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Supercritical PArticle formation (SPAF) process for the versatile production of ready-to-market drug delivery systems

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ABSTRACT

Supercritical PArticle Formation has been proposed for the entrapment of ferrous sulphate in liposomes, overcoming drawbacks linked to conventional processes, such as residual solvents and low versatility. The innovation of this process was the combined integration of supercritical assisted production with drying techniques (freeze and spray-drying) and final drum-grinding, being effective in preserving active ingredients while eliminating liquid content. Liposomes powder was stable over 24 months, mean size down to 3.5 μm was achieved, with a Drug to Lipid Ratio of 6. Encapsulation efficiency up to 94 ± 4 % was obtained without significant loss during drying. Supernatant and lipidic solids were separated and analyzed, demonstrating presence of lightweight floating liposomes in the aqueous part and larger lipo-complexes among the solids. During the production of a pilot-scale batch of 25 kg, liquid-to-powder yield of 0.179 kg/L was obtained. Drying was successful to produce narrow vesicles in absence of pesticides, bacteria and heavy metals.

1. Introduction

The use of liposomes and other lipidic vesicles in the pharmaceutical and nutraceutical fields introduced innovative ways of administration of drugs and bioactives (Bernela et al., 2018). Liposomes are spherical vesicles constituted by at least one double lipidic layer, in which it is possible to incapsulate a wide range of molecules, protecting them from enzymatic or thermal degradation, while increasing their bioavailability (Cardoso-Daodu et al., 2022). The unique behavior of liposomes stands in their structure, which allows for the transportation of both hydrophilic and hydrophobic compounds, facilitating targeted and controlled drug release with reduced side effects (Trucillo et al., 2020).

During 40 years since their discoveries by Dr. Bangham (in 1960′s), liposomes were produced using conventional methods, that were characterized by significant drawbacks (Antimisiaris et al., 2021; Raucher et al., 2018), primarily related to the use of organic solvents (Amalia et al., 2022; Han et al., 2023; Valle and Navarro, 2015). These solvents, apart from being potentially harmful to health and the environment, can leave residues in the final products, causing side effects and compromising treatment safety and efficacy (Çağdaş et al., 2014; Cheng et al., 2017). Moreover, conventional methods suffer from issues of repeatability and versatility, making it challenging to standardize production processes for different types of drugs or active molecules (Shah et al., 2020). The significant achievement in liposomal field gave the possibility to develop also other types of vesicles such as niosomes and nanostructured lipidic carriers. In this study, liposomes were preliminary compared with NLC and niosomes in order to find the best lipid-drug couple for this specific nutraceutical application.

A powerful solution to overcome conventional methods' drawbacks was proposed with the use of supercritical fluids (Karn et al., 2013; William et al., 2020). Supercritical carbon dioxide is generally employed in liposomes production due to its easy-to-reach critical temperature and pressure (73 bar and 31 °C), which guarantees high diffusivity (such as gases) and high density (comparable to liquids) during mixing with other preparation components (Falconer et al., 2015; Lim et al., 2019). The main advantage deriving by the use of CO_2 is the substitution with organic solvents characterized by high toxicity, that generally have a quite larger environmental impact (Arévalo-Pérez et al., 2020; Miljković, 2017; Pande, 2023). Furthermore, not only supercritical fluid technology allows a green (Domingo, 2015; Pascual et al., 2015) and improved control on the particle size distribution of liposomes, but it also guarantees the repeatability and versatility of the process

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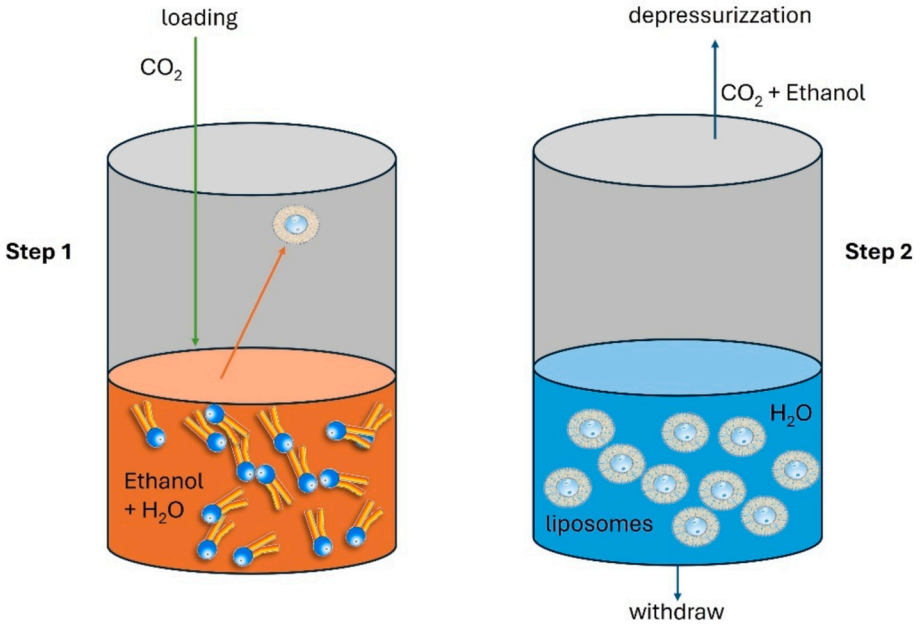

Fig. 1. Mechanism of particle formation using SPAF.

(Chakravarty et al., 2019; Maja et al., 2020; Naik et al., 2010; Santo et al., 2015). An additional advancement in liposome production technology is represented by spray-drying and freeze-drying techniques (Huang et al., 2014; Yu et al., 2021; Filipczak et al., 2020; Chen et al., 2010). These post-treatment processes become fundamental to improve the stability and shelf life of liposomes (Sopyan et al., 2020; Franzé et al., 2018), while removing any possibility of degradation due to the presence of liquids. Lyophilization removes water from frozen liposomes through sublimation, turning them into a fine and homogeneous powder. This not only facilitates the storage and transformation of liposome-based formulations (Mohammed et al., 2006), but also ensures greater stability of the final product, allowing for a long-term preservation of the entrapped drug, without significant loss of efficacy (Wang and Grainger, 2019; Arshinova et al., 2012; Arshinova et al., 2012).

Spray-drying and freeze-drying techniques have been employed in the production of liposomes, with the declared aim to enhance their stability, storage, and delivery efficiency (Wessman et al., 2010). For instance, spray drying has proven to be effective in producing liposomal powders with good re-dispersibility in water, while maintaining the structural integrity of the liposomes and encapsulated compounds (Maa et al., 1999). Freeze drying, on the other hand, has been widely used to preserve liposomes by forming stable lyophilized formulations, minimizing leakage of encapsulated drugs, and improving shelf life (Ola et al., 2010). Both techniques allow for the transformation of liposomal suspensions into dry formulations, which facilitates long-term storage and ease of transportation.

