Check for updates

Original Research Paper

Natural Product Communications
Volume 17(7): 1–12
© The Author(s) 2022
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/1934578X221112811
journals.sagepub.com/home/npx

Fast Inverted Photoprotective o/w Emulsions Loaded With Dihydroquercetin and β-Carotene: An Innovative Approach to *In Vitro* Assessment of Antioxidant Activity in a Bioenvironment

Radava Martić[1], Jelena Kotur-Stevuljević[2], Anđelija Malenović[3], Ljuboš Ušjak[4], Silvana Petrović[4], Bojan Čalija[1], Jela Milić[1], and Danina Krajišnik[1]

Abstract

Fast inverted, oil-in-water (o/w) emulsions, also known as SWitch-Oil-Phase (SWOP) emulsions, express the performances of both o/w and water-in-oil (w/o) emulsions during application to the skin, favoring their use as cosmetic carriers in sunscreen products. The objective of this study was to investigate the antioxidant potential (by 2 different methods) and the ultraviolet (UV) absorption ability of SWOP emulsion (S) with incorporated plant-based antioxidants dihydroquercetin (DHQ) and β-carotene (βC), using quercetin (Q) in a reference emulsion, in addition to the evaluation of their physicochemical properties and stability. A new biochemical extracellular model for *in vitro* assessment of antioxidative properties for the SWOP emulsions (S, S_{Q}, S_{DHQ}, and $S_{DHQ\beta C}$) was developed and compared with the results of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay. The analyses were performed at 20 °C and 37 °C, and oxidative stress parameters were monitored and statistically analyzed. The sun protection factor (SPF) of the samples was determined *in vitro*. Q and DHQ incorporated into the SWOP emulsion exhibited a strong DPPH radical scavenging ability. Neither incorporated nor pure βC showed DPPH radical scavenging ability at the tested concentrations. Contrary to that, in the bioenvironment conditions, $S_{DHQ\beta C}$ showed minor antioxidative effects increase and also a significant decrease in exogenous pro-oxidative effects, caused by pro-oxidant, when compared to S_{DHQ}. The obtained SPFs of $S_{DHQ\beta C}$, S_{DHQ}, and S_{Q} were 5.19, 4.65, and 3.35, respectively. The physicochemical stability of the emulsions was satisfactory during 1 month storage. The presented results demonstrated that the SWOP emulsion is a suitable carrier for antioxidants with a photoprotective ability. The novel biochemical approach could be used in addition to DPPH assay with several advantages, relevant for the testing of antioxidant activity of potential active ingredients in cosmetic products.

Keywords

SWOP emulsion, plant-based antioxidants, flavonoids, antioxidant activity, bioenvironment, photoprotection, statistics

Received: March 24th, 2022; Accepted: June 21st, 2022.

Introduction

As the largest organ in the human body, skin is exposed to numerous exogenous environmental factors, but ultraviolet (UV) radiation is recognized as an essential risk factor affecting skin physiology. Based on photon wavelength, the UV radiation from the sun consists of UVA (315-400 nm), UVB (280-315 nm), and UVC (100-280 nm) bands.[1] Chronic exposure to UV radiation leads to many side effects on the skin (thickening of the stratum corneum, epidermis, and dermis,[2] wrinkling of the skin and photoaging,[3] that are directly related to the formation of reactive oxygen species (ROS),[4] supporting the rational use of antioxidants in topical formulations.[5] The use of antioxidants in skin care is further supported by the reported efficacy in the enrichment of the endogenous cutaneous protection system after topical administration.[5] Additionally, the use of natural ingredients incorporated in innovative and efficient formulations becomes a world tendency along with cosmetic product development, especially in the

[1]Department of Pharmaceutical Technology and Cosmetology, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia
[2]Department of Medical Biochemistry, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia
[3]Department of Drug Analysis, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia
[4]Department of Pharmacognosy, University of Belgrade – Faculty of Pharmacy, Belgrade, Serbia

Corresponding Author:
Danina Krajišnik, Department of Pharmaceutical Technology and Cosmetology, University of Belgrade – Faculty of Pharmacy, Vojvode Stepe No. 450, Belgrade, 11221, Serbia.
Email: danina.krajisnik@pharmacy.bg.ac.rs

Creative Commons Non Commercial CC BY-NC: This article is distributed under the terms of the Creative Commons Attribution-NonCommercial 4.0 License (https://creativecommons.org/licenses/by-nc/4.0/) which permits non-commercial use, reproduction and distribution of the work without further permission provided the original work is attributed as specified on the SAGE and Open Access page (https://us.sagepub.com/en-us/nam/open-access-at-sage).

photoprotection market, reinforced by population acceptance and the media. Active ingredients from natural products containing chromophores, aromatic rings, and compounds with antioxidant properties provide novel possibilities for the treatment and prevention of UV-caused oxidative damages.[6]

Flavonoids, a group of polyphenolic compounds originating from plants, are interesting for the pharmaceutical, cosmetic, and food industries because of their variety of possible beneficial properties. Antioxidant activity is one of the most important properties of flavonoids.[7] Besides being scavengers of free radicals, flavonoids reduce their production due to UV absorption ability. Flavonoids cannot replace traditional synthetic sunscreens; however, in combination with commercial sunscreens, they could significantly improve the sun protection factor (SPF), the UVA protection factor (UVA-PF), and the photostability of formulations due to their additive and synergistic effects.[2]

Dihydroquercetin (DHQ) is a flavonoid that acts as a powerful antioxidant having a similar antioxidant activity profile to that of α-tocopherol.[8] It is known that DHQ absorbs over a wide range of the UV spectrum, showing maximum absorption at 225 nm and 325 nm and helps prevent skin damage caused by external agents, alleviates skin inflammation, soothes irritated skin,[9] and even suppresses UV-induced skin carcinogenesis.[3] These properties of DHQ, as well as its strong safety profile, indicate that it could be interesting for further investigations, especially in combination with other UV filters and/or antioxidants incorporated into a suitable cosmetic carrier.

Vitamins A, C, E, carotenoids, and various enzymes, such as glutathione peroxidase (GPx), catalase (CAT), and superoxide dismutase (SOD), are the most common antioxidants in human skin.[10] Applied topically, these antioxidants are able to enrich the defense potential of the upper layers of human skin against the action of environmental hazards and irradiations.[11] Additionally, antioxidants are added to cosmetic products to increase their stabilization and protect active ingredients from oxidation/degradation.[5]

Various *in vivo*, *ex vivo*, and *in vitro* methods have been developed for the evaluation of antioxidant activity, but many of them cannot be used in the cosmetic industry. Whenever possible, it is recommended to use noninvasive instrumental methods or *in vitro* tests according to the EU Regulation (EC) No. 1223/2009 and this provision is accepted by many other countries worldwide. Among *in vitro* free radical scavenging methods, the 2, 2-diphenyl-1-picrylhydrazyl (DPPH) assay is the most commonly used. However, standard *in vitro* tests cannot provide data on activity in contact with a bioenvironment and do not reflect the actual biological processes in skin cells.[12, 13]

Classical sunscreen formulations include the most popular, worldwide, emulsion products such as creams/gel creams and lotions. Water-in-oil (w/o) emulsions are preferred for sunscreens since they can provide high SPF and water resistance. Despite many desirable characteristics, w/o emulsions generally contain more oils than water and their greasiness and a heavier feel makes them less favored in comparison with oil-in-water (o/w) emulsions.[14] Therefore, fast inverted o/w emulsions, also known as SWitch-Oil-Phase (SWOP) emulsions, which invert into w/o emulsions during application to the skin and form a lipophilic, water-resistance layer, are especially suitable as carriers in sun protection products.[15]

The aim of this study was the preparation of SWOP emulsions with incorporated plant-derived antioxidants (DHQ and β-carotene [βC]) and the investigation of their antioxidant potential and UV absorption ability in addition to physicochemical properties and stability. For these purposes, a comparative SWOP emulsion with the well-known plant-derived antioxidant quercetin (Q) was used as a reference emulsion. Antioxidant potential of the incorporated and pure plant-derived antioxidants was determined using the DPPH radical scavenging assay. To estimate and evaluate the antioxidant activity in a bioenvironment, a new *in vitro* extracellular biochemical model was developed. Antioxidant properties of different plant-based ingredients were monitored regarding their influence on oxidative stress parameters and the parameters of antioxidative protection. The UV absorption ability of the SWOP emulsions was estimated by an *in vitro* spectrophotometric method.

