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Journal of Chromatography A, 758 (1997) 145–157

JOURNAL OF CHROMATOGRAPHY A

# Novel methods for identification and quantification of the mushroom nephrotoxin orellanine
# Thin-layer chromatography and electrophoresis screening of mushrooms with electron spin resonance determination of the toxin

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Received 15 August 1995; revised 23 April 1996; accepted 7 August 1996

**Abstract**

Orellanine, (2,2′-bipyridine)-3,3′,4,4′-tetrol-1,1′-dioxide, the toxin from several *Cortinariaceæ* species, induces an acute renal failure which can be very severe or even irreversible and fatal. It is therefore important to be able to quickly and simply identify orellanine in mushroom samples with classical methods, readily available in any laboratory, such as anti-poison centers. This article reports the results of three analytical methods: classical TLC on cellulose plates in *n*-butanol–acetic acid–water and two original methods, electrophoresis on agarose gel and direct electron spin resonance (ESR) after enzymatic oxidation. They were applied to detect orellanine in 34 *Cortinariaceæ* and 4 other species of toadstools. Our three sets of results are convergent. TLC (detection limit: 15 ng with fluorescence densitometry), electrophoresis (25 ng) and even ESR (5 μg), are sensitive enough for our purpose, and a sophisticated method like HPLC (detection limit: 50 pg) is not required. As the ESR spectrum of the toxin semiquinone is highly specific, TLC or electrophoresis coupled with ESR are a convenient alternative to liquid chromatography coupled with mass spectrometry, with the same specificity, for a confirmation or with samples such as ours with high toxin contents. ESR unambiguously confirms the relatively high contents of orellanine, from 0.45% (*C. henrici*) to 1.1–1.4% (*C. orellanus*), found in five *Cortinarius* from the subgenus *Leprocybe*, section *Orellani*. The five species, though they are from different geographic origins, have a more or less common pattern of fluorescent compounds, among which orellinine and orelline beside orellanine. It can be useful to note that orellanine semiquinone can be easily detected by ESR directly in the fresh mushroom. The toxin is absent in the other mushrooms we tested, especially in *D. cinnamomea* and *C. splendens*, which have been claimed as toxic and suspected to contain orellanine.

*Keywords:* Electron spin resonance; Orellanine; Toxins

## 1. Introduction

Orellanine, (2,2′-bipyridine)-3,3′,4,4′-tetrol-1,1′-dioxide (Fig. 1), is the toxin from several *Cortinariaceæ* species, among which are contained *C. orellanus* and *C. speciosissimus* [1,2]. Very severe or even fatal human intoxications have still been reported every autumn in the last few years in Europe and North America. After a few days delay, the lethal toxin induces an acute renal failure which

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0021-9673/97/17.00 
*PII* S0021-9673(96)00695-4

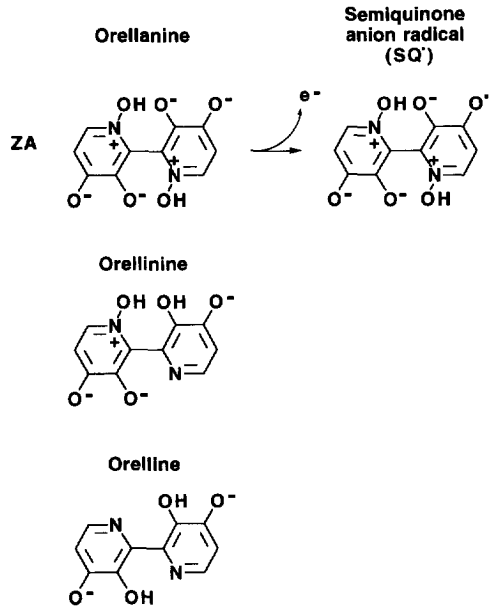



Fig. 1. Chemical structures of orellanine, its orthosemiquinone anion radical and its reduction products, orellinine and orelline. ZA: predominant pseudo-zwitterionic species of orellanine at pH 5.8 to 11 [12].

can be severe. Neither antidote nor specific therapy are known at present. In the case of irreversible renal failure, only chronic hemodialysis or renal transplantation are efficient.

We have recently shown that the toxicity of orellanine might be due to its ability to form an orthosemiquinone anion radical after a one-electron oxidation under various physiological conditions [3]. This activation results in the formation of toxic molecular products, such as orthoquinone, and in the formation of semiquinone and oxygen free radicals, which then induce oxidative stress and various disorders in the cellular physiology [3,4]. Interestingly, orellanine is the only aminoxidized bipyridine we studied which is able to form such a semiquinone radical (Fig. 1) under physiological conditions and to develop severe nephrotoxicity [5].

The mycological literature concerning the toxicity of the numerous species of *Cortinariaceæ* is uncertain and often misleading. It is therefore important to be able to quickly and simply identify orellanine in mushroom samples. Screenings were achieved by a few authors, but only with thin-layer chromatography (TLC) applied to crude extracts of several mushrooms. No specific identification of the detected compounds with the toxin or its degradation or metabolization products was made.

Tebbett et al. screened 61 *Cortinariaceæ* species by TLC with UV light detection at 254 nm [6]. They aimed to determine the presence, in these species, of the compounds they extracted from *C. speciosissimus* and they identified as cyclic decapeptides called cortinarins A, B (both claimed as toxic) and C (non-toxic). These compounds were also determined in 43 *Cortinariaceæ* species using high-performance liquid chromatography (HPLC) [7] and in 10 species with reversed-phase HPLC [8]. However, even the existence of the cortinarins was strongly questioned recently [9], the toxicity of the extracts being attributed to orellanine and their fluorescence together to a steroid and to the photoreduction products of orellanine (Fig. 1), orellinine and the atoxic compound orelline [2].

Keller-Dilitz et al. [10] subjected ethanolic extracts of 14 samples of five *Cortinarius* species (*C. fluorescens, C. orellanoides, C. orellanus, C. rainierensis* and *C. speciosissimus*) to TLC on cellulose plates in *n*-butanol–acetic acid–water (BAW) (3:1:1, v/v). On the basis of colour, fluorescence and $R_F$ value (0.69) of a co-chromatographed reference sample, spots were attributed to orellanine in *C. orellanoides, C. orellanus, C. rainierensis* and *C. speciosissimus*, but not in *C. fluorescens*.

