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Influence of medicinal and aromatic plants into risk assessment of a new bioactive packaging based on polylactic acid (PLA)

Georgiana-Luminita Gavril[a], Magdalena Wrona[b], Anis Bertella[c], Michał Świeca[d], Maria Râpă[e], Jesús Salafranca[b], Cristina Nerín[b,*]

[a] Department of Bioinformatics, National Institute of Research and Development for Biological Sciences, 296 Splaiul Independentei, sector 6, 060031, Bucharest, Romania
[b] Department of Analytical Chemistry, Aragon Institute of Engineering Research I3A, EINA-University of Zaragoza, Torres Quevedo Building, María de Luna 3, 50018, Zaragoza, Spain
[c] Laboratory of Applied Microbiology, Department of Biology, Faculty of Life and Natural Sciences, University of Oran 1 Ahmed BenBella, 31100, Algeria
[d] Department of Biochemistry and Food Chemistry, University of Life Sciences in Lublin, 8 Skromna Street, 20-704, Lublin, Poland
[e] Faculty of Materials Science and Engineering, University Politehnica from Bucharest, 313 Splaiul Independentei, 060042, Bucharest, Romania

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ABSTRACT

A new biodegradable antioxidant active packaging for food applications based on antioxidants from medicinal and aromatic plants incorporated into a polylactic acid matrix was designed and developed. Melt blending processing technique was applied to prepare polylactic acid films loaded by sage and lemon balm leaves. Antioxidant properties of developed active films were investigated using the following methods: 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl and a home-made generator of hydroxyl radicals. In addition, reducing power and total phenolic content of polylactic acid films were checked. The results of antioxidant capacity showed that percentage of hydroxylation for active film with lemon balm and sage was 55.5% ± 0.1% and 67.4% ± 0.3%, respectively. The reducing power of active films increased 8 times in comparison to the blank samples.

Moreover, extensive investigation of influence of sage and lemon balm leaves on material safety and type of migrants was performed using migration assays. The composition of both non-volatile and volatile compounds of different active packaging films was compared with neat polylactic acid film. Three different food simulants such as 95% (v/v) ethanol, 10% (v/v) ethanol and 3% (w/v) acetic acid were checked. It was shown that the addition of sage and lemon balm leaves into a polylactic acid structure decreased the migration of both linear and cyclic polylactic acid oligomers, currently not legislated by European Union. Besides, total absence or decrease of migration of volatile compounds were observed when using the active films. Both thermal and mechanical properties of films were also evaluated.

1. Introduction

Plastics have become the world's most chosen materials in food packaging applications due to its performance, cost effectiveness and durability (Majid et al., 2018). Unfortunately, they have resulted in a global transboundary pollution problem (The Lancet Planetary Health, 2018). Therefore, researches of the last decade have focused on finding solutions for replacing traditional plastic with bio-plastics that could be applied for bio-packaging applications.

The bio-packaging and the active packaging with antioxidant properties are new areas of technology. Antioxidants are a group of food preservatives that delay or prevent food deterioration through oxidative mechanisms. They can work as scavengers, either by preventing the formation of reactive species, or by removing them before they start damaging processes of food (Lin et al., 2018). There is currently a general tendency in the food industry to replace the use of synthetic antioxidants with natural ones, such as flavonoids present in medicinal plants (Lin et al., 2018). Natural compounds have been recently studied and proven to be effective antioxidants and once they are incorporated into packaging materials these could be utilised as antioxidant stuffs in different fields like food, pharmaceuticals or cosmetics (Masek et al., 2018; Wrona et al., 2015, 2017a, 2017b).

Extensive studies on medicinal plants have been performed and published. Either fresh or dried leaves of *Melissa officinalis* L. (lemon

* Corresponding author.
E-mail addresses: georgi.gavril@yahoo.com (G.-L. Gavril), magdalenka.wrona@gmail.com (M. Wrona), bertella.anis@gmail.com (A. Bertella), michal.swieca@up.lublin.pl (M. Świeca), rapa_m2002@yahoo.com (M. Râpă), fjsl@unizar.es (J. Salafranca), cnerin@unizar.es (C. Nerín).

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balm) are used as medicine and should be harvested before flowering. However, the amount of essential oils in the plant is very small and the storage conditions of the dry matter have a huge influence on it. According to the literature, the extract of lemon balm waste improved the quality and shelf life of bread (Vasileva et al., 2018). Besides, the extracts of lemon balm incorporated into cupcakes added advantageous functional properties to them (Caleja et al., 2018). In the case of *Salvia officinalis* L. (sage), their main components described in literature are citronellal, citral, citronellol, linalool and geraniol (Avci and Giachino, 2016). Sage can be used in form of extracts and essential oil from the leaves for pharmaceutical, perfumery and food industries applications (Vosoughi et al., 2018). Sage is characterised by a high content of oxygenated monoterpenes (e.g. camphor, eucalyptol) and monoterpene hydrocarbons (e.g. pinene, linalool) (El Euch et al., 2018). Several authors have demonstrated both the antioxidant and antimicrobial effectiveness of the *Salvia officinalis* essential oil in maintaining the freshness of chicken meat, pork sausages, cooked meat balls and fried potatoes (Akcan et al., 2017; Oudjedi et al., 2018; Petrová et al., 2013; Šojić et al., 2018). Moreover, extracts from the leaves of *Salvia officinalis* tested on mice have shown anti-inflammatory activity twice stronger than indomethacin, which was used as reference (Baricevic et al., 2001).

Food packaging protects foodstuffs, but it can also constitute a source of chemical food contamination due to migration processes. Migration is defined as the mass transfer between the packaging material and the packaged food. Materials for food packaging must not under any circumstances cause unacceptable changes in the composition, taste and odour of the product, nor may release substances in quantities that are dangerous to the health of consumers. Especially plastics must meet strict formal requirements. In Europe national legislation and community level legislation continue to coexist. At the Union level, food contact materials are regulated under the EU Framework Regulation (EC) No 1935/2004 (EU, 2004) and the Specific Regulation (EU) No 10/2011 (EU, 2011) applies on plastics.

The objective of this study was to develop new active and sustainable packaging based on the combination of a biodegradable polymer (polylactic acid, PLA) with powdered dry leaves of two medicinal plants, sage and lemon balm, incorporated as source of antioxidants. Moreover, biodegradable polymers with incorporated natural matter can be characterised by lower cost, better mechanical properties (Rapa et al., 2016; Vasile et al., 2017, 2018) and higher biodegradability (Tawakkal et al., 2012, 2014). Also, it should be highlighted that active material with application of dry medicinal and aromatics plants (MAPs) instead of essential oil is cheaper due to absence of the extraction process of active agent. To the best of our knowledge, no antioxidant and biodegradable food packaging based on MAPs leaves has been created thus far. Moreover, in the literature there is a lack of information about the influence of active matter into biodegradable polymer structure. The study of antioxidant capacity, migration assays and risk assessment are presented and discussed in this work.

2. Material and methods

2.1. Chemicals

Methanol (HPLC grade; CAS 67-56-1) and ethanol absolute (GC-MS grade; CAS 64-17-5) were supplied by Scharlau Chemie S.A (Barcelona, Spain). 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS, ≥98%; CAS 30931-67-0), 2,2-diphenyl-1-(2,4,6-trinitrophenyl)hydrazyl (DPPH, ≥98%; CAS, 1898-66-4), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 97%; CAS 53188-07-1), potassium persulfate (≥99.0%; CAS 7727-21-1), sodium acetate (> 99%; CAS 127-09-3), sodium salicylate (> 99.5%; CAS 54-21-7), hydrogen peroxide (> 30%; CAS 7722-84-1), orthophosphoric acid (85%; CAS 7664-38-2), potassium ferricyanide (≥99.0%; CAS 13746-66-2), trichloroacetic acid (≥99.0%; CAS 76-03-9), 3,6-dimethyl-1,4-dioxane-2,5-dione (lactide, 99%; CAS 95-96-5); ethyl 3-hydroxyhexanoate (≥98%; CAS 2305-25-1), methyl stearate (≥96%; CAS 112-61-8), ethyl laurate (≥98%; CAS 106-33-2), 1-dodecanol (≥98%; CAS 112-53-8), humulene (trans, trans,trans-2,6,6,9-tetramethyl-1,4,8-cycloundecatriene, ≥96%; CAS 6753-98-6), naphthalene (99%, CAS 91-20-3) and acetic acid (≥99.8%; CAS 64-19-7) were from Sigma-Aldrich Química S.A. (Madrid, Spain). Ultrapure water was obtained from a Wasserlab Ultramatic GR system (Navarra, Spain).

