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Enantiomeric separation of panthenol by Capillary Electrophoresis. Analysis of commercial formulations and toxicity evaluation on non-target organisms

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ABSTRACT

The first CE methodology enabling the enantiomeric separation of panthenol was developed in this work. Electrokinetic chromatography with cyclodextrins (CD-EKC) was the CE mode employed for this purpose. The effect of different experimental variables such as the nature and concentration of the cyclodextrin, the temperature and the separation voltage was investigated. The best enantiomeric separation was obtained with 25 mM (2-carboxyethyl)-β-CD (CE-β-CD) in 100 mM borate buffer (pH 9.0), with a separation voltage of 30 kV and a temperature of 30 °C. Under these conditions, an enantiomeric resolution of 2.0 in an analysis time of 4.2 min was obtained, being the biologically active enantiomer D-panthenol (dexpanthenol) the second-migrating enantiomer. The analytical characteristics of the method were evaluated in terms of precision, accuracy, selectivity, linearity, LOD, and LOQ, showing a good performance for the quantitation of dexpanthenol in cosmetic and pharmaceutical formulations. The enantiomeric impurity (L-panthenol) could be detected at a 0.1% level with respect to the majority enantiomer, allowing to accomplish the requirements of the ICH guidelines. The method was also successfully applied to study the stability of panthenol under abiotic and biotic conditions and its toxicity on non-target organisms (the aquatic plant *Spirodela polyrhiza*).

1. Introduction

Chirality is currently a topic of high relevance in different fields such as the pharmaceutical, agrochemical or environmental, among others, since enantiomers can show different biological, pharmacodynamic and pharmacokinetic activities in addition to different toxicity, degradation rates and persistence in the environment [1,2]. In some cases, only one enantiomer produces the desired activity, while the other one might have a different activity, be inactive or even toxic [3]. Thus, racemic mixtures should only be used when the different enantiomers exhibit complementary biological activities [1]. However, nowadays, an 88% of the chiral marketed drugs are administered in their racemic forms [1]. In the cosmetic field, the use of products containing chiral compounds in an enantiomeric pure form is preferred, mainly in order to prevent allergic reactions, penetration issues and their ineffectiveness [4]. As a consequence, the development of analytical methodologies enabling the enantiomeric determination of chiral drugs, cosmetics and personal-care products is of crucial importance.

The wide use of pharmaceuticals and personal care products has led to the presence of amounts of residues in the environment, what has caused them to be considered as emerging pollutants [5], with undesirable effects not only for animals and humans, but also towards non-target organisms [6,7]. Although the presence of these products in the environment has been an increasingly active area of research over the last 20 years [8,9], low attention has been payed in the case of toxicity studies at the enantiomeric level [10]. Data regarding the effects of human pharmaceuticals upon aquatic organisms are available for fish, daphnia and algae. Vita et al. [9] reviewed the ecotoxicity evaluation studies of cosmetics in aquatic environment, concluding that more effort is needed to

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understand the environmental risk of cosmetic ingredients due to the different ways in which these compounds can affect aquatic organisms. Low attention has been payed to toxicological studies of cosmetics using plants as non-target organisms. Only the work of Azizullah et al. [11] reported the effect of shampoo formulations on the aquatic plant *Lemna minor*. However, none of these previous works evaluated the role of enantiomers on the ecotoxicity observed.

Panthenol (2,4-dihydroxy-*N*-(3-hydroxypropyl)−3,3-dimethylbutanamide), also known as provitamin B_5, pantothenyl alcohol or pantothenol [12], is the biologically active alcohol analogue of pantothenic acid (vitamin B_5) [13]. Once panthenol penetrates the skin, it oxidizes into pantothenic acid, which is a constituent of skin and hair [14], and a component of coenzyme A that plays an important role in the metabolism of proteins, fatty acids, sterols, carbohydrates, steroid hormones, gluconeogenesis and porphyrins, because is an essential nutrient [4,14–16]. Panthenol has two enantiomers, D and L, having both moisturizing properties [12]. However, L-panthenol is inactive while D-panthenol (dexpanthenol) is biologically active [17], and, in some cases, the effect of D-panthenol can be blocked by L-panthenol, resulting in the elimination from the human body of the active form [14]. Due to its soothing and restorative properties and its skin regeneration and moisturizing effects, dexpanthenol is frequently used in personal-care and cosmetic products [4,14]. In addition, it is used in dietary supplements, pharmaceuticals and lozenges [14] (the use of dexpanthenol in cosmetics and pharmaceutical products was approved by the European Commission on Cosmetic Ingredients and the Food and Drug Administration [17]). For example, it is used in nail care products (enhancing flexibility and hydration), as a component in hair conditioner products (conferring sheen and long lasting moisture), or in skin care products (acting as conditioning agent, providing deep penetrating moisture to the skin, accelerating wound healing and improving skin elasticity), among others [17]. Enantioselective methodologies are necessary to achieve the chiral separation of panthenol and to detect low amounts of the enantiomeric impurity (L-panthenol).

Separation techniques mainly employed in chiral analysis are HPLC, GC, SFC and CE. Although HPLC has been the most employed technique, CE has demonstrated to be a powerful tool to achieve enantiomeric separations in a variety of samples [1,2,18–20], due to numerous advantages such as its high peak efficiency, low consumption of chiral selectors, reagents and samples, the easy change of chiral selector without the need of using a chromatographic column, among others.

Since the first topical dexpanthenol-containing formulation was developed [21], several analytical methodologies were reported enabling the enantioseparation of panthenol [4,14,15,17,22,23]. Konig and Sturm developed in 1985 a GC methodology enabling the enantioseparation of panthenol in <11 min [22]. Four works described the enantiomeric separation of panthenol by HPLC [14,15,17,23] with UV detection [15,23] or UV and polarimetric detection [14,17]. Under the best conditions, the chiral separation was obtained in 8.2 min, with resolution values lower than 2.7 and detection limits in the order of μg mL^{-1} for both enantiomers. In three of these articles, the chiral methodologies developed were applied to the determination of panthenol enantiomers in different samples such as bulk drugs [15], hair care products [17], and dietary supplements [14]. Khater and West carried out the chiral separation of panthenol by SFC using a Chiralpak IA stationary phase in less than 6 min, although the values for resolution or detection limits were not provided [4]. The methodology was applied to the determination of the enantiomeric purity of panthenol in different cosmetic formulations marketed as enantiomerically pure in dexpanthenol. In spite of the interesting characteristics of CE to achieve chiral analyses, the enantiomeric separation of panthenol by CE has not been reported before.