Rapior et al. [11] sought orellanine and its photodecomposition product orelline in 49 *Cortinariaceæ* species, including the eight species of the section *Orellani*. They analyzed methanol–water extracts by TLC on cellulose in two different solvent systems: *n*-butanol–hydrochloric acid–chloroform–water (BCCE) (40:20:15:3.8, v/v) and BAW (3:1:1, v/v). An orellanine standard was co-chromatographed. Only the *Cortinarius* species in the section *Orellani* except *C. fulvaureus* showed spots, visualized by UV light at 366 nm, similar to the ones of orellanine ($R_F$=0.70 in BAW and 0.57 in BCCE, claimed detection limit 10 to 20 ng). Other spots ($R_F$=0.57 in BAW and 0.42 in BCCE) were attributed to orelline, but without referring to any orelline standard.

Taking into account our knowledge about the acido–basic properties of orellanine [12] and about its behaviour in TLC, we optimized two sensitive HPLC methods for the separation and determination

of the toxin in pure aqueous solution [13]. Only one of these methods is suitable for the determination of orellanine in mushroom extracts. However, this method is delicate and time consuming as it requires extraction, delicate dissolution of the heavily laden extracts in phosphoric acid pH 0, addition of an organic counter ion ($2.5 \cdot 10^{-3}$ mol $l^{-1}$ 1-octanesulphonic acid) in the mobile phase (phosphoric acid pH 1–acetonitrile, 94:6, v/v) and delicate and time consuming maintenance of the $SiC_{18}$ bonded phase with this pH 1 mobile phase. Not every commercial column stands up to such a treatment, and polymeric columns, which could be an alternative choice, are still expensive. The length of time needed in addition for the stabilization process (half a day before each use) and for the analyses (10 min at least for a single assay) almost precludes the use of this HPLC method for a large, but not routine, screening.

Searching for another method using common equipment without a complex extraction procedure and readily available in any laboratory, such as anti-poison centers in case of emergency, we thought of electrophoresis [14]. The aim of this paper was to develop and apply a rapid, sensitive and specific method to determine the toxin in various samples of mushrooms. To ensure specificity in the detection of orellanine, we have used its property to form an orthosemiquinone anion radical after enzymatic oxidation [3]. This radical can be detected with very high specificity and sensitivity by electron spin resonance (ESR). This article reports the results of three methods used with the suitable standards: classical TLC on cellulose plates in BAW and our two original methods, electrophoresis on agarose gel and direct ESR after enzymatic oxidation. These methods were applied to detecting orellanine in 34 *Cortinariaceæ* and 4 other species of toadstools. On the basis of the results obtained, we have been able to unambiguously state the species among those tested which actually do contain the toxin.

## 2. Experimental

### 2.1. Samples, chemicals and reagents

Water was deionized and twice distilled in a quartz glass still. Sodium acetate and acetic acid were analytical-reagent grade (Merck). Phosphoric acid, potassium hydroxide, potassium dihydrogen phosphate and disodium hydrogen phosphate were Suprapur reagent grade (Merck). Trizma base (molecular biology grade) and tyrosinase (EC 1.14.18.1) from mushroom (4200 Units/mg) were supplied by Sigma. Ethylenediaminetetraacetic acid disodium salt (EDTA) was from Aldrich-Europe. Methanol (Chromanorm grade) and diethyl ether (RP grade) were purchased from Prolabo and *n*-butanol (RPE grade) from Carlo Erba.

The fungal material (Table 1) was collected in different regions of Europe (except for *C. rainierensis* which is a North American species). Due to the photosensitivity of orellanine, all handling was done in the dark. Orellanine was extracted from dry powdered carpophores of *C. orellanus* mushrooms collected locally and purified as previously described [13]. Reference solutions of orellanine 50 mmol $l^{-1}$ were prepared in the dark by dissolving the toxin in 200 mmol $l^{-1}$ potassium hydroxide (for TLC or ESR) or in 100 mmol $l^{-1}$ Tris buffer [Tris(hydroxymethyl)aminomethane], pH 10, (for electrophoresis). Then, the pH was adjusted to neutrality with acetic acid (for TLC or electrophoresis) or phosphoric acid (for ESR). Stock solutions of this photosensitive and easily oxidable product were stored in the dark at 4°C and used within two days. Orelline was prepared by one of us [17].

### 2.2. Extraction procedure

Desiccated carpophores of mushrooms were finely powdered. Fatty material and apolar pigments were removed from the samples (30 to 600 mg desiccated powder) by a preliminary extraction for 1 h at 20°C with 3 ml diethyl ether. After decantation, orellanine and by-products were extracted from the dried samples for 1 h with 3 ml methanol. Insoluble material was removed by centrifugation for 20 min at 5000 *g*. Lastly, the methanolic extract was evaporated to dryness in a centrifuge vacuum evaporator (Speed-Vac AES 2000 Savant).

### 2.3. Thin-layer chromatography (TLC)

As previously shown, Si–CN and cellulose layers are the only ones which allow a good separation of

Table 1
Occurence of fluorescent compounds and of radical signals in the mushrooms tested[a]

