



# UNIVERSITÀ DI PARMA

## ARCHIVIO DELLA RICERCA

University of Parma Research Repository

Mechanisms of imiquimod skin penetration

This is the peer reviewed version of the following article:

*Original*

Mechanisms of imiquimod skin penetration / Telo', Isabella; Pescina, Silvia; Padula, Cristina; Santi, Patrizia; Nicoli, Sara. - In: INTERNATIONAL JOURNAL OF PHARMACEUTICS. - ISSN 0378-5173. - 511:1(2016), pp. 516-523. [10.1016/j.ijpharm.2016.07.043]

*Availability:*

This version is available at: 11381/2817270 since: 2021-10-18T17:04:59Z

*Publisher:*

Elsevier B.V.

*Published*

DOI:10.1016/j.ijpharm.2016.07.043

*Terms of use:*

Anyone can freely access the full text of works made available as "Open Access". Works made available

*Publisher copyright*

note finali coverpage

(Article begins on next page)

Manuscript Number: IJP-D-16-01224R1

Title: Mechanisms of imiquimod skin penetration

Article Type: Research Paper

Section/Category:

Keywords: Skin delivery,  
Imiquimod,  
Stratum Corneum,  
Solubility,  
Microemulsion,  
Solvent Uptake

Corresponding Author: Prof. sara Nicoli,

Corresponding Author's Institution: Department of Pharmacy, University of  
Parma

First Author: Isabella Telò

Order of Authors: Isabella Telò; Silvia Pescina, PhD; Cristina Padula;  
Patrizia Santi, Prof.; sara Nicoli

Abstract: Imiquimod (IMQ) is an immunostimulating drug used for the treatment of neoplastic skin diseases, such as actinic keratosis (AK) and superficial basal cell carcinoma (sBCC), and as adjuvant for vaccination. Imiquimod formulation and skin delivery is highly challenging because of its very low solubility in most pharmaceutical excipients and poor penetration properties. Objectives of the work were: 1) to evaluate IMQ solubility in different solvents and pharmaceutical excipients; 2) to evaluate IMQ skin retention after the application of simple saturated solutions; 3) to evaluate the role of stratum corneum and solvent uptake on IMQ skin retention and 4) to formulate IMQ in microemulsions - prepared using previously investigated components - and compare them with the commercial formulation. The results show that IMQ solubility is not related to the solubility parameter of the solvents considered. The highest solubility was found with oleic acid (74 mg/ml); in the case of PEGs, the solubility increased linearly with MW (PEG 200: 1.9 mg/ml; PEG 400 7.3 mg/ml, PEG 600 12.8 mg/ml). Imiquimod skin retention from saturated solutions (Tween 80, oleic acid, propylene glycol, PEG 200, PEG 400, PEG 600, Transcutol, 2-pyrrolidone, DMSO) resulted relatively similar, being 1.6 µg/cm<sup>2</sup> in case of oleic acid (solubility 74 mg/ml) and 0.18 µg/cm<sup>2</sup> in case of propylene glycol (solubility 0.60 mg/ml). Permeation experiments on stripped skin (no stratum corneum) and isolated dermis as well as uptake experiments on isolated stratum corneum sheets demonstrated that IMQ accumulation is related to skin solvent uptake. Finally, microemulsions (MEs) prepared with the above-studied components demonstrated a very good performance attributed to an increased solvent

uptake. In particular, a ME composed of 10% oleic acid, 35% Transcutol, 35% Tween 80 and 20% water is able to accumulate the same amount of drug as the commercial formulation but with far more efficiency, since its concentration was 12 times lower.



UNIVERSITA' DEGLI STUDI DI PARMA  
Dipartimento di Farmacia

---

Dear Editor,

I am writing to submit the paper entitled "Mechanisms of imiquimod skin penetration" that has been revised accordingly to the reviewer's comments. A point-by-point answer to the comments has been attached and the revisions have been marked in red in the text.

I confirm that this work is original and has not been published elsewhere nor is it currently under consideration for publication elsewhere. All authors approved the manuscript and its submission to this journal.

Looking forward to receiving the editorial decision

Sincerely,

Sara Nicoli

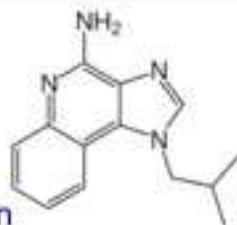
Parma 18/07/2016

Sara Nicoli PhD  
Associate Professor of Pharmaceutical Sciences  
Department of Pharmacy  
University of Parma  
Parco Area delle Scienze, 27/A  
43124 Parma, Italy  
Telefono +39 0521 905065/71  
Fax +39 0521 905006  
E-mail: [sara.nicoli@unipr.it](mailto:sara.nicoli@unipr.it)

### IMIQUIMOD

Immunostimulant drug

- Actinic keratosis
- Basal cell carcinoma
- Adjuvant for vaccination



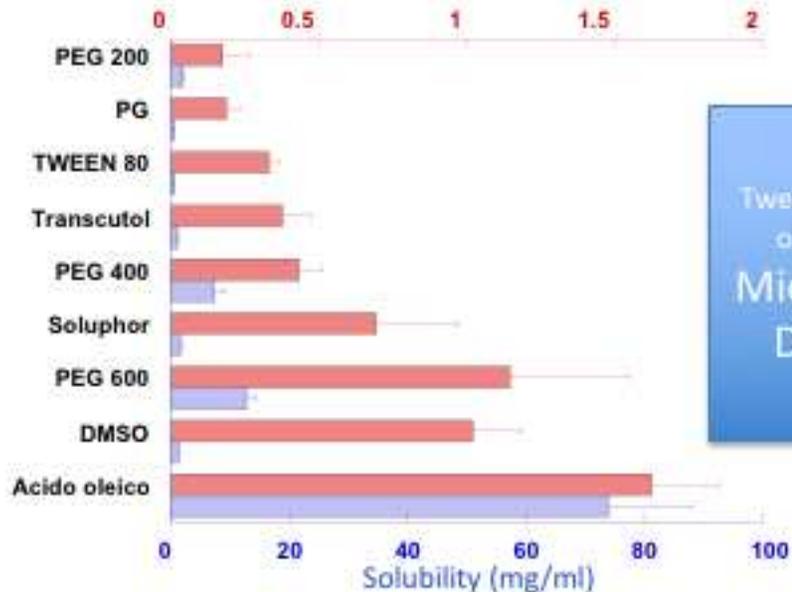
Low solubility in most solvents and excipients

Low skin permeability

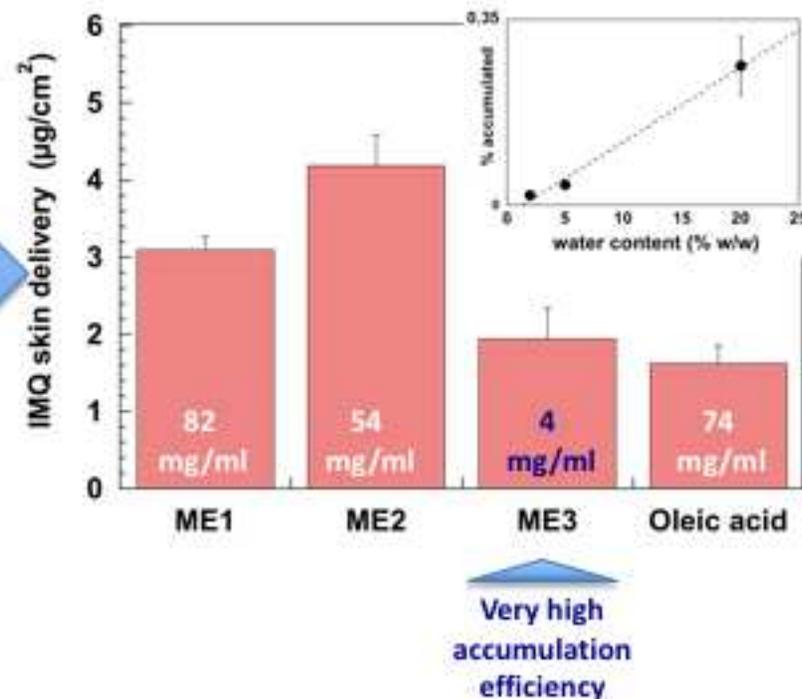


- Solubility studies
- Skin delivery from saturated solutions
- Barrier role of SC and epidermis
- Stratum corneum uptake experiments
- Microemulsion formulation and evaluation

Skin accumulation from saturated solutions ( $\mu\text{g}/\text{cm}^2$ )



Tween 80/transcutol/  
oleic acid/water  
**Microemulsion**  
Drug effect



Ms. Ref. No.: IJP-D-16-01224

Title: Mechanisms of imiquimod skin penetration and enhanced skin delivery using microemulsions

International Journal of Pharmaceutics

Reviewers' comments:

Reviewer #2: I was asked to review the article entitled Mechanisms of imiquimod skin penetration and enhanced skin delivery using microemulsions. According to the authors objectives of the work were as follows: 1) to evaluate IMQ solubility in different solvents and pharmaceutical excipients; 2) to evaluate IMQ skin retention after the application of simple saturated solutions; 3) to evaluate the role of stratum corneum and solvent uptake on IMQ skin retention and 4) to formulate IMQ in microemulsions prepared using previously investigated components - and compare them with the commercial formulation.

The addressed topic is interesting from conceptual as well as from methodological point of view. The contents is rationally presented, conclusions are sufficiently supported by presents data. Well written.

However, I have some comments:

\* Title - ME are used as delivery systems only in last part to confirm previously offered mechanism and for comparison with commercial product. The formulation is already known (Bhatia, 2013), so I think there is no need to be emphasized also in title. Focus of the article was namely to underline the role of solvent in IMQ skin penetration and reception.

We agree on the reviewer's comment. The title has been changed to "Mechanisms of imiquimod skin penetration"

\* The ME formulation are irrationally listed in table 3 as ME 1, ME 2 and ME 3. It will be easier for readers if they were list in ascending (or descending) line according to oleic acid or water content

The ME are now presented in ascending line according to water content

\* Justify the use of IMQ saturated solutions for accumulation and permeation experiments more precisely.

The justification have been added (page 12 lines 262-264)

1 **Mechanisms of imiquimod skin penetration and enhanced skin delivery**  
2 **using microemulsions**

3 **Mechanisms of imiquimod skin penetration**

4 **Isabella Telò<sup>a</sup>, Silvia Pescina<sup>a</sup>, Cristina Padula<sup>a</sup>, Patrizia Santi<sup>a</sup>, Sara Nicoli<sup>a\*</sup>**

5

6 **<sup>a</sup> Department of Pharmacy, University of Parma, Parco Area delle Scienze 27/A,**

7 **43124 Parma, Italy**

8

9

10 \*Corresponding author  
11 Sara Nicoli PhD  
12 Department of Pharmacy  
13 University of Parma  
14 Parco Area delle Scienze, 27/A  
15 43124 Parma, Italy  
16 Telefono +39 0521 905065/71  
17 Fax +39 0521 905006  
18 E-mail: sara.nicoli@unipr.it  
19

20

21

22 **Keyword**

23 Skin delivery, Imiquimod, Stratum Corneum, solubility, Microemulsion, Solvent uptake

24

25

26 **Abstract**

27 Imiquimod (IMQ) is an immunostimulating drug used for the treatment of neoplastic skin  
28 diseases, such as actinic keratosis (AK) and superficial basal cell carcinoma (sBCC), and  
29 as adjuvant for vaccination. Imiquimod formulation and skin delivery is highly challenging  
30 because of its very low solubility in most pharmaceutical excipients and poor penetration  
31 properties. Objectives of the work were: 1) to evaluate IMQ solubility in different solvents  
32 and pharmaceutical excipients; 2) to evaluate IMQ skin retention after the application of  
33 simple saturated solutions; 3) to evaluate the role of stratum corneum and solvent uptake  
34 on IMQ skin retention and 4) to formulate IMQ in microemulsions – prepared using  
35 previously investigated components - and compare them with the commercial formulation.  
36 The results show that IMQ solubility is not related to the solubility parameter of the  
37 solvents considered. The highest solubility was found with oleic acid (74 mg/ml); in the  
38 case of PEGs, the solubility increased linearly with MW (PEG 200: 1.9 mg/ml; PEG 400  
39 7.3 mg/ml, PEG 600 12.8 mg/ml). Imiquimod skin retention from saturated solutions  
40 (Tween 80, oleic acid, propylene glycol, PEG 200, PEG 400, PEG 600, Transcutol, 2-  
41 pyrrolidone, DMSO) resulted relatively similar, being  $1.6 \mu\text{g}/\text{cm}^2$  in case of oleic acid  
42 (solubility 74 mg/ml) and  $0.18 \mu\text{g}/\text{cm}^2$  in case of propylene glycol (solubility 0.60 mg/ml).  
43 Permeation experiments on stripped skin (no stratum corneum) and isolated dermis as  
44 well as uptake experiments on isolated stratum corneum sheets demonstrated that IMQ  
45 accumulation is related to skin solvent uptake. Finally, microemulsions (MEs) prepared  
46 with the above-studied components demonstrated a very good performance. In particular,  
47 a ME composed of 10% oleic acid, 35% Transcutol, 35% Tween 80 and 20% water is able  
48 to accumulate the same amount of drug as the commercial formulation but with far more  
49 efficiency, since its concentration was 12 times lower.