2.2. Antioxidants

Two MAPs species from the Lamiaceae family with recognized antioxidant capacity, lemon balm and sage, were selected for this study. They were harvested in the summer of 2016, from culture of the experimental field of NIRDBS/'Stejarul'Biological Research Centre, Romania. The plants were slowly dried in the open air, protected from direct sunlight and then stored in special spaces for the preservation of the MAPs performance. After drying, lemon balm and sage were processed in a planetary ball mill Retsch PM 100 (Haan, Germany) and sieved to obtain 62 μm powder.

2.3. PLA – polymeric matrix

PLA (trade name Ingeo™ biopolymer 4032D) was provided by NatureWorks LLC (Minnetonka, Minnesota, USA). PLA was characterised by a good processing (due to the included plasticizer), a residual monomer content of 0.21%, relative viscosity of 4.02 and a D-lactide content of 1.5%.

2.4. Active film preparation

PLA samples loaded with 3% (w/w) lemon balm and sage powders were prepared by melt blending processing technique using a Brabender Plastograph (Duisburg, Germany) under a mixing temperature of 180 °C for 10 min at 40 rpm. The rationale for using 3% (w/w) content of each powdered leaves was based on the melt processing behaviour of PLA composites, since higher contents led to considerable increase of the torque during melting of PLA. Additionally, agglomeration of powdered leaves was also observed. In contrast, melt processing of PLA composites with powdered leaves contents lower than 3% (w/w) was reproducible but their active properties were not evidenced. Before melting, PLA was oven-dried at 80 °C for 4 h. Square sheets (150 × 150 × 1 mm) and films (thickness ≤100 μm) were prepared by pressing (Collin P 200 E, Maitenbeth, Germany) at a temperature of 185 °C, preheating time of 5 min, pressing time of 10 min at 147 bars, followed by a sudden cooling of the mould under pressure. Neat PLA was used as reference. Obtained PLA active films are presented in Fig. 1.

2.5. Antioxidant properties

2.5.1. ABTS

The experiments were carried out using an improved antiradical activity (ABTS, see Fig. 2 for chemical structure) decolourisation assay (Re et al., 1999). The ABTS radical cation ($ABTS^{+\bullet}$) was produced by reacting 7 mM stock solution of ABTS with 2.45 mM potassium persulfate (final concentration) and allowing the mixture to stand in the dark for at least 6 h at room temperature prior to use. The $ABTS^{+\bullet}$ solution was diluted to obtain an absorbance value of 0.7 ± 0.05 at 734 nm measured with a PerkinElmer Lambda 40 UV–Vis spectrophotometer (Waltham, USA). For assay, 20 mg of film were mixed with 2 mL of ABTS solution. The samples were shaken in darkness at 20 °C. The affinity of test material to quench ABTS free radical after 1, 2, 18 and 24 h, respectively, was evaluated according to equation (1) where the percentage of scavenging (S%) was calculated.

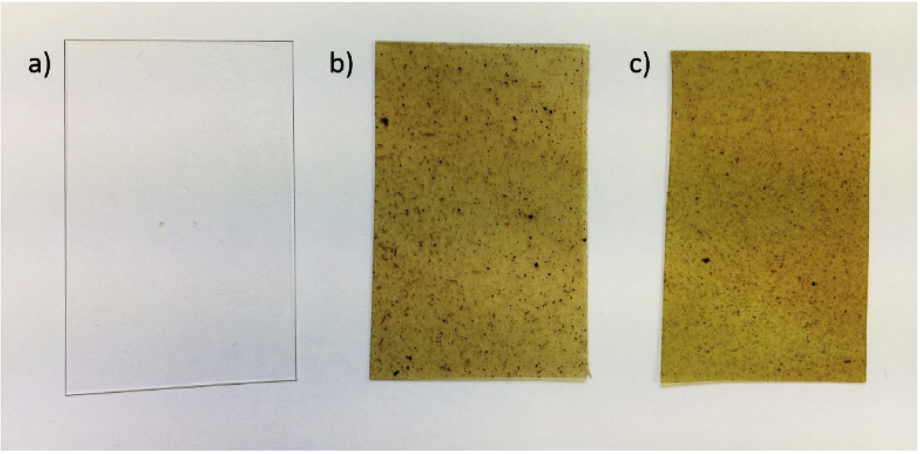


Fig. 1. Images of obtained PLA films where a) neat film (blank); b) film with incorporated lemon balm; c) film with incorporated sage.

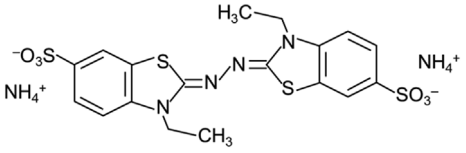


Fig. 2. Chemical structure of ABTS.

$$S\% = [(A_C - A_A) / A_C] \cdot 100 \quad (1)$$

where A_C is the absorbance of control and A_A the absorbance of sample. Free radical scavenging ability was expressed as Trolox equivalent (TE) in μg per g of film (Prior et al., 2005). For this purpose, a calibration graph with Trolox as standard was prepared with five points in triplicate covering the range 5–150 μg g^{-1} in methanol (r^2 = 0.998).

2.5.2. DPPH

The DPPH radical-scavenging activity was determined using the method proposed by W. Brand-Williams et al. (1995). The radical stock solution (100 μM) was prepared fresh daily by dissolving 4 mg of DPPH in 100 mL of methanol. For assay, 20 mg of film were mixed with 2 mL of DPPH solution. The samples were shaken in darkness at 20 °C. The affinity of test material to quench DPPH free radical (see Fig. 3 for chemical structure) was evaluated after 1, 2, 18 and 24 h according to equation (1). The determination was carried out spectrophotometrically by measuring the absorbance at 517 nm. As in the previous section, free radical scavenging ability was expressed as TE in μg per g of film. In this case the calibration graph with Trolox covered the range 2–400 μg g^{-1} (r^2 = 0.996).

2.5.3. Method based on generation of hydroxyl radicals

The method is based on the generation of gas-phase hydroxyl radicals that let to assess directly the antioxidant capacity of materials with incorporated active agents (Pezo et al., 2006, 2008). This method involves the in-situ, vapour-phase generation of OH· radicals and their quantification in the presence and absence of potential antioxidant extracts. The oxidant atmosphere generated from aqueous hydrogen peroxide (0.29 mol L^{-1}) was carried, by an air stream, through an empty quartz chamber in which UV radiation promotes the formation of free radicals by a photochemical reaction. The products then pass through a bag containing a piece of active material, finally bubbling into an impinger containing an aqueous solution of salicylic acid at pH 4.5, which reacts with the OH· radicals forming 2,5-dihydroxybenzoic acid. The described reaction was performed for 24 h. This solution was quantified by HPLC with fluorescence detector.

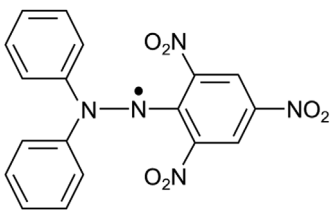


Fig. 3. Chemical structure of DPPH free radical.