| Species[b] | Spot No. | TLC | | | | Electrophoresis | | | ESR |
|---|---|---|---|---|---|---|---|---|---|
| | | $R_F$ | Daylight Colour | Fluorescence | | Migration (mm) | Fluorescence | | Signal[c] intensity (arbitrary units) |
| | | | | Colour | Intensity[c] (pixels) | | Colour | Intensity[d] (pixels) | |
| 1 *Paxillus involutus* (Batsch) Fr. | - | - | - | - | - | - | - | - | 31 |
| 2 *Lepista nebularis* Fr. | - | - | - | - | - | - | - | - | 2 |
| 3 *Lepista gilva* (Pers. ex Fr.) Roze. | - | - | - | - | - | - | - | - | 37 |
| 4 *Entoloma sinuatum* (Bull. ex Fr.) Kumm. | - | - | - | - | - | - | - | - | 5 |
| 5 *Inocybe cervicolor* (Pers. ex Pers.) Quél. | - | - | - | - | - | - | - | - | 9 |
| 6 *Inocybe geophylla* (Sow. ex Fr.) Kumm. | 1 | 1.00 | y | y-r | 13 042 | 16/54 | w | 4 771 | 9 |
| | 2 | 0.36 | lt y | bt t | 7 822 | 2 | w | 3 259 | |
| | 3 | 0.30 | | ft b | 2 802 | | | | |
| | 4 | 0.22 | | ft b | 2 496 | | | | |
| | 5 | 0.10 | | b | nm | | | | |
| 7 *Inocybe virgatula* Kühn. | - | - | - | - | - | - | - | - | 8 |
| 8 *Inocybe sp.* | 1 | 1.00 | oc | y | 14 891 | 48 | w | 9 196 | 14 |
| | 2 | streak | | ft | 4 955 | 2 | w | 4 808 | |
| | 3 | 0.36 | | bt t | 13 423 | | | | |
| | 4 | 0.23 | | b | nm | | | | |
| 9 *Hebeloma sp.* | - | - | - | - | - | - | - | - | 10 |
| *Dermocybe cinnamomea* Fr. 10a, 10b, 10c | 1 | 1.00 | oc | o | 6 252 | 58 | r | 574 | |
| | 2 | streak | y | p | nm | 45 | w | 4 728 | 21; 24; 17 |
| | 3 | 0.84 | | b | nm | -5/+11 | w | 5 362 | |
| | 4 | 0.60 | | r-p | 4 138 | 1 | w | 1 900 | |
| | 5 | 0.44 | y | b | 5 510 | | | | |
| | 6 | 0.31 | y | r-o | 1 863 | | | | |
| | 7 | 0.26 | | b | 464 | | | | |
| 11 *Dermocybe cinnabarina* Fr. Mos. | 1 | 1.00 | ro | | | 1 | w | 1 013 | 19 |
| | 2 | 0.97 | bn | ro | 1 549 | | | | |
| | 3 | 0.93 | ro | ft b | 10 196 | | | | |
| | 4 | 0.41 | | ft b | 1 827 | | | | |
| | 5 | streak | | y | nm | | | | |
| 12 *Dermocybe sanguinea* Fr. | 1 | 1.00 | o | o | 2 775 | 45 | w | 14 486 | 18 |
| | 2 | 0.75 | ct | du o | 17 898 | 1 | w | 928 | |
| | 3 | 0.55 | r | ft | 4 261 | | | | |
| | 4 | 0.43 | y | ft | 656 | | | | |
| | 5 | 0.37 | y | p | 935 | | | | |
| | 6 | 0.34 | b | b | 2 439 | | | | |
| | 7 | 0.30 | o-r | r-o | 366 | | | | |
| 13 *Dermocybe semisanguinea* Fr. | 1 | 1.00 | oc-r | y | 1 228 | 28 | r | 5 238 | 23 |
| | 2 | 0.92 | y | du r | 1 775 | 5 | w | 2 304 | |
| | 3 | 0.78 | y | ro-p | 3 055 | | | | |
| | 4 | 0.62 | y | cy y | 4 106 | | | | |
| | 5 | 0.52 | r | oc-ro | 6 728 | | | | |
| | 6 | 0.44 | y | oc | 3 624 | | | | |
| | 7 | 0.35 | y | ft | 1 924 | | | | |
| | 8 | 0.23 | y | t | 4 893 | | | | |
| | 9 | 0.11 | | y-gn | 6 929 | | | | |
| | 10 | 0.03 | | ft y | 3 056 | | | | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 14 *Cortinarius infractus* (Pers. ex Fr.) Fr. | 1 | 1.00 | oc | y | 7 782 | 59 | w | 4 435 | 51 |
| | 2 | 0.79 | ft y | bt lt b | 13 570 | 30 | w | 40 000 | |
| | 3 | 0.61 | ft y | ct | 15688 | -4 | w | 6 875 | |
| | 4 | 0.54 | | bn | 3 941 | -45 | w | 3 750 | |
| | 5 | 0.49 | y | y | 1 482 | | | | |
| | 6 | 0.44 | ft y | b | 2 540 | | | | |
| | 7 | 0.37 | ft y | y-gn | 2 614 | | | | |
| | 8 | 0.33 | | y | 1 537 | | | | |
| 15 *Cortinarius subtortus* (Pers. ex Fr.) Fr. | 1 | 0.96 | y | y | 1 438 | 57 | w | 10 792 | 11 |
| | 2 | 0.85 | y | y | 4 848 | 32/48 | w | 2 495 | |
| | 3 | 0.76 | y | y | 4 448 | 14/32 | w | 4 480 | |
| | 4 | 0.60 | | ft gn | 3 301 | 0 | w | 948 | |
| | 5 | 0.47 | | t | 2 396 | | | | |
| | 6 | 0.26 | | ft y | 1 652 | | | | |
| *Cortinarius violaceus* (L. ex Fr.) Fr. | 1 | 0.93 | y | cy y | 2 674 | 16/61 | w | 2 258 | |
| | 2 | 0.79 | | mv | 1 110 | 2 | w | 1 940 | |
| 16a, 16b | 3 | 0.33 | y | b | 361 | | | | 7, 9 |
| | 4 | 0.27 | | ft y | 235 | | | | |
| 17 *Cortinarius henrici* Reum. | 1 | 1.00 | be | y | 640 | | | | nt |
| | 2 | 0.75 | y/gn | de t | 4 150 | | | | |
| | 3 | 0.58 | y | b | 218 | | | | |
| | 4 | 0.52 | y | b | 1 419 | | | | |
| | 5 | 0.4 | | t-b | 659 | | | | |
| | 6 | 0.16 | y | y-t | 4 900 | | | | |
| 18 *Cortinarius orellanoides* Hy. | 1 | 1.00 | be | y | 407 | | | | nt |
| | 2 | 0.75 | y/gn | de t | 2 291 | | | | |
| | 3 | 0.58 | y | b | 101 | | | | |
| | 4 | 0.52 | y | b | 376 | | | | |
| | 5 | streak | | ft | 577 | | | | |
| | 6 | 0.14 | y | b-vt | 1 636 | | | | |
| 19 *Cortinarius orellanus* (Fr.) Fr. | 1 | 1.00 | be | y | 6 905 | 67 | de t | 10 349 | 14 |
| | 2 | 0.75 | y/gn | de t | 10 735 | 52 | b | 5 566 | |
| | 3 | 0.58 | y | b | 5 540 | 44 | b | 2 951 | |
| | 4 | 0.52 | y | b | 6 469 | 36 | y | nm | |
| | 5 | 0.35 | y | t | 5 225 | 14/32 | y | 7 388 | |
| | 6 | streak | y | ft | 1 905 | 2 | y | 2 007 | |
| | 7 | 0.20 | y | ft | 1 037 | | | | |
| | 8 | 0.14 | | t-b | 1 050 | | | | |
| 20 *Cortinarius rainierensis* Smith | 1 | 1.00 | be | y | 2 054 | | | | nt |
| | 2 | 0.75 | y/gn | de t | 8 654 | | | | |
| | 3 | 0.58 | y | b | 1 135 | | | | |
| | 4 | 0.52 | y | b | 8 171 | | | | |
| | 5 | 0.39 | | ft | 3 341 | | | | |
| | 6 | 0.15 | y | y-t | 11 712 | | | | |
| 21 *Cortinarius speciosissimus* Kühn. & Romagn. | 1 | 1.00 | be | y | 3 320 | 67 | de t | 4 248 | 7 |
| | 2 | 0.76 | y/gn | de t | 5 727 | 53 | b | 3 731 | |
| | 3 | 0.58 | y | b | 1 034 | 45 | b | 933 | |
| | 4 | 0.52 | y | b | 1 549 | 37 | y | nm | |
| | 5 | 0.34 | y | t | 1 746 | 12/32 | y | 6 013 | |
| | 6 | 0.28 | | ft | 856 | 10 | y | 1 112 | |
| | 7 | | | | | 2 | y | 2 656 | |
| 22 *Cortinarius claricolor* Fr. ss Mos. | 1 | 0.91 | y | y | 18 484 | 16/48 | w | 3 804 | 4 |
| | 2 | 0.57 | | y | 6 758 | 2 | w | 945 | |
| | 3 | 0.36 | | y-gn | 3 856 | | | | |
| 23 *Cortinarius præstans* (Cord.) Gill. | - | - | - | - | - | - | - | - | 9 |
| 24 *Cortinarius splendens* Hy. | 1 | 1.00 | o-r | bt o | 5 781 | 45 | w | 4 078 | 3 |
| | 2 | 0.86 | o-r | bt y-o | 9 862 | 3/18 | w | 2 537 | |
| | 3 | streak | y-ro | y | 10 298 | 3 | w | 849 | |
| | 4 | 0.52 | y | o | 1 922 | 2 | w | 960 | |
| | 5 | 0.45 | y | mv | 1 400 | 1 | w | 3 841 | |
| | 6 | 0.38 | ro-o | ro | 2 959 | -0.5 | w | 747 | |
| | 7 | 0.30 | r-o | bt o | 5 628 | -1/-5 | w | 1317 | |