Materials and Methods

Materials

SWOP emulsion base (labeled as S) and SWOP emulsions with incorporated Q, DHQ, and a combination of DHQ and βC (labeled as S_Q, S_{DHQ}, and $S_{DHQ\beta C}$, respectively) were prepared according to the compositions presented in Table 1. As stated in our previous works,[16, 17] the SWOP emulsions were stabilized with a combination of the nonionic emulsifier polyglyceryl-2 dipolyhydroxystearate (Dehymuls® PGPH, BASF, Germany), an anionic surfactant that is a mixture of lauryl glucoside and sodium lauryl glucose carboxylate (Plantapon® LGC Sorb, BASF, Germany), and the polymeric stabilizer sodium polyacrylate (Cosmedia® SP, BASF, Germany).

The oil phase (Phase I) consisted of liquid paraffin (RA.M.Oil S.p.A., Italy), caprylic/capric triglycerides (Myritol® 318, BASF, Germany), *Helianthus annuus* seed oil (Dermafeel Sunflower Oil, Amedeo Brasca & C.S.r.l., Italy), cetearyl alcohol (Lanette® O, BASF, Germany) and, as a preservative, propylparaben (Schülke & Mayr, Germany). Glycerin (Cremer Oleo GmbH & Co. KG, Germany), propylene glycol (PG) (BASF, Germany), methylparaben (Schülke & Mayr, Germany), as a preservative, and purified water were used as ingredients of the aqueous phase (Phase II). DHQ (Taxifoliol®, 90%), Q, and βC (Beta Carotene CWD®, 10%), all donated by Kingherbs Limited, China, were used as cosmetic active ingredients.

All reagents used for antioxidant capacity measurement were of the highest purity available and obtained from Sigma Aldrich (Dorset, United Kingdom).

Methods

Preparation of the Emulsions. The SWOP emulsions, labeled as S, S_Q, S_{DHQ}, and $S_{DHQ\beta C}$, were prepared by hot–hot emulsification,

Table 1. Compositions of the Investigated Emulsions According to[16, 17].

Composition		Emulsions (% w/w)			
		S	S_Q	S_{DHQ}	$S_{DHQ\beta C}$
Phase I	Dehymuls® PGPH *Polyglyceryl-2-dipolyhydroxystearate*	4.0	4.0	4.0	4.0
	Lanette® O *Cetearyl alcohol*	2.0	2.0	2.0	2.0
	Liquid paraffin *Paraffinum liquidum*	8.0	8.0	8.0	8.0
	Myritol® 318 *Caprylic/capric triglyceride*	8.0	8.0	8.0	8.0
	Dermafeel sunflower oil *Helianthus annuus (Sunflower) seed oil*	8.0	8.0	8.0	8.0
	Propylparaben	0.05	0.05	0.05	0.05
Phase II	*Glycerin*	3.0	3.0	3.0	3.0
	Propylene glycol (PG)	10.0	8.0	8.0	8.0
	Plantapon® LGC Sorb *Sodium lauryl glucoside carboxylate, lauryl glucoside*	1.5	1.5	1.5	1.5
	Methylparaben	0.1	0.1	0.1	0.1
	Cosmedia® SP *Sodium polyacrylate*	0.8	0.8	0.8	0.8
	Water *Aqua*	54.55	54.05	54.05	46.05
Phase III	Taxifoliol® *Dihydroquercetin (DHQ)*	0	0	0.5	0.5
	Quercetin (Q)	0	0.5	0	0
	PG	0	2.0	2.0	2.0
	Beta Carotene CWD® *β-carotene (βC)*	0	0	0	2.0
	Water *Aqua*	0	0	0	6.0

as previously reported for SWOP emulsion base.[16, 17] The active substances Q, DHQ, and βC, were dispersed in an appropriate solvent (Table 1, Phase III), and added to the emulsions while stirring at 40 °C. All the prepared samples were left for 48 h to equilibrate before further testing.

Physicochemical Properties of Prepared Emulsions. Freshly prepared emulsions were investigated by visual monitoring (general appearance, color, consistency of the emulsions, homogeneity of the emulsions, and occurrence of phase separation).

pH and conductivity measurements, rheological characterization, and differential scanning calorimetry were performed in a similar manner, as previously described.[16, 17] pH and conductivity measurements were performed in triplicate at room temperature (20 ± 2 °C). Continuous flow tests for the prepared emulsions (S, S_{DHQ}, and $S_{DHQ\beta C}$) were performed in triplicate after 48 h and 30 days' storage at room temperature (20 ± 2 °C). Finally, the samples analyzed by differential scanning calorimetry (DSC) were heated from 25 °C to 130 °C, at a heating rate of 5 K min^{-1} to compare the endothermic transition enthalpies of the test and reference samples. All the samples were measured in duplicate at least.[17]

Having applied a comprehensive analysis that included the aforementioned methods, alongside contact angle measurements and polarization microscopy, the colloidal structure, the phase inversion, and the sensorial properties of SWOP emulsions were discussed and explained in our previous papers.[16, 17]

In Vitro Determination of Antiradical Activity by DPPH Assay. The radical scavenging ability of Q, DHQ, βC, and SWOP emulsions containing Q (S_Q), DHQ (S_{DHQ}), and DHQ-βC ($S_{DHQ\beta C}$) was tested by DPPH assay.[18] Different aliquots of stock solutions of pure flavonoids Q and DHQ (0.25 mg mL^{-1}), βC (1 mg mL^{-1}), and SWOP emulsions (5 mg mL^{-1}) in PG (or water in the case of βC and SWOP emulsion containing βC) were diluted to 2 mL with 99.9% ethanol, and then 0.5 mL of 0.5 mM DPPH solution in ethanol was added to each dilution. Mixtures were vigorously shaken and left in the dark for 30 min. Absorbances were measured at 517 nm (and additionally at 540 nm for pure βC and SWOP emulsion containing βC) using corresponding mixtures of ethanol and PG (or water) as blanks. One mL of 0.5 mM DPPH solution diluted with 4 mL of the corresponding solvent mixture was used as a negative control. Scavenging of DPPH radical, S(%), was calculated using the following equation:

$$S(\%) = 100 \times (A_0 - A_s) / A_0 \quad (1)$$

where A_0 is the absorbance of the control and A_s is the absorbance of the tested sample. The SC_{50} value represented the concentration of the sample that caused the scavenging of 50% DPPH radicals. The results were expressed as the means of three determinations ± standard deviation. To determine whether there is a statistically significant difference ($\alpha = 0.05$) between the SC_{50} values of pure compounds and compounds incorporated into SWOP emulsions, one-way ANOVA, followed by Tukey's post hoc test was carried out using SPSS version 20.0.

