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Optimization, characterization and *in vitro* evaluation of curcumin microemulsions

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ABSTRACT

The purpose of this study was to improve the solubility and the stability and oral uptake of curcumin by developing an o/w microemulsion, using food grade components. Three microemulsions were developed and characterized, stabilized by non ionic surfactants Cremophor EL, Tween 20, Tween 80 or Lecitin and containing a variety of oils, namely olive oil, wheat germ oil, vitamin E. Chemical and physical stabilities of three systems was also evaluated within two months. The oral absorption of curcumin from the best microemulsion was investigated *in vitro* using parallel artificial membrane permeability assay (PAMPA). The optimal formulation consisted of 3.3 g/100 g of vitamin E, 53.8 g/100 g of Tween 20, 6.6 g/100 g of ethanol and water (36.3 g/100 g), with a maximum solubility of curcumin up to 14.57 mg/ml and a percentage of permeation through the artificial membrane of about 70%.

1. Introduction

Curcumin, is a natural poliphenolic compound (Fig. 1) isolated from the rhizomes of *Curcuma longa* L. Generally, it has been associated with a large number of biological and cellular activities, including antioxidant, anti-inflammatory and hypocholesterolemic properties. In addition, it is able to induce apoptosis in human cancer cells of different tissues origin, including B and T cells and cells from colon, epidermis, prostate, breast and head (Aggarwal, Kumar, & Bharti, 2003).

Although curcumin is pharmacologically safe (Kunnumakkara, Anand, & Aggarwal, 2008), the use of this molecule in therapy is limited due to its low water solubility at acid or physiological pHs and, consequently, due to its poor bioavailability (Tønnesen, Másson, & Loftsson, 2002). Another drawback for clinical application is its rapid hydrolysis in alkaline media and its photochemical degradation (Tønnesen et al., 2002).

Microemulsions have attracted much interest in recent years as drug delivery systems being highly dispersed, stable and transparent systems and easy to prepare (Kesisoglou & Panmai, 2007). Furthermore, their nanoscopic dimensions allow a better absorption in to the cells. Finally, microemulsions show the ability to overcome the problems of solubility and stability of many phytotherapeutic, nutraceuticals and food additives (Spernath & Aserin, 2006). In fact, oral absorption of hydrophobic drugs can be significantly improved using lipid-based non-particulate drug delivery systems, which avoid the dissolution step and provide significant improvement of oral absorption in comparison with an oral solid or suspension dosage form (Kreilgaard, 2002; Narang, Delmarre, & Gao, 2007).

These systems have dispersed phase size lower than 100 nm, differently from classical emulsions. This characteristic gives them transparent and allows to formulate the drug either as ready-to-use aqueous solutions and as non-aqueous concentrates. The concentrate may be a microemulsion, which is diluted with water immediately before administration, or administered as it is and gets diluted with gastric fluids *in vivo* (Ajit, Narang, Delmarre, & Gao, 2007; Garti, Yaghmur, Leser, Clement, & Watzke, 2001). Several reviews have summarized physical and biopharmaceutical aspects of these systems (Flanagan & Singh, 2006; Gursoy & Benita, 2004; Lawrence & Rees, 2000; Pouton, 2000).

The main aim of this study is to formulate an o/w microemulsion of curcumin for oral administration, using food acceptable components, to increase solubility, stability and ameliorate oral absorption of this compound. The study is part of the objective to find a specific formulation and proper dosage for a possible pediatric dietary supplement containing curcumin. The European Food Safety Authority (EFSA, 2010) allocated an ADI of curcumin of 0–3 mg/kg by/day.

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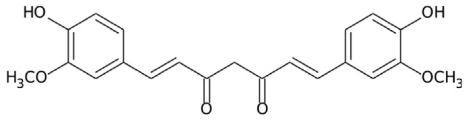

Fig. 1. Chemical structure of curcumin.

Recent studies have reported that emulsion-based delivery systems can also be used to encapsulate curcumin for increasing its bioavailability (Ahmed, Li, McClements, & Xiao, 2012; Bisht et al., 2007; Cui et al., 2009; Li et al., 2005; Lin, Lin, Chen, Yu, & Lee, 2009; Mukerjee & Vishwanatha, 2009; Setthacheewakul, Mahattanadul, Phadoongsombut, Pichayakorn, & Wiwattanapatapee, 2010; Wang et al., 2008; Yu & Huang, 2010).

Many formulations were investigated by solubility study and pseudo-ternary phase diagrams. Three microemulsion systems were developed and characterized, stabilized by the non ionic surfactants Cremophor EL, Tween 20, Tween 80 or Lecitin, and containing a variety of oils, namely olive oil, wheat germ oil, vitamin E.

The chemical and physical stabilities are also tested and the oral absorption of curcumin from optimized formulation was investigated by using *in vitro* parallel artificial membrane permeability assay (PAMPA).