The aim of this work was to develop the first analytical methodology by CE enabling the enantiomeric separation of panthenol and to apply it to the determination of dexpanthenol and its enantiomeric impurity in commercial formulations, and to evaluate, for the first time, its stability and toxicity, under biotic and abiotic conditions, assessing its toxicity on a non-target organism, the aquatic plant *Spirodela polyrhiza.*

2. Materials and methods

2.1. Reagents and samples

All chemicals and reagents employed were of analytical grade. Sodium hydroxide and boric acid were obtained from Sigma-Aldrich (St. Louis, MO, USA). Methanol was purchased from Scharlau (Barcelona, Spain). The chiral selectors carboxymethyl-α-CD (CM-α-CD, DS ~ 3.5), carboxymethyl-γ-CD (CM-γ-CD, DS ~ 3.5), succinyl-β-CD (Succ-β-CD, DS ~ 3.4), succinyl-γ-CD (Succ-γ-CD, DS ~ 3.5), (2-carboxyethyl)-β-CD (CE-β-CD, DS ~ 3.5), (2-carboxyethyl)-γ-CD (CE-γ-CD, DS ~ 3.5), phosphated β-CD (Ph-β-CD, DS ~ 4), sulfated α-CD (S-α-CD, DS ~ 12), sulfated γ-CD (S-γ-CD, DS ~ 10) and sulfobutylated β-CD (SB-β-CD, DS ~ 6.3) were acquired from Cyclolab (Budapest, Hungary). Carboxymethyl-β-CD (CM-β-CD, DS ~ 3) and sulfated β-CD (S-β-CD, DS ~ 18) were from Sigma-Aldrich (St. Louis, MO, USA). Heptakis-(2,3-di-O-acetyl-6-O-sulfo)-β-CD (HDAS-β-CD) was provided by AnaChem (Budel, The Netherlands) and sulfobutileter-β-CD (Captisol) was from Cydex Pharmaceuticals (Lawrence, Kansas). Water used to prepare solutions was purified through a Milli-Q system from Millipore (Bedford, MA, USA).

Panthenol racemate and dexpanthenol were from Merck (Darmstadt, Germany). The commercial cosmetic (mouthwash) and pharmaceutical (topic gel) formulations were acquired in a drug store from Ávila (Spain). According to the labeled data, the cosmetic formulation contains 0.5% of dexpanthenol (and 0.1% of allantoin) while the pharmaceutical formulation contains 1.25% of dexpanthenol.

Duckweed turions and concentrated solution of Steinberg medium were purchased from MicroBioTests (Gent, Belgium).

2.2. Apparatus

Electrophoretic experiments were carried out in an Agilent 7100 CE system from Agilent Technologies (Waldbronn, Germany) with a diode array detector (DAD). The electrophoretic system was controlled with the HP ^{3D}CE ChemStation software that included the data collection and analysis. Separations were performed in uncoated fused-silica capillary of 50 μm I.D. and a total length of 58.5 cm (50 cm effective length) provided by Polymicro Technologies (Phoenix, AZ, USA). Injections were made applying a pressure of 50 mbar for 10 s.

To weigh the different reagents, standards and the pharmaceutical formulation, an OHAUS Adventurer Analytical Balance (Nänikon, Switzerland) was used. pH measurements were performed in a pH-meter model 744 from Metrohm (Herisau, Switzerland). All solutions were sonicated using an ultrasonic bath B200 from Branson Ultrasonic Corporation (Danbury, USA).

To carry out the germination of turions an IBERCEX growth chamber (Madrid, Spain) was employed.

The quantification of chlorophyll fluorescence (CF) was carried out using Leica TCS SP5 confocal imaging (Germany). The software Image J (National Institute of Health, USA) was used to analyze images.

2.3. CE procedure

Before its first use, the new capillary was conditioned with 1 M sodium hydroxide for 30 min, followed by 15 min with Milli-Q

water and finally with buffer solution for 60 min. At the beginning of each working day, the capillary was rinsed with 0.1 M sodium hydroxide for 10 min, Milli-Q water for 5 min, buffer solution for 20 min and background electrolyte (BGE) for 10 min. In order to ensure the repeatability between injections, the capillary was flushed with 0.1 M sodium hydroxide (4 min), Milli-Q water (2 min), buffer solution (4 min) and BGE (3 min). All stages were carried out applying 1 bar of pressure.

Buffer solutions (100 mM, pH 9) were prepared by dissolving the appropriate amount of boric acid in Milli-Q water to reach a concentration of 100 mM and adjusting the pH with sodium hydroxide 1 M to the desired value (pH 9) before completing the volume with water. BGEs were prepared by dissolving the appropriate amount of the different CDs in the borate buffer solution.

Stock standard solutions of panthenol or dexpanthenol were prepared by dissolving the appropriate amount in methanol and stored at 4 °C. Standard working solutions of panthenol racemic and/or dexpanthenol were prepared from the stock standard solutions by dilution in water. Stock solutions of the commercial formulations were prepared by diluting the appropriate amount in methanol. Working solutions of the commercial formulations (mouthwash and topic gel) were prepared from the stock solutions by dilution in water. All solutions were filtered through disposable nylon 0.45 μm pore size filters purchased from Scharlau (Barcelona, Spain) and sonicated before their injection in the CE system.

2.4. Toxicity tests

Toxicity tests with *Spirodela polyrhiza* were performed according to International Standard ISO 20,079 (ISO DIS 2005) with some modifications. Prior to the toxicity assessment, turions were germinated in Steinberg medium, for 72 h, at a temperature of 23 ± 2 °C, under continuous irradiation and light intensity of 6000 lux.

Fresh solutions (stock solution) of panthenol racemic and dexpanthenol, were prepared in the same medium used for the growth of *Spirodela polyrhiza* at a concentration of 2000 mg L^{-1}. Those stock solutions were further serial diluted with the Steinberg medium to make working (tested) concentrations. For each, panthenol and dexpanthenol, nine concentrations ranging from 800 to 0.79 mg L^{-1}, were tested in comparison to the control (0 mg L^{-1}).