Therefore, the combination of lipidic vesicles production with supercritical fluids and lyophilization represents a significant progress in the creation of safer, more effective, and sustainable dietary supplements, but also for the design of delivery systems (Đorđević et al., 2014; Klettenhammer et al., 2020; Ajeeshkumar et al., 2021).

In this work, the Supercritical Particle Formation (SPAF) process has been proposed for the first time to provide a unique technique including all the transformation steps from supercritical assisted mixing in liquid form to complete drying and powder transformation. This simultaneous result was achieved with an acceptable control on particle size distribution, organic solvent removal and stability over time. Indeed, the conventional and supercritical processes nowadays employed are characterized by low stability of liposomes, when stocked in aqueous suspension at 4 °C. However, this requires high storage volumes and energy to provide a constant refrigeration temperature. The alternative proposed by authors with SPAF consists in the total elimination of liquids from the liposomal preliminary suspension. The transformation in powder will guarantee larger stability over years of storage at room temperature, without significant exposition to heat sources, thus facilitating the transition of supercritical assisted process from lab-scale to industrial level and massive production (Farmacia, 2016).

The mechanism of liposome formation (Fig. 1) can be described as follows: in a 1 bar closed environment containing water, ethanol, phospholipids, and iron sulfate, the system is mixed and heated to 40 °C. A supercritical CO_2 flow is introduced into the reactor (step 1 in Fig. 1), increasing the pressure to 100 bar at 40 °C. Due to the immiscibility of water and CO_2 in this ternary system (water-carbon dioxide-ethanol, with negligible other contributes), water is dispersed turbulently, forming droplets. CO_2 solubilizes the ethanol, allowing phospholipids to diffuse to the water droplet' surface, forming multilayers (step 2 of Fig. 1). During depressurization, CO_2 and ethanol are removed, leaving liposomes suspended in water.

The advantages of this process include versatility in molecule-carrier systems and rapid production, driven by pressure variation from 1 bar to 100 bar, which creates a turbulent environment that enhances the homogeneity of the reaction chamber. The batch processing also allows for the separation of production batches, which is beneficial for pharmaceutical and nutraceutical applications.

In this work, ferrous sulphate has been a good candidate for the encapsulation in liposomes of different nature. Ferrous sulfate is an important dietary supplement primarily because it is a source of iron, a critical mineral that is a key component of hemoglobin. The protein in red blood cells carries oxygen from the lungs to the rest of the body; therefore, without adequate iron, the body cannot produce enough healthy red blood cells, leading to iron deficiency anemia. During pregnancy, women iron needs increase significantly to support the growing fetus and to increase maternal blood volume.

As defined above, traditional methods are often characterized by potentially harmful residual solvents, while supercritical assisted methods generally eliminate almost all residual organic co-solvents. SPAF process not only creates an alternative to conventional method, but also achieves a second goal, with the elimination of all liquid content, resulting in the production of dried powder, that is safer than liquid liposome suspension. The repeatability of this process will be another

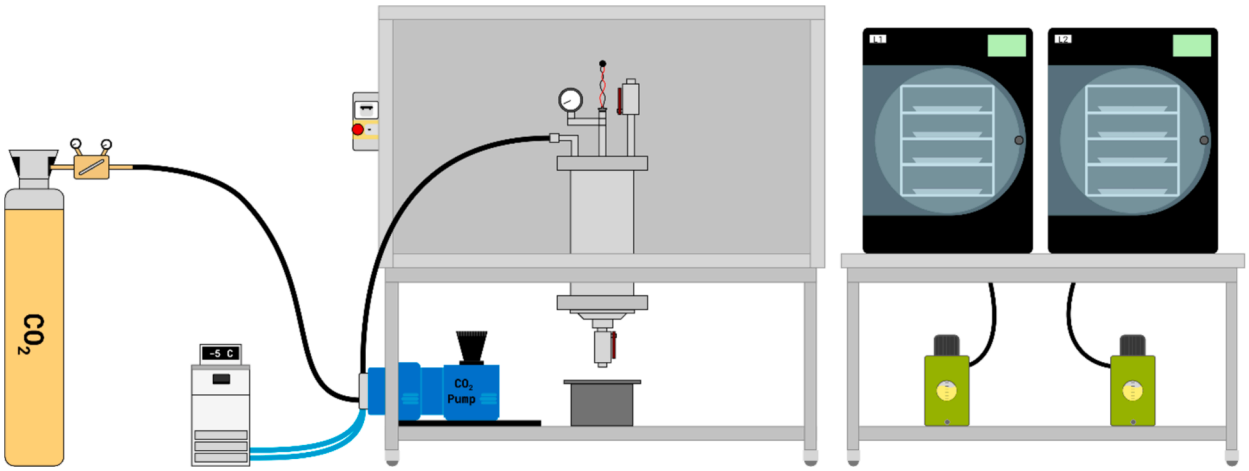

Fig. 2. Sketch of SPAF process.

Table 1
Description of different attempted formulations produced using SPAF at 40 °C and 100 bar.

Core materials	Drug dissolved	Encapsulation efficiency, EE ± SD
Niosomes	Fluorescein	91 ± 6
Niosomes	Ferrous sulfate	80 ± 3
Niosomes	Metformin	89 ± 7
Liposomes	Fluorescein	92 ± 2
Liposomes	Ferrous sulfate	90 ± 3
Liposomes	Metformin	85 ± 5
NLC	Fluorescein	93 ± 2
NLC	Ferrous sulfate	91 ± 7
NLC	Metformin	89 ± 3

Table 2
Iron-loaded liposomes at different drug-to-lipid ratios. EE: Encapsulation Efficiency.

Drug-to-lipid ratio	D10, μm	D [3:2] , μm	D90, μm	EE ± SD
0	0.10	0.25	10	——
2	0.20	5	123	30 ± 3
5	0.15	10	258	90 ± 5
6	0.25	12	325	94 ± 4

essential feature to ensure consistent and effective delivery of bioactives and dietary supplements. The proper design of SPAF process contributed to the reduction of its carbon footprint and environmental impact, while increasing the production yield on mass basis. This makes SPAF not only greener, but also more cost-effective, with a significant reduction of waste generated. The samples drying was performed with a lyophilization step and, as an alternative, using spray drying technique, that are often employed for vesicles production (Huang et al., 2014; Yu et al., 2021; Filipczak et al., 2020; Chen et al., 2010; Wessman et al., 2010; Maa et al., 1999; Ola et al., 2010).

The final aim of this work is to demonstrate that a particularly unstable molecule such as ferrous sulphate can be entrapped in liposomes to create a stable and efficient ready-to-market delivery system. However, the supercritical assisted process does not represent the novelty itself, but its combination with other post-processing steps such as lyophilization or spray drying, that guarantees the novelty of this work, by preserving good qualities of supercritical assisted production, such as particle size distribution, encapsulation efficiency, stability, sterility and safety over time.

