Spray-drying microencapsulation of synergistic antioxidant mushroom extracts and their use as functional food ingredients

Running title: Mushroom functional food ingredients

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Abstract

In this work, hydroalcoholic extracts of two mushrooms species, *Suillus luteus* (L.: Fries) (Sl) and *Cropinopsis atramentaria* (Bull.) (Ca), were studied for their synergistic antioxidant effect and their viability as functional food ingredients tested by incorporation into a food matrix (cottage cheese). In a first step, the individual extracts and a combination of both showing synergistic effects (Sl:Ca, 1:1) were microencapsulated by spray-drying using maltodextrin as the encapsulating material. The incorporation of free extracts resulted in products with higher initial antioxidant activity (t0) but declining after 7 days (t7), which was associated with their degradation. However, the cottage cheese enriched with the microencapsulated extracts, that have revealed a lower activity at initial time, showed an increase at t7. This improvement can be explained by an effective protection provided by the microspheres together with a sustained release. Analyses performed on the studied cottage cheese samples showed the maintenance of the nutritional properties and no colour modifications were noticed.

Keywords: Mushroom extracts; antioxidant activity; synergistic effects; microencapsulation; cottage cheese

1. Introduction

Mushrooms are widely appreciated all over the world for their nutritional properties (Kalač, 2009). They have a low fat content but are rich in water, minerals, proteins, fibres and carbohydrates (Heleno, Barros, Martins, Queiroz, Santos-Buelga & Ferreira, 2012; Kalač, 2009; Reis et al., 2011; Reis, Barros, Martins & Ferreira, 2012;). Besides their nutritional value, it has been demonstrated that mushrooms have health promoting benefits (Palacios et al., 2011). They are effective as anti-inflammatory (Ma, Chen, Dong & Lu, 2013), antitumor (Heleno, Ferreira, Calhelha, Esteves, Martins & Queiroz, 2014), antibacterial (Alves, Ferreira, Martins & Pintado, 2012) and antioxidant agents (Reis et al., 2011), extending their potential use to functional foods and applications in the biomedical field.

During natural cellular metabolism, reactive oxygen (ROS), nitrogen (RNS) and sulphur (RSS) species are produced (Carocho & Ferreira, 2013), being ROS the most abundant ones (Ferreira, Barros & Abreu, 2009). When high concentrations of these species are present, an oxidative stress is generated. If in excess, ROS may oxidize and damage cellular lipids, proteins and DNA, leading to their modification and inhibition of normal functions (Ferreira et al., 2009). Given this scenario, the organism develops defence mechanisms such as endogenous defences, which can be of enzymatic type, leading to the production of superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px) and glutathione reductase; or non-enzymatic, resulting in species such as glutathione (GSH), α-tocopherol (vitamin E), ascorbic acid (vitamin C) and lipoic acid (Carocho & Ferreira, 2013; Ferreira et al., 2009). Both mechanisms are able to provide cells protection against excessive levels of free radicals (Carocho & Ferreira, 2013).

The exogenous antioxidant defence promoters can be ingested as part of the daily diet to help fight against high ROS contents. Therefore, mushrooms can play an important role since they contain diverse phenolic compounds, known to be excellent antioxidants due to their capacity for capturing free radicals by electron transferring, and to the excellent redox properties of the phenolic hydroxyls groups (Ferreira et al., 2009).

Apart from their instability at high temperatures, and in the presence of oxygen and light, some mushroom extracts are characterized by a strong odour and flavour. One way to ensure their viability as functional food ingredients is to proceed with their microencapsulation, providing protection against oxidation and masking odour and flavour (Ersus & Yurdagel, 2007; Fang & Bhandari, 2010). Despite the numerous available microencapsulation possibilities, spray-drying is still one of the most widely used processes to encapsulate food ingredients (Fang & Bhandari, 2010). Among the main advantages of this technique are its easy industrialization and possibility of continuous production. Nevertheless, prolonged contact with high temperatures can compromise the bioactive properties of the mushroom extracts, and should be avoided.

Among several possibilities, maltodextrin (MD), a hydrolysed starch, offers advantages as microencapsulation material (Gharsallaoui, Roudaut, Chambin, Voilley & Saurel, 2007). It is a low cost material with neutral aroma and flavour, high water solubility, low viscosity at high solids content, being able to provide an effective protection against oxidation (Ersus & Yurdagel, 2007; Saénz, Tapia, Chávez & Robert, 2009).