The experimental setup was conducted with two replicates in transparent sterile 24 well plates. Each well was filled with 2 mL of tested concentration and inoculated with a total of 1 duckweed. Freshly and healthy plants with a uniform frond size as per visual observation were used. The exposure experiments were conducted at the same operational conditions previously described for the pre-culture. The duration of toxicity exposure was 96 h. The effects in panthenol and dexpanthenol exposed duckweed specimens were assessed by determining the frond area as well as plant photosynthesis efficiency.

The changes in foliar growth of duckweed was digitally recorded during 96 h. The images were subsequently analysed with the software Image J and used to determine frond area. After 96 h of exposure, the specimens were collected from each setup and used for the quantification of CF by confocal imaging (λexc/λem = 488/595–700 nm). The intensity of CF was evaluated by processing images of the plant components (bud, leave, root) with ImageJ software.

Calculation of toxicological parameters was done according to the procedure reported in [6].

2.5. Stability of exposure concentration

The stability of compounds under biotic and abiotic conditions was evaluated. The experimental conditions were the same used in ecotoxicity test (Section 2.4). Briefly, abiotic setups were performed in the absence of duckweed to check the effect of light and hydrolysis of compounds into aqueous reaction media under continuous light and also in darkness. Each experimental condition was replicated twice.

2.6. Analytical data treatment

The values of migration times, resolution values (Rs) and area values were obtained using the Chemstation software from Agilent Technologies. In order to compensate the differences in the electrophoretic conditions and to obtain better reproducibility of data, corrected peak areas (Ac) were used for data treatment. Experimental data analysis and composition of graphs were carried out using Excel Microsoft, Origin Pro8 and Statgraphics Centurion XVII software. Calculation of toxicity parameters was carried out using CompuSyn software.

3. Results and discussion

3.1. Development of an analytical methodology for the enantiomeric separation of panthenol by CE

Since panthenol is a neutral compound except at very basic pH (pKa 13.033), a screening test with a set of fourteen negatively charged CDs at pH 9.0 was carried out (CM-α-CD, CM-β-CD, CM-γ-CD, Succ-β-CD, Succ-γ-CD, CE-β-CD, CE-γ-CD, Ph-β-CD, S-α-CD, S-β-CD, S-γ-CD, SB-β-CD, HDAS-β-CD and Captisol) in order to evaluate their discrimination power and select the most appropriate chiral selector to achieve the enantioselective separation of panthenol. In these experiments, all CDs were tested at a concentration of 10 mM (except CM-β-CD, Succ-γ-CD, S-β-CD and captisol which were prepared at a concentration of 2% w/v) in 100 mM borate buffer, using a voltage of 20 kV, a temperature of 20 °C and a detection wavelength of 205 nm (bandwidth 4 nm). Among the fourteen CDs studied, only two of them, CE-β-CD and Ph-β-CD, enabled the partial separation of panthenol enantiomers with resolution values of 1.2 in 7.6 min and 0.6 in 8.2 min, respectively. Therefore, CE-β-CD was selected as the chiral selector. In order to improve the shape of the peaks, different experimental detection parameters such as the bandwidth, the possibility of using a reference length and the bandwidth of the reference were optimized. Three different bandwidths (4, 15 and 30 nm) were studied with and without reference (300 nm with a bandwidth of 100 nm). As the highest peak heights were obtained with a bandwidth of 30 nm and without reference, these values were taken as the most appropriate.

The influence of the CE-β-CD concentration was investigated in the range from 10 to 25 mM (10, 15, 20 and 25 mM). Resolution values increased from 1.2 for a 10 mM concentration of CE-β-CD to 2.2 for 25 mM CE-β-CD. As the analysis time differed just in 2 min for these two CE-β-CD concentrations, a value of 25 mM was chosen for the concentration of the chiral selector. The effect of the temperature was studied (20, 25 and 30 °C). Shorter analysis times (9.5 min at 20 °C and 7.5 min at 30 °C) and higher resolution values (2.2 at 20 °C and 2.5 at 30 °C) were obtained when increasing the temperature. For this reason, a temperature of 30 °C was chosen. Then, in order to reduce the analysis time, the influence of the voltage on the enantiomeric separation was evaluated at 20, 25 and 30 kV. Since an increase in the separation voltage gave rise to shorter analysis times (7.5 min at 20 kV and 4.2 min at 30 kV), a voltage of 30 kV was chosen as the optimum although a lower resolution value was obtained (2.5 at 20 kV and 2.0 at 30 kV).

The migration order for panthenol enantiomers was established by injecting a solution of racemic panthenol spiked with dexpanthenol. It was possible to assign dexpanthenol as the

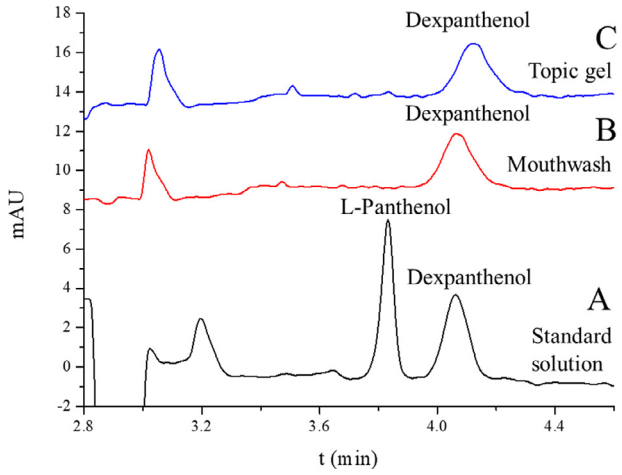

Fig. 1. Electrophoregrams corresponding to the enantiomeric separation of panthenol under the optimized conditions, (A) in a standard solution of 200 mg L^{-1}, (B) in a cosmetic formulation (mouthwash) containing 100 mg L^{-1} of dexpanthenol (according to the label of the commercial formulation) and (C) in a pharmaceutical formulation (topic gel) containing 100 mg L^{-1} of dexpanthenol (according to the label of the commercial formulation). Experimental conditions: 25 mM CE-β-CD in 100 mM borate buffer (pH 9.0); uncoated fused-silica capillary 50 µm id × 50 cm (58.5 cm to the detector); injection by pressure 50 mbar × 10 s; applied voltage 30 kV; temperature 30 °C; UV detection 205 ± 30 nm.

second-migrating enantiomer (being the enantiomeric impurity the first-migrating enantiomer). In this way, it is possible to avoid the overlapping of the peak of the active enantiomer (dexpanthenol) with that of the impurity (L-panthenol). Fig. 1**A** shows the separation of panthenol enantiomers from a standard solution under the optimized conditions in 4.2 min and a resolution value of 2.0.