(*Contnd.*)

Table 1 (*contnd.*)

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| 25 *Cortinarius subfulgens* Ort. ss Mos. | - | - | - | - | - | - | - | - | 17 |
| 26 *Cortinarius herbarum* Hy. | 1 | 0.92 | y | y | 13 994 | 0/59 | w | 12 614 | 12 |
| | 2 | 0.75 | ft y | ft b | 6 083 | 0 | w | 3 886 | |
| | 3 | 0.61 | | ft y | 6 931 | | | | |
| | 4 | 0.36 | ft y | y-gn | 4 295 | | | | |
| | 5 | 0.25 | ft y | ft y | 3 248 | | | | |
| | 6 | 0.18 | | ft y | 1 465 | | | | |
| | 7 | 0.12 | | ft y | 1 027 | | | | |
| 27 *Cortinarius purpurascens* Fr. | 1 | 1.00 | oc | y | 2 637 | 0/59 | w | 8 154 | 13 |
| | 2 | 0.47 | y | y-gn | 3 447 | 2 | w | 2 661 | |
| | 3 | 0.38 | ft y | ft y-gn | 1 267 | | | | |
| | 4 | 0.29 | ft y | ft y-gn | 1 116 | | | | |
| | 5 | 0.23 | ft y | ft y-gn | 1 024 | | | | |
| | 6 | 0.19 | ft y | ft y-gn | 660 | | | | |
| 28 *Cortinarius subtriumphans* Mos. | - | - | - | - | - | - | - | - | 7 |
| 29 *Cortinarius variecolor* Fr. | - | - | - | - | - | - | - | - | 8 |
| 30 *Cortinarius varius* Fr. ss Mos. | - | - | - | - | - | - | - | - | 7 |
| 31 *Cortinarius brunneus* Fr. | 1 | 1.00 | be | y | 6 042 | 18/64 | w | nm | 30 |
| | 2 | 0.82 | | b | 9 654 | 3/13 | w | 5 076 | |
| | 3 | 0.64 | | y-gn | 11 112 | 2 | w | 4 239 | |
| | 4 | 0.49 | | y-gn | 4 870 | | | | |
| | 5 | 0.37 | | y-gn | 3 880 | | | | |
| 32 *Cortinarius armillatus* Fr. | - | - | - | - | - | - | - | - | 24 |
| 33 *Cortinarius bulliardi* Fr. | - | - | - | - | - | - | - | - | 16 |
| 34 *Cortinarius flexipes* Fr. (Kühn.) ss Mos. | - | - | - | - | - | - | - | - | 7 |
| 35 *Cortinarius traganus* Fr. | - | - | - | - | - | - | - | - | 9 |
| 36 *Cortinarius caninus* (Fr.) Fr. | 1 | 0.96 | y | y | 9 569 | 23/70 | w | 3 014 | 15 |
| | 2 | 0.37 | | y-gn | 3 163 | 10/22 | w | 1 123 | |
| | 3 | | | | | | w | 2 821 | |
| 37 *Cortinarius sp* | - | - | - | - | - | - | - | - | 11 |
| 38 *Rozites caperata* (Pers. ex Fr.) Karst. | - | - | - | - | - | - | - | - | 8 |
| **Standards** | | | | | | | | | |
| Orellanine | | 0.75 | y/gn | de t | | 67 | de t | | |
| Orelline | | 0.52 | y | b | | 45 | t | | |

[a] All voucher specimens are deposited in the Herbarium at the University of Grenoble, F, except 17 and 18 (University of Montpellier, F) and 20 (University of Washington, USA).

[b] According to the classification by Moser [15].

[c] All fluorescence intensities ($\lambda_{ex}$ 366 nm), determined with NIH image, are expressed for 100 mg dried mushroom powder giving 200 $\mu$l methanolic extract, 10 $\mu$l of which are applied on the cellulose layer. Elution with *n*-butanol–acetic acid–water (4:1:5, v/v).

[d] All fluorescence intensities ($\lambda_{ex}$ 312 nm), determined with NIH Image, are expressed for 100 mg dried mushroom powder giving dried methanolic extract redissolved in 500 $\mu$l TAE buffer (pH 7.8), 25 $\mu$l of which are applied on the 5 mm thick 0.8% agarose gel. Migration duration 60 min at 6 V/cm.

[e] ESR signal amplitude of immobilized organic radicals. All signal intensities are expressed for 10 mg dried mushroom powder and the following spectrometer settings: cell temperature: 20°C; microwave power: 5 mW; modulation frequency: 100 kHz; modulation amplitude: 0.125 mT; receiver gain: 8 to $10\times10^5$; time constant: 0.5 s; scan range: 10 mT; scan time: 500 s.

Abbreviations:

/=to; b=blue; be=beige; bn=brown; bt=bright; ct=citron; cy=creamy; de=delayed; du=dull; ft=faint; gn=green; lt=light; mv=mauve; nm=not measurable; nt=not tested; o=orange; oc=ochre; p=purple; r=red; ro=rose; t=turquoise; w=white; y=yellow.

orellanine [13]. Butanolic eluents, added with acetic acid, give successful separations on the cellulose layer with the water–butanol ratio ranging from 1:3 to saturation. A low pH value is required so that the fully protonated form of orellanine is highly predominant [12]. The $R_F$ values obtained for orellanine with *n*-butanol–acetic acid–water eluents (3:1:1 to 4:1:5, v/v) are between 0.85 and 0.70 respectively on cellulose TLC-plates without fluorescence indicator (Merck). Each dried methanolic extract was dissolved again in 100 to 200 μl methanol. Ten μl of each methanolic extract were applied to the cellulose layer and eluted with *n*-butanol–acetic acid–water (4:1:5, v/v). Reference solutions of orellanine and orelline were co-chromatographed. Location of the compounds on the TLC plates was by visible light and by UV light at 366 nm.

