Research Article

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# Protein matrices ensure safe and functional delivery of rosmarinic acid from marjoram (*Origanum majorana*) extracts

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## Abstract

**BACKGROUND: To understand the interactions between carriers and functional ingredients is crucial when designing delivery systems, to maximize bioefficacy and functionality. In this study, two different protein matrices were evaluated as means to protect the extract isolated from marjoram leaves (*Origanum majorana*), casein micelles from fresh skim milk and soy protein isolate (SPI).**

**RESULTS: Marjoram extract was obtained from pressurization of ethanol and water solvent. Protein dispersions of casein and SPI (5 g $L^{-1}$ each) with or without marjoram extract (0.1–3 mg $mL^{-1}$) were prepared and homogenized. The physicochemical characterization of charge and entrapment efficiency were conducted. The results demonstrated that entrapment efficiency was highly dependent on the carrier itself where SPI formulations showed 20% higher affinity when compared to casein micelles. To investigate the physiological behaviour of the marjoram–protein dispersions, human macrophages were employed. A non-specific inflammatory response of macrophages stimulated with bacterial lipopolysaccharide was measured for TNF-$\alpha$, IL-1$\beta$ and IL-6 cytokine secretion.**

**CONCLUSION: Casein and SPI protein formulations warranted high bioefficacy of marjoram extract, showing their potential as safe carriers.**


**Keywords:** marjoram; rosmarinic acid; entrapment; soy protein isolate; caseins; anti-inflammatory activity

## INTRODUCTION

Health promoting benefits of aromatic plants and spices have been extensively described. Among them marjoram (*Origanum majorana* L.) is recognized for being used as a food additive with flavouring properties in addition to promoting digestive system well-being. Marjoram extract (ME) is known for its antioxidant and antimicrobial properties[1,2] and for its anti-inflammatory activity.[3] This activity is contributed to the composition of the ME mainly phenolic acids like rosmarinic, caffeic, carnosic and gallic, as well as other phenolic compounds such as luteolin, apigenin or carnosol.[4] Rosmarinic acid has been reported as the main compound detected in hydroalcoholic MEs.[5]

Environmental clean technologies for extraction and concentration of compounds from plant origin are well established and offer reduced toxicity usually associated with traditional solid–liquid extractions where methanol, hexane or acetone is used as a solvent. More advanced techniques have become available aiming to reduce losses on the bioactive quantities extracted from plants and increase their purity and maximize their functionality *per se*. Consequently, ultrasound-assisted extraction (UAE) and microwave-assisted extraction (MAE) are techniques that offer high reproducibility in short time, simple manipulation and low energy input.[6] Potential industrial application of UAE to plant materials has been previously described providing higher extraction yield than classic methods (e.g. solid–liquid extraction).[7] Pressurized liquid extraction (PLE) is also employed in food technology and is considered a safe and clean technique. Extraction process using PLE occurs at solvent temperature between 50–200 °C and pressure around 6–12 MPa.[8] Optimization of extraction process has been achieved using higher temperatures to increase solubility and extraction rate, for example, water can behave as an organic solvent at certain temperature that affects its dielectric constant.[9]

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Despite the advantages of applying these techniques in ingredient development, they are mainly and widely used in pharmaceutical applications. Nevertheless their incorporation in food products represent a challenge due to low solubility of final products in water or complex matrices and the level of solvent traceability in the final product. Therefore, protein-based nanostructures are ideal alternative carriers of bioactive compounds including carotenoids or phenolic compounds. Compounds, especially poor water-soluble molecules enclosed in milk or plant-based proteins have shown improved solubility and stability during processing or transit through the gastro intestinal tract.[10,11] Milk components, including milk proteins, have been described as delivery carriers for biologically active molecules. Caseins represent more than 80% of total milk proteins where about 95% is present as colloidal aggregates, so called casein micelles, due to the presence of calcium and phosphate linked to serine-phosphate residues in native milk. It is described that the hydrophobic environment of the core of casein micelles offers the possibility to entrap non-polar molecules.[12] Examples of successful delivery of low water-soluble molecules in casein micelles are curcumin or vitamin D.[13]

Noteworthy, plant-based proteins are an abundant low-cost source of bioactive peptides, such as soy proteins. Soy proteins exposure to alkali solubilization and acid precipitation process outcomes protein denaturation, and as a result aggregation in isolates with an average size of nanometres and low water solubility, highly dependent of preparation conditions is achieved.[14] Soy protein isolate (SPI) is the most commercially available soy protein and commonly recognized for good ability to adsorb and stabilize the interphase of oil-in-water emulsions, owing to the amphiphilic properties of isolates. Moreover, SPI has been described as carrier of lipophilic compounds (e.g. $\beta$-carotene or curcumin).[15,16]

The objective of this study was to evaluate the bioefficacy and functionality of ME entrapped in protein matrices, respectively, casein micelles and SPI. Physicochemical characterization of the formulations was conducted and entrapment efficiency was determined by means of high-performance liquid chromatography (HPLC). To determine the potential bioefficacy of the encapsulated ME an *in vitro* model of inflammation was employed. Immuno-modulatory response was conducted in human macrophages to confirm the hypothesis that ME entrapment in protein matrices may ensure safe delivery and therefore functionality.

## MATERIALS AND METHODS

### Marjoram samples preparation

Dried marjoram leaves (*Origanum majorana* L.) were obtained from Herboristeria Murciana company (Murcia, Spain), certified ISO 9001:2008. The sample was ground in a knife mill (Grindomix GM 200, Restch, Llanera, Spain) and the particle size was determined by sieving the ground plant material to the appropriate size (< 500 μm).