2. Materials and methods

2.1. Materials

Dry extract of *Curcuma longa* L. (curcumin content 95.0% of total curcuminiods) was purchased from Galeno (Comeana, Prato, Italy).

Olive oil was from Oljvia (Badia a Settimo, Florence, Italy); wheat germ oil (*Triticum aestivum*) was purchased from Aboca (Sansepolcro, Arezzo, Italy); sunflower oil (*Helianthus Annus*) was from Coop (Massarosa, Lucca, Italy); soybean oil, Tween® 20, Tween® 80 and Cremophor EL were from Sigma–Aldrich (Steinheim, Germany); DL-α-Tocopherol acetate (Vitamin E) was purchased from Sigma–Aldrich (Milan, Italy); Lecitin was from Galeno (Comeana, Prato, Italy).

2.2. Solvent and reagents

Ethanol analytical reagent grade was from Riedel-de Haen Laborchemikalien GmbH & Co. KG, Seelze, Germany. All solvents were HPLC grade; CH_3CN and MeOH for HPLC and HCOOH (≥98%) were purchased from Sigma–Aldrich (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA). Phosphotungstic acid (PTA) was from Electron Microscopy Sciences (Hatfield, USA). Cholesterol, Dichloromethane, DMSO, 1,7-Octadiene (≥98%) were purchased from Sigma–Aldrich (Milan, Italy). Hydrion Buffer chemvelope pH 7.40 ± 0.02 was purchased from Micro Essential laboratory (Brooklin, New York). KH_2PO_4, NaOH, NaCl, HCl were purchased from Sigma–Aldrich (Milan, Italy).

2.3. HPLC-DAD analysis of curcumin

All samples were analyzed by HPLC using a reverse-phase *LUNA® Phenomenex-C_{18}* column (5 μm, 2.00 mm × 150 mm, Phenomenex, Casalecchio di Reno, Bologna, Italy) at 25 °C. Curcuminoids were detected at 420 nm by a gradient elution method (Table 1). Flow rate 0.4 ml/min.

2.4. Solubility studies

To find out appropriate constituents of microemulsions, the solubility of curcumin were determined in different oils, surfactants and co-surfactants. An excess amount of curcumin was added to 5 ml of oil or surfactant. Each mixture was shaken reciprocally at 25 °C for 24 h, then was centrifugate at 13,148 × *g* for 10 min. The supernatant was taken and the drug concentration was quantified by HPLC-DAD, after 10-folds dilution with methanol/dichloromethane (6:4). The analyses were performed in triplicate.

Table 1
Gradient elution method.

Time (min)	H_2O/HCOOH (%) pH = 3.2	CH3CN (%)
0.1	82.0	18.0
10	56.0	44.0
13	56.0	44.0
27	52.0	48.0
32	20.0	80.0
35	82.0	18.0

2.5. Construction of pseudo-ternary phase diagrams

Pseudo-ternary phase diagrams were constructed in order to obtain the concentration range of all components in which they form microemulsions. The pseudo-ternary phase diagrams were constructed using the water titration method.

Surfactant and co-surfactant were mixed at different weight ratios (S_{mix}). For each S_{mix} ratio, pseudoternary phase diagram was elaborated by testing weight ratio of oil/S_{mix} of 0:100, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20 and 90:10. Each oil-S_{mix} mixture was diluted under vigorous stirring dropwise at 50 °C with water. After equilibrium, each sample was visually checked and determined as being clear microemulsion, emulsion or gel.

Curcumin-loaded microemulsions were prepared by dissolving drug powder into the oil-S_{mix} mixture, by adding the required quantity of water, and stirring to form a clear and transparent dispersion. The resulting microemulsions were tightly sealed and stored at +4 °C temperature.

2.6. Solubility and encapsulation efficacy of curcumin into microemulsions

The capacity of selected microemulsions of solubilizing curcumin was investigated and compared with respective micellar solution of surfactant or oil solution.

In order to determine the maximum loading capacity of curcumin in microemulsion, increasing amounts of curcumin (ranging from 7 to 90 mg) were loaded to the previous formulations. The mixture was stirred for 24 h at 25 °C under light shielding. The undissolved drug was removed by centrifugation at 13,148 × *g* for 10 min, then supernatant was taken and the encapsulation efficiency was quantified by HPLC-DAD at 420 nm after 15-folds dilution with methanol. The analyses were performed in triplicate.

2.7. Particle size analysis

The droplet sizes of microemulsions with or without drug were measured by a Dynamic Light Scattering (DLS, Zetasizer® Nano ZS90, Malvern Instruments, Malvern, UK), after 20-fold dilution. Time correlation functions were used to obtain the hydrodynamic diameter of the particles and the particle size distribution (Polydispersity, Pd) of the different formulations using the ALV-60X0 software V.3.X provided by Malvern. Autocorrelation functions were analyzed by the cumulants method (fitting a single exponential to the correlation function to obtain the mean size) and distribution method (to fit a multiple exponential to the correlation function to obtain particle size distributions). Pd is a dimensionless

measurement of the broadness size distribution calculated from distribution algorithm and values were calculated for each peak as peak width/mean diameter. Scattering was measured in an optical quality 4 ml borosilicate cell at a 90° angle. Measurements were carried out at the set temperature of +25° C.

