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# Development of topical nanoemulsions containing quercetin and 3-*O*-methylquercetin

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This study describes the physico-chemical properties and the skin permeation profile of quercetin (Q) and 3-*O*-methylquercetin (MQ) from lipid nanoemulsions. Formulations composed of octyldodecanol, egg lecithin, water (NE) and cetyl trimethyl ammonium bromide (CNE) were obtained by spontaneous emulsification. This procedure yielded monodisperse nanoemulsions exhibiting a mean droplet size of approximately 200–300 nm. Nanoemulsions were further characterized in terms of ζ-potential, surface tension, and morphology by transmission electron microscopy. The amount of flavonoids incorporated into nanoemulsions reached nearly 100% (at 1 mg/mL). The permeation studies were carried out using ear pig skin mounted in Franz diffusion cells. The overall results have shown a slow permeation profile of both Q and MQ from nanoemulsions. However, a higher permeation flux rate of flavonoids from CNE (~0.2 μg/cm$^2$/h) as compared to NE (~0.08 μg/cm$^2$/h) was observed, showing the effect of the positively charged surface of CNE on this parameter. Such results open interesting perspectives for the topical administration of the flavonoids Q and MQ.

## 1. Introduction

The antiviral activity of polyphenol compounds isolated from medicinal plants has been investigated at length. Several studies have especially addressed the effect of flavonoids against enveloped viruses, such as the herpes simplex virus, since they are important human pathogens (Khan et al. 2005). For instance, it was shown that natural occurring quercetin causes a concentration-dependent reduction in the infectivity of herpes simplex virus type-1 and reduces the intracellular virus replication (Kaul et al. 1985). The main mechanism of antiviral activity of quercetin seems to be related to its ability to bind to viral protein and to interfere with viral nucleic acid synthesis (Middleton et al. 2000).

A key consideration for an effective topical therapy of herpes simplex viruses is the drug permeation profile. Drug must reach in fact the basal epidermis which is considered as the main site of the infection (Parry et al. 1992). However, only few reports have described the permeation profile of flavonoids such as quercetin throughout the skin. To date, Saija et al. (1998) have reported the low skin permeation profile of quercetin from a volatile reservoir even after a previous treatment of excised human skin with penetration enhancers such as lecithin and limonene. More recently, the inability of quercetin to permeate well through pig ear skin was shown from topical non-ionic- and anionic emulsions since it could not be detected in the receptor compartment of Franz diffusion cells (Casagrande et al. 2007).

In spite of the potential interest in the use of flavonoids in the treatment of topical virus infections, the very poor water solubility of some nonglycosidic flavonoids could represent a major drawback. In this context, the design of nanoemulsions intended for the topical administration of flavonoids has recently been described (Silva et al. 2007, 2009; Fasolo et al. 2007). In fact, such systems offer an interesting alternative to the administration of lipophilic molecules due to their effectiveness in drug solubilization (Friedman et al. 1995; Piemi et al. 1999; Fernandez et al. 2000; Fang et al. 2004; Alves et al. 2005; Yilmaz et al. 2006; Hoeller et al. 2009), representing a potential for improving the efficacy and even the anticipated positive acceptance by patients due to its hydrophilic character. Nanoemulsions are fine dispersions of well-tolerated lipids in water where the poorly-soluble drugs can be dissolved in the oil core and/or adsorbed on the oil-water interface of nanoemulsions. The incorporation of drugs in such systems can increase the skin permeation rate and enhance the topical effect caused by prolonged residence time in the uppermost skin layers due to both the large surface area and the low surface tension of the nanodroplets (Klang et al. 1998; Bouchemal et al. 2004).

The development of topical nanoemulsions containing quercetin (Q) and 3-*O*-methylquercetin (MQ) is currently under study by our research group. In a recent article, we reported a liquid chromatography method for the determination of both flavonoids incorporated in topical nanoemulsions (Fasolo et al. 2007). In this study, the purpose was to investigate the main physico-chemical properties and the permeation profile of Q and MQ from these nanoemulsions.

