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Effects of UV Radiation on Usnic Acid in *Xanthoparmelia microspora* (Müll. Arg. Hale)

Ernesto Fernández*[1], Wanda Quilhot[1], Cecilia Rubio[1], María Eliana Hidalgo[2], Rodrigo Diaz[1] and Juan Ojeda[1]

[1]Facultad de Farmacia, Universidad de Valparaíso, Valparaíso, Chile
[2]Facultad de Ciencias, Universidad de Valparaíso, Valparaíso, Chile

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INTRODUCTION

An important decrease in the level of stratospheric ozone has been observed during the past decade (1), with accompanying increases in UV-B radiation (2) and some UV-A radiation (3), thus leading to probable damage to aquatic and terrestrial ecosystems (4–7). Inhabitants of terrestrial and aquatic ecosystems have developed strategies to cope with the potential stress induced by UV radiation. One of these strategies is the synthesis of photoprotector compounds derived mainly from the biosynthetic route of the polymalonate acetate (8–13). As levels of UV radiation increase, accumulation rates of some photoprotective compounds in lichen species from the Chilean Alpine zones also increase (14). Photodegradation of depsides may have accounted for the low levels of phenolics in lichens at higher elevations (15). It was observed that, in *Xanthoparmelia* lichens from the Alpine regions of Chile, the higher the altitude where the lichen was found, the greater amount of usnic acid it produced (16). This increase of usnic acid is the result of the lichen's ability to adapt to radiation, which ensures its survival in extreme environmental conditions. In thalli from *Xanthoparmelia oleosa* collected at 4690 m no usnic acid was detected, which could be proof of the presence of a photodegradation product of usnic acid (17) but which has not been identified to date. In growth-chamber experiments lasting 1 week, thalli of *Umbilicaria americana* always produced smaller amounts of phenolic residue when exposed to both UV-B and UV-A radiation, compared with exposure to UV-A alone. In one field experiment exclusion of UV-B by light filtration produced significant increases in phenolic compounds. This increase occurs only during spring and autumn, when lichens are metabolically more active (18).

In this article we evaluated the accumulation rates of usnic acid in *Xanthoparmelia microspora* (Müll. Arg. Hale) exposed to doses of solar radiation and to additional doses of UV-A and UV-B radiation. Furthermore, we report on the photolysis of usnic acid in nonnucleofílic solvents during exposure to higher doses of UV-A and UV-B radiation.

MATERIAL AND METHODS

Effects of UV-A and UV-B radiation doses on usnic acid photodegradation. Thalli from *X. microspora* (Müll. Arg. Hale) were collected from their substrate (rock) in Granizo, Valparaíso (lat 33°S, long 71°W). The samples were carried to the laboratory and exposed to natural conditions of solar light, temperature and humidity. The samples were divided into three treatment groups: the control group, which was exposed only to natural solar radiation; experimental group 1, which was exposed to solar light supplemented with 70% additional UV-A radiation; and experimental group 2, which was exposed to solar light supplemented with 70% additional UV-B radiation.

Daily doses of solar light were measured by a Solar Light radiometer equipped with a UV-A and UV-B detector. Additionally, supplemental doses of UV-A were applied daily by means of a Philips TLK 40W/10R photoreactor (λ_{max} 360 nm; irradiance, 7.5 mW cm^{-2}) and supplemental doses of UV-B were applied daily by means of a Philips TLK 40W/12R photoreactor (λ_{max}, 310 nm; irradiance, 1.46 mW cm^{-2}). The maximum irradiation time for the samples was 45 days.

Determination of the rates of accumulation of usnic acid. Usnic acid concentrations were determined at 0, 15, 30 and 45 days of treatment. The thalli were submerged in chloroform for 48 h at room temperature. The extracts were filtered and taken to a fixed volume. Aliquots of the extracts were applied to a 60HF254 Merck thin-layer silica gel by means of a Camag Linomat III applicator and were eluted with toluene-ethyl acetate-formic acid (35 : 5 : 1). Usnic acid was quantified by high-performance thin-layer chromatography (HPTLC) and its remission was recorded at wavelength of 313 nm by a Camag photodensitometer. The concentration variability of usnic acid was determined by means of analysis of variance and the differences between the means were determined by the Turkey test.

