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Note

Improved analytical and preparative methods for necatorin from *Lactarius necator* (Fr.) Karst mushroom

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It has been shown that the mushroom *L. necator*, which until recently was regarded in Finland and some other countries as a first class edible mushroom contains one of the strongest mutagenic compounds found in nature[1–4]. This compound, called necatorin, has been tentatively identified as 7-hydroxycoumaro[5,6-*c*]cinnoline[5]. The preliminary characterization of its mutagenic properties[2–4] revealed it to be comparable to aflatoxin B_1 (6000 revertants per μg in the Ames *Salmonella* assay with test strain TA 100). Although no positive response was observed in the host-mediated assay or in the micronucleus test with the pure compound, necatorin has been shown to give a positive response in the DNA repair test with mammalian cells[6]. The compound has been found in *L. necator* in concentrations ranging from 3 to 20 mg/kg, and has been shown to be stable[7]. No loss was observed in samples stored in the dark for 6 days under conditions ranging from 0.5 *M* hydrochloric acid to 0.5 *M* sodium hydroxide solution. The compound was however susceptible to decomposition by light, especially at high pH values. The boiling of necatorin in 0.5 *M* sulphuric acid or in 0.5 *M* sodium hydroxide solution for 1 h had very little effect.

The published isolation method[2] is rather laborious, requiring preparative high-performance liquid chromatography (HPLC) after extraction of mushroom samples overnight. An even more important deficiency of the method was its low and variable yield. The results of quantitative analysis performed previously[7], the chemical identity of the compound and its properties provide a basis for both an improved HPLC method and an improved preparative method. The former is necessary for a study of the occurrence and distribution of necatorin and the latter is required to provide material for further evaluation of the toxicological and possible carcinogenic properties.

MATERIALS AND METHODS

The mushroom samples

L. necator mushrooms were collected in September 1983 in the neighbourhood of this laboratory or from elsewhere in southern Finland. They were identified by

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Mr. M. J. Pellinen in this laboratory. Some mushrooms were freshly treated for the quantitative analysis but most were stored at −20°C.

HPLC

The equipment consisted of a M-6000A pump, M-710B automatic injector, a Radial-Pak C_{18} column and a Nova-Pak C_{18} column (both 100 × 8 mm with 5-μm particles) in an RCM-100 module and a dual-channel M-440 UV–VIS detector monitoring at 254 nm and 405 nm (all equipment from Millipore-Waters, U.S.A.). The eluent was methanol–water–acetic acid (54:44:2) at a flow-rate of 2 ml/min.

Analysis of necatorin. A 0.5-g sample of fresh or thawed mushrooms was homogenized with 1 ml water. The homogenate was centrifuged and the water was discarded. The precipitate was then extracted twice with 0.75 ml of 3% (w/v) aqueous sodium carbonate. The extracts were combined, 300 μl acetonitrile were added and the samples were brought to 2 ml volume with water and centrifuged*.

Aliquots (50 μl) were injected for HPLC. The standard employed was calibrated as described earlier[2] and dissolved in 3% (w/v) aqueous sodium carbonate. The quantifications were based on peak-height measurements. The adequacy of the extraction was confirmed by doubling the volumes of sodium carbonate solutions used and also by a third extraction.

Preparative isolation of necatorin

Mushrooms (800 g) were homogenized with 1 l cold water and centrifuged. The precipitate was extracted twice with 750 ml and 500 ml of 3% (w/v) aqueous sodium carbonate with subsequent centrifugations*. The carbonate solutions were combined and acidified to pH 6.0 with hydrochloric acid, after which 50 ml acetic acid were added and the solution was extracted for 48 h in a Kutcher-Steudel apparatus with *ca.* 15 l diethyl ether. The final volume of the extract was 0.5 l which was then extracted with 50 ml of 5 *M* sodium hydroxide solution.