## 2. Investigations, results and discussion

Table 1 shows the physico-chemical properties of nanoemulsions. As can be seen, the droplet size of blank NE was

**Table 1: Physico-chemical properties of nanoemulsions**

| | Droplet size (nm) | ζ-potential (mV) | Surface tension (mN/m) | Flavonoid content (mg/ml)[b] |
|---|---|---|---|---|
| NE | 253 ± 10 | −37.5 ± 1.4 | 28.87 ± 0.12 | - |
| $NE_Q$ | 307 ± 19 | −27.4 ± 6.0 | 28.93 ± 0.12 | 0.995 ± 0.003 |
| $NE_{MQ}$ | 305 ± 14 | −29.0 ± 4.8 | 28.17 ± 0.06 | 0.993 ± 0.008 |
| CNE | 177 ± 10 | 75.7 ± 1.2 | 26.50 ± 0.1 | - |
| $CNE_Q$ | 188 ± 2 | 76.3 ± 2.1 | 26.53 ± 0.06 | 0.991 ± 0.006 |
| $CNE_{MQ}$ | 263 ± 2 | 79.2 ± 3.9 | 26.27 ± 0.06 | 0.990 ± 0.005 |

[a] Results are the mean ± standard deviation of three different batches
[b] Flavonoid content for an initial amount of 1 mg/ml

approximately 250 nm, which is in accordance with results previously reported for nanoemulsions obtained by a spontaneous emulsification procedure (Fasolo et al. 2007; Silva et al. 2007, 2008). Transmission electron microscopy investigations (Fig. 1) of the oil droplets showed the typical appearance of an o/w emulsion with droplets displaying a size of nearly 200–300 nm, according to the PCS experiments (Teixeira et al. 2000, 2001; Yilmaz et al. 2005). The addition of CTAB significantly reduces ($p < 0.05$) the droplet size of formulations up to approximately 180 nm. Regardless of the formulation, the addition of either Q or MQ seems to increase the droplet size of nanoemulsions, whereas surface tension remained unchanged (26–28 mN/m). Concerning ζ-potential measurements, NE exhibits a negative value at a pH of approximately 6, which could be attributed to the presence of negatively-charged lipids in egg-lecithin (Yang and Benita 2000; Li and Tian 2002). However, nanoemulsions containing CTAB (CNE) displayed positive values of higher than +70 mV, indicating the location of this cationic surfactant at the o/w interface of nanoemulsions.

The incorporation of either Q or MQ in nanoemulsions was close to 100% (at 1 mg/mL) since no free flavonoid was detected in the water phase. This result was as expected due to the low water solubility of aglycones, which have in turn favored both flavonoids association into the oil phase of nanoemulsions. This is consistent with partition coefficient of Q and MQ (log P-value of ∼2–3) reported in previous literature (Rothwell et al. 2005; Montenegro et al. 2007; Schwingel et al. 2008). However, even if the partition coefficient could be considered favorable to flavonoid incorporation into nanoemulsions, our results clearly show the low solubility of both Q and MQ into the oil core composed by ODD. The solubility of Q and MQ was 56.01 ± 0.32 μg/ml and 37.76 ± 0.77 μg/ml, respectively. This finding suggests a possible role of egg-lecithin on the incorporation of the flavonoids in the nanoemulsions. Previous literature has in fact shown some