2. Material

The following materials were employed in the present work: Ferrous sulphate Ph.Eur. (CAS 7782-63-0), Ascorbic acid Ph. Eur. (CAS 50–81-7), maltodextrins Ph.Eur. (CAS 9050-36-6), sodium ascorbate Ph.Eur. (CAS 134-03-2) were Purchased from Galeno Srl, Comeana (PO), Italy. Fatfree soybean lecithin (non-GMO) with 45 % phosphatidylcholine (PC) named "Lipoid P45" was produced by Lipoid AG, Germany and purchased by the Italian retailer AVG Srl, Bollate (MI), Italy. Bi-distilled water (CAS 7732-18-5) was purchased by MPIM Srl, San Giovanni Teatino (CH), Italy. Ethanol food grade (purity 95 %, CAS 64-17-5) was purchased from Lider Srl, Mercato San Severino (SA), Italy. Carbon dioxide (CAS 124–38-9) was purchased from SOL Spa, (Monza-Brianza), Italy.

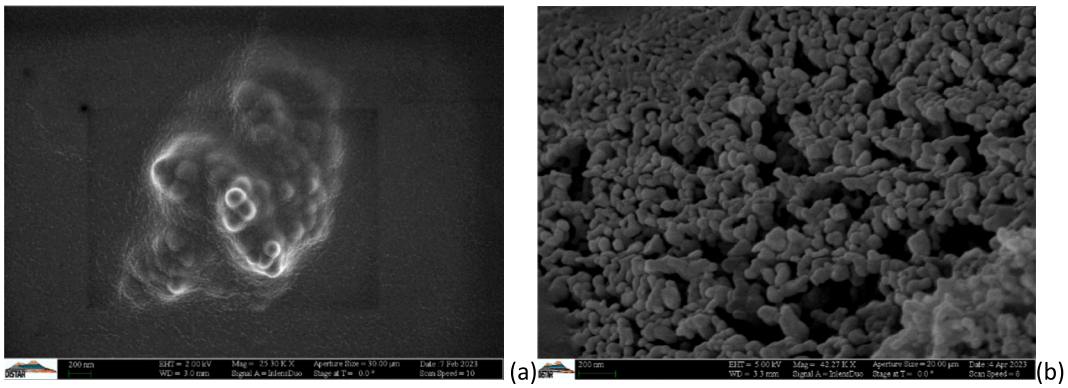

Fig. 3. SEM observations of empty (a) and DLR of 2 (b).

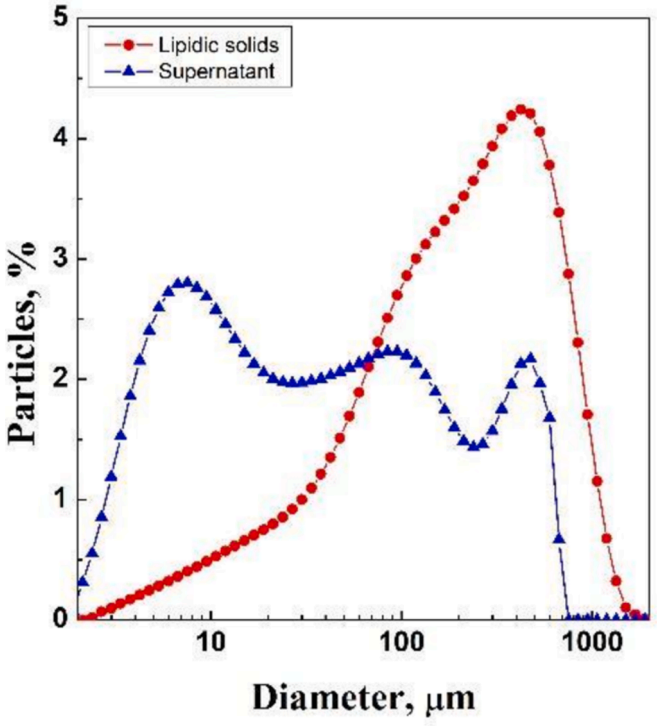

Fig. 4. Granulometric analysis of supernatant and separated lipidic solids.

Table 3
Comparison in terms of mean size and encapsulation efficiency.

Type of formulations	D10, μm	D [3:2], μm	D90, μm	encapsulation efficiency, EE ± SD
Liposomes in supernatant	4	13	368	90 ± 5
Liposomes in solids	29	63	589	83 ± 4

Once received, raw materials were stocked in proper conditions: in particular, phospholipids (PC) were stocked in refrigerator, at the temperature of −20 ± 5 °C, using an automatized control system and sensor (Tapo T310_V1, TP-Link, Tsim Sha Tsui, Hong Kong). Before performing the experiments, PC was withdrawn from the refrigerator and kept at room conditions until reaching almost a viscous aspect. Then, the PC was processed together with the other raw materials in the main reactor. After the production of liquid suspension of liposomes and before the lyophilization step, the suspension was stocked at the temperature of 4 °C, to avoid thermal and/or photo degradation. Indeed, PC is generally sensitive to oxygen and light (Cui and Decker, 2016). Ethanol and distilled water were stocked at room temperature.

Further tests were performed on the produced samples in order to detect and, quantify eventual residues of pesticides, further underlining the commitment to ensuring the product's adherence to safety standards, establishing powder compliance for the food industry.

3. Equipment

The process developed for the production of liposomes is called Supercritical PArticle Formation (SPAF). This technique has been conceived with the aim to design drug delivery systems of lipidic, polymeric, or hybrid nature, improving the bioavailability and therapeutic efficacy of the entrapped active principles. A sketch of the process is proposed in Fig. 2. The cylindric reactor represents the heart of the SPAF process and consists of a process chamber made of stainless steel capable of withstanding high pressures (up to 500 bar), having a height of 40 cm, an external diameter of 18 cm and internal diameter of 15 cm. Before pressurizing, the reactor is opened and fed with phospholipids dissolved in ethanol and ferrous sulphate + ascorbic acid dissolved in water. Then, the reactor is closed and pressurized with carbon dioxide. Carbon dioxide, used at supercritical conditions (over 31 °C and 73 bar), helps to obtain micro and nanometric particles with precise control over size and morphology. As shown in Fig. 2, carbon dioxide is stored in a cylinder at about 60 bar and room temperature. Before feeding it to the reactor pump (Lewa, Leonberg, Germany, mod. LDB1), CO_2 is refrigerated using a cooling bath (Julabo, Milan, Italy, mod. Corio CD 200F) in order to transform eventual gaseous molecules in liquid phase.

Supercritical CO_2 acts as a co-solvent, helping the diffusion of phospholipids in the reactor chamber. Since the operating conditions have been set at 100 bar and 40 °C, ethanol and carbon dioxide have been mixed during the formation of liposomes, thus guaranteeing a fast diffusion of lipids to the water droplets.

A liquid aqueous bulk is created at the bottom of the formation chamber. After waiting 1 h contact time, the carbon dioxide is slowly released by an on/off valve set at the top of the chamber. Since ethanol is mixed with carbon dioxide, it is released from the top of the formation reactor, transported by CO_2. Once completed depressurization, liposomes floating in the aqueous bulk are discharged using another on/off valve valve set at the bottom of the reactor. Contact time of 1 h has been set after several tests conducted at lower time of contact, that resulted in an unstable and poorly controlled suspension.