### Pressurized liquid extraction

Extraction of marjoram was performed in a Dionex ASE 350 (Dionex Corporation. Sunnyvale, CA, USA) system equipped with a solvent controller unit. Three different ratios of ethanol/water (*v*/*v*) solutions were applied (50:50, 70:30, 100:0) as extraction solvent. Powdered marjoram sample (1.0 g) was mixed with sea sand (4.0 g) and placed into an 11 mL Dionex (ASE 350) stainless-steel cell. The extraction was performed at 100 °C for 10 min at 10.34 MPa, in duplicates. Prior to freeze-drying (Labconco Corporation, Kansas City, MO, USA) the extracts, the solvent was evaporated in a Rotovapor IKA RV 10 (VWR International, Barcelona, Spain). All the lyophilized samples were stored at −20 °C until use.

### Ultrasound-assisted extraction (UAE)

Ground marjoram (40 g) with the corresponding concentration of ethanol/water solution (50:50, 70:30, 100:0) in a ratio 1:10 (bark/solvent) were submitted to ultrasound extraction for 30 min using a 1/2 diameter disruptor horn probe at 70% amplitude (maximum power output of 400 W at 60 Hz) (Branson Digital Sonifier, Branson Ultrasonics, model 250; Danbury, CT, USA) maintaining the temperature at 35 °C with an ice bath and assisted with a stir plate. After sonication, the samples were filtrated, evaporated and freeze-dried. All samples were stored under −20 °C prior to analysis.

### Rosmarinic acid quantification, total phenolic content and antioxidant activity determination

HPLC-~~pulsed amperometric detection~~ (PAD) analysis of rosmarinic acid in marjoram extracts was performed as previously described.[17]

Total phenolic content (TPC) was determined using the Folin–Ciocalteu's colorimetric method developed by Singleton *et al.*[18] A standard curve was calculated using gallic acid, and results were expressed as gallic acid equivalents (GAE) (milligrams of gallic acid per gram of dried extract). The antioxidant activity of the ME was determined by the $ABTS^{\bullet+}$ assay. This method was applied according to Re *et al.*[19] protocol. The results were expressed as TEAC values (millimoles of Trolox equivalents per gram of dried extract).

### Entrapment of marjoram pressurized liquid extract in protein aggregates

Two different protein matrices, caseins and SPI were employed as carriers of marjoram pressurized liquid extract. Preliminary studies were performed to choose a SPI concentration with minimum insoluble fraction (less than 10%). A range of SPI concentrations were prepared in 50 mM sodium phosphate buffer pH 7.4, stirred for 1 h at 40 °C and stored overnight at 4 °C for complete hydration. Conventional homogenization was then performed using the protein solutions at 450 kPa for four passes followed by low speed centrifugation (100×*g* for 5 min) (Eppendorf, Brinkmann Instruments, Westbury, NY, USA). Supernatant aliquots were collected and protein content was determined by Lowry assay (DC Protein Assay, BioRad Laboratories, Mississauga, ON, Canada), using BSA as standard.

Caseins were isolated from skim milk by centrifugation at 62 000×*g* for 30 min and 20 °C (OptimaTM LE-80 K, with a Ti-45 rotor, Beckman–Coulter, Mississauga, ON, Canada). Protein analysis of the pellets was measured using a Dumas combustion method nitrogen analyser (FP-528, Leco Inc., St Joseph, MI, USA). Casein pellets were dissolved at 5 g $L^{-1}$ (based on protein) in 20 mM imidazole buffer (pH 7.0) containing 5 mM calcium chloride to ensure the isotonic environment using a hand-held homogenizer (Polytron PT 1200, Kinematica, Fisher Scientific, Mississagua, ON, Canada). ME stock solutions were dissolved in ethanol/imidazole buffer (1:3), final volume 1 mL, and added dropwise to achieve 0.1, 0.25, 0.5, 1, 2 and 3 mg $mL^{-1}$ in the casein solution. The mixtures



  

**Table 1.** Evaluation of extraction yield [% dry weight ± standard deviation (SD)], total phenolic content [TPC (mg GAE $g^{-1}$ dry extract ± SD)], antioxidant activity [TEAC (mmol TE $g^{-1}$ dry extract ± SD)] and quantification of rosmarinic acid (RA) (mg RA $g^{-1}$ of dry extract ± SD) for marjoram extracts obtained by ultrasound-assisted extraction (UAE) and pressurized liquid extraction (PLE) using different percentages of ethanol (% *v*/*v*) during the extraction process[A,B]