Microencapsulation can be applied to protect bioactive natural extracts and some examples calling up this thematic can be found in literature (Dias, Ferreira & Barreiro, 2015). Nevertheless, these studies are mainly related with the microencapsulation process development and do not proceed with the implementation of a final application

(functional food) (Ersus & Yurdagel, 2007; Kha, Nguyen & Roach, 2010; Silva, Stringheta, Teófilo & Oliveira, 2013; Saénz et al., 2009; Wu, Zou, Mao, Huang & Liu, 2014). In fact, and according to the performed research, the examples dealing with the full process development are scarcer. In this context, Çam, Içyer & Erdoğna, (2014) tested the incorporation of microencapsulated *Punica granatum* L., an extract from pomegranate, in ice creams, and Martins et al., (2014) studied the incorporation of *Rubus ulmifolius* Schoot (Rosaceae), a species of wild blackberry, microencapsulated in alginate microparticles, in a dairy product, yogurt. The obtained results, rather preliminary, are motivating corroborating the interest of developing foods enriched with natural extracts that are often referred as health promoters (Ramalingum & Mahomoodally, 2014).

In this work, extracts of two mushroom species, *Suillus luteus* (L.: Fries) and *Cropinopsis atramentaria* (Bull.) were investigated for their synergistic antioxidant effects and a promising combination of both (in similar proportions) was chosen to be microencapsulated by spray-drying using maltodextrin as the encapsulating material. The obtained powders were characterized by Scanning Electron Microscopy (SEM) (to inspect morphology and particle size) and for their antioxidant activity (free radicals scavenging activity and reducing power). Encapsulation yield and efficiency were also estimated. As a final step of this work the produced microspheres were incorporated in cottage cheese samples and its antioxidant activity, nutritional value and colour were determined and compared with the counterparts using extracts in its free form and a control (sample with no added extracts).

2. Materials and methods

2.1. Standards and reagents

For antioxidant tests, 2,2-dipheny-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). For chromatographic analysis, HPLC-grade acetonitrile was from Fisher Scientific (Lisbon, Portugal). The standards, such as fatty acids methyl ester (FAME) reference standard mixture 37 (standard 47885-U), β-carotene (98%) and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, MO, USA), as also formic acid. Maltodextrin (MD) was provided by Cargill (Wayzata, MN, USA) with dextrose equivalent of 18. All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, SC, USA).

2.2. Preparation of mushroom extracts and evaluation of synergistic effects

2.2.1. Extracts preparation

Mushroom samples, *Suillus luteus* (Sl) and *Cropinopsis atramentaria* (Ca), were harvested in Bragança region located at the North-eastern of Portugal, according to a previous report of the authors (Heleno et al., 2012; Reis et al., 2011). The chemical characterization of both species can be found in the cited references.

Samples of individual species and combinations in three different proportions (Sl:Ca 1:2, 1:1 and 2:1, w/w) were extracted. To prepare the extracts, the lyophilized mushroom samples (1.5 g) were extracted with methanol/water (80:20, v/v, 30 mL) at room temperature during 1h under stirring. The extract was filtered through a Whatman paper filter Nº 4 and the remaining residue subjected to an additional extraction. The

combined extracts were evaporated under reduced pressure in a rotatory evaporator (Büchi R-210, Flawil, Switzerland) until complete removal of methanol, lyophilized (FreeZone 4.5, Labconco, Kansas City, MO, USA) and stored in a desiccator protected from light until use.

2.2.2. Evaluation of antioxidant activity

The antioxidant activity of the obtained extracts was evaluated by using the DPPH radical scavenging activity, reducing power and inhibition of β-carotene bleaching assays. For the assays, the extracts were re-dissolved in methanol/water (80:20, v/v) at 20 mg/mL, and stored at 4 ºC. These stock solutions were successively diluted to determine EC_{50} values (sample concentration providing a value of 50% in the DPPH and β-carotene bleaching assays or an absorbance value of 0.5 in the reducing power assay).

DPPH radical scavenging activity (Heleno et al., 2012) was evaluated using an ELX800 microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA), and calculated as a percentage of DPPH discoloration using the formula: $[(A_{DPPH} - A_S)/A_{DPPH}] \times 100$, where A_S is the absorbance of the solution containing the sample at 515 nm, and A_{DPPH} is the absorbance of the DPPH solution. Reducing power (Heleno et al., 2012) was evaluated by the capacity to convert Fe^{3+} into Fe^{2+}, measuring the absorbance at 690 nm in the microplate reader mentioned above. Inhibition of β-carotene bleaching (Heleno et al., 2012) was evaluated by the β-carotene/linoleate assay; the neutralization of linoleate free radicals avoids β-carotene bleaching, which is measured by the formula: (β-carotene absorbance after 2 h of assay/initial absorbance) × 100. Trolox was used as positive control.

