Mechanisms of imiquimod skin penetration

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Abstract: Imiquimod (IMQ) ia 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/cm2 in case of oleic acid (solubility 74 mg/ml) and 0.18 µg/cm2 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 performanceattributed 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.

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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

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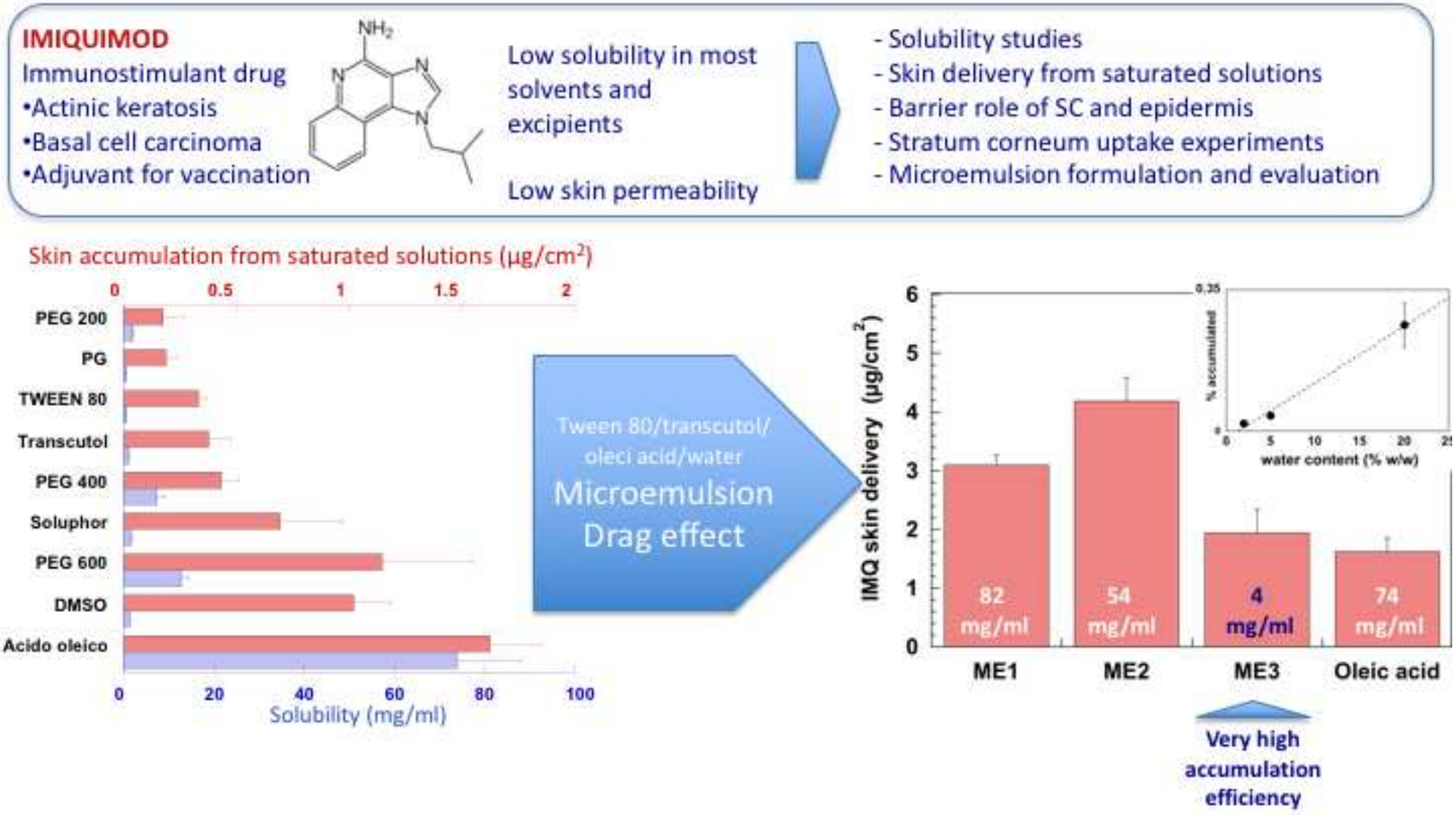
*Graphical Abstract (for review)



*Response to Reviewers

Ms. Ref. No.: IJP-D-16-01224
Title: Mechanisms of imiquimod skin penetration and enhanced skin delivery using microemulsions
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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)

~~Mechanisms of imiquimod skin penetration and enhanced skin delivery using microemulsions~~

Mechanisms of imiquimod skin penetration

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Keyword

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

Abstract

Imiquimod (IMQ) ia 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^2 in case of oleic acid (solubility 74 mg/ml) and 0.18 μg/cm^2 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. 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.

1. INTRODUCTION

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

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

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

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

The aim of the present paper is the comprehension of the mechanisms underlying imiquimod skin penetration and retention, so as to be able to formulate the drug in an efficient vehicle. Thus, the specific objectives of the work were: 1) to evaluate IMQ solubility in different solvents, vehicles 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.

2. MATERIALS AND METHODS

2.1. Materials

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

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

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

2.2. Analytical method

Imiquimod quantification was performed by HPLC using a Flexar instrument (Perkin Elmer, Waltham, MA, USA) and a C18 column (Kinetex C18 2.6µ 100Å 75x4.6 mm, Phenomenex, Torrance, CA, USA), equipped with column guard (Widepore C18 4x3 mm, Phenomenex). In case of samples from extraction and permeation experiments, fluorescence detection (λ_{exc}260 nm, λ_{em}340 nm) was used (injection volume: 1 µl), while solubility samples were analysed by UV absorbance (λ 242 nm; injection volume: 10 µl).