| Extraction technique | Ethanol/ water (% *v*/*v*) | Extraction yield (%) | TPC (mg GAE $g^{-1}$ dry extract) | TEAC (mmol TE $g^{-1}$ dry extract) | RA (mg RA $g^{-1}$ dry extract) |
|---|---|---|---|---|---|
| UAE | 50:50 | $11.56 \pm 0.7^{b\,2}$ | $233.2 \pm 2.1^{b\,1}$ | $1.44 \pm 0.02^{b\,2}$ | $33.62 \pm 1.18^{b\,1}$ |
| | 70:30 | $15.6 \pm 0.3^{a\,2}$ | $256.6 \pm 3.4^{a\,1}$ | $1.52 \pm 0.04^{a\,2}$ | $35.87 \pm 1.89^{a\,1}$ |
| | 100:0 | $5.86 \pm 0.4^{c\,2}$ | $143.4 \pm 1.7^{c\,2}$ | $0.54 \pm 0.02^{c\,2}$ | $23.36 \pm 0.82^{c\,2}$ |
| PLE | 50:50 | $22.9 \pm 0.1^{a\,1}$ | $237.5 \pm 2.2^{b\,1}$ | $1.49 \pm 0.02^{b\,1}$ | $31.48 \pm 0.26^{c\,1}$ |
| | 70:30 | $23.3 \pm 0.1^{a\,1}$ | $265.9 \pm 4.8^{a\,1}$ | $1.81 \pm 0.04^{a\,1}$ | $33.94 \pm 0.75^{a\,1}$ |
| | 100:0 | $11.1 \pm 0.34^{b\,1}$ | $201.2 \pm 4.9^{c\,1}$ | $0.81 \pm 0.02^{c\,1}$ | $32.36 \pm 0.39^{b\,1}$ |

[A]Within an extraction technique, different superscript lowercase letters indicate statistical differences between ethanol/water composition at $P < 0.05$.
[B]Within the same ethanol/water composition, different superscript numbers indicate statistical differences between extraction technique at $P < 0.05$.

were further kept for 1 h on a magnetic stirrer at 37 °C. Casein formulations were then submitted to high-pressure homogenization at 475 kPa for four passes using a microfluidizer (model M-110Y, Microfluidics Corporation, Newton, MA, USA).

Protein solutions containing 5 g $L^{-1}$ SPI were chosen to incorporate ME. Stock extract solutions were dissolved in ethanol/sodium phosphate buffer (1:3), final volume 1 mL, to achieve 0.1, 0.25, 0.5, 1, 2 and 3 mg $mL^{-1}$ in the SPI formulations. Protein solutions were prepared as described earlier and after overnight storage at 4 °C, extract solutions were added dropwise. The mixtures were further kept for 1 h on a magnetic stirrer at 37 °C. High-pressure homogenization was then performed at 475 kPa for four passes using a microfluidizer (model M-110Y).

Zeta ($\zeta$)-potential of the fresh casein and SPI formulations was measured by dynamic light scattering (Zetasizer Nano, Malvern Instruments, Malvern, UK). Casein formulations were diluted in 20 mM imidazole buffer (pH 7.0) containing 5 mM calcium chloride (1:1000) while SPI formulations were diluted in 50 mM sodium phosphate buffer pH 7.4 (1:100).

### Rosmarinic acid entrapment efficiency

Entrapment efficiency of rosmarinic acid in casein and SPI formulations was measured after homogenization. Samples were priory filtered (0.45 µm PVDF filters, Fisher Scientific) and aliquots of 500 µL were centrifuged in concentrator microcentrifuge tubes (Spin-x UF 500 10 K MWCO PES 500 µL, Corning, NY, USA), for 15 min at 3000×*g* (benchtop Eppendorf centrifuge 5415D, Brinkmann Instruments). Collected permeate was further analysed for rosmarinic acid quantification by means of HPLC-PAD as previously described.[17]

### *In vitro* immunomodulatory activity of marjoram formulations

Human THP-1 monocytes (American Type Culture Collection, ATCC, CEDARLANE Corporation, Burlington, ON, Canada) were cultured in RPMI 1640 culture medium supplemented with 10% foetal bovine serum (FBS), 100 U $mL^{-1}$ penicillin, 100 mg $mL^{-1}$ streptomycin, 2 mM L-glutamine and 0.05 mM $\beta$-mercaptoethanol (Sigma-Aldrich, Oakville, ON, Canada) at 37 °C in 95% humidified air containing 5% $CO_2$. Cells were plated at a density of $5 \times 10^5$ cells $mL^{-1}$ in 24 well plates. Differentiation to macrophages was induced by incubating the cells with 100 ng $mL^{-1}$ phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich) for 48 h.

The toxic effect of the marjoram formulations (50, 100 and 200 µL) on differentiated macrophages was tested using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay (Sigma-Aldrich) following Mosmann's method.[20]

For immunomodulatory assay, cells were washed with phosphate-buffered saline (PBS) solution and incubated with 0.05 µg $mL^{-1}$ lipopolysaccharide (LPS) (Sigma-Aldrich) in the presence of either 100 µL of casein or SPI formulations containing, respectively, 0, 0.5 and 1 mg $mL^{-1}$ of ME for 24 h. Formulations were tested along with a control of ME 100 and 50 µg $mL^{-1}$. Then, the supernatants were kept frozen at −80 °C. The release of cytokines IL-1$\beta$, IL-6 and TNF-$\alpha$ was measured in the supernatants of macrophages cells using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Bio-Techne Corporation, Minneapolis, MN, USA), according to the manufacturer's instructions.

### Statistical analysis

For each sample, duplicate extractions were performed and the analysis of phenolic compounds was carried out in triplicate expressed as mean values and standard deviation. The results were analysed using one-way analysis of variance (ANOVA) followed by LSD (least significant difference) test with a $P \leq 0.05$ using Statgraphics Centurion XVI (Statpoint Inc., Washington, DC, USA) software.