Once the liquid suspension has been withdrawn from the reactor, it was stored at 4 °C for 24 h. Then, the liquid suspension containing the liposomes is transferred to the post-processing section, depicted in the right part of Fig. 2. During this part, any eventual residue of ethanol is fast removed, while all the content of water is eliminated from liposomes suspension. The monitoring of this step is crucial to obtain liposomes containing drug in powder form. The separation occurs through

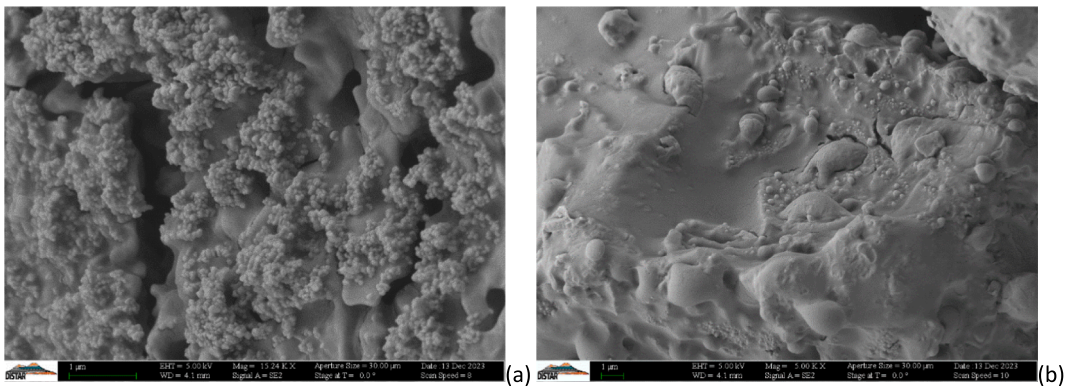

Fig. 5. SEM analysis of supernatant (a) and separated solids (b).

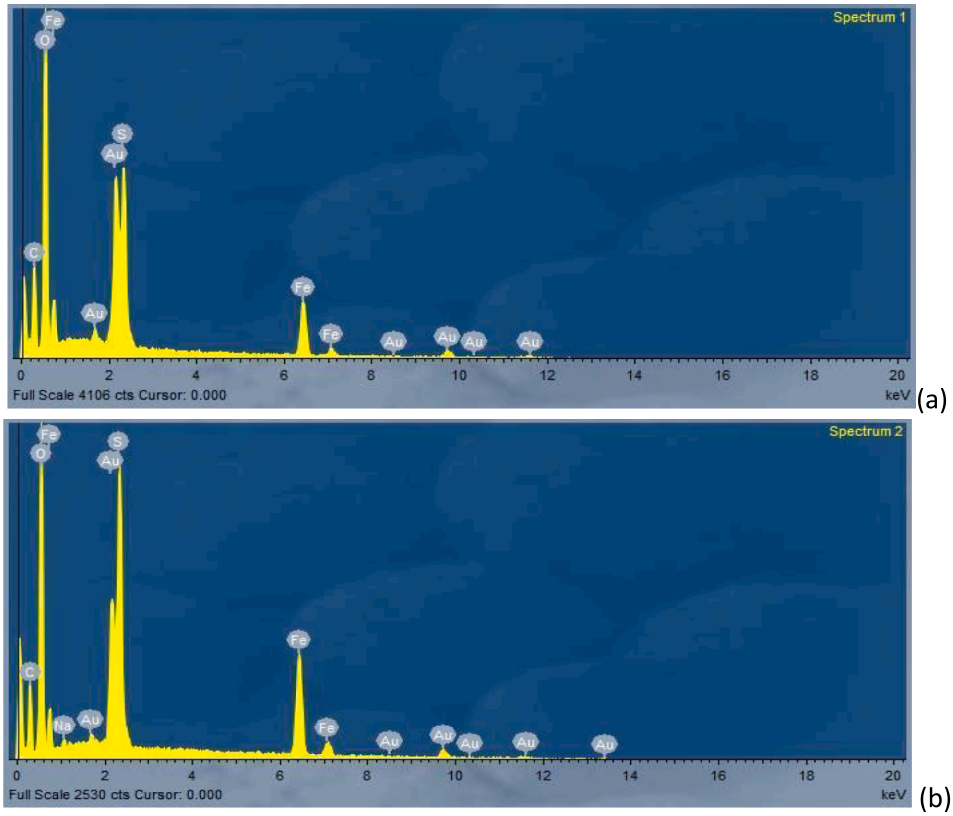

Fig. 6. EDX analysis of separated solids (a) and supernatant (b).

lyophilization in vacuum conditions, by setting temperature conditions depending on the specific needs of the formulation. The formulation of liposomes produced with the SPAF process was designed to have an 80 % ferrous sulfate content.

Freeze-drying (Harvest right, California, USA) process was carried out, using a standard medium-sized freeze dryer with the following technical specifications: dimensions and volume of 63.5 x 50.8 x 76.2 cm^3 and a total weight of 96.16 kg. The freeze dryer was equipped with a vacuum pump to create the necessary vacuum conditions for water sublimation. Specific oil and an oil filter were used to ensure the pump's efficiency. Inside the freeze dryer, samples were placed on stainless steel trays (set of 4 trays) to ensure uniform distribution of the material to be processed. Before the freeze-drying process began, the products were frozen to a temperature of −40 °C to facilitate the rapid transition of ice from the solid to the gaseous phase during sublimation. During the process, the vacuum pump created a low-pressure environment, facilitating the sublimation of ice. The freeze-drying cycle, with a total duration of approximately 20 h, was capable of processing up to 4–6 kg of material per cycle. If pre-frozen material was used, the cycle duration was reduced by approximately 3–4 h. At the end of the process, the freeze-dried products were packaged in aluminized containers or storage bags, along with an oxygen absorber (e.g., Air Out Oxygen) to preserve the product's freshness until consumption. This equipment allowed for the preservation of the nutritional and organoleptic characteristics of the treated products, ensuring superior quality compared to traditional drying methods. A freeze-drying guide was utilized to optimize operational parameters and ensure process reproducibility.