Isolation and identification of a photoproduct of usnic acid. Usnic acid in acetonitrile (0.02%) was irradiated with UV-A or UV-B as previously described. The irradiated solutions were concentrated until dry. The dried residue was dissolved in acetone and eluted in a chromatographic column with toluene-ethyl acetate-formic acid (35 : 5 : 1) and silica gel 60 as the adsorbent.

The photoproduct with the highest concentration was isolated and purified. An usnic acid solution (5.8×10^{-4} *M* in acetonitrile) was irradiated and concentrated at reduced pressure until dry and then eluted in a chromatographic column (silice gel 60) with toluene-ethyl acetate-formic acid (35 : 5 : 1). Its chemical structure was determined by means of UV spectroscopy (Cecil CE 2041). IR spectroscopy was performed to record the photoproduct's spectra in KBr pellets (FT-IR Nicolet Impact 420); NMR (Bruker AC-200) with TMS as an internal standard and $CDCl_3$ as solvent and AM-500, Mass spectra (MS) were recorded on a Fison TRIO 1000 (70 eV). Fluorescence spectral analysis of the photoproduct was performed in a spectrofluorometer (Shimadzu RF540).

SPF determination. The photoprotector capacity of the photoproduct was determined by measuring the transmittance (area under the curve) of the solutions, using homosalate as a reference sunscreen (19). Usnic acid solutions in a universal solvent, which was made by mixing 12.5 g of methylene chloride, 37.5 g of cyclohexane and 50 g of isopropanol (12), were irradiated using a narrow-band UV-B Philips TL40W/01 RS lamp that provided a mean irradiance of 1.46 mW cm^{-2}.

Photodegradation mechanism (oxygen involved). Usnic acid solutions (5.8×10^{-4} *M* in acetonitrile and in methanol) were irradiated with UV-A light (irradiance, 7 mW cm^{-2}) in tubes exposed to environmental atmospheric conditions (21% O_2 and 78% N_2) and to nitrogen atmospheric conditions for 2 h.

*Corresponding author email: maria.hidalgo@uv.cl (María Eliana Hidalgo)

Table 1. Usnic acid rates in thallus of *Xanthoparmelia microspora* exposed to sunlight and 70% additional UV-A or UV-B irradiances.*

Days of Treatment	Samples	Usnic Acid (g 100 mL^{-1}) Additional UV-A (mW cm^{-2})	Usnic Acid (g 100 mL^{-1}) Additional UV-B (mW cm^{-2})
0	Control	0.91 ± 0.010	0.91 ± 0.010
15	Control	1.90 ± 0.110	1.90 ± 0.110
	Irradiated	6.20 ± 0.155	4.16 ± 0.053
30	Control	2.46 ± 0.028	2.46 ± 0.028
	Irradiated	2.55 ± 0.011	2.42 ± 0.015
45	Control	2.73 ± 0.145	2.73 ± 0.145
	Irradiate	1.64 ± 0.020	1.33 ± 0.060

*Data are mean values of three lichen samples ± SEM.

Hemolysis determination. Red blood cell suspensions (2% in PBS) were incubated in the dark at room temperature and at 37°C in the presence of 10^{-7} *M* usnic acid solutions. The hemolysis percentage was determined by measuring the hemoglobin liberated. The percentages of hemoglobin, hemichrome, and methemoglobin were evaluated according to the procedures given in Winterbourn (20). The measurements were carried out spectrophotometrically.

Photohemolysis determination. Red blood cell (RBC) suspensions (2% in PBS) were irradiated in the presence of usnic acid solution (10^{-7} *M*) for a period of 3 h (dose, 16 J m^{-2}).

Determination of the toxicity in Artemia saline. Nauplii of *A. saline* were incubated in usnic acid solutions with concentrations of 10, 100 and 1000 ppm. Survival of the species was determined after 48 h.

RESULTS

Additional UV-A or UV-B dose radiation effects on usnic acid accumulation rates

Table 1 shows usnic acid concentrations in samples of *X. microspora* exposed to sunlight radiation with 70% additional UV-A or UV-B irradiances.

Photoproduct isolation from usnic acid solution

No photodegradation was observed when a chloroform solution of usnic acid was irradiated for 3 h (dose, 154 J cm^{-2}).