2.2.3 Classification of additive, synergistic or antagonistic effects

Theoretical values of the antioxidant activity of the assayed mixture extracts were calculated as the weighted mean of the experimentally determined EC_{50} values of the individual extracts and considering additive contributions, e.g., for a mixture comprising 33% (w/w) of *S. luteus* and 67% (w/w) of *C. atramentaria*, $EC_{50} = EC_{50}$ *S. luteus* × 0.33 + EC_{50} *C. atramentaria* × 0.67.

The classification as additive (AE), synergistic (SE) or antagonistic (negative synergistic (NS)) effects was performed as follow: AE: EC_{50} theoretical and experimental values revealed differences lower than 5%; SE: EC_{50} experimental values were at least 5% lower than theoretical values; AN: EC_{50} experimental values were at least 5% higher than theoretical values. The limit of 5% was chosen taking into account the coefficients of variation obtained in the replications of each antioxidant activity assay. It should be noted that lower EC_{50} values means higher antioxidant activity.

2.3. Microencapsulation of mushroom extracts and characterization

2.3.1. Microencapsulation

Microencapsulation was performed by spray-drying using the lyophilized extracts and maltodextrin (MD) as the encapsulating material. The used spray-drying conditions were adapted from the best ones achieved in the work of Wu and co-workers (Wu et al., 2014) that comprise the preparation of a solution with an extract/MD of 1/20 (w/w) and a solids content of 21% (w/w) and the following atomization conditions: inlet temperature 170 ºC, outlet temperature 95 ºC, aspiration at 90% and pump at 20% (6 mL/min). To prepare the atomizing solution, the extracts (1 g) were dissolved firstly in

ethanol (10 mL) followed by water addition (90 mL) to achieve a homogenous solution. Thereafter, 20 g of MD were added under stirring until total dissolution. The equipment was a Mini Spray Dryer B-290 from Büchi (Flawil, Switzerland) used in combination with the Inert Loop B-295 to provide a closed loop circulation under nitrogen atmosphere. The nozzle size was 0.7 mm and the atomized volume 100 mL.

Microcapsules incorporating the individualized extracts and the chosen synergistic combination of both (Sl:Ca, 1:1) have been prepared. Pure MD microcapsules were also produced to be used as control.

2.3.2. Microcapsules characterization

The microencapsulation process was characterized in terms of yield and efficiency. Additionally, the obtained powders were analysed by SEM (to inspect morphology and particle size) and for their antioxidant activity.

The encapsulation yield was determined gravimetrically as the ratio between the weight of microspheres obtained at the end of the process and the weight of the atomized materials (encapsulation material plus extract). It gives an estimative of the material losses during the process.

Encapsulation efficiency (EE) was determined by HPLC-DAD (Heleno et al., 2012; Martins et al., 2014) as the ratio between the experimentally determined encapsulated extract, in terms of cinnamic acid (the major compound identified in the extracts), and the theoretical one. Quantification was based in a calibration curve obtained at 280 nm by injecting known concentrations (0.3-50 µg/mL) of cinnamic acid (y=972179x+174853; R^2=0.999). The results were expressed as µg per mg of extract.

The antioxidant activity, used to inspect the maintenance of synergistic effects was evaluated by DPPH radical scavenging activity and reducing power assays, according to the methodologies described above. The synergistic effect of the encapsulated extracts was evaluated by testing the microcapsules incorporating the chosen synergistic mixture and the corresponding combination prepared from the two individually microencapsulated extracts.

2.4. Incorporation of mushroom extracts into cottage cheese and evaluation of antioxidant activity, nutritional composition and colour of the final products

2.4.1 Preparation of the cottage cheese samples

All the cottage cheese samples were prepared by Queijos Casa Matias Lda (Seia, Portugal). Three groups of samples, each one comprising two ewe cottage cheeses (100 g each), were prepared: (i) control sample (cottage cheeses without added mushroom extracts); (ii) cottage cheeses added with microencapsulated extracts (3 g/cheese); and (iii) cottage cheeses added with free extracts (80 mg/cheese). All the prepared samples contain an equivalent amount of extract (Sl:Ca, 1:1), either in its free or encapsulated form.

2.3.1. Evaluation of cottage cheese antioxidant, nutritional and colour properties

The samples (control sample, cottage cheeses added with microencapsulated extracts and cottage cheeses added with free extracts) were evaluated in what concerns antioxidant activity, nutritional composition and colour, immediately after preparation (t0) and after seven days of storage at 4 ºC (t7).

The antioxidant activity was evaluated using DPPH radical scavenging activity and reducing power, following the extraction process and methodologies described above.

The nutritional composition (moisture, protein, fat, carbohydrates and ash) was evaluated using the AOAC procedures (AOAC, 2005): the protein content (Nx6.38) was determined using the Kjeldahl method; the fat was determined by Soxhlet extraction with petroleum ether; the ash content was determined by incineration at 600±15 ºC; and total carbohydrates were calculated by difference. Total energy was calculated as Energy (kcal)=4×(protein weight (g)+carbohydrate weight (g))+9×(lipid weight (g)).