2.8. Morphology

Morphology of curcumin microemulsions was observed using a transmission electron microscope (TEM) (TEM CM12 Philips with Gatan Uhrst 3500, Eindhoven, The Netherlands).

Curcumin microemulsions were diluted 10-folds with distilled water and mixed by slightly shaking. One drop of sample was deposited on a *formuvar* film-coated copper grid and then stained with one drop of 1 g/100 ml of aqueous solution of phosphotungstic acid (PTA), allowing to dry before TEM observation.

2.9. Stability studies

In order to evaluate the stability of microemulsions, they were inserted into sealed glass vials and stored at 4 °C for 2 months. Their chemical and physical stabilities were studied by monitoring the occurrence of phase separation, dispersed phase size and drug content at predetermined intervals by DLS and HPLC/DAD analyses.

Furthermore, in an effort to mimic physiological dilution process after oral administration, the microemulsions were diluted 10, 20 and 30-fold with distilled water (pH = 5.5). The dilutions were followed by gentle vortexing for 2 min at ambient temperature. The samples were analyzed by DLS to confirm the physical stability of the systems in terms of size, Pd and homogeneity.

Microemulsion C1 were also diluted up to 20-folds in media with different pH to mimic diverse environments met after oral administration: simulated intestinal fluid (6.8 g KH_2PO_4 and 77 ml aqueous NaOH 0.2 mol/l in 1 l of water − final pH = 6.8) and simulated gastric fluid (2.0 g NaCl and 7.0 ml HCl (37 ml/100 ml of water), were dissolved in 1 l of water − final pH = 1.2), both without the presence of enzymes. The physical stability of microemulsion was checked by DLS analysis.

2.10. In vitro parallel artificial membrane permeability assay (PAMPA)

The PAMPA is a method for predicting passive intestinal absorption. The assay is carried out in a 96-well, MultiScreen-IP PAMPA filter plate with a Transport Plates Multiscreen (Millipore corporation, Tullagreen, Carrigtwohill, County Cork, Ireland). The ability of compounds to diffuse from a donor compartment into an acceptor compartment is evaluated, by placing a polyvinylidene difluoride (PVDF) membrane filter pretreated with a lipid-containing organic solvent between the two compartements. A mixture of lecithin (1 g/100 ml) and cholesterol (0.8 g/100 ml) in 1,7-octadiene was prepared and sonicate to ensure complete dissolution. 5 μL of the lipidic mixture were added to the filter of each well. Immediately after the application of the artificial membrane, 150 μL of drug containing donor solutions (free drug or curcumin loaded microemulsions diluted in a solution of 5 ml DMSO in 100 ml of PBS pH 7.4) were added to each well of the donor plate. 300 μL of buffer (5 ml DMSO in 100 ml of PBS) were added to each well of the acceptor plate. The acceptor plate was then placed into the donor plate, ensuring that the underside of the membrane was in contact with buffer. The plate was covered and incubated at room temperature under shaking for 24 h and permeation was evaluated after 1, 2, 4, 6, 19, 24 h.

3. Results and discussion

3.1. Solubility study in oils and surfactants

The purpose of this study was to formulate microemulsions suitable for oral administration. It was therefore decided to use oils of vegetable origin, although these are less valid as regards the solubility of curcumin. The chosen compounds are all food grade. The solubility of curcumin in various oils and surfactants is reported in Table 2. Based on the results, curcumin showed the highest solubility in DL-α-tocopherol acetate. Despite that the solubility in oils is not very high, it is considerably higher than that in water, corresponding to 1.003×10^{-3} mg/ml. Other oils in which curcumin shows an increased solubility were olive oil, wheat germ oil and soybean oil.

Among the surfactants, Tween 20 and Cremophor EL, showed better solubility for curcumin than others. Both these substances are non ionic and GRAS (generally-recognized-as-safe) excipients and are widely used in pharmaceutical preparations. The solubility values are higher compared to oils and this is probably because they are amphiphilic molecules with affinity for both polar and apolar groups of curcumin. The hydrophilicity of the surfactant influences, as expected, the amount of curcumin and of oil phase solubilized in the aqueous surfactant phase (Garti et al., 2001).

In contrast to completely lipophilic molecules that are sensitive to the hydrocarbon chain length, the curcumin shows an optimal interaction with surfactants more hydrophilic, because the molecule shows low water solubility but it has a polyphenolic structure.

Tween 20 is the most hydrophilic surfactant among the Tweens and it showed increased solubilization capacity. Cremophor EL and Tween 80 are more hydrophobic, but the first shows more polar groups in the chemical structure (OH− and $-OCH_2CH_2-$) and it resulted capable to solubilize a larger quantity of curcumin (Garti et al., 2001; Hou & Shah, 1987; Spernath, Yaghmur, Aserin, Hoffman, & Nissim, 2002).