The water phase was kept overnight at 4°C during which time necatorin was precipitated and could be filtered from the solution as its sodium salt. Necatorin was identified on the basis of HPLC and the Ames *Salmonella* assay[8,9].

RESULTS AND DISCUSSION

The calibration curve for necatorin is shown in Fig. 1 and the curve of injection volume *versus* peak height in Fig. 2. The calibration curve was linear over the concentration range required for quantitative analysis of necatorin in mushrooms (1–40 mg/kg, which means 12.5–500 ng per injection). Fig. 3 shows that necatorin is easily quantified, especially at 405 nm.

The curve in Fig. 2 shows that the sensitivity could of course be further enhanced by injecting larger volumes if necessary. It also shows that injecting necatorin as its sodium salt in 3% sodium carbonate with 15% acetonitrile has an on-column enrichment effect, indicated by the linearity of the curve from 20 to 200 μl. The on-column enrichment is further demonstrated by the 10% higher peaks obtained upon injection of necatorin in alkaline solutions as compared with in acidic or neutral

* Sample preparation was carried out and samples were stored in cold.

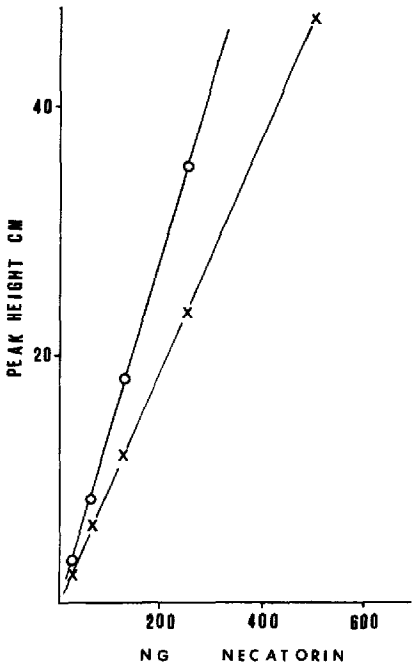
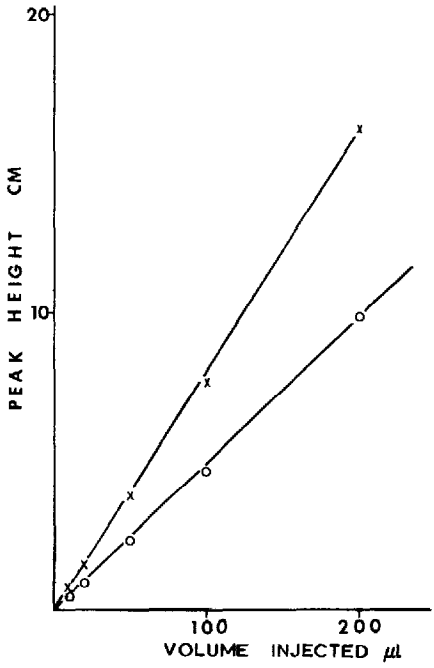


Fig. 1. Calibration curve for necatorin. Detection wavelengths: 254 (○) and 405 nm (×). For details see text.



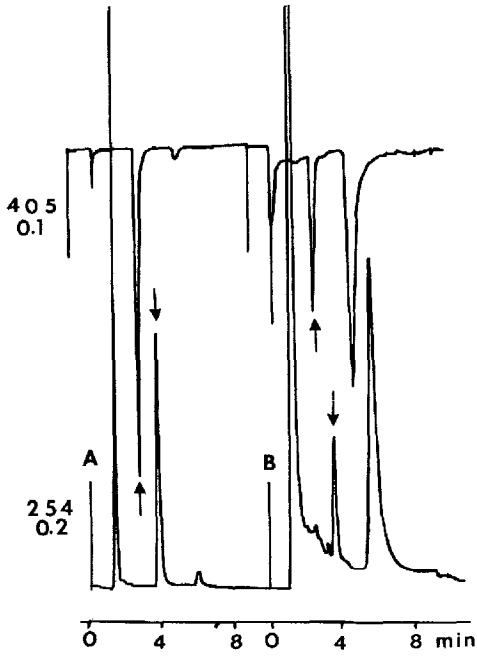


Fig. 2. Effect of varying the injection volume on peak height of necatorin. Detection wavelengths: 254 (×) and 405 nm (○).