Usnic acid solutions (5.8×10^{-4} *M* in acetonitrile and methanol; λ_{max}, 232 and 281) irradiated with UV-A and UV-B radiation produced the same photoproducts. Usnic acid solutions in acetonitrile irradiated for 3 h (irradiance, 7 mW cm^{-2}) generated 37% of the photoproduct with the highest concentration. A progressive increase in photoproduct concentration and a growing decrease of usnic acid concentration was observed (Fig. 1). The presence of a nonlinear relationship between the increase in concentration of the main photoproduct and the decrease in the concentration of the usnic acid indicates the possibility of secondary photolitic processes (Fig. 2).

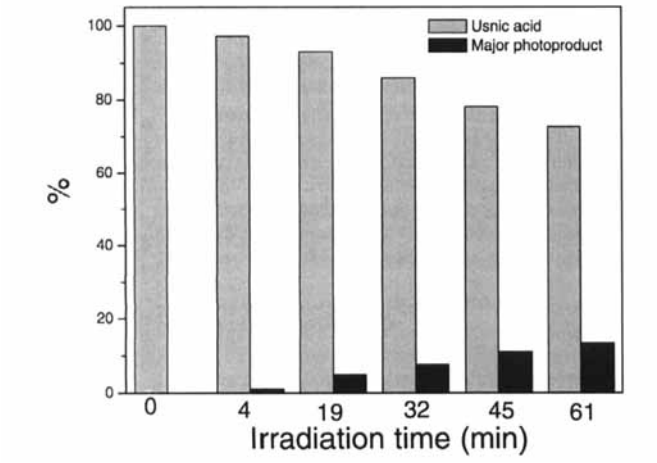


Figure 1. Usnic acid photodegradation (0.02 % in acetonitrile).

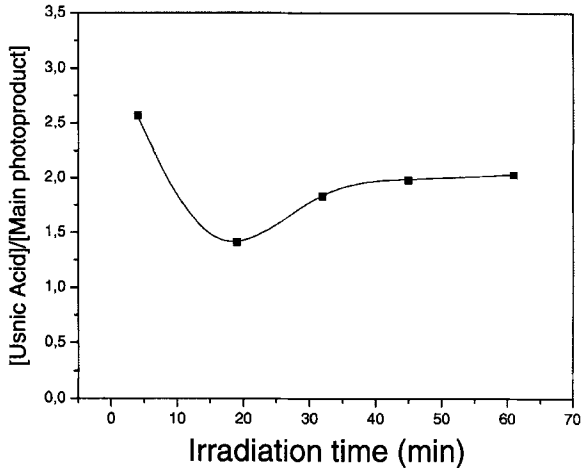


Figure 2. Relationship between usnic acid concentration and principal photoproduct.

Identification of the principal photoproduct

The principal photoproduct obtained under the described experimental conditions had the following properties: molecular weight, 318 g mol^{-1}; Rf, 0.42 (toluene-ethyl acetate-formic acid [35 : 5 : 1]) and melting point (mp) 220°C (Fig. 3). The UV spectrum of the photocompound in acetonitrile, compared with usnic acid, had three maximum levels of absorption—λ_{max} 241, 294.5, and 344 nm—which corresponded to the transitions π-π* and n-π* of aromatic ketones. The fluorescence spectrum of the photocompound in acetonitrile presented an emission band centered at a λ of 512 nm (λ_{exit} 344 nm), in contrast to usnic acid, which did not emit fluorescence. The IR spectrum in the KBr presented a wide band at 3420 cm^{-1}, corresponding to an associate tension band (OH, C2′), and a band at 1620 cm^{-1}, which was probably assigned to a chelated ketone (enol of 1,3-diketone, C4′). The RMN-H1 spectrum in CDC_{13} showed the following signals: 2.4 ppm (s, 3H; C3-CH_3), 2.0 ppm (s, 3H; C5-CH_3), 2.7 ppm (s, 3H; C7-$COCH_3$), 4.3 ppm (s, 2H; CH_2, C1′), 6.1 ppm (s, 1H; OH, C4), 7.9 ppm (s, 1H; OH, C6) and 14.3 ppm (s, 1H; OH, C2′). The mass spectrum showed the following signals: $C_{17}H_{18}O_6$ 318 (M+ corresponding to the MW), the fragment 233 that was formed from the rupture of the compound M+ with loss of the ring

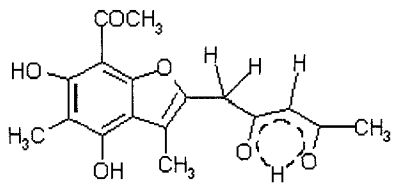
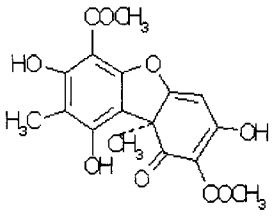


Figure 3. Principal photoproduct. Molecular weight = 318 g mol^{-1}; mp = 220°C.