3.2. Analytical characteristics of the chiral CE method developed

In order to apply the developed method to the quality control of cosmetic and pharmaceutical formulations and to the study of the stability of panthenol and dexpanthenol and their toxicity on the non-target organism *Spirodela Polyrhiza*, the analytical characteristics of the methodology were evaluated in terms of linearity, accuracy, precision, selectivity, limits of detection (LOD) and limits of quantification (LOQ). The results obtained are grouped in Table 1.

The linearity of the method was established from twelve standard solutions at different concentration levels. Corrected peak areas (Ac) were plotted as a function of the analyte concentration in mg L^{-1}. As it can be seen in Table 1, a linear range from 1 to 25 mg L^{-1} for L-panthenol and from 15 to 150 mg L^{-1} for D-panthenol enantiomers, was obtained, with satisfactory results in terms of linearity as R^2 values were higher than 99% for both enantiomers, confidence intervals for the slopes did not include the zero value, and confidence intervals for the intercept included the zero value (in both cases for a 95% confidence level). The Response Relative Factor (RRF), which is calculated dividing the slopes of the calibration lines, (slope minor component/slope major component) obtained was 1.1. This was in accordance with what the European Pharmacopeia establishes (RRF values between 0.8 and 1.2) [24] and demonstrates that the response of the enantiomeric impurity (L-panthenol) is equivalent to that of the active enantiomer (D-panthenol), thus, the chiral method developed responds in an equivalent way for both enantiomers.

Comparison of the slopes obtained by the external standard calibration method and the standard additions calibrations method for each commercial formulation (eleven known amounts of panthenol were added to the commercial formulation samples containing a constant concentration of dexpanthenol) and for the plant samples (thirteen known amounts of panthenol were added to the culture medium) showed that there were no statistically significant differences between the slopes of each calibration straight line (for a 95% confidence level). Thus, there are no matrix interferences so the external calibration method can be used to quantify the con-

Table 1
Analytical characteristics of the developed CE methodology for the determination of panthenol enantiomers with CE-β-CD.

	L-Panthenol	Dexpanthenol
External standard calibration method[a]		
Range	**1–25 mg L^{-1}**	**15–150 mg L^{-1}**
Slope ± $t \cdot S_{slope}$	0.068 ± 0.003	0.057 ± 0.002
Intercept ± $t \cdot S_{intercept}$	0.01 ± 0.04	0.1 ± 0.1
R^2	99.6%	99.7%
Standard additions calibration method for the cosmetic formulation[b]		
Range	**0–25 mg L^{-1}**	**0–100 mg L^{-1}**
Slope ± $t \cdot S_{slope}$	0.065 ± 0.002	0.059 ± 0.001
R^2	99.8%	99.7%
ACCURACY[c]		
Recovery (%)	102 ± 3	99 ± 3
Standard additions calibration method for the pharmaceutical formulation[d]		
Range	**0–25 mg L^{-1}**	**0–100 mg L^{-1}**
Slope ± $t \cdot S_{slope}$	0.065 ± 0.002	0.058 ± 0.003
R^2	99.7%	99.1%
ACCURACY[e]		
Recovery (%)	99 ± 3	94 ± 6
Standard additions calibration method for toxicity studies[f]		
Range	**0–25 mg L^{-1}**	**0–100 mg L^{-1}**
Slope ± $t \cdot S_{slope}$	0.063 ± 0.006	0.054 ± 0.001
R^2	99.0%	99.8%
ACCURACY[g]		
Recovery (%)	97 ± 3	98 ± 2
PRECISION		
Instrumental repeatability[h]		
t, RSD (%)	0.6	0.3
A_c, RSD (%)	1.5	1.5
Method repeatability[i]		
t, RSD (%)	0.7	0.4
A_c, RSD (%)	2.2	2.7
Intermediate precision[j]		
t, RSD (%)	0.9	0.5
A_c, RSD (%)	3.0	3.3
LOD[k]	1.0 mg L^{-1}	4.0 mg L^{-1}
LOQ[l]	3.3 mg L^{-1}	13.3 mg L^{-1}

A_c: corrected area.
[a] Twelve standard solutions at different concentration levels injected in triplicate.
[b] Addition of eleven known amounts of panthenol standard solution to a cosmetic sample solution containing a constant concentration of dexpanthenol.
[c] Accuracy was evaluated as the mean recovery obtained from six cosmetic samples solutions ($n = 6$) containing 40 mg L^{-1} of dexpanthenol (as labelled amount) spiked with 5 and 40 mg L^{-1} of L- and dexpanthenol, respectively.
[d] Addition of eleven known amounts of panthenol standard solution to a pharmaceutical sample solution containing a constant concentration of dexpanthenol.
[e] Accuracy was evaluated as the mean recovery obtained from six pharmaceutical samples solutions ($n = 6$) containing 40 mg L^{-1} of dexpanthenol (as labelled amount) spiked with 5 and 40 mg L^{-1} of L- and dexpanthenol, respectively.
[f] Addition of thirteen known amounts of panthenol standard solution to the culture medium of the plant samples.
[g] Accuracy was evaluated as the mean recovery obtained from three culture medium of plant samples solutions ($n = 3$) spiked with 15 and 50 mg L^{-1} of L- and dexpanthenol, respectively.
[h] Instrumental repeatability was calculated from six consecutive injections of panthenol standard solution ($n = 6$) at a concentration of L-panthenol of 5 mg L^{-1} and a concentration of dexpanthenol of 75 mg L^{-1}.
[i] Method repeatability was determined by using the value obtained for three replicates of panthenol standards solutions injected in triplicate on the same day ($n = 9$) at a concentration of L-panthenol of 5 mg L^{-1} and a concentration of dexpanthenol of 75 mg L^{-1}.
[j] Intermediate precision was calculated by using the value obtained for three replicates (injected in triplicate during three consecutive days) of panthenol standard solution ($n = 9$) at a concentration of L-panthenol of 5 mg L^{-1} and a concentration of dexpanthenol of 75 mg L^{-1}.
[k] LOD obtained experimentally for a S/N = 3.
[l] LOQ obtained experimentally for a S/N = 10.