Furthermore, olive oil, DL-α-tocopherol and wheat germ oil were chosen as oily phase and Tween 20, Cremophor EL and Tween 80 as surfactant were still selected for pseudo-ternary phase diagram study. In a previous publication α-tocopherol acetate has been used successfully as oil excipient to formulate a cyclosporine A microemulsion (Hirunpanich & Sato, 2009). The vitamin E is on behalf of oil component and has antioxidant properties to increase curcumin stability.

3.2. Pseudo-ternary phase diagram study

The construction of phase diagram allows to find out the right proportion of components for the existence of microemulsions (Piao et al., 2010). The diagrams were constructed using water

Table 2
Solubility of curcumin in different oils and surfactants. (Mean ± S.D.; $n = 3$).

	Curcumin solubility (mg/ml)
Oils	
Olive	1.182 ± 0.112
Wheat germ	1.247 ± 0.028
Soybean	1.286 ± 0.088
Sunflower	1.077 ± 0.062
DL-α-Tocopherol	1.719 ± 0.062
Surfactants	
Tween 20	17.614 ± 1.141
Cremophor EL	16.103 ± 2.423
Tween80	3.194 ± 0.476
Lecitin	0.785 ± 0.101
Water	$1.003 \times 10^{-3} \pm 3.84 \times 10^{-4}$

titration method. The samples were defined as microemulsions when they appeared as clear liquids (Fig. 2). Three microemulsions were selected from the phase diagrams and their composition was reported in Table 3.

3.2.1. C1 (Tween 20/Ethanol)

The phase diagram obtained was reported in Fig. 2. In this case, the system is not a mixture of two surfactants, but rather between a surfactant and ethanol. Tween 20 forms more easily microemulsions in the presence of a short-chain alcohol and this latter acts as cosolvent. A weight ratio surfactant/ethanol 10/90 g/g was used. During the titration, we observed a net color change and the formation of a transparent system. The area corresponding to the microemulsion is very similar to that already reported for a different drug (Hirunpanich & Sato, 2009), but in our study, the area corresponding to the formation of gels was also inserted, due to the presence of birefringent structures with a certain viscosity.

3.2.2. C2 (Tween 20/Cremophor EL)

The microemulsion is present for both weight ratio of Tw20/CremEL 1/2 and 1/1 and their area of existence are similar. Furthermore, it can be concluded that Tw20/Cremophor EL 1/2 is the best formulation (C2, Fig. 2) because the system showed good stability, also after dilution. The ratio oil/S_{mix} is maintained around 10/90.

3.2.3. C3 (Lecitin/Tween 80)

From the analysis of the phase diagrams the best formulation resulted Lc/Tw80 with a molar ratio of 1/4, because it provides a more distinct color change and a wider area of existence (Fig. 2). So, for subsequent analysis of characterization of the microemulsion, we have chosen a point of the phase diagram elaborated with this S_{mix} ratio (C3).

Table 3
Composition of three selected microemulsions. C1: Tween 20/Ethanol; C2: Tween 20/Cremophor EL; C3: Lecitin/Tween80.

Microemulsions	Constituent (g/100 g)
C1	DL-α-Tocopherol 3.3 Tween 20 53.8 Ethanol 6.6 Water 36.3
C2	Wheat germ oil 20 S_{Mix} 2 Water 78
C3	Olive oil 3 S_{Mix} 26 Water 71

3.3. Characterization of microemulsions

Microemulsions were characterized by DLS analysis, in order to assess the mean diameter (size) and the homogeneity of the sample (polydispersity, Pd). A lower diameter of the drops of oil (<100 nm) (Tenjarla, 1999) and the transparency of the system confirm the presence of microemulsions. DLS evidenced the presence of homogeneous systems, with a narrow size distribution for all samples (Table 4), low values of Pd (about 0.25) and mean diameter values ranging between 5.26 nm (C3) and 14.67 nm (C2).

