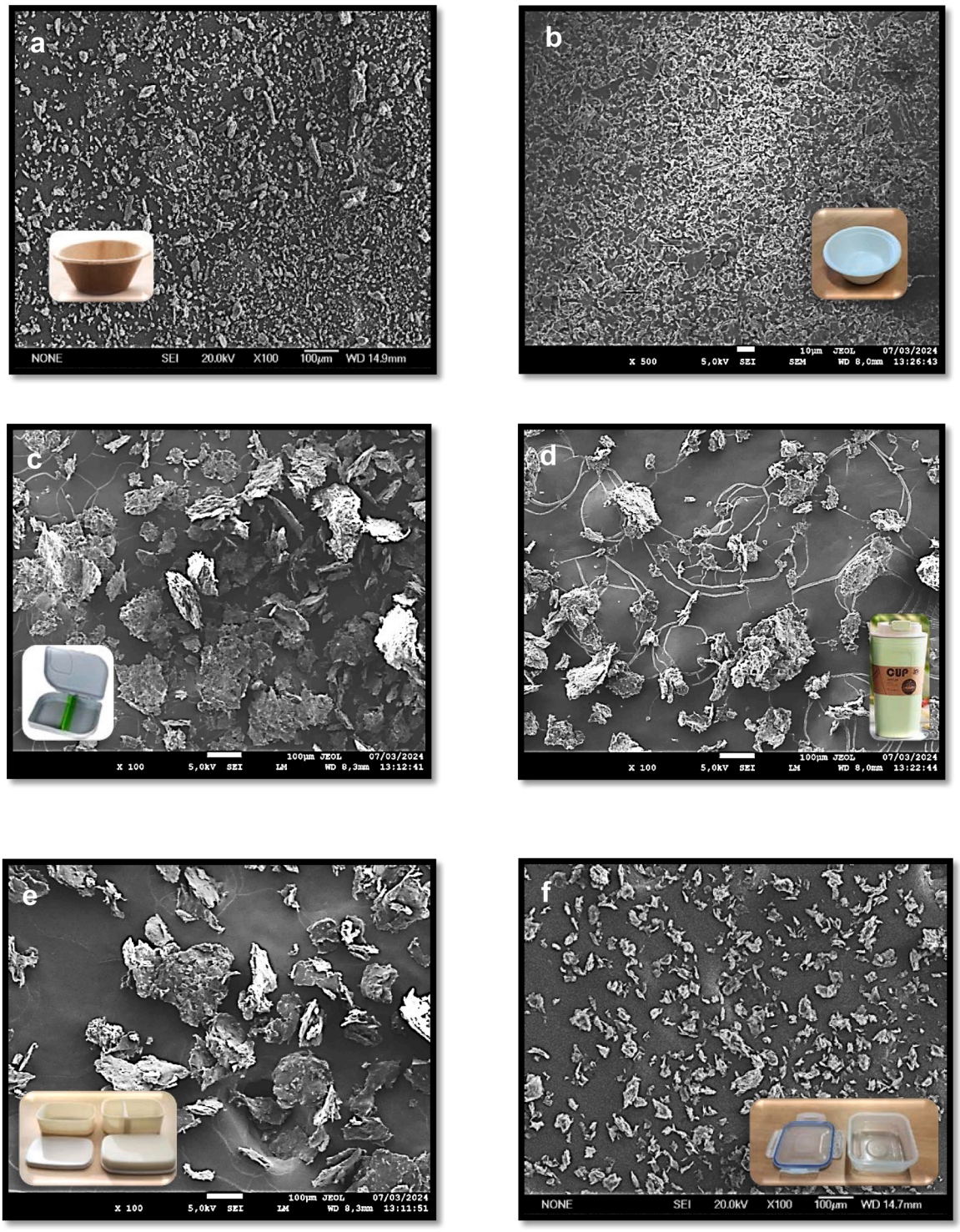

Fig. 2. SEM images of micro(bio)plastics obtained by cryomilling of: a) Häago™ PL bowl; b) Häago™ SCB bowl; c) Ajaa™ lunchbox; d) coffee cup; e) Harko™ lunchbox; f) Lock & Store™ lunchbox. SEM operating parameters: acceleration voltage 5–20 kV; working distance 8–14 mm; magnification range 100–500 X.