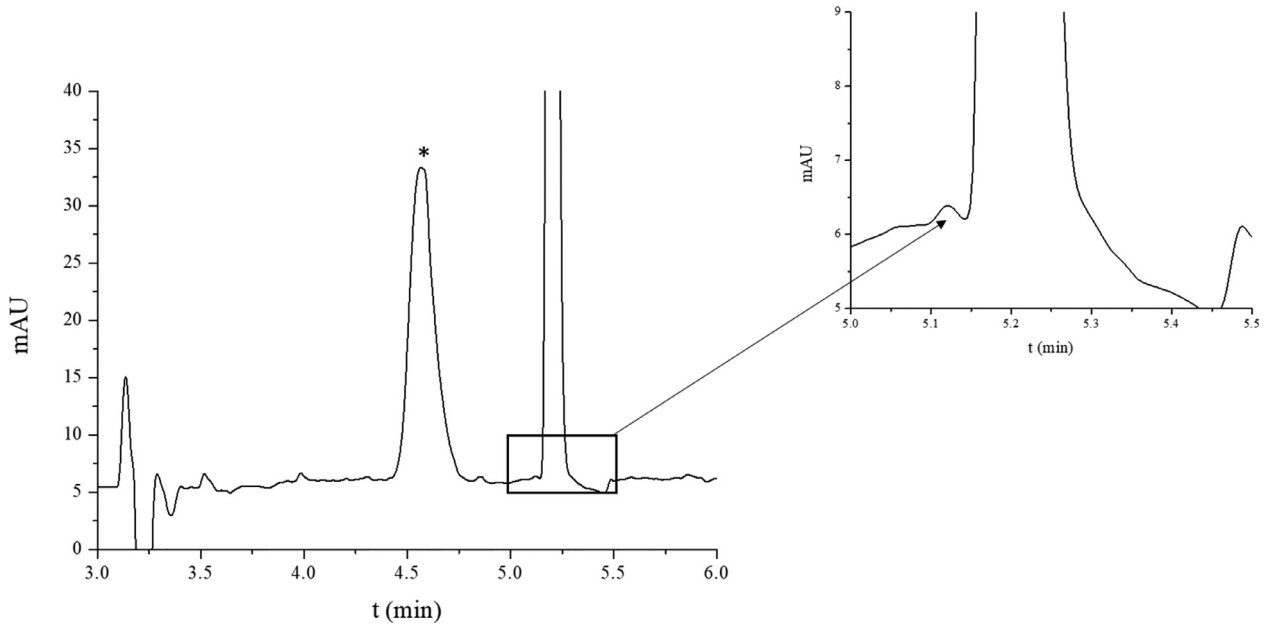

Fig. 2. Electrophoregram corresponding to the LOD of L-panthenol (1 mg L^{-1} standard solution) in the presence of 1000 mg L^{-1} of dexpanthenol according to the label of the commercial formulation (mouthwash). Asterisk could correspond to allantoin. Experimental conditions as in Fig. 1.

tent of dexpanthenol in the cosmetic and pharmaceutical formulations and to perform stability and toxicity studies.

Accuracy of the developed methodology (Table 1) was evaluated as the recovery (%) obtained for panthenol enantiomers when the commercial formulation solutions containing, each, 40 mg L^{-1} of dexpanthenol were spiked with 5 and 40 mg L^{-1} of L- and D-panthenol, respectively, and when the culture medium of the plant samples was spiked with 15 and 50 mg L^{-1} of L- and D-panthenol, respectively. The mean recovery values obtained for the cosmetic formulation were 102 ± 3 for L-panthenol and 99 ± 3 for dexpanthenol, and for the pharmaceutical formulation, the mean recovery values obtained were 99 ± 3 for L-panthenol and 94 ± 6 for dexpanthenol. In the case of plant samples, the recovery values were 97 ± 3 for L-panthenol and 98 ± 2 for dexpanthenol. Then, the recovery values obtained were acceptable as the 100% value is included in all cases.

Precision of the method was evaluated as instrumental repeatability, method repeatability and intermediate precision. In all three cases, racemic panthenol standard solution at two different concentration levels, 10 mg L^{-1} (for L-panthenol) and 150 mg L^{-1} (for D-panthenol) was employed. Instrumental repeatability was determined from six repeated injections on the same day of the standard solutions of racemic panthenol. RSD values (%) were lower than 0.6% for migration times and 1.5% for corrected peak areas. Regarding method repeatability, it was assessed with three replicates of the standard solutions of panthenol injected in triplicate on the same day. RSD values obtained in this case, were lower than 0.7% and 2.7% for migration times and corrected peak areas, respectively. Finally, intermediate precision was evaluated by injecting in triplicate three replicates of the standard solutions of racemic panthenol during three consecutive days. RSD values for migration times were lower than 0.9% whereas for peak areas were lower than 3.3% (see Table 1).

The estimation of the detection (LOD) and quantification (LOQ) limits for the enantiomers of panthenol calculated theoretically ($LOD=(3.29^{*}s_{intercept})/slope$ and $LOQ=(10^{*}s_{intercept})/slope$) were 0.5 and 1.5 mg L^{-1} for L-panthenol, respectively and 3.5 and 10.6 mg L^{-1} for D-panthenol, respectively. However, when the LOD of L-panthenol was experimentally verified (Fig. 2), it was found that this value was underestimated, and a more realistic value could be considered to be 1.0 mg L^{-1} when considering a signal/noise ratio (S/N) of 3, and therefore, the value for the LOQ became 3.3 mg L^{-1} considering in this case a S/N ratio of 10 (see Table 1). In the same way, the LOD and LOQ for the D-enantiomer were experimentally verified, obtaining a LOD of 4.0 mg L^{-1} and a LOQ of 13.3 mg L^{-1}. According to these values, the relative limit of detection (RLOD, calculated as (LOD for the minor enantiomer/concentration of the major enantiomer injected) x 100)) obtained was 0.1%, which is in accordance to the legal ICH regulations [25]. Thus, the developed methodology can be applied to the quality control to the cosmetic and pharmaceutical formulation marketed as enantiomerically pure in dexpanthenol (Fig. 2).