## RESULTS AND DISCUSSION

### Comparison of marjoram extracts and rosmarinic acid determination

Two extraction techniques (PLE and UAE) were used to obtain a ME with a high quantity of rosmarinic acid and a significant antioxidant activity using three different concentrations of ethanol/water like solvent extraction. Both ethanol and methanol have been widely used to extract phenolic compounds from plant material due to their polarity and good solubility, although, for industrial purposes ethanol is preferable since it is considered GRAS.[9,21] The effect of ethanol on yield extraction, TPC and TEAC value of marjoram extract for both techniques are presented in Table 1. The higher extraction yield occurred when a mixture of ethanol/water was used as solvent extraction, instead of absolute ethanol. Moreover, the values were significantly higher when the extraction was carried out by the PLE technique, in comparison with UAE, up to 23% of yield when 50% and 70% ethanol was used.

For the TPC determination, the values ranged from 143.4 to 265.9 mg GAE $g^{-1}$ of dry extract for both techniques, PLE and UAE. However, at absolute ethanol as extraction phase, PLE yields

the highest activity of ME (201.2 ± 4.9 versus 143.4 ± 1.7 mg GAE $g^{-1}$ of dry extract). The highest TPC value was achieved for a mixture of ethanol/water (70:30) (*v/v*). Meanwhile, the antioxidant activity of MEs, expressed as Trolox equivalent (TE) per gram of dry matter, ranged from 0.54 to 1.52 mmol TE $g^{-1}$ for those obtained with the UAE technique and from 0.81 to 1.81 mmol TE $g^{-1}$ for the PLE technique. Curiously, the effect of ethanol in the TEAC values in both techniques, was higher for those extractions performed with an ethanol/water of 70:30 composition rather than 100% ethanol. Particularly in this condition, the use of PLE allowed a slightly elevated value than UAE. In addition, in this study, a strong correlation between the TPC and the antioxidant activity is exhibited, as the higher TPC values corresponding to the higher TEAC values. Other researchers have reported a positive correlation between the TPC and antioxidant activity of herbs.[22]

Rosmarinic acid quantification and its potential antioxidant activity in MEs is shown in Table 1. It can be observed that the values were similar in ME with 50% and 70% of ethanol, unlike for the absolute ethanol condition where PLE showed a better result. Thereby, PLE and UAE seem to represent an appropriate approach to obtain MEs with optimum quantity of rosmarinic acid, although a better extraction yield was obtained by the PLE.

Based on the earlier results we selected the PLE technique as an extraction technique using 70% ethanol, due to the advantages that PLE presents, like a remarkable higher extraction yield and a slightly higher antioxidant activity when compared with UAE in the studied conditions.

### Entrapment of rosmarinic acid from marjoram pressurized extract in delivery systems

A number of delivery systems were designed to maximize entrapment of rosmarinic acid from marjoram pressurized extract. Preliminary experiments were conducted in oil-in-water emulsions. Previous studies have demonstrated that tea polyphenols are able to associate at the interface of sodium caseinate stabilized soybean oil emulsions.[23] Different concentrations of ME were studied in 10% soybean oil and 0.5% sodium caseinate formulated emulsions, however less than 10% rosmarinic acid was adsorbed at the interface. In addition, low solubility of the extract was observed in soybean oil and emulsions were not further considered as carriers of marjoram pressurized extract. Rosmarinic acid has low solubility in water and low partition coefficient, which complicates its formulation.[24]

Since marjoram pressurized extracts showed slight solubility in water, entrapment of PLE ME was assessed in protein carriers, caseins and SPI. Complexation of low water-soluble compounds with SPI has been described to improve water dispersibility and stability to processing treatments.[15] Previous research from our group demonstrated that the commercial SPI employed in this study has lower water solubility than that reported in the literature.[25] A range of SPI solutions in water (0.1 – 200 g $L^{-1}$) were prepared to determine protein insoluble fraction using the Bradford protein assay.[26] Results showed that protein concentrations below 5 g $L^{-1}$ assure an insoluble fraction lower than 10%. As for the caseins dispersions, higher solubility in water was observed. Hence, protein dispersions of caseins and SPI were employed at 5 g $L^{-1}$ along the study.

Table 2 shows $\zeta$-potential results of casein and SPI formulations determined by dynamic light scattering. Furthermore, $\zeta$-potential of formulations with SPI were not affected by incorporation of ME. Similar values were obtained in the presence of the highest concentration of ME (−14.56 ± 1.01 mV) and without extract (−13.77 ± 0.61 mV). The same effect was found in casein formulations, no differences in surface charge caused by the addition of extract (−19.96 ± 2.08 mV) and (−20.55 ± 1.49 mV) for 0 and 1 mg $mL^{-1}$ of ME, respectively. Hence, $\zeta$-potential results without the extract are consistent with those previously reported in the literature for SPI (−13.40 mV) and casein micelles (−21.7 mV).[10,27] Therefore, the presence of ME did not compromise physical stability of SPI and casein.

**Table 2.** Measurements of $\zeta$-potential of protein suspensions (5 g $L^{-1}$), caseins (CAS) or soy protein isolate (SPI), containing 0, 0.5 or 1 mg $mL^{-1}$ of marjoram extract (ME)[A]

| Sample | $\zeta$-Potential (mV) |
|---|---|
| CAS (0 mg $mL^{-1}$ ME) | −19.96 ± 2.08[a] |
| CAS (0.5 mg $mL^{-1}$ ME) | −20.06 ± 1.05[a] |
| CAS (1 mg $mL^{-1}$ ME) | −20.55 ± 1.49[a] |
| SPI (0 mg $mL^{-1}$ ME) | −13.77 ± 0.61[a] |
| SPI (0.5 mg $mL^{-1}$ ME) | −14.70 ± 0.30[a] |
| SPI (1 mg $mL^{-1}$ ME) | −14.56 ± 1.01[a] |

[A]Within the same protein suspension, different superscript lowercase letters indicate statistical differences between 0, 0.5 and 1 mg $mL^{-1}$ of marjoram at $P < 0.05$.