Fatty acids were determined by analysing the petroleum ether extract previously obtained, by gas-chromatography coupled with a flame ionization detector (GC-FID), according to the procedure described by the authors (Reis et al., 2011). The identification was made by comparison of the relative retention times of fatty acid methyl esters with standards. The results were expressed as relative percentages.

Sample colour was determined in a colorimeter (model CR-400, Konica Minolta Sensing, Inc., Japan), using the illuminant C and a diaphragm aperture of 8 mm. The CIE *L*a*b** colour space values were registered using the data software "Spectra Magic Nx" (version CM-S100W 2.03.0006) (Caleja et al., 2015). Three readings on the sample top and bottom parts, were used.

2.5. Statistical analysis

For each group, two samples were used and antioxidant evaluation assays, nutritional determinations and colour analysis were carried out in triplicate. The results were expressed as mean values ± standard deviation (SD) and further analysed using one-way

analysis of variance (ANOVA) followed by Tukey's HSD Test (α=0.05). This analysis was carryout out using SPSS v. 22.0 program (IBM Corp., Armonk, NY, USA).

3. Results and discussion

3.1. Synergistic effects between mushroom extracts

The results of the antioxidant activity (measured by DPPH radical scavenging activity, reducing power and inhibition of β-carotene bleaching assays) of the free extracts, *S. luteus* (Sl) and *C. atramentaria* (Ca), and of the prepared mixtures (Sl:Ca 1:2, 1:1 and 2:1, w/w) are shown in **Table 1**. Comparing the two individualized mushrooms extracts, the lower EC_{50} values were obtained for Sl extract, indicating that this mushroom has higher antioxidant activity than Ca. This tendency was also corroborated in previous studies carried out by the authors using different extracting solvents, namely pure methanol (Heleno et al., 2012; Reis et al., 2011). The combination Sl:Ca (1:2) presented the highest EC_{50} values and, thus, it was discharged for microencapsulation studies. In what concerns to the combinations Sl:Ca (1:1) and Sl:Ca (2:1), both presented propensity for synergistic effects based on DPPH radical scavenging activity and β-carotene bleaching inhibition assays, even DPPH pointed out for a higher synergistic effect for the Sl:Ca (2:1) (a lower EC_{50} value was achieved for this combination). In the case of the reducing power assay, the achieved values were quite similar. Analysing the obtained results and taking into account practical aspects of sample preparation, the combination Sl:Ca (1:1) was chosen to proceed with microencapsulation and, thereafter, with food incorporation studies.

3.2. Microencapsulation of mushroom extracts

3.2.1. Encapsulation yield, encapsulation efficiency and SEM analysis

The lyophilized extracts of Sl and Ca alone, as well as their mixture in similar proportions (Sl:Ca, 1:1), were encapsulated by spray-drying with maltodextrin (MD) as the encapsulating material. The main purpose of the microencapsulation was the stabilization of the extracts in order to avoid their oxidation and loss of bioactivity, as also to remove their strong odour. The encapsulation yield (EY) of the spray-drying process was around 50%, in accordance with other published works using MD as the encapsulating material (Wu et al., 2014). Thus the lower value was achieved for the Sl:Ca (1:1) mixture (47.1%), the highest value was Sl extract (55.2%) and the intermediate value of EY corresponds to the Ca extract (51.8%). The encapsulation efficiency (EE), determined in terms of cinnamic acid (a compound present in the extracts of both mushroom species) was comprised between 43.5-62.6%. The lower value was achieved with Ca extract (43.5%) and the highest value with Sl extract (62.6%). For the Sl:Ca (1:1) mixture an intermediate value was attained (59.8%). As an illustrative example, **Figure 1** shows the chromatograms obtained with pure MD (**Figure 1A**), Sl extract in its free form (**Figure 1B**) and Sl extract in its encapsulated form (**Figure 1C**). As can be observed, cinnamic acid is present in the chromatograms assigned to free and encapsulated Sl extracts but not in the one of pure MD. This evidence corroborates an effective encapsulation of the Sl extract. The same tendency was observed with Ca extract and Sl:Ca (1:1) mixture (data not shown).

SEM micrographs are shown in **Figure 2** and evidence a heterogeneous particle size distribution (2-50 μm). In a general way particles presented a round shape with both, smooth and rough, surfaces. The appearance of teeth or invaginations on the particles surface, which gives rise to a rough surface, can be attributed to a rapid evaporation of

water during the drying process (Rosenberg, Kopelman, Talmon, 1985; Wu et al., 2014). Differences were also observed as a function of the encapsulated extracts, particles with rough surface are predominant in the assay performed with the Sl extract (**Figure 2A**), being almost absent in the one prepared with the mixture Sl:Ca (1:1) (**Figure 2C**). Also, a larger particle size was registered for this last case.

