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Ethylidene Gyromitrine and N-methyl-N-formylhydrazine in Commercially Available Dried False Morels, Gyromitra esculenta Fr. ex Pers.

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Introduction

The False morel, Gyromitra esculenta, is a mushroom that grows from April till May in sandy soils, preferably under conifers. Its geographical distribution includes the whole of Central and Eastern Europe. In Poland and Western Russia it is the wild edible mushroom that is most often collected, dried and exported in large amounts. It is generally known that fresh G. esculenta is highly poisonous and many fatalities occurring after eating raw or improperly cooked mushrooms have been reported (1, 2).

The principal toxic compound in G. esculenta has been identified by *List* and *Luft* (3) as a derivative of monomethylhydrazine, i. e. the N-methyl-N-formylhydrazone of acetaldehyde, which they named gyromitrine. This compound being very sensitive to oxygen and somewhat volatile, it is rapidly lost on cooking and drying of the mushrooms.

This explains why canned and dried False morels have always been considered harmless. In most European countries these products are even commercially available.

However, in Germany G. esculenta has been removed from the list of marketable edible mushrooms (4) and, recently, *Schmidlin-Mészáros* (5) has even seriously doubted the innocousness of dried False morels.

It should be pointed out that the available information on the quantities of the toxic principle in the mushroom is rather confusing.

List and *Luft* found up to 1600 ppm of gyromitrine in fresh carpophores by volumetric estimation of the potassium iodate reducing materials obtained from an ethyl ether extract (2, 6).

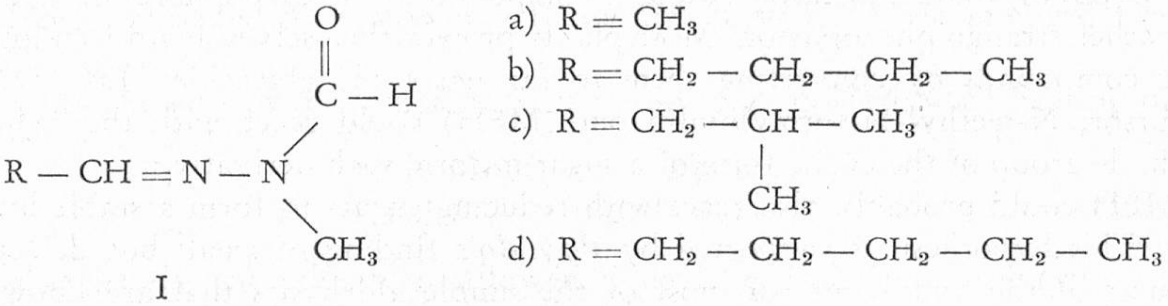
Schmidlin-Mészáros analysed commercially available dried False morels by thin-layer chromatography of ether extracts and found levels of gyromitrine ranging from 1000—3000 ppm. In addition, she observed that this compound was

accompanied by 250—600 ppm of N-methyl-N-formylhydrazine, which she considered to be a decomposition product of gyromitrine (5).

Pyysalo used gas chromatography coupled with mass spectrometry for his research on the volatile compounds of a number of edible mushrooms (7). During his studies on the volatiles of G. esculenta, he not only found gyromitrine, but also small quantities of higher homologues, i. e. the N-methyl-N-formylhydrazones of pentanal, 3-methylbutanal and hexanal (8).

Pyysalo named these compounds according to the alkylidene side chain in the general formula I.

Thus, the original gyromitrine, derived from acetaldehyde was named ethylidene gyromitrine (a) and the others pentylidene (b), 3-methylbutylidene (c) and hexylidene gyromitrine (d).



I

During GLC-MS of an ether-pentane extract of a steam distillate obtained in vacuum from False morels, it was found that these toxic hydrazones contributed only about 5 percent to the total amount of volatiles, the principal aroma compound being 1-octen-3-ol at a concentration of 72% (7). Considering that fresh mushrooms generally contain between 20 and 100 mg/kg of 1-octen-3-ol (9), the quantities of N-methyl-N-formylhydrazones in Pyysalo's extracts amounted to a few ppm.

This is considerably less than the high concentrations reported earlier (2, 5, 6).

Later, *Pyysalo* studied the stability of ethylidene gyromitrine (EtG) during cooking and drying of False morels. In the course of this work, he found that fresh mushrooms contained 800 mg/kg of the toxic hydrazon, whereas the concentrations in dried False morels from Finnish households varied from «not detectable» to 200 mg/kg.