3.3. Quantitative analysis of dexpanthenol in pharmaceutical and cosmetic formulations

Once demonstrated the suitability of the developed methodology for the enantioselective determination of dexpanthenol, the CE method was applied to the analysis of a commercial cosmetic formulation (mouthwash) and a commercial pharmaceutical formulation (topic gel) by injecting a diluted sample of these products containing each dexpanthenol at a concentration of approximately 40 mg L^{-1} according with their labels.

Fig. 1**B** and 1**C** show the electrophoregrams corresponding to the commercial pharmaceutical formulations analyzed under the optimized conditions. As can be seen, the developed methodology shows an adequate selectivity due to the absence of interferences. As can be seen in Table 2, the mouthwash sample revealed a con-

Table 2
Results obtained in the analysis of a cosmetic and a pharmaceutical formulation with an established content of 0.5% and 1.25% of dexpanthenol, respectively. Experimental conditions as in Fig. 1.

	Dexpanthenol content (mg of dexpanthenol per 100 mg of sample)	% Established dexpanthenol content
Cosmetic formulation (mouthwash)	0.51 ± 0.02	103 ± 4
Pharmaceutical formulation (topic gel)	1.24 ± 0.05	99 ± 4

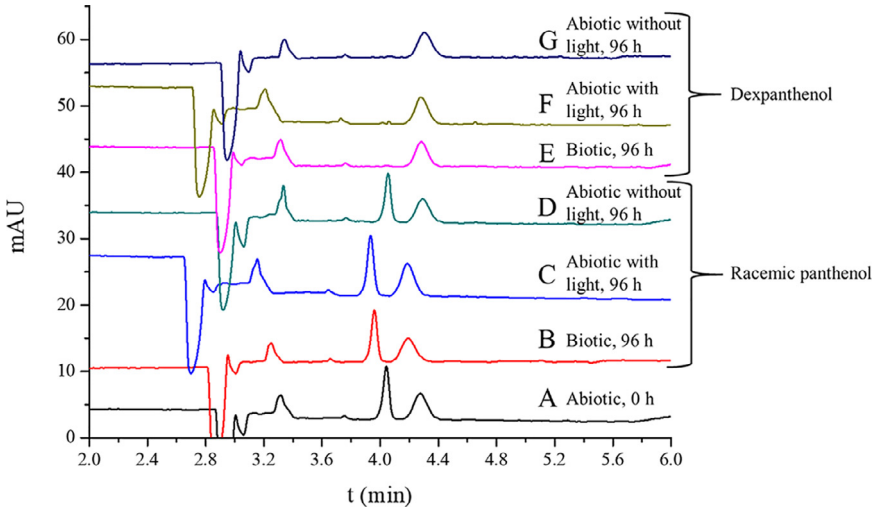

Fig. 3. Electrophoregrams obtained when an abiotic plant sample spiked with 200 mg L^{-1} of racemic panthenol was analyzed at 0 h of exposure (A) and when biotic, abiotic with light and abiotic without light plant samples spiked with 200 mg L^{-1} of racemic panthenol (B, C, D) and 100 mg L^{-1} of dexpanthenol (E, F, G) were analyzed after 96 h of exposure. Experimental conditions as in Fig. 1.

tent of dexpanthenol of 0.51 ± 0.02 mg per 100 mg of sample (corresponding to 103 ± 4 of the labeled content) and the topic gel sample a content of dexpanthenol of 1.24 ± 0.05 mg per 100 mg of sample (corresponding to 99 ± 4 of the labeled content). The results showed a good agreement with the labeled values in both cases, demonstrating the presence of the pure enantiomer of the active principle at > 99.9% and that L-panthenol was not detected.

3.4. Stability study for panthenol racemate and dexpanthenol under biotic and abiotic conditions

Stability of panthenol racemate and dexpanthenol was studied in the range of 12.5–300 mg L^{-1} for panthenol racemate and 6.25 to 150 mg L^{-1} for dexpanthenol enantiomer using individual standard solutions in each case in plant culture medium. Initial and final concentrations (after three days of incubation) were determined by CD-EKC. As an example, Fig. 3 shows the electrophoregrams obtained at 0 h under abiotic conditions and after 96 h of exposure of racemic panthenol (200 mg L^{-1}) and dexpanthenol (100 mg L^{-1}) under biotic (plant samples) and abiotic (culture medium; with and without light) conditions. As can be seen in Fig. 4, no significant variations in the concentrations were observed for the compounds regardless of the different abiotic (with and without light) and biotic conditions. Neither the continuous light irradiation nor the Steinberg culture medium employed for the plant cultivation promote the degradation of racemic panthenol and dexpanthenol. In addition, the presence of the plant *Spirodela polyrhiza* does not induce the degradation. Similar results were obtained for each of the concentrations tested.

Attending to all the data obtained for enantiomers, the variation of the concentration with respect to the nominal value for each