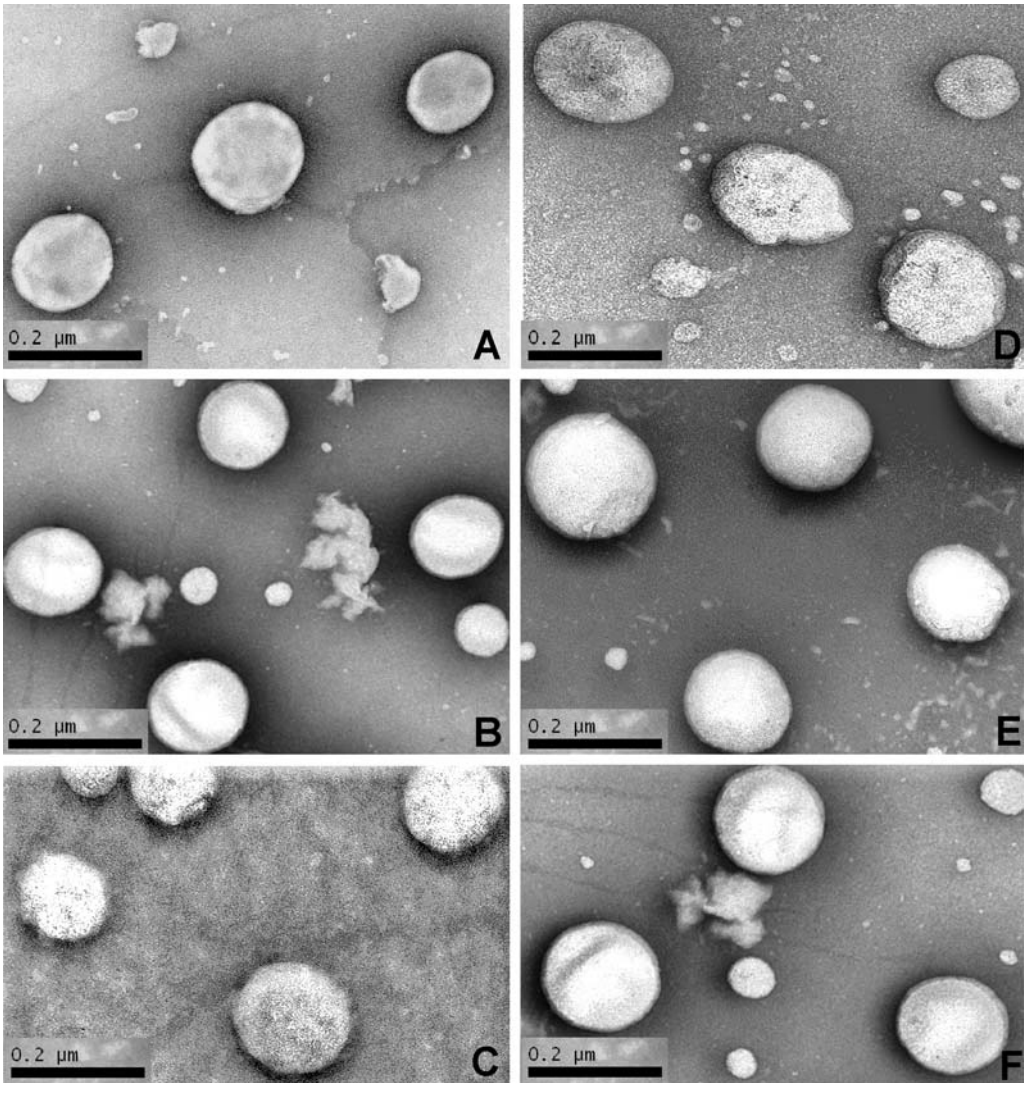


Fig. 1: TEM micrographs of nanoemulsions after negative staining with uranyl acetate. Key: Left column: blank NE (A), NEQ (B) and NEMQ (C); Right column: blank CNE (D), NEQ (E) and NEMQ (F)