As an alternative, the spray drying (mod. SP 2 L, Xiamen Ollital Technology Co.) process was conducted at 50 °C, using a new, industrial-grade spray dryer machine. In particular, at 50 °C, solvent removal mechanism is triggered. Increasing the temperature would lead to degradation of phospholipids and trapped molecules. Below 50 °C, on the contrary, the solvent evaporates too slowly, significantly extending the processing time. The mass flow rate ratio between the liposomal suspension and hot air was set at 1:2. Increasing air flow rate, an excessive turbulent flow would be generated, which could damage the liposomes through mechanical breakage. If the ratio is lower than 1:2, solvent cannot be completely removed. The glass wall is maintained at 110 °C on its surface to ensure a continuous thermal flow within the chamber. At the center of the chamber, the air inlet was guaranteed at 50 °C. Therefore, the resulting temperature differential (ΔT) was kept constant to promote the removal of all liquids (water and any residual solvents) without damaging the product. This equipment is designed for applications in chemical processing, food processing, and various other industries, including hotels, manufacturing plants, food and beverage factories, and energy and mining sectors. The spray dryer machine features an electric heating method, utilizing 316L stainless steel materials for construction, ensuring durability and resistance to corrosion. The equipment operates on a voltage of 220 V, with a power consumption of 2.2KW. The machine's dimensions are 650 mm in length, 800 mm in width, and 1250 mm in height, with a total weight of 120 kg. The machine's core components include a pump, PLC (Programmable Logic Controller), engine, and pressure vessel.

4. Methods

4.1. Particle size distribution

Samples were characterized in terms of their average size and size distributions, using a Mastersizer 2000, Malvern Instruments, United Kingdom. Refractive index used for liposomes was set at 1.45, as suggested by the literature (Hupfeld et al., 2009; Sebaaly et al., 2016; Belkilani et al., 2021). An obscuration of around 12–15 % has been reached for the measurement of the samples. The range of measurement for this instrument is 20 nm to 2 mm, according to the Fraunhofer approximation (Martín et al., 2020; Figueiredo et al., 2021). Sample

Table 4
Yield of the lyophilization process on mass basis.

Data of production	Volume of liquid Produced*, L	Mass of powder Recovered**, kg	mass yield, kg/L
14/07/2023	7	1.139	0.1627
17/07/2023	8	1.316	0.1645
18/07/2023	8	1.606	0.2007
19/07/2023	8	0.862	0.1077
20/07/2023	8	1.368	0.1710
21/07/2023	8	0.732	0.0914
24/07/2023	6	0.971	0.1617
25/07/2023	8	1.482	0.1852
26/07/2023	4	0.727	0.1817
27/07/2023	4	0.731	0.1827
06/09/2023	2	0.485	0.2427
11/09/2023	4	0.755	0.1889
12/09/2023	4	0.688	0.1720
15/09/2023	4	0.755	0.1889
18/09/2023	4	0.740	0.1851
19/09/2023	4	0.719	0.1797
20/09/2023	4	0.745	0.1862
21/09/2023	4	0.703	0.1757
22/09/2023	2	0.382	0.1910
27/09/2023	4	0.706	0.1765
28/09/2023	6	1.053	0.1756
29/09/2023	4	0.780	0.1951
02/10/2023	2	0.398	0.1993
03/10/2023	4	0.849	0.2122
04/10/2023	4	0.344	0.0860
05/10/2023	4	1.049	0.2624
06/10/2023	2	0.368	0.1840
07/10/2023	6	1.136	0.1894
08/10/2023	2	0.367	0.1835
09/10/2023	4	0.695	0.1737
10/10/2023	4	0.785	0.1964
SUM	147	25.440	—

*Volume of liposome liquid suspension collected from the bottom of the reaction, after all the cycles of production performed during the indicated day of production.
**Mass of all the volume produced in the indicated day, recovered after its full drying. This does not imply that the entire volume produced that day has been dried in the same day.

measurements were reported in terms of D(10 %), D(90 %) and D[3:2], representing the 10 % average dimensions, 90 % average dimensions and surface particle mean dimensions. Samples were grinded using a Mambo 11090, Cecotech, Valencia, Spain. As an alternative to grinding, powder was also spray dried, using MiniSprayDryer S-300 (Buchi, Cornaredo (MI), Italy).

4.2. Encapsulation efficiency determination

To determine the $FeSO_4$ content in liposomes, a spectrophotometer operating in the visible range of 325–1000 nm (Onda V10 Plus, Giorgio Bormac S.r.L., Milan, Italy) has been used. This aspect represents an important phase in the development of drug delivery systems, since it is necessary to derive the encapsulation of the Fe species thourh the entrapment of $FeSO_4$ molecule. Therefore, the accurate measurement of the active principle's concentration in solution is calculated as reported in Equation (1):

$$EE,\% = \left(1 - \frac{C_{supernatant}}{C_{total}}\right) * 100 \quad (1)$$

where $C_{supernatant}$ is the concentration of drug measured in the supernatant, while C_{total} is the total concentration of ferrous solphate entrapped in liposomes.

Initially, it was necessary to prepare a reference aqueous solution containing a known concentration of ferrous sulfate and absorbance was measured. Then, various known dilutions of ferrous Sulfate solutions were prepared, and the absorbance was measured as well. Once obtained at least 4 points, a calibration line has been determined. The maximum absorbance peak of ferrous sulphate was 710 nm, as reported by the literature (Cheng et al., 2022). Once determined the calibration line (with R^2 value of 98 %), it was possible to analyze each sample from the supernatant. The obtained measurements were then compared with the calibration curve to determine the concentration of the molecule in the tested solutions. By analyzing the concentration of the active principle available in solution and comparing it with the concentration effectively encapsulated in the liposomes (measured separately), it was possible to evaluate the encapsulation efficiency of the process. Based on this analysis, it was also possible to make improvements to the liposome preparation conditions, such as modifying the ratios between the lipid components and the active principle, optimizing the hydration conditions of the lipids, while adapting the liposome separation methodologies.

4.3. SEM analysis

Liposomes suspensions were analyzed after drying. Clearly, liposomes formulations needed to be metallized to become electrically conductive. In particular, liquid suspensions were adapted on aluminum stub and left drying before metallization and SEM observation. This required a specific preparation protocol: a drop of liposome suspension was spread on an aluminum stub and allowed to dry at room temperature for over 3 days. Moreover, powder of liposomes has been also analyzed using SEM, after freeze-drying or spray-drying steps. Due to the sub-micrometric and nanometric dimensions, it was necessary to use scanning electron microscope for a more precise determination of shape and control of vesicles dimensions. Dry samples were covered with a thin gold layer, using a sputter coater. A scanning electron microscope, model EL30 LaB6, Philips, has been employed to observe samples, after they were dried and metallized.

4.4. EDX analysis

Liposomal powders were further analyzed using Energy Dispersive Spectroscopy (EDS) characterizations, performed using a X-Max 50 detector (Oxford Instruments NanoAnalysis, High Wycombe, UK). A software (Integrated Calibration and Application Tool (INCA), Etas Group, Stuttgart, Germany) was employed to obtain distribution maps of the basic elements for the automatic acquisition of the sample composition. Qualitative information concerning the peaks defined the presence of the specific element on the sample spot, that in this case was focused on the detection of ferrous species.

4.5. External characterizations

External laboratory was contacted to perform analysis on heavy metals (protocolos UNI EN 13805:2014 + UNI EN 15763:2010), bacteriological growth (UNI ISO 16649–2:2010, UNI EN ISO 11290–1:2017, UNI EN ISO 6579–1:2020, API ®, UNI EN ISO 7932:2020, ISO 21569:2005) and iron content (UNI EN 13805:2014 + UNI EN 15763:2010).