In Vitro Antioxidant Activity of the Tested Emulsions in a Bioenvironment. Serum samples were collected from healthy individuals after regular daily work in a clinical chemistry laboratory of the Military Medical Academy (Belgrade) in order to make the serum pool. Healthy volunteers, who had attended regular medical check-ups and had given written approval that any serum leftover after biochemical analyses ordered by physicians could be used for our antioxidative potency investigation, were included in this study. The members of the study team did not use any of the data connected with subjects whose serum leftovers were used. The Ethical Committee (decision No. 797/2, May 5, 2012. University of Belgrade – Faculty of Pharmacy) has verified this procedure for sample collection. Samples were selected according to basic biochemical parameter results, ie, those whose metabolites were within reference ranges were used, as an additional confirmation about the subject's health status. Serum samples were pooled and aliquoted, then frozen at −80 °C and used for redox status analytics no more than 2 weeks after the collection.[19]

The test samples were prepared by diluting each emulsion (S, S_Q, S_{DHQ}, and $S_{DHQ\beta C}$) to 1% in PG, and dissolving antioxidants (Q and DHQ) to 0.005% in PG. Serum volume was 450 μL and the added volume of each previously prepared test sample was 50 μL (1:10 ratio), ie, the exogenous addition did not exceed 10% of the total sample volume, which is of the utmost importance to samples' bio-matrix quality provision. For probes with the well-known pro-oxidant tert-butyl-hydroperoxide (TBH, conc. 0.25 mmol L^{-1}), 25 μL of each previously described sample (prepared in double concentration) and 25 μL TBH were added to 450 μL serum samples. All the samples were incubated in duplicate for 2 h, at either 20 °C or 37 °C. This model enabled us to estimate antioxidative potency of carotenoid and polyphenolic compound-enriched emulsions in a bioenvironment by measuring several redox status parameters: pro-oxidants (total oxidant status—TOS, pro-oxidant-antioxidant balance—PAB, and advanced oxidation protein products—AOPP) and antioxidants (total antioxidant status—TAS, serum paraoxonase-1—PON1, and total protein sulfhydryl groups—SHG), to some extent, similar to human skin.

The colorimetric assay based on the oxidation of ferrous ion to ferric ion in an acidic medium in the presence of different oxidant species such as H_2O_2 and lipid hydroperoxides, was used for TOS measurement.[20] AOPP were determined by the Witko-Sarsat method, in a reaction with glacial acetic acid and potassium iodide.[21] The assay used for measurement of PAB is based on complex reactions of 3,3′, 5,5′-tetramethylbenzidine and its cation used as a redox indicator, which is simultaneously developing.[22] TAS was determined with a novel automated colorimetric method with ABTS cation developed by Erel.[23] PON1 activity was measured as the rate of paraoxon (POase) hydrolysis, according to Richter and Furlong.[24] SHG in serum (mmol/L) was determined by the method of Ellman,[25] using 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB). Values of the monitored parameters were analyzed using Kruskal–Wallis test and Mann–Whitney post hoc test, and the statistical significance was set at the 0.05 value.

Pro-oxidative score, antioxidative score, and oxy score (OS) were calculated from separate redox status parameters using Z score statistics. OS was calculated as the difference between pro-oxidative score (average value of Z scores of all measured pro-oxidants and their products, ie, TOS, AOPP, and PAB) and antioxidative score (average value of Z scores of all measured antioxidants, ie, TAS, PON1, and SHG). Z scores represent the difference between the original value and the control value divided by the SD of control values (or population means and SDs). A higher OS of substance means weaker antioxidative protection and higher pro-oxidants content and vice versa.[26]

Spectrophotometric Determination of SPF. A quantity of 0.25 g of the emulsions S, S_Q, S_{DHQ}, and $S_{DHQ\beta C}$ were weighed, transferred to a 25 mL volumetric flask, and diluted to volume with ethanol. Further, it was kept for ultrasonication for 5 min and then filtered through cotton, disposing of the first 10 mL. Then, a 2 mL aliquot (filtered through filter paper) was transferred to a 10 mL volumetric flask and the volume was adjusted with ethanol.

The absorption spectra of the prepared samples were obtained in the range of 290 to 320 nm at intervals of 5 nm using a UV/Visible spectrophotometer (UV-VIS Cintra 202, GBC Scientific Equipment, Australia). Three determinations were made at each point using ethanol as a blank.

SPF values were calculated using a mathematical equation (Eq. 2)[2] developed by Mansur et al[27] with an intention to substitute the *in vitro* method proposed by Sayre et al[4, 28]:

$$SPF_{spectrophotometric} = CF \times \sum_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda) \qquad (2)$$

where EE—erythemal effect spectrum; I—solar intensity spectrum; Abs—absorbance of sunscreen product; and CF – correction factor (= 10).

The values of EE × I, presented in Table 2, are constants and predetermined by Sayre et al[28]

Results and Discussion

Evaluation of Physicochemical Properties of the SWOP Emulsions

Organoleptic properties and several parameters which include pH value, conductivity, and viscosity of the emulsions were

Table 2. Normalized Product Function Used in the Calculation of Sun Protection Factor (SPF).[4, 28]

λ (nm)	290	295	300	305	310	315	320
EE(λ) × I(λ)	0.0150	0.0817	0.2874	0.3278	0.1876	0.0839	0.0180

Abbreviations: EE, erythemal effect spectrum; I, solar intensity spectrum.

regularly monitored in the previous researches of SWOP emulsions under normal storage conditions and gave the first insight into the structure and stability of an emulsion.[16, 17] The prepared emulsions were shiny, smooth, and light creams, but varied in color (S—white, S_{DHQ}—yellowish, and $S_{DHQ\beta C}$—yellow to orange) due to the color of the incorporated antioxidants. The emulsion $S_{DHQ\beta C}$ had the most intense color, due to the high βC concentration, which was used to achieve a better antioxidant effect. The consistency of the SWOP emulsion S was soft semisolid, while the emulsions with incorporated antioxidants had lower viscosity due to the presence of flavonoid DHQ[29] and the method of antioxidant incorporation.

The measured pH values of the prepared emulsions stored under normal conditions slightly changed during 30 days; 48 h after preparation, pH values for S, S_{DHQ}, and $S_{DHQ\beta C}$ were 5.83, 5.82, and 5.82, respectively, while 30 days after preparation the alterations in these values were negligible (5.84, 5.80, and 5.78, respectively). The conductivity measurements demonstrated differences between the emulsions during the testing period. The initial conductivities were higher than 1000 μS cm^{-1} and in accordance with our previous findings.[16] The SWOP emulsion base S had higher and less changeable conductivities (1665 μS cm^{-1} after 48 h, ie, 1686 μS cm^{-1} after 30 days) than the emulsions with antioxidants: 1675 μS cm^{-1} after 48 h, ie, 1598 μS cm^{-1} after 30 days for sample S_{DHQ}, and 1162 μS cm^{-1} after 48 h, ie, 725 μS cm^{-1} after 30 days for sample $S_{DHQ\beta C}$. Although the conductivity of sample $S_{DHQ\beta C}$ decreased more significantly during the testing period, it was still about fifteen times higher compared to that of the conventional o/w emulsions (~50 μS cm^{-1}). The obtained results indicated changes in the structure of SWOP emulsion with both antioxidants ($S_{DHQ\beta C}$), which could affect its long-term stability.

The rheological profiles (Figure 1, Table 3) indicated "shear-thinning" flow behavior with a moderately pronounced thixotropy, ie, these emulsions are pseudoplastic non-Newtonian semisolid systems with hysteresis, which is of high importance for sunscreen formulations. Namely, the sunscreen formulations with a pseudoplastic flow produce a coherent protective film covering the skin surface with evenly distributed UV filters and this characteristic helps to promote a higher SPF.[30] Additionally, the formulations with lower hysteresis area (HA) are ideal for cosmetic actives that should stay on the skin surface, such as sunscreens.[31] It was noticed that the apparent viscosity (maximal—η_{max} and minimal—η_{min}) of the emulsions (S, S_{DHQ}, and $S_{DHQ\beta C}$) decreased during the storage time (48 h and 30 days) at room temperature (Figure 1, Table 3). However, a pronounced decrease in rheological parameter values was observed for both S_{DHQ} and $S_{DHQ\beta C}$, which could be an indicator of changes in the structure as a result of the addition of DHQ and βC, and also of stability of these emulsions that should be investigated in further research.

Due to its sensitivity to thermal changes in materials, in the previous research, DSC was used as a tool for the investigation of colloidal structure and stability of the SWOP emulsion.[17]

DSC curves of all the investigated samples exhibited similar behavior characterized by a series of intensive endothermic events ranging from 100 °C to 120 °C (Figure 2). Namely, the endothermic events were split into several sharp peaks suggesting intensive structural changes due to the gradual evaporation of water and PG. Loss of water at such high temperatures implies excellent thermal stability of all the investigated formulations, which could be ascribed to the strong interactions with emulsifiers and stabilizers.