Bags (dimensions 10 × 10 cm, volume 135 ± 5 mL determined by filling bags with water) were made using 40 μm thickness low density polyethylene film by thermosealing. In each bag a piece of test material (3 × 5 cm) was placed. All samples were prepared in triplicate. A neat film of PLA was used as blank sample.

Next, a Waters 2795 Series HPLC system (Milford, USA) coupled to a Waters 474 fluorescence detector (λ_{ex} = 324 nm, λ_{em} = 448 nm) were used for the chromatographic analysis of 2,5-dihydroxybenzoic acid resulting from the hydroxylation of salicylic acid. A Waters Atlantis dC18 column of 5 μm particle size (100 mm × 4.6 mm) was used to separate both compounds. Injection volume was 10 μL and flow was 1 mL min^{-1} and. A mixture of 35 mmol L^{-1} acetate buffer at pH 5.9 with methanol (9:1 v:v) was applied in isocratic mode.

2.5.4. Reducing power

Reducing power was determined by the method of Oyaizu (1986). The sample (2.5 mL) was mixed with phosphate buffer (2.5 mL, 200 mM, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). The mixture was incubated at 50 °C for 20 min. Reactions were stopped with 0.5 mL 10% trichloroacetic acid and centrifuged for 10 min at 6500 × *g*. The upper layer of the solution (2.5 mL) was mixed with 2.5 mL of ultrapure water and 0.5 mL of 0.1% $FeCl_3$, and the absorbance was measured at 700 nm. Reducing power was expressed as Trolox equivalents in μg per g of film.

2.5.5. Determination of total phenolic compounds (TPC)

The amount of total phenolics was determined using the Folin-Ciocalteau reagent (Singleton et al., 1999). To 0.5 mL of the sample, 0.5 mL H_2O and 2 mL Folin-Ciocalteau reagent (1:5 H_2O) were added. After 3 min, 10 mL of 10% aqueous Na_2CO_3 (w/v) was added and the contents were mixed and allowed to stand for 30 min. The absorbance at 725 nm was measured in a UV–Vis spectrophotometer. The amount of total phenolics was calculated as gallic acid equivalents in μg per g of film.

2.6. Migration assays

Migration assays were performed by total immersion of 2 × 3 cm pieces of active material in different aqueous food simulants: 3% acetic

Table 1
List of standards used for quantification of volatile compounds.

Nº	Compound	Standard	Linear range (μg·g^{-1})	LOD (μg·g^{-1})	LOQ (μg·g^{-1})
10% ethanol					
1	humulene	humulene	0.0035–5.1	0.0010	0.0035
2	viridiflorene (*(1aR,7R,7aS,*7bR*)-1,1,4,7-tetramethyl-1a,2,3,5,6,7,7a,7b-octahydrocyclopropa[e] azulene*)	naphthalene	0.32–2.5	0.010	0.032
3	ethyl dodecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
4	isopropyl myristate	ethyl laurate	0.0093–5.01	0.0028	0.0093
5	ethyl tetradecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
6	ethyl 13-methyl-tetradecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
7	ethyl pentadecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
8	1-tetradecanol	1-dodecanol	0.0037–2.5	0.0011	0.0037
9	ethyl hexadecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
10	ethyl 14-methyl-hexadecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
11	ethyl heptadecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
12	1-hexadecanol	1-dodecanol	0.0037–2.5	0.0011	0.0037
13	1-nonadecanol	1-dodecanol	0.0037–2.5	0.0011	0.0037
14	2-(hexadecyloxy)-ethanol	ethyl laurate	0.0093–5.01	0.0028	0.0093
15	(Z)-6-octadecenoic acid	octadecanoic acid	0.0035–5.4	0.0011	0.0035
16	ethyl linoleate	ethyl laurate	0.0093–5.01	0.0028	0.0093
3% acetic acid					
1	bis(2-ethylhexyl) hexanedioate	ethyl laurate	0.0093–5.01	0.0028	0.0093
2	ethyl tetradecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
3	methyl hexadecanoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
4	isopropyl palmitate	ethyl laurate	0.0093–5.01	0.0028	0.0093
5	ethyl 9-hexadecenoate	ethyl laurate	0.0093–5.01	0.0028	0.0093
6	methyl stearate	methyl stearate	0.0031–5.02	0.00094	0.0031

Table 2
Antioxidant capacity, reducing power and phenolics content of PLA films.

Analysis		Neat PLA film	PLA film loaded with 3% (w/w) lemon balm	PLA film loaded with 3% (w/w) sage
ABTS (μg TE·g^{-1}) *[see* section 2.5.1*]*	1h	5.0 ± 1.6	37.1 ± 0.5	38.2 ± 0.4
	2 h	5.8 ± 1.4	48.8 ± 1.3	63.3 ± 0.6
	18 h	24.3 ± 0.8	118.6 ± 2.3	83.1 ± 5.8
	24 h	24.3 ± 0.8	138.3 ± 3.0	139.6 ± 1.2
DPPH (μg TE·g^{-1}) *[see* section 2.5.2*]*	1 h	8 ± 2	128 ± 13	108 ± 5
	2 h	44 ± 2	248 ± 10	138 ± 2
	18 h	277 ± 7	1106 ± 12	827 ± 6
	24 h	691 ± 9	1208 ± 4	969 ± 10
Hydroxylation percentage (%) *[see* section 2.5.3*]*		100.0 ± 0.0	55.5 ± 0.1	67.4 ± 0.3
Reducing power (μg TE·g^{-1}) *[see* section 2.5.4*]*		11.47 ± 4.42	85.38 ± 4.04	73.73 ± 10.96
Total phenolics (μg·g^{-1}) *[see* section 2.5.5*]*		0.091 ± 0.061	4.27 ± 0.12	6.48 ± 0.08

acid (w/v), 10% ethanol (v/v) and 95% ethanol (v/v). Samples of 95% ethanol were five times concentrated under gentle stream of nitrogen. The tests were carried out for 10 days at 60 °C to simulate the worst possible conditions of migration. All samples were prepared in triplicate. Also, samples of neat PLA film were analysed. Pure food simulants were used as blanks.

2.7. Analysis of non-volatile compounds. UHPLC-ESI-Q-TOF-MS^E conditions

Chromatographic separation of the non-volatile compounds present in the simulant solutions after the migration tests was performed using an Acquity UPLC from Waters (Milford, USA) with an Acquity UHPLC BEH C18 column of 1.7 μm particle size (2.1 mm × 100 mm). The following parameters were set up: column temperature was 35 °C; column flow rate was 0.3 mL min^{-1}; injection volume was 10 μL and the gradient of mobile phase ranged from 5% to 95% of methanol from 0 to 10 min.

A time-of-flight Waters Xevo G2 mass spectrometer with atmospheric pressure ionization (API) operated as source in the electrospray ionization (ESI) was selected for detection.

The mass spectrometer was operated in both positive (ESI+) and negative (ESI–) mode, with mass range from 10 to 1200 Da. The following parameters were used: 2.5 kV for ESI+ and 0.5 kV for ESI– (corona voltage); 40 V (sampling cone voltage); nitrogen (desolvation gas) at 500 L h^{-1} (gas flow rate) and 20 L h^{-1} (cone gas flow rate); MS^E (acquisition mode); 5–30 V (collision energy ramp); centroid (data collection mode), sensitivity (analyser mode). Moreover, leucine-enkephalin solution was used for LockSpray. Data analysis was performed with the software MassLynx version 4.1 from Waters.

2.8. Analysis of volatile compounds

The determination and quantification of volatile compounds migrated to simulants was performed in an Agilent Technologies 6890 Series GC system (Madrid, Spain) connected to a 5973 series mass detector and CTC Analytics system autosampler. Chromatographic conditions were as follows: BP20 column (30 m × 0.25 mm × 0.25 μm); the initial temperature was 40 °C held for 5 min, then it was increased with a rate of 10 °C·min^{-1} up to 220 °C, held for 5 min. Helium was used as carrier gas at 1 mL min^{-1}.