It should, however, be mentioned that he determined the EtG levels according to the afore-mentioned titration method, of which he doubted the selectivity (10).

Recently, *Pyysalo* analysed extracts of fresh False morels by high resolution capillary GLC and found not less than 9 volatile methylformylhydrazones at an average combined level of 57 mg/kg, the principal being EtG at a concentration of 50 mg/kg (11).

Using the same analytical technique for more accurately monitoring the levels of the toxicant during processing, *Pyysalo* found that prolonged outdoor drying in the sun was more effective in removing EtG from the mushrooms than drying in a stream of warm air at 50°C.

Furthermore, he established that the volumetric potassium iodate method of List and Luft was definitely not suitable for the determination of the toxic hydrazones: in press juice obtained from 0.5 kg of fresh carpophores, he found 350 mg of potassium iodate reducing materials, calculated as EtG, whereas gas chromatography indicated the presence of only 25 mg of this compound.

The major cause of the striking differences between the reported EtG levels can undoubtedly be traced to the use of inappropriate analytical methods. Virtually no research has been done on finding the most suitable analytical procedure for the determination of the N-methyl-N-formylhydrazones.

Since *List* and *Luft* (2, 3, 6), all research workers on G. esculenta have extracted the poison with water saturated ethyl ether, and the efficiency of this extraction method has not been questioned.

Moreover, it seems to have been overlooked that the free occurrence of a highly (phyto) toxic and easily oxidable compound in the carpophores of a fungus is a rather strange phenomenon. Most plants protect themselves against endogenic toxic compounds by converting them to far less toxic glycosides. EtG and its precursor, N-methyl-N-formylhydrazone (MFH) could react with the reducing aldehyde group of the cyclic form of a sugar to form such derivatives.

MFH could probably also react with reducing sugars to form a stable hydrazone. This hypothesis is supported by *Pyysalo's* finding of small but detectable amounts of the hydrazones of most of the simple aldehydes that are known to occur in mushrooms (11). Among these hydrazones, EtG is probably predominant because during its biosynthesis acetaldehyde is more readily available than the other alkanals.

This paper reports the results of an investigation concerning the occurrence of free and chemically bound methylhydrazine derivatives in commercially available dried False morels.

It also proposes a simple method for the selective estimation of both ethylidene gyromitrine and its precursor, N-methyl-N-formylhydrazine.

Experimental

Materials

Commercially available dried False morels were obtained from different shops in the Lake of Geneva region in Switzerland. Some fresh, fully grown Gyromitras were gathered on a site near the city of Bern and lyophilized upon receipt.

Analytical standards

MFH and its hydrazones were synthesized as described by *List* and *Luft* (3). Standard solutions of these compounds in ethyl ether and chloroform are stable for months if kept at 4°C.

Solutions of methylhydrazine are degraded rapidly and have to be prepared fresh every other day.

Extraction procedures

Soxhlet method: 2 g of the finely ground material were extracted during 8—16 hours, using ethyl ether saturated with water. The extract was concentrated to 5 ml.

Chloroform method: 1—2 g of the dried material was rehydrated with distilled water overnight.

To prevent oxidation of the hydrazones, the vessel was filled with nitrogen. Subsequently, 100 ml chloroform were added and the slurry was mixed at high speed with an ultra turrax during 3 minutes. After elimination of water with anhydrous sodium sulfate, the extract was filtered over a folded paper filter and concentrated in a Rotavapor apparatus at 30°C under slight vacuum.

Liberation of chemically bound MFH and EtG: 1 g of finely ground material was brought into a thick-walled glass tube and 20 ml distilled water were added. The tube was sealed in a blowpipe flame and heated for the required time at 120°C.

Maximum amounts of the toxicants were found after the following heating times:

For MFH	2 hours
For EtG	6—8 hours

Subsequently, the tubes were allowed to cool, opened, and the entire contents were extracted with cloroform as described above.

Gas-liquid chromatography (GLC)

All GLC analyses were performed on a Perkin Elmer F-11 apparatus equipped with a 5 ft x $^1/_8$″ glass column filled with 10% Carbowax 20 M, coated on Chromosorb W, 100—120 mesh, and a flame ionisation detector.