(structure 1 breaks into fragment 85). The fragment 233 is present in the mass spectra of usnic acid (Fig. 4).

Therefore, on the basis of this spectroscopic evidence, we postulate that the principal photoproduct has the following chemical structure: (2-[2′-Hydroxy-4′-oxopentyl]-3,5-dimethyl-4,6-dihydroxy-7-acetylbenzofuran) (Fig. 3).

Photodegradation mechanism (oxygen involved)

The chromatographic (HPTLC) results for both 5.8×10^{-4} *M* solutions of usnic acid in methanol and acetonitrile under atmospheric conditions and nitrogen, using as a mobile-phase toluene-ethyl acetate-formic acid (139 : 83 : 8), showed the same Rf.

Toxicity and phototoxicity determination in RBC

Figure 5 shows the hemolysis percentages of controls and usnic acid solutions (10^{-7} *M*) in conditions of darkness, 37°C and irradiation.

A low photohemolysis percentage (1.47% after 3 h of irradiation) was observed in usnic acid under the different conditions.

Toxicity and phototoxicity determination in *A. saline*

The survival of *A. saline* after 48 h of incubation demonstrated that usnic acid is not toxic for this species (Fig. 6).

Principal photoproduct SPF: test *in vitro*

The photoprotection capacity of the previously isolated principal photoproduct was evaluated by measuring the transmittance of the solution in a universal solvent (as previously described) using homosalate as a reference (SPF = 4).

An inverse correlation was detected between the area under the curve of the transmittance spectra and the SPF of the species in solution (Table 2).

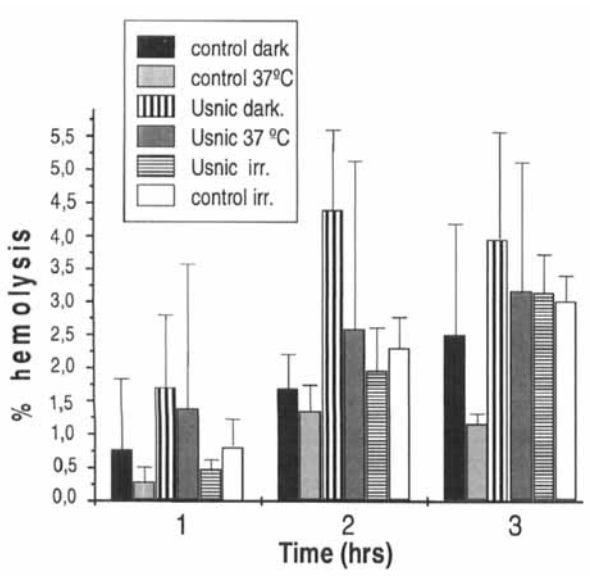


Figure 5. Hemolysis and photohemolysis of usnic acid solutions.

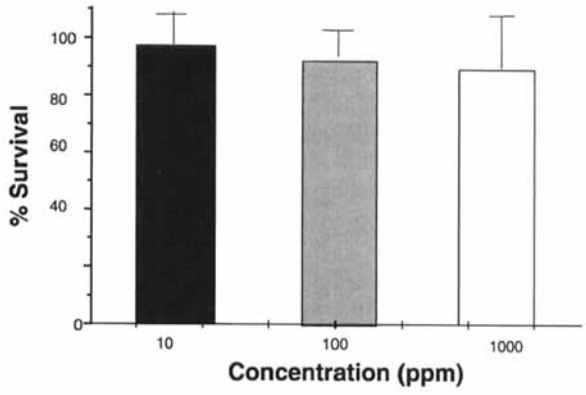


Figure 6. Percentage survival of *Artemia salina* in the presence of usnic acid.