3.2.2. *In vitro* antioxidant properties

The produced microparticles containing Sl, Ca and Sl:Ca (1:1) extracts were evaluated for their antioxidant activity using two different assays (DPPH radical scavenging activity and reducing power). Additionally, a mixture prepared with the individually encapsulated extracts, trying to mimicking the used synergistic mixture (Sl:Ca, 1:1), was also prepared and tested. The objective was to check the possible advantages of using this strategy over the previous one (both extracts encapsulated in the same microparticle).

The obtained EC_{50} values for the individually microencapsulated extracts (Mic Sl), Ca (Mic Ca) and for the two synergistic mixtures (Mic Sl:Ca, 1:1 and Mic Sl+Mic Ca, 1:1) are shown in **Table 2**. In what concerns the individualized encapsulated extracts, Sl revealed the higher antioxidant activity, confirming the tendency already observed with the free extract forms. No significant advantage was noticed as a result of the used methodology for mixtures preparation. The obtained EC_{50} values were quite similar for both preparations, giving the Sl:Ca (1:1) slight better results. In fact, for this mixture the assumption of synergistic effects was achieved with both DPPH radical scavenging activity and reducing power assays. These microspheres, corresponding to a more

attractive preparation procedure based in a unique step, were then selected for incorporation into cottage cheese.

3.3. Cottage cheese enriched with mushroom extracts

3.3.1. *In vitro* antioxidant properties

Table 3 shows the antioxidant activity results of the prepared cottage cheese samples (control cottage cheese without extracts; cottage cheese enriched with Sl:Ca (1:1) extract in its free form; and cottage cheese enriched with Sl:Ca (1:1) extract in its microencapsulated form) obtained according with DPPH radical scavenging activity and reducing power assays. It was observed that the incorporation of free extracts resulted in products with higher initial antioxidant activity (t0) but declining after seven days (t7), which can be associated with their degradation. However, the cottage cheese enriched with the microencapsulated extracts, that have revealed a lower activity at initial time, showed an increase at t7. This improvement can be explained by an effective protection provided by the used microencapsulation process (spray-drying with MD) together with a sustained release.

The antioxidant increment observed for the control sample, according to the reducing power assay, is compatible with the formation of antioxidant (in this case, reducing) compounds as a result of lipid peroxidation process occurring during cottage cheese storage. Nevertheless, both antioxidant evaluation assays, show a clear cut of tendency pointed out for higher antioxidant preservation over time if microencapsulated extracts are used.

3.3.2. Colour and nutritional evaluation

Nutritional evaluation in cottage cheese samples comprised the assessment of protein, fat (including fatty acids profile), carbohydrate and ash contents, as well as, total energy. **Table 4** shows the obtained results that pointed out, comparatively with the control sample, for nutritional properties maintenance after the incorporation of mushroom extracts (both in its free and microencapsulated forms). Cottage cheese samples, as expected, showed lipids as the major macronutrients with special contribution from saturated fatty acids. Moreover, the same fatty acids profile was observed in all the samples, with palmitic acid (C16:0) presented at higher content. Oleic (C18:1) and linoleic (C18:2) acids were the major MUFA and PUFA, respectively. Moisture, ash, carbohydrates and energy values are within the range of the reference values for this type of cottage cheese. In same table, colours evaluation of samples indicated no changes due to the extracts incorporation (free and microencapsulated forms) in cottage cheese. In general, the introduction of free and encapsulated extracts did not affect the nutritional value of the cottage cheese when comparing with the control sample.

Conclusions

In this work, the antioxidant activity of Sl and Ca extracts was evaluated being observed that their combination results in synergistic effects. The spray-drying of the extracts using an extract/maltodextrin ratio of 1/20 and an inlet temperature of 170 ºC resulted in good encapsulation yield (around 50%) and efficiency (43.5-62.6%.).

The microencapsulated extracts with maltodextrin did not lose antioxidant activity, being the combination Sl:Ca (1:1) the best as it revealed synergistic effects. The microspheres with Sl:Ca (1:1) and with free extract (in the same proportion) were

incorporated into cottage cheese. The results showed that, in comparison with the free form, the encapsulated extracts became more effective since the antioxidant activity was preserved along time. The evaluation of nutritional value revealed that the introduction of free and encapsulated extracts did not affect the contents of proteins, fat (and fatty acids), carbohydrates, and ash, when comparing with the control cottage cheese. Also, no colour changes were detected.

Competing interests

The authors declare no competing financial interest.