50

## 51 1. INTRODUCTION

52 Imiquimod (IMQ, Figure 1), an immunomodulating drug member of the imidazoquinoline  
53 amine family, exerts its activity via binding to the toll-like receptors 7 and 8, involved in the  
54 innate immune system response. Their activation leads to the transcription of different pro-  
55 inflammatory mediators, such as tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), interferon  $\alpha$  (IFN- $\alpha$ ),  
56 different interleukins (IL-1, IL-6, IL-8, IL-10 and IL-12), which trigger the immune system to  
57 recognize the presence of foreign agents (Schon and Schon, 2007). When applied  
58 topically, imiquimod activates Langheran's cells, which migrate to local lymph nodes to  
59 activate the adaptive immune system (Miller et al., 1999).

60 Imiquimod was approved in 1997 by the U.S. Food and Drug Administration for treating  
61 external genital and perianal warts. In 2004 its use has then been extended to the  
62 treatment of actinic keratosis (AK) and superficial basal cell carcinoma (sBCC). For this  
63 indication, its efficacy resulted superior to topical fluorouracil and photodynamic therapy  
64 (PDT) (Arits et al., 2013; Lecluse and Spuls, 2015). Together with the approved  
65 indications, several off-label applications have been reported (David et al., 2011) and  
66 some of them underline the important potential of this compound toward skin infections  
67 and skin cancers, such as nodular basal cell carcinoma (nBCC), squamous cell carcinoma  
68 *in situ* (Bowen's diseases) and lentigo maligna (Ellis et al., 2012; Mora et al., 2015). The  
69 interest for this molecule is witnessed also by the large number of clinical trials (*approx.* 20  
70 open/recruiting): some of them concern the treatment of neoplastic skin diseases, but  
71 great attention is also dedicated to the use of topical IMQ as adjuvant for vaccination  
72 (Fehres et al., 2014; Stein et al., 2014) (National Institutes of Health, 2016).

73 Regardless of the clinical application, imiquimod applied topically has to cross the stratum  
74 corneum and interact with the epidermal cells - in particular with Langheran's cells - to  
75 elicit its action. Imiquimod formulation and skin delivery is highly challenging because of its

76 very low solubility in either hydrophilic or lipophilic vehicles. Additionally, despite the low  
77 molecular weight, IMQ has very poor skin penetration properties, probably due to the very  
78 low solubility in the stratum corneum (SC) and underlying tissues. The epidermal  
79 concentration required for therapeutic efficacy is not known (and probably disease-related)  
80 but *in vitro* studies on cultured cells showed that the lowest effective concentration  
81 inducing cytokine production is between 0.1 and 0.5 µg/ml (Miller et al., 1999), while  
82 higher concentrations (25-50 µg/ml) are required to observe a direct pro-apoptotic activity  
83 (Schon et al., 2003).

84 The aim of the present paper is the comprehension of the mechanisms underlying  
85 imiquimod skin penetration and retention, so as to be able to formulate the drug in an  
86 efficient vehicle. Thus, the specific objectives of the work were: 1) to evaluate IMQ  
87 solubility in different solvents, vehicles and pharmaceutical excipients; 2) to evaluate IMQ  
88 skin retention after the application of simple saturated solutions; 3) to evaluate the role of  
89 stratum corneum and solvent uptake on IMQ skin retention and 4) to formulate IMQ in  
90 microemulsions – prepared using previously investigated components - and compare them  
91 with the commercial formulation.

92

## 93 **2. MATERIALS AND METHODS**

### 94 **2.1. Materials**

95 IMQ (MW=240.3 g/mol; pKa: 7.3) was purchased from TCI Europe N.V. (Zwijndrecht,  
96 Belgium) or Hangzhou Dayangchem, (Zhejiang, China). Oleic acid was obtained from Alfa  
97 Aesar (Karlsruhe, Germany). Transcutol and Capryol 90 were a gift from Gattefossè (Lyon,  
98 France). 70% Perchloric acid solution, Brij 78, albumin from bovine serum, trypsin from  
99 bovine pancreas (activity 8550 BAEE units/mg, corresponding to 2850 USP units/mg)

100 were purchased from Sigma Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO),  
101 PEG 200, 400 and 600, were obtained from A.C.E.F. (Fiorenzuola, Italy). Soluphor P (2-  
102 pyrrolidone), Lutrol F-68, Lutrol F-127, and Kolliphor-TPGS were a gift from BASF  
103 (Ludwigshafen, Germany). Sorbitan monooleate 80 (Span80) was a gift from Croda Ibérica  
104 SA, Spain.

105 For HPLC analysis, bidistilled water was used. Acetonitrile and methanol were of HPLC  
106 grade; all other reagents were of analytical grade.

## 107 **2.2. Analytical method**

108 Imiquimod quantification was performed by HPLC using a Flexar instrument (Perkin Elmer,  
109 Waltham, MA, USA) and a C18 column (Kinetex C18 2.6 $\mu$  100Å 75x4.6 mm,  
110 Phenomenex, Torrance, CA, USA), equipped with column guard (Widopore C18 4x3 mm,  
111 Phenomenex). In case of samples from extraction and permeation experiments,  
112 fluorescence detection ( $\lambda_{exc}$ 260 nm,  $\lambda_{em}$ 340 nm) was used (injection volume: 1  $\mu$ l), while  
113 solubility samples were analysed by UV absorbance ( $\lambda$  242 nm; injection volume: 10  $\mu$ l).

114 Stock solution was prepared by dissolving approx.. 2 mg of imiquimod in 20 ml of HCl  
115 0.1M. Calibration curves were prepared in the interval 0.05 – 3  $\mu$ g/ml in the case of  
116 fluorescence and 5-50  $\mu$ g/ml for UV detection. The methods were validated for precision  
117 and accuracy. LOD and LOQ for fluorescence were respectively 0.01 and 0.05  $\mu$ g/ml  
118 (RSD=2.5%; RE=16%).

## 119 **2.3. Solubility studies**

120 2 mg of IMQ was added to 2 ml of the following vehicles: H<sub>2</sub>O, PBS pH 7.4, PBS pH 4,  
121 propylene glycol, ethanol, octanol, Capryol 90, silicon oil, oleic acid, DMSO, Tween 80,  
122 PEG 200, PEG 400, PEG 600, miglyol, soybean oil, liquid paraffin, 2-pyrrolidone,

123 Transcutol (diethylene glycol monoethyl ether), water solution of 1% w/v  $\beta$ -CD, 1% w/v  
124 albumin, 0.55 w/v Tween 20, 50 and 200 mg/ml TPGS, 5 mg/ml Lutrol F68, 15 mg/ml  
125 Lutrol F127, 10 mg/ml Brij 78, 4% w/v lauric acid in EtOH:H<sub>2</sub>O (50:50). The samples were  
126 left overnight at room temperature, under magnetic stirring. The following morning, the  
127 vehicles in which IMQ was not dissolved (solubility < 1 mg/ml), were discarded. On the  
128 contrary, if IMQ was completely dissolved (solubility > 1 mg/ml), an excess amount of IMQ  
129 was added, and, after 24 hour mixing, the suspension was centrifuged and/or filtered  
130 (0.45 $\mu$ m), diluted and analysed by HPLC-UV for the accurate determination of the  
131 solubility.

#### 132 **2.4. Accumulation and permeation experiments**

133 For permeation experiments, porcine skin was used. The skin was excised from the outer  
134 part of pig ears within 3 hours from animal death, separated from the underlying cartilage  
135 with a scalpel and frozen at -20°C until use. All tissues were used within 3 months. The  
136 skin, once thawed, was mounted on vertical diffusion cells (DISA, Milano, Italy; 0.6 cm<sup>2</sup>  
137 surface area) with the stratum corneum facing the donor compartment. The receptor  
138 compartment was filled with 1% w/v albumin solution in PBS pH 7.4 (IMQ solubility: 143  $\pm$   
139 3  $\mu$ g/ml). IMQ saturated solutions in Transcutol, Tween 80, 2-pyrrolidone, propylene  
140 glycol, PEG 200, PEG 400, PEG 600, DMSO and oleic acid were tested. The commercial  
141 formulation Imunocare (IMQ 5% w/w, Difa Cooper, Caronno Pertusella, Italy) and three  
142 microemulsions, whose composition is reported in Table 3, were also evaluated. The  
143 donors were applied for 6 hours at infinite dose (200 mg/cm<sup>2</sup>, occluded). Some  
144 experiments were performed on tape-stripped skin (i.e. skin without the SC) and across  
145 isolated dermis. In order to prepare stripped skin, the tissue was tape-stripped (Scotch  
146 Booktape #845, 3M Co., St Paul, MN USA) until the complete removal of the stratum  
147 corneum (20-28 strips). In order to prepare isolated dermis, fresh skin samples were

148 immersed in hot water (60°C) for two minutes, and epidermis was peeled-off. The  
149 conditions tested are listed in detail in Table 2.

150 At the end of the experiments, the receptor solution was sampled, the donor formulation  
151 was removed, the tissue was rinsed with distilled water, blotted dry with filter paper and  
152 tape-stripped twice to remove possible traces of the formulation. Skin samples were then  
153 heated (hairdryer for 60 seconds) and separated into epidermis and dermis with the help  
154 of a spatula. Extraction was performed overnight at room temperature using two different  
155 mixtures: epidermis was extracted with 1 ml of oleic acid: methanol (1:3), dermis with 1 ml  
156 of PEG 400 : methanol : HCl 1M (1:2:2). To measure IMQ permeation, 1 ml of the receptor  
157 solution was transferred in an Eppendorf tube, added of 50 µl of 70%v/v perchloric acid to  
158 precipitate albumin and centrifuged (12000 rpm, 15 minutes). Extraction and permeation  
159 samples were analysed by HPLC-fluorescence.

160 In order to set up and validate the procedure for IMQ skin extraction, skin samples isolated  
161 from the outer part of pig ear were separated into dermis and epidermis by immersion for 2  
162 minutes in distilled water at 60°C. Tissues were spiked with a known amount of IMQ and  
163 then extracted using different temperature, time, and mixtures. IMQ quantification was  
164 then performed by HPLC with fluorescence detection. The recovery from the receptor  
165 solution after albumin precipitation was evaluated was well.

## 166 **2.5. Stratum corneum uptake experiments**

167 In order to probe any correlation between skin solvent uptake and the penetration of drug  
168 into the skin from specific vehicles, stratum corneum uptake experiments were performed.