The enthalpies of these thermal transitions were $-0.66 \cdot 10^3$ J g^{-1}, $-1.01 \cdot 10^3$ J g^{-1}, and $-0.9 \cdot 10^3$ J g^{-1} for S, S_{DHQ}, and $S_{DHQ\beta C}$, respectively, implying a decrease in thermal stability upon addition of DHQ, which was in agreement with a previously reported decrease in rheological parameters of the emulsions containing flavonoids.[16, 17] However, the effect of DHQ, and even βC, on the thermal stability of emulsions was negligible at temperatures below 80 °C.

In Vitro Antioxidant Activity and Determination of SPF

The DPPH test is a method for evaluation of the antioxidant potential indirectly by spectrophotometry used to measure the color change that occurs during the reaction between the purple, stable, free radical DPPH and the antioxidant.[12] DHQ and Q incorporated into SWOP emulsion exhibited strong DPPH radical scavenging ability, without significant statistical difference compared to the pure compounds. The SC_{50} values of incorporated and pure Q were 3.48 ± 0.10 μg mL^{-1} and 3.37 ± 0.03 μg mL^{-1}, respectively, and the SC_{50} values of DHQ incorporated in S_{DHQ} and $S_{DHQ\beta C}$, and of pure DHQ were 5.36 ± 0.27 μg mL^{-1}, 5.06 ± 0.14 μg mL^{-1}, and 5.02 ± 0.10 μg mL^{-1}, respectively (Table 4). However, there was a statistically significant difference between the SC_{50} values of Q (pure and incorporated into SWOP emulsion) and DHQ (pure and incorporated into SWOP emulsion) indicating better antioxidant activity of Q. On the other hand, although known for its antioxidant activity, neither incorporated nor pure βC showed any DPPH radical scavenging ability at the tested concentrations (0.40-8.00 μg mL^{-1}), which is in

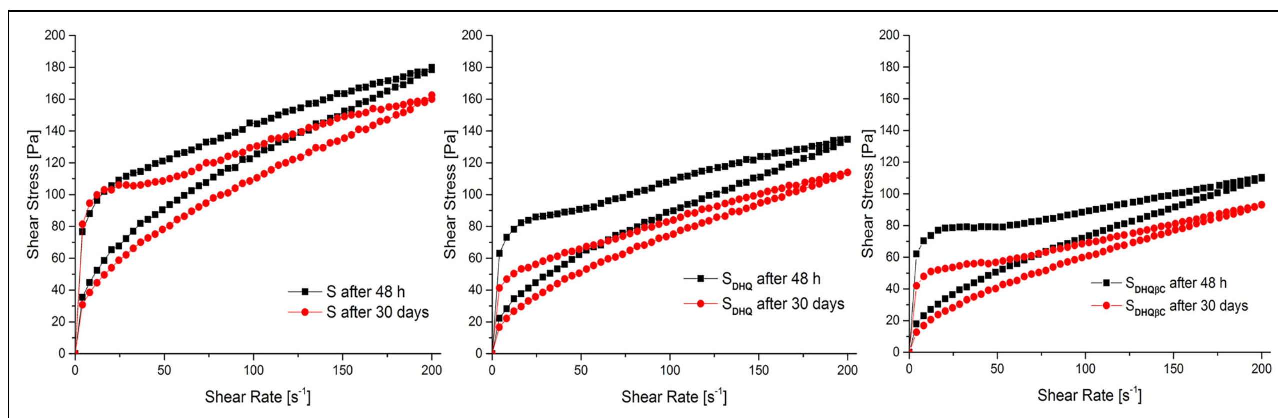


Figure 1. Flow curves of the investigated emulsions plotted with shear stress as a function of the increasing/decreasing shear rate, after 48 h and 30 days storage at room temperature (n = 3, all points in each flow curve have a coefficient of variation lower than 3%).

Table 3. Values of Maximal (η_{max}) and Minimal (η_{min}) Apparent Viscosity, Yield Stress (σ_0), and Hysteresis Area (HA) for the Investigated Emulsions During Testing Period.

Sample	Storage time	Parameters			
		η_{max} [Pas]	η_{min} [Pas]	*HA* [Pa s^{-1}]	σ_0 [Pa]
S	48 h	18.8 ± 0.3	0.9 ± 0.01	4162 ± 73.4	69.5 ± 2.8
	30 days	19.8 ± 0.4	0.81 ± 0.01	4485.1 ± 74.0	88.5 ± 0.7
S_{DHQ}	48 h	15.31 ± 0.75	0.674 ± 0.005	4120 ± 264.3	66.2 ± 3.5
	30 days	10.1 ± 0.5	0.57 ± 0.01	2081.5 ± 176.3	40.5 ± 1.7
$S_{DHQ\beta C}$	48 h	15.23 ± 0.58	0.55 ± 0.05	3804.5 ± 134.5	70.8 ± 1.0
	30 days	10.31 ± 1.07	0.46 ± 0.02	2182.4 ± 461.9	47.0 ± 0.7

agreement with the findings of Müller et al.[32] The same results were also obtained when the measurements were made at 540 nm, to minimize the interference at 517 nm between the absorbance of DPPH and carotenoids. Therefore, DPPH assay is not suitable for the testing of antioxidant activity of βC incorporated into SWOP emulsion, since carotenoids are not able to scavenge the DPPH radical.[32]

The SC_{50} values of the tested SWOP emulsions S_Q, S_{DHQ}, and $S_{DHQ\beta C}$ were 0.70 ± 0.02 mg mL^{-1}, 1.07 ± 0.05 mg mL^{-1}, 1.01 ± 0.03 mg mL^{-1}, respectively. Therefore, there was no significant statistical difference between the SC_{50} values of the two DHQ containing SWOP emulsions, ie, emulsion with and without βC (S_{DHQ} and $S_{DHQ\beta C}$).

We presumed that the *in vitro* antioxidant activity of the tested emulsions in a bioenvironment could give a more realistic insight into the human *in vivo* reaction toward the investigated emulsions. The main way was to estimate the changes in antioxidant capacity, ie, the sum of all antioxidant activities of the serum (a human serum mixture containing many antioxidants)[33] in the presence of the investigated antioxidants. The reactions were performed at two different temperatures (20 °C and 37 °C) and five subgroups (Figure 3). Antioxidative score values at 20 °C were ranked as follows: Q/PG and S_Q/PG −0.33 (−5.11–6.17) > $S_{DHQ\beta C}$/PG −5.50 (−14.67–3.67) > DHQ/PG, and S_{DHQ}/PG −9.33 (−9.89– −7.50), and the difference between last two subgroups was insignificant (Figure 3A). At 37 °C, the rank order was the same: Q/PG and S_Q/PG −7.34 (−14.50– −3.00) > $S_{DHQ\beta C}$/PG −33.50 (−45.67– −21.33) > DHQ/PG, and S_{DHQ}/PG −37.33 (−39.00– −34.67), with a significant difference between the last two subgroups (Figure 3B). The comprehensive OS, reflecting the balance between pro-oxidants and antioxidants, was the lowest in Q containing samples, which indicates the superiority of this antioxidant compared to the other tested substances. The superiority of Q containing serum samples was also observed after incubation at 37 °C. A marginally significant difference between Q samples and DHQ-βC combination sample speaks in favor of the antioxidant capability improvement after the βC integration in the complex mixture of the skin protective emulsion (Figure 3B). The βC inclusion in the DHQ containing sample leads to the improvement of its antioxidative potential.