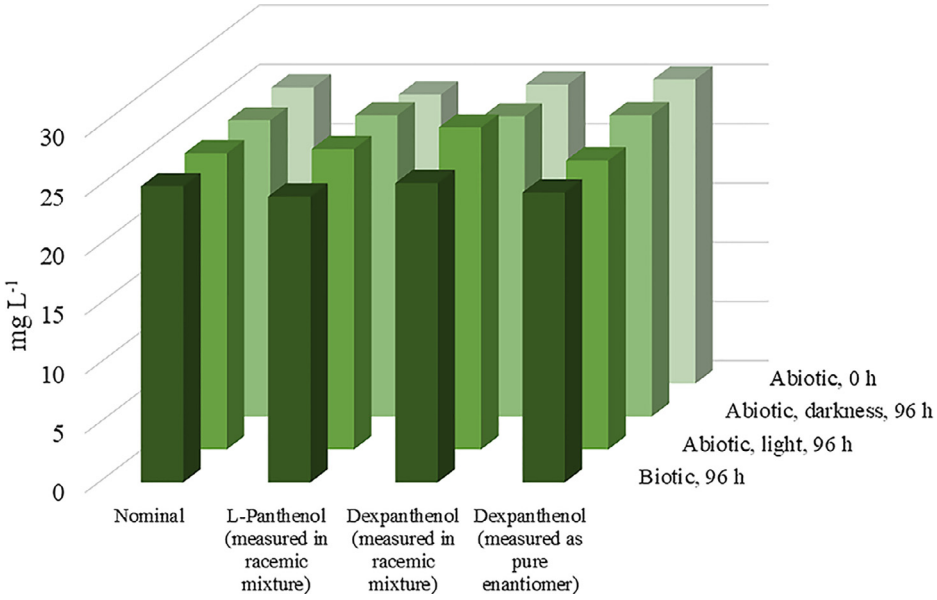

Fig. 4. Stability of L- and D-panthenol (values obtained from measures carried out using 50 mg L^{-1} of racemic panthenol standard solution) and dexpanthenol (values obtained from measures carried out using 25 mg L^{-1} of dexpanthenol standard solution) evaluated under biotic and abiotic (with and without light) conditions. Nominal enantiomer concentration: 25 mg L^{-1}.

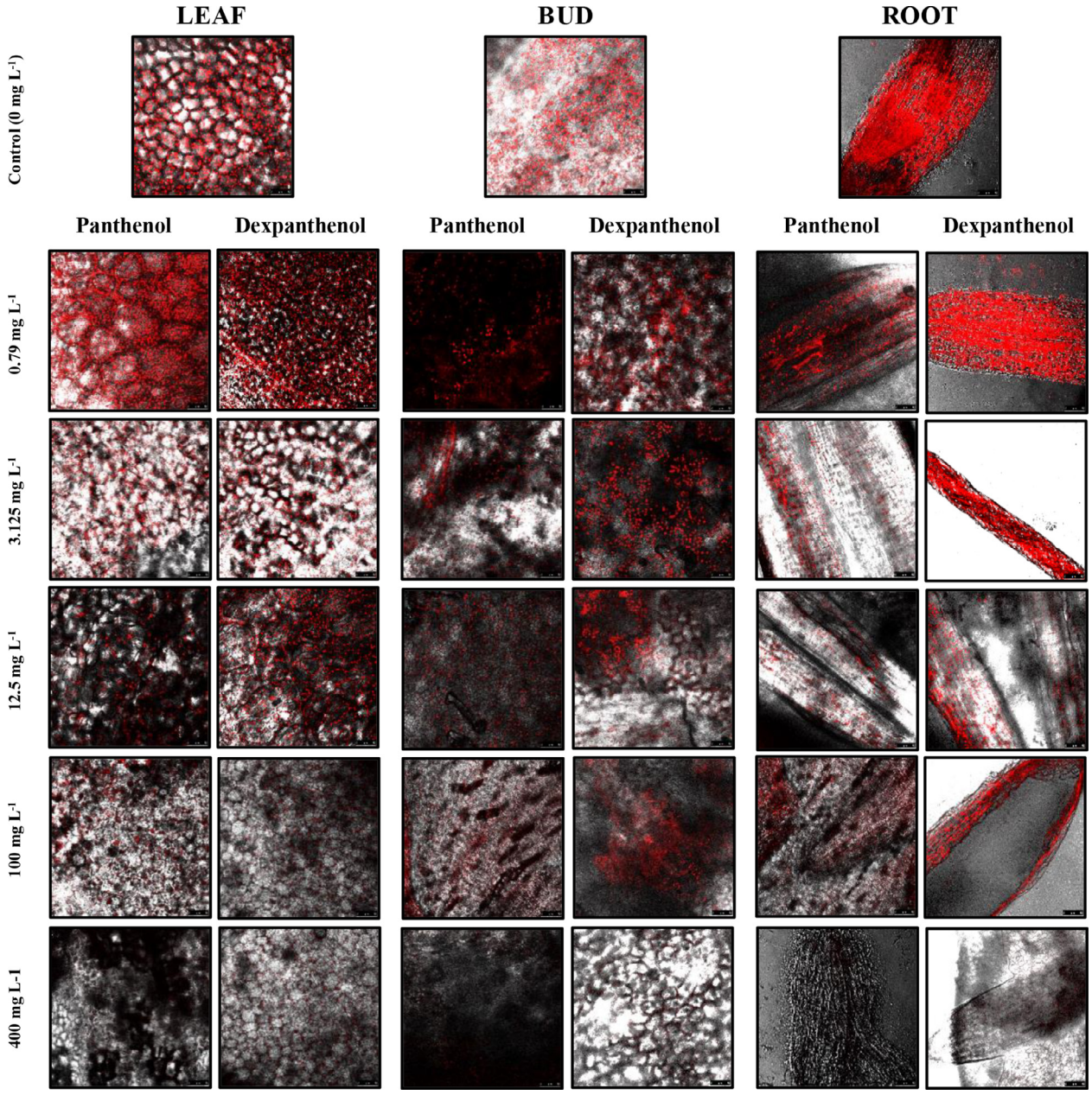

Fig. 5. Confocal images of the aquatic plant *Spirodela polyrhiza* at 96 h of exposition time to panthenol and dexpanthenol.

enantiomer was lower than 5% for L-panthenol and lower than 9% for dexpanthenol (as racemic as well as pure enantiomer), independently of the assay conditions, so both enantiomers can be considered stable during 96 h of cultivation of *Spirodela polyrhiza* in the standard fitotoxicty test medium.

Stability and biodegradability of target compounds were reported in the registration dossiers of the European Chemical Agency (ECHA), which are mandatory for the commercial use of chemicals in the EU region [26,27] (DL panthenol EC number: 240–540–6; Dexpanthenol EC number: 201–327–3). According to these documents, compounds are stable to hydrolysis at pH 4, 7 and 9. Biodegradation was almost complete in contact to aerobic sludge during 28 days of exposure for racemate and 15 days of exposure for dexpanthenol. These data agree with those obtained in this work and both compounds can be considered stable in presence of plants.

3.5. Ecotoxicity of racemic panthenol and dexpanthenol on Spirodela polyrhiza

The ecotoxicity of racemic panthenol and dexpanthenol was determined for the first time in this work on the aquatic plant *Spirodela polyrhiza*. The toxicological parameters (EC20 and EC50) were evaluated using two end-points: the size of the first frond and the natural chlorophyll fluorescence emission by buds, leaves and roots. Table 3 shows the values obtained for these parameters.