2.8.1. GC-MS

When using 95% ethanol as simulant, samples were analysed by direct injection. The following parameters were applied: 1 μL (injection volume in splitless mode); 250 °C (injector temperature); SCAN (acquisition mode); 50–350 m/z (mass range); 230 °C (MS Source) and 150 °C (MS Quad).

2.8.2. SPME-GC-MS

Migration samples of 3% acetic acid and 10% ethanol simulants were analysed by SPME-GC-MS. Analyses were carried out with a DVB/CAR/PDMS fibre from Supelco (Madrid, Spain) previously selected.

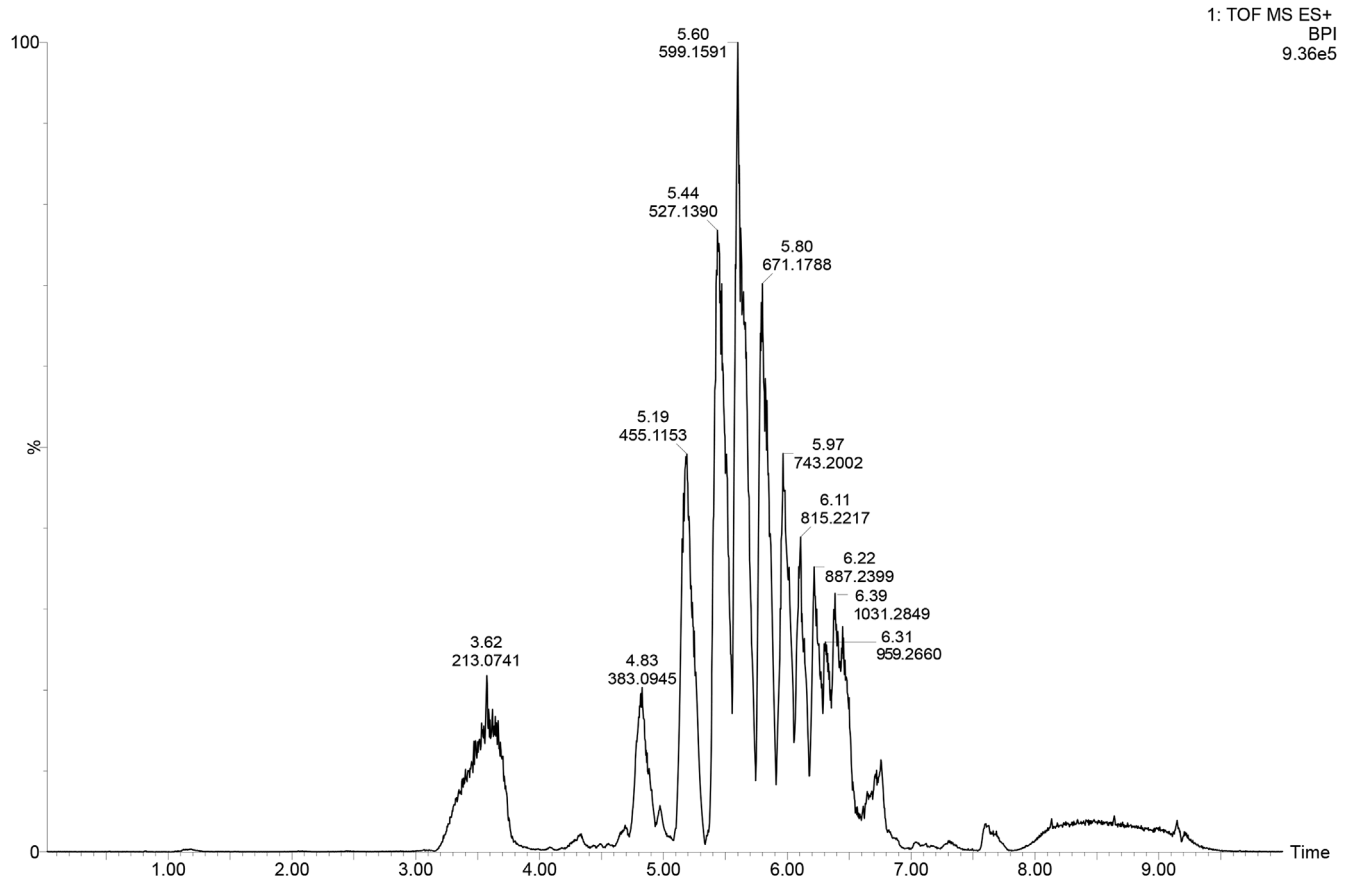


Fig. 4. Chromatogram of non-volatile migrants detected in the neat PLA film (after subtracting the chromatogram of 95% ethanol used as blank) by UPLC-Q-TOF-MS^E using ESI+.

Injection was performed in splitless mode and extraction conditions were as follows: 80 °C (extraction temperature); 15 min (extraction time) and 2 min at 250 °C (desorption time); SCAN (acquisition mode); 50–350 m/z (mass range); 230 °C (MS Source) and 150 °C (MS Quad).

2.9. Quantitative analysis

External standard calibration was used to perform quantitative analysis of both non-volatile and volatile compounds. In addition, analytical parameters were determined.

In the case of non-volatile compounds, ethyl 3-hydroxyhexanoate and lactide were used for quantification of linear and cyclic oligomer of PLA, respectively. Standards were prepared in 95% ethanol. The linearity of the calibration curve was determined by preparing seven working standards from the stock solutions in the range of 0.1–10 μg g^{-1} for ethyl 3-hydroxyhexanoate and 0.1–30 μg g^{-1} for lactide.

When considering the volatile compounds, standards used for quantification and their linear ranges are shown in Table 1. Depending on the sample, the standards were prepared in 3% acetic acid or 10% ethanol.

Blank solutions of the liquid simulants were also analysed. The concentration of all detected migrants was calculated and expressed as that corresponding to 6 dm^2 of material per 1 kg of food simulant (6:1). The analytical features of the method such as the linear range, limits of detection (LOD) and quantification (LOQ) were determined. LOD and LOQ were calculated by using the signal-to-noise method (3:1 and 10:1, respectively).

2.10. Physical and mechanical properties

In order to investigate the thermal degradation, thermogravimetric analyses (TGA) were performed in a Netzsch STA 449 F3 Jupiter (Selb, Germany) simultaneous thermal analyser. The samples (40 mg) were placed in alumina slip-on plates and heated under a nitrogen atmosphere (20 mL min^{-1}) from room temperature to 450 °C at 10 °C·min^{-1}. Both the extrapolated onset-temperature (according to ISO 11357–1 and the temperature of maximum differential thermogravimetric analysis (DTG) were calculated.

Tensile tests were carried out in reference to ISO 527, but with slight modifications due to the limited amount of materials. Three 115 mm × 10 mm rectangular strips (test specimen type 2) were cut from each sample. Tensile strength and elongation at break were measured using an openLETT tensile tester (Rotterdam, The Netherlands) with a load cell of 5000 N, at 10 mm min^{-1} and 50 mm as initial distance between grips.

2.11. Statistical analysis

All data are presented as the mean ± standard deviation for three independent measurements (n = 3). When considering the antioxidant performance of active materials in comparison to neat PLA, an increase in measured properties is always expected (with the only exception of hydroxylation percentage). Consequently, statistically significant differences among samples has been evaluated by means of paired one-tailed *t*-test analysis ($P < 0.05$). In the case of mechanical properties, two-tailed *t*-test have been considered since the direction of the hypothetical difference cannot be predicted a priori.

Table 3
Characterisation of compounds determined in migration assays by UPLC-Q-TOF-MS^E using ESI+.