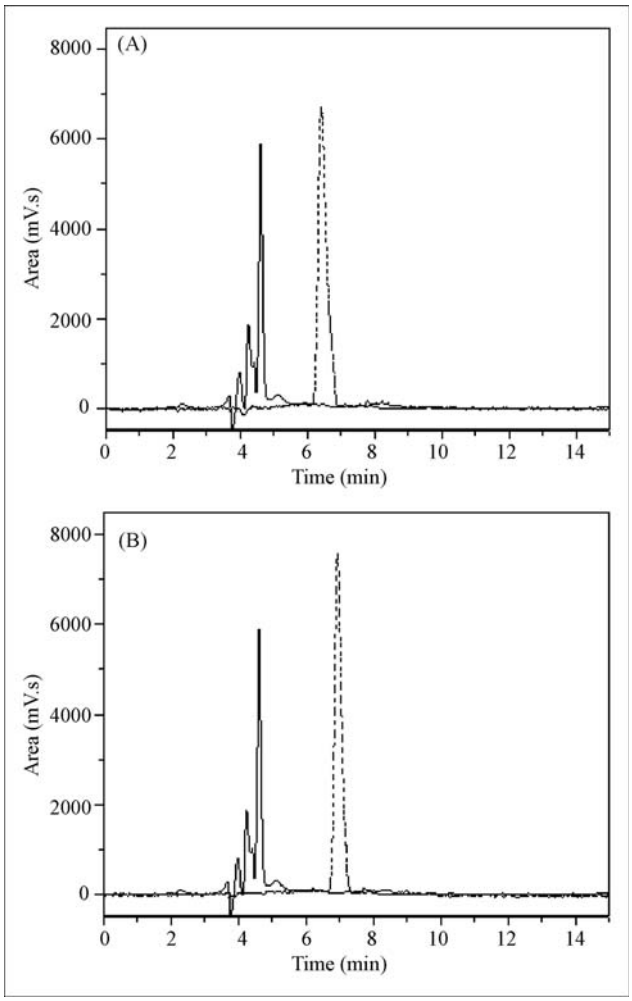


Fig. 2: Specificity study. Typical chromatograms of skin extract (solid lines) and flavonoids (dashed lines) for Q (A) and MQ (B). Detection of 368 for Q and 354 nm for MQ

evidence of interactions between flavonoids, such as Q and phospholipids through DSC experiments (Saija et al. 1995). Such interactions most likely lead to a homogeneous dispersion of flavonoids into nanodroplets as we did not detect any crystals on the nanoemulsions by microscopy (Fig. 1). Taking such considerations into account, it was no surprise to observe an increase in ζ-potential in NE nanoemulsions, suggesting the presence of Q and MQ at the interface of nanoemulsions. It can, therefore, be reasonably assumed that Q and MQ might be distributed in both the oil core and o/w interface of nanoemulsions, thus interacting with phospholipid molecules of egg-lecithin. The revalidation of a previously reported LC method (Fasolo et al. 2007) allows us to evaluate the Q and MQ permeation profile by monitoring their concentrations in the receptor compartment of Franz diffusion cells. First, the specificity of the method was carried out through the comparison of the peak retention time of either Q or MQ and a pig ear skin extract. No interference of the skin components was noticed since no overlaps of peaks were detected after the injection of either Q or MQ at the set wavelengths (Fig. 2). Q and MQ presented retention times of approximately 6.4 and 7 min, respectively. The accuracy of the method was determined by recovery test. As can be seen in Table 2, whatever the amount of flavonoid added to the skin extract was, the recoveries ranged from 97.46% to 99.24% for Q and from 96.01% to 99.77% for MQ, indicating a satisfactory agreement between amounts added and found.

Fig. 3 shows the total amount of permeated Q and MQ per unit surface area (μg/cm$^2$) *versus* time (h). Whatever the formulation, flavonoids can reach the receptor compartment through both NE and CNE formulations. These results are in agreement with favorable physico-chemical properties of nanoemulsions which present a low viscosity, a low interfacial tension, and a high surface area from the reduced droplet size (Table 1). Such properties may play an important role in the increase in oil droplets binding to epithelial cells, thus favoring flavonoid permeation.

However, after 8 h, the total amount of flavonoids permeated from NE was approximately 0.6 μg/cm$^2$, while from CNE it was

**Table 2: Accuracy of LC assay for Q and MQ**

| | Q | | MQ | |
|---|---|---|---|---|
| Added (μg/mL) | Peak area (mV.s) | Recovery (%) | Peak area (mV.s) | Recovery (%) |
| 0.50 | 73017 ± 361 | 98.81 | 40516 ± 273 | 97.54 |
| 0.90 | 129636 ± 759 | 97.46 | 71786 ± 98 | 96.01 |
| 1.125 | 165004 ± 1327 | 99.24 | 93243 ± 311 | 99.77 |