Fig. 3. HPLC chromatogram of necatorin standard (10 mg/l) (A) and extract of *L. necator* mushroom (B). The arrows denote necatorin peaks. Values below the wavelengths give sensitivity in a.u.f.s.

TABLE I

YIELDS FROM THE PREPARATIVE EXPERIMENTS IN MILLIGRAMS OF NECATORIN AND AS A PERCENTAGE (IN PARENTHESES) OF THE ORIGINAL AMOUNT PRESENT IN FRESH MUSHROOMS MEASURED BY HPLC

Original	*Extraction with diethyl ether*		*Precipitate*
	0–24 h	*24–48 h*	
23.8	17.9 (75)	4.7 (20)	16.3 (67)
25.3			20.8 (82)
25.3			17.5 (69)

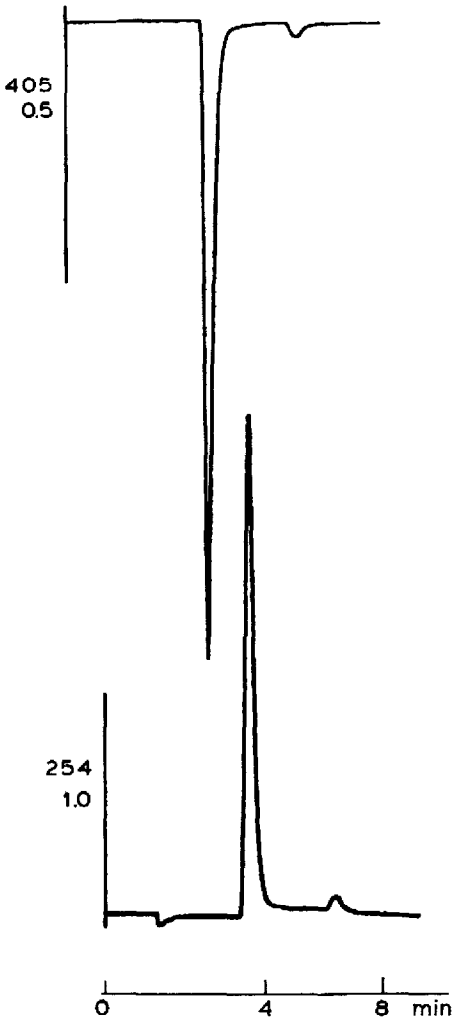


Fig. 4. A 25-μl injection of necatorin isolated by the preparative method described. The sample was dissolved in 100 ml of water.

solutions. The change from the Radial-Pak C_{18} column, which has no endcapping of residual silanol groups, to the Nova-Pak C_{18} column having endcapping reduced the tailing of the necatorin peak, which indicates that the residual silanol groups interact with the azo group of necatorin.

More than 200 injections had no deleterious effect on the column, despite the high pH of the injected samples and the relatively simple purification of necatorin from the biological matrix. The quantity of necatorin in the samples was in the range of 20–25 mg/kg. Necatorin was detected in all the samples studied, which indicates that it is a normal constituent of *L. necator*, not an accidental contamination. Fresh and frozen samples gave similar results.

The system can also be applied to smaller sample sizes, simply by scaling down to 50-mg samples, without losing the simplicity and reliability. The yields in the preparative system from three experiments are given in Table I. The system seems to work well, bearing in mind its simplicity and the high purity of the material (Fig. 4).

The analytical and preparative methods described, which are both based on the extraction of otherwise water-insoluble necatorin from the mushroom matrix as a soluble sodium salt, will be employed in further studies of the properties and occurrence of this compound in conjunction with future harvests.

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