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

2.3. Solubility studies

2 mg of IMQ was added to 2 ml of the following vehicles: H_2O, PBS pH 7.4, PBS pH 4, propylene glycol, ethanol, octanol, Capryol 90, silicon oil, oleic acid, DMSO, Tween 80, PEG 200, PEG 400, PEG 600, miglyol, soybean oil, liquid paraffin, 2-pyrrolidone,

Transcutol (diethylene glycol monoethyl ether), water solution of 1% w/v β-CD, 1% w/v albumin, 0.55 w/v Tween 20, 50 and 200 mg/ml TPGS, 5 mg/ml Lutrol F68, 15 mg/ml Lutrol F127, 10 mg/ml Brij 78, 4% w/v lauric acid in EtOH:H_2O (50:50). The samples were left overnight at room temperature, under magnetic stirring. The following morning, the vehicles in which IMQ was not dissolved (solubility < 1 mg/ml), were discarded. On the contrary, if IMQ was completely dissolved (solubility > 1 mg/ml), an excess amount of IMQ was added, and, after 24 hour mixing, the suspension was centrifuged and/or filtered (0.45µm), diluted and analysed by HPLC-UV for the accurate determination of the solubility.

2.4. Accumulation and permeation experiments

For permeation experiments, porcine skin was used. The skin was excised from the outer part of pig ears within 3 hours from animal death, separated from the underlying cartilage with a scalpel and frozen at -20°C until use. All tissues were used within 3 months. The skin, once thawed, was mounted on vertical diffusion cells (DISA, Milano, Italy; 0.6 cm^2 surface area) with the stratum corneum facing the donor compartment. The receptor compartment was filled with 1% w/v albumin solution in PBS pH 7.4 (IMQ solubility: 143 ± 3 µg/ml). IMQ saturated solutions in Transcutol, Tween 80, 2-pyrrolidone, propylene glycol, PEG 200, PEG 400, PEG 600, DMSO and oleic acid were tested. The commercial formulation Imunocare (IMQ 5% w/w, Difa Cooper, Caronno Pertusella, Italy) and three microemulsions, whose composition is reported in Table 3, were also evaluated. The donors were applied for 6 hours at infinite dose (200 mg/cm^2, occluded). Some experiments were performed on tape-stripped skin (i.e. skin without the SC) and across isolated dermis. In order to prepare stripped skin, the tissue was tape-stripped (Scotch Booktape #845, 3M Co., St Paul, MN USA) until the complete removal of the stratum 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) trypsine in pH
170 7.4 PBS, at 4°C for 15 hours. Epidermis was removed with a cotton swab and SC sheets

obtained were individually rinsed 3 times with distilled water, dried in oven at 37°C for 1 h, and kept in a dessiccator on $CaCl_2$ until use.

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

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

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

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

2.6. Preparation of microemulsions

Microemulsions were prepared by mixing oleic acid, Transcutol, Tween 80 and water in the proportions (w/w) indicated in Table 3. An eccess amount of IMQ was then added and the suspention was stirred overnight at room temperature, after which the ME were centrifuged, filtered (0.45 µm) and analysed by HPLC-UV.

2.7. Statistical analysis

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

193 experimental points are represented as mean value ± 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±6 %) and PEG400 : methanol : HCl 1M (1:2:2) for dermis (recovery 97±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

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

3.2. Imiquimod solubility

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

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

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

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

DMSO	78.13	13.4 [b]	1.29 ±0.13
PG	76.09	14 [b]	0.60 ±0.03

[a]Average MW

[b](Vaughan, 1985)

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

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

3.3. Imiquimod skin accumulation and permeation

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

Table 2. Amount of IMQ accumulated from saturated solutions applied for 6 h in infinite dose conditions (average ± SD)-

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

Some general considerations can be made: despite the relatively low molecular weight and the favourable log P (calculated log D pH 7.4 : 2.65 (Advanced Chemistry Development Inc., 2015)), IMQ skin penetration properties are very poor and the data are very variable. The presence of albumin in the receptor compartment ensured sink conditions (IMQ solubility: 143 ± 3 µg/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 µg/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, µg/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 µg/cm^2 in case
275 of oleic acid (solubility 74 mg/ml), and 0.18 µg/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 ≈ 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 termodynamic activity (and skin interaction - if any), generate
283 concentration-dependent skin accumulation.

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

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

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

3.4. SC uptake experiments

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

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

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

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

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

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

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

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

3.5. Formulations containing IMQ

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

We formulated IMQ into a vehicle suitable for skin administration, taking into account the information collected until here on the role of solvent uptake. We decided to prepare microemulsions, since Hatout *et al*. have studied the penetration of ME components into the SC and the results suggest a more efficient uptake of the components when they are 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

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

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

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

4. CONCLUSION

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

The work on imiquimod skin retention from pure solvents represented the starting point for the preparation of microemulsions. The results show that 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 is 12 times lower.

