

Liposomal incorporation of Lavandin essential oil by a conventional method and by PGSS

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Essentials oils had been long used as natural preservatives, flavourings and fragrances. Some essential oils, as for example lavandin oil, have been found to have biocide and antiviral properties. The aim of this paper is to achieve an adequate formulation that allows the use of this oil in livestock by oral or parental administration as a substitute of synthetic drugs. Liposomes are promising drug carriers in antiviral therapy due to their capability to enhance skin penetration of drugs, deliver the entrapped drugs across cell membranes, improved essential oil stability and bioavailability. The feasibility of using commercially available lecithins and cholesterol for produce liposomes by a modification of the Bangham method and by PGSS drying has been tested. The liposomes obtained by modified film method were multivesicular or unilamellar/multilamellar with a mean diameter of 420-1290 nm and incorporation oil efficiency between 6-66% wt. Cholesterol results to decrease liposome size, improve vesicle stability, homogeneity and encapsulation yield. Formulations were examined for their physic stability by following for one month the evolution of released essential oil, and it was found that liposomes were stable at least for this period. On the other hand, by application of the PGSS process, dry and fine but aggregated particles of soy lecithin, with size ranging from 7-3 μm were obtained. The obtained powder was then dispersed in water for the elaboration of multilamellar liposomes whose size ranged between 1 - 1.5 μm .

1. Introduction

The overall objective of the present research was to formulate lavandin essential oil (approximate composition: 45% linalool and 25% linalyl acetate) in soya lecithin-based liposomes for applications as antimicrobial and antiviral agent in livestock

Liposomes are formed spontaneously by self-assembly of phospholipids in aqueous solution, producing vesicles (aqueous medium surrounded by a lipid membrane). These structures can retain water-soluble substances in the inner aqueous phase and oil-soluble substances in the bilayer wall. Lavandin essential oil is hydrophobic. Therefore, it accumulates in the lipid bilayer. Liposomes enhance the targeting of essential oils to cells because lipid bilayer of liposomes can protect encapsulations substances from digestion [1], bind to intestinal mucosa [2] and allow the deliver of antiviral agents that cannot cross the cytoplasmatic barrier [3].

In recent years liposomes have been extensively studied as carrier systems but for essentials oil few studies have been published. For example, *A. arborescens* was incorporated in soya phosphatidylcholine and hydrogenated phosphatidylcholine multilamellar liposomes greatly improving its antiviral activity [4], *Santolina insularis* was incorporated in hydrogenated soya phosphatidylcholine and cholesterol liposomes enhancing its antiviral efficacy [5] and carvacrol and thymol isolated from *Origanum dictamnus* L essential oil were successfully encapsulated in phosphatidyl choline-based liposomes [6].

Soybean lecithin is a product accepted by consumers and legislators as being natural, therefore it has been used as emulsifier in this work. Soybean lecithine is a complex mixture of 98% phospholipids like phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol [7].

A manufacturing method is desirable to formulate liposomes with high entrapment efficiency, narrow size distribution, long-term stability and protective properties. In this work, the suitability of three different techniques for liposomes generation, based on Bangham method, has been studied. The Bangham method or thin hydration method is one of the most widely used and simplest techniques for the formulation of liposomes [8], and because of this it was used in this work as a reference method. However this method has limited use due to low entrapment efficiency, organic solvent traces, homogenisation and small scale production. On the other hand, in this work liposomes were also obtained by PGSS drying of a lavender oil lecithin emulsion and later hydration of the obtained powder. In the particular case of interest, a few references about soy lecithin micronization had been found. According to previous literature, RESS [9] way was not effective in micronizing soy lecithin, but the SAS [9,10] method was effective producing microspheres in a wide range of operating conditions whose diameters ranged from 1 to 40 μm . Precipitation of phospholipids complex of puerarin was achieved by GAS (Gas antisolvent solutions) and SEDS (solution enhanced dispersions with supercritical fluids) processes, obtaining aggregated particles with a diameter around 5.5 μm [11].

2. Materials and Methods

2.1. Materials

Lavandin essential oil “super” used in this project was purchased from COCOPE (Valladolid, Spain), which is a manufacturer of essential oils in Spain. This lavender oil was produced by steam distillation. Soybean lecithin (97% phospholipids) was obtained from Glama-sot (SOTYA, Spain). CO_2 was provided by Carburos Metálicos (Barcelona, Spain).