DISCUSSION

Thalli from *X. Microspora* exposed to sunlight and to 70% additional doses of UV-A showed an increase in usnic acid concentration from 1.9 to 6.2% w/w after 15 days of treatment. Under the same conditions, the thalli exposed to UV-B radiation showed an increase in usnic acid concentration from 1.9 to 4.16% w/w. These results agree with those of Swanson and Fahselt (15) for the lichen *Umbilicaria antartica*; they postulated an interaction between UV-A photons and a photoreceptor mechanism that affected phenolic metabolism. Nevertheless, we found that, once a certain radiation threshold is reached (45 days), the photodegradation process occurs. The increase of the usnic acid concentration with UV-A light corresponds to its λ_{max} absorption (430 nm) in the solid-state, as found in the lichen (11).

Takani and Takahashi (21) isolated and characterized a photoproduct of MW 360 g mol^{-1} (from irradiated usnic acid methanolic solutions with all regions of the Hg lamp spectrum), which had a structure similar to that of the photoproduct isolated by us.

According to the chromatographic and spectroscopic results, the photodegradation mechanism of usnic acid in acetonitrile occurs by means of a nucleophilic attack by water molecules, which are present in a low percentage in the acetonitrile (0.3%). The mechanism proposed is similar to those reported in Takani and Takahashi (21), which involves a decarboxilation on the photoproduct generation. This photoreaction is quite common in the photodegradation of quinolone (22).

Table 2. Value of the SPF in vitro of usnic acid and the main photoproduct.

Filter	Concentration (mg 10 mL^{-1})	SPF
Homosalate	0.4	4.00
Photoproduct	0.4	7.35
Usnic acid	0.4	7.40

No photoproduct formation was observed in the chloroformic solution of usnic acid. This is because the chloroform is a non-nucleophilic solvent (maximum water percentage, 0.01%).

In lichens, usnic acid is immersed in an organic matrix in the presence of water and solar radiation; thus, one could propose a photodegradative mechanism similar to the one proposed in this article, which is supported by results obtained by Ramirez (17) in thalli from *X. oleosa* collected at 4690 m, where no usnic acid was detected.

On the other hand, evidence exits that oxygen does not participate in the photolytic mechanism and that, in this mechanism, methanol and water could act as nucleophiles. Usnic acid photostability depends on the nucleophilic nature of the solvent or the dispersant phase.

In relation to the value of SPF *in vitro,* it is postulated that the orthohydroxycarbonilic group is the one that characterizes the commercial solar filters (23). Usnic acid turned out to be one of the best UV-B natural filters, with an *in vivo* protection factor similar to that of Nivea Sun Spray LSF 5 (24). Therefore, a 50% decrease in the SPF would have been expected for the principal photoproduct, because its two orthohydroxycarbonilic units are stabilized by resonance and hydrogen bonding, thus producing a highly stable chelate structure. However, the experimental evidence demonstrates that the photoproduct has the same SPF as that of usnic acid; thus, no relationship exists between the number of orthohydroxycarbonilic units and SPF.

CONCLUSION

The photoprotection demonstrated by usnic acid and the characteristics exhibited by the photoproduct, such as no toxicity, no phototoxicity and photoprotection capacity, could suggest a potential use of such a compound as a solar protector in cosmetics. Further experiments are in progress.