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Table 1. Antioxidant activity, expressed as EC_{50} values (mg/mL), of individual and combined extracts prepared from *S. luteus* (Sl) and *C. atramentaria* (Ca). Results are presented as mean values±SD.

Assay	Sl	Ca	Sl:Ca(1:1)			Sl:Ca(1:2)			Sl:Ca(2:1)		
			Theoretical*	Experimental	Effect	Theoretical**	Experimental	Effect	Theoretical***	Experimental	Effect
DPPH radical scavenging activity	2.86±0.02	4.62±0.14	3.74	3.49±0.13	SE	4.04	4.07±0.06	AE	3.44	2.72±0.02	SE
Reducing power	0.97±0.02	1.11±0.02	1.04	1.05±0.02	AE	1.06	1.20±0.04	NS	1.02	0.94±0.01	SE
Inhibition of β-carotene bleaching	1.64±0.20	5.28±0.36	3.46	1.46±0.04	SE	4.08	1.51±0.07	SE	2.84	1.47±0.04	SE

Theoretical values were calculated as weighted mean experimental EC_{50} values available in individual mushrooms: *$EC_{50} = EC_{50}Sl\times0.50 + EC_{50}Ca\times0.50$; ** $EC_{50} = EC_{50}Sl\times0.33 + EC_{50}Ca\times0.67$; *** $EC_{50} = EC_{50}Sl\times0.67 + EC_{50}Ca\times0.33$.

AE- additive effect: theoretical and experimental values reveal differences lower than 5%; SE- synergistic effect: experimental values are more than 5% lower for EC_{50} when compared with theoretical values; NS- negative synergistic (antagonistic) effect: experimental values are more than 5% higher for EC_{50} when compared with theoretical values.

Table 2. Antioxidant activity, expressed as EC_{50} values (mg/mL), of microencapsulated (Mic) individual and combined extracts prepared from *S. luteus* (Sl) and *C. atramentaria* (Ca). Results are presented as mean values±SD.

Assay	Mic Sl	Mic Ca	Theoretical	Mic Sl:Ca (1:1)		Mic Sl+Mic Ca (1:1)	
				Experimental		Experimental	Effect
DPPH radical scavenging activity	2.13±0.06	2.36±0.16	2.25	1.96±0.06	SE	2.04±0.04	SE
Reducing power	0.85±0.01	0.78±0.01	0.82	0.87±0.01	NS	0.83±0.02	AE

Theoretical value was calculated as weighted mean experimental EC_{50} values available in individual mushrooms: $EC_{50} = EC_{50}Sl \times 0.50 + EC_{50}Ca \times 0.50$.
AE- additive effect: theoretical and experimental values reveal differences lower than 5%; SE- synergistic effect: experimental values are more than 5% lower for EC_{50} when compared with theoretical values; NS- negative synergistic (antagonistic) effect: experimental values are more than 5% higher for EC_{50} when compared with theoretical values.
Mic Sl + Mic Ca (1:1) – mixture prepared with the individually encapsulated extracts for mimicking microspheres with combination Sl:Ca (Mic Sl:Ca).

Table 3. Antioxidant activity, expressed as EC_{50} values (mg/mL), of cottage cheese control and samples enriched with free and microencapsulated extracts prepared from *S. luteus* (Sl) and *C. atramentaria* (Ca) combined in similar proportions (Sl:Ca, 1:1). Results are presented as mean values±SD.

	Control cottage cheese		Cottage cheese with free extract		Cottage cheese with microencapsulated extract	
Storage days	0 days	7 days	0 days	7 days	0 days	7 days
DPPH radical scavenging activity	>200	>200	83.61±2.57^{c}	161.86±7.28^{a}	133.44±4.09^{b}	96.96±9.33^{c}
Reducing power	>200	56.58±0.18	14.49±0.19^{c}	19.96±0.70^{b}	20.46±0.49^{a}	13.67±0.18^{d}

In each line, different letters mean statistical significant differences ($p<0.05$) between samples.

Table 4. Nutritional value and colour parameters of the cottage cheese control and samples enriched with free and microencapsulated extracts prepared from *S. luteus* (Sl) and *C. atramentaria* (Ca) combined in similar proportions (Sl:Ca, 1:1), along storage time. Results are presented as mean values±SD.