At a column temperature of 110°C and a carrier gas flow of 30 ml N_2/min, the following retention times were observed:

EtG	9 min
MFH	19 min
3-methylbutylidene gyromitrine	21 min
hexylidene gyromitrine	52 min

The three hydrazones chromatographed without apparent decomposition. Methylhydrazine did not yield a response, not even at the 10 µg level. MFH showed a tailing peak. A reproducible response was only obtained after priming the column with mcg loads of the compound. Sensitivity remained rather low: the detection limit was approximately 0,2 µg whereas as little as 10 ng of EtG still yielded a measurable peak.

Several other stationary phases were tested for their suitability to chromatograph MFH and methylhydrazine, but no improvement compared to Carbowax 20 M was observed.

Thin-layer chromatography (TLC)

The TLC system used for the analyses of the mushroom extracts are listed in table 1. In all cases, ready made plates were used.

Table 1. RF values of N-methyl-formylhydrazine (MFH) and its derivatives in four thin-layer chromatography systems using SiO_2 Merck ready made plates

	Dichloro-methane-methanol 9 : 1 (2)	Chloroform-acetone-diethylamine 5 : 4 : 1	Toluene-acetone 9 : 1	Chloroform-acetone-pyridine 4 : 1 : 1
MFH	0.23	0.30*	0.03	0.45
Ethylidene gyromitrine	0.62	0.65	0.20	0.77
3-Methylbutylidene gyromitrine	0.70	—	0.32	—
Hexylidene gyromitrine	0.72	—	0.38	—
Methylhydrazine	0.02	0.60	—	0.34
Methylhydrazine hydrochloride	—	0.40	—	—

* MFH partly decomposed to methylhydrazine in this system

Chromogenic reagents

Pyridine pyridinium chloride:

The chromatogram was sprayed with a 1% solution of this reagent in equal parts of aqueous NaOH 2 N and 95% ethanol. After heating for 5 minutes at 80°C, the plate was sprayed with concentrated hydrochloric acid and ethanol 1 : 1 v/v.

Reddish brown spots turned up slowly against a yellow background. Visibility improved with time. The limit of detection was 0.5—1 µg, but sometimes even as little as 0.1—0.2 µg was visible.

Cinnamyl aldehyde:

0.3 ml of this compound was dissolved in a freshly prepared mixture of 15 ml methanol and 5 ml of concentrated hydrochloric acid, providing an excellent reagent that yielded yellow spots for all the hydrazine derivatives at room temperature.

EtG and the other hydrazones appeared rather slowly, presumably because they first had to be hydrolyzed to methylhydrazine. Heating at 80°C for a few minutes improved visibility. The limit of detection was 0.1 µg for all compounds mentioned in table 1.

Confirmation of identity by derivatization to methylhydrazine

The fact that MFH and its hydrazones are easily hydrolyzed to methylhydrazine, allowed a simple confirmatory TLC procedure in situ: suitable aliquots

of the sample extracts were applied on the start line of the silica gel layer and overspotted with ethanol-concentrated HCl 5 : 1 v/v. The same was done with the reference compounds, including methylhydrazine.

Hydrolysis was allowed to take place for 15 minutes at room temperature, after which the plate was developped in chloroform-acetone-diethylamine 5 : 4 : 1 v/v.

Volumetric estimation of potassium iodate reducing substances

This determination was performed as described by *List* and *Luft* (3, 6).

Results

Free EtG

Initially, seven samples of dried False morels were extracted with water saturated ethyl ether and the extracts analysed for EtG by gas- and thin-layer chromatography. In addition, we also analysed the lyophilized mushrooms gathered in the Bern region.

The results of these analyses are given in table 2.

Table 2. Free ethylidene gyromitrine in commercially available dried False morels

Sample no	Free ethylidene gyromitrine in ppm
1	106*
2	4
3	35*
4	3
5	79*
6	12
7	< 1
8 (lyophilized G. esculenta gathered in the Bern region)	< 1

* Values confirmed by TLC

Only 3 samples of dried material contained significant amounts of the toxic compound, i. e. concentrations that could unequivocally be determined by both chromatographic methods. Most extracts contained many co-extracted compounds, as indicated by the presence of a considerable number of peaks on the chromatogram and a pronounced odour of lower fatty acids. However, the EtG peak was never overlapped by extraneous material and even small quantities could be readily observed.