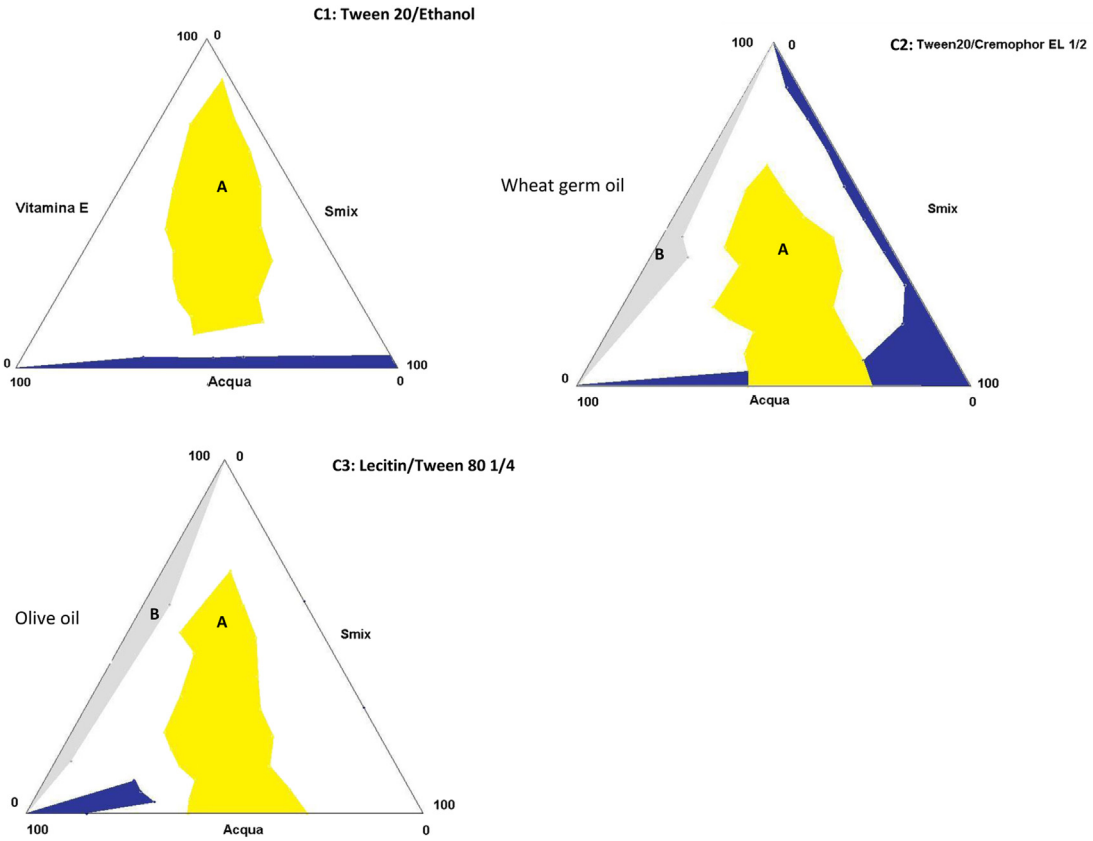

Fig. 2. Pseudo-ternary phase diagrams composed of various oils and surfactants/cosurfactants. The black area represents microemulsion existence ranges, the A area represents the gel-like phase and the B area represents phase separation.

The microemulsions loaded with curcumin were also characterized by DLS, to highlight if the presence of this molecule in oil droplets may affect on size, polidispersity and stability. In fact, some drugs tend to localize more to the interface and this depends on both their chemical structure and the presence of surfactants/co-surfactants at the interface. This would lead to a greater solubility of the substance in the system, but at the same time, could destabilize the microemulsions. The localization of the compound at the interface may partially move the surfactants/cosurfactants from the interface making the system less stable, thereby increasing the size of the dispersed phase.

From these analyses we can see that after the addition of curcumin, the droplet size of microemulsions and their homogeneity remain unchanged. Thus, the encapsulation of curcumin in microemulsions no affects these systems and the microemulsions resulted stable, with low tendency to aggregate. The dimensional data obtained so far were compared with those obtained by TEM analysis. TEM results confirmed the presence of droplets with size less 30 nm (Fig. 3). Microemulsion C2 showed sphere-shaped and uniform globules, with mean diameter of 18–30 nm, uniformly dispersed, which did not tend to form aggregates. Also in the case of the microemulsion C1, no aggregate were evidenced and the globules showed size less than 30 nm (25–30 nm).

TEM analysis of C3 microemulsion showed the presence of small spherical drops of oil with a mean diameter of about 20 nm, coherent with nanometric dimensions obtained by DLS.

3.4. Solubility of curcumin into microemulsions

The amount of drug solubilized in the selected microemulsions was reported in Table 5. The solubility of curcumin was improved considerably by all formulations. Among the three microemulsions, C1 showed the highest solubilization capacity (14.57 mg/ml). Also C3 microemulsion increased the solubility of drug to 5.15 mg/ml. The effect on solubility also affected all other curcuminoids (demethoxycurcumin and bisdemethoxycurcumin) probably due to the different co-surfactants used in the formulations.

In the case of C1 formulation, this is probably due to the presence in the system of ethanol. In fact, the penetration of alcohol in the interfacial film determined a greater fluidity of the interface thus allowing long hydrophobic chains of the surfactant to move freely. Consequently, the penetration of oils between lipophilic structures was facilitated and thus increased the solubility of curcumin. In addition, ethanol acts as a co-solvent. Finally, from the studies of phase solubility, it was observed the maximum solubility of curcumin in tocopherol acetate oil phase and Tween 20 as surfactant, respectively. These two components showed a synergic effect when they are present in the same formulation.