Taking into account the EC50 values derived from the first frond at exposure times of 48, 72 and 96 h, which ranged from 2.18 to 6.37 mg L^{-1}, racemic panthenol and dexpanthenol can be classified as toxic to aquatic environment in accordance to the European Regulation (EC1272/2008). In contrast, after 24 h of contact, only racemic panthenol resulted toxic for the plant, while dexpanthenol resulted harmful (EC50 of 25.15 mg L^{-1}). Similar profile was

Table 3
Toxicological parameters of panthenol racemate and dexpanthenol on *Spirodela polyrhiza*.

Evaluation of first frond				
	Panthenol racemate		Dexpanthenol	
Exposure time (h)	EC20 (mg L^{-1})	EC50 (mg L^{-1})	EC20 (mg L^{-1})	EC50 (mg L^{-1})
24	1.20 ± 0.02	5.56 ± 0.01	7.63 ± 0.03	25.15 ± 0.02
48	0.99 ± 0.01	4.55 ± 0.03	1.42 ± 0.01	6.37 ± 0.02
72	0.46 ± 0.01	2.40 ± 0.02	0.61 ± 0.02	3.35 ± 0.01
96	0.41 ± 0.01	2.18 ± 0.01	0.37 ± 0.01	2.22 ± 0.01
Evaluation of chlorophyll fluorescence				
Part of the plant, 96 h exposure time	Panthenol racemate		Dexpanthenol	
	EC20 (mg L^{-1})	EC50 (mg L^{-1})	EC20 (mg L^{-1})	EC50 (mg L^{-1})
Bud	$(9.40 \pm 0.01) \times 10^{-5}$	0.03 ± 0.01	0.03 ± 0.01	1.10 ± 0.01
Leaves	0.38 ± 0.02	5.04 ± 0.02	0.41 ± 0.02	4.94 ± 0.03
Roots	0.01 ± 0.01	0.26 ± 0.01	4.22 ± 0.01	12.81 ± 0.01

EC20 and EC50 are the concentration of compounds that decreased the foliar growth or the autofluorescence emission by 20% and 50%, respectively. These values were reported with 95% of confidence intervals.

observed for the EC20 values obtained from the plants growing, which denoted higher negative effect of the racemate compared to dexpanthenol.

Evaluation of natural chlorophyll fluorescence at 96 h of exposure time clearly showed severe reduction in this biological response of the aquatic plants. Fig. 5 shows this effect. Attending to the parameters estimated for each part of the plant (Table 3), the reduction in autofluorescence occurred at different levels. Low EC50 values were obtained for racemic panthenol on buds and roots, showing high toxicity to the aquatic plant. However, for dexpanthenol, only EC50 values obtained from buds of *Spirodela polyrhiza* showed high toxicity. The autofluorescence obtained from leaves was not affected at the same level as could be observed from the parameters calculated, which also resulted similar for both racemic panthenol and dexpanthenol. Considering the EC20 values, the differences in sensitivity is even more clear, showing lower values of this parameter for contaminants on buds.

Ecotoxicity of pharmaceuticals was reported by ECHA in the registration dossier of compounds [26,27] and in the database of the United States Environmental Protection Agency (EPA) [28,29]. Ecotoxicity for racemic panthenol and dexpanthenol was reported for fish (rainbow trouts), green algae (*Desmodesmus subspicatus, Selenastrum capricornutum*) and microinvertebrate (*Daphnia magna*), resulting nontoxic for all organisms in acute and chronic tests [26–29]. Unfortunately, no data was previously reported for ecotoxicity of panthenol and dexpanthenol on aquatic or terrestrial plants, so this is the first work in which the negative effect on the foliar growth and autofluorescence behavior of duckweed species was evaluated.

Azizullah et al. evaluated the effect of anti-dandruff shampoos containing panthenol (among other compounds) on the duckweed *Lemna minor* [11]. Authors found that concentrations above 0.01% volume of shampoos caused inhibition on biomass growth and light harvesting pigments. In addition, authors found that chlorophyll a and b were more sensitive than biomass growing, similar to the results obtained in this work.

Our results show that both pollutants can cause inhibition of foliar growth of *Spirodela polyrhiza*, but the negative effect was better observed measuring the chlorophyll fluorescence emission. Special attention should be paid to the early-state development of plant, due to the extremely low values of the EC50 and EC20 obtained for buds. In contrast, leaves were minus affected. In fact, the foliar development continues despite the affection in the photosynthetic apparatus of the plant.

4. Conclusions

An analytical CE methodology enabling the chiral separation of panthenol in an analysis time of 4.2 min and with a resolution value of 2.0 has been developed in this work for the first time. These results were obtained employing 25 mM CE-β-CD in 100 mM borate buffer (pH 9.0), with a separation voltage of 30 kV and a temperature of 30 °C. The analytical characteristics of the method were adequate to achieve the quantitation of dexpanthenol in commercial formulations and enabled to detect up to 0.1% of L-panthenol, the enantiomeric impurity, allowing to accomplish the legal ICH regulations. Thus, the developed methodology can be considered a powerful tool to achieve the quality control of cosmetic and pharmaceutical formulations marketed as enantiomerically pure in dexpanthenol. The chiral method enabled to show that racemic panthenol and dexpanthenol were stable under the different abiotic and biotic conditions assayed in this work. Toxicity studies reported in this work for the first time on aquatic plants demonstrate that toxicity of racemic panthenol is considerably higher than that of dexpanthenol for short exposure times (24 h) although the toxicity differences are minor for larger exposure times.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

CRediT authorship contribution statement

Sara Jiménez-Jiménez: Investigation, Methodology, Formal analysis, Validation, Data curation, Visualization, Writing - original draft. **Georgiana Amariei:** Investigation, Data curation, Visualization. **Karina Boltes:** Formal analysis, Resources, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **María Ángeles García:** Conceptualization, Methodology, Formal analysis, Resources, Supervision, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition. **María Luisa Marina:** Conceptualization, Resources,

Supervision, Writing - original draft, Writing - review & editing, Project administration, Funding acquisition.

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