Cholesterol (grade $\geq 99\%$) was purchased from Sigma-Aldrich (Spain). Chloroform (PA Grade) was supplied by Panreac (Barcelona, Spain). Trans-2-Hexen-1-ol 98% provided by Sigma-Aldrich (Spain).

2.2. Liposomes preparation

Vesicles were prepared according to the thin hydration method. Soybean lecithin and cholesterol were dissolved together with 10 ml of chloroform in a 50 ml round-bottom flask. Organic solvent was evaporated by a rotary evaporator at 40°C until a thin film was formed on the walls. When lavender essential oil was used it was dissolved in 10 ml of chloroform and then mixed in a round bottom flask with soybean lecithin and cholesterol. The lipid film was suspended in 1-2 ml of water and three different procedures were developed. In the first method, the lipid film was heated at 60°C (above lipid transition temperature) for 20 minutes and then sonicated in an ultrasound bath (J.P Selecta, 50Hz, 100W) during 30 minutes to reduce size and homogenize liposomes). In the second method, the lipid film was suspended in water and then shaken in a vortex mixer (Vortex V-3, 50-3400 r.p.m.) at 1700 r.p.m. for 15 min and this suspension was allowed to hydrate for 2h in the dark at room temperature, in order to avoid any structural defects. In a third method, the lipid film was heated at 60°C (above lipid transition temperature) for 20 minutes and then shaken in a vortex mixer at 17000 rpm for 15 min.

2.3. Emulsion preparation

Water in oil emulsions were prepared by a two step process. Lecithin suspension was initially prepared in deionized water (Milli-Q, Millipore) at 50°C with the aid of a Magnetic stirrer (IKA). Afterwards the oil in corresponding ratio was gradually added to the suspension under continuous agitation at 11085 r.p.m. for 5 min with the mechanical stirrer

and a crude emulsion was obtained. The resulting coarsely-dispersed raw emulsion is then fed into the rotor-stator machine (IKA[®] LABOR PILOT 2000/4) whose capacity is 200ml and processed for 4 min for fine emulsification. The stability of lavandin oil emulsions in a CO₂ environment at different pressures (1-120 bar) and temperatures (273-373°C) was studied using a high-pressure view cell.

2.4. PGSS-Drying

Once stable oil-in-water emulsion had been obtained, PGSS drying was applied to dry them. In this process the emulsion is saturated with CO₂ causing a decrease of the emulsion viscosity. The emulsion saturated with CO₂ is contacted with the supercritical CO₂ in a static mixer and then expanded in a nozzle, which facilitates the formation of extremely fine droplets which dry very fast resulting in fine powders. The conditions in the spray tower have to be above the dew line (temperature composition diagram for CO₂ and water), in order to remove water and carbon dioxide from the powder and achieve a superheating of the mixture. The residual humidity in the dried powder depends on the relative humidity in the spray tower. Considerable amount of the solvent is already extracted into the gas in the static mixer before expansion. Therefore after expansion only a small amount of solvent has to be evaporated. By the combination of co-extraction in the static mixer and evaporation of residual solvent the demand of CO₂ is reduced [12].

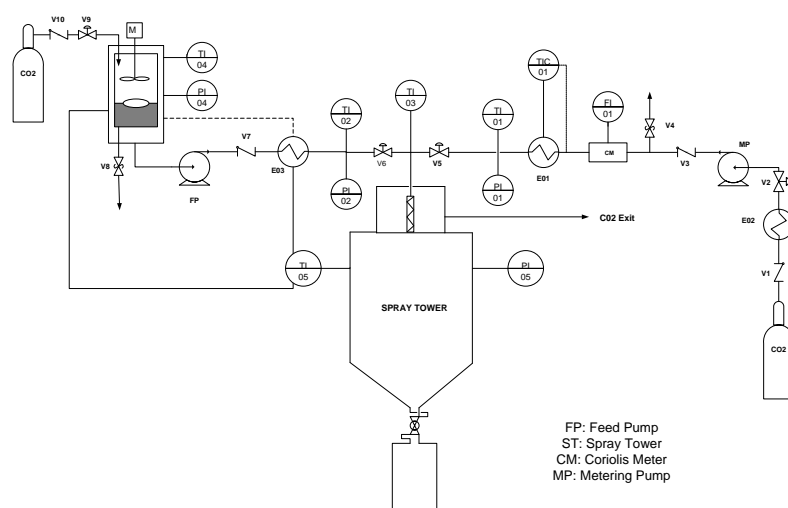


Figure 1. Schematic scheme of a PGSS plant.