Curcumin solubility in microemulsion C3 was slightly higher than ones in the every single constituent of microemulsion: it is increased approximately 5-folds compared to that one in olive oil, about 7 times in comparison with lecithin and about 1.5 times compared to Tween 80 (Table 2). This is probably due to the ability of curcumin of solubilizing at the interface.

The solubility of curcumin in the C2 microemulsion was the lowest, and this despite the fact that this substance showed a rather good solubility both in Tween 20 and in Cremophor EL (see phase solubility). Nevertheless, the solubility was greatly increased compared to that one in water.

The encapsulation efficiency results indicated that the encapsulated concentration increased in a dose-dependent manner respect to the amount of added curcumin. The encapsulation efficiency was exceeded 90% for C1 microemulsion, corresponding to a total amount of encapsulated curcuminoids of 10.45 mg/ml.

Even in the case of C2 the encapsulation efficiency was high around 68%. The maximum loading capacity was obtained at the

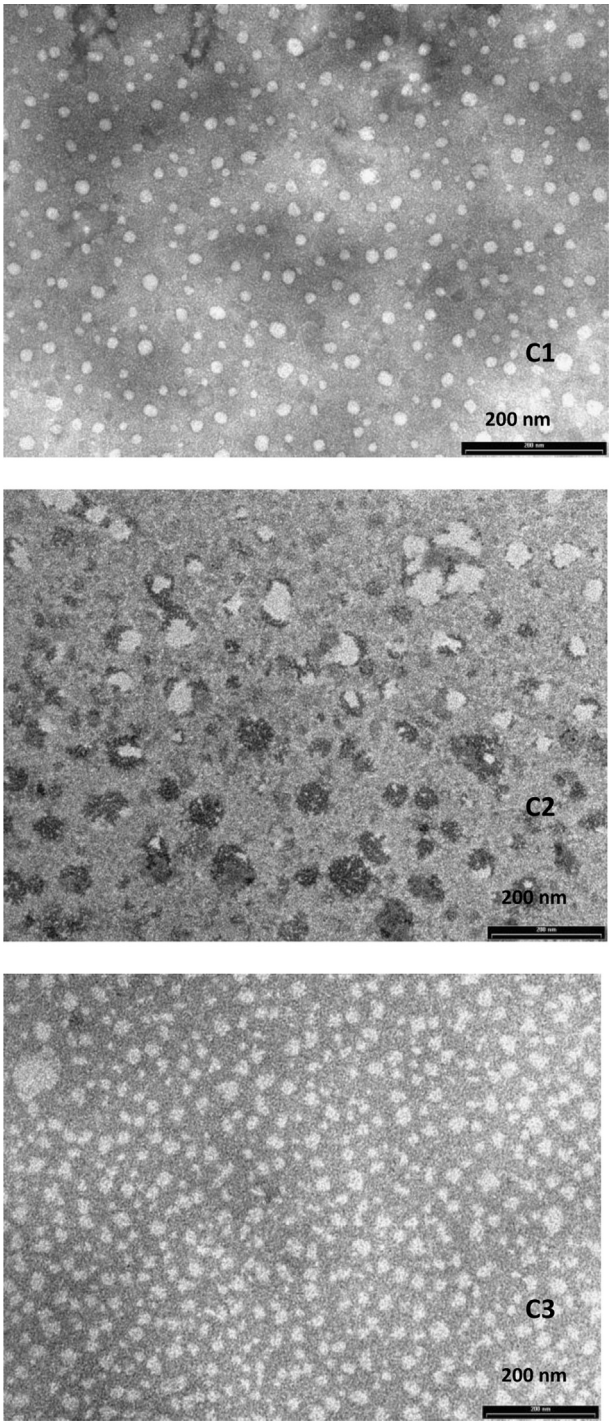

Fig. 3. Morphology of curcumin microemulsions, observed using a transmission electron microscope.

Table 4
Physical characterization of empty or curcumin loaded microemulsions. Data were shown as mean ($n = 3$) ± S.D.; Pd: polydispersity.

Sample	Size (nm)	Pd	Sample	Size (nm)	Pd
C1	9.73 ± 0.08	0.26 ± 0.01	C1 + curcumin	10.07 ± 0.45	0.18 ± 0.03
C2	14.73 ± 0.26	0.26 ± 0.007	C2 + curcumin	13.11 ± 0.91	0.15 ± 0.07
C3	5.26 ± 0.04	0.25 ± 0.03	C3 + curcumin	5.33 ± 0.15	0.12 ± 0.05

Table 5
Solubility of curcumin and curcuminoids into microemulsions. (Mean ± S.D., $n = 3$).

Sample	Curcumin (mg/ml)	Curcuminoids (mg/ml)
C1	14.57 ± 0.992	21.80 ± 2.627
C2	2.93 ± 0.098	4.43 ± 0.336
C3	5.15 ± 0.028	9.52 ± 0.084
Water	$1.003 \times 10^{-3} \pm 3.84 \times 10^{-4}$	N.D.

lower amount of encapsulated curcuminoids (3.84 mg/ml). Compared to the other two formulations, the encapsulation efficiency of C3 formulation is the lowest and reaches the value of 56.87% corresponding to 4.93 mg/ml of curcuminoids.