manner as pristine-PP MPs, while MPs containing plant fibers (bamboo or wheat straw) did not significantly affect ROS levels. The antibacterial additive triclosan (TCS) has been reported to cause oxidative stress in normal and cancer human cell lines due to ROS production mechanisms [31]. In this study, no cytotoxicity or oxidative stress was observed after 24 h of exposure of Caco-2 cells to very low levels of TCS (2.6 µM) (data not shown); however other authors have reported that dietary concentrations $\geq$ 10 µM for 3 weeks can promote mild colonic inflammation in mice [56]. Previous results obtained from *in vitro* gastrointestinal digestion assays have estimated a bioaccessible intake of 300 µg/week of TCS from MPs released from microwaved PPTCS lunchboxes [29], which could trigger proinflamatory or cytotoxic effects after chronic exposure.

After observing a significant decrease in ROS levels following exposure to MBs of sugarcane bagasse (SCB) and palm leaf (PL), additional experiments were conducted to evaluate their potential as ROS scavengers. Therefore, cell viability and ROS assays were performed by exposing Caco-2 cells to a positive control (e.g. t-BOOH, 200 µM) in the

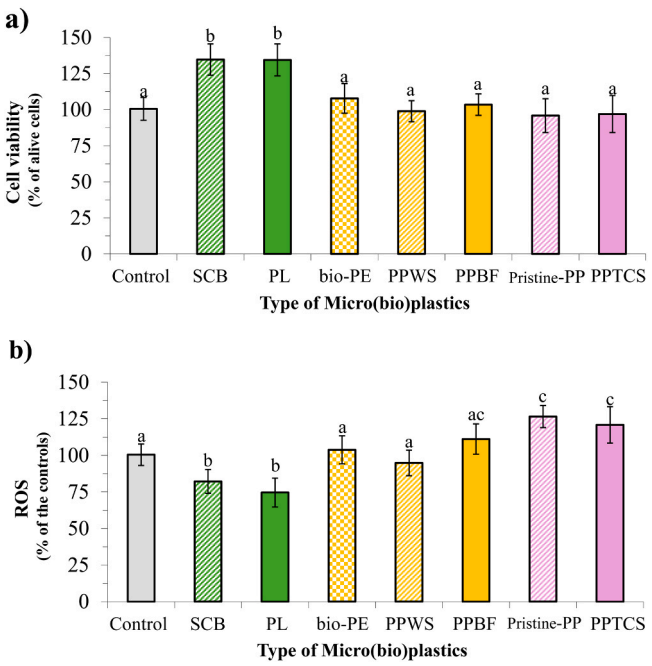

Fig. 3. a) Cell viability and b) ROS generation in Caco-2 cells after exposure to micro(bio)plastics for 24 h. Data are calculated as percentage of the relative increase or decrease with respect to control values and are expressed as mean ± standard deviation of three biological replicates. Different letters at the top of each bar indicate statistically significant differences ($p < 0.05$).

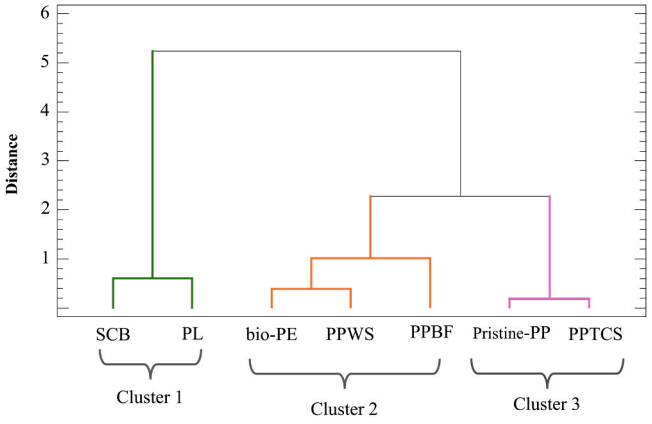

Fig. 4. Dendrogram of clusters, grouped according to the results obtained from cell viability and ROS generation, using the squared Euclidean distance measure and Wards agglomerative procedure.

absence/presence of different concentrations (0.1–2.0 mg/mL) of MBs.