2.5. Measurement of the trapping efficiency of liposomes.

The trapping efficiency of soybean lecithin based liposomes for lavandin essential oil was estimated by subjecting them to centrifugation (Microcentrifuge 24D Labnet) at 13000 r.p.m. for 30 minutes. Then the supernatant (water with non encapsulated oil) was separated with the aid of a syringe and 1ml acetone was added to the residual liposomes to disrupt the vesicles. The sample was filtrated to eliminate the lecithin and then heated at 60°C for one hour in an oven to eliminate the acetone. The obtained essential oil was analysed by gas-chromatograph coupled with a mass spectrometer (GC-MS) Agilent 6890/5973 (Agilent Technologies, Palo Alto, CA, USA) and an Agilent HP-5ms Capillary GC column. The sample was diluted in Hexane including n-hexenal as internal standard, in order to be able to determine the amount of essential oil through a previous calibration. The operating conditions are the followings: Helium was the carrier gas at 0,7 ml/min, split mode injection (200:1), injection temperature 250°C and injection volume 1µl. The oven temperature was programmed as follows: 5 min at 65°C and 4°C/min to 220°C. Identification of compounds was based on relative retention times, matching with Nist MS

library or comparison of their relative retention times with those of authentic samples.

2.6. Encapsulated and superficial oil in soybean lecithin microparticles.

The encapsulation efficiency of soybean lecithin microparticles was determined by dissolving 0,5 g of the powder in 2 ml of acetone. The mixture was vigorously vortexed for 1 minute and the filtrated to eliminated soybean particles. Lavandin oil extracted was analysed by gas-chromatograph. Surface oil is another important characteristic of soybean microparticles and was determined by washing 0,5g of powder with 2 ml of a dissolution of 0,5 % w/w n-Hexenal in Hexane. The suspension was the filtered and the samples were analyzed by gas-chromatograph.

2.7. Observation of liposomes.

Automated Upright Microscope system for Life Science Research Leica DM4000 B was used to determinate the structure of lecithin based liposomes.

2.8. Measurement of particle size of liposomes.

Particle size of liposomes was estimated by Dynamic Light scattering (Autosizer Lo-C (Manern instruments) at 20°C. Sampling was carried out after a gentle rotation of the liposomes container in order to obtain an ever dispersion of the droplets and further dilution with deionized water to less than 0,4% w/w to prevent multiple scattering effects. Particle size measurements are reported as volume distribution as defined as the average emulsion diameter (d_{32}) and are average values of measurements made ten times on at least three freshly prepared emulsion samples. The following optical parameters were applied: lavandin oil refractive index: 1,46 and water refractive index: 1,3310.

3. Results

3.1. Liposomes

In order to study the influence of vesicle structure on the liposome properties, different vesicular formulations were prepared by three different methods based on the film method. Vesicles were obtained from lavandin oil (0.1-0.4 mg), soybean lecithin (0.1-0.4 mg), Cholesterol (0.013-0.052 mg) and hydration water (1-2 ml). Their influence on liposome structure and entrapped essential oil were determined. First, the influence of lavandin oil to lecithin ratio was evaluated by adding a fix amount lecithin (0.1 g) and cholesterol (0.013 g) and different amounts of lavandin oil (0.1-0.4 g). The morphology of prepared vesicles was evaluated using optical microscopy. Different vesicular formulations like small unilamellar/multilamellar vesicles (SUV/SMV) and multivesicular vesicles (MVV) were obtained, as it is reported in table 1. Usually SUV/SMV were obtained, however, when the ratio lavandin oil/lipids was smaller than 1.8 MVV were obtained. As hydration water increases SUV/SMV appears at higher lavandin/lipids ratio, for example when 2 ml of water were added MVV were obtained at lavandin oil to lipids ratio of 2. Liposome size is strongly dependent on the inclusion of essential oil (increasing with the lavandin oil/lipids ratio), amount of cholesterol (decreasing with the amount of cholesterol) and liposome preparation technique. The method which enhances to obtain the smaller liposomes was method 3 which involve vortex mixing of the liposomes and two hours of subsequent hydration in darkness. The concentration of lecithin also influenced the size of liposomes, and it was found that liposome diameter decreases with the concentration of cholesterol for all the liposome preparation methods, as shown in Table 1.