Entrapment efficiency of rosmarinic acid in caseins and SPI solutions was determined by means of HPLC analysis. Rosmarinic acid concentration was measured in permeate samples obtained after centrifugation in concentrator tubes. Figure 1 illustrates the results obtained for entrapment efficiency in casein (Fig. 1A) and SPI (Fig. 1B) formulations. Caseins micelles entrapped 56.40 ± 4.82% of rosmarinic acid contained in 0.1 mg $mL^{-1}$ of ME. Similar results were found with 0.25 mg $mL^{-1}$ of the extract (57.40 ± 10.02%), however the entrapment efficiency rapidly dropped to 20% at 1 mg $mL^{-1}$ of extract that remained stable at 2 and 3 mg $mL^{-1}$. Our previous studies also demonstrated successful delivery of aromatic plant extracts in casein micelles, particularly the two main compounds, carnosic acid and carnosol, presented in rosemary supercritical extracts.[10] Encapsulation of hydrophobic pure compounds as curcumin and vitamin D in casein micelles has also been described.[13,28] Moreover, encapsulation in casein micelles provides protection from degradation of $\beta$-carotene exposed to common industrial treatments as pasteurization, sterilization or baking.[29]

From the SPI results obtained, it is interesting to point out that at the lowest concentrations of ME (0.1 mg $mL^{-1}$), the entrapment efficiency of rosmarinic acid in SPI reached the highest value (87.11 ± 8.51%). As the extract concentration increased, the entrapment progressively decreased and the amount detected in the aqueous phase increased to 67.54 ± 2.58% at the highest analysed concentration of 3 mg $mL^{-1}$. However, at 1 mg $mL^{-1}$ of marjoram in SPI an entrapment efficiency of 45.07 ± 6.79% rosmarinic acid was detected. Similarly, decay in encapsulation efficiency of curcumin in SPI solutions while the concentration of curcumin was increased was also described.[11] In the study by Chen *et al.*,[11] complexation of curcumin was assessed using 50 g $L^{-1}$ SPI solution and the maximum encapsulation efficiency was obtained at 0.0315 mg $mL^{-1}$ of curcumin. Teng *et al.*[30] described the same trend of encapsulation efficiency that increased with decreasing curcumin and protein ratio. A ratio of 10 g curcumin $kg^{-1}$ protein provided an encapsulation efficiency of 97.2% while when increased to 50 g curcumin $kg^{-1}$ protein, the encapsulation efficiency decreased to just 52.8%.

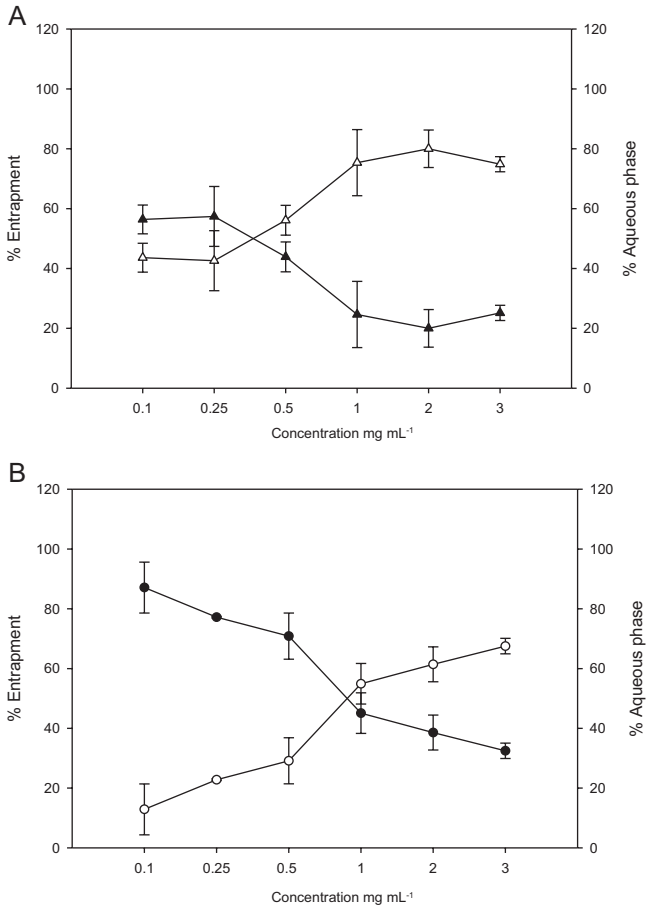


**Figure 1.** Percentage of rosmarinic acid entrapment (black triangles and circles) and detected in the aqueous phase (white triangles and circles) in caseins (A) and soy protein isolate (B) formulations at different marjoram pressurized extract concentrations. Entrapment was calculated based on the amount of rosmarinic acid detected in the aqueous phase. Results are the average of three independent experiments, with bars representing standard deviation.