As shown in Fig. 5a, cell viability decreased by approximately 40 % when cells were exposed to positive control, compared to the untreated control. However, when cells were exposed to positive control in the presence of SCB-MBs at concentrations above 0.1 mg/mL, a partial recovery of cell viability of up to 70 % was observed. Meanwhile, in the presence of PL-MBs, cell viability increases progressively, reaching values of around 115 % at the highest concentration tested (2.0 mg/mL).

On the other hand, a clear pattern of increased ROS production is observed after exposure of Caco-2 cells to the positive control (Fig. 5b), which could not be prevented by incubation with SCB-MBs, regardless of the concentration tested. In contrast, PL-MBs induced a gradual decrease in ROS levels, until they were restored to the control group level at a concentration of 2 mg/mL. These results indicate that PL-MBs in

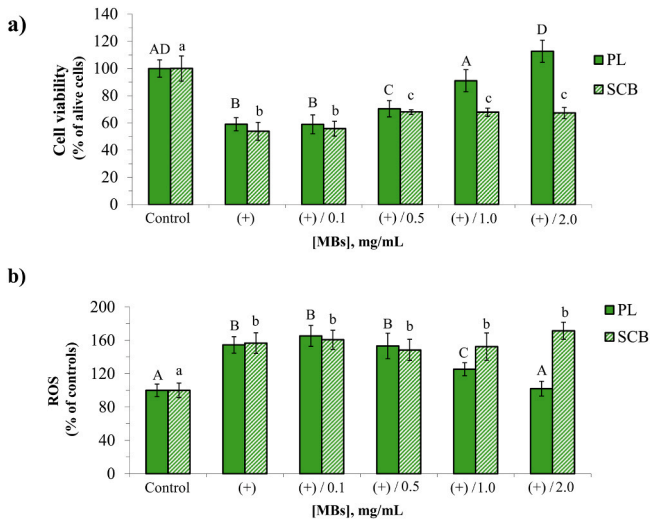

Fig. 5. a) Cell viability and b) ROS generation in Caco-2 cells after 24 h of exposure to different concentrations (0.1–2.0 mg/mL) of PL and SCB-MBs in the presence of 200 μM t-BOOH (+). Data are calculated as percentage of the relative increase or decrease with respect to the control values and are expressed as mean ± standard deviation of three biological replicates. Different letters at the top of each bar indicate statistically significant differences ($p < 0.05$).

particular, behave as potential ROS scavengers, showing a dose-dependent protective effect against oxidative stress.

Plant-derived MBs are naturally hydrophilic, unlike petroleum-derived MPs; therefore, the leaching of antioxidant bioactive compounds (if present) from MBs into the cell culture medium, could contribute to the scavenging of ROS species and exert a potential cytoprotective effect [27].

3.2.2. *Determination of the (poly)phenol composition in MBs of plant origin*

Both sugarcane bagasse and palm leaves are plant biomass rich in phytochemicals (e.g. (poly)phenols), known for their potent antioxidant and anti-inflammatory activities, demonstrated in *in vitro* and *in vivo* assays [23,27,30]. Therefore, to investigate the potential correlation between the reduction in ROS levels observed in *in vitro* assays and the presence of antioxidant bioactive compounds in MBs, the total (poly) phenol content (TPC) was measured in SCB and PL-MBs extracts, according to the Folin-Ciocalteau assay. As shown in Table S1 (Supplementary Material), the results, expressed as μg of gallic acid equivalents (GAE) per g of MBs, indicate a relatively high content (700 ± 100 μg/g) of antioxidant compounds in PL-MBs extracts compared to SCB-MBs extracts (undetectable).