	Control cottage cheese		Cottage cheese with free extract		Cottage cheese with microencapsulated extract	
Storage days	0 days	7 days	0 days	7 days	0 days	7 days
Nutritional value						
Ash (g/100 g fw)	1.48±0.02^{cd}	1.69±0.03^{ab}	1.35±0.14^{d}	1.80±0.09^{a}	1.62±0.10^{bc}	1.56±0.05^{bc}
Fat (g/100 g fw)	16.56±0.04^{a}	16.21±0.00^{bc}	15.77±0.08^{de}	17.67±0.39^{a}	15.51±0.18^{e}	16.04±0.04^{cd}
Protein (g/100 g fw)	12.48±0.26^{ab}	12.31±0.19^{ab}	12.60±0.03^{a}	12.43±0.03^{ab}	12.10±0.00^{b}	12.45±0.13^{ab}
Carbohydrates (g/100 g fw)	1.15±0.29^{c}	2.02±0.20^{a}	1.03±0.03^{c}	2.38±0.33^{a}	1.29±0.06^{bc}	1.84±0.13^{ab}
Energy (Kcal/100 g fw)	203.56±0.09^{bc}	203.23±0.07^{b}	196.44±0.70^{d}	218.27±1.11^{a}	193.17±0.93^{e}	201.53±0.27^{c}
Fatty acids						
C4:0	6.56±0.83	8.55±0.48	9.51±0.50	7.98±0.81	8.07±0.85	8.34±0.21
C6:0	4.67±0.42	5.44±0.52	6.11±0.23	5.24±0.49	4.84±0.02	5.06±0.37
C8:0	3.08±0.26	3.59±0.47	3.83±0.15	3.50±0.22	3.11±0.09	3.27±0.31
C10:0	6.64±0.35	7.77±0.96	7.81±0.26	7.73±0.32	6.78±0.27	7.25±0.53
C12:0	3.26±0.07	3.67±0.25	3.54±0.07	3.71±0.09	3.38±0.13	3.48±0.09
C14:0	7.83±0.04	8.45±0.06	8.12±0.07	8.51±0.06	8.11±0.29	8.12±0.09
C16:0	20.90±0.02	20.44±0.66	19.98±0.36	20.74±0.43	20.55±0.36	20.74±0.33
C18:0	13.06±0.16	11.50±0.55	11.25±0.11	11.84±0.48	12.26±0.06	12.43±0.13
C18:1n9	24.99±0.06	22.45±0.97	21.82±0.50	22.64±0.85	24.21±0.21	23.02±0.98
C18:2n6	3.46±0.04	3.09±0.18	3.00±0.03	3.14±0.10	3.30±0.05	3.23±0.04
SFA (relative %)	68.60±0.15^{a}	71.81±1.32^{a}	72.52±0.61^{a}	71.64±1.02^{a}	69.62±0.28^{a}	71.04±0.90^{a}
MUFA (relative %)	26.13±0.08^{a}	23.55±1.01^{a}	22.95±0.55^{b}	23.70±0.87^{a}	25.40±0.23^{a}	24.12±1.00^{a}
PUFA (relative %)	5.27±0.07^{a}	4.65±0.31^{a}	4.53±0.07^{c}	4.65±0.15^{a}	4.98±0.06^{b}	4.85±0.11^{a}
Colour parameters						
L^*	91.50±0.85^{a}	91.05±2.00^{a}	91.48±0.63^{a}	90.74±0.87^{a}	91.10±0.93^{a}	90.98±0.83^{a}
a^*	-2.12±0.14^{b}	-2.07±0.38^{a}	-1.90±0.08^{a}	-1.74±0.39^{a}	-1.93±0.11^{a}	-1.74±0.30^{a}
b^*	9.86±0.31^{b}	11.43±1.60^{a}	10.23±0.80^{b}	12.39±1.79^{a}	11.11±0.46^{a}	11.21±1.58^{a}

L^*, a^* and b^* represent colour parameters. Butiric acid (C4:0), caproic acid (C6:0); caprylic acid (C8:0); capric acid (C10:0); lauric acid (C12:0); myristic acid (C14:0); palmitic acid (C16:0); stearic acid (C18:0); oleic acid (C18:1n9); linoleic acid (C18:2n6); SFA- Saturated fatty acids; MUFA- Monounsaturated fatty acids; PUFA- Polyunsaturated fatty

acids. The difference to 100% corresponds to other 19 less abundant fatty acids (data not shown). In each line, different letters mean statistical significant differences ($p<0.05$) between samples.