169 To prepare SC sheets, isolated epidermis samples were soaked in 1% (w/v) trypsin in pH  
170 7.4 PBS, at 4°C for 15 hours. Epidermis was removed with a cotton swab and SC sheets

171 obtained were individually rinsed 3 times with distilled water, dried in oven at 37°C for 1 h,  
172 and kept in a dessiccator on CaCl<sub>2</sub> until use.

173 SC sheets ( $\approx 1.6$  mg/cm<sup>2</sup>, area of approximately 2.5 cm<sup>2</sup>) were first weighted (Mettler  
174 Toledo, sensitivity 0.001 mg) and then individually soaked for 6h in 2 ml of vehicle in a  
175 temperature-controlled oven at 32±1°C. SC sheets were then removed from the vehicle,  
176 carefully dried using filter paper and re-weighted. Solvent uptake was expressed both as  
177 moles/cm<sup>2</sup> and as percentage of weight increase using the following equation:

$$178 \quad \% \text{Weight increment} = ((W_f - W_i) / W_i) \times 100 \quad \text{Equation 1}$$

179 Where  $W_f$  is final weight and  $W_i$  is initial weight of SC sheet.

180 SC uptake experiments were performed also using IMQ solutions in different vehicles, so  
181 as to measure both solvent uptake and IMQ retention. In this case, after blotting and re-  
182 weighting, IMQ was extracted from the SC using 1 ml of oleic acid: methanol mixture (1:3)  
183 overnight at room temperature.

## 184 **2.6. Preparation of microemulsions**

185 Microemulsions were prepared by mixing oleic acid, Transcutol, Tween 80 and water in  
186 the proportions (w/w) indicated in Table 3. An excess amount of IMQ was then added and  
187 the suspension was stirred overnight at room temperature, after which the ME were  
188 centrifuged, filtered (0.45  $\mu$ m) and analysed by HPLC-UV.

## 189 **2.7. Statistical analysis**

190 The significance of the differences between conditions was assessed using *Student's t-*  
191 *test*. Differences were considered statistically significant when  $p < 0.05$ . Table 2 reports all  
192 skin retention data as mean value  $\pm$  SD. In Figures 3, 4, 8, for sake of clarity, the

193 experimental points are represented as mean value  $\pm$  standard error of the mean (sem),  
194 as indicated in the legend.

195

### 196 **3. RESULTS AND DISCUSSION**

#### 197 **3.1. Validation of IMQ skin extraction and analysis**

198 Initial efforts were directed towards the development of a reliable procedure for the  
199 extraction of IMQ from the different skin layers, i.e. SC, epidermis and dermis.  
200 Unfortunately, the quantification of IMQ in the SC, removed by tape stripping, was not  
201 possible due to the presence, regardless of the type and brand of scotch tape and of the  
202 extraction mixture used, of an analytical fluorescent interference with variable retention  
203 time. For this reason, IMQ was quantified in the dermis and in the whole epidermis (SC  
204 plus viable epidermis), after heat separation. Prior to epidermis-dermis separation, the skin  
205 was wiped cleaned and stripped twice, to remove IMQ retained in the most superficial SC  
206 layers, considered not available for absorption (OECD, 2011). This procedure was  
207 essential also to avoid the contamination of the deeper layers, in particular when vehicles  
208 in which IMQ has high solubility (such as oleic acid) were used. Preliminary experiments  
209 demonstrated that this procedure was able to reduce significantly the variability of the  
210 experimental data.

211 Several extracting conditions were evaluated for epidermis and dermis; a satisfactory  
212 recovery was obtained using a mixture oleic acid:methanol (1:3) for epidermis (recovery  
213  $98\pm 6$  %) and PEG400 : methanol : HCl 1M (1:2:2) for dermis (recovery  $97\pm 6$  %) overnight  
214 at room temperature. The stability of standard solutions of IMQ in these conditions was  
215 tested and no degradation was observed. IMQ recovery from the receptor solution after

216 albumin precipitation was 91±6%. IMQ peak was efficiently separated from other peaks  
217 deriving from the biological matrix.

218

### 219 3.2. Imiquimod solubility

220 IMQ is a molecule highly insoluble in aqueous media; its solubility increases slightly at  
221 acidic pH because it is a weak base (pKa=7.3) (Chollet et al., 1999). We have evaluated  
222 IMQ solubility in different vehicles: it resulted lower than 1 mg/ml in the case of surfactants  
223 and cyclodextrin in aqueous solution (50 and 200 mg/ml TPGS, 1% w/v β-CD, 0.5% w/v  
224 Tween 20, 5mg/ml lutrol F68, 15 mg/ml lutrol F127, 10 mg/ml Brij 78, 4%w/v lauric acid in  
225 1:1 ethanol:water), hydrophilic solvents such as PG, ethanol, capryol 90 and lutrol L44, but  
226 also lipophylic vehicles such as silicon oil, octanol, paraffin oil, miglyol and soybean oil.

227 When the solubility resulted higher than 1 mg/ml, the equilibrium solubility after 24 h was  
228 determined and the results obtained are reported in Table 1 together with the MW and  
229 solubility parameter of solvents. Despite the low solubility, PG and Tween 80 solutions  
230 were analysed since these excipients are frequently used in dermal application.

231 *Table 1. Solubility of IMQ in the different excipients used (average± SD)*

	MW	Solubility Parameter ( $\delta$ ) (cal cm <sup>-3</sup> ) <sup>1/2</sup>	Solubility (mg/ml)
OLEIC ACID	286.46	7.91 <sup>b</sup>	73.86 ±14.2
TWEEN 80	1310 <sup>a</sup>	9.74	0.66 ±0.02
TRANSCUTOL	134.17	10.9	1.11 ±0.07
PEG 600	600 <sup>a</sup>	≈11 <sup>b</sup>	12.83 ±1.58
PEG 400	400 <sup>a</sup>	≈11 <sup>b</sup>	7.3 ±1.84
PEG 200	200 <sup>a</sup>	≈11 <sup>b</sup>	1.98 ±0.38
2-PYRROLIDONE	85.1	11.9	1.64 ±0.12

DMSO	78.13	13.4 <sup>b</sup>	1.29 ±0.13
PG	76.09	14 <sup>b</sup>	0.60 ±0.03

232

<sup>a</sup>Average MW

233

<sup>b</sup>(Vaughan, 1985)

234

235 IMQ solubility is not related to the solubility parameter of the solvents considered, even  
 236 when taking into account the individual dispersion, polarity and hydrogen bonding  
 237 components (Hansen, 2007). The highest solubility was found with oleic acid, probably due  
 238 to its lipophilicity and the possibility of hydrogen bond formation with IMQ. In the case of  
 239 PEGs (200, 400, 600) the solubility increased linearly with PEG MW (Figure 2), suggesting  
 240 a hydrophobic interaction of IMQ with ethyl groups and/or formation of hydrogen bonds.

241 Few literature papers report imiquimod solubility data in pharmaceutical excipients.  
 242 Chollet *et al.* (Chollet et al., 1999) screened different excipients to be used for cutaneous  
 243 formulations, and found that only fatty acids (namely oleic acid, isostearic acid and linoleic  
 244 acid) gave an IMQ concentration higher than 1 mg/ml. In the case of oleic acid, however,  
 245 they found a much lower solubility value (20 mg/ml), probably because they determined  
 246 the solubility after only 30 minutes of contact.

### 247 3.3. Imiquimod skin accumulation and permeation

248 IMQ accumulation was studied starting from different vehicles and using different  
 249 membranes (full thickness porcine skin, stripped skin, isolated dermis). The results  
 250 obtained are reported in Table 2 as amount accumulated per cm<sup>2</sup> after 6 hours of skin  
 251 contact.

252

253 Table 2. Amount of IMQ accumulated from saturated solutions applied for 6 h in infinite dose conditions  
 254 (average  $\pm$  SD)-

n	Solvent	Skin	EPIDERMIS ( $\mu\text{g}/\text{cm}^2$ )	SD	DERMIS ( $\mu\text{g}/\text{cm}^2$ )	SD	TOTAL SKIN ( $\mu\text{g}/\text{cm}^2$ )	SD
3	<b>TWEEN 80</b>	Full thickness	0.26	$\pm 0.08$	0.06	$\pm 0.03$	0.33	$\pm 0.06$
3	<b>2-pyrrolidone</b>	Full thickness	0.48	$\pm 0.27$	0.22	$\pm 0.22$	0.69	$\pm 0.48$
3	<b>PG</b>	Full thickness	0.14	$\pm 0.05$	0.05	$\pm 0.01$	0.19	$\pm 0.06$
3	<b>Transcutol</b>	Full thickness	0.32	$\pm 0.14$	0.06	$\pm 0.04$	0.38	$\pm 0.17$
4	<b>PEG 200</b>	Full thickness	0.12	$\pm 0.12$	0.06	$\pm 0.07$	0.17	$\pm 0.19$
3	<b>PEG 400</b>	Full thickness	0.20	$\pm 0.05$	0.23	$\pm 0.13$	0.43	$\pm 0.16$
4	<b>PEG 600</b>	Full thickness	0.79	$\pm 0.63$	0.36	$\pm 0.20$	1.14	$\pm 0.81$
5	<b>PEG 600</b>	Stripped skin	1.39	$\pm 1.05$	0.40	$\pm 0.12$	1.79	$\pm 0.98$
3	<b>PEG 600</b>	Isolated dermis			61.90	$\pm 20.79$	61.90	$\pm 20.79$
3	<b>Oleic Acid</b>	Full thickness	1.02	$\pm 0.38$	0.61	$\pm 0.10$	1.62	$\pm 0.40$
5	<b>Oleic Acid</b>	Stripped skin	3.16	$\pm 0.93$	1.05	$\pm 0.74$	4.03	$\pm 1.20$
3	<b>Oleic Acid</b>	Isolated dermis			2.80	$\pm 2.44$	2.80	$\pm 2.44$
4	<b>DMSO</b>	Full thickness	0.61	$\pm 0.08$	0.41	$\pm 0.33$	1.02	$\pm 0.27$
5	<b>Imunocare</b>	Full thickness	1.27	$\pm 0.60$	0.62	$\pm 0.30$	1.89	$\pm 0.77$
4	<b>Imunocare</b>	Full thickness - Finite dose	1.67	$\pm 0.14$	1.04	$\pm 0.43$	2.71	$\pm 0.42$
3	<b>Imunocare</b>	Stripped skin	1.16	$\pm 0.48$	0.73	$\pm 0.16$	1.89	$\pm 0.61$
4	<b>ME1</b>	Full thickness	1.85	$\pm 0.67$	1.25	$\pm 0.51$	3.09	$\pm 0.38$
7	<b>ME2</b>	Full thickness	1.17	$\pm 0.54$	0.76	$\pm 0.59$	1.94	$\pm 1.07$
5	<b>ME3</b>	Full thickness	2.41	$\pm 0.72$	1.77	$\pm 0.23$	4.18	$\pm 0.90$

255

256 Some general considerations can be made: despite the relatively low molecular weight  
 257 and the favourable log P (calculated log D pH 7.4 : 2.65 (Advanced Chemistry  
 258 Development Inc., 2015)), IMQ skin penetration properties are very poor and the data are  
 259 very variable. The presence of albumin in the receptor compartment ensured sink  
 260 conditions (IMQ solubility:  $143 \pm 3 \mu\text{g}/\text{ml}$ ) however IMQ permeation was extremely low, if

261 any. In most of the cases, the concentration of IMQ in the receptor compartment was  
262 included between LOD and LOQ, indicating that the amount permeated after 6 hours was  
263 between 0.08 and 0.3  $\mu\text{g}/\text{cm}^2$ .