Results are the mean ± standard deviation of three experiments

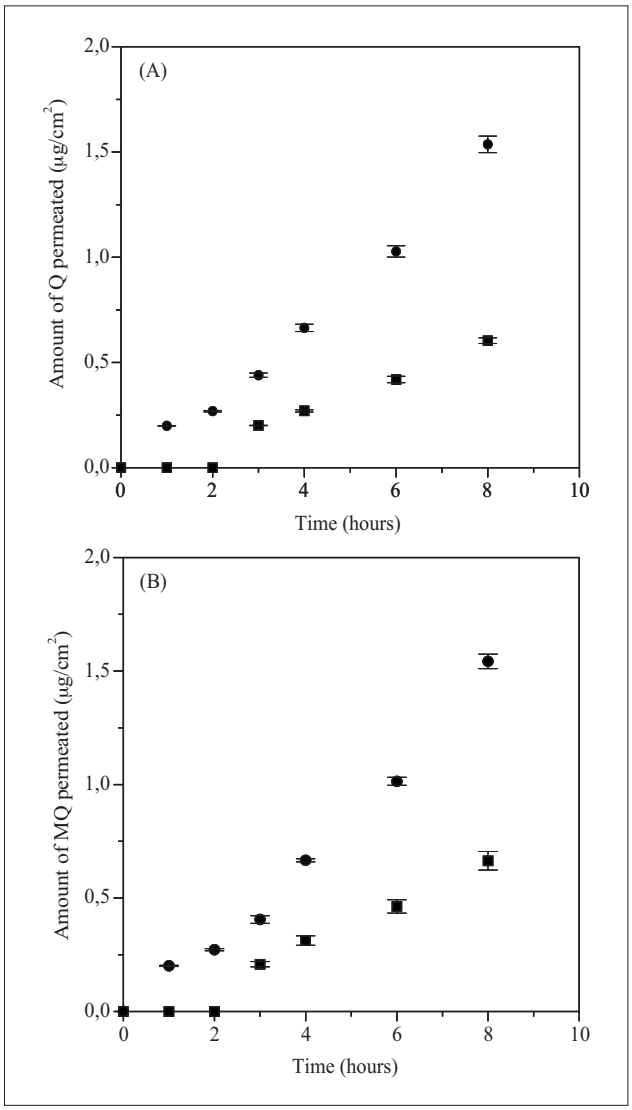


Fig. 3: Amount of flavonoids permeated from nanoemulsions through excised porcine skin. (A): Q permeation from NE (■) or CNE (●). (B): MQ permeation from NE (■) or CNE (●). The values were the mean ± S.D. of three individual experiments

**Table 3: Permeation parameters in percutaneous permeation of Q or MQ from nanoemulsions**

| | Flux ($\mu g/cm^2/h$) | Lag time (h) |
|---|---|---|
| $NE_Q$ | $0.08 \pm 0.003$ | $0.60 \pm 0.077$ |
| $CNE_Q$ | $0.20 \pm 0.010$ | $0.45 \pm 0.038$ |
| $NE_{MQ}$ | $0.09 \pm 0.006$ | $0.62 \pm 0.097$ |
| $CNE_{MQ}$ | $0.20 \pm 0.003$ | $0.50 \pm 0.040$ |

Results are the mean ± standard deviation of three experiments

**Table 4: Final composition of nanoemulsion (%,w/w)**

| | $NE_Q$ | $NE_{MQ}$ | $CNE_Q$ | $CNE_{MQ}$ |
|---|---|---|---|---|
| Q | 0.1 | - | 0.1 | - |
| MQ | - | 0.1 | - | 0.1 |
| ODD | 8.0 | 8.0 | 8.0 | 8.0 |
| Egg-lecithin | 2.0 | 2.0 | 2.0 | 2.0 |
| CTAB | - | - | 0.5 | 0.5 |
| Purified water to | 100.00 | 100.00 | 100.00 | 100.00 |