### 2.4. Electrophoresis

Each dried methanolic extract was dissolved in 500 μl TAE buffer (40 mmol $l^{-1}$ Tris–acetate, 1 mmol $l^{-1}$ EDTA, pH 7.8). Then 15 to 30 μl of each sample were applied into the slots of a 5 mm thick 0.8% agarose gel (Agarose NA from Pharmacia). Reference solutions of orellanine and orelline were co-electrophoresed. Gels were run in a horizontal submarine ASA Midigel cell for 60 min at 6 V $cm^{-1}$ in TAE buffer (pH 7.8). Immediately after the run, the gels were laid onto a UV transilluminator (312 nm).

### 2.5. Image processing and fluorescence densitometry

Under UV light, orellanine successively turns into the products of reduction of one and both of its N-oxide groups (Fig. 1), orellinine and lastly orelline which is fluorescent [2]. So orellanine appears as a dark zone immediately after exposure to UV light and as a bright turquoise–blue fluorescent spot after about 1 min.

TLC plates and electrophoresis gels were first irradiated for 30 s to 1 min with UV light (366 nm for TLC, 312 nm for electrophoresis). Then they were photographed (1 min exposure under UV light) using a black-and-white film type 667 and a Polaroid camera equipped with an orange filter (Kodak Wratten 22A). The plates or gels were digitalized with an Imager TM (Appligene) high-performance CCD video camera equipped with an orange filter (Kodak Wratten 22A) and a video copy processor P 68 E (Mitsubishi). The image analysis was performed on a Macintosh LC3 computer using the public domain NIH Image 154 program (written by Wayne Rasband at the US National Institute of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov or on floppy disk from NTIS, 5285 Port Royal Rd., Springfield, VA 22 161, part number PB93-504868).

TLC plates were scanned with an LS-50 luminescence spectrometer (Perkin-Elmer) equipped with a fluorescence plate reader, using 400 nm as excitation wavelength and 450 nm as emission wavelength.

### 2.6. Direct ESR

Preliminary assays were made with samples of cap and stalk of a fresh carpophore of *C. orellanus*. Fresh sample (12 mg) was introduced into the ESR cell. After running a first spectrum, 50 μl phosphate buffer was added into the cell which was then subjected to sonication for 5 min in an ultrasonic bath (Branson) before recording a second spectrum.

Each dried extract obtained after methanolic extraction (see Section 3.2) was dissolved in 200 mmol $l^{-1}$ disodium hydrogen phosphate. The pH value should be 8.5 to 9. Then, the pH was adjusted to neutrality with phosphoric acid and the volume was made up to 100 μl with 200 mmol $l^{-1}$ phosphate buffer (pH 7). Assays with one-step extraction were made with 100 mg mushroom powder swollen overnight at room temperature in 700 μl 100 mmol $l^{-1}$ phosphate buffer (pH 8). The supernatents (100 μl) were used to run ESR spectra. Dioxygen was bubbled for 1 min in each sample. Then tyrosinase (4200 U/ml) was added. Dioxygen was bubbled again for 1 min at room temperature. After pipetting the samples into the cell, they were ready for scanning within 30 s.

X-Band ESR spectra were obtained using a flat cell, a Bruker cavity and a Bruker ER 100 D spectrometer (9.3 GHz). The *g* measurements were made with tannol (2,2,6,6,tetramethylpiperidine-4-ol-1-oxide) as a reference.

## 3. Results and discussion

### 3.1. Thin-layer chromatography (TLC)

TLC was used to achieve a first screening of the mushrooms. Among the 38 species investigated, only 20, i.e. the *Cortinariaceæ* mushrooms from the generæ *Inocybe*, *Dermocybe* and *Cortinarius*, showed fluorescent compounds on TLC plates. The results are shown in Table 1. To illustrate the relative visual importance of all fluorescent spots or streaks observed on the chromatograms, Table 1 gives their fluorescence intensities expressed in pixels by image analysis. We have preferred this method to fluorimetric scanning because of its convenience, flexibility and rapidity. No significant difference has been observed between the results obtained for the mushrooms composition with the two modes of detection. The compositions of *I. geophylla* and *I. sp.* seem to be very similar on the basis of their chromatograms, with a bright turquoise fluorescent compound at $R_F$ 0.36. Thus the TLC of *I. sp.* should be compared to that of other *Inocybes* in order to complete its identification. A substance with a yellow–green fluorescence and a $R_F$ value of about 0.37 is seen on the chromatograms of 6 *Cortinarius* mushrooms (*C. brunneus, caninus, claricolor, herbarum, infractus* and *purpurascens).*

In the same way, the *Cortinarius* mushrooms from the subgenus *Leprocybe*, section *Orellani*, seem to have very similar compositions. To allow its quantification, orellanine has to be turned, under UV light, into its final product of reduction, orelline (Fig. 1), which is fluorescent [2]. The overall yield of this photochemical conversion in the spots on the TLC plates depends on the density of the spots, its reproducibility is not very good with large amounts of toxin. Thus, the linearity of the response requires the application of suitable quantities of mushroom extracts so that orellanine spots do not contain more than 1 nmol orellanine (250 ng, 100 μmol $l^{-1}$ in the spot) [14]. As the detection limit of orellanine after migration is not much lower (0.20 nmol using image processing), we chose to use relatively high quantities of mushroom extracts so as to have a good chance of detecting even traces of orellanine, but there was a poor chance of correctly quantifying high amount samples. Due to the limited quantities of our mushroom samples, we could achieve a rough quantitative determination of the toxin with *C. orellanus* and *C. speciosissimus* only. We found with image processing, orellanine contents of about 1.2 and 0.4%, respectively, in *C. orellanus* and *C. speciosissimus*. In the same way as for orellanine, the orelline contents were estimated to about 0.07 and 0.03%, respectively. A mere detection of orellanine was ensured by TLC in the other mushrooms.