264 The retention of IMQ in the skin was at first evaluated from saturated solutions in pure  
265 solvents so as to keep the formulation as simple as possible and try to elucidate the  
266 mechanisms underlying IMQ dermal delivery. In fact, **regardless the solvent, saturated**  
267 **drug solutions have the same thermodynamic activity (equal to 1), thus the same tendency**  
268 **to “escape” from the vehicle.** Figure 3 shows the total amount accumulated (epidermis +  
269 dermis,  $\mu\text{g}/\text{cm}^2$ ) and the % accumulated with respect to the amount applied (volume 0.2  
270 ml; for concentrations see Table 1). This percentage can be useful to compare the  
271 different vehicles and to have information concerning the efficiency of delivery.

272 The solvents used are very different in terms of physico-chemical characteristics, IMQ  
273 solubility (Table 1) as well as possible effect on SC structure. Despite this diversity, the  
274 amounts of IMQ accumulated are relatively similar, being for instance 1.6  $\mu\text{g}/\text{cm}^2$  in case  
275 of oleic acid (solubility 74 mg/ml), and 0.18  $\mu\text{g}/\text{cm}^2$  in case of PG (solubility 0.60 mg/ml)  
276 (Figure 3). Some of the vehicles tested are known to modify the SC structure, increasing  
277 its permeability, however this effect is not evident in the data here collected, with the  
278 exception of DMSO, characterised by the highest accumulation %.

279 Considering PEGs, liquids characterised by a similar structure and lipophilicity (solubility  
280 parameter  $\approx 11$ ), a reasonably good linear correlation is present between donor  
281 concentration (corresponding to solubility) and skin retention (insert in Figure 3). These  
282 solutions, despite the same thermodynamic activity (and skin interaction - if any), generate  
283 concentration-dependent skin accumulation.

284 In order to clarify the role of SC on IMQ skin retention, further experiments were performed  
285 across tape stripped skin (skin deprived of SC). These experiments were done starting  
286 from PEG 600 and oleic acid, vehicles with very different physico-chemical properties and  
287 characterised by a relevant IMQ solubility. The result obtained, expressed as total amount  
288 accumulated in the skin ( $\mu\text{g}/\text{cm}^2$ ) is reported in Figure 4: no difference was found in IMQ  
289 accumulation between stripped and intact skin starting from PEG 600, while a 2-fold  
290 difference was found starting from oleic acid. We also studied the penetration across  
291 isolated dermis, in order to assess the role of viable epidermis. While no further difference  
292 was found in the case of oleic acid, a very high accumulation was found from PEG 600.  
293 The results illustrated in Figure 4 suggest that the fate of IMQ is linked to the affinity of the  
294 vehicle itself for the different barriers considered. PEG 600, highly hydrophilic, has a very  
295 low tendency to permeate both SC and viable epidermis (a relatively lipophilic tissue),  
296 while it diffuses easily across the dermis (hydrophilic tissue). On the contrary, oleic acid,  
297 having a strong lipophilic character penetrates better in the tape stripped skin but no  
298 further enhancement is found across/into isolated dermis.

299 From the data obtained it is possible to hypothesize that the transport of IMQ is linked, at  
300 least to a certain extent, to the diffusion of the solvent. This can be due to two different  
301 phenomena (Moser et al., 2001): 1) the modification of IMQ solubility in the different skin  
302 layers due to the solvent uptake 2) the solvent drag effect, a non-specific mechanism  
303 where solute and solvent permeate simultaneously (Bendas et al., 1995; Schneider et al.,  
304 1996).

305 In order to further investigate this phenomenon, studies of solvent and IMQ uptake into the  
306 SC were performed.

### 307 **3.4. SC uptake experiments**

308 We have evaluated the uptake of IMQ and solvent into sheets of isolated SC; these  
309 experiments were performed using either pure solvent or IMQ solutions. Since the solvent  
310 uptake was measured using a gravimetric method, the studies were performed using  
311 completely de-hydrated SC sheets. The results of solvent uptake are illustrated in Figure 5  
312 as nmoles/cm<sup>2</sup> and as % of SC weight increase. Even if the method used for measuring  
313 the uptake is relatively rough, the data are characterised by a relatively small variability,  
314 with the exception of Transcutol and PG where the RSD is equal or higher than 50%.  
315 However, the average value found for Transcutol ( $1.44 \pm 0.92 \cdot 10^{-6}$  mol/cm<sup>2</sup>) is reasonably  
316 in line with the data obtained by GC-MS by Oliveira et al using human SC (10% water  
317 content) and a 24 h period of equilibration ( $6.17 \pm 1.95 \cdot 10^{-6}$  mol/cm<sup>2</sup>) (Oliveira et al., 2012).

318 The uptake results obtained are consistent with the MW of the vehicle, except for DMSO  
319 that, despite MW and solubility parameter similar to pyrrolidone and PG, showed a 6-fold  
320 higher skin retention. This result is in agreement with the known property of DMSO to  
321 interact with both the lipid polar head groups (perhaps replacing water) causing expansion  
322 of the hydrophilic domains, and with keratin, displacing bound water (Anigbogu et al.,  
323 1995; Barry, 1987; Greve et al., 2008); the % of increase of SC weight was about 200%.  
324 In the case of the other solvents, a weight increase of *approx.* 30% was found, with  
325 the exception of Transcutol, characterized by a low and very variable uptake.

326 Then, IMQ was added to the different vehicles at different concentrations and the  
327 uptake was measured for both the solvent (gravimetric method as before) and IMQ  
328 (extraction and HPLC analysis). The paired data obtained are represented in Figure 6  
329 where the amount of IMQ experimentally determined ( $\mu\text{g}/\text{cm}^2$ ) is plotted versus the  
330 theoretical one ( $\text{IMQ}_{\text{theo}}$ ,  $\mu\text{g}/\text{cm}^2$ ), calculated taking into account the concentration of  
331 IMQ in the vehicle ( $\mu\text{g}/\text{ml}$ ) and the experimentally determined vehicle uptake ( $\text{ml}/\text{cm}^2$ ).

332 In the case of Transcutol, the data are characterised by a considerable dispersion,  
333 mainly due to the variability in solvent uptake data.

334 From the linearity of the data presented in Figure 6, it is evident that IMQ SC accumulation  
335 is directly related to the solvent uptake. A similar result has been previously found in case  
336 of methyl paraben dissolved in isopropylmyristate, methyl ether of isosorbide (DMI) and  
337 Transcutol using human SC (Oliveira et al., 2012). The easiest explanation is that the  
338 uptake of the solvent has dragged IMQ into the SC. However, IMQ solubility into the  
339 vehicle-containing stratum corneum does not coincide with IMQ solubility in the vehicle,  
340 since the slope is not always equal to one. Apparently, the presence of SC together with  
341 the vehicle has not modified the solubility of IMQ in oleic acid (slope = 1), while it has  
342 significantly increased it in the case of PG (slope > 1) and reduced it in case of DMSO,  
343 pyrrolidone and PEG 600 (slope < 1). Perhaps, the solvent-stratum corneum interaction  
344 alters the capability of the solvents to form hydrogen bonds with IMQ, or the stratum  
345 corneum solubility parameter has some sort of influence.

346 Then, an attempt was done to correlate the SC solvent uptake with the results obtained in  
347 the permeation experiments (data in Table 1). Figure 7 represent the total amount of IMQ  
348 accumulated in the skin during permeation experiments (Table 1) as a function of the  
349 product between solvent uptake and IMQ solubility (the permeation experiments were  
350 performed from saturated solutions).

351 The result illustrated in Figure 7 suggests a correlation between the two experiments,  
352 indicating that solvent uptake does play a role in IMQ skin retention. However, the  
353 correlation is not direct (log scale on x axis). This can be explained considering that the  
354 conditions of the SC in the two set-up are different: SC is completely dehydrated in the  
355 uptake experiment, while it is characterised by a water gradient (from 15-20% at the  
356 surface to 70% at the interface with the granular layer (Crowther et al., 2008) in the case of

357 permeation/retention experiments. SC water content can impact on solvent uptake. An  
358 additional reason that can explain the lack of linear correlation between the two  
359 experimental set-up is the elimination of the first two SC strips after the retention  
360 experiment with a consequent reduction of the IMQ accumulated.

### 361 **3.5. Formulations containing IMQ**

362 The following step was to develop an innovative formulation of IMQ, based on the results  
363 obtained in the first part of the work. As a reference, we tested the 5% w/w IMQ  
364 commercial cream containing as excipients isostearic acid, benzyl alcohol, cetyl alcohol,  
365 stearyl alcohol, white soft paraffin, polysorbate 60, sorbitan stearate, glycerol, methyl  
366 hydroxybenzoate, propyl hydroxybenzoate, xanthan gum and purified water (Haque et al.,  
367 2015). Isostearic acid is used as main component of the oily phase for its solubilisation  
368 properties (Chollet et al., 1999) and has been recently found to contribute to inflammatory  
369 responses via inflammasome activation (Walter et al., 2013). This formulation was  
370 evaluated in three different conditions: infinite dose, finite dose, and finite dose on stripped  
371 skin. The results, reported in Figure 8, indicate that 1) the skin delivery is low, despite the  
372 high concentration of the vehicle (50 mg/g) 2) the amount of formulation applied does not  
373 influence IMQ accumulation, suggesting that IMQ permeation is not sensitive to skin  
374 occlusion 3) the SC is not a significant barrier toward the diffusion of IMQ since only a  
375 limited accumulation increase was found in the case of stripped skin, likewise the  
376 behaviour of oleic acid and PEG 600 saturated solutions (Figure 4).

377 We formulated IMQ into a vehicle suitable for skin administration, taking into account the  
378 information collected until here on the role of solvent uptake. We decided to prepare  
379 microemulsions, since Hatout *et al.* have studied the penetration of ME components into  
380 the SC and the results suggest a more efficient uptake of the components when they are  
381 applied in a ME, compared to their separate application (Hathout et al., 2010).

382 *Table 3. Composition of the prepared microemulsions and solubility of IMQ*

% w/w	ME1	ME2	ME3
Oleic Acid	70	50	10
Transcutol	14	22.5	35
Tween 80	14	22.5	35
Water	2	5	20
IMQ solubility (mg/ml)	82 ± 9	54 ± 1	3.7 ± 0.7

383

384 The composition of the MEs (Table 3) was selected in the ME region of a ternary diagram  
 385 reported in the literature (Bhatia et al., 2013), using Tween 80 (polyoxyethylene- sorbitan  
 386 monooleate) instead of Tween 20 (polyoxyethylene-sorbitan monolaurate) so as to deal  
 387 with the same excipients previously investigated. The formulations were transparent,  
 388 indicating that the change in the surfactant (and in particular the in length and saturation of  
 389 the hydrophobic portion of the surfactant) did not impact on the ME structure.

390 The MEs were saturated with IMQ and the concentrations found (Table 3) were  
 391 proportional to the oleic acid content, suggesting that the drug is localised in the oily  
 392 phase.

393 The accumulation results (Figure 8) obtained with ME1 and ME2, containing respectively  
 394 70% and 50% oleic acid (Table 3), are significantly higher ( $p < 0.01$ ) compared to the  
 395 saturated oleic acid solution. This effect cannot be attributed neither to the thermodynamic  
 396 activity of the drug (both solution and MEs are saturated) nor to a direct effect of ME  
 397 components on the SC permeability with the consequent higher diffusion of IMQ, as  
 398 reported in case of many other drugs formulated in ME (Lopes, 2014). In fact, as  
 399 demonstrated with the commercial cream (Figure 8) and with saturated solutions of PEG  
 400 600 and oleic acid (Figure 4) the SC represent only a modest barrier for IMQ permeation, if

401 any. Or better, it represents a barrier in so far as it hinders the penetration of the solvent.  
402 We can hypothesize that in the case of MEs, a high penetration of the ME component  
403 takes place, thus “dragging” IMQ into the skin. Literature data obtained with a ME very  
404 similar to **ME3** (15.4% oleic acid; 30.8% Tween 20; 30.8% Transcutol; 23% water)  
405 confirms this hypothesis: the concentration profiles of oleic acid, Tween 20 and Transcutol  
406 into the SC show a deeper and faster penetration when these compounds are applied in a  
407 ME than as pure solvents (Hathout et al., 2010). Additionally, Mahrhauser et al have  
408 studied the penetration of both the drug (diclofenac) and the surfactant from a  
409 microemulsion (10% oleic acid; 32.5% fluorosurfactant; 32.5% isopropanol; 25% water)  
410 and demonstrated their simultaneous penetration into the SC, suggesting also in this case  
411 a “drag” (or “push” ) effect (Mahrhauser et al., 2014).