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

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532

533

FIGURE LEGEND

Figure 1. *Imiquimod*

Figure 2*. IMQ solubility as a function of the average PEG MW (average± SD).*

Figure 3*. IMQ skin (epidermis+dermis) retention (µg/cm^2) and % of IMQ accumulated starting from saturated solutions. The data are represented as mean ± sem. In the insert, IMQ skin accumulation is represented as a function of solubility in PEGs.*

Figure 4*. IMQ skin accumulation (µg/cm^2; average* ± sem*) starting from saturated solutions in PEG (12.8 mg/ml) and oleic acid (73.8 mg/ml) following the application on intact skin (white bar), SC stripped skin (grey bar) and isolated dermis (black bar). Asterisks indicate that the accumulation is statistically higher with respect to full thickness skin *p<0.05;** p<0.01.*

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

Figure 6. *Correlation between the amount of IMQ extracted from the SC and the theoretical amount calculated considering the solvent uptake (ml/cm^2) and the IMQ concentration (µg/ml)*

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

Figure 8. *IMQ accumulation in the skin (µg/cm^2; average* ± sem*; gray bars) from the commercial formulation and from the saturated ME. As a comparison, the value obtained from the oleic acid saturated solution is also reported. * significantly different form oleic acid saturated solution (p<0.01); # significantly different from Imunocare (p<0.05). Red circles indicate on Y2 axis the IMQ concentration (% w/w) in the formulations tested. The insert illustrates the accumulation efficiency (% accumulated) of MEs as a function of water content.*