When comparing entrapment efficiency of ME using caseins and SPI, SPI noted 20% to 30% higher entrapment efficiency than caseins at the studied concentrations. SPI nanoparticles seemed to provide a more favourable environment for rosmarinic acid than casein micelles. A similar effect was previously reported in a comparison of caseins and SPI as delivery carriers for curcumin. Chen *et al.*[11] noted in their study higher encapsulation of curcumin in SPI nanoparticles than casein micelles by spray-drying, 96% of encapsulation efficiency compared to 83.1% reported by Pan *et al.*[31]

### Immunomodulatory activity of marjoram–protein formulations

To evaluate the bioefficacy and functionality of marjoram–protein formulations, their *in vitro* immunomodulatory activity was assessed using human macrophages differentiated from THP-1 monocytes cell line. Preliminary data was obtained to determine cytotoxicity of protein formulations containing ME (10, 50, 100 and 200 µL) and 100 µL was the maximum volume that did not induced cytotoxicity on the cells (data not shown). Figure 2 illustrates the results for TNF-$\alpha$, IL-1$\beta$ and IL-6 secretion. As shown, the incorporation of LPS (Control+) increased the secretion of the three measured cytokines compared with basal levels of secretion in untreated cells (Control−). Formulated marjoram protein carriers, caseins and SPI, and ME solution significantly reduced TNF-$\alpha$ secretion. In particular, caseins formulations showed a significant higher effect in reduction of TNF-$\alpha$ secretion compared to SPI formulations. Marjoram solutions (100 and 50 µg mL$^{-1}$) reduced TNF-$\alpha$ secretion to similar levels than those obtained with SPI formulations containing 1 and 0.5 mg mL$^{-1}$ of ME. Secretion of the pro-inflammatory cytokine IL-1$\beta$ was only reduced in marjoram solution treated cells up to 50% with 100 µg mL$^{-1}$. Neither casein alone formulations or SPI solutions with or without marjoram encapsulated showed any effect on suppressing IL-1$\beta$ secretion. Both protein formulations showed elevated level of the cytokine from 130 to 180%. The presence of marjoram triggered reduction of IL-1$\beta$ secretion caused by casein and SPI solutions alone. Similar to TNF-$\alpha$ secretion, IL-6 secreted levels were reduced in cells treated with marjoram solutions, casein and SPI formulations, compared to activated cells (Control+). However, the SPI empty solution seemed to reduce the secretion of IL-6 (83% secretion), no statistical differences were found. When comparing casein and SPI marjoram formulations, only at the highest concentration of marjoram (1 mg mL$^{-1}$), casein formulation showed a higher reduction of IL-6 secretion. Studies have shown the potential of rosmarinic acid to induce anti-inflammatory effects on different cell lines. Thus, Jiang *et al.*[32] showed evidence of rosmarinic acid down regulating the levels of TNF-$\alpha$, IL-6 and high mobility box 1 protein in LPS induced RAW264.7 cells, indicating that rosmarinic acid might inhibit activation of the nuclear factor-$\kappa$B pathway by inhibiting I$\kappa$B kinase activity. Accordingly, rosmarininc acid inhibited LPS-induced up-regulation of IL-1$\beta$, IL-6, TNF-$\alpha$ and suppressed expression of iNOS in human gingival fibroblasts.[33] Further, Lembo *et al.*[34] indicated that rosmarinic acid produced a significant reduction in IL-1$\beta$, IL-6, IL-8 and TNF-$\alpha$ gene expression in HaCat cells after UVB irradiation.

Our results showed that empty casein and SPI suspensions reduced TNF-$\alpha$ and IL-6 secretion. Anti-inflammatory properties of sodium caseinate has also been described in cell models.[35] TNF-$\alpha$ activated Caco-2 cells reduced IL-8 secretion after exposure with sodium caseinate hydrolysates for 24 h. In addition, casein derived peptides as glycomacropeptide are described in the literature for their immunomodulatory properties.[36] Lunasin, known as a bioactive polypeptide identified in soybean with chemopreventive properties, has also been described as anti-inflammatory in RAW 264.7 macrophages.[37,38] Similar to our study, lunasin reduces secretion of TNF-$\alpha$ and IL-6 in LPS activated RAW 264.7 macrophages.[39] Peptides obtained from pepsin and pancreatin hydrolysates of soy products also showed anti-inflammatory activity by means of inhibition of NO production, TNF-$\alpha$ and IL-1$\beta$ secretion.[40]

## CONCLUSION

The findings indicated that PLE and UAE are adequate techniques to obtain MEs with a high content of rosmarinic acid and consequently antioxidant activity. Among extracts, PLE with a solvent mixture of 70:30 (*v*/*v*) ethanol/water presented the highest yield and antioxidant activity. Entrapment of PLE MEs in SPI provided 20% to 30% higher entrapment efficiency than caseins. The complexes of ME with caseins or SPI did not alter the immunomodulatory response of the extract itself. The results of this study would suggest that SPI and caseins could be safely used as carriers of herb extracts for applications in food product development.