412 By comparing the different MEs it is evident the important role played by water content, in  
413 fact the accumulation efficiency resulted proportional to water % in the ME (see insert in  
414 Figure 8).

415 Similar results were observed for other permeants (sucrose, ketoprofen, lidocaine, alpha-  
416 tocopherol (Lopes, 2014)) and it has been suggested that the content of water can  
417 influence the microemulsion internal structure, with consequent change in drug mobility  
418 (Mahrhauser et al., 2015; Zhang and Michniak-Kohn, 2011). It is worth highlighting that  
419 **ME3**, containing only 0.37% imiquimod, is able to accumulate the same amount of drug  
420 as the commercial formulation (5% w/w) and can represent an interesting starting point for  
421 the further optimisation of an IMQ based topical formulation.

422

#### 423 **4. CONCLUSION**

424 The results obtained in the present work underline the role of solvent in imiquimod skin  
425 penetration and retention. It was found that drug skin uptake is strictly related to solvent  
426 uptake, suggesting the relevance of solvent drag effect in imiquimod skin delivery.  
427 Concerning the localization of the barrier for transport, the stratum corneum represents a  
428 barrier towards the solvent more than towards the drug. In fact, when the stratum corneum  
429 was removed by tape stripping, imiquimod retention increased from a very lipophilic  
430 vehicle, such as oleic acid, but not from the hydrophilic vehicle propylene glycol.

431 The work on imiquimod skin retention from pure solvents represented the starting point for  
432 the preparation of microemulsions. The results show that a ME composed of 10% oleic  
433 acid, 35% Transcutol, 35% Tween 80 and 20% water is able to accumulate the same  
434 amount of drug as the commercial formulation but with far more efficiency, since its  
435 concentration is 12 times lower.

436 The data here collected can be a valuable base to optimize imiquimod-loaded  
437 microemulsions for both the treatment of neoplastic skin diseases and as adjuvant for  
438 vaccination; future studies will address the need for adequate rheological properties  
439 together with the possibility of further increase drug uptake.

#### 440 **ACKNOWLEDGEMENTS**

441 We are grateful to Dott. Pierugo Cavallini and Macello Annoni S.p.A. (Madonna dei Prati,  
442 Busseto, PR) for providing fresh pig ears.

443 The financial support of Italian Ministry of Education, University and Research  
444 (PRIN2010H834LS) is gratefully acknowledged.

#### 445 **REFERENCES**

446 Advanced Chemistry Development Inc., 2015. ACD/Percepta, Version 2015, Pack 2,  
447 Toronto, On, Canada, [www.acd.labs.com](http://www.acd.labs.com).

448 Anigbogu, A.N.C., Williams, A.C., Barry, B.W., Edwards, H.G.M., 1995. Fourier transform  
449 raman spectroscopy of interactions between the penetration enhancer dimethyl sulfoxide  
450 and human stratum corneum. *Int J Pharm* 125, 265-282.

451 Arits, A.H., Mosterd, K., Essers, B.A., Spoorenberg, E., Sommer, A., De Rooij, M.J., van  
452 Pelt, H.P., Quaedvlieg, P.J., Krekels, G.A., van Neer, P.A., Rijzewijk, J.J., van Geest, A.J.,  
453 Steijlen, P.M., Nelemans, P.J., Kelleners-Smeets, N.W., 2013. Photodynamic therapy  
454 versus topical imiquimod versus topical fluorouracil for treatment of superficial basal-cell  
455 carcinoma: a single blind, non-inferiority, randomised controlled trial. *Lancet Oncol* 14,  
456 647-654.

457 Barry, B.W., 1987. Mode of action of penetration enhancers in human skin. *J Control*  
458 *Release* 6, 85-97.

459 Bendas, B., Schmalfuß, U., Neubert, R., 1995. Influence of propylene glycol as cosolvent  
460 on mechanisms of drug transport from hydrogels. *Int J Pharm* 116, 19-30.

461 Bhatia, G., Zhou, Y., Banga, A.K., 2013. Adapalene Microemulsion for Transfollicular Drug  
462 Delivery. *J Pharm Sci* 102, 2622-2631.

463 Chollet, J.L., Jozwiakowski, M.J., Phares, K.R., Reiter, M.J., Roddy, P.J., Schultz, H.J., Ta,  
464 Q.V., Tomai, M.A., 1999. Development of a topically active imiquimod formulation. *Pharm*  
465 *Dev Technol* 4, 35-43.

466 Crowther, J.M., Sieg, A., Blenkiron, P., Marcott, C., Matts, P.J., Kaczvinsky, J.R.,  
467 Rawlings, A.V., 2008. Measuring the effects of topical moisturizers on changes in stratum  
468 corneum thickness, water gradients and hydration in vivo. *Br J Dermatol* 159, 567-577.

469 David, C.V., Nguyen, H., Goldenberg, G., 2011. Imiquimod: a review of off-label clinical  
470 applications. *J Drugs Dermatol* 10, 1300-1306.

471 Ellis, L.Z., Cohen, J.L., High, W., Stewart, L., 2012. Melanoma In Situ Treated  
472 Successfully Using Imiquimod After Nonclearance with Surgery: Review of the Literature.  
473 *Dermatol Surg* 38, 937-946.

474 Fehres, C.M., Bruijns, S.C., van Beelen, A.J., Kalay, H., Ambrosini, M., Hooijberg, E.,  
475 Unger, W.W., de Gruijl, T.D., van Kooyk, Y., 2014. Topical rather than intradermal  
476 application of the TLR7 ligand imiquimod leads to human dermal dendritic cell maturation  
477 and CD8+ T-cell cross-priming. *Eur J Immunol* 44, 2415-2424.

478 Greve, T.M., Andersen, K.B., Nielsen, O.F., 2008. Penetration mechanism of dimethyl  
479 sulfoxide in human and pig ear skin: An ATR–FTIR and near-FT Raman spectroscopic in  
480 vivo and in vitro study. *J Spectrosc* 22.

481 Hansen, C.M., 2007. *Hansen Solubility Parameters: A User's Handbook, Second Edition*.  
482 CRC press, Taylor&Francis Group, Boca Raton, FL.

483 Haque, T., Rahman, K.M., Thurston, D.E., Hadgraft, J., Lane, M.E., 2015. Topical  
484 therapies for skin cancer and actinic keratosis. *Eur J Pharm Sci* 77, 279-289.

485 Hathout, R.M., Mansour, S., Mortada, N.D., Geneidi, A.S., Guy, R.H., 2010. Uptake of  
486 microemulsion components into the stratum corneum and their molecular effects on skin  
487 barrier function. *Mol Pharm* 7, 1266-1273.

488 Lecluse, L.L., Spuls, P.I., 2015. Photodynamic therapy versus topical imiquimod versus  
489 topical fluorouracil for treatment of superficial basal-cell carcinoma: a single blind, non-  
490 inferiority, randomised controlled trial: a critical appraisal. *Br J Dermatol* 172, 8-10.

491 Lopes, L., 2014. Overcoming the Cutaneous Barrier with Microemulsions. *Pharmaceutics*  
492 6, 52.

493 Mahrhauser, D.-S., Kählig, H., Partyka-Jankowska, E., Peterlik, H., Binder, L., Kwizda, K.,  
494 Valenta, C., 2015. Investigation of microemulsion microstructure and its impact on skin  
495 delivery of flufenamic acid. *Int J Pharm* 490, 292-297.

496 Mahrhauser, D., Hoppel, M., Scholl, J., Binder, L., Kahlig, H., Valenta, C., 2014.  
497 Simultaneous analysis of skin penetration of surfactant and active drug from  
498 fluorosurfactant-based microemulsions. *Eur J Pharm Biopharm* 88, 34-39.

499 Miller, R.L., Gerster, J.F., Owens, M.L., Slade, H.B., Tomai, M.A., 1999. Imiquimod applied  
500 topically: a novel immune response modifier and new class of drug. *Int J*  
501 *Immunopharmacol* 21, 1-14.

502 Mora, A.N., Karia, P.S., Nguyen, B.M., 2015. A quantitative systematic review of the  
503 efficacy of imiquimod monotherapy for lentigo maligna and an analysis of factors that  
504 affect tumor clearance. *J Am Acad Dermatol* 73, 205-212.

505 Moser, K., Kriwet, K., Naik, A., Kalia, Y.N., Guy, R.H., 2001. Passive skin penetration  
506 enhancement and its quantification in vitro. *Eur J Pharm Biopharm* 52, 103-112.

507 National Institutes of Health, 2016. ClinicalTrials.gov, p. <http://clinicaltrials.gov/>.

508 OECD, 2011. ENV/JM/MONO(2011)36 Guidance notes on dermal absorption - Series on  
509 testing and assessment n. 156, Paris.

510 Oliveira, G., Hadgraft, J., Lane, M.E., 2012. The role of vehicle interactions on permeation  
511 of an active through model membranes and human skin. *Int J Cosmet Sci* 34, 536-545.

512 Schneider, I.-M., Dobner, B., Neubert, R., Wohlrab, W., 1996. Evaluation of drug  
513 penetration into human skin ex vivo using branched fatty acids and propylene glycol. *Int J*  
514 *Pharm* 145, 187-196.

515 Schon, M., Bong, A.B., Drewniok, C., Herz, J., Geilen, C.C., Reifenberger, J., Benninghoff,  
516 B., Slade, H.B., Gollnick, H., Schon, M.P., 2003. Tumor-selective induction of apoptosis  
517 and the small-molecule immune response modifier imiquimod. *J Natl Cancer Inst* 95,  
518 1138-1149.

519 Schon, M.P., Schon, M., 2007. Imiquimod: mode of action. *Br J Dermatol* 157 Suppl 2, 8-  
520 13.

521 Stein, P., Gogoll, K., Tenzer, S., Schild, H., Stevanovic, S., Langguth, P., Radsak, M.P.,  
522 2014. Efficacy of Imiquimod-Based Transcutaneous Immunization Using a Nano-  
523 Dispersed Emulsion Gel Formulation. PLoS ONE 9, e102664.

524 Vaughan, C.D., 1985. Using solubility parameters in cosmetics formulation. J Soc Cosmet  
525 Chem 36, 319-333.

526 Walter, A., Schafer, M., Cecconi, V., Matter, C., Urosevic-Maiwald, M., Belloni, B.,  
527 Schonewolf, N., Dummer, R., Bloch, W., Werner, S., Beer, H.D., Knuth, A., van den Broek,  
528 M., 2013. Aldara activates TLR7-independent immune defence. Nat Commun 4, 1560.

529 Zhang, J., Michniak-Kohn, B., 2011. Investigation of microemulsion microstructures and  
530 their relationship to transdermal permeation of model drugs: ketoprofen, lidocaine, and  
531 caffeine. Int J Pharm 421, 34-44.

532

533

534 **FIGURE LEGEND**

535 **Figure 1.** Imiquimod

536 **Figure 2.** IMQ solubility as a function of the average PEG MW (average $\pm$  SD).

537 **Figure 3.** IMQ skin (epidermis+dermis) retention ( $\mu\text{g}/\text{cm}^2$ ) and % of IMQ accumulated  
538 starting from saturated solutions. The data are represented as mean  $\pm$  sem. In the insert,  
539 IMQ skin accumulation is represented as a function of solubility in PEGs.