5. Results

5.1. Comparative preliminary experiments

Supercritical Particle Formation (SPAF) has been developed with the aim of creating different drug delivery systems by changing the materials used to transport molecules. This cradle-to-gate process concerns the production of particles whose external layer can be either phospholipid, polymeric, or a hybrid composition of both. The versatility of SPAF not only stands on the vehicle materials, but also on the transported molecules.

To demonstrate the "double" versatility of the process, a preliminary

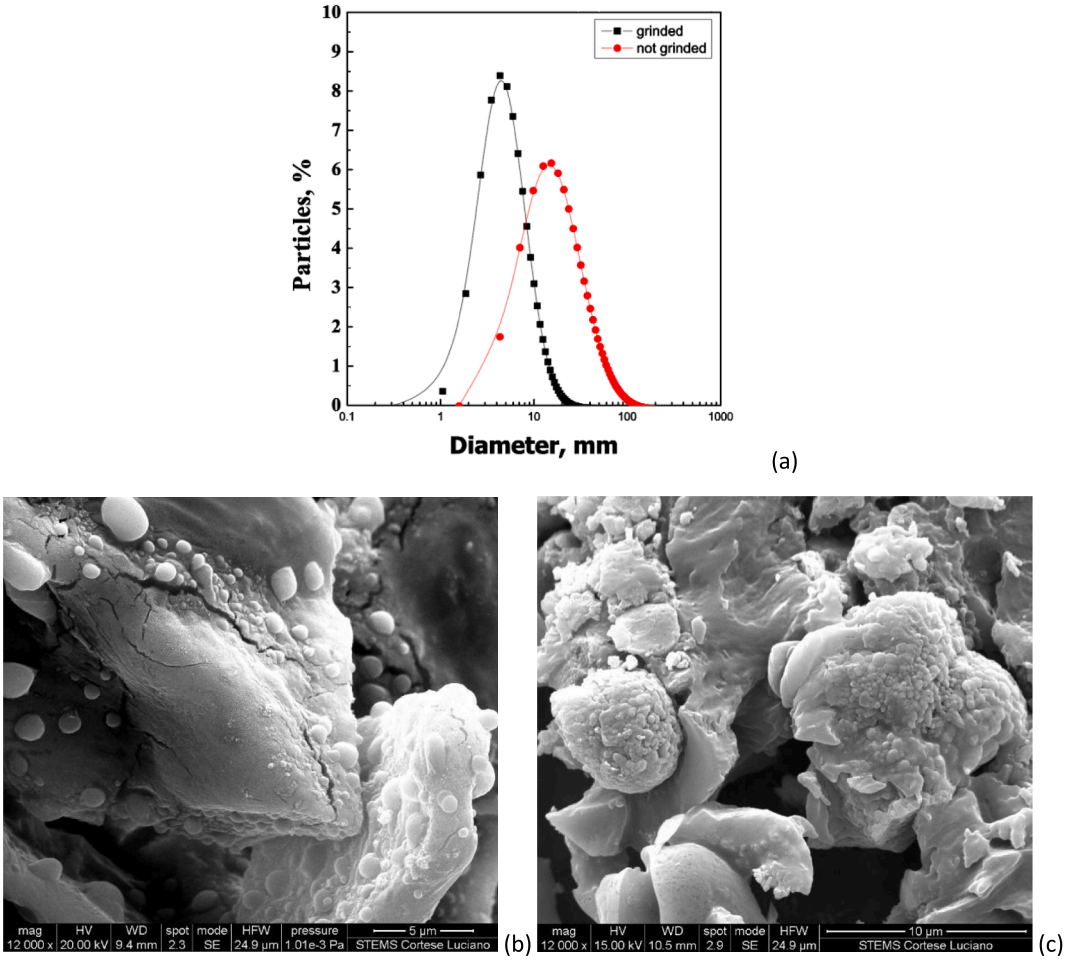

Fig. 7. Comparison of PSDs of full sample (supernatant + liquid bulk) before and after grinding step (a) and SEM images of the bulk sample after grinding step (b, c) – 12kx magnitude.

experimental campaign was conducted using three different molecules and three different delivery structures. A fluorescent material, ferrous sulfate, and metformin were encapsulated in three distinct formulations: niosomes, liposomes, and Nanostructured Lipidic Complexes (NLC). Niosomes are spherical vesicles composed of non-ionic surfactants, used to encapsulate drugs and other active ingredients, very similar to liposomes; nanostructured lipidic complexes are macroscopic delivery systems composed of nanometric objects and aggregates. These formulations have been produced in liquid form and have been analyzed without performing a drying step. In the following table, it is possible to find their main characteristics of the preliminary attempted formulations, in which the ratio among core materials and drug mass was set at 10 % w/w. Temperature was set at 40 °C, pressure at 100 bar and time contact at 1 h. Results are summarized in Table 1.

Analyzing the core/drug coupling shown in Table 1, a core-drug materials formulation was identified, addressing this choice to the most underexplored couple for a process operating with supercritical fluids. Fluorescein is a molecule commonly used as a tracer (Mehvar and Shepard, 1992; Fan et al., 2018), as reported in the literature; however, it is less suitable for food applications, due to its toxicity effects. On the other hand, metformin is a pharmaceutically interesting compound (Glossmann and Lutz, 2019; Cuyàs et al., 2018), but the mechanisms of cellular transport still need to be understood and optimized (Markowicz-Piasecka et al., 2016). Ferrous sulfate, on the other hand, is a molecule finding widespread use in the dietary supplements market, which is currently experiencing significant global expansion (Zakrzewski et al., 2022; Bryszewska, 2019).

Regarding the choice of core materials, nanostructured lipidic complexes (NLCs) need further investigation from a morphological perspective, particularly concerning the reproducibility of macroscopic structures in relation to the distribution of microscopic ones (Tang et al., 2023). Similarly, niosomes (Shaker et al., 2015; Ag Seleci et al., 2016) need to be furthermore tested for cellular biocompatibility. Liposomes, whose stability has already been well demonstrated, can be the right choice for this study. Indeed, a formulation of iron loaded liposomes has been selected from the above reported table and proposed in this study for scalable industrial production.

Formulations reported in Table 1 were stored at 4 °C and monitored over time; liquid suspensions of liposomes, niosomes and NLC had an average stability of at least 8 months. Once transformed in powder through spray drying or freeze-drying steps, the chances of stability significantly increased. Liposomes formulations have been monitored over 2 years in powder form, confirming a stability of 15 ± 4 months; in particular, ferrous sulfate loaded liposomes had a stability value of at least 24 months, demonstrating their applicability to market standards and requirements.