Figure 1

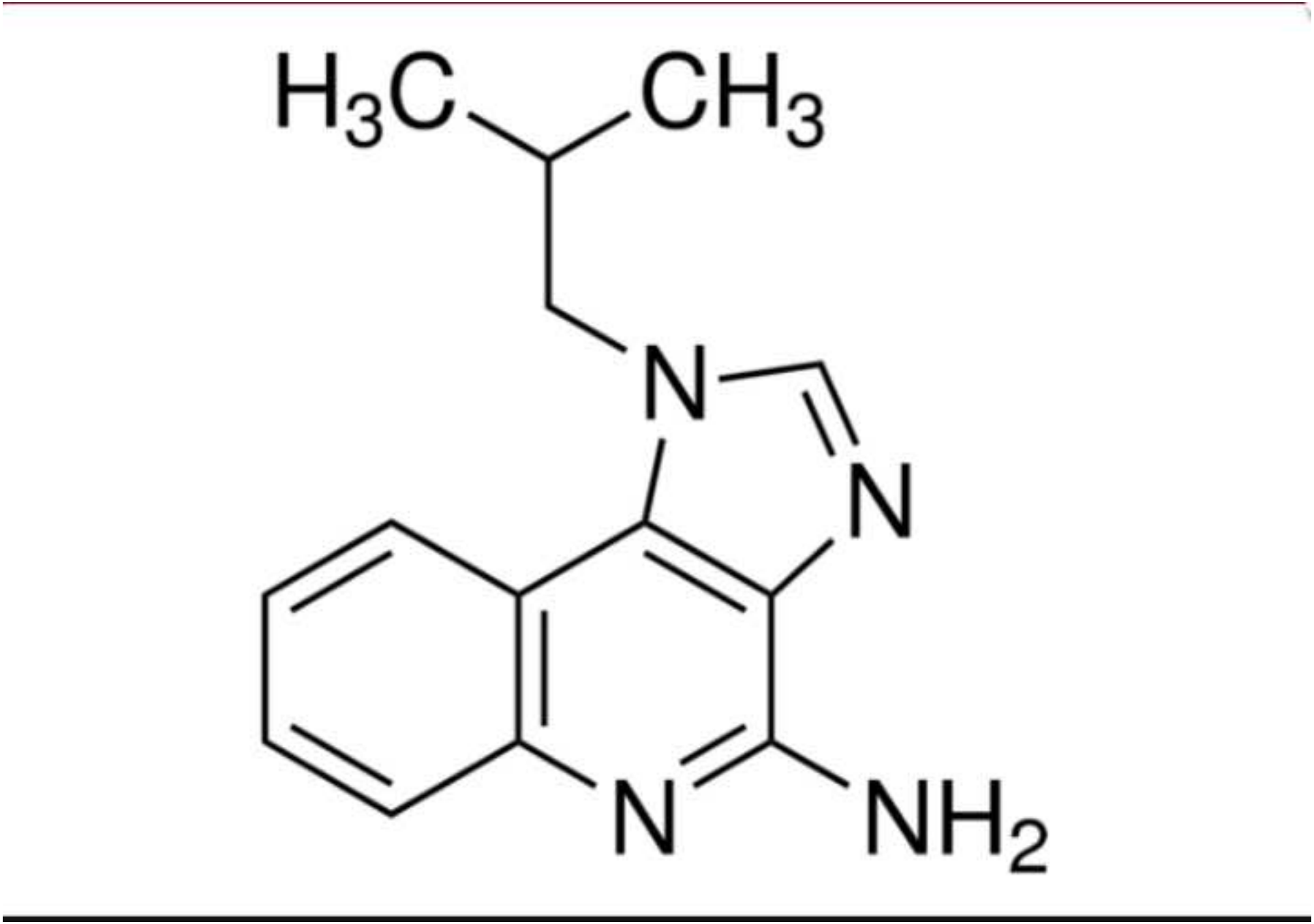


Figure 2

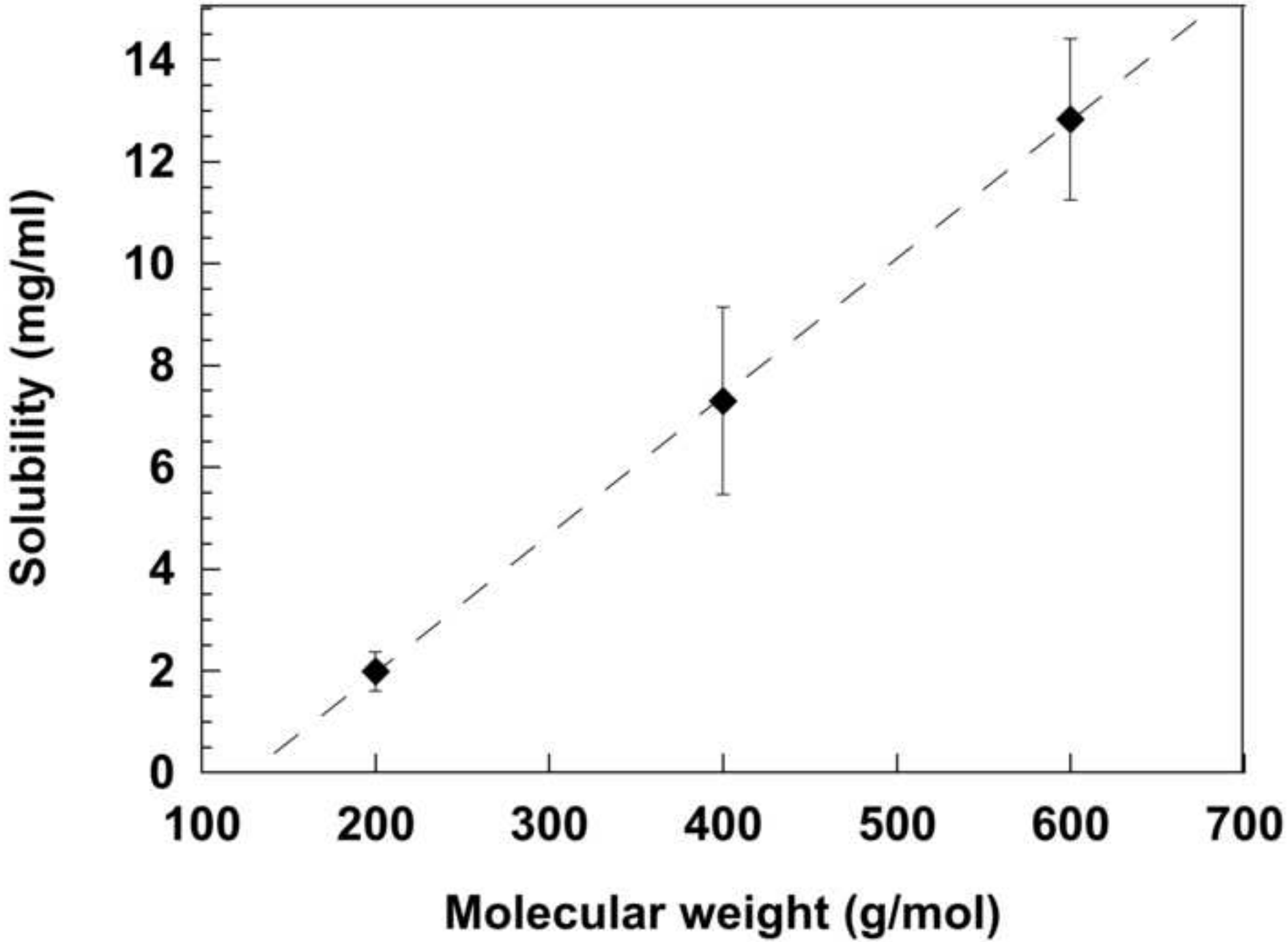

Solubility (mg/ml)
0
2
4
6
8
10
12
14
100
200
300
400
500
600
700
Molecular weight (g/mol)