540 **Figure 4.** IMQ skin accumulation ( $\mu\text{g}/\text{cm}^2$ ; average  $\pm$  sem) starting from saturated  
541 solutions in PEG (12.8 mg/ml) and oleic acid (73.8 mg/ml) following the application on  
542 intact skin (white bar), SC stripped skin (grey bar) and isolated dermis (black bar).  
543 Asterisks indicate that the accumulation is statistically higher with respect to full thickness  
544 skin \* $p<0.05$ ; \*\*  $p<0.01$ .

545 **Figure 5.** SC uptake of different vehicles obtained by soaking dehydrated SC sheets for 6  
546 hours at 37°C in an excess of each solvent (moles/ $\text{cm}^2$ ; average $\pm$ sd); In the insert, the:  
547 SC weight increase (%) is presented.

548 **Figure 6.** Correlation between the amount of IMQ extracted from the SC and the  
549 theoretical amount calculated considering the solvent uptake ( $\text{ml}/\text{cm}^2$ ) and the IMQ  
550 concentration ( $\mu\text{g}/\text{ml}$ )

551 **Figure 7.** Correlation between the amount of IMQ accumulated into the skin during  
552 permeation experiments (data in Table 1) and the product of solvent uptake\* solubility (log  
553 scale).

554 **Figure 8.** IMQ accumulation in the skin ( $\mu\text{g}/\text{cm}^2$ ; average  $\pm$  sem; gray bars) from the  
555 commercial formulation and from the saturated ME. As a comparison, the value obtained  
556 from the oleic acid saturated solution is also reported. \* significantly different from oleic  
557 acid saturated solution ( $p<0.01$ ); # significantly different from Immunocare ( $p<0.05$ ). Red  
558 circles indicate on Y2 axis the IMQ concentration (% w/w) in the formulations tested. The  
559 insert illustrates the accumulation efficiency (% accumulated) of MEs as a function of  
560 water content.