5.2. *Preparation of iron-based liposomes*

As indicated above, SPAF process is characterized by two main steps: production of lipidic colloidal suspensions in liquid form, followed by complete elimination of water and other eventual residual liquid solvents content, through lyophilization. In this section, two formulations of liposomes loaded with ferrous sulfate and sodium ascorbate, as

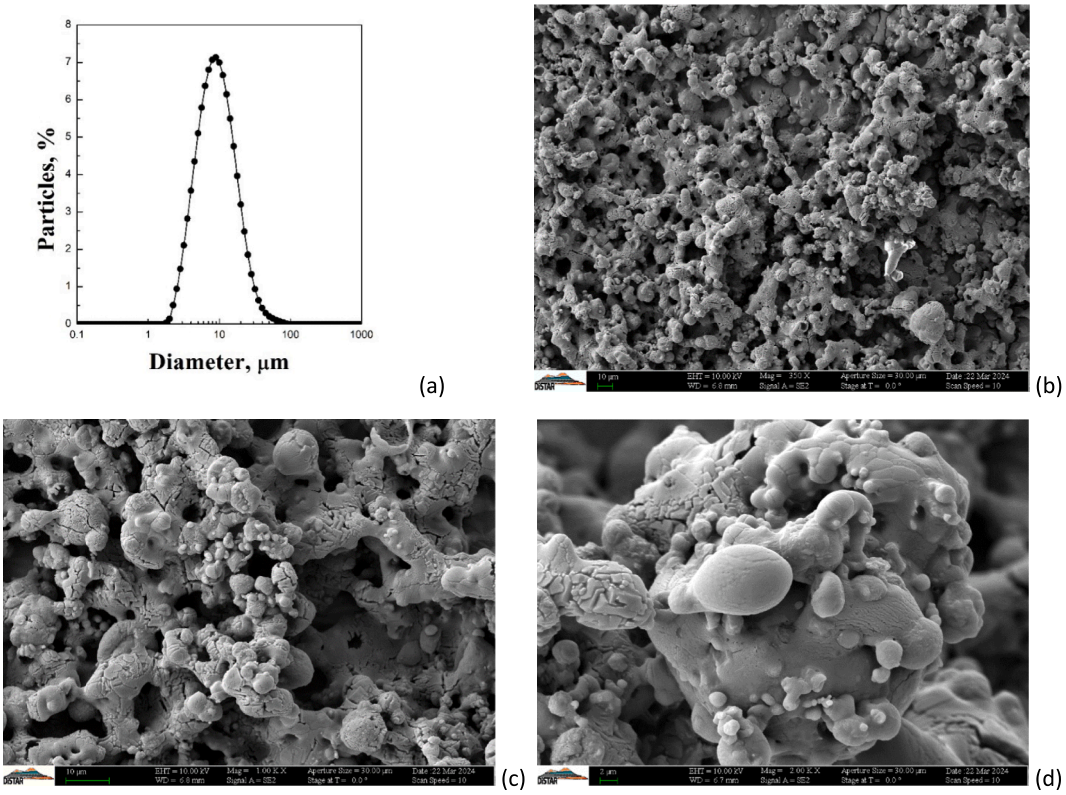

Fig. 8. Particle Size Distribution (a) and SEM observations (b-d) after direct spray drying of samples.

oxidative protectant, have been prepared. $FeSO_4$ has been utilized in this work in its hydrate form, that is characterized by crystals of non-homogeneous shapes, irregular forms and smooth surfaces. While operating parameters such as temperature, pressure and time contact were respectively 40 °C, 100 bar and 1 h, the ratio among ferrous sulfate and phospholipids was varied, according to Table 2. This parameter was defined as Drug-to-Lipid ratio (DLR); in particular, the effect of 3 different drug-to-lipid concentrations was studied, assuming as control value a reference sample produced without the entrapment of ferrous sulphate. Conditions were determined in consideration of our previous studies performed on Nanostructured Lipidic Carriers, as shown in a previous work (Trucillo et al., 2022).

As it is possible to see, the encapsulation of ferrous sulphate is having a significant effect on the aggregation phenomena of liposomes, that have a mean value of 250 nm without the addition of the bioactive molecule. Empty liposomes show lower phenomena aggregation also due to the larger dilution in aqueous medium, in total absence of ferrous sulphate dissolved in water. On the other side, the encapsulation of ferrous sulfate is favoring aggregation phenomena, that in certain cases may result also in the formation of ferrous complexes. The drug-to-lipid ratio (DLR) has an effect on liposomes mean size and dispersion of sample, having an increasing trend of D90 value from 123 µm (DRL of 2) to 325 µm for DRL 6. The increase of DRL also resulted in a significant increase of the encapsulation efficiency, that was detected as 94 ± 4 % for the highest DRL.

In Fig. 3**a**, empty liposomes are shown, while in Fig. 3**b**, samples loaded with drug to lipid ratio of 2 are shown. Concerning the other DLR, higher encapsulation efficiency was achieved, and particle size distributions were significantly affected by the formation of numerous lipid aggregates. However, the increase of DLR also resulted in a larger capability to retain drug, reducing leakage.

5.3. Analysis of supernatant and solids

Liposomes were first proposed in liquid suspension; therefore, it was necessary to analyze the aqueous and the solid phase. To separate them, a centrifugation step was performed at 6000 rpm. The supernatant was then withdrawn and analyzed for granulometric properties. Granulometric analysis was also conducted on the dried solid powder. Specifically, particle size distributions considered the mean dimensions of lightweight particles floating in the aqueous medium. In contrast, the analysis of the solid powder also accounted for the agglomeration phenomena typically occurring with phospholipids. This analysis has been performed on the sample produced at DLR of 6, being the sample with the highest encapsulation efficiency and with the largest phenomena of aggregation. From the SEM and PSD analysis performed on this sample, it was demonstrated that liposomes of significant different dimensions are contained in both supernatant and liquid, as shown in Fig. 4 and Table 3.

As it is possible to see in Fig. 4 and Table 3, the separation of supernatant by supernatant determined significant difference in terms of mean size and number of most representative peaks. However, the encapsulation efficiency has been monitored not only in solid forms, but also in the supernatant floating particles, in order to evaluate eventual loss of compounds during the process of total liquids eliminations.

To further validate the scientific evidence presented in Table 3, a 25 kg bulk sample was produced. A huge amount has been centrifuged and separated from supernatant, to compare dried supernatant and solids, in terms of SEM morphology (see Fig. 5). This study was useful not only for the determination of production yield, but also to understand the necessity to lyophilize both lipidic solids and supernatant, simultaneously, without previous elimination of liquids containing light-weight liposomes component.

In order to assess the presence of ferrous compounds and liposomes in the supernatant and solids samples, the following EDX analysis have

been determined (see Fig. 6).

As it is possible to see in Fig. 6, ferrous species were detected in both figures, thus indicating that it is not possible to eliminate neither supernatant, neither solid liposomes from the production, that could result into loss of bioactive and reduction of ferrous content of the finite sample. Sulphur has also been detected in both samples, being part of the ferrous sulphate entrapped molecule. Na has been detected since in the formulation sodium ascorbate has been included as protectant against ferrous oxidation.