Nonparametric repeated measures ANOVA (Friedman's test) was used for the comparison of antioxidative potency of the investigated samples (Figure 4), in a native state and upon TBH influence. Antioxidative potency of the investigated samples, regarding the separate oxidative stress parameters

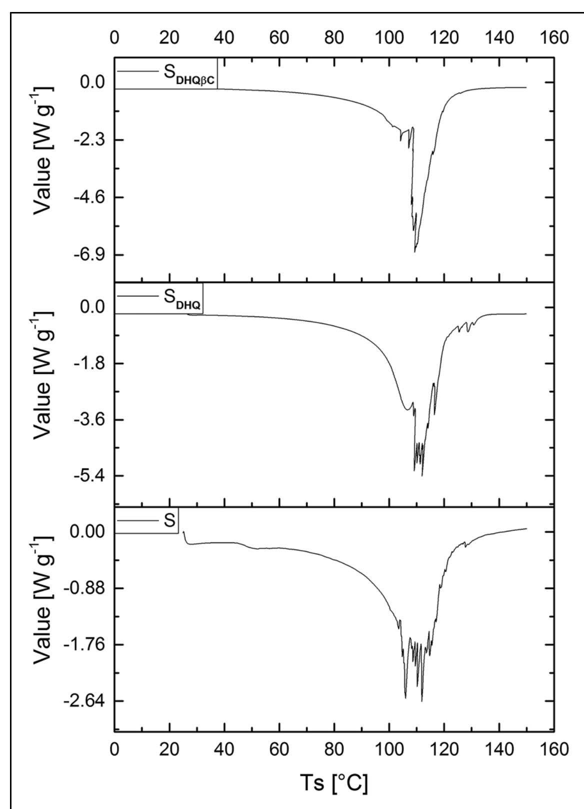


Figure 2. DSC curves of the investigated emulsions.

Table 4. DPPH Radical Scavenging Activity of Pure Flavonoids and Flavonoids Incorporated in SWOP Emulsions Expressed as the Concentrations That Scavenge 50% of DPPH Radicals (SC_{50}).

Flavonoids	SC_{50} (μg mL^{-1})
Quercetin (Q)	3.37 ± 0.03^a
Q incorporated into SWOP	3.48 ± 0.10^a
Dihydroquercetin (DHQ)	5.02 ± 0.10^b
DHQ incorporated into SWOP	5.36 ± 0.27^b
DHQ incorporated into SWOP containing *β*-carotene (βC)	5.06 ± 0.14^b

[a,b]Different letters in superscript indicate significant differences between the SC_{50} values (determined by one-way ANOVA, followed by Tukey's post hoc test, $P < 0.05$).
Abbreviations: DPPH, 2, 2-diphenyl-1-picrylhydrazyl; SWOP, Switch-Oil-Phase.

(PON1, SHG, AOPP, and TOS) (Figure 4A-C and E), in a native state (labeled as 20 °C) was statistically different in comparison to the antioxidative potency under TBH influence (labeled as 37 °C + TBH). The parameter TAS did not show statistical difference under the introduced conditions (Figure 4D).

The comparison of the results for the samples SerEDP and SerEQP (containing the SWOP emulsion) and the samples SerDP and SerQP (without the SWOP emulsion) (Figure 4) imply that the SWOP emulsion is a suitable carrier for the investigated antioxidants and that it shows beneficial effects, especially in the state with exogenous pro-oxidant TBH.

In native bioenvironmental conditions and room temperature, the best overall antioxidant power was shown by SerEQP, followed by SerEDP, and the weakest was SerEDβC. However, upon the pro-oxidant challenge and body temperature (37 °C), ie, the conditions imitating damaging processes caused by external harmful influences with oxidative stress involvement, the sample with DHQ-βC combination became a stronger antioxidant compared to the sample with DHQ only. This implies that some level of this antioxidant mixture activation could be expected upon ROS confrontation, as well as the increase of its effect in contact with body temperature.

The higher antioxidant potency of Q compared to DHQ, ie, the emulsion S_Q compared to the emulsion S_{DHQ}, could be explained by differences in the chemical structures of these antioxidants (Figure 5A and B). DHQ meets two of the three criteria for effective radical scavenging ability. The first criterion is the presence of the *o*-dihydroxy structure in the B ring responsible for its stability, and the second one is the 5- and 7-OH groups with a 4-oxo function in the A and C rings, responsible for a maximum radical scavenging potential. However, DHQ lacks the 2, 3 double bond in conjunction with the 4-oxo function in the C ring (Figure 5A), which makes it less potent than its oxidation product and planar hydrophobic equivalent Q, which contains this 2, 3 double bond.[8] Even the addition of βC did not increase the antioxidative potency of DHQ enough to reach the one of Q. It is possible that a combination of another antioxidant with DHQ would have a more synergistic effect or that some multicomponent mixture of antioxidants at a low, physiological concentration would be more efficient, since it is known that there is a critical concentration at which the antioxidants can start to produce radicals, instead of acting as neutralizers.[11]

The SWOP emulsion containing a combination of DHQ and βC showed minor antioxidative effect amplification compared to S_{DHQ}, which was not in agreement with the results obtained by DPPH radical assay. This disagreement could be explained by the lack of DPPH assay ability to test the antioxidant activity of βC incorporated into the SWOP emulsion. Additionally, this analysis indicated that $S_{DHQβC}$ showed a significant decrease in exogenous pro-oxidative effects caused by TBH, when compared to S_{DHQ}, which could not be revealed using the DPPH radical assay test. Determination of the *in vitro* antioxidant activity of the antioxidants/emulsions tested in the bioenvironment has another advantage over the DPPH radical assay. Namely, using the former method, it is possible to monitor the influence of temperature (room temperature vs higher temperature corresponding to skin temperature) on the antioxidant power of the tested antioxidants/emulsions.

Spectrophotometric determination of *in vitro* SPF by applying the Mansur equation is a simple, easily reproducible, and economical, and it is usually performed as a screening method for a selection of the best photoprotective substance, ie, the best formulation.[4] S_{DHQ} showed better UV absorption