N°	t_{Ret} (min)	Compound	Formula	m/z	Adduct	Fragments
1	3.62	PLA linear oligomer, n = 2	$C_{8}H_{14}O_{5}$	213.0741	$[M+Na]^+$	174.0853
2	4.83	PLA cyclic oligomer, n = 5	$C_{15}H_{20}O_{10}$	383.0945	$[M+Na]^+$	145.0917 217.0123
3	5.19	PLA cyclic oligomer, n = 6	$C_{18}H_{24}O_{12}$	455.1153	$[M+Na]^+$	145.0917 217.0123 289.0893
4	5.44	PLA cyclic oligomer, n = 7	$C_{21}H_{28}O_{14}$	527.1390	$[M+Na]^+$	217.0123 289.0893 361.1012
5	5.60	PLA cyclic oligomer, n = 8	$C_{24}H_{32}O_{16}$	599.1591	$[M+Na]^+$	289.0893 361.1012 433.1312
6	5.80	PLA cyclic oligomer, n = 9	$C_{27}H_{36}O_{18}$	671.1788	$[M+Na]^+$	361.1012 433.1312 505.2312
7	5.97	PLA cyclic oligomer, n = 10	$C_{30}H_{40}O_{20}$	743.2002	$[M+Na]^+$	433.1312 505.2312 577.4315
8	6.11	PLA cyclic oligomer, n = 11	$C_{33}H_{44}O_{22}$	815.2217	$[M+Na]^+$	505.2312 577.4315 649.2362
9	6.22	PLA cyclic oligomer, n = 12	$C_{36}H_{48}O_{24}$	887.2399	$[M+Na]^+$	577.4315 649.2362 721.1232
10	6.31	PLA cyclic oligomer, n = 13	$C_{40}H_{52}O_{26}$	959.2660	$[M+Na]^+$	649.2362 721.1232 793.5431
11	6.39	PLA cyclic oligomer, n = 14	$C_{43}H_{56}O_{28}$	1031.2849	$[M+Na]^+$	721.1232 793.5431 865.1453

3. Results and discussion

3.1. Antioxidant properties

Phenolics content, reducing power and antioxidant capacity of films are presented in Table 2.

In all cases, the addition of functional components significantly increased the phenolics content and improved the antioxidant capacity of films ($p = 0.0000004$ for lemon balm and $p = 0.00000002$ for sage). The films enriched with sage contained about 1.5 times more phenolics than those with lemon balm. An 8-fold increase of the reducing power of films, compared to the control, was observed without significant difference ($p = 0.080$) between sage and lemon balm films. In the case of antioxidant properties, a positive effect of herbs addition was also observed. As can be seen in Table 2, the percentage of hydroxylation (calculated as the area of 2,5-dihydroxybenzoic acid divided by the sum of areas of 2,5-dihydroxybenzoic acid and salicylic acid multiplied by 100) for active film with lemon balm (55.5%) was lower than the one prepared with sage (67.4%). The lower the hydroxylation percentage (formation of 2,5-dihydroxybenzoic acid), the stronger antioxidant capacity of the film will be (scavenging of OH· radicals). In addition, despite its lower content of phenolics, lemon balm-based films were characterised by significantly higher ability to scavenge DPPH radicals (by about 25% more than sage-based films, $p = 0.036$), whereas no significant difference in the ability of quenching of ABTS radicals ($p = 0.347$) was noticed after 24 h between the active films. The differences in the free radical scavenging capacity of PLA films loaded with MAP powdered leaves seem to be opposite to their total content of phenolics, and could be attributed to the singular scavenging capacity of individual phenols present in lemon balm and sage.

3.2. Analysis of non-volatile compounds by UHPLC-ESI-Q-TOF-MS^E

First of all, the following analytical parameters were determined: linear range from 0.029 to 30.02 μg g^{-1} with $r^2 = 0.999$ for lactide was obtained. In the case of 3-hydroxyhexanoate, linear range was from 0.0028 to 10.2 μg g^{-1} with $r^2 = 0.999$. Regarding minimum detectable concentrations, LOD for lactide and ethyl 3-hydroxyhexanoate were 0.0087 and 0.00085 μg g^{-1}, respectively, whereas the LOQ for lactide and ethyl 3-hydroxyhexanoate were 0.029 and 0.0028 μg g^{-1}, respectively.

Migration of non-volatile compounds was not detected in the case of 10% ethanol and 3% acetic acid using both ESI+ and ESI– ionization. Therefore, the application of developed packaging to real food such as molasses, sugar syrups, honey, sugar and sugar products group and fresh vegetables, peeled or cut (processed vegetables group) is free of migration of non-volatile compounds. Nevertheless, some migration was observed in the case of 95% ethanol (fatty simulant) due to its organic character, as can be seen in Fig. 4.

Table 3 shows all the detected migrants, which were essentially the same in all of the analysed samples. However, their concentration differed depending on the type of sample.

According to the results from Table 3, it can be observed that 10 different PLA cyclic oligomers (n = 5–14) and one linear oligomer (n = 2) were detected. These findings are consistent with literature (Aznar et al., 2019). The structures of the detected non-volatile compounds in 95% ethanol are shown in Fig. 5.

Quantitative analysis of non-volatile migrants is presented in Fig. 6 (a), where the following tendency was observed: migration of cyclic PLA oligomers from neat film was higher in the case of compounds with molar masses between 360 g mol^{-1} (n = 5) and 576 g mol^{-1} (n = 8). The migration of cyclic PLA oligomers from neat film decreased for the compounds starting with the molar mass 648 g mol^{-1} (n = 9) and it was stabilized, reaching approximately 2 mg kg^{-1} (6:1) in the case of the compound with molar mass 792 g mol^{-1} (n = 11).

When comparing the results of neat PLA film with active films it can be seen that the addition of sage and lemon balm leaves into the polymer matrix notoriously decreased the migration of both PLA linear and cyclic PLA oligomers, with better results in sage-based films. When checking the concentration of migrants in comparison with the blanks, it can be seen that the highest reduction in the case of sage was obtained for cyclic oligomers n = 13 and n = 14 (83.3% and 76.9%, respectively). On the other hand, in the case of lemon balm the highest reduction was obtained for the cyclic oligomers n = 8 (53.4%) and n = 7 (51.7%) and the linear oligomer (51.4%).

The plastic packaging materials must be manufactured in accordance with specific regulation (EU) No 10/2011 which contains a positive list of monomers and additives that can be used as starting materials. Both linear and cyclic PLA oligomers are not present in such positive list. To assess the health risks of migrants not included on the list of permitted substances, the software Toxtree based on Cramer's rules was used to estimate their toxicity. The Cramer classification is a theoretical approach to classify chemicals based on the expected level of oral toxicity into one of the three toxicity classes and proposes a maximum daily intake. The theoretical maximum migration amounts (mg·kg^{-1}) of the migrants are deducted from equation (2).

$$EDI = \text{migration} \times \text{food intake} \times CF \quad (2)$$

where EDI: estimated daily intake (maximum daily intake for each substance per person, considered 1 kg·person^{-1}·day^{-1} in Europe); CF: consumption factor (fraction of the daily diet for specific materials, only used in USFDA but not in Europe).