Table 1. Characterization of prepared formulations which contain 0.1g of lecithin and 0.013g of cholesterol.

Lav/Lipids	Water (ml)	Method 1		Method 2		Method 3
		Diameter (μm)	Structure	Diameter (μm)	Structure	Diameter (μm)
1.0	1.0	0.68 \pm 0.04	MVV	0.42 \pm 0.01	MVV	0.75 \pm 0.09
1.8		0.781 \pm 0.04	SUV/SMV	0.65 \pm 0.07	SUV/SMV	0.77 \pm 0.02
2.4		0.77 \pm 0.17	SUV/SMV	0.75 \pm 0.02	SUV/SMV	0.81 \pm 0.03
3.5		0.94 \pm 0.09	SUV/SMV	1.26 \pm 0.08	SUV/SMV	0.94 \pm 0.03
1.0	1.5	0.75 \pm 0.05	MVV	0.84 \pm 0.02	MVV	0.60 \pm 0.09
1.8		0.75 \pm 0.04	SUV/SMV	0.92 \pm 0.02	SUV/SMV	0.73 \pm 0.03
2.4		0.99 \pm 0.02	SUV/SMV	1.05 \pm 0.09	SUV/SMV	0.80 \pm 0.02
3.5		1.03 \pm 0.04	SUV/SMV	1.09 \pm 0.02	SUV/SMV	0.89 \pm 0.02
0	2	2.47 \pm 0.24	SUV/SMV	1.32 \pm 0.07	SUV/SMV	
1.0		0.94 \pm 0.01	MVV	0.78 \pm 0.12	MVV	0.66 \pm 0.02
1.8		1.07 \pm 0.03	SUV/SMV	0.87 \pm 0.02	MVV	0.81 \pm 0.04
2.4		1.22 \pm 0.05	SUV/SMV	0.89 \pm 0.05	SUV/SMV	0.88 \pm 0.04
3.5		1.29 \pm 0.42	SUV/SMV	0.93 \pm 0.02	SUV/SMV	0.87 \pm 0.06

Incorporation efficiency of lavandin essential oil in the liposomes was calculated as a percentage of the total amount of Lavandin oil founded at the end in the product. The influence of lavandin/lipid ratio on the lavandin oil and linalool encapsulation efficiency for each one of the processes is reported in figure 2. It can be appreciated that for method 1 and 2 the encapsulation efficiency increases with the lavandin/lipids ratio, while method 3 follows an opposite trend. Method 2 results to be the most efficient in terms of encapsulation efficiency of lavandin oil.