561

Figure 1

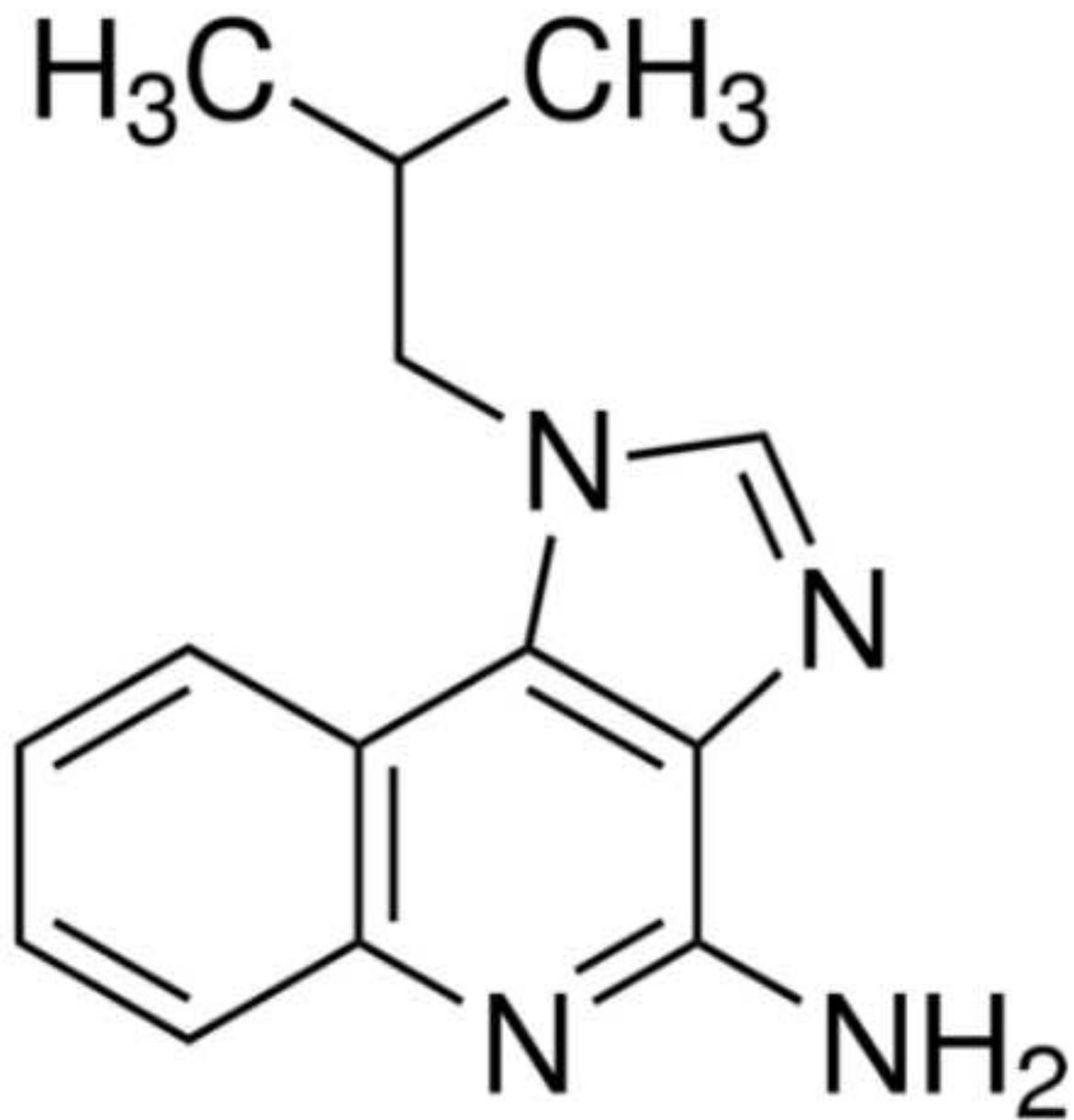


Figure 2

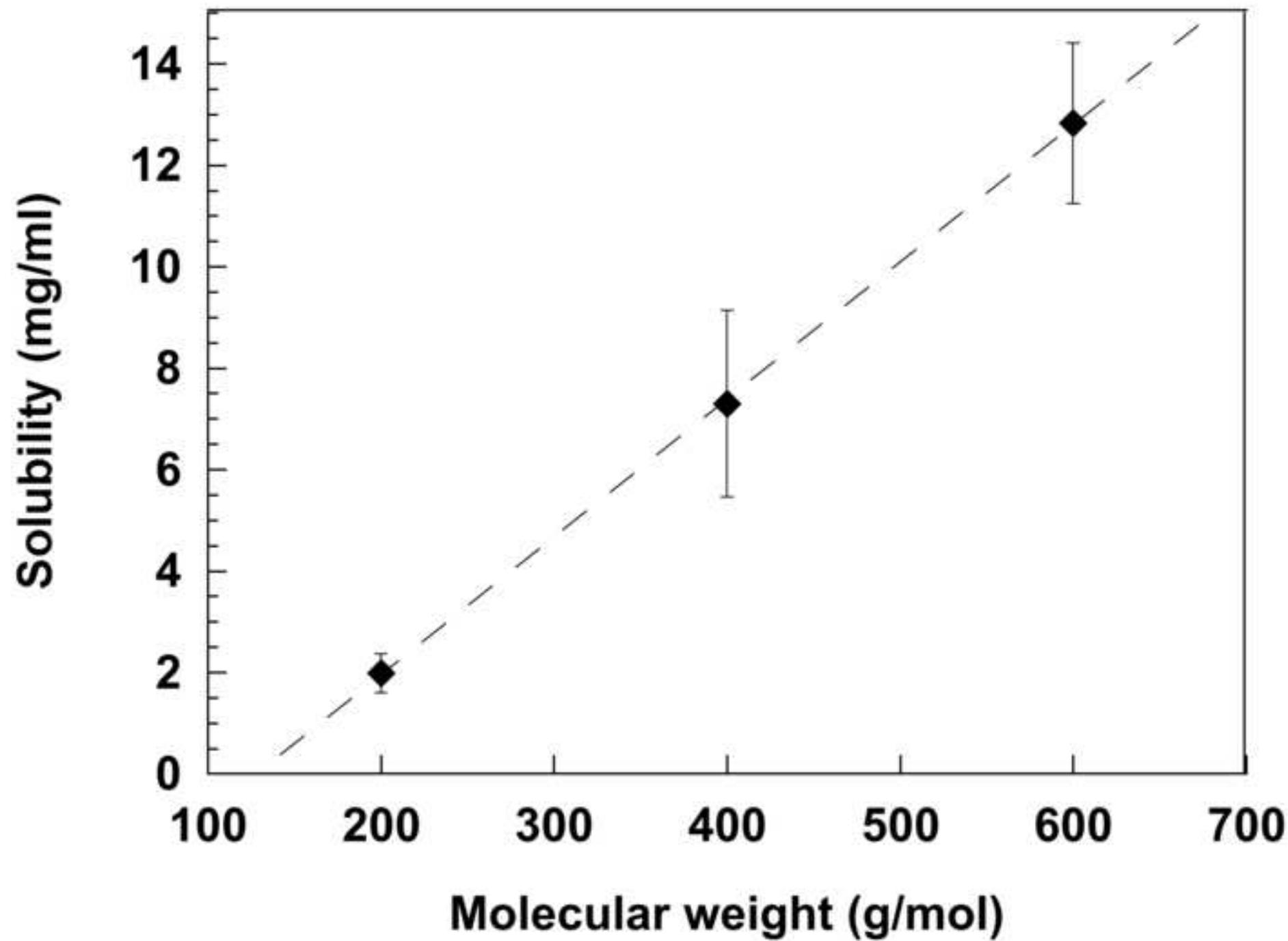


Figure 3

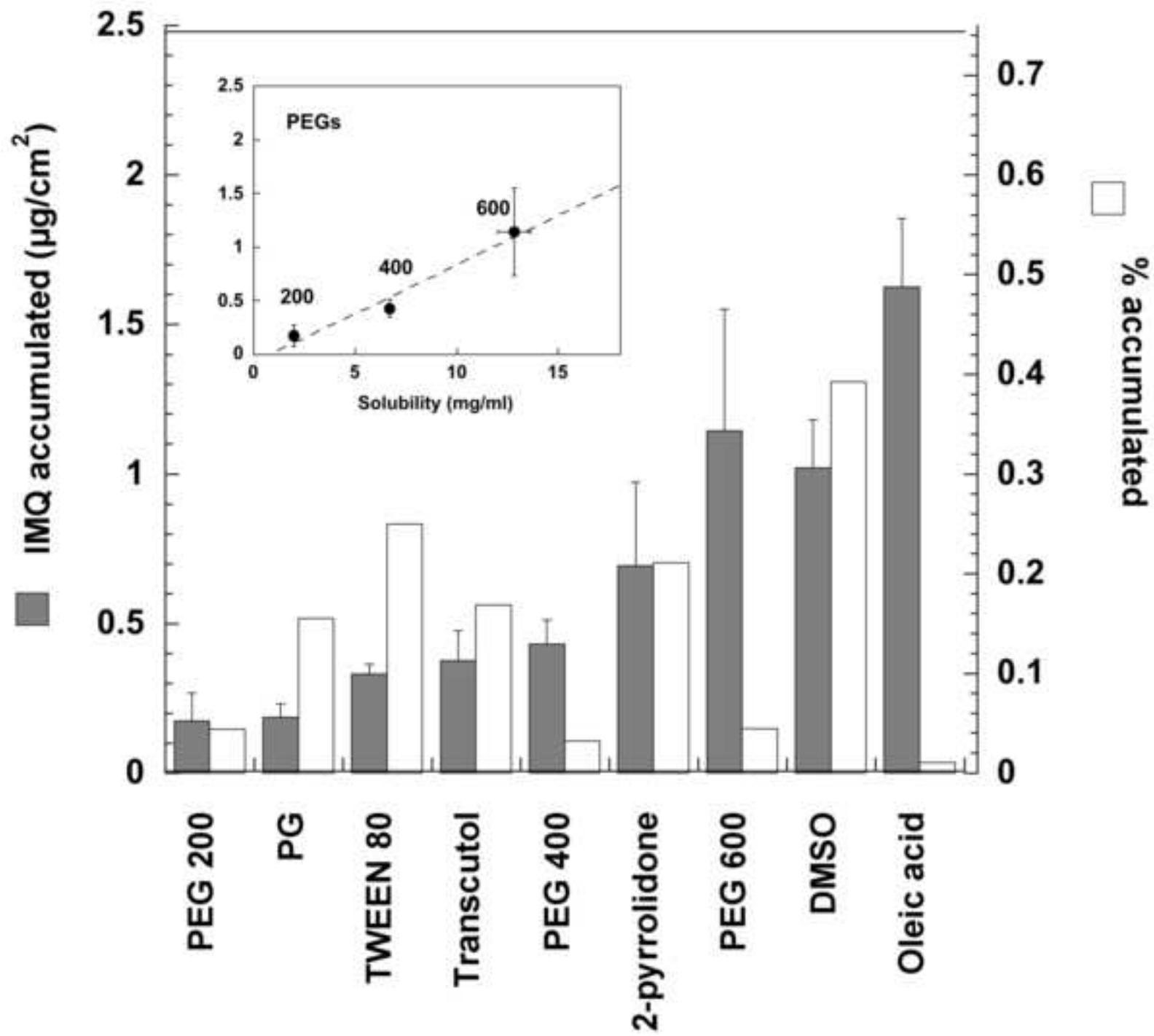


Figure 4

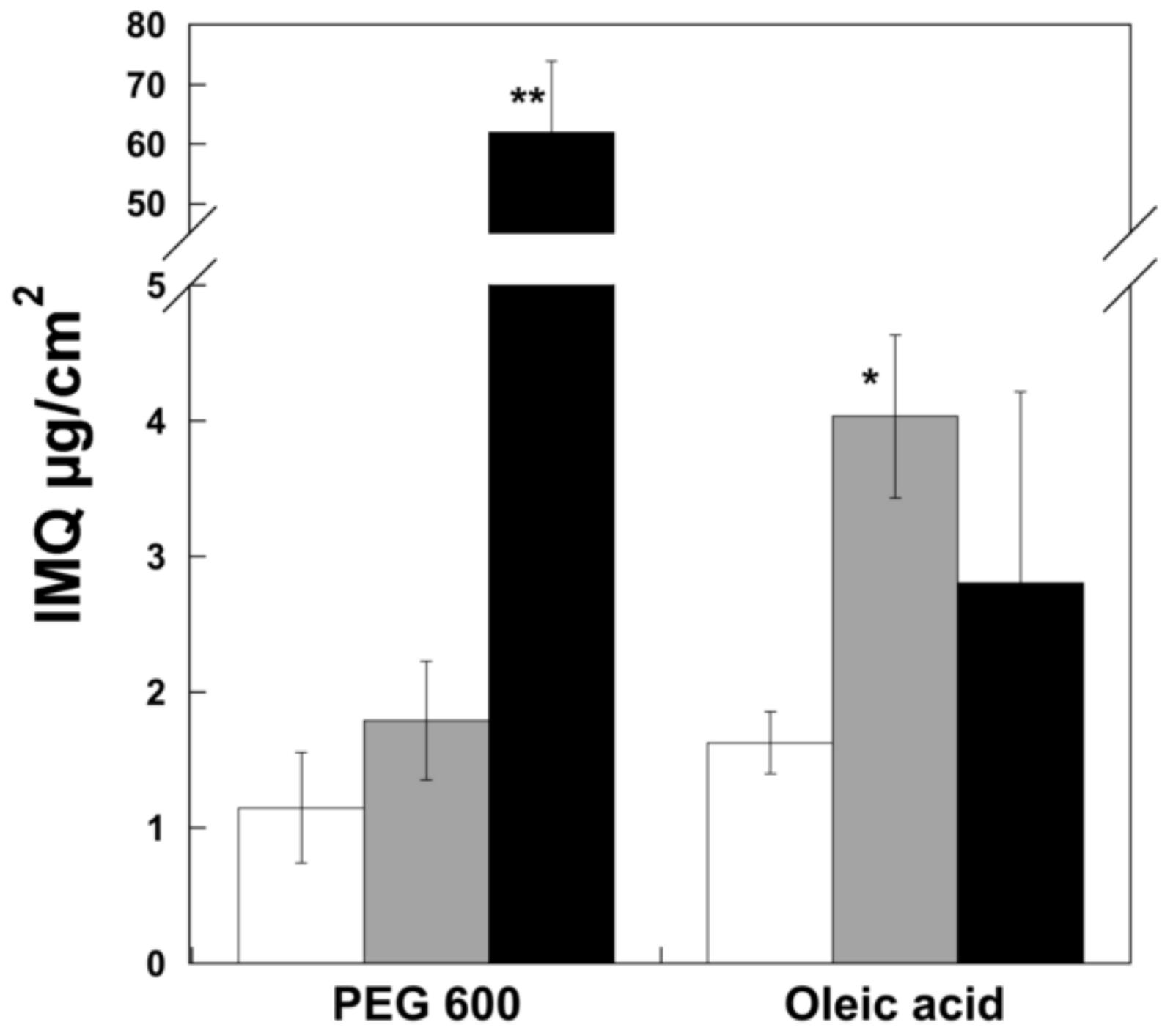


Figure 5

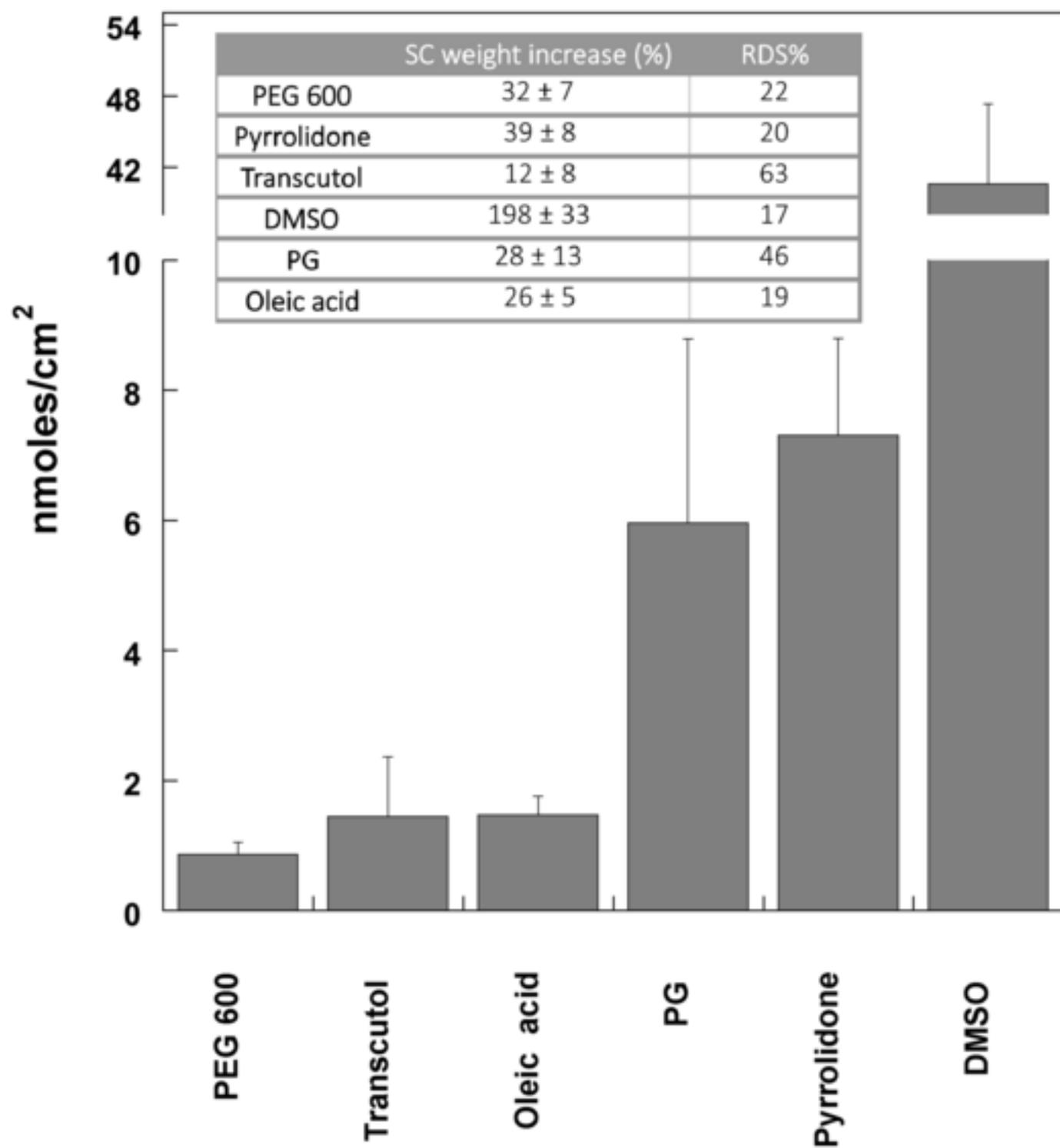


Figure 6

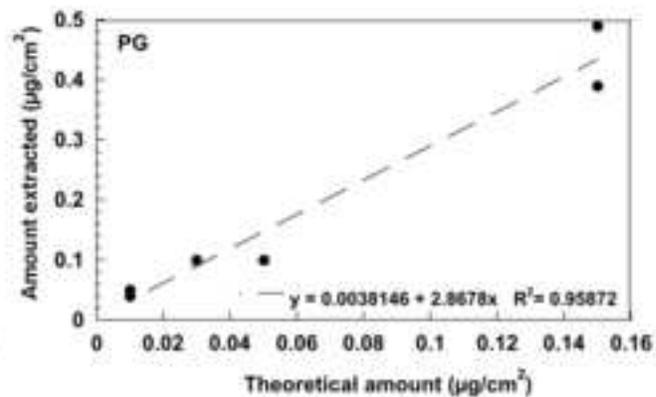
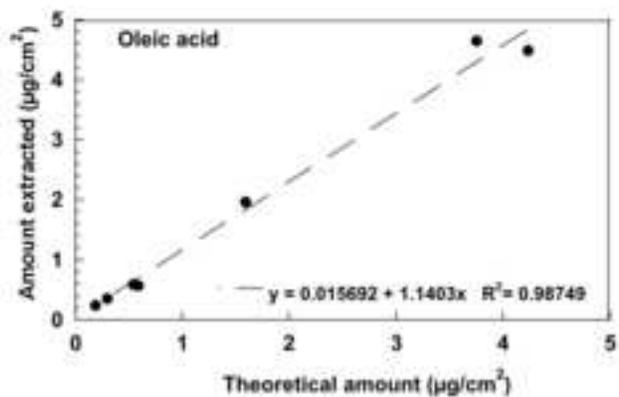
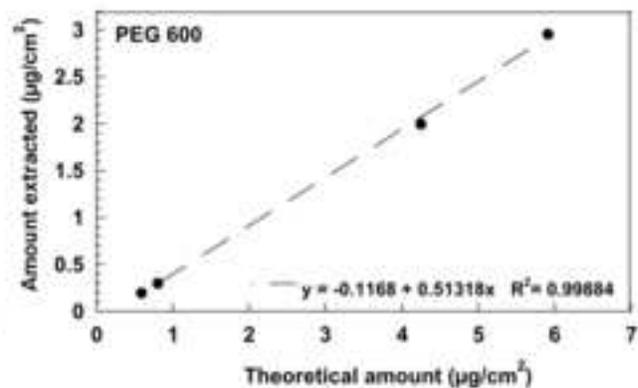
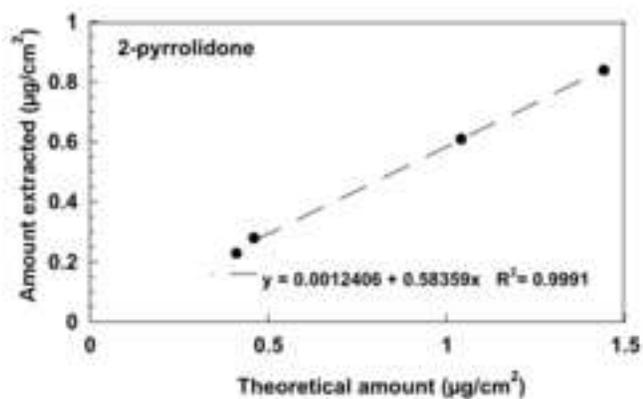
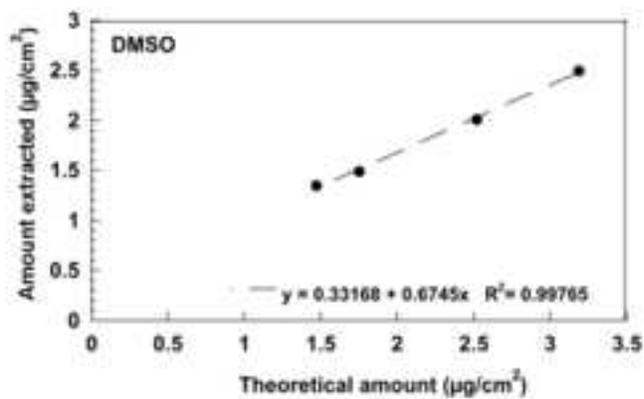
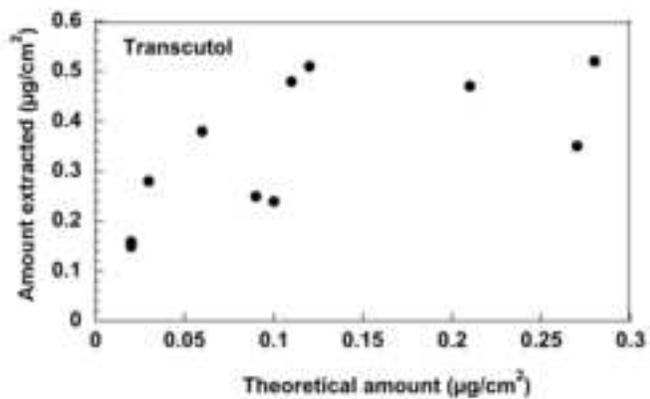