3.5. Stability studies

Based on visual identification, the three microemulsions loaded or not with curcumin remained transparent for 2 months without the occurrence of phase separation at 4 °C.

Furthermore, microemulsions possessed an ability to be diluted with aqueous solution without destroying their structure for 24 h. Thus, it is important to determine the solubilization capacity, because microemulsions will be diluted by water in the gastrointestinal tract upon oral administration, with possible drug precipitation.

To determine the stability during time, microemulsions were stored away from light and at 4 °C. A withdrawal was made at regular intervals (every 5–7 days) within 60 days in order to analyze the chemical stability by HPLC-DAD analysis and to measure the size and Pd of the dispersed phase by DLS technique. Furthermore, for each timepoint, macroscopic observations were performed to check the transparency of the sample and the occurrence of possible coalescence, creaming and separation phenomena.

The C1 microemulsion appears to be stable for 60 days. The samples analyzed showed a good physical and chemical stabilities: they remained transparent during the whole period of the test and the amount of loaded curcumin (14.90–14.41 mg/ml), droplet size (9.44–9.74 nm) and the Pd (0.16–0.19) were unaffected during two months (Fig. 4).

The C2 microemulsion tended to form flocs in the surface, but the preparation backs to transparent after agitation. The amount of curcumin remained unchanged during stability test as confirmed by HPLC-DAD analysis (2.48–2.22 mg/ml). The size of droplets was around 14 nm, and the Pd around 0.10 (Fig. 4). Therefore, it is possible to conclude that this microemulsion meet long-term physical stability criteria when kept at a temperature of 4 °C and protected from light.

C3 preparation became cloudy already after 10 days, but it return a homogeneous formulation after stirring. After two weeks, the formulation remained opalescent and not became again transparent. Also we evidenced the formation of mold and, in some samples, of gelatinous bodies probably due to molecular rearrangement of the lecithin at low temperatures.

On the contrary, the amount of curcumin contained in microemulsion (5.35–4.84 mg/ml), size of oily droplets and polydispersity were constant (Fig. 4). In conclusion, this microemulsion was not considered stable as evidenced by macroscopic observations.

Furthermore, in an effort to mimic physiological dilution process after oral administration, the microemulsion C1, used *in vitro* test,

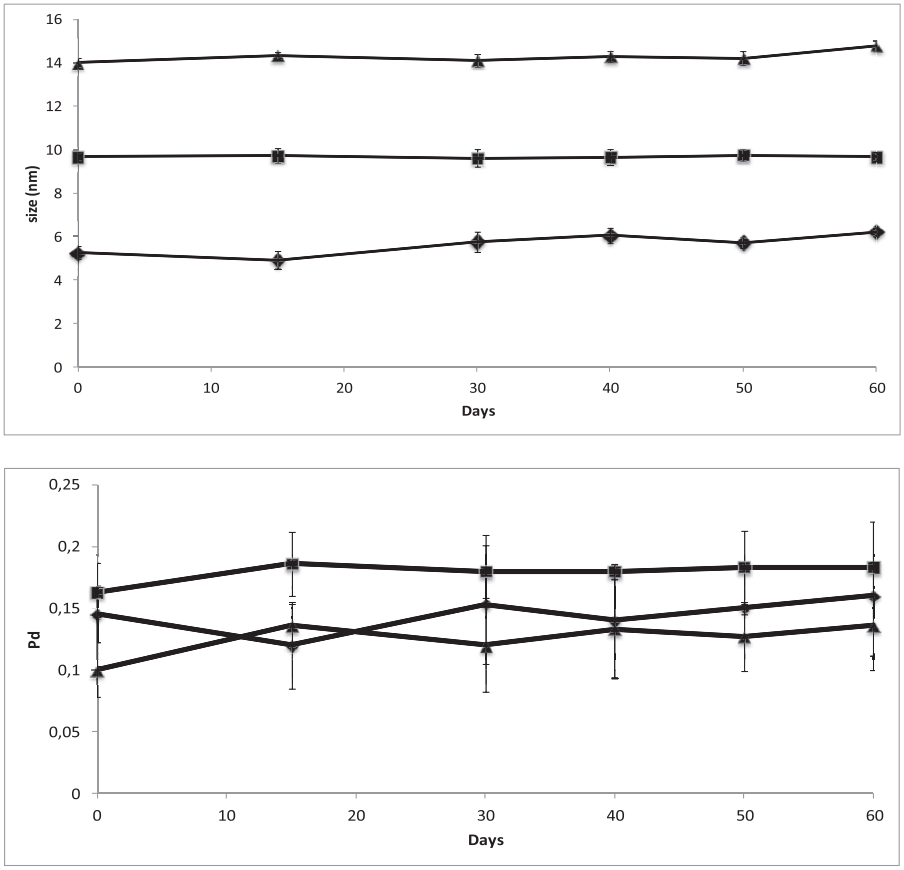

Fig. 4. Physical stability of microemulsions C1 (-■-), C2 (-▲-), C3 (-◆-): variation of size and polydispersity (Pd), after storing at 4 °C for 2 months. Data were shown as mean diameter ± S.D. ($n = 3$).