However, the Folin-Ciocalteau assay, despite being a reference method for the quantification of total (poly)phenols, is not specific for these natural compounds, as other easily oxidizable non-phenolic compounds present in plant extracts can also be quantified, leading to an overestimation of the TPC [23]. Therefore, the extracts were subsequently analyzed by LC-QTOF-MS/MS. As summarized in Table 2, the (poly)phenolic compounds identified in these extracts were predominantly phenolic acids (hydroxybenzoic acids and hydroxycinnamic acids) and flavonoids (e.g. flavanols and flavonols). These results are consistent with previous studies on extracts of sugarcane bagasse, date palm and other plant leaves [1,23,5]. The total (poly)phenol content was 18 times higher in PL-MBs extracts than in SCB-MBs extracts, which might be due to the fact that heat and other pretreatments applied during SCB processing can lead to the destruction of certain (poly) phenols [27].

Furthermore, differences were observed in the relative amounts of the different (poly)phenol classes. For example, phenolic acids were the most abundant (90 % of the total) in SCB-MBs extracts, and among

Table 2
List of (poly)phenols identified and quantified[a] in extracts of plant fiber MBs by LC-QTOF-MS/MS.

Compound	Retention time (min)	Formula	Molecular Mass	[M-H]⁻, *m/z*	MS/MS, *m/z*	µg/g PL-MBs	µg/g SCB-MBs
Hydroxybenzoic acids							
Gallic acid*	0.8	$C_7H_6O_5$	170.0215	169.0142	125	3.2 ± 0.1	0.33 ± 0.01
Protocatechuic acid*	1.6	$C_7H_6O_4$	154.0266	153.0193	109	4.4 ± 0,8	1.7 ± 0.3
Hydroxybenzoic acid	2.6	$C_7H_6O_3$	138.0317	137.0244	93	1.10 ± 0. 08	8.9 ± 0.5
4-hydroxybenzoic acid*	2.8	$C_7H_6O_3$	138.0317	137.0244	93	15.2 ± 0.9	n.d
Syringic acid	5.8	$C_9H_{10}O_5$	198.0528	197.0455	123	17.0 ± 1,2	3.6 ± 0.8
Class Total						**41 ± 3 (9.1 %)**	**14 ± 2 (28.3 %)**
Hydroxycinnamic acids and derivatives							
Caffeoylquinic acid	2.1	$C_{16}H_{18}O_9$	354.0951	353.0878	191	n.d	0.47 ± 0.09
Coumaric acid	4.2	$C_9H_8O_3$	164.0473	163.0401	119	13.9 ± 0.5	n.d
5-*O*-Caffeoylquinic acid (chlorogenic acid) *	4.3	$C_{16}H_{18}O_9$	354.0951	353.0878	191	18.6 ± 0.5	0.8 ± 0.1
Caffeic acid*	4.7	$C_9H_8O_4$	180.0423	179.0350	135	1.2 ± 0.2	1.14 ± 0.05
Caffeoylquinic acid	4.8	$C_{16}H_{18}O_9$	354.0951	353.0878	191; 173	2.4 ± 0.1	0.6 ± 0.3
Coumaroylquinic acid	6.1	$C_{16}H_{18}O_8$	338.1002	337.0929	191; 163	232 ± 9	7.3 ± 0.7
p-Coumaric acid*	6.5	$C_9H_8O_3$	164.0473	163.0401	119	7 ± 1	n.d
Feruloylquinic acid	7.1	$C_{17}H_{20}O_9$	368.1107	367.1035	193	10.2 ± 0.6	n.d
Ferulic acid*	7.8	$C_{10}H_{10}O_4$	194.0579	193.0506	134	0.8 ± 0.2	n.d
Feruloylquinic acid	8.1	$C_{17}H_{20}O_9$	368.1107	367.1035	193; 191	n.d	5.8 ± 0.1
Rosmarinic acid	11.4	$C_{18}H_{16}O_8$	360.0845	359.0772	161;135;72	11 ± 3	16 ± 1
Class total						**297 ± 15 (66.1 %)**	**32 ± 2 (61.7 %)**
Flavonols							
Kaempferol-3,7-*O*-diglucoside	7.7	$C_{27}H_{30}O_{16}$	610.1534	609.1461	447;285	11.1 ± 0.7	n.d
Quercetin-rhamnoside (quercitrin)	8.1	$C_{21}H_{20}O_{11}$	448.1006	447.0933	300;301;284	10.9 ± 0.3	n.d
Quercetin-galactoside-rhamnoside	8.2	$C_{27}H_{30}O_{16}$	610.1534	609.1461	301	9.0 ± 0.9	n.d
Kaempherol-rutinoside	8.8	$C_{27}H_{30}O_{15}$	594.1585	593.1512	285;257	42 ± 2	0.31 ± 0.03
Quercetin-3-*O*-rutinoside (rutin)*	9.0	$C_{27}H_{30}O_{16}$	610.1534	609.1461	300	n.d	1.7 ± 0.2
Quercetin-3-*O*-glucoside (isoquercitrin)*	9.2	$C_{21}H_{20}O_{12}$	464.0955	463.0882	301; 300	0.42 ± 0.04	0.45 ± 0.02
Kaempherol-*O*-galactoside	9.7	$C_{21}H_{20}O_{11}$	448.1006	447.0933	285	37 ± 3	0.29 ± 0.06
Kaempherol-3-*O*-glucoside*	10.7	$C_{21}H_{20}O_{11}$	448.1006	447.0933	285	n.d	1.2 ± 0.4
Kaempherol*	13.8	$C_{15}H_{10}O_6$	286.0477	285.0405	257	0.03 ± 0.01	0.06 ± 0.01
Class total						**110 ± 7 (24.7 %)**	**4.0 ± 0.7 (8.0 %)**
Flavanols							
Catechin*	3.8	$C_{15}H_{14}O_6$	290.079	289.0718		n.d	0.22 ± 0.01
Epicatechin*	5.9	$C_{15}H_{14}O_6$	290.079	289.0718		0.20 ± 0.05	0.30 ± 0.07
Procyanidin B1*	5.6	$C_{30}H_{26}O_{12}$	578.1424	577.1351		n.d	0.50 ± 0.03
Class total						**0.20 ± 0.05 (0.1 %)**	**1.0 ± 0.1 (2.0 %)**
Total (POLY)phenolic compounds						**448 ± 25**	**51 ± 5**