Figure 7

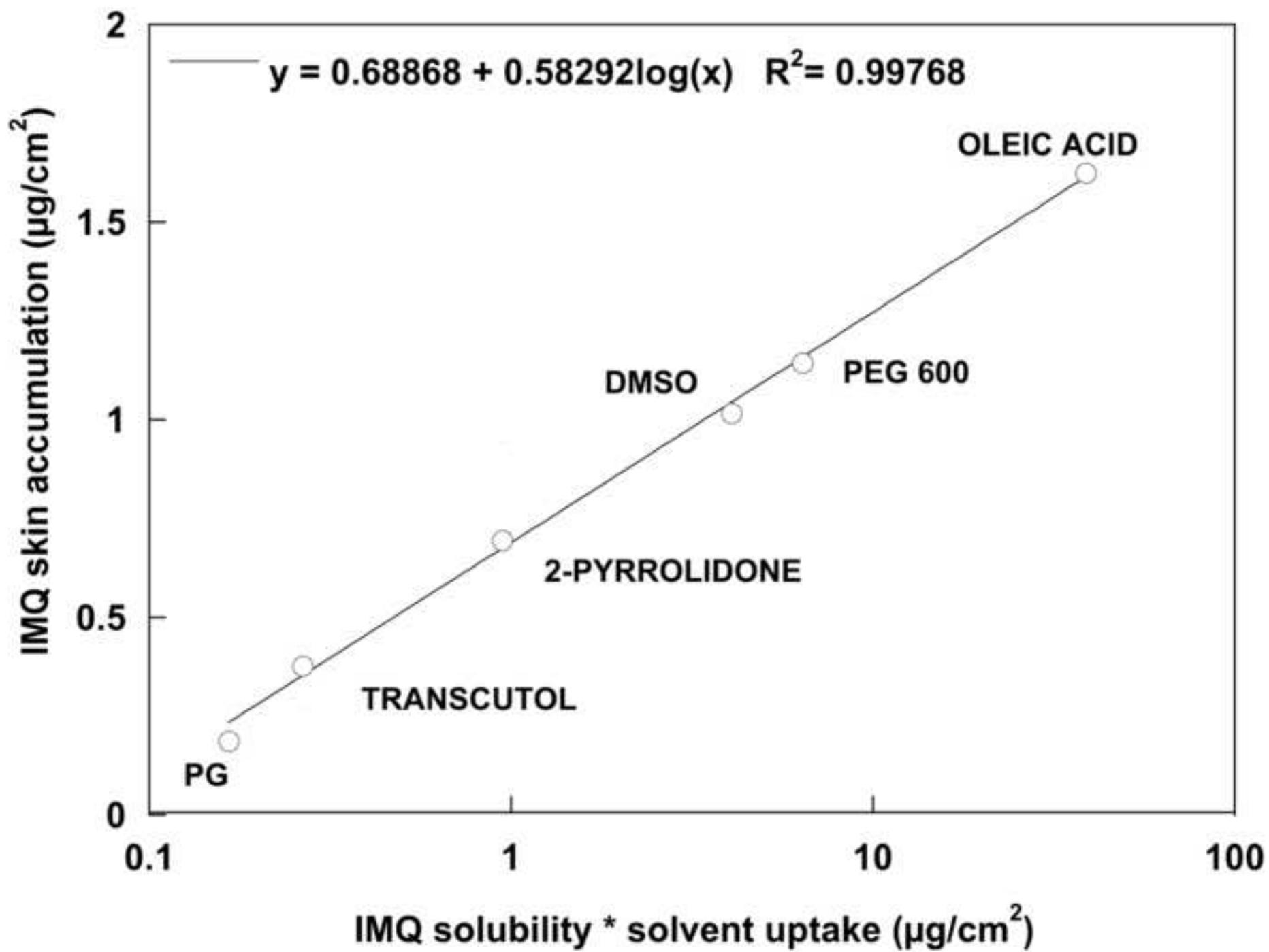
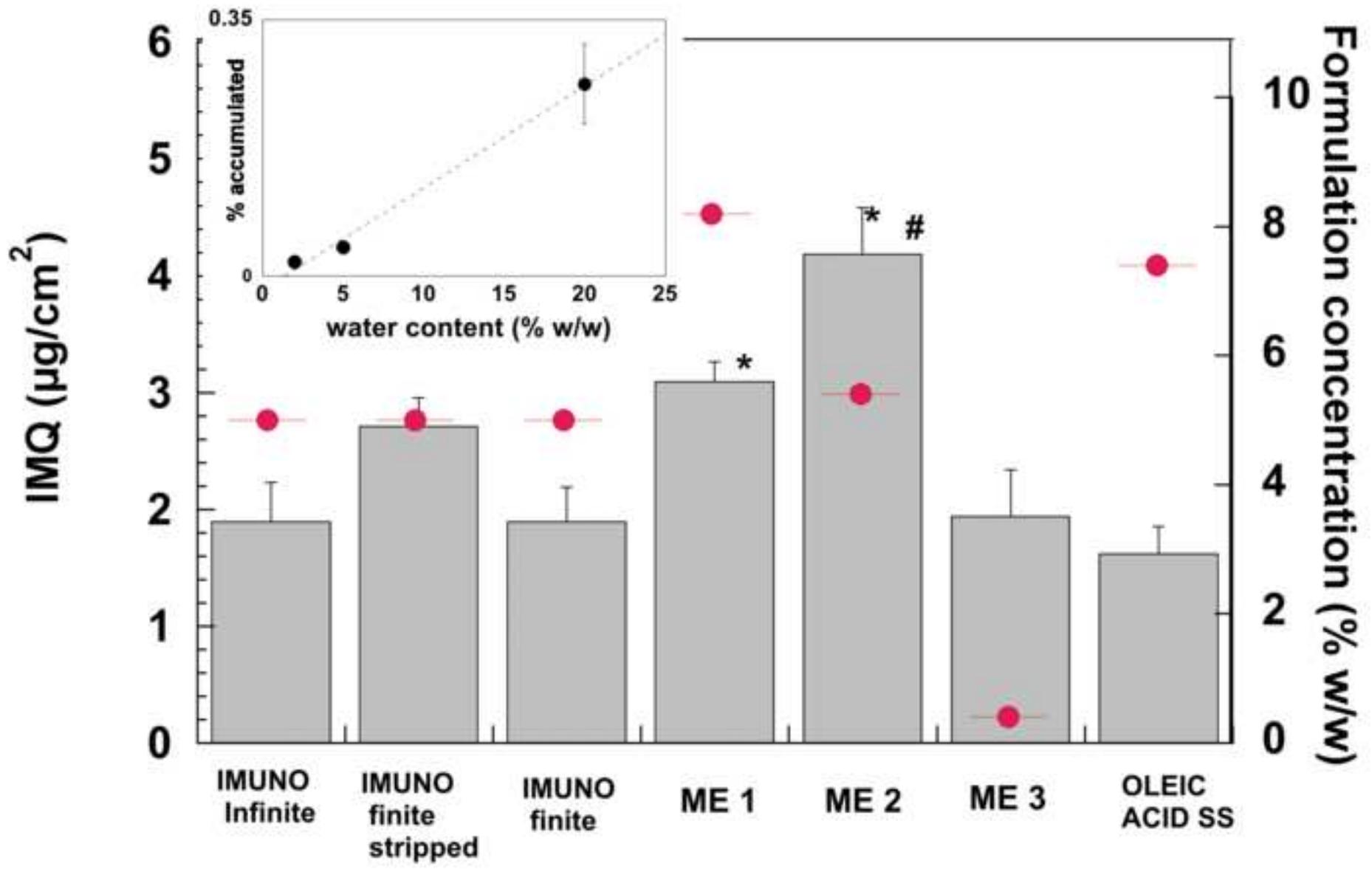


Figure 8 revised



## IJP AUTHOR CHECKLIST

Dear Author,

It frequently happens that on receipt of an article for publication, we find that certain elements of the manuscript, or related information, is missing. This is regrettable of course since it means there will be a delay in processing the article while we obtain the missing details.

In order to avoid such delays in the publication of your article, if accepted, could you please run through the list of items below and make sure you have completed the items.

### Overall Manuscript Details

- Is this the final revised version? Yes
- Are all text pages present? Yes
- Are the corresponding author's postal address, telephone and fax numbers complete on the manuscript? Yes
- **Have you provided the corresponding author's e-mail address?** Yes
- **Manuscript type – please check one of the following:**
  - Full-length article X
  - Review article
  - Rapid Communication
  - Note
  - Letter to the Editor
  - Other
- **Manuscript section – paper to be published in:**
  - Pharmaceutical Nanotechnology section
  - Personalised Medicine section

### Manuscript elements

- Short summary/abstract enclosed? Yes
- 3-6 Keywords enclosed? Yes
- Complete reference list enclosed? Yes
- Is the reference list in the correct journal style? Yes
- Are all references cited in the text present in the reference list? Yes
- Are all **original** figures cited in the text enclosed? Yes
  - Electronic artwork format? -----
- Are figure legends supplied? Yes
- Are all figures numbered and orientation provided? Yes
- Are any figures to be printed in colour? NO
  - If yes, please list which figures here:-----
- If applicable, are you prepared to pay for reproduction in colour? Not applic.
- Are all tables cited in the text supplied? Yes

### General

- Can you accept pdf proofs sent via e-mail? Yes