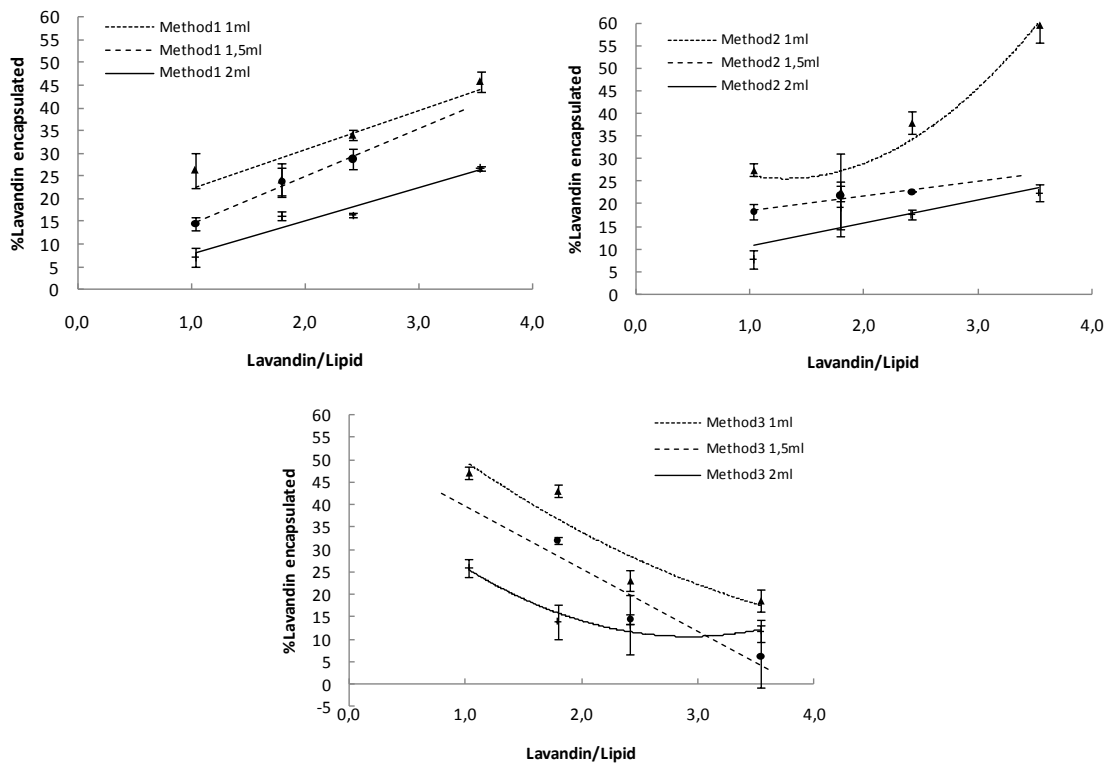


Figure 3. Encapsulated lavandin oil as a function of Lavandin/Lipids ratio for different methods and amounts of hydration water.

Physical stability of vesicles was studied over one month. Essential oil content of the different preparations was determined and analyzed as it is described in section 2.5. Lavandin oil encapsulated in liposomes was released slowly, since after fifty days released oil ranged between 5,2-0,2% (table 2). Linalool and linalyl acetate were faster released but slight differences related to the vesicle composition or liposome process method were obtained. It can be concluded that method 2 resulted most efficient in terms of lavandin control released and physical stability of liposomes. The main parameters affecting liposome physical stability are the content of essential oil and the process used to generate the liposomes.

Table 2. Delivered oil, linalool and linalyl acetate (LA) for different liposomes formulations.

	Method 1		Method 2		Method 3	
	0.2	0.4	0.2	0.4	0.2	0.4
Initial Lavandin (g)	0.2	0.4	0.2	0.4	0.2	0.4
% Encapsulated oil	16	27	19	32	14	12
% Encapsulated linalool	6	0	3	7	7	3
% Encapsulated LA	1.8	2.1	0	2.3	0.9	0.8
% Delivered oil 50 days	0.3	0.2	2.7	1.8	5.2	4.7
% Delivered linalool 50 days	17	25	21	20	20	19
% Delivered LA 50 days	23	25	25	24	24	24

3.2. PGSS-Drying

PGSS drying experiments were performed drying a lavandin oil lecithin emulsion, in which lavandin oil concentration was 5% wt. and soybean lecithin concentrations ranged between 5-20% wt. Firstly, the emulsion was pressurised at 10 bar and 30°C before expansion, in order to saturate emulsion with CO₂ and reduced the emulsion viscosity. Different conditions of pre-expansion pressure, pre-expansion temperature, gas to product ratio (GPR) were selected in order to study their influence in the particles characteristics. The correspondent results are shown in table 3. From the results it can be observed that dried particles can be only obtained in a narrow range of conditions. Entrapped efficiency of lavandin oil was not high. On the other hand, lavandin oil was detected in the surface of particles, implying that all the lavandin oil is encapsulated inside of the particle. GPR results to be an important variable, because when it was increased linalool was not encapsulated at all and lavandin oil entrapment efficiency decreases.

Particles obtained from PGSS processes were dissolved in water (0.5 g in 2ml water) in order to verify the formation of liposomes. Liposomes obtained were multilamellar and its size range between 1.05±0.04 µm (Exp. 4) and 1.47±0.12 µm (Exp. 5).