*Identification of compounds was confirmed by the authentic standard. All other compounds were tentatively identified by comparing their MS characteristics with those reported in the literature and MS databases (e.g. MassBank and PubChem).
[a]Quantitative results are expressed as mean ± standard deviation of three replicates.
n.d: Content below the limit of detection (LOD)

them, hydroxycinnamic acids were the predominant group (62 %), which behave more efficiently as antioxidants than hydroxybenzoic acids [38]. In contrast, in PL-MBs extracts, the percentage of phenolic acids was 75 %, and hydroxycinnamic acids accounted for 66 % of the total phenolic content. Among them, coumaric acid and its derivatives were the most abundant in PL-MBs extracts, while rosmarinic acid was the main compound in SCB-MBs extracts, followed by ferulic and coumaric acid derivatives. The PL-MBs extracts also showed a significant amount of rosmarinic acid, which is particularly relevant due to its potent antioxidant activity provided by the double dihydroxyl group in the ortho position.

Flavonoids, particularly flavonols, were the second most abundant group of phenolic compounds in PL-MBs extracts representing 24.7 % of the total, with kaempherol derivatives being more abundant than quercetin derivatives. In contrast, in SCB-MBs extracts the flavonol content was much lower (8 %), with a slightly higher content of quercetin derivatives compared to kaempherol. Finally, flavanols were the least abundant (poly)phenolic compounds, representing 2.0 and 0.1 % of the total in SCB and PL-MBs extracts, respectively.