Table 6
Physicochemical properties of microemulsion C1 at different pH. (Mean ± S.D., $n = 3$; Pd: polydispersity).

pH	Size (nm)	Pd
5.5	9.67 ± 0.46	0.24 ± 0.01
1.2	9.60 ± 0.91	0.23 ± 0.01
6.8	8.98 ± 0.35	0.15 ± 0.04

were diluted up to 20-folds in diverse environments met after oral administration: simulated intestinal fluid (pH = 6.8) and simulated gastric fluid (pH = 1.2), both without the presence of enzymes. DLS analyses confirm the physical stability of microemulsion C1. In particular, mean diameters and polydispersity values were unmodified at tested pHs in comparison with empty formulation (not diluted or diluted up to 20-folds in water, pH 5.5) as reported in Table 6. Physicochemical properties of microemulsion will be maintained constant when they will pass through the whole gastrointestinal tract.

3.6. In vitro permeation study

The parallel artificial membrane permeability assay (PAMPA) represents a potential approach for rapid assessment of absorption (Kansy, Senner, & Gubernator, 1998). PAMPA is based on a 96-well microplate technology and allows reasonable throughput. It enables fast determination of the trends in the ability of the compounds to permeate membrane by passive diffusion and is thus suited for screening of large libraries. The experiment was carried out measuring the ability of curcumin to diffuse from C1 formulation to a donor compartment through a PVDF membrane. This microemulsion was selected because it appeared the best in terms of encapsulation efficiency and stability. Results (Table 7) showed a strong increasing of the amount of curcumin permeated in comparison with a saturated aqueous solution of curcumin used as control.

It was possible to measure a quantity of curcuminoids in the acceptor compartment already after 6 h: the value (17.44 μg) is about 10% of the curcuminoids present in the donor compartment (176.10 μg) and the percentage of permeation after 24 h was approximately of 70% (corresponding to 120.12 μg).

Conversely, free curcuminoids in PBS showed a permeation trend more linear with a progressive increase in their amount. Initially, the amount of permeated curcuminoids from the microemulsion or from PBS buffer solution was similar. However, there is a remarkable difference after 6 h, in fact the amount of curcuminoids permeated from microemulsion is 10.000 times higher than that from buffer solution (17.44 μg vs. 0.17×10^{-3} μg), proving a high solubilizing capacity of microemulsion. In the case of buffer solution the total permeated curcuminoids after 24 h resulted 1.66 μg.

Moreover, the presence of surfactants at the interface could facilitate the absorption through the phospholipid layer, the curcumin contained in C1 formulation has a capacity of greater permeation compared to curcumin in aqueous solution.

Table 7
Quantity of curcuminoids permeated from the microemulsion C1 and from buffer (5 ml of DMSO in 100 ml of PBS, 7.4). (Mean ± S.D., $n = 3$).

Incubation time (h)	C1 (μg)	Buffer (μg)
1	0	$0.08 \pm 6 \times 10^{-4}$
2	$0.07 \pm 2.1 \times 10^{-3}$	$0.09 \pm 5 \times 10^{-4}$
4	$0.08 \pm 1.8 \times 10^{-3}$	$0.11 \pm 4.6 \times 10^{-3}$
6	17.44 ± 10.73	$0.17 \pm 6 \times 10^{-3}$
24	120.12 ± 4.51	$0.47 \pm 1.7 \times 10^{-3}$

4. Conclusions

Curcumin was formulated into microemulsions to improve its oral bioavailability. The physical nature of these systems, mechanism of drug entrapment, as well as the physico–chemical interactions of constituents determined drug solubilization capacity and physical stability during storage and upon dilution.

The optimized formulation was obtained with vitamin E (3.3 g/100 g), Tween 20 (53.8 g/100 g), ethanol (6.6 g/100 g) and water (36.3 g/100 g). It resulted the best in terms of size, polidispersity, encapsulation efficiency and positive influence on the solubility of curcumin, that was increased approximately 10,000 fold compared to its water solution. The microemulsion can be diluted with aqueous buffer and it was stable for at least two months. The optimized microemulsion showed a maximum solubility of curcumin of 14.57 mg/ml and a percentage of permeation through the artificial membrane of about 10% of curcumin after 6 h and about 70% after 24 h.

The results have significant and potential relevance for future pharmacological applications, in particular for the preparation of a possible dietary supplement for pediatric administration. In fact, the developed formulation allows to obtain a very high solubility of curcumin, able to meet the ADI of this molecule fixed by EFSA in 0–3 mg/kg by/day.

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