Figure 3

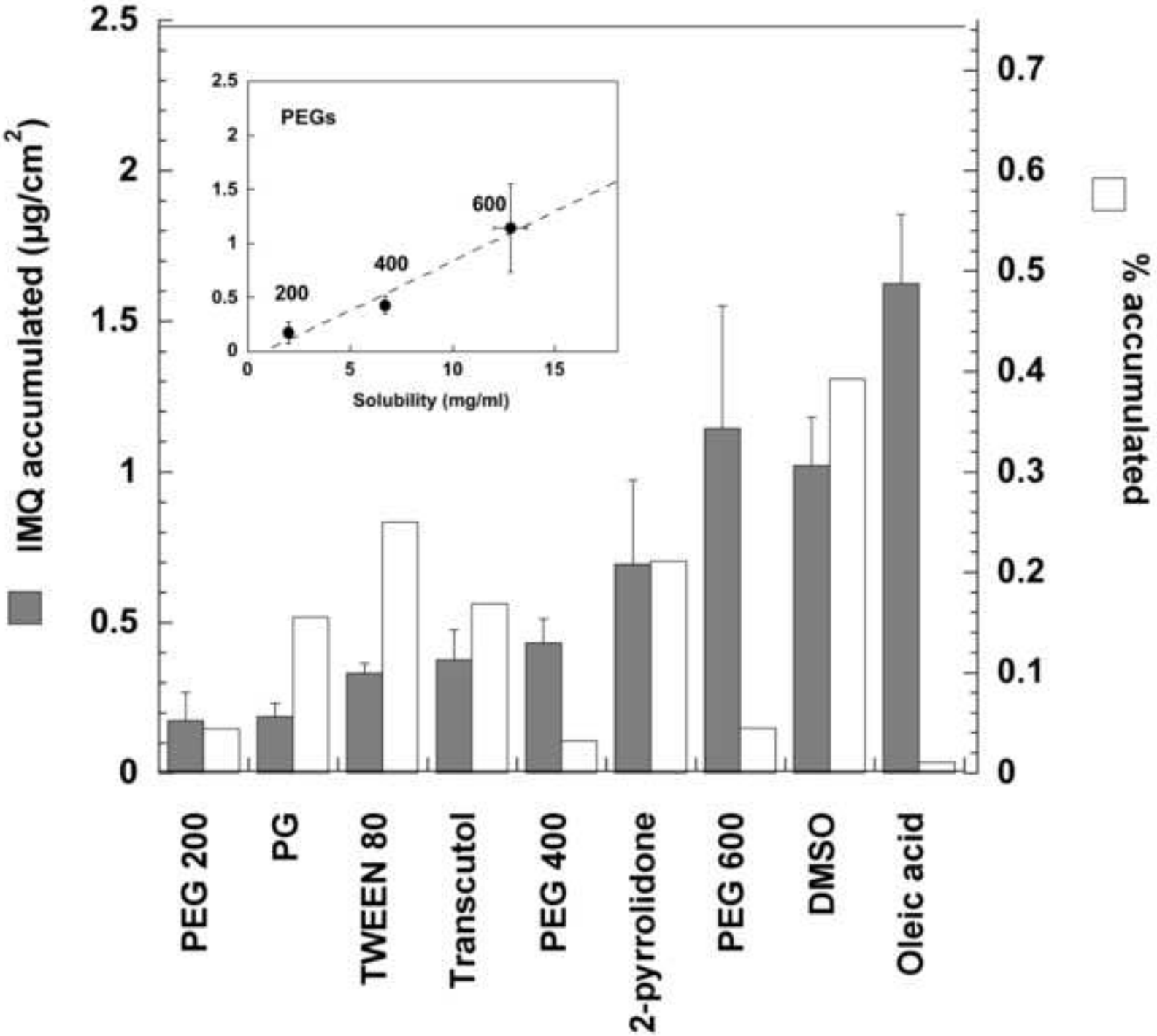

IMQ accumulated (µg/cm²)
% accumulated
2.5
2
1.5
1
0.5
0
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PEGs
200
400
600
Solubility (mg/ml)
PEG 200
PG
TWEEN 80
Transcutol
PEG 400
2-pyrrolidone
PEG 600
DMSO
Oleic acid