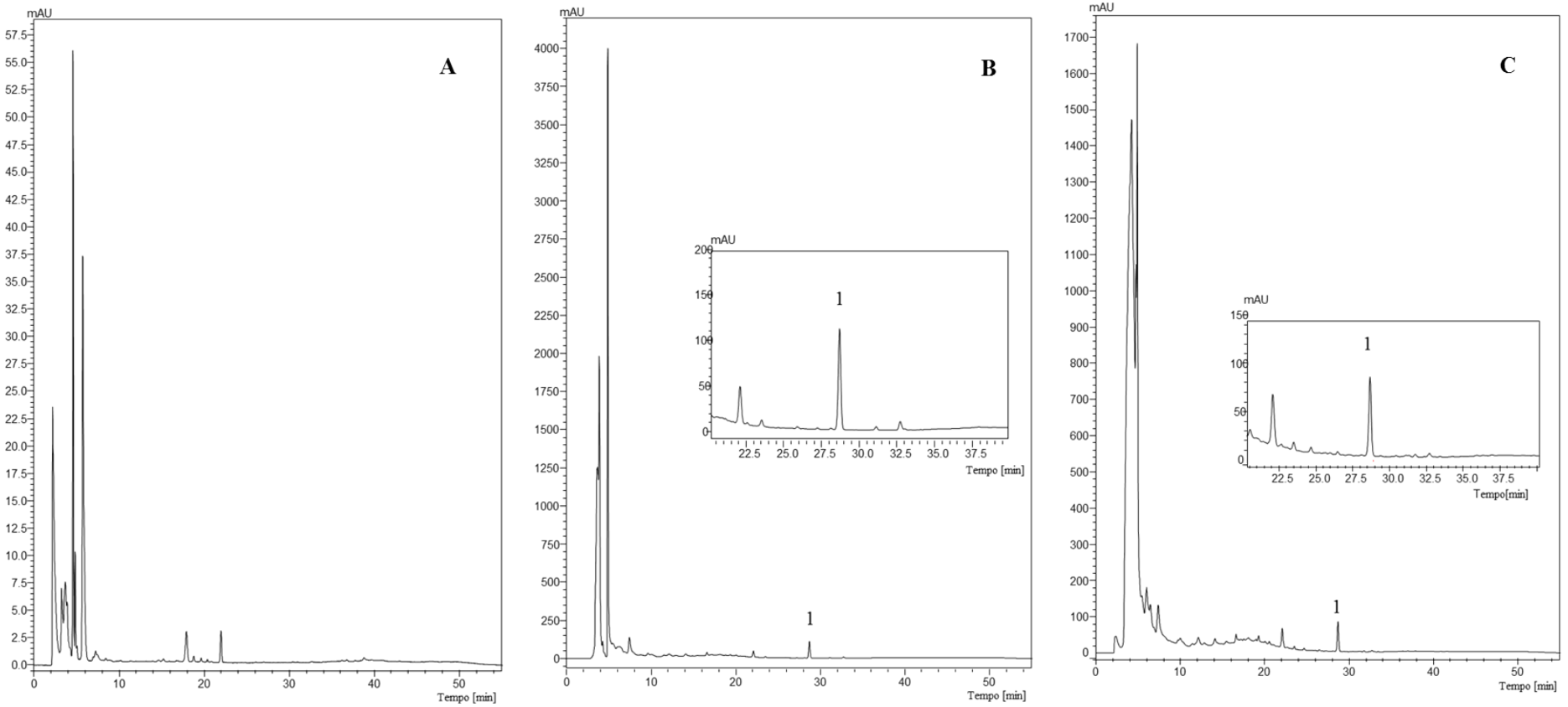

Figure 1. Chromatograms obtained at 280 nm of maltodextrin (A),free *S. luteus* (Sl) extract (B) and microspheres with Sl extract (C). 1- Cinnamic acid.

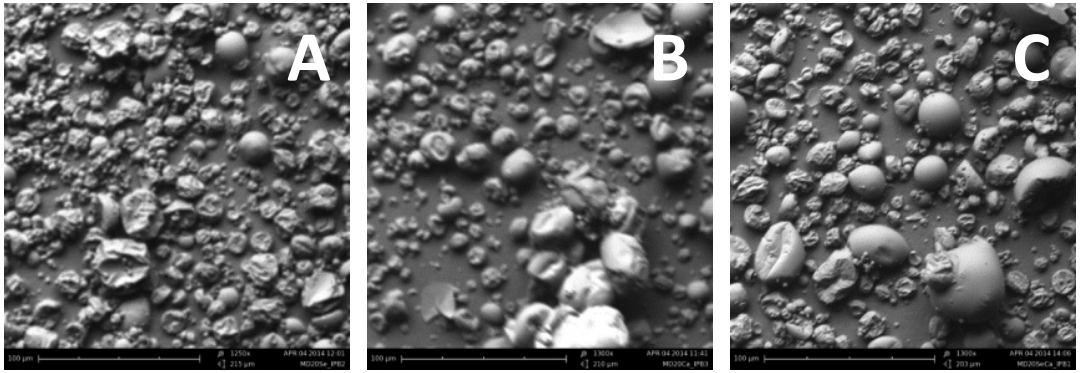

Figure 2. SEM analysis of microparticles containing *S. luteus* extract (Sl) (**A**), *C. atramentaria* extract (Ca) (*B*) and the mixture Sl:Ca (1:1) under a magnification of 1300x.