Class I contains substances with simple chemical structures, which suggest a low oral toxicity, with theoretical maximum migration amounts of 1.80 mg kg^{-1}. Class II contains substances that have structures with higher risk than Class I substances, but do not have

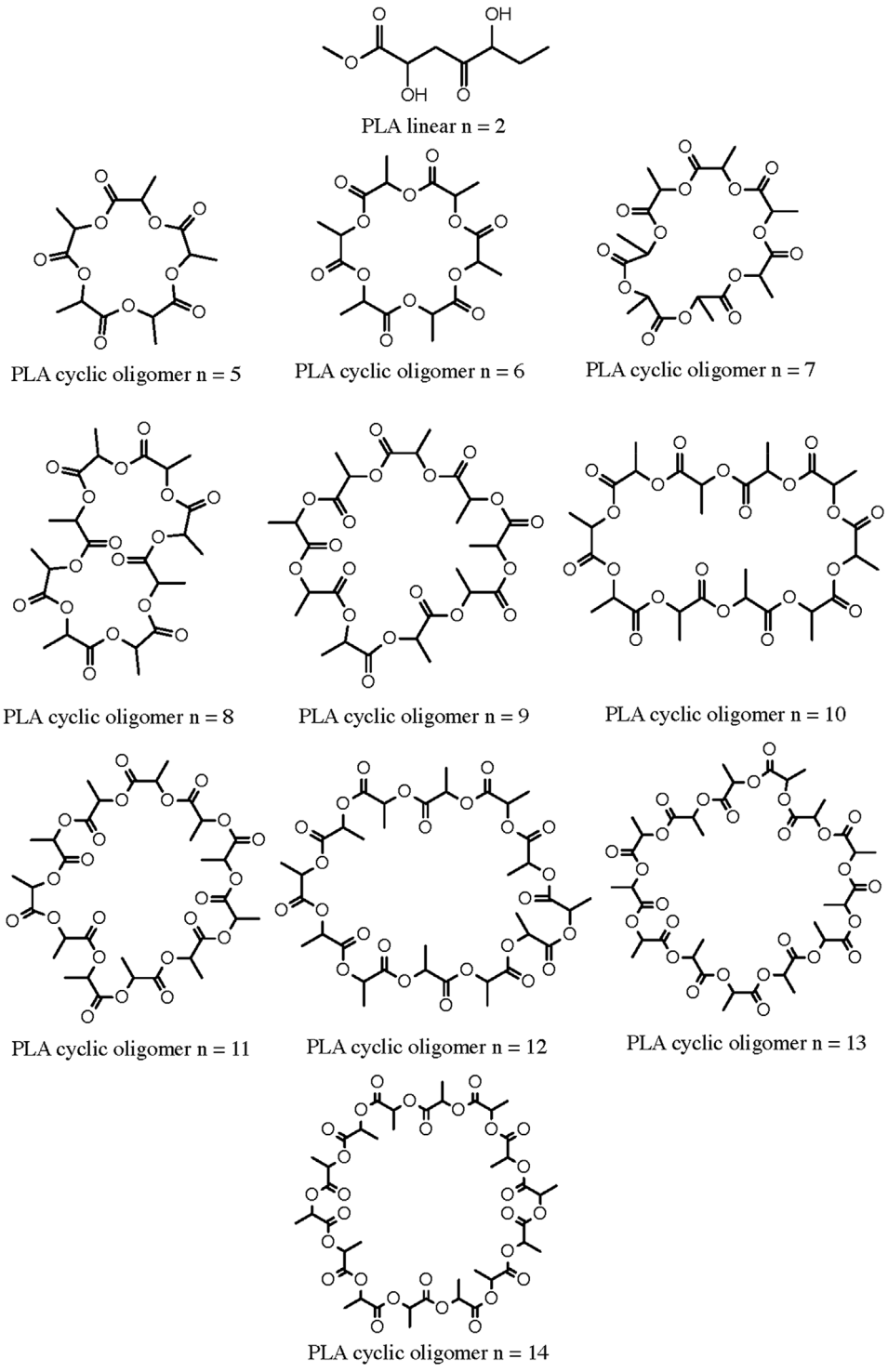
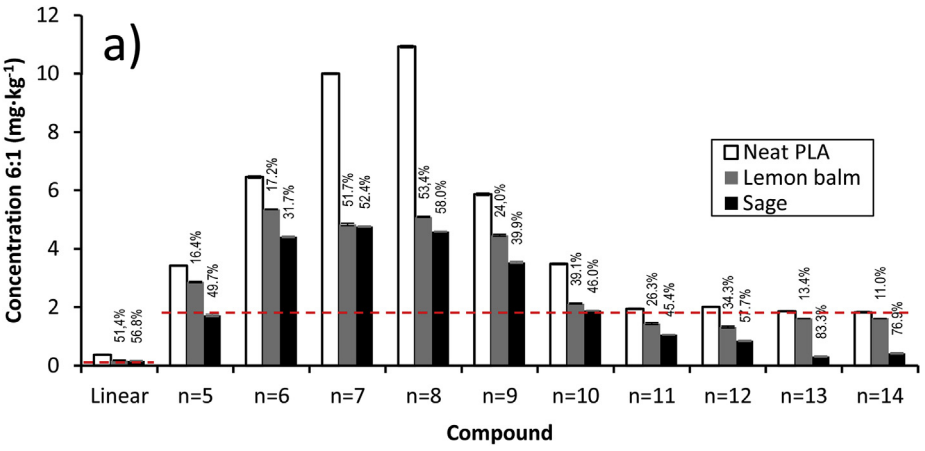


Fig. 5. Structures of detected PLA oligomers.

structural features suggestive of toxicity, such as those in Class III. Theoretical maximum migration amounts for substances from classes II and III are 0.54 and 0.09 mg kg^{-1}, respectively. The linear PLA oligomer belongs to class III since it has an open chain with 3 functional groups (oxo-, hydroxy- and methoxy-). In contrast, all the detected cyclic PLA oligomers belong to class I because they are heterocyclic with less than 3 functional groups.

When analysing the results from Fig. 6 (a), it can be concluded that none of the films comply with (EU) No 10/2011 legislation in the case of migration in 95% ethanol that corresponds to such types of food as: fruits, vegetables, fish, crustaceans and molluscs, marinated meat products and cheese all preserved in oily medium; nuts in paste or cream form; animals and vegetable fats and oils, whether natural or treated (including cocoa butter, lard, resolidified butter). Nevertheless, (EU) No 10/2011 legislation recommends dividing the results of migration in fatty simulant by a factor of 2, 3, 4 or 5 depending on the type of food. It is because the result obtained with simulant D2 may in certain cases significantly overestimate the migration into food. The value of correction factor depends on time and temperature of the migration test and how good simulant D2 mimics real fatty food. Results for factor 3



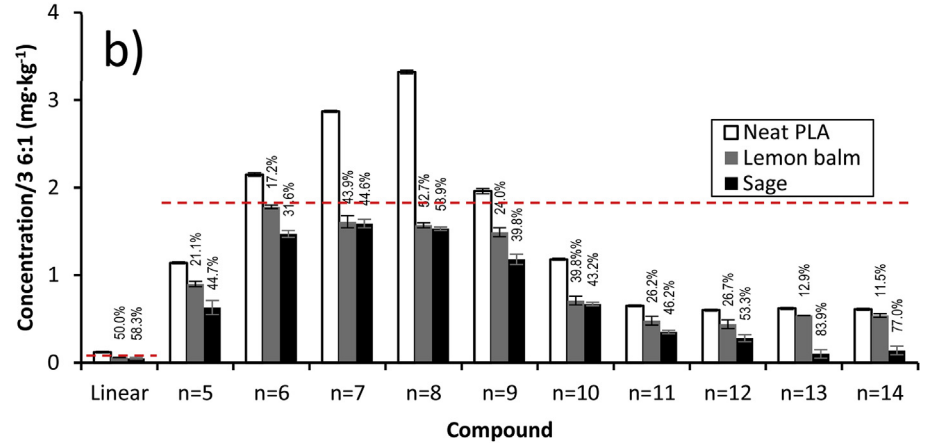


Fig. 6. Quantitative analysis of non-volatile migrants in 95% ethanol, where: a) concentration of migrants 6:1 (mg·kg^{-1}); b) concentration of migrants 6:1 (mg·kg^{-1}) divided by 3 according to (EU) No 10/2011. Red dashed lines indicate theoretical maximum migration limits (0.09 and 1.80 mg kg^{-1} for linear and cyclic PLA oligomers, respectively). The values over bars indicate the percentage of reduction in comparison to neat PLA. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