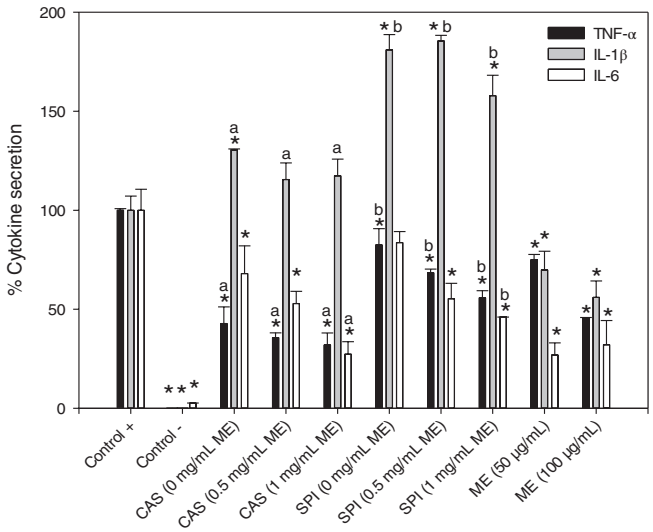


**Figure 2.** Effect of casein (CAS) and soy protein isolate (SPI) formulations versus marjoram extract (ME) in cytokines secretion determined by enzyme-linked immunosorbent assay (ELISA). Percentage of secretion was determined after 24 h incubation. A control lipopolysaccharide (LPS) activated macrophages (Control+) was used for comparison. TNF-$\alpha$ (black bars), IL-1$\beta$ (grey bars) and IL-6 (white bars). Results are the average of three independent experiments, with bars representing standard deviation. Asterisk (*) denotes statistical differences between Control+ and each other sample per cytokine analysed $P < 0.05$. Lowercase letters a and b indicate statistical differences between pair comparison of casein and SPI formulation at the same concentration $P < 0.05$.

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## REFERENCES

1 Ouedrhiri W, Balouiri M, Bouhdid S, Moja S, Chahdi FO, Taleb M *et al.*, Mixture design of *Origanun compactum, Origanum majorana* and *Thymus serpyllum* essentials oils: optimization of their antibacterial effect. *Ind Crop Prod* **89**:1–9 (2016).

2 Roby MHH, Sarhan MA, Selim KAH and Khalel KI, Evaluation of antioxidant, total phenols and phenolic compounds in thyme (*Thymus vulgaris* L.), sage (*Salvia officinalis* L.) and marjoram (*Origanum majorana* L.) extracts. *Ind Crop Prod* **43**:827–831 (2013).

3 Mueller M, Hobiger S and Jungbauer A, Anti-inflammatory activity of extracts from fruits, herbs and spices. *Food Chem* **122**:987–996 (2010).

4 Hossain MB, Camphuis G, Aguiló-Aguayo I, Gangopadhyay N and Rai DK, Antioxidant activity guided separation of major polyphenols of marjoram (*Origanum majorana* L.) using flash chromatography and their identification by liquid chromatography coupled with electrospray ionization tandem mass spectrometry. *J Sep Sci* **37**:3205–3213 (2014).

5 Jungbauer A and Medjakovic S, Anti-inflammatory properties of culinary herbs and spices that ameliorate the effects of metabolic syndrome. *Maturitas* **71**:227–239 (2012).

6 Chemat F, Rombaut N, Sicaire AG, Meullemiestre A, Fabiano-Tixier AS and Abert-Vian M, Ultrasound assisted extraction of food and natural products. Mechanisms, techniques, combinations, protocols and applications. A review. *Ultrason Sonochem* **34**:540–560 (2017).

7 Khan MK, Vian MA, Tikier F, Dangles AS and Chemat F, Ultrasound assisted extraction of polyphenols (flavone glycosides) from orange (*Citrus sinensis* L) peel. *Food Chem* **119**:851–858 (2010).

8 Herrero M, del Pilar Sánchez-Camargo A, Cifuentes A and Ibáñez E, Plants, seaweeds, microalgae and food by-products as natural sources of functional ingredients obtained using pressurized liquid extraction and supercritical fluid extraction. *Trends Analyt Chem* **71**:26–38 (2015).

9 Herrero M, Cifuentes A and Ibañez E, Sub-and supercritical fluid extraction of functional ingredients from different natural sources: plants, food-by-products, algae and microalgae: a review. *Food Chem* **98**:136–148 (2006).

10 Arranz E, Santoyo S, Jaime L, Fornari T, Reglero G, Guri A *et al.*, Improved bioavailability of supercritical rosemary extract through encapsulation in different delivery systems after *in vitro* digestion. *Food Dig Res Curr Opin* **6**:30–37 (2015).

11 Chen FP, Li BS and Tang CH, Nanocomplexation between curcumin and soy protein isolate: influence on curcumin stability/bioaccessibility and *in vitro* protein digestibility. *J Agric Food Chem* **63**:3559–3569 (2015).

12 Dalgleish DG, On the structural models of bovine casein micelles – review and possible improvements. *Soft Matter* **7**:2265–2272 (2011).

13 Livney YD, Milk proteins as vehicles for bioactives. *Curr Opin Colloid Interface Sci* **15**:73–83 (2010).

14 Tang CH, Emulsifying properties of soy proteins: a critical review with emphasis on the role of conformational flexibility. *Crit Rev Food Sci Nutr* **57**:2636–2679 (2017).

15 Deng XX, Zhang N and Tang CH, Soy protein isolate as a nanocarrier for enhanced water dispersibility, stability and bioaccessibility of $\beta$-carotene. *J Sci Food Agric* **97**:2230–2237 (2017).

16 Tapal A and Tiku PK, Complexation of curcumin with soy protein isolate and its implications on solubility and stability of curcumin. *Food Chem* **130**:960–965 (2012).

17 Villalva M, Jaime L, Aguado E, Nieto JA, Reglero G and Santoyo S, Anti-inflammatory and antioxidant activities from the basolateral fraction of Caco-2 cells exposed to a rosmarinic acid enriched extract. *J Agric Food Chem* **66**:1167–1174 (2018).