ODD: octyldodecanol, CTAB: cetyl trimethylammonium bromide

approximately 1.6 mg/cm$^2$, indicating that a significant difference in permeation profiles exists. In fact, the positive charge of CNE formulations could increase the interaction with epithelial cells which carry a negative charge upon their surface due to the presence of negatively charged residues in the outer cell membranes. Flux values were in fact up to 3.5-fold higher for CNE as compared to NE (Table 3), indicating a positive charge effect on the permeation of flavonoids. These results are in agreement with data published by Piemi et al. (1999) for nanoemulsions containing the antifungical drugs econazol and miconazol nitrate.
Finally, the results of this study show that the composition of nanoemulsions represents the main parameter which influences the flavonoid permeation through excised porcine skin. For a given formulation, no significant differences were detected neither in the total amount of Q and MQ permeated through the skin (Fig. 3) nor in the flux and lag-time values (Table 3), indicating no effect of the 3' substitution (OH or $OCH_3$). However, the overall results of this study showed a slow permeation profile for both aglycones, which are in agreement with previous literature (Saija et al. 1998; Casagrande et al. 2007). It must be mentioned that the permeation profile of both flavonoids contradicted their octanol−water partition coefficient, which could be considered as favorable to skin permeation. A higher permeation profile for both flavonoids would have been more commonly expected result. Saija et al. (1998) have attributed the inability of quercetin to permeate well through the skin to its very low water solubility, which may have a negative effect on the skin permeation process.
In conclusion, this study demonstrates the potential of nanoemulsions as a topical delivery system for both Q and MQ. The physico-chemical studies suggest the role of lecithin on their incorporation into nanoemulsions. Considering the slow permeation flux of both flavonoids from nanoemulsions, such a system could be useful for local skin administration.

## 3. Experimental

### 3.1. Chemicals and reagents

Egg-lecithin (Lipoid E-80®) was kindly donated by Lipoid GmbH (Ludwigshafen, Germany). Octyldodecanol was obtained from Delaware (Porto Alegre, Brazil). Cetyl trimethylammonium bromide (CTAB) and the Q reference standard (>98%) were purchased from Sigma-Aldrich (São Paulo, Brazil). Q raw material was purchased from Galena (Campinas, Brazil). MQ (>99%) was isolated from *Achyrocline satureioides* Lam D.C. - Asteraceae (Fasolo et al. 2007). Ultrapure water was obtained from a Milli-Q apparatus (Millipore, Billerica, USA). Methanol LC grade was obtained from Merck (Darmstadt, Germany). Trifluoracetic acid was obtained from Nuclear (Diadema, Brazil).

### 3.2. Preparation of nanoemulsions

Nanoemulsions containing both flavonoids (Q or MQ) were prepared using a spontaneous emulsification procedure. Briefly, the method consists of injecting an organic phase containing components of the oil core into the water phase under magnetic stirring. Subsequently, the organic solvent was removed by evaporation under reduced pressure at 40–45 °C. The final composition (%, w/w) of nanoemulsions was presented in Table 4. Typically, two kinds of formulations containing either Q or MQ were prepared in the absence (NE) or in the presence of the cationic surfactant CTAB (CNE). Either Q or MQ was added to the ethanol phase to produce a final concentration in nanoemulsions of 1 mg/mL. Blank nanoemulsions were prepared under the same conditions, but in the absence of flavonoids as control formulations.

### 3.3. Characterization of nanoemulsions

The mean droplet size and ζ-potential of the nanoemulsions were determined at 25 °C by photon correlation spectroscopy (PCS) and electrophoretic mobility, respectively (3000HS Zetasizer, Malvern Instruments, England). The samples were adequately diluted in water for size determinations or in 1 mM NaCl solution for ζ-potential measurements. The bulk pH of the nanoemulsions was directly recorded using a pH-meter B474 (Micronal, Brazil) in recently prepared emulsions. The surface tension was directly measured in nanoemulsions using a *Lecompte du Nouy* ring by means of a tensiometer Krüss K8600 (Krüss GmbH, Germany). Morphological examination of nanoemulsions was performed by means of transmission electron microscopy (TEM). The nanoemulsions were diluted at a 1:10 ratio, obtaining an oil phase concentration equal to 1%. Specimens for TEM visualization were prepared by mixing samples with one droplet of 2% (w/v) uranyl acetate solution. The samples were then adsorbed to the 200 mesh formvar-coated copper grids, left to dry, and examined by TEM (JEM-1200 ExII, Jeol, Japan).