On all chromatograms showing an appreciable peak of EtG, there were also small, but distinct peaks having the same retention times as 3-methyl-butylidene

and hexylidene gyromitrine. These peaks were not evaluated as the quantities were too small to allow confirmation of identity. Upon subjecting the concentrated extracts to TLC, we found that only a small volume could be spotted without difficulty. The chromatogram was easily overloaded and several co-extracted compounds also yielded reddish spots with pyridine pyridinium reagent.

Convincing chromatograms were only obtained by applying the lowest possible quantity of the extract that would yield a clearly visible spot of EtG and by using cinnamyl aldehyde as a chromogenic reagent.

Somewhat surprized about the absence of EtG in the lyophilized mushrooms and about the generally low levels encountered in the samples, we tried out other methods for the isolation of the toxic principle.

Prolonged Soxhlet extraction with water saturated ethyl ether did not improve the yield, nor did steam distillation of aqueous sample slurries under reduced pressure.

In trying out several solvents, we found that EtG was most easily extracted with chloroform, provided that the samples were first rehydrated by soaking in a tenfold weight of distilled water.

These chloroform extracts were far more pure than those obtained by ether extraction as described above.

In adopting extraction with chloroform, considerable time was gained and TLC analysis of the extracts was greatly facilitated. However, upon re-analysing the samples mentioned it table 2 by the chloroform procedure, no significant increase in EtG content was observed.

Aliquots of the three ethyl ether and chloroform sample extracts, containing the highest amount of the toxicant, were subjected to the derivatization procedure as described under «Confirmation of identity by derivatization to methylhydrazine». After hydrolysis with hydrochloric acid, all sample aliquots were found to yield a quantity of methylhydrazine that was roughly proportional to the initially observed EtG concentration.

Chemically bound MFH and EtG

Two samples of dried False morels and the lyophilized material from the Bern region, in which free EtG was respectively low or absent, were subjected to the aqueous extraction procedure at 120°C in sealed tubes as described under «Experimental». 1 g quantities of the powdered carpophores were heated with water during 0, 1, 2, 3, 4 and 6 hours, and in some cases even longer.

Chloroform extracts of the hydrolysates were analysed for both EtG and MFH. (During analysis for free EtG, extracts had not been pure enough to permit detection of low levels of the latter compound.)

As it could not be excluded that prolonged heating in aqueous medium would hydrolyze EtG and/or MFH to methylhydrazine, we paid special attention to the possible presence of this compound during TLC analysis. However, the results were negative.

The results listed in table 3 show that both EtG and MFH are gradually liberated on prolonged heating: the highest level of MFH is observed after about 2 hours, whereas, the EtG concentration attains its maximum only after 6 hours.

Table 3. Liberation of chemically bound N-methyl-N-formylhydrazine and ethylidene gyromitrine from False morels by heating of aqueous suspensions at 120°C

Hydrolysis time in hours	Sample 6		Sample 4		Sample 8 lyophilized carpophores	
	MFH	EtG	MFH	EtG	MFH	EtG
0	40	12	~ 10	3	< 10	< 1
1	75	18	20	3	< 10	< 1
2	200	20	30	5	< 10	< 1
3	150	25	30	6	< 10	< 1
4	80	57	20	11	< 10	< 1
6	40	74	10	30	< 10	< 1
8	—	—	—	53	—	—
16	—	—	—	48	—	—
24	—	—	—	44	—	—

All values expressed in mg/kg

Potassium iodate reducing materials

Five chloroform extracts obtained as described above, were subjected to extraction with hydrochloric acid and to subsequent titration of the potassium iodate reducing material (3).

In table 4 the results are compared with the MFH and EtG levels actually found by chromatography. The volumetrically estimated levels are about 50—70 times higher than the sum of the toxicants determined by the more specific methods. Obviously, the extracts contained strongly reducing material other than MFH or its hydrazone.

Absence of the toxicants in four species of related fungi

Gyromitra esculenta holds a special position among the higher Ascomycetes. It has only one close relative, G. infula and it would be of chemotaxonomical interest to analyse this species for EtG and MFH. However, G. infula, being very rare in Switzerland, was not available for analysis.

Other, more distantly related Ascomycetes, such as Helvella lacunosa, H. crispa, H. elastica and even Sarcosphaera eximia have all been considered to be more or less poisonous when eaten raw and it has been supposed that these fungi contain the same toxic principle as G. esculenta (12).