Figure 4

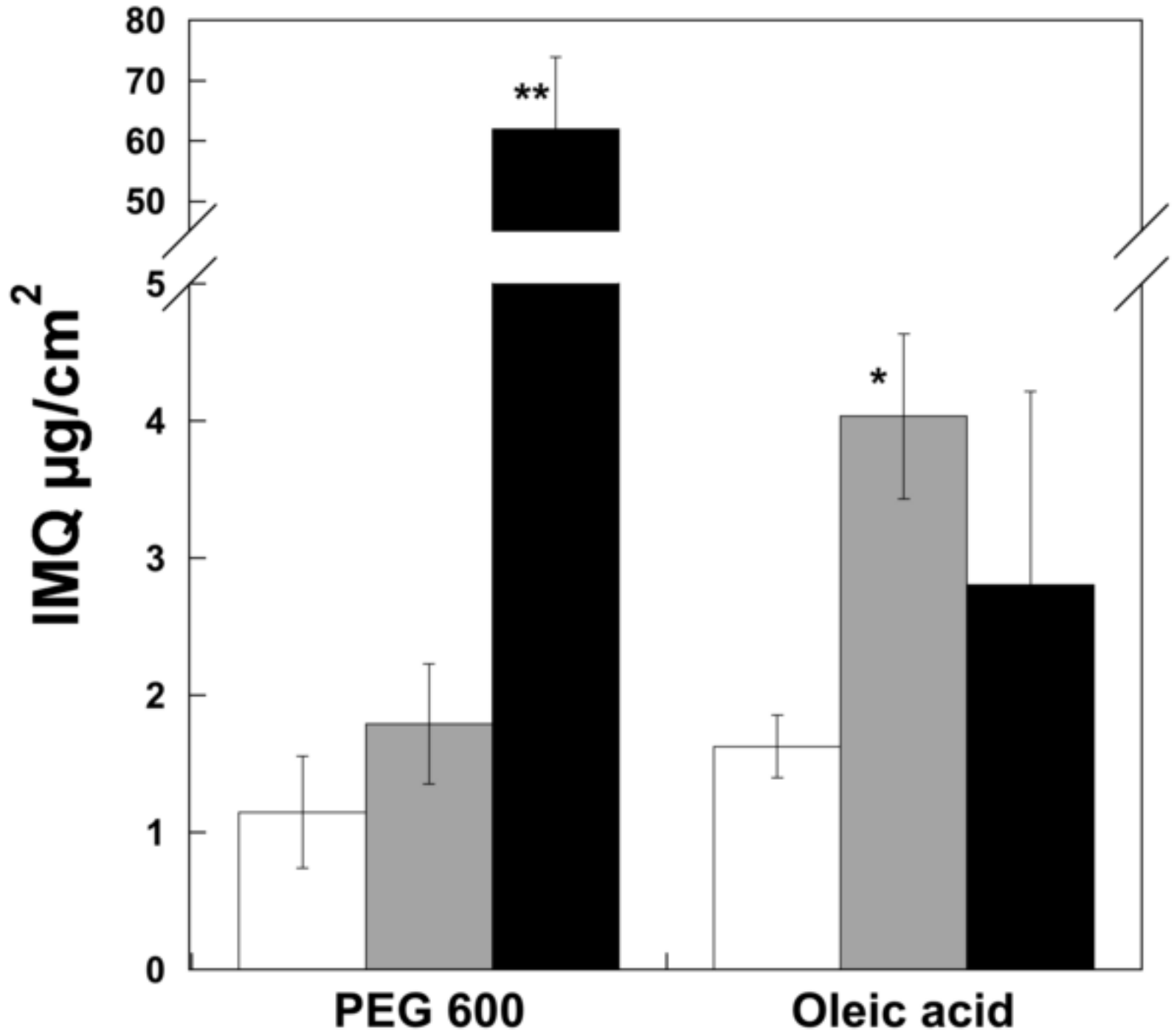


Figure 5

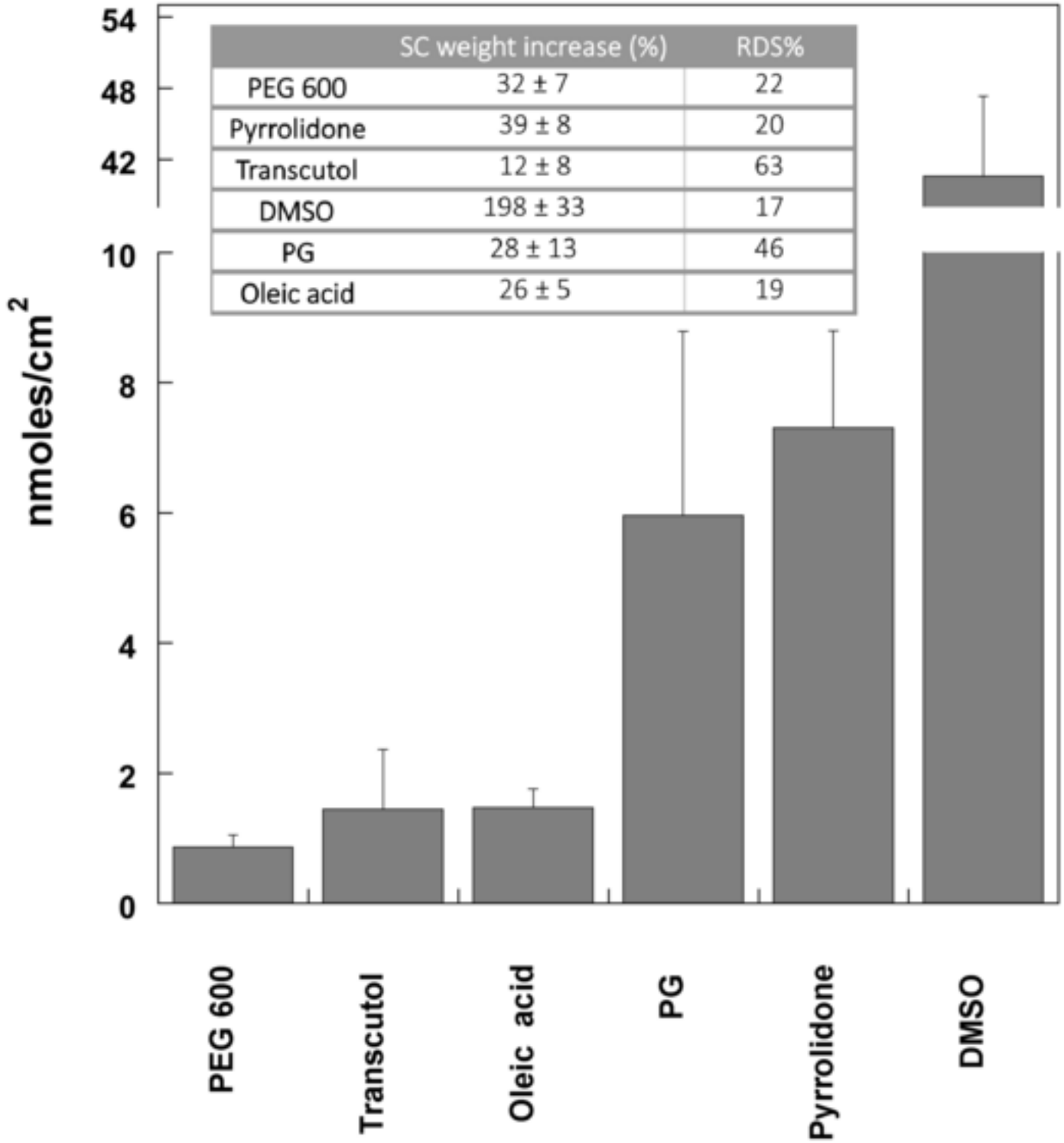

SC weight increase (%)
RDS%
PEG 600
32 ± 7
22
Pyrrolidone
39 ± 8
20
Transcutol
12 ± 8
63
DMSO
198 ± 33
17
PG
28 ± 13
46
Oleic acid
26 ± 5
19
nmoles/cm2
54
48
42
10
8
6
4
2
0
PEG 600
Transcutol
Oleic acid
PG
Pyrrolidone
DMSO