5.4. Lyophilization step

As it was clarified, 25 kg of lyophilized liposomes has been produced in bulk and furthermore analyzed to demonstrate the industrial scalability of the process. Therefore, these formulations have been studied deeply in terms of yield on mass basis. As it was already described in Material and Methods sections, the liquid yield of the reactor (left part of Fig. 2) is almost 100 %, since it is possible to consider negligible the loss of aqueous content during pressure release. Additionally, water has a low affinity with carbon dioxide, thus not being released from the top of the reactor. The yield was also calculated on a mass basis, accounting for the transformation from liquid suspension to powder, specifically considering the lyophilization step. The mass yield was monitored for each productive session and an average has been provided in the last line of Table 4. Table 4 reports the volume of liquid suspension of liposomes produced day by day, during this batch of production. Please note that these values may vary depending on several factors, such as operators' availability, process issues and availability of raw materials, that generally may affect also industries. Note also that these values of volumes were not necessarily dried in the same day. The productivity conditions were standardized to 100 bar, 40 °C and 1 h contact time. As indicated in the following characterization steps, each production daily batch was analyzed and demonstrated the repeatability of the process.

On average, on a total batch of 147 L of colloidal suspension produced from the reactor (left part of Fig. 2), 25.44 kg were recovered from the lyophilization step (right part of Fig. 2), thus corresponding to an average mass yield of 0.179 kg/L. In particular, the repeatability of the lyophilization step was almost constant, with a few exceptions related to a maximum value of about 0.262 kg/L and a minimum of about 0.09 kg/L. These exceptions are probably due to operators' errors during manipulations thus concentrating or diluting too much the plates of the lyophilization unit operations.

5.5. Post-processing alternative: Grinding vs. Spray drying steps

The first proposed alternative to enhance particle size distribution of the produced liposomes was achieved adding a post-treatment step. In particular, the combination of reactor + lyophilization steps was improved by mechanical grinding of the lyophilized powder, thus obtaining a reduction of lipidic agglomerates size and size distribution. Fig. 7 contains a SEM comparison of the same sample before (Fig. 7**b**) and after (Fig. 7**c**) this processing steps. As it is possible to see, the dispersion of liposome powder size was characterized by a qualitative significant reduction after grinding step. In order to verify if the grinding step damaged samples, SEM observations have been performed after grinding.

As we can see in Fig. 7, many aggregates have been separated, if compared with Fig. 3 and Fig. 5. Moreover, smaller liposomes were obtained after size reduction of larger phospholipids complexes, as also obtained elsewhere in our previous work for nanostructured lipidic carriers (Tang et al., 2023). As it is possible to see, a significant reduction of liposomes mean size was obtained to 3.5 μm, considering the still larger presence of aggregates of reduced dimensions. An encapsulation efficiency of 91 ± 2 % of ferrous sulphate has been detected using spectrophotometer after grinding step, confirming that almost negligible was the loss of ferrous content.

As an alternative to lyophilizing + grinding step, the use of a spray drier has been considered, obtaining a narrower particle size distribution, as reported in Fig. 8. This means that liquid suspension of samples has been directly processed through spray drying, without following the lyophilization and grinding steps.

As shown in Fig. 8**a**, liposomes loaded with ferrous sulphate have a mean diameter of 7.9 μm, with a D10 value of 4.3 μm and a D90 of 21.2 μm. The distribution of samples is more uniform, with evidence of a larger numerosity of spherical vesicles. Aggregates are still present, but of significantly lower dimensions.

5.6. Food grade analysis: heavy metals and microbiology

A significant achievement of this study is the unequivocal demonstration that the produced sample complies with food-grade standards, making it suitable for industrial food applications. The presentation of these results is giving more strength to the versatility and efficacy of the SPAF process to the market.

The content of heavy metals was first analyzed on liposomal powder; in particular, the absence of heavy metals is a common required characteristic for the product to attain food-grade status. This type of analysis is essential as the absence of specific heavy metals is a critical condition for ensuring the compliance of the product with the standards associated with food-grade products and their suitability for the food-related industrial applications. Powdered liposomes loaded with 80 % ferrous sulfate were first analyzed for their content of Hg, Cd, Pb, and As, none of which were detected. However, since iron-loaded liposomes are typically sold with various excipients, a 1:4 mass-based dilution with maltodextrins was also analyzed to verify whether the maltodextrin used could contaminate the samples. Also in this case, not-detectable values of heavy metals were registered. This analysis was performed considering a LOQ (Limit Of Quantification) value of 0.02 mg/kg for Hg, 0.01 mg/kg for Cd, 0.02 mg/kg for Pb and 0.02 mg/kg for As. No toxic effects have been detected on all analyzed samples.

Other aliquots of the same batch samples were subjected to microbiological analysis, to assess any potential bacteria growth. This test appeared to be fundamental to food-grade standards, as the absence of undesirable microbial contaminants is a necessary condition for food applications. Three different types of formulations (80 % concentrated ferrous sulphate liposomes, 1:4 w/w diluted samples with maltodextrins and the same diluted sample after 3 months stored at 20 °C) were put in contact with *E.coli*, *Listeria monocytogenes*, *Salmonella spp*, *Bacillus cereus*. No microbiological growth nor activity has been detected after performing these characterizations. Pesticides were also analyzed, without finding any presence of chemical substances used to kill, repel, or control pests, including insects, weeds and fungi.

The systematic assessment of microbiological parameters further confirms the safety and quality of the product, thereby contributing to its qualification as a reliable and compliant material for use in the food industry.

6. Conclusions

SPAF process has been proposed in this work as an overall process that combines the high control of particle size distribution and encapsulation efficiency due to supercritical fluids, with the high stability of powder obtained after post-processing steps of lyophilization or spray drying. The storing and transportation of liposomes in aqueous form could result in particle size modification, drug degradation, formation of microorganisms and, thus, degradation of the products. In order to propose high quality products on the market, the combined effect of supercritical fluids and powder transformation ensured high versatility, replicability, and control of mean size, while spray drying and lyophilization techniques allowed for finer adjustments of particle size distribution and enhanced stability over time. The powder form was shown to be stable for months, and even years for ferrous sulfate-loaded

liposomes. The combination of production and post-processing steps significantly increased the process efficiency, including yield on a mass basis. The versatility of the supercritical-assisted process for producing dietary supplements demonstrated not only that SPAF ensures a high level of sterility, but also a considerable potential for scalability to an industrial level. This work could represent a basis for further studies and combination of different hydrophilic and lipophilic drugs over different biomaterials for core formation.

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Daniele Sofia: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Massimo Moffa:** Visualization, Validation, Software, Resources, Data curation, Conceptualization. **Paolo Trucillo:** Writing – original draft, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Paolo Trucillo, Daniele Sofia, Massimo Moffa are co-inventors of the following patent number #IT202100012851A1 licensed to IODO Srl, a company own by the co-inventors. Authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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