Table 4. Potassium iodate reducing materials as determined by titration (6) in dried and lyophilized False morels

Sample no	Quantity found volumetrically (calculated as EtG)	Concentrations actually found by GLC / TLC	
		MFH	EtG
A	1600	30	5
B	2000	20	11
C	3700	—	53
D	3600	—	48
E	500	< 10	< 1

All values expressed in mg/kg

The three above-mentioned Helvellaceae were gathered in the Lake of Geneva region and S. eximia was collected in the nearby Val d'Anniviers in Valais.

All four species were found to be exempt of free and bound EtG and MFH.

Discussion

The free EtG values reported in this paper are in agreement with those found by *Pyysalo* during his drying experiments with Finnish mushrooms (11).

Considering that these levels depend greatly upon the drying conditions, it is only normal to find commercially available dried False morels with free EtG contents ranging from «not detectable» to more than 100 ppm (table 2).

Pyysalo did dot include the occurrence and fate of possible present MFH in his investigations. It could well be that he did not observe it on his slightly alkaline GLC column, because MFH is hydrolyzed most easily under alkaline conditions, especially at high temperature.

The concentrations of free EtG found by *Pyysalo* and the levels of the free and bound toxicants reported in this paper are too low to be associated with the numerous cases of fatal poisoning described in the literature (1).

Some lyophilized fresh False morels from the Bern region in Switzerland were even found to be exempt of both EtG and MFH! (table 2 and 3).

It is, of course, possible that G. esculenta from different countries contains widely different concentrations of the toxic principle(s).

This might explain why fatalities have been quite frequent in Russia and in Germany (1, 2), whereas none occurred in Finland or France (10, 12).

It should also be borne in mind that the present procedure to liberate chemically bound EtG and MFH is at best a tentative one.

Considering that both compounds are easily decomposed by bases and acids, hydrolysis of the hypothetic precursor(s) was performed by simply heating with water under pressure.

The method worked, but the hydrolysis might not be quantitative; nor has the possible partial decomposition of the released compounds been fully taken into account.

Table 3 shows that MFH and EtG were liberated at different rates. When liberation of MFH already decreased after 2—3 hours, the release of EtG was only just starting.

This suggests that both compounds were released from different precursors, i. e. MFH was not formed through hydrolysis of EtG.

However, under the drastic conditions of hydrolysis at 120°C, MFH may have been partly hydrolyzed to methylhydrazine. We have tried to take this possibility into account, but methylhydrazine is a powerful reducing agent and, therefore, easily lost during extraction and concentration.

Moreover, our work on the estimation of this compound was seriously hampered by the fact that we could not subject it to gas chromatography.

Work is in progress on a determinative method for both MFH and methylhydrazine based on the procedure of *Timbrell* et al. for the gas chromatographic estimation of hydrazine metabolites of the drug isoniazid in human urine (13).

In this method the reaction with p-chlorobenzaldehyde yields stable hydrazones that can be chromatographed in the gas phase without irreversible adsorption phenomena and without decomposition.

This approach would probably also permit to perform hydrolysis of the hypothetic precursors by treatment with acid and to determine the liberated methylhydrazine by derivatization with p-chlorobenzaldehyde.

Conclusions

The fact that the greater part of EtG and MFH in Gyromitra esculenta is chemically bound casts considerable doubt upon all published data for the poison content.

Toxicological considerations are also of limited value until our knowledge about the true nature of the toxicants and their concentrations in the carpophores becomes more meaningful.

However, the findings reported in this paper could explain why there have been cases of poisoning even after the prescribed twice boiling of the mushrooms and discarding of the water (1).

Considering that both EtG and MFH are only slowly released during hydrolysis at 120°C, ordinary cooking procedures may not always have provided optimal conditions for removing the toxic compounds.

There is little doubt that ingestion of the chemically bound toxicants is dangerous for the eater of False morels: upon contact with hydrochloric acid present in the gastric juices, glycosides and/or hydrazones will be hydrolyzed, yielding EtG and MFH as shortlived intermediates, which in turn will be converted to highly toxic methylhydrazine.

In order to complete our knowledge on the poison content of G. esculenta and its preserves, the following research is necessary:

Improvement of analytical methodology by gas chromatography determination of MFH and methylhydrazine as their p-chlorobenzaldehyde or similar derivatives.

Finding out in what form EtG and MFH occur in the carpophores, i. e. establishing the chemical nature of the precursor(s).