On each of the chromatograms of the *Orellani* mushrooms, a major fluorescent spot showed colour, fluorescence properties and $R_F$ values which were identical with the ones of the reference orellanine (yellow to green under visible light, delayed turquoise fluorescence and $R_F$=0.75). Another fluorescent spot showing similar characteristics to the ones of the orelline standard could be observed on these chromatograms (yellow under visible light, immediate blue fluorescence and $R_F$=0.52). Orellanine and orelline were determined by chromatography of mushroom samples spiked with one or the other standards (Fig. 2) and recording the fluorescence spectra of the spots of the samples directly on the TLC layer. The spectra of the reference samples of orellanine and orelline had identical characteristics

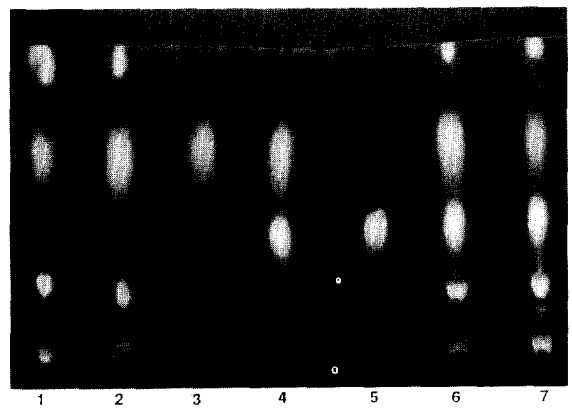



Fig. 2. TLC determination of orellanine and orelline. 1: *C. orellanus* extract, 2: *C. orellanus* extract+orellanine, 3: orellanine, 4: orellanine+orelline, 5: orelline, 6: *C. orellanus* extract+orellanine+orelline, 7: *C. orellanus* extract+orelline. Fluorescence intensities ($\lambda_{ex}$ 366 nm), determined by image analysis with NIH Image, are expressed for 100 mg dried mushroom powder in 200 μl methanolic extract, 10 μl of which is applied on the cellulose layer. Orellanine in the standard spots: 5 nmol. Orelline in the standard spots: 2.5 nmol. Elution with *n*-butanol–acetic acid–water (4:1:5, v/v).

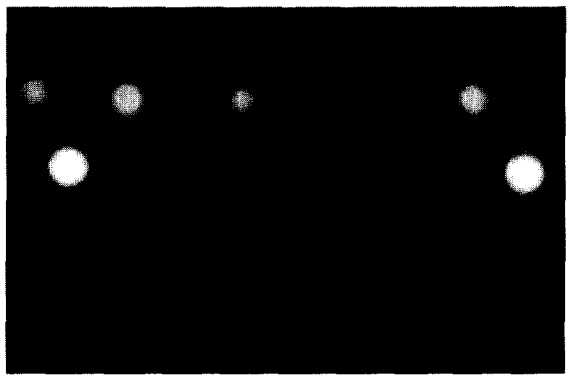
(excitation wavelength 400 nm and emission wavelength 450 nm) and shapes. On this basis, the two most significant chromatographic fractions of these five *Orellani* might be attributed to orellanine and orelline. A third fluorescent spot (yellow under visible light, immediate blue fluorescence and $R_F$= 0.58) was observed in front of that of orelline. Its spectrum, identical to that of orelline, induced us to attribute this spot to orellinine.

### 3.2. Electrophoresis

Only the 20 mushrooms showing fluorescent compounds with TLC were subjected to electrophoresis, except for 3 of them, *C. henrici*, *C. orellanoides*, and *C. rainierensis*, the samples of which were insufficient in quantity. Table 1 summarizes the results. For all mushrooms tested, all the fluorescent compounds detected had an anodic migration at the slightly alkaline pH used, except for *C. infractus* which had some fluorescent compounds with anodic migration and two with cathodic migration. Four *Cortinariaceæ* mushrooms, *Dermocybe cinnamomea*, *D. sanguinea*, *D. semisanguinea* and *C. splendens*, showed a bright white fluorescent spot with identical electrophoretic migration (45 mm under our conditions, see Table 1). For 9 *Cortinariaceæ* mushrooms, *I. geophylla*, *D. cinnabarina*, *C. brunneus*, *C. caninus*, *C. claricolor*, *C. herbarum*, *C. purpurescens* and *C. violaceus*, most of the fluorescent compounds shown by TLC were about electrically neutral and/or had high molecular masses: migration was short during electrophoresis and the spots were observed near the starting dot. The other compounds migrated as diffuse streaks in our system.

A common migration pattern was observed only for the *Cortinarius* mushrooms from the subgenus *Leprocybe*, section *Orellani*. Orellanine and orelline were determined by electrophoresis of mushroom samples spiked with one or the other standards. With image processing, the detection limit of orellanine after run was better than with TLC: 0.1 nmol (25 ng, 3 μmol $l^{-1}$ in the spot), it was very bad with fluorimetric scanning (0.45 μmol, 115 μg, 15 mmol $l^{-1}$). The migration of the toxin was consistent with its p*K* values (1.5, 5.8 and 11.0 [12]) and its two negative charges at the pH used. A major spot of orellanine was found in the extracts of both of the *Orellani* tested. The toxin content of different samples of *C. orellanus* dry powder, calculated with image processing in relation to a standard range, was 1.4±0.5%. The orellanine content of caps was found to be about 2.5 to 3 times the one of stalks, as illustrated in Fig. 3. Likewise, the toxin content of *C. speciosissimus* was 0.6±0.2%. No significant difference was observed between the contents of caps and stalks for this mushroom.

Spots with immediate blue fluorescence could be seen at the level of the orelline standards for the same mushrooms. Due to the bad detection obtained with fluorimetric scanning on gel, the fluorescence spectra of these spots did not allow the unambiguous identification of them as orelline spots. In the same way as for orellanine, the orelline contents of the dry powders of *C. orellanus* and *C. speciosissimus* could be estimated with image processing to 0.07±0.01 and 0.05±0.03%, respectively. As with TLC, a spot with immediate blue fluorescence was observed in front of that of orelline. It could be attributed to orellinine on the basis of the results obtained with



1 2 3 4 5 6 7 8 9 10

Fig. 3. Electrophoresis detection of orellanine and orelline. 1: orellanine, 2: orelline, 3: extract from *C. orellanus* cap, 4: extract from *C. orellanus* stalk, 5: extract from whole *C. orellanus* body, 6: extract from *C. speciosissimus* cap, 7: extract from *C. speciosissimus* stalk, 8: extract from whole *C. speciosissimus* body, 9: orellanine, 10: orelline. Fluorescence intensities ($\lambda_{ex}$ 312 nm), determined with NIH Image, are expressed for 100 mg dried mushroom powder in dried methanolic extract redissolved in 500 μl TAE buffer (pH 7.8), 25 μl of which is applied on the 5 mm thick 0.8% agarose gel. Orellanine in the standard spots: 20 nmol. Orelline in the standard spots: 10 nmol. Migration time: 60 min at 6 V $cm^{-1}$.

samples of orellanine, which turns into orellinine and orelline when irradiated with UV light [2].