18 Singleton VL, Orthofer R and Lamuela-Raventos RM, Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin–Ciocalteu reagent. *Methods Enzymol* **199**:152–179 (1999).

19 Re R, Pellegrini N, Proteggente A, Pannala A, Yang M and Rice-Evans C, Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med* **26**:1231–1237 (1999).

20 Mosmann T, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *J Immunol Methods* **65**:55–63 (1983).

21 Jaime L, Mendiola JA, Herrero M, Soler-Rivas C, Santoyo S, Señorans FJ *et al.*, Separation and characterization of antioxidants from Spirulina platensis microalga combining pressurized liquid extraction, TLC, and HPLC-DAD. *J Sep Sci* **28**:2111–2119 (2005).

22 Kaliora AC, Kogiannou DA, Kefalas P, Papassideri IS and Kalogeropoulos N, Phenolic profiles and antioxidant and anticarcinogenic activities of Greek herbal infusions; balancing delight and chemoprevention? *Food Chem* **142**:233–241 (2014).

23 Sabouri S, Geng J and Corredig M, Tea polyphenols association to caseinate-stabilized oil–water interfaces. *Food Hydrocoll* **51**:95–100 (2015).

24 Casanova F, Estevinho BN and Santos L, Preliminary studies of rosmarinic acid microencapsulation with chitosan and modified chitosan for topical delivery. *Adv Powder Technol* **297**:44–49 (2016).

25 Fernandez-Avila C, Arranz E, Guri A, Trujillo AJ and Corredig M, Vegetable protein isolate-stabilized emulsions for enhanced delivery of conjugated linoleic acid in Caco-2 cells. *Food Hydrocoll* **55**:144–154 (2016).

26 Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248–254 (1976).

27 Zhao Z and Corredig M, Influence of sodium chloride on the colloidal and rennet coagulation properties of concentrated casein micelle suspensions. *J Dairy Sci* **99**:6036–6045 (2016).

28 Sahu A, Kasoju N and Bora U, Fluorescence study of the curcumin–casein micelle complexation and its application as a drug nanocarrier to cancer cells. *Biomacromolecules* **9**:2905–2912 (2008).

29 Sáiz-Abajo MJ, González-Ferrero C, Moreno-Ruiz A, Romo-Hualde A and González-Navarro CJ, Thermal protection of $\beta$-carotene in re-assembled casein micelles during different processing technologies applied in food industry. *Food Chem* **138**:1581–1587 (2013).

30 Teng Z, Luo Y and Wang Q, Nanoparticles synthesized from soy protein: preparation, characterization, and application for nutraceutical encapsulation. *J Agric Food Chem* **60**:2712–2720 (2012).
31 Pan K, Zhong Q and Baek SJ, Enhanced dispersibility and bioactivity of curcumin by encapsulation in casein nanocapsules. *J Agric Food Chem* **61**:6036–6043 (2013).
32 Jiang WL, Chen XG, Qu GW, Yue XD, Zhu HB, Tian JW *et al.*, Rosmarinic acid protects against experimental sepsis by inhibiting proinflammatory factor release and ameliorating hemodynamics. *Shock* **32**:608–613 (2009).
33 Zdařilová A, Svobodová A, Šimánek V and Ulrichová J, *Prunella vulgaris* extract and rosmarinic acid suppress lipopolysaccharide-induced alteration in human gingival fibroblasts. *Toxicol In Vitro* **23**:386–392 (2009).
34 Lembo S, Balato A, Di Caprio R, Cirillo T, Giannini V, Gasparri F *et al.*, The modulatory effect of ellagic acid and rosmarinic acid on ultraviolet-B-induced cytokine/chemokine gene expression in skin keratinocyte (HaCaT) cells. *Biomed Res Int* **2014**:1–8 (2014).
35 Mukhopadhya A, Noronha N, Bahar B, Ryan MT, Murray BA, Kelly PM *et al.*, Anti-inflammatory effects of a casein hydrolysate and its peptide-enriched fractions on TNF$\alpha$-challenged Caco-2 cells and LPS-challenged porcine colonic explants. *Food Sci Nutr* **2**:712–723 (2014).
36 Chatterton DE, Nguyen DN, Bering SB and Sangild PT, Anti-inflammatory mechanisms of bioactive milk proteins in the intestine of newborns. *Int J Biochem Cell Biol* **45**:1730–1747 (2013).
37 Dia VP, Wang W, Oh VL, De Lumen BO and De Mejia EG, Isolation, purification and characterisation of lunasin from defatted soybean flour and *in vitro* evaluation of its anti-inflammatory activity. *Food Chem* **114**:108–115 (2009).
38 Hernández-Ledesma B, Hsieh C-C and de Lumen BO, Seed components in cancer prevention, in *Nuts and Seeds in Health and Disease Prevention*, ed. by Preedy VR, Watson RR and Patel VB. Academic Press, San Diego, CA, pp. 101–109 (2011).
39 Hernández-Ledesma B, Hsieh CC and Ben O, Antioxidant and anti-inflammatory properties of cancer preventive peptide lunasin in RAW 264.7 macrophages. *Biochem Biophys Res Commun* **390**:803–808 (2009).
40 Dia VP, Bringe NA and de Mejia EG, Peptides in pepsin–pancreatin hydrolysates from commercially available soy products that inhibit lipopolysaccharide-induced inflammation in macrophages. *Food Chem* **152**:423–431 (2014).