Optimizing conditions to release chemically bound toxicants.

Determination of free and chemically bound EtG and MFH in fresh and dried carpophores of G. esculenta, preferably analysing many samples from widely different origin.

Studying the fate of the toxicants during processing conditions as boiling, canning and drying, using improved analytical methods.

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Summary

Ethylidene gyromitrine (EtG) or acetaldehyde N-methyl-N-formylhydrazine, the toxic principle of False morel mushrooms, Gyromitra esculenta, has so far been determined by solvent extraction, followed by gas chromatography or titrimetric analysis of the extract.

In using such methods, considerable quantities of the toxic compound escape detection, because the greater part of EtG occurs as a precursor, probably a glycoside from which it can only be released by prolonged heating with water at 120°C in sealed tubes.

During this treatment, appreciable levels of N-methyl-N-formylhydrazine (MFH) are also liberated, indicating that this compound may also be present as a glycoside or possibly as a hydrazone of a reducing sugar.

Final determination of both compounds was performed by simple gas- and thin-layer chromatographic methods.

Free EtG levels in commercially available dried False morels varied from «not detectable» to 106 ppm. The amount of chemically bound EtG in two samples was 6 and 17 times higher. A similar phenomenon was observed for the MFH content.

The research work necessary for improving our knowledge about the true nature of the toxic compounds in G. esculenta is briefly outlined.

Zusammenfassung

Aethyliden-Gyromitrin (AEG) oder Acetaldehyd N-methyl-N-formylhydrazin, das Gift der Frühjahrslorchel, Gyromitra esculenta, wurde bisher mit Lösungsmitteln extrahiert und im Extrakt durch Gaschromatographie oder Titration bestimmt.

Bei diesen Methoden wird jedoch ein beträchtlicher Teil des Giftes nicht erfaßt. Die Hauptmenge des AEGs liegt als ein Precursor vor, wahrscheinlich in Form eines Glyco-

sides. Es kann daher nur durch längeres Erhitzen mit Wasser auf 120°C im geschlossenen Rohr freigemacht werden.

Während dieser Behandlung werden ebenfalls bedeutende Mengen an N-methyl-N-formylhydrazin (MFH) freigesetzt. Diese Substanz dürfte auch als Glycosid oder als Hydrazon eines reduzierenden Zuckers vorliegen.

Die Bestimmung der beiden Verbindungen erfolgte nach einfachen gas- und dünnschichtchromatographischen Methoden.

Gehalte an freiem AEG in im Handel erhältlichen Trockenlorcheln variierten von «nicht nachweisbar» bis 106 mg/kg. In zwei Proben waren die Mengen an chemisch gebundenem AEG 6- bzw. 17mal höher. Analoge Feststellungen wurden für MFH gemacht.

Zum Schluß werden einige kurze Hinweise bezüglich weiterer Erforschung der Natur der Lorchel-Giftstoffe gegeben.

Résumé

L'éthylidène gyromitrine (EtG) ou N-méthyl-N-formylhydrazine acétaldéhyde est le principe toxique des fausses morilles, Gyromitra esculenta. On l'a déterminé jusqu'ici en faisant une extraction par solvant suivie d'un dosage de cet extrait par chromatographie en phase gazeuse ou par titrimétrie.

Avec ces méthodes, des quantités considérables du composé toxique échappent à la détection. En effet, la plus grande partie de l'EtG existe sous forme combinée, probablement un glycoside. Pour la libérer, il faut un chauffage prolongé en présence d'eau à 120°C en tube scellé.

Au cours de ce traitement, des teneurs appréciables en N-méthyl-N-formylhydrazine (MFH) sont aussi libérées, ce qui indique que ce composé doit aussi exister sous forme combinée comme glycoside ou même comme hydrazone d'un sucre réducteur.

Le dosage final des deux composés a été fait par simple chromatographie en phase gazeuse et sur couche mince.

La teneur en EtG libre des échantillons de fausses morilles deshydratées du commerce a varié de «non détectable» à 106 ppm. La teneur en EtG chimiquement lié de deux échantillons fut 6 à 17 fois plus élevée. Un phénomène analogue fut observé pour la teneur en MFH.

L'auteur esquisse brièvement le travail de recherche qui est encore nécessaire pour améliorer nos connaissances sur la vraie nature des composés toxiques de la Gyromitra esculenta.

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