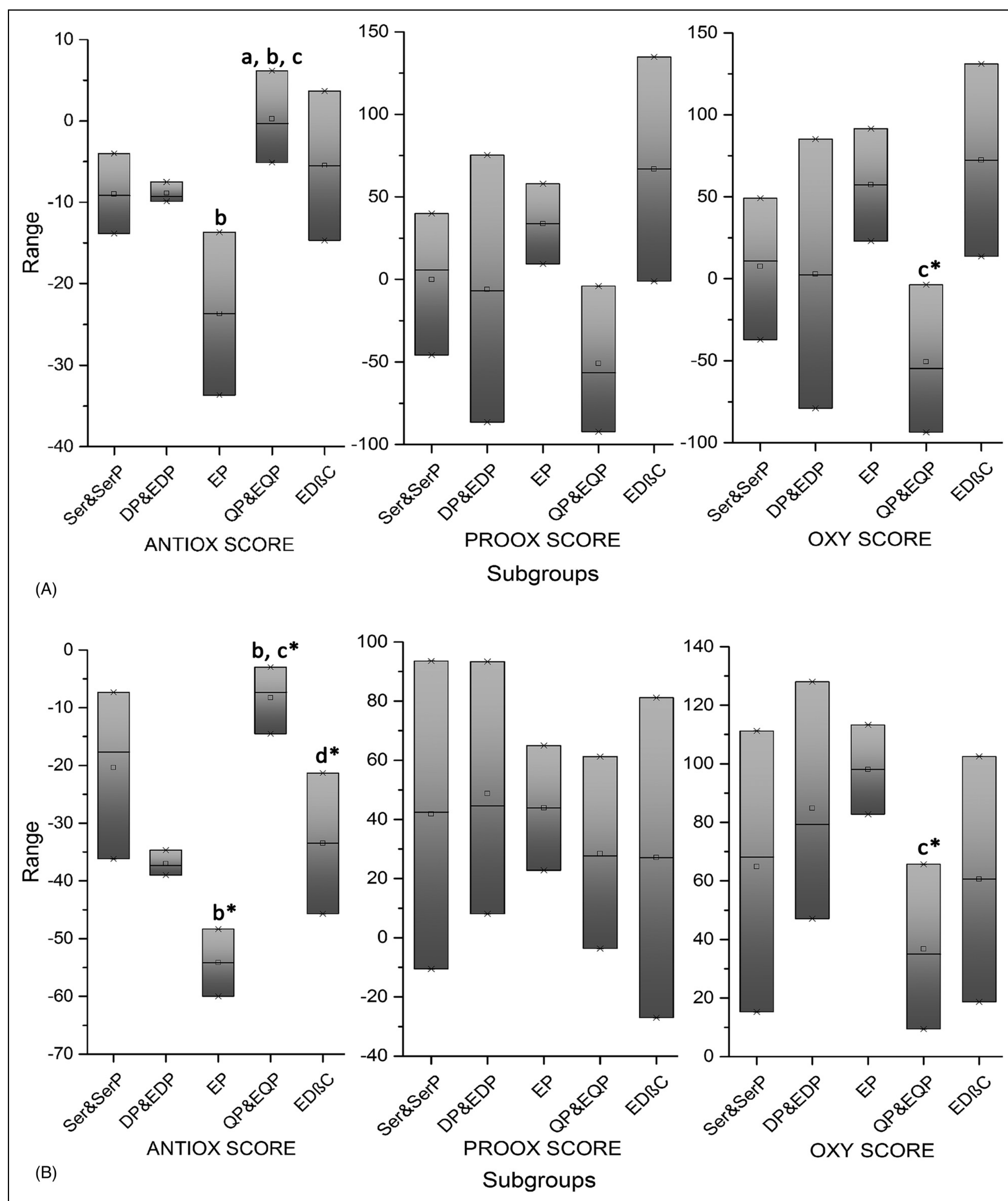
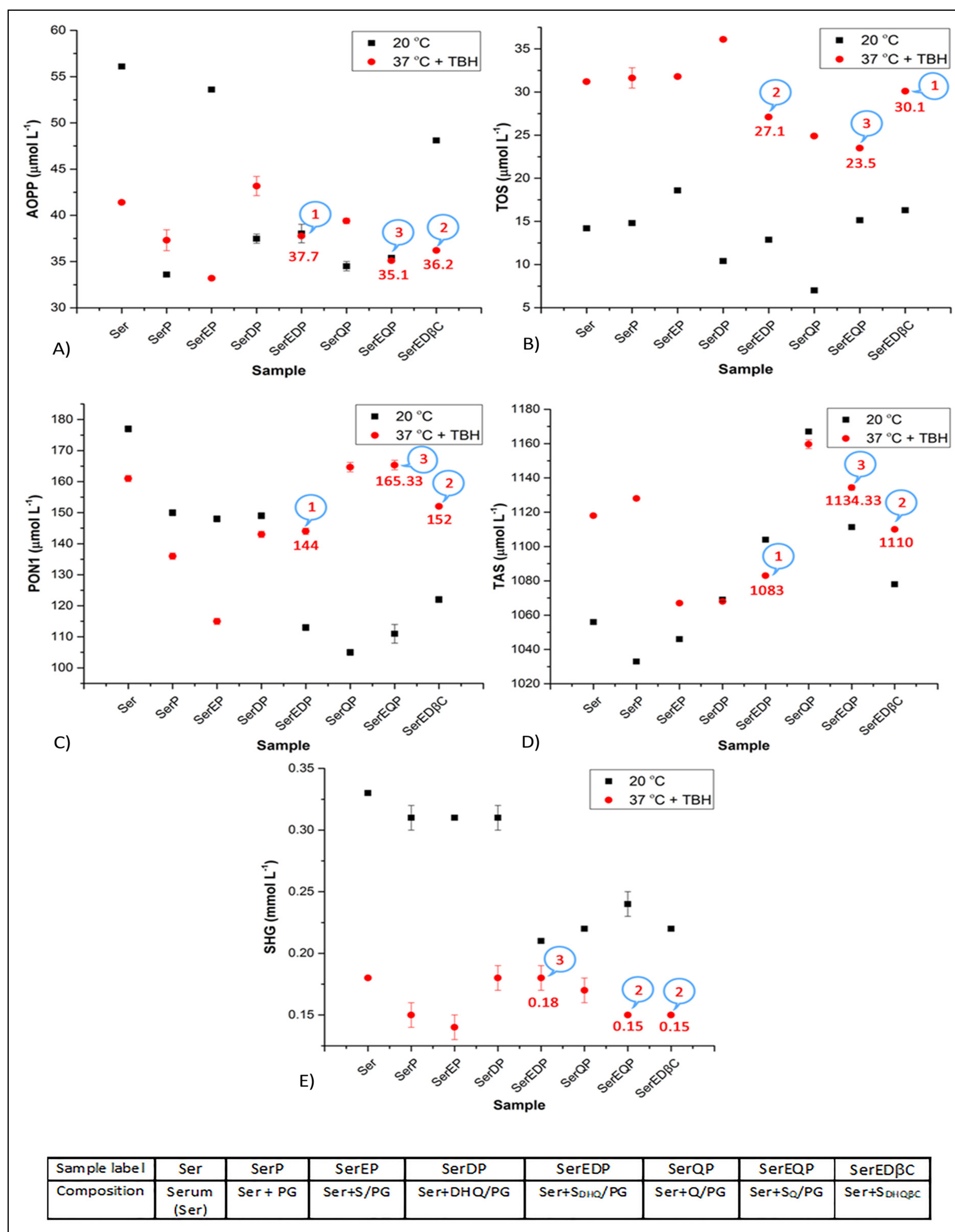


Figure 3. Range of pro-oxidative, antioxidative, and oxidative scores for different subgroups at (A) 20 °C and (B) 37 °C. Box plots range from the 25th to the 75th percentile, the 50th percentile is drawn inside the box, the whiskers present minimal and maximal values and squares presents mean values.

Different letters indicate significant difference or marginally significant difference (letters with an asterisk) between the score values of subgroups ([a] toward Ser&SerP, [b] toward DP&EDP, [c] toward EP, and [d] toward QP&EQP) determined by Mann–Whitney post hoc test, $P < 0.05$).

Subgroups: Ser&SerP, Serum and Serum/PG; DP&EDP, DHQ/PG and S_{DHQ}/PG; EP, S/PG; QP&EQP, Q/PG and S_{Q}/PG; EDβC, $S_{DHQ\beta C}$/PG.



Sample label	Ser	SerP	SerEP	SerDP	SerEDP	SerQP	SerEQP	SerEDβC
Composition	Serum (Ser)	Ser + PG	Ser+S/PG	Ser+DHQ/PG	Ser+S_{DHQ}/PG	Ser+Q/PG	Ser+S_Q/PG	Ser+$S_{DHQ\beta C}$

Figure 4. Presentation of separate oxidative stress parameters: (A) AOPP, (B) TOS, (C) PON1, (D) TAS, and (E) SHG for the investigated samples under introduced conditions (20 °C and 37 °C + TBH).

The numbers in callouts (1, 2, or 3) are assigned to the samples of interest. The higher the assigned number, the stronger is the antioxidative protection and the lower the pro-oxidants content, and *vice versa*.

Abbreviations: AOPP, advanced oxidation protein products; PON1, serum paraoxonase-1; SHG, total protein sulfhydryl groups; TAS, total antioxidant status; TBH, tert-butyl-hydroperoxide; TOS, total oxidant status.

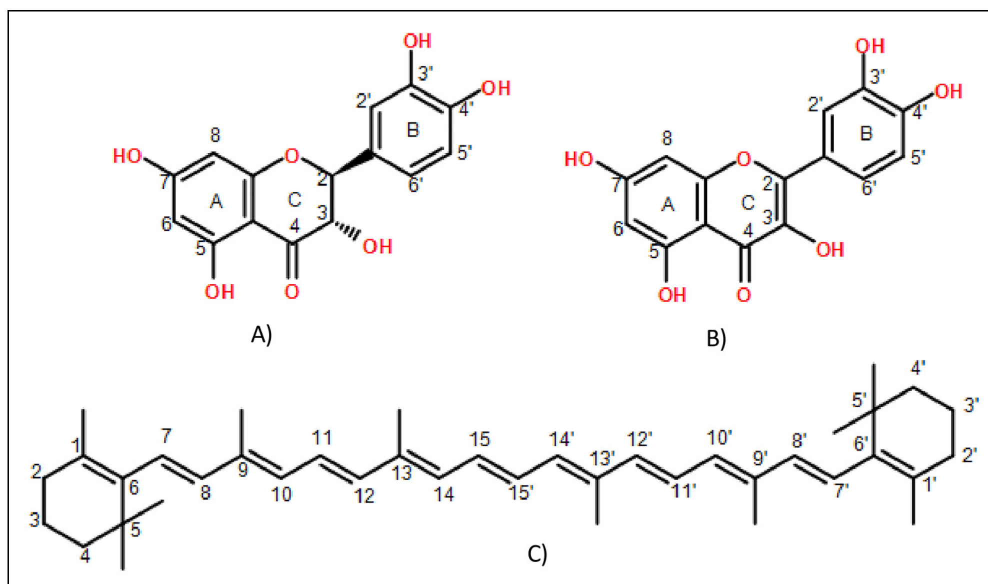


Figure 5. Chemical structure of (A) dihydroquercetin (DHQ), (B) quercetin (Q), and (C) β-carotene (βC).