### 3.4. Liquid chromatography assay of Q and MQ

Q and MQ assay was performed by liquid chromatography (LC) as recently reported (Fasolo et al. 2007). The apparatus consisted of a Shimadzu LC-10A system (Kyoto, Japan) equipped with a model LC-10AT pump, an SPD-10AV UV-VIS variable-wavelength detector (set at 354 nm for MQ and 368 nm for Q), a SCL-10Avp system controller, and a Rheodyne 7725 injection valve with a 50 μL loop. Flavonoids were analyzed using a Shimpack column CLC-ODS (M) RP-18, 5 μm, 250 mm × 4 mm i.d., connected with a precolumn Waters RP-18, 10 μm. The mobile phase consisted of a methanol-water (70:30 v/v) mixture acidified with 0.1% of trifluoracetic acid (TFA), which was filtered and degassed by suction-filtration through a nylon membrane, in isocratic flow. The liquid chromatography system was operated at a flow-rate of 0.8 mL·min$^{-1}$ and at a sensitivity of 0.5 AUFS, at room temperature.
A linear response was obtained in the evaluated concentration range (0.05–1.5 μg/mL), with a determination coefficient of $r^2 = 0.9976$ and $r^2 = 0.9991$, for Q and MQ, respectively. The method proved to have a satisfactory precision for both intra-day (RSD <1.83% for Q and <1.88 for MQ) and inter-days variation (RSD< 1.72% for Q and < 1.73% for MQ).

### 3.5. Determination of Q or MQ incorporated into nanoemulsions

Nanoemulsion aliquots of 0.5 mL, containing Q or MQ, were appropriately diluted in methanol (0.5 μg/mL), filtered, and analyzed by LC. Free Q or MQ was determined in an ultrafiltrate obtained through separation of the water phase using an ultrafiltration/ centrifugation procedure (Ultrafree-MC 10,000 MW, Millipore). Samples were added to ultrafiltration membranes and centrifuged at 5.000 rpm. The concentration of Q or MQ was determined in the ultrafiltrates. The association efficiency (%) was estimated by the difference between the total and free drug concentrations.

### 3.6. Determination of Q and MQ solubility

For the determination of the solubility of either Q or MQ in ODD, an excess of each flavonoid was added to the oil. The mixtures were shaken for 24 h and centrifuged. The supernatants were then diluted in methanol for further analysis of soluble flavonoids by LC.

### 3.7. *In vitro percutaneous permeation study*

Prior to the evaluation of skin permeation, the analytical method (Fasolo et al. 2007) was revalidated according to ICH (ICH 2005). The specificity of the method was evaluated by analyzing solutions of the pig ear skin extracts. The system response was examined through the presence of interference or overlaps with the Q or MQ responses. The accuracy was determined applying the method to quantify Q or MQ in the presence of pig ear skin extracts and expressed as recovery. Next, the extract was spiked with known amounts of Q or MQ at different levels: low, medium, and high, corresponding, respectively, to 0.5, 0.9, and 1.125 μg/mL. Samples were appropriately diluted and analyzed by LC using the conditions described above.

Percutaneous permeability of either Q or MQ was then assessed using Franz type diffusion cells which presented a surface area for diffusion of 2.54 $cm^2$ and a receptor volume of 9.0 ml. Excised circular pig ear skins were mounted between donor and receptor compartments, with the inner portion facing the inside part of the cell. The skin was hydrated with a phosphate buffer (pH 7.4) for 12 h at 37 °C. After the phosphate buffer in the receptor chamber was replaced by a hydroethanol solution (50%, v/v) so as to ensure sink conditions. The bathing solution was kept under a controlled temperature (37 ± 1.0 °C) and stirred throughout the entire time of the experiment (8 h). Nanoemulsions were placed in the donor compartment in a theoretical concentration of both Q and MQ of 1.0 mg. Samples of 2.0 ml were withdrawn at hourly intervals, and the same volume of fresh receptor fluid was added in order to keep a constant volume. The aliquots were diluted in methanol and analyzed by LC. The results were expressed as mean ± standard deviation of permeated Q or MQ per unit surface area (μg/$cm^2$) as the time function (h), and a graph was plotted. The steady-state flux was calculated from the slope of the resulting linear profile. The lag-time of diffusion was determined by the intercept of the linear profile on the x-axis.

### 3.8. *Statistical analysis*

Results are expressed as mean ± standard deviation of three independent experiments and were analyzed by the Student *t*-test with $p < 0.05$ significance.

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