### 3.3. Direct ESR

For a preliminary assay, we tried to detect the orthosemiquinone radical of orellanine without any extraction in a fresh *Cortinarius* mushroom known to contain relatively large amounts of orellanine. With crude solid samples of a fresh carpophore of *C. orellanus*, a one-broad-line ESR signal was observed. Its $g$-value was characteristic of an immobilized organic radical. After sonicating the sample in phosphate buffer (pH 7), the signal turned into the isotropic nine-line one of the orellanine semiquinone (Fig. 4) [3]. This result suggests that the semiquinone form of orellanine was present in the fresh mushroom. With an equal mass of cap and stalk, the intensity of the signal for cap was a little more than twice the one for stalk. Thus, it is possible to detect the orthosemiquinone radical of orellanine in fresh mushrooms without a difficult extraction and the intensity of the signal is related to the concentration of the toxin. Note that the signal of the immobilized organic radical in the dry powder was not altered by methanolic extraction and thus could be attributed to another compound other than orellanine.

Most dry powdered samples investigated in this study gave a one-broad-line ESR signal characteristic of an immobilized organic radical (Table 1). Thus, it is not characteristic of mushrooms containing orellanine. Two species, *C. violaceus* and *I. geophylla*, showed a spectrum superimposed on the latter. This second spectrum is the one of iron or of copper (Fig. 5). Unfortunately, we were unable to observe any isotropic semiquinone signal with desiccated samples of the mushrooms, even after overnight rehydration before sonication. The characteristic semiquinone signal was found only after methanolic or phosphate buffer extraction and enzymatic oxidation of the extract with the tyrosinase–$O_2$ system (Fig. 4). The better results were obtained using one-step extraction with 100 mmol $l^{-1}$ phosphate buffer (pH 8). The detection limit of ESR for orellanine was determined with reference solutions of the toxin. Several dilutions (0 to 10 mmol $l^{-1}$) were treated with the tyrosinase–$O_2$ system as described for mushroom extracts in the experimental section. The detection

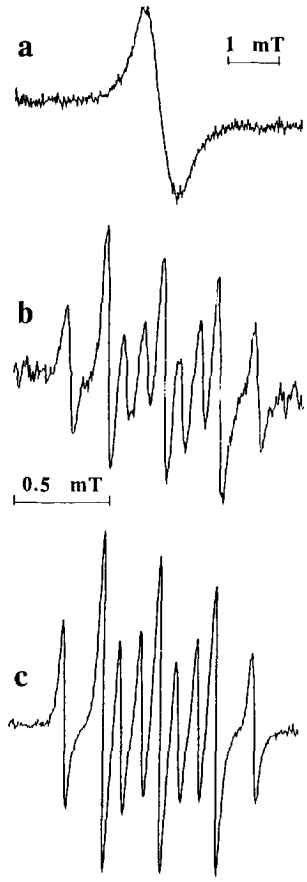



Fig. 4. ESR spectra of *C. orellanus* and of orellanine. Spectrometer settings: microwave power: 5 mW; modulation frequency: 100 kHz; scan time: 500 s. (a) ESR signal of an immobilised organic radical in dry *C. orellanus* powder. Particular spectrometer settings: cell temperature: 20°C; modulation amplitude: 0.125 mT; receiver gain: $10 \cdot 10^5$; time constant: 0.5 s; scan range: 10 mT. (b) ESR signal of the orthosemiquinone anion radical generated by $O_2$–tyrosinase-catalyzed oxidation of orellanine in the supernatents (100 μl) of *C. orellanus* powder (100 mg) swollen overnight at room temperature in 700 μl of 100 mmol $l^{-1}$ phosphate buffer (pH 8). Reaction mixture: 100 μl supernatent at room temperature, $O_2$ bubbling 1 min, tyrosinase (4200 U/ml) added, again $O_2$ bubbling 1 min. After being pipetted into the spectrometer cell, samples were ready to scan within 30 s. Particular spectrometer settings: cell temperature: 20°C; modulation amplitude: 0.080 mT; receiver gain: $1.6 \cdot 10^6$; time constant: 0.2 s; scan range: 3 mT. (c) ESR signal of the orthosemiquinone anion radical generated by $O_2$–tyrosinase-catalyzed oxidation of reference orellanine in pure solution ($g$=2.0053, $a_N$ =0.31 mT, $a_H$ =0.21 mT). Reaction mixture: 100 μl 25 mmol $l^{-1}$ orellanine in 100 mmol $l^{-1}$ Tris buffer (pH 7.4) at room temperature, $O_2$ bubbling 1 min, tyrosinase (4200 U/ml) added, again $O_2$ bubbling 1 min. After being pipetted into the spectrometer cell, samples were ready to scan within 30 s. Particular spectrometer settings: cell temperature: 37°C; modulation amplitude: 0.063 mT; receiver gain: $3.2 \cdot 10^5$; time constant: 1 s; scan range: 3 mT.

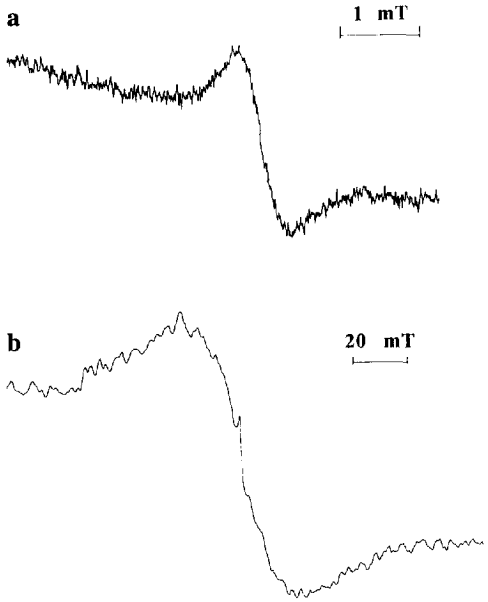



Fig. 5. ESR spectra of *C. violaceus*. Spectrometer settings: cell temperature: 25°C; microwave power: 5 mW; modulation frequency: 100 kHz; modulation amplitude: 0.125 mT; receiver gain: $10 \cdot 10^5$; time constant: 0.5 s. (a) ESR signal of an immobilised organic radical in dry *C. violaceus* powder. Particular spectrometer settings: scan range: 10 mT; scan time: 500 s. (b) ESR signal of an immobilised metal in dry *C. violaceus* powder. Particular spectrometer settings: scan range: 300 mT; scan time: 100 s.

limit, based on a signal-to-noise ratio of 3, was estimated to be 0.2 mmol $l^{-1}$, i.e. 20 nmol (5 μg) orellanine in the 100-μl aliquot of extract in the cell. This relatively low sensitivity was due to the fact that the radical species accounts for only about 0.02% of the total orellanine concentration [3].