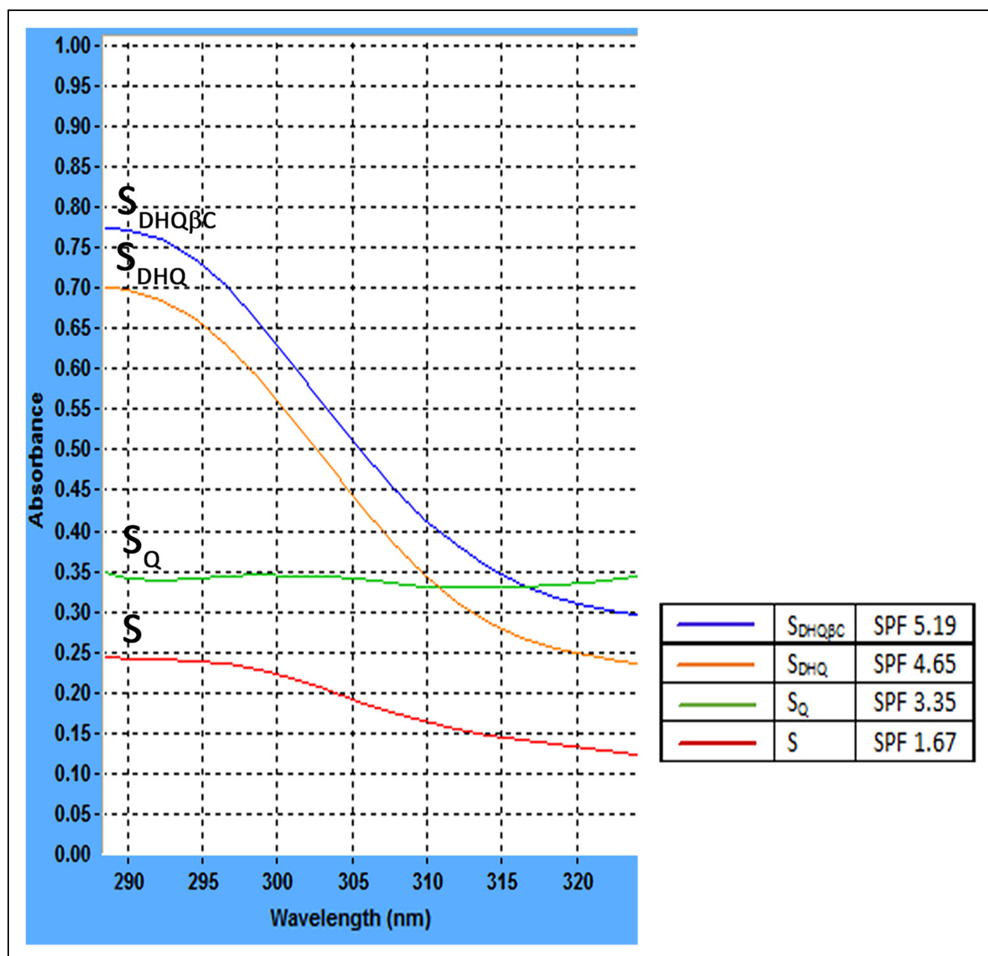


Figure 6. Absorbance spectra of the investigated emulsion samples.

than S_Q (SPF 4.65, ie, 3.35, respectively) (Figure 6) indicating that DHQ absorbed better in the UV spectrum than Q. The SPF of $S_{DHQ\beta C}$ was the highest (5.19), probably due to the contribution of both active ingredients. Although βC is not a typical UV absorber, it probably protects DHQ from oxidation due to its antioxidant activity, and supports DHQ absorbance in the UV spectrum. The SPF of the emulsion base S was negligible (1.67) (Figure 6).

The SPF of Q and rutin were also investigated using another method, ie, the transmittance method proposed by Diffey et al[34, 35] Both Q and rutin (10%) formulations yielded similar SPF values (4.52 ± 0.38, ie, 4.72 ± 0.20, respectively).[35] In our study, the SPF values of the emulsions with Q, DHQ, and βC (S_Q, S_{DHQ}, and $S_{DHQ\beta C}$) were similar to the reported results, despite the different SPF determination methods and lower concentrations of flavonoids. Thus, these results indicate the good photoprotective potential of the selected antioxidants in combination with a prospective cosmetic vehicle such as SWOP emulsion.

Conclusions

The objective of this work was the investigation of SWOP emulsion base as a carrier for natural ingredients DHQ and βC, used in cosmetic products due to their antioxidant and UV protection activity. The results of the antioxidant effects of the tested samples revealed that the innovative biochemical approach could be used as a simple, economical, and easy achievable additional test to basic *in vitro* DPPH radical assay with a few advantages, especially when it comes to a testing of antioxidant activity in cosmetic formulations with complex structure such as emulsion carriers. Principally, this analysis could be more suitable for the evaluations of antioxidants and/or cosmetic products with antioxidants such as βC, since the DPPH assay was not suitable for the evaluation of its antioxidant activity. Furthermore, the analysis in a bioenvironment indicated that the emulsion with a combination of DHQ and βC exhibited a significant decrease in exogenous pro-oxidative effects. The SPF of the $S_{DHQ\beta C}$ emulsion was the highest because of βC addition, which was expected. The physicochemical stability of the investigated samples was satisfactory during 30 days storage at room temperature. The presented results demonstrated that the SWOP emulsion could be used as a cosmetic vehicle for the antioxidants with UV protection ability such as DHQ and βC. However, the development of each new cosmetic product has to be accompanied by an assessment of its overall complex composition and stability.

Acknowledgements

This research was funded by the Ministry of Education, Science and Technological Development, Republic of Serbia through Grant Agreement with the University of Belgrade – Faculty of Pharmacy No. 451-03-68/2022-14/200161. The authors thank the Kingherbs Limited, China for the free samples of cosmetic active ingredients.

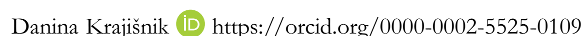
Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by the Ministarstvo Prosvete, Nauke i Tehnološkog Razvoja (grant number 451-03-68/2022-14/200161).