Figure 6

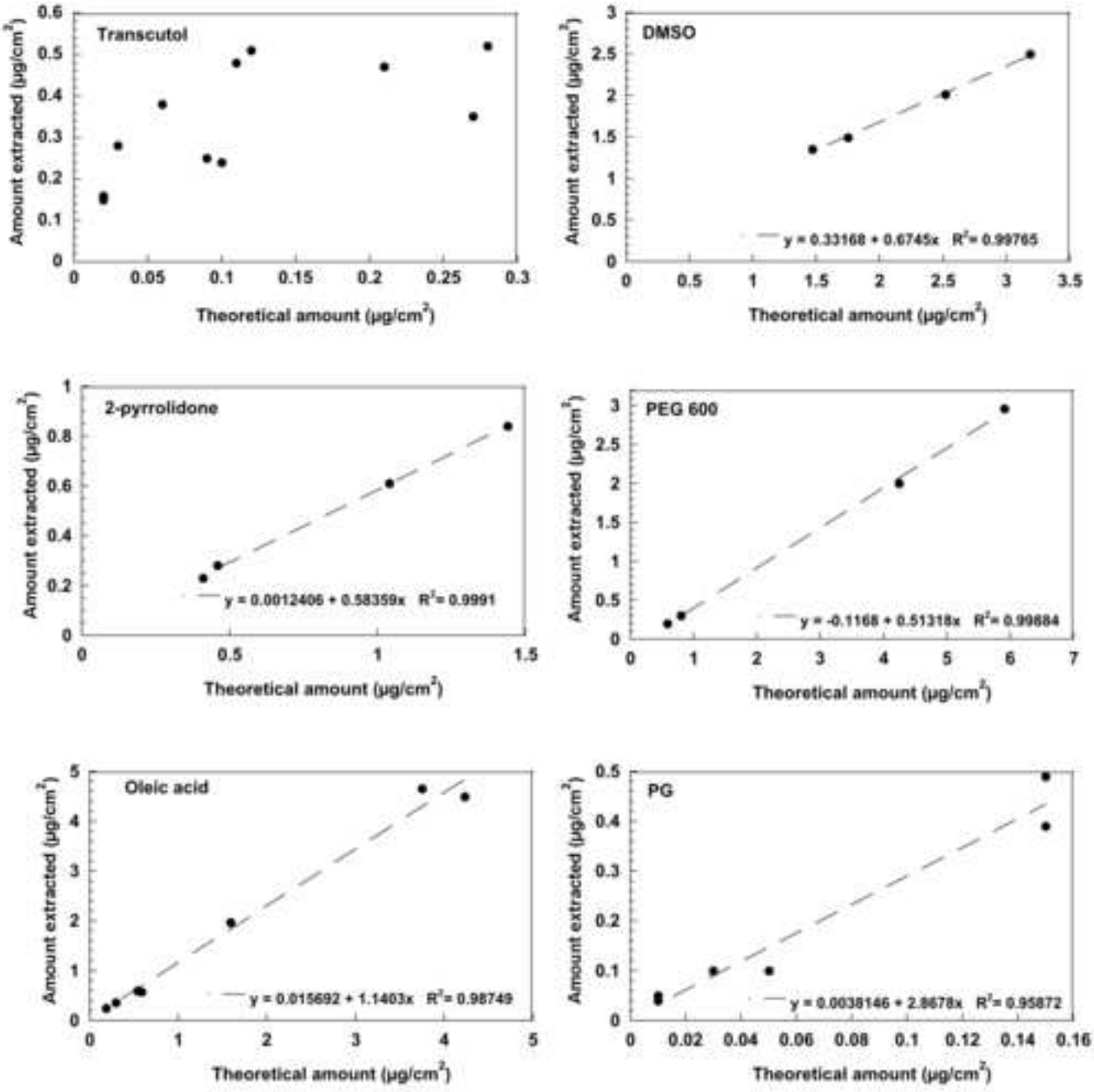

Transcutol
Amount extracted (µg/cm²)
Theoretical amount (µg/cm²)
DMSO
y = 0.33168 + 0.6745x R²= 0.99765
2-pyrrolidone
y = 0.0012406 + 0.58359x R²= 0.9991
PEG 600
y = -0.1168 + 0.51318x R²= 0.99884
Oleic acid
y = 0.015692 + 1.1403x R²= 0.98749
PG
y = 0.0038146 + 2.8678x R²= 0.95872