REFERENCES

1. Bjorn, L. O. (1996) Effects of ozone depletion and increased UV-B on terrestrial ecosystem. *Int J Environ Stud* **51**, 217–243.
2. McKenzie R. L., L. O. Björn, A. Bais and M. Ilyas (2003) Changes in biologically active ultraviolet radiation reaching the earth's surface. In *Environmental Effects on Ozone Depletion, 2002 Assesment.* United Nations Environment Programme (UNEP), Nairobi, Kenya, pp. 1–23.
3. Madronich S., R. L. Mckenzie, L. O. Björn and M. M. Caldwell (1998) Changes in biological active ultraviolet radiation reaching the Earth's surface. *J. Photochem. Photobiol. B.* **46**, 5–19.
4. Tevini M. (1994) UV-B effects on terrestrial plants and aquatic organisms. *Prog. Bot.* **55**, 174–190.
5. Mazza C. A., H. E. Boccalandro, C. V. Giordano, D. Battista, A. L. Scopel and L. Balare (2000) Functional significance and induction by solar radiation of ultraviolet-absorbing sunscreens in field grown soybean crops. *Plant Physiol.* **122**, 117–126.
6. Searles P. S., S. D. Flint and M. M. Caldwell (2001) A meta-analysis of plant field studies simulating stratospheric ozone depletion. *Oecologia* **127**, 1–10.
7. Flint S. D. and M. M. Caldwell (2003) Field testing of UV biological spectral weighting functions for higher plants. *Physiologia Plantarum* **117**, 137–144.
8. Asahina Y. and S. Shibata (1954) *Chemistry of Lichen Substances.* A. Asher and Co Ltd, Vaals-Amsterdam.
9. Vicente C. (1975) *Fisiología de las Sustancias Liquénicas.* Alhambra, Madrid.
10. Culberson C. F. and J. A. Elix (1989) Lichens substances. In *Methods in Plant Biochemistry,* Vol. 1 (Edited by P. M. Day and J. B. Harbone), pp. 509–535. Academic Press, London.
11. Quilhot W., E. Fernández and M. E. Hidalgo (1994) Photoprotection mechanisms in lichens against UV-radiation. *Br. Lichen Soc. Bull.* **75**, 1–5.
12. Fernández E., W. Quilhot, I. González, M. E. Hidalgo, X. Molina and I. Meneses (1996) Photoprotector capacity of lichen metabolites against UV-B radiation. *Cosmetics and Toiletries* **111**, 69–74.
13. Quilhot W., E. Fernández, C. Rubio, F. Cavieres, M. E. Hidalgo and D. J. Galloway (1996) Preliminary data on the accumulation of usnic acid related to ozone depletion in two antarctic lichens. *Ser. Cient. INACH* **461**, 105–111.
14. Rubio C., E. Fernández, M. E. Hidalgo and W. Quilhot (2002) Effects of solar UV-B radiation in the accumulation of Rhizocarpic acid in a lichen species from alpine zones of Chile. *Bol. Soc. Chil. Quim.* **47**, 067–072.
15. Swanson A., D. Fahselt and D. Smith (1996) Phenolic levels in *Umbilicaria americana* in relation to enzyme polymorphism, altitude and sampling data. *Lichenologist* **28**, 331–339.
16. Fernández E., W. Quilhot, C. Rubio and E. Barre (1998) Lichen's adaptation to altitude. In *Photosynthesis Mechanisms and Effects,* Vol. 5 (Edited by G. Garab), pp. 4093–4096. Kluwer, Dordrecht.
17. Ramírez J. (1997) *Tasas de acumulación de ácido úsnico y su relación con la irradiancia solar UV en líquenes de altura.* Tesis, Escuela de Química y Farmacia, Universidad de Valparaíso, Chile.
18. Swanson A., D. Fahselt and D. Smith (1997) Effects of ultraviolet on polyphenolics of *Umbilicaria americana. Can. J. Bot.* **75**, 284–289.
19. Meybeck A. (1983) Objective methods for the evaluation of sunscreen. *Cosmetics and Toiletries* **98**, 51–60.
20. Winterbourn C. (1990) Oxidant reactions of hemoglobin. *Methods Enzymol.* **186**, 265–272.
21. Takani M., and K. Takahashi (1985) The photolysis of usnic acid and its derivatives. *Chem. Pharm. Bull.* **33**, 2772–2777.
22. Fernandez E, G. Sánchez, E. Navarrete and F. del Alcázar (2004) UVA-induced loss of quinolone antibacterial activity. *Ars Pharmaceutica* **45**, 111–119.
23. Hidalgo M. E., E. Fernández, W. Quilhot, and E. A. Lissi (1992) Solubilization, photophysical and photochemical behaviour of depsides and depsidones in water and Brij-35 solutions at different pH values. *J. Photochem. Photobiol. A.* **67**, 245–254.
24. Rancan F., S. Rosan, K. Boehm, E. Fernández, M. E. Hidalgo, W. Quilhot, C. Rubio, F. Boehm, H. Piazena and U. Oltmanns (2002) Protection against UVB irradiation by natural filters extracted from lichens. *J. Photochem. Photobiol. B.* **68**, 133–139.