In view of the lower sensitivity of ESR for tyrosinase activated orellanine, the latter technique was used with the 12 mushrooms only, showing fluorescent compounds with migration and detection characteristics close to that of orellanine. Table 2 summarizes the outcomes. Among these mushrooms, only five (*C. henrici, C. orellanoides, C. orellanus, C. rainierensis* and *C. speciosissimus*) appeared to contain orellanine, identified by the characteristic ESR spectrum of its semiquinone form. All five belong to the subgenus *Leprocybe*, section *Orellani*. Among them, *C. orellanus* had the highest orellanine semiquinone content. In this range of concentration (0.1 to 0.8 μmol orellanine in the ESR cell, i.e. 1 to 8 mmol $l^{-1}$), the signal intensity was proportional to

Table 2
Determination of orellanine by measurement of the ESR signal of its orthosemiquinone anion radical in 5 *Cortinariacæ* belonging to the subgenus *Leprocybe*, section *Orellani*

| Species | Orellanine content (% dry weight) |
|---|---|
| *C. henrici* | 0.45 |
| *C. orellanoides* | 0.20 |
| *C. orellanus* | 1.10 |
| *C. rainierensis* | 0.85 |
| *C. speciosissimus* | 0.50 |

Experimental conditions and spectrometer settings: see Fig. 4b and c.

the concentration of orellanine [3]. Thus, the latter could be estimated with a standard range (Table 2). We have found values of 1.1 and 0.5% for the orellanine contents of the dry powders of *C. orellanus* and *C. speciosissimus*, respectively. The other mushrooms, *D cinnamomea* and *D. semisanguinea, C. brunneus, C. herbarum, C. infractus, C. subtortus* and *C. violaceus* did not show any isotropic nine-line orellanine semiquinone signal and thus, do not contain orellanine detectable in our conditions.

## 4. Conclusion

The results of our three methods are convergent. Only the *Cortinariaceæ* mushrooms from the genera *Inocybe, Dermocybe* and *Cortinarius* contain fluorescent compounds. Two very different separation methods, TLC and electrophoresis, show the presence of compounds with similar behaviour to that of orellanine and orelline in the five tested *Cortinarius* mushrooms from the subgenus *Leprocybe*, section *Orellani*. The fluorescent spectrum of the corresponding spots may be attributed to orelline. As the action of tyrosinase has a relative specificity for the oxidation of catechols into semiquinones and the ESR spectrum of the semiquinone of the toxin is highly specific [3], our ESR work unambiguously confirms the presence of orellanine in these *Cortinarius* mushrooms. The toxin is present in relatively high amounts in these mushrooms. This fact is interesting and it can be useful to note that orellanine semiquinone can be detected by ESR directly in the fresh mushroom, just after sonicating it in buffer.

The orellanine content of the dry powder of *C.*

*orellanus* determined by electrophoresis (1.4±0.5%) and by ESR (1.1%) is in good agreement with the one found by us [13] using HPLC (1.2±0.1%). The relatively higher values found for *C. orellanus* and *C. speciosissimus* by electrophoresis with image analysis could be explained by heterogeneity of sampling. For *C. orellanus*, this value is identical to the one found by others [16] using TLC (1.4%). However, the latter authors found more orellanine (0.9%) than us (0.6±0.2% by electrophoresis, 0.5% by ESR) in *C. speciosissimus*, most likely due to sample variation. Both ESR and electrophoresis show that orellanine is unequally distributed in *C. orellanus*, with 2 to 3 times more toxin in the caps than in the stalks. This can be related to our quantitative determination of orellanine by HPLC or pulse polarography in the two parts of the mushroom (unpublished results). The toxin is absent in the other *Cortinariaceæ* mushrooms and other toadstools we tested, especially in *D. cinnamomea* and *C. splendens* which have been claimed as toxic and suspected to contain orellanine. It shows that the fluorescent compounds content of the mushrooms is not an indicator of their toxicity, as claimed by several mushroom handbooks.

The nephrotoxicity of two of the five *Orellani* mushrooms, *C. orellanus* and *C. speciosissimus*, is already well known to be related to orellanine [2]. Although no mushroom poisonings by the other species have been reported, our work shows that *C. henrici, C. orellanoides* and *C. rainierensis* should undoubtedly be considered potentially lethal, like the preceding ones. The latency period before the nephrotoxicity appears can be extremely long (2 to 17 days). It could explain that the toxicity of these mushrooms has passed unnoticed, as has long been the case for *C. orellanus* [1]. The five species have a more or less common pattern of fluorescent compounds, among which are orellanine, orellinine and orelline, even though they are from different geographic origins. One of them, *C. rainierensis*, is the first North American species proven to contain orellanine. It was considered as belonging to the section *Orellani* because of morphological similarities with European taxa in this group [10]. This taxonomic relationship is strongly supported by the presence of orellanine proved by our ESR analysis, the toxin being absent in all the other *Cortinarius* subgenus. Lastly, the orellanine content of our almost fifty year old sample of *C. rainierensis* is very high, of the same order of magnitude as the one of our sample of *C. orellanus*. Orellanine, although it is a photosensitive and easily oxidable compound, proves to be extremely stable in the mushroom.

All the mushrooms tested that contain orellanine have a very high toxin content, so that a sophisticated method like HPLC (detection limit: 50 pg) is not required. The three methods described here, TLC (detection limit: 15 ng with fluorescence densitometry), electrophoresis (25 ng) and even ESR (5 μg), are sensitive enough for our purpose. The first two methods are classical ones, rapid and readily available in any laboratory such as anti-poison centers. Thus, for confirmation and for samples such as ours with high toxin contents, TLC or electrophoresis coupled with ESR can indeed be a convenient alternative with the same specificity, instead of using liquid chromatography coupled with mass spectrometry (LC–MS), a method which is long for a screening, expensive and not anywhere available.

## Acknowledgments

We are indebted to Professor C. Andary and Dr. S. Rapior (Laboratoire de Botanique, Phytochimie et Mycologie, Faculté de Pharmacie de Montpellier) for giving samples of *C. henrici, C. orellanoides* and *C. rainierensis*. Thanks are due to Dr. P. Block (Laboratoire des Systèmes Adhésifs Cellulaires, LESAC, CNRS), to Professor J. Lhomme and to Dr. P. Michon (Laboratoire d'Etudes Dynamiques et Structurales de la Sélectivité, URA CNRS 332, Université J. Fourier de Grenoble) for allowing us to use, respectively, the image processing station, the ESR spectrometer and the fluorimetric gel and TLC scanning station.

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