Figure 7

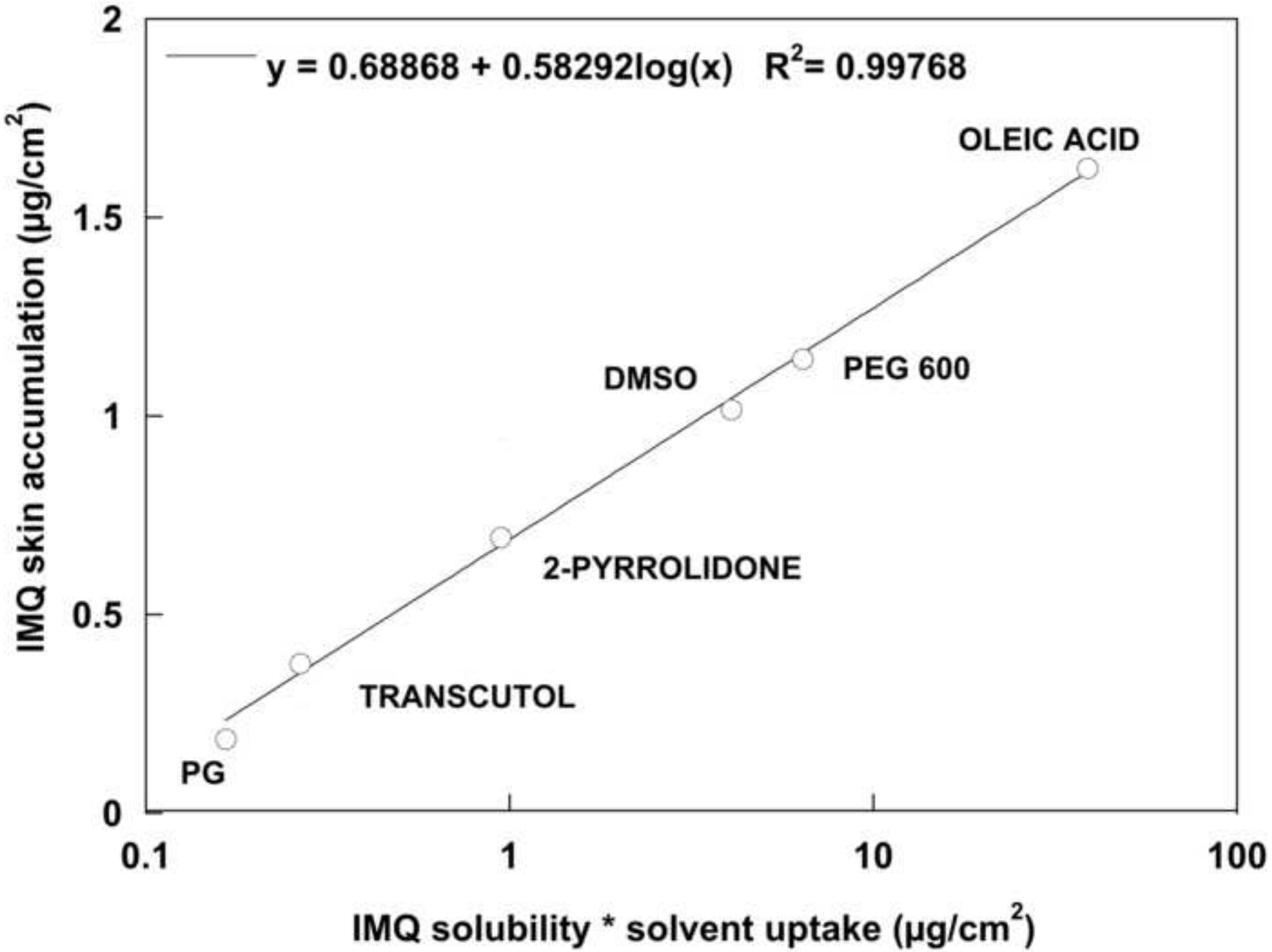
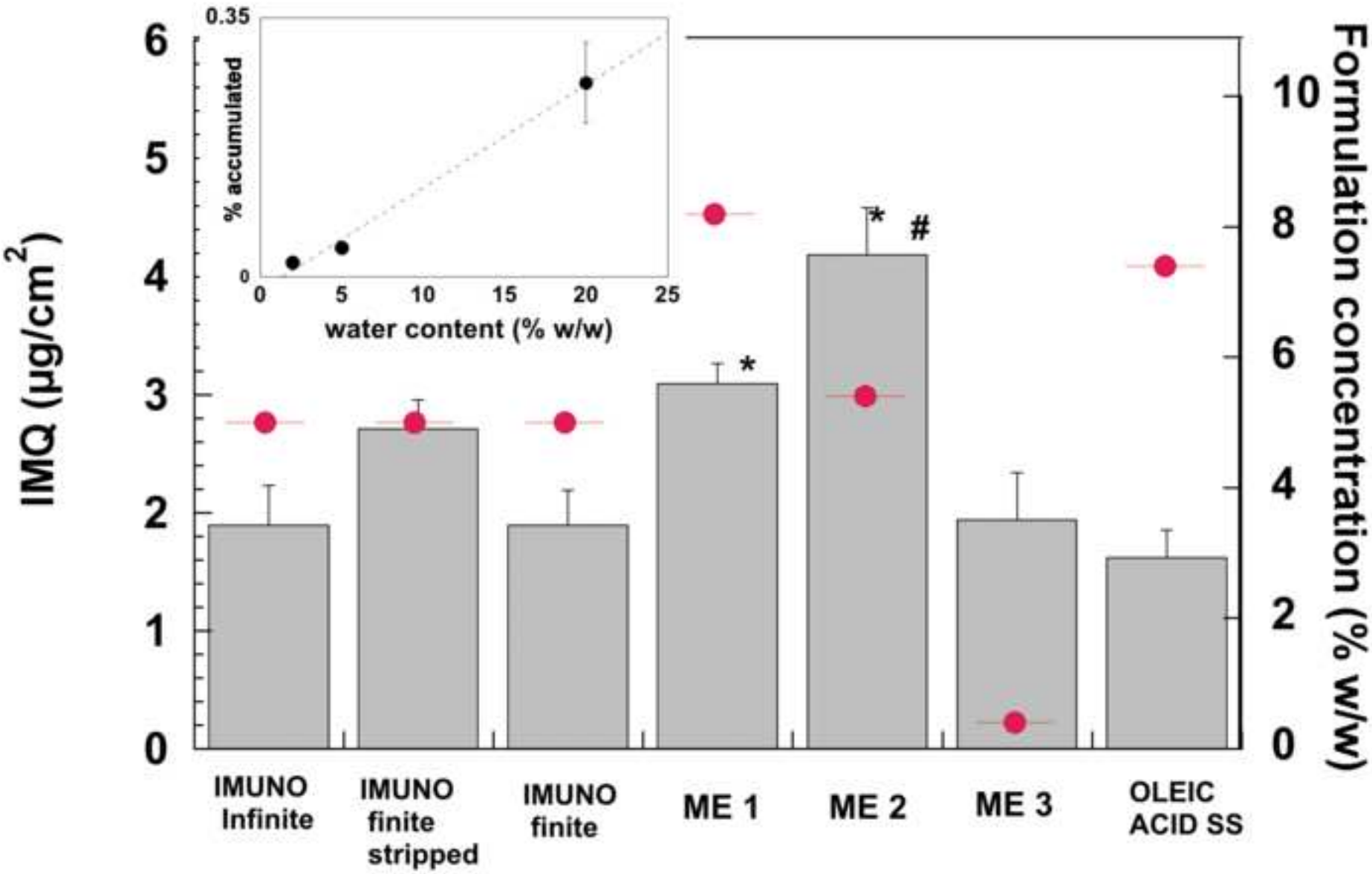

y = 0.68868 + 0.58292log(x) R²= 0.99768
OLEIC ACID
PEG 600
DMSO
2-PYRROLIDONE
TRANSCUTOL
PG
IMQ skin accumulation (µg/cm²)
IMQ solubility * solvent uptake (µg/cm²)
2
1.5
1
0.5
0
0.1
1
10
100

Figure 8 revised



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