Furthermore, the higher content of (poly)phenols (particularly hydroxycinnamic acids) in PL-MBs extracts could be responsible for the dose-dependent ROS scavenging and cytoprotective potential observed in *in vitro* assays. This effective protection has also been observed in previous studies with other cell lines (e.g. human HepG-2 cells), subjected to oxidative stress and treated with sugarcane extracts rich in (poly)phenolic compounds [51].

However, translating the results of *in vitro* research to humans is challenging, due to the highly complex mechanisms of absorption, biotransformation in the digestive tract and action of MPs in the human body environment [32,35,34]. Therefore, the differences between cellular models and the human body undoubtedly constitute a limitation of *in vitro* testing.

Overall, plant-based food packaging can be considered a sustainable alternative to single-use plastics, which often release significant amounts of MPs and additives, such as synthetic phenolic antioxidants that have been linked to cytotoxic, carcinogenic and endocrine-disrupting effects [12,20,26].

However, further research is needed to elucidate the benefits and limitations of plant-active packaging under real-use conditions. Indeed, further experiments will be conducted to evaluate polyphenol migration in different food simulants (e.g., acidic, lipophilic) or foods and packaging uses (e.g. microwave heating, hot or cold foods), as well as to assess the stability, bioaccessibility, and bioavailability of polyphenolic compounds using *in vitro* gastrointestinal digestion procedures.

4. Conclusions

Commercial food packaging labelled as “eco-friendly” or “natural” often contains a high proportion of non-biodegradable conventional plastics. This study investigated the nature of several food contact items derived from conventional plastics and plant biomass, purchased from e-commerce platforms. ATR-FTIR characterization identified misleading “eco-friendly” labelling on some of the packaging analyzed, as well as non-compliance with the EU regulatory framework prohibiting the marketing of plastic FCMs containing plant fiber additives (e.g. bamboo fiber). The European Union recently took a leading role by prohibiting false or misleading environmental claims and unproven sustainability labels on products, while other countries lack such regulations. This study demonstrates the need for global regulations to ensure proper labelling of food packaging, prevent unfair trade practices, and help consumers make informed choices.

Furthermore, the extensive use of single-use plastics contributes to global MPs pollution. However, while biomass-derived FCMs are considered sustainable alternatives, risk assessment studies for MBs are needed. This work has revealed the dose-dependent antioxidant potential of MBs (particularly, those derived from palm leaf food packaging) in *in vitro* studies with Caco-2 cells subjected to oxidative stress. This effect can largely be attributed to the high content of (poly)phenols present in PL-MBs extracts, which are natural antioxidant compounds.

These findings contribute to the risk assessment of plant-derived FCMs, which could help establish future regulatory guidelines on permissible levels of MBs in foods and have valuable implications for their potential use as active food packaging.

Nevertheless, future research on the human toxicity assessment of MPs/MBs should explore novel experimental models combined with machine learning computer simulations to improve the predictive capacity and accuracy of toxicity assessment.

Environmental implications

Food packaging accounts for 60 % of global plastic waste, contributing to the rise of microplastics (MPs), a major environmental concern. Compostable/biodegradable food packaging is a sustainable alternative to mitigate plastic pollution. This research identified mislabeling on some so-called plant-based food packaging, made of non-biodegradable polypropylene (PP) and containing plant-based additives banned in the EU. This study compared the cytotoxicity of conventional PP-MPs with that of microbioplastics (MBs) derived from truly plant-based food containers. Palm leaf-MBs showed dose-dependent protection against oxidative stress, attributable to natural antioxidant antioxidant polyphenols. Therefore, there are sustainable alternatives to mitigate the risks associated with MPs.

CRediT authorship contribution statement

M. Dolores Marazuela: Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Miguel Klaiber:** Writing – review & editing, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. **Adrián Domene:** Validation, Software, Methodolog, Investigation, Data curation. **Raquel Mateos:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation. **Sonia Ramos:** Writing – review & editing, Supervision, Resources, Methodology, Investigation, Formal analysis, 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 the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jhazmat.2025.140782.

Data availability

Data will be made available on request.

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