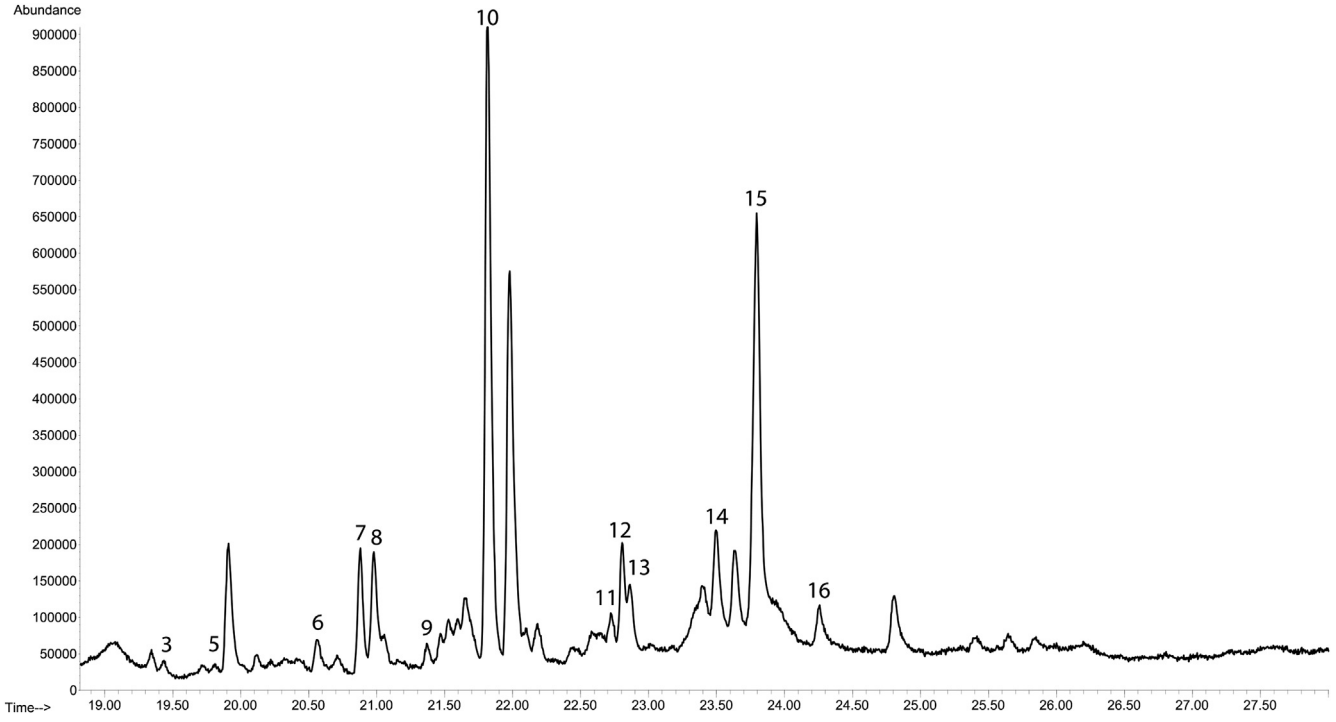


Fig. 7. Chromatogram of migration of neat PLA into 10% ethanol acquired by SPME-GC-MS.

Table 4
Qualitative results of migration study in 10% ethanol and 3% acetic acid.

Nr.	t_{Ret} (min)	Compound	CAS	Neat PLA	PLA + 3% (w/w) lemon balm	PLA + 3% (w/w) sage
10% ethanol						
1	15.82	humulene	6753-98-6			•
2	16.11	viridiflorene	21747-46-6			•
3	19.43	ethyl dodecanoate	106-33-2	•		
4	19.84	isopropyl myristate	110-27-0		•	
5	19.91	ethyl tetradecanoate	124-06-1	•		
6	20.56	ethyl 13-methyl-tetradecanoate	64317-63-1	•	•	
7	20.88	ethyl pentadecanoate	41114-00-5	•	•	
8	20.98	1-tetradecanol	112-72-1	•	•	
9	21.37	ethyl hexadecanoate	628-97-7	•		
10	21.82	ethyl 14-methyl-hexadecanoate	1000336-64-7	•	•	•
11	22.73	ethyl heptadecanoate	14010-23-2	•		
12	22.81	1-hexadecanol	36653-82-4	•	•	•
13	22.86	1-nonadecanol	1454-84-8	•		
14	23.49	2-(hexadecyloxy)-ethanol	2136-71-2	•		
15	23.79	(Z)-6-octadecenoic acid	593-39-5	•		
16	24.25	ethyl linoleate	544-35-4	•		
3% acetic acid						
1	19.12	bis(2-ethylhexyl) hexanedioate	103-23-1		•	•
2	19.91	ethyl tetradecanoate	106-33-2	•		
3	21.47	methyl hexadecanoate	110-27-0	•		
4	21.71	isopropyl palmitate	124-06-1	•		
5	21.93	ethyl 9-hexadecenoate	64317-63-1	•		
6	23.30	methyl stearate	41114-00-5	•	•	

Table 5
Quantitative results of migration study in 10% ethanol and 3% acetic acid.

Nr	t_{Ret} (min)	Compound	Concentration 6:1 ($mg \cdot kg^{-1}$)			SML ($mg \cdot kg^{-1}$)	Cramer Class
			Neat PLA	PLA + 3% (w/w) lemon balm	PLA + 3% (w/w) sage		
10% ethanol							
1	15.82	humulene	–	–	0.34 ± 0.01	–	I
2	16.11	viridiflorene	–	–	0.71 ± 0.01	–	I
3	19.43	ethyl dodecanoate	0.36 ± 0.02	–	–	–	I
4	19.84	isopropyl myristate	–	0.34 ± 0.01	–	–	I
5	19.91	ethyl tetradecanoate	0.39 ± 0.06	–	–	–	I
6	20.56	ethyl 13-methyl-tetradecanoate	0.36 ± 0.01	0.35 ± 0.01	–	–	I
7	20.88	ethyl pentadecanoate	0.36 ± 0.04	0.33 ± 0.01	–	–	I
8	20.98	1-tetradecanol	< LOQ	< LOQ	–	–	I
9	21.37	ethyl hexadecanoate	0.47 ± 0.18	–	–	–	I
10	21.82	ethyl 14-methyl-hexadecanoate	0.56 ± 0.03	0.37 ± 0.01	0.36 ± 0.02	–	I
11	22.73	ethyl heptadecanoate	0.52 ± 0.22	–	–	–	I
12	22.81	1-hexadecanol[a]	< LOQ	< LOQ	< LOQ	[a]	–
13	22.86	1-nonadecanol	< LOQ	–	–	–	I
14	23.49	2-(hexadecyloxy)-ethanol	< LOQ	–	–	–	I
15	23.79	(Z)-6-octadecenoic acid	0.68 ± 0.01	–	–	–	I
16	24.25	ethyl linoleate	0.37 ± 0.02	–	–	–	I
3% acetic acid							
1	19.12	bis(2-ethylhexyl) hexanedioate	–	0.70 ± 0.03	0.49 ± 0.01	–	I
2	19.91	ethyl tetradecanoate	0.76 ± 0.15	–	–	–	I
3	21.47	methyl hexadecanoate	1.35 ± 0.06	–	–	–	I
4	21.71	isopropyl palmitate	1.03 ± 0.12	–	–	–	I
5	21.93	ethyl 9-hexadecenoate	0.84 ± 0.24	–	–	–	I
6	23.30	methyl stearate	0.59 ± 0.08	0.93 ± 0.27	–	–	I

[a] The compound appears on the positive list of (EU) No 10/2011 without specific migration limit.

are shown on Fig. 6 (b). The factor of 3 was chosen because it represents the minimum value with which both active materials comply with (EU) No 10/2011 legislation, while neat PLA still cannot be used for oily food contact applications. It was demonstrated that developed antioxidant materials based on MAP powdered leaves can be used for packaging in a huge range of applications: pastry, biscuits, cakes, bread (dry and fresh) all with fatty substances on the surface; chocolate, chocolate-coated products; confectionery products with fatty substances on the surface; fresh chilled, processed, salted or smoked fish and meat and processed meat products; natural cheese without rind or with edible rind (gouda, camembert, and the like) and melting cheese; fried potatoes, fritters and the like; fried or roasted foods of animal origin; dried foods with fatty substances on the surface and cocoa powder.