ORCID iD


Danina Krajišnik https://orcid.org/0000-0002-5525-0109

References

1. Basen-Enquist K, Brown P, Coletta AM, Savage M, Maresso KC, Hawk E. Lifestyle and cancer prevention. In: Niederhuber JE, Armitage JO, Doroshow JH, Kastan MB, Tepper JE, eds. *Abeloff's Clinical Oncology*, 6th ed. Elsevier Health Sciences; 2020:337-374. https://doi.org/10.1016/C2015-0-05400-4.
2. Monsalve-Bustamante YA, Puertas-Mejia MA, Mejia-Giraldo JC. Comparison of the photoprotective effect between hydrolyzed and aglycones flavonoids as sunscreen: a systematic review. *J Appl Pharm*. 2020;10(1):116-123. https://doi.org/10.7324/JAPS.2020.101016
3. Oi N, Chen H, Ok Kim M, Lubet RA, Bode AM, Dong Z. Taxifolin suppresses UV-induced skin carcinogenesis by targeting EGFR and PI3K. *Cancer Prev Res (Phila)*. 2012;5(9):1103-1114. https://doi.org/10.1158/1940-6207.CAPR-11-0397
4. Mansur MCPPR, Leitão SG, Cerqueira-Coutinho C, et al. *In vitro* and *in vivo* evaluation of efficacy and safety of photoprotective formulations containing antioxidant extracts. *Rev Bras Farmacogn*. 2016;26(2):251-258. https://doi.org/10.1016/j.bjp.2015.11.006
5. Silva S, Ferreira M, Oliveira AS, et al. Evolution of the use of antioxidants in anti-ageing cosmetics. *Int J Cosmet Sci*. 2019;41(4):378-386. https://doi.org/10.1111/ics.12551
6. Milani LPG, Garcia NOD, Morais MC, Dias ALS, Oliveira NL, Conceição EC. Extract from byproduct *Psidium guajava* standardized in ellagic acid: additivation of the in vitro photoprotective efficacy of a cosmetic formulation. *Rev Bras Farmacogn*. 2018;28(6):692-696. https://doi.org/10.1016/j.bjp.2018.08.005
7. Malinowska P. Effect of flavonoids content on antioxidant activity of commercial cosmetic plant extracts. *Herba Pol*. 2014;59(3):63-75. https://doi.org/10.2478/hepo-2013-0017
8. Weidmann AE. Dihydroquercetin: more than just an impurity? *Eur J Pharmacol*. 2012;684(1-3):19-26. https://doi.org/10.1016/j.ejphar.2012.03.035
9. Philippov SV, Bogorodov IM. Invertors. Method of using wood extracts in cosmetic and hygiene products. US 20130295036 A1, 2013.
10. Woodby B, Penta K, Pecorelli A, Lila MA, Valacchi G. Skin health from the inside out. *Annu Rev Food Sci Technol*. 2020;11(1):235-254. https://doi.org/10.1146/annurev-food-032519-051722

11. Lademann J, Meinke MC, Sterry W, Darvin ME. Carotenoids in human skin. *Exp Dermatol*. 2011;20(5):377-382. https://doi.org/10.1111/j.1600-0625.2010.01189.x
12. Alam MN, Bristi NJ, Rafiquzzaman M. Review on *in vivo* and *in vitro* methods evaluation of antioxidant activity. *Saudi Pharm J*. 2013;21(2):143-152. https://doi.org/10.1016/j.jsps.2012.05.002
13. Ratz-Lyko A, Arct J, Pytkowska K. Methods for evaluation of cosmetic antioxidant capacity. *Skin Res Technol*. 2012;18(4):421-430. https://doi.org/10.1111/j.1600-0846.2011.00588.x
14. Li H, Colantonio S, Dawson A, Lin X, Beecker J. Sunscreen application, safety, and sun protection: the evidence. *J Cutan Med Surg*. 2019;23(4):357-369. https://doi.org/10.1177/1203475419856611
15. Beuché M, Amela CC, Domingo M. Inventors. Aqueous metastable oil-in-water emulsions. EP1917954A1, 2008.
16. Korać R, Krajišnik D, Milić J. Sensory and instrumental characterization of fast inverting oil-in-water emulsions for cosmetic application. *Int J Cosmet Sci*. 2016;38(3):246-256. https://doi.org/10.1111/ics.12282
17. Korać R, Krajišnik D, Savić S, et al. A new class of emulsion systems – fast inverted o/w emulsions: formulation approach, physical stability and colloidal structure. *Colloids Surf A Physicochem Eng Asp*. 2014;461(1):267-278. https://doi.org/10.1016/j.colsurfa.2014.08.005
18. Casagrande R, Georgetti SR, Verri Jr WA, Borin MF, Lopez RF, Fonseca MJ. *In vitro* evaluation of quercetin cutaneous absorption from topical formulations and its functional stability by antioxidant activity. *Int J Pharm*. 2007;328(2):183-190. https://doi.org/10.1016/j.ijpharm.2006.08.006
19. Janković T, Turković N, Kotur-Stevuljević J, Vujić Z, Ivković B. Differences in antioxidant potential of chalcones in human serum: *in vitro* study. *Chem Biol Interact*. 2020;324:109084. https://doi.org/10.1016/j.cbi.2020.109084
20. Erel O. A new automated colorimetric method for measuring total oxidant status. *Clin Biochem*. 2005;38(12):1103-1111. https://doi.org/10.1016/j.clinbiochem.2005.08.008
21. Witko-Sarsat V, Friendlander M, Capeillère-Blandin C, et al. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. *Kidney Int*. 1996;49(5):1304-1313. https://doi.org/10.1038/ki.1996.186
22. Alamdari DH, Paletas K, Pegiou T, Sarigianni M, Befani C, Koliakos G. A novel assay for the evaluation of the prooxidant-antioxidant balance, before and after antioxidant vitamin administration in type II diabetes patients. *Clin Biochem*. 2007;40(3-4):248-254. https://doi.org/10.1016/j.clinbiochem.2006.10.017
23. Erel O. A novel automated direct measurement method for total antioxidant capacity using a new generation, more stable ABTS radical cation. *Clin Biochem*. 2004;37(4):277-285. https://doi.org/10.1016/j.clinbiochem.2003.11.015
24. Richter RJ, Furlong CE. Determination of paraoxonase (PON1) status requires more than genotyping. *Pharmacogenetics*. 1999;9(6):745-753. https://doi.org/10.1097/00008571-199912000-00009
25. Ellman GI. Tissue sulfhydryl groups. *Arch Biochem Biophys*. 1959;82(1):70-77. https://doi.org/10.1016/0003-9861(59)90090-6
26. Gjorgieva Ackova D, Kotur-Stevuljevic J, Bhushan Mishra C, Mehta Luthra P, Saso L. Antioxidant properties of synthesized bicyclic thiazolopyrimidine derivatives as possible therapeutic agents. *Appl Sci*. 2018;9(1):113. https://doi.org/10.3390/app9010113
27. Mansur JS, Breder MNR, Manusur MCA, Azulay RD. Determinação do fator de proteção solar por espectrofotometría. *An Bras Dermatol*. 1986;61(3):121-124.
28. Sayre RM, Agin PP, Levee GJ, Marlowe EA. Comparison of *in vivo* and *in vitro* testing of sun screening formulas. *Photochem Photobiol*. 1979;29(3):559-566. https://doi.org/10.1111/j.1751-1097.1979.tb07090.x
29. Zillich OV, Schweiggert-Weisz U, Eisner P, Kerscher M. Polyphenols as active ingredients for cosmetic products. *Int J Cosmet Sci*. 2015;37(5):455-464. https://doi.org/10.1111/ics.12218
30. Sarruf FD, Peres DA, Nishikawa DO, et al. Assessment of *in vitro* sun protection factor (SPF) and rheological profile of commercial infant's sunscreens. *Rev Ciênc Farm Básica Apl*. 2013;34(1):33-36.
31. Isaac VLB, Moraes JDD, Chiari BG, et al. Determination of the real influence of the addition of four thickening agents in creams using rheological measurements. *J Disper Sci Technol*. 2013;34(4):532-538. https://doi.org/10.1080/01932691.2012.683759
32. Müller L, Fröhlich K, Böhm V. Comparative antioxidant activities of carotenoids measured by ferric reducing antioxidant power (FRAP), ABTS bleaching assay (aTEAC), DPPH assay and peroxyl radical scavenging assay. *Food Chem*. 2011;129(1):139-148. https://doi.org/10.1016/j.foodchem.2011.04.045
33. Antolovich MI, Prenzler PD, Patsalides E, McDonald S, Robards K. Methods for testing antioxidant activity. *Analyst*. 2002;127(1):183-198. https://doi.org/10.1039/b009171p
34. Diffey BL, Robson J. A new substrate to measure sunscreen protection factors throughout the ultraviolet spectrum. *J Soc Cosmet Chem*. 1989;40(5):127-133.
35. Choquenet B, Couteau C, Paparis E, Coiffard LJM. Quercetin and rutin as potential sunscreen agents: determination of efficacy by an *in vitro* method. *J Nat Prod*. 2008;71(6):1117-1118. https://doi.org/10.1021/np7007297