Table 3. Results of PGSS drying of a lavandin oil emulsion using soybean lecithin as amulgent

Exp	T _{CO2} (°C)	T _{Mixer} (°C)	T _{tower} (°C)	P _{before exp} (bar)	GPR	%Lecithin emulsion	Particles	Diameter (µm)	%Lavandin encapsulated	%Linalool encapsulated
1	150	108	43	70	15	5	No			
2	151	118	45	65	31	7	Wet	-	-	-
3	144	112	44	77	18	7	Wet	-	-	-
4	146	113	50	73	16	7	Dried	2,99±0,65	9,6±0,3	8±0,17
5	146	110	47	73	33	7	Dried	6,63±0,55	8,4±0,2	0
6	148	110	41	75	17	7	Wet	-	-	-
7	151	113	42	77	14	7	Wet	-	-	-
8	123	82	37	75	36	9	Wet	-	-	-
9	129	104	39	75	36	8	Wet	-	-	-
10	129	105	44	75	35	9	Wet	-	-	-
11	132	116	45	81	37	9	Wet	-	-	-
12	150	90	49	85	41	9	Burnt	-	-	-
13	153	116	50	70	16	8	Burnt	-	-	-
14	142	108	44	110	48	18	Wet	-	-	-
15	145	111	40	104	46	18	Wet	-	-	-
16	151	117	47	89	37	18	Wet	-	-	-

4. Conclusion

Vesicles sizes are strongly dependent on bilayer composition, lavandin oil load and the experimental method used for its production. Firstly, the mean size of the vesicles was strongly influenced by the inclusion of essential oil (increasing with the lavandin oil/lipids ratio) and the amount of cholesterol (decreasing with the amount of cholesterol). Actually, empty vesicles (1.32-2.87µm) are always larger than essential oil loaded vesicles (0.42-1.29 µm), which can be explained based on the higher cohesion packing among the apolar chains in vesicles membrane [13]. The addition of cholesterol in the liposome composition, caused changes in the degree of head group dissociation and interaction with lipophilic compounds. A Cholesterol molecule will be oriented with its steroid nucleus among the fatty acid chains of phospholipid molecules and its hydroxyl group facing towards the face water. Therefore cholesterol is often added to improve liposomes *in vitro* and *in vivo* stability and decreases membrane permeability [14]. The hydration water also plays an important role, since diameter increases as the amount of water increases. This can be explained because for entrap a higher amount of water with the same quantity of emulgent, main drop diameter has to decrease.

Uni/multilamellar and multivesicle vesicles were obtained, showing both a good entrapping efficiency (6-60%). It can be observed that for liposomes generated by method 1 or 2 entrapping efficiency increases with lavandin/lipids ratio, otherwise for method 3 entrapping efficiency decreases with lavandin/lipids ratio. The amount of hydration water affects entrapping efficiency, increasing as the amount of water decrease.

The structure of liposomes depends mainly on the lavandin/lipids ratio and hydration water, as can be observed in table 1. For lavandin oil/lipids ratio smaller than 1, generally MVV were formed and higher ratios give SUV/SMV, regardless the method used.

Vesicle dispersions showed a very high stability for at least one month. Average size distribution changes during the first ten days and remain stable since then, confirming that fusion and breakage of vesicles did not appreciably occurs. The release rate of lavandin oil

was slow. In all cases, this parameter results to be independent of liposome process or formulation.

It can be concluded that method 2 results the most efficient in terms of essential oil load and vesicles stability. In fact, vortex mixing gives small uni/multilamellar vesicles whose size ranges from 0,6 to 0,94 μm , while sonicated oil-loaded vesicles range from 0,68 to 1,29 μm . Hydration of the liposomes in darkness for one hour, improve their stability and encapsulation efficiency. That could be explained because the higher time to layer formation allows layer defects to be repaired.

PGSS was effective in micronizing filled soy lecithin, forming spherical aggregate particles. GPR seems to have an important effect upon the particle size and entrapped oil, as well as pre-expansion temperature. Soy lecithin concentration of the emulsion plays an important role, because soy lecithin is known to give highly viscous media in presence of supercritical mixture and diffusional effects might take place during the micronization process. As a consequence it is not possible to work with high concentrate emulsions. Anyway, more experiments had to be developed in order to confirm these trends and optimize the process.

Acknowledgments

Authors acknowledge Country Council of Valladolid for financing this project. S. Varona performed this work with a FPI-UVa research fellowship.

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