3.3. Analysis of volatile compounds in ethanol 95% as simulant by GC-MS

No migration of volatile compounds was detected when analysing the test solutions in ethanol 95% by direct injection in the GC-MS system.

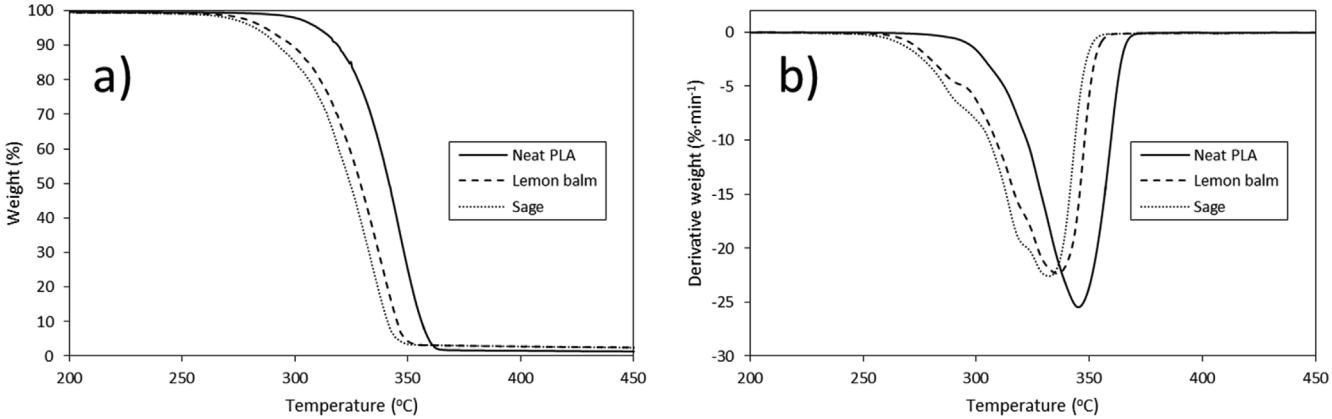


Fig. 8. TGA (a) and DTG (b) thermograms of neat PLA and PLA containing powdered MAPs films.

Table 6
Extrapolated onset-temperature (T_{onset}), temperature of maximum DTG analysis (T_{DTG}) and percentage of weight variation (ΔW) of PLA films calculated from TGA/DTG tests.

Film	T_{onset} (°C)	T_{DTG} (°C)	ΔW (%)
Neat PLA	322.7	345.3	98.92
PLA + 3% (w/w) lemon balm	308.6	335.9	98.07
PLA + 3% (w/w) sage	305.2	332.2	98.08

Table 7
Tensile strength (TS) and elongation at break of PLA samples.

Film	TS (MPa)	Elongation (%)
Neat PLA	49.6 ± 4.2	4.39 ± 0.85
PLA + 3% (w/w) lemon balm	45.3 ± 2.9	5.31 ± 0.17
PLA + 3% (w/w) sage	38.7 ± 2.1	5.12 ± 0.42

3.4. Analysis of volatile compounds in 3% acetic acid and 10% ethanol as simulants by SPME-GC-MS

An example of chromatogram of migration of neat PLA into 10% ethanol is presented in Fig. 7. Table 4 shows the volatile compounds in 10% ethanol and 3% acetic acid for all analysed samples.

Sixteen and eight different volatile compounds were determined in samples of 10% ethanol and 3% acetic acid, respectively. The highest number of volatile migrants was detected in the case of neat PLA for both types of simulants. The addition of MAP powdered leaves prevented the migration of compounds such as unsaturated fatty acid, alcohols and ethyl esters.

A higher inhibitory effect of migration, reflected by the results of quantitative analysis (Table 5), was observed in the case of sage-based films. Also, volatile compounds originally present in plants were detected: humulene, viridiflorene (sage) and isopropyl myristate (lemon balm), in agreement with other studies reported in the literature (Bogdanovic et al., 2016; Cai et al., 2006; Hussain et al., 2011). All detected phenolic compounds stem from plants (sage and lemon balm).

When analysing the quantitative results of volatile compounds (Table 5) it can be seen that none of the migrants exceeds the set limits that are expressed as theoretical maximum migration amounts (see section 3.2.). However, it should be highlighted that none of the volatile compounds, with the only exception of 1-hexadecanol, have been found on the positive list of (EU) No 10/2011. Therefore, Cramer rules described before were applied to determine their permitted limits of migration. The analytical parameters of the method for quantification of volatile compounds are shown in Table 1.

3.5. Evaluation of physical and mechanical properties

From room temperature to 200 °C no thermal change for analysed films was noted, proving their thermal stability during melt processing. An onset temperature decrease of 14.1 and 17.5 °C for PLA containing lemon balm and sage, respectively in comparison to neat PLA was noticed, as shown in Fig. 8 (a) and Table 6. This effect is also evident, as Fig. 8 (b) shows, when considering the temperature of maximum DTG, with −9.4 °C (lemon balm) and −13.1 °C (sage) vs. neat PLA, thus indicating that the powdered MAPs are less thermally stable than PLA (Rizvi et al., 2011). Finally, the percentage of weight variation shows about 1% of difference between PLA films with MAP powdered leaves incorporated, due to the presence of mineral elements (Pytlakowska et al., 2012) that are not degraded at high temperatures during TGA.

With regard to mechanical tests, as Table 7 shows, no significant differences in tensile strength (TS) were found between the neat PLA and the film containing lemon balm ($p = 0.109$). Nevertheless, a significant decrease of 17.5% in TS of the film prepared with sage vs. the neat PLA was noticed ($p = 0.008$), probably due to the presence of higher number of agglomerations (Baheti et al., 2013). Moreover, no significant differences in elongation at break were observed among films ($p > 0.140$ in all the cases), thus indicating that the incorporation of sage and lemon balm leaves into the PLA matrix did not substantially modify the mechanical polymer properties.

4. Conclusions

Two new antioxidant PLA biofilms based on incorporation of MAP powdered leaves were successfully developed, and an 8-fold increase of the reducing power of films in comparison to the control was registered, without statistically significant difference between sage and lemon balm films. On the other hand, the percentage of hydroxylation for active films with lemon balm and sage was 55.5% ± 0.1% and 67.4% ± 0.3%, respectively, thus indicating a very good antioxidant capacity.

Migration of non-volatile compounds was observed in the case of ethanol 95%. Cyclic and linear oligomers of PLA were detected as migrants which are not included in the Regulation EU No 10/2011. The linear PLA oligomer found belongs to III class of toxicity of Cramer, while all the detected cyclic PLA oligomers belong to class I. In comparison with neat PLA, active films containing powdered MAPs into the polymer matrix decreased significantly the migration of linear and cyclic PLA oligomers. The highest reduction of migrants was obtained in the case of sage, where the results obtained for cyclic oligomers $n = 13$ and $n = 14$ were 83.3% and 76.9%, respectively. In the case of lemon balm, the highest reduction of migrants was obtained for the linear oligomer (51.4%) and cyclic oligomers $n = 7$ and $n = 8$ (51.7% and 53.4%, respectively).

Sixteen and eight different volatile compounds were determined in samples of 10% ethanol and 3% acetic acid, respectively. The highest number of volatile migrants was detected in the case of neat PLA in both types of simulant. The addition of powdered MAPs decreased or prevented the migration of volatile compounds.

A small decrease in thermal stability of films has been noticed due to the incorporation of MAP powdered leaves to the PLA matrix, whereas the tensile properties remain almost unaffected.

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Transparency document

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