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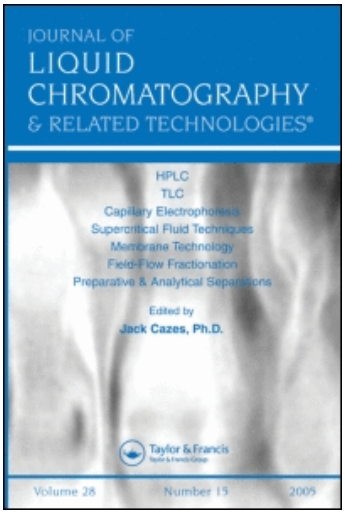

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Hallucinogenic Species in Amanita Muscaria. Determination of Muscimol and Ibotenic Acid by Ion-Interaction HPLC

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HALLUCINOGENIC SPECIES IN AMANITA MUSCARIA. DETERMINATION OF MUSCIMOL AND IBOTENIC ACID BY ION-INTERACTION HPLC

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ABSTRACT

Ibotenic acid and muscimol are considered the principal components responsible of the hallucinogenic properties of *Amanita muscaria*. In this paper a sensitive ion-interaction HPLC method is presented which permits the simultaneous determination of ibotenic acid and muscimol. A C_{18} reverse phase is the stationary phase and the mobile phase is an aqueous solution of 5.0 mM octylammonium o-phosphate. A spectrophotometric comparative detection performed at 230 and 254 nm is employed. Detection limits are of 18 μg/L for ibotenic acid and of 30 μg/L for muscimol.

Different sample pretreatment processes were compared. The highest recovery yields together with the minimum matrix effect were obtained by a procedure which only consists in the sonication of the fresh sample and its squeezing. The extract obtained is then diluted as required and filtered (0.2 μm) before injection. This preparation offers the further advantage to maximally preserve the native composition.

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The method was applied in the analysis of *Amanita muscaria* and permitted to evaluate the content of muscimol and ibotenic acid in heads and stems of the mushrooms. The same analysis performed for samples of *Amanita citrina* confirmed the absence in this species of both ibotenic acid and muscimol.

INTRODUCTION

Amanita muscaria is generally more known for its toxical properties than for the psychotropic ones and the literature methods concerning the analysis of *Amanita* mostly regard identification and determination of its toxic components, such as muscarine, amatoxins and phallotoxins.[1-5]

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It is on the other hand reported[6,7] that in North Europe countries, and especially in Siberia, *Amanita muscaria* hallucinogenic properties were known since 4.000 b.C. The "Rig-Veda", which is considered the first politeist text, mentions *Amanita* as the "God plant" because of its exceptional properties to transmit prodigious powers.[8] The species responsible for the psychotropic effects, which have been now identified are: ibotenic acid (amino-(3-hydroxy-5-isoxazolyl)acetic acid) and its decarboxylation product muscimol (3-hydroxy-5-aminomethylisoxazole).[8-10] Their structures are presented in Fig.1.

These compounds are receiving great interest in psychiatric medicine because of their potential ability to be employed as an alternative to synthetic drugs or to be used as possible models for the development and preparation of new drugs devoted to the treatment of psycho-diseases.[9] Analytical methods are therefore required for extraction, purification and determination of these compounds in *Amanita* mushrooms.

Only two determination methods are reported in literature. One makes use of a microbore column and UV detection after reaction with ninhydrin in presence of potassium cyanide.[9] The other (direct source[10] is not easily available) is a HPLC method, which makes use of an amino-bonded stationary phase; the preparation of the sample requires two SPE separation processes respectively performed with Dowex 1-X8 resin (acetate form) for the extraction of ibotenic acid and with alumina-silica gel for muscimol. Detection limits are respectively 18 and 10 mg/L of ibotenic acid and muscimol.

This paper presents a sensitive HPLC ion-interaction method for the simultaneous separation and determination of muscimol and ibotenic acid in the extract which can be easily obtained by squeezing the fresh sample of

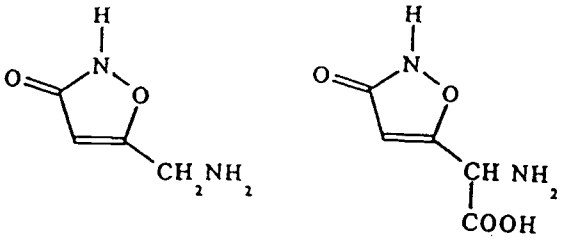

Figure 1. Chemical structures of the analytes.

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Amanita muscaria. No other pretreatment is required (a part a suitable dilution and 0.22 μm filtration) and this feature is of particular importance because the native composition is, as far as possible, preserved and conditions for the taking place of decarboxylation reactions (which transform ibotenic acid into muscimol) are prevented. Measurements are also performed for samples of *Amanita citrina*, which is reported not to contain psychotropic compounds.[8]

EXPERIMENTAL

Chemicals and Reagents

Ultrapure water from Millipore MilliQ (Milford, MA) system was used for the preparation of solutions. Ibotenic acid and muscimol are Sigma Chemical Co. reagents. Pentylamine, hexylamine, heptylamine, octylamine, nonylamine, ortho-phosphoric acid and HPLC-grade acetonitrile were Fluka (Buchs, Switzerland) chemicals. All other reagents were C.Erba (Milano, Italy).

Apparatus

The chromatographic analyses were performed with a Merck-Hitachi (Tokyo, Japan) Lichrograph chromatograph Model L- 6200 equipped with a two channel D-2500 chromato-integrator interfaced with a UV-Vis detector L-4200.

The absorbance spectra A/λ were recorded by a Hitachi (Tokyo, Japan) mod. 150-20 spectrophotometer. The analyses were performed at two different wavelenghts, namely 230 nm, which corresponds to the maximum absorbance for the two analytes, and 254 nm, at which absorptivity coefficient for ibotenic acid is much lower than at 230 nm. The choice of the latter wavelenght was due to: a) a lower matrix interference effect as concerns muscimol determination and b) to confirm, under different conditions, the analyte identification and determination.

For pH measurement a Metrohm (Herisau, Switzerland) 654 pH-meter provided with a combined glass-calomel electrode was employed.

Chromatographic Conditions

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A Phase Separations (Desidee, CLWYD, UK) Spherisorb S5 ODS-2 (5 μm, 250 x 4.6 mm) cartridge was used together with a guard pre-column Merck (Darmstadt, Germany) Lichrospher RP-18 (5 μm).

The mobile phases were aqueous solutions of the ion-interaction reagents. The use of orthophosphate and salicylate of different alkylamine (namely pentylamine, hexylamine, heptylamine, nonylamine and decylamine) at different pH values (3.0, 6.4 and 8.0) was compared. The solutions were prepared by adding to the amount of the amine (weighted to prepare the desidered molar concentration), o-phosphoric acid or salicylic acid up to the desidered pH value.

The chromatographic system was conditioned by passing the eluent through the column until a stable baseline signal was obtained; a minimum of 1 hour was necessary at flow-rate of 1.0 mL/min. When in use, overnight the flow-rate of the mobile phase was maintained at 0.1 mL/min. After use or anyway after three days of use, the column was washed and a new mobile phase freshly prepared.

The washing steps were performed with water (1.0 mL/min for 30 min), a 50/50 v/v water/acetonitrile mixture (1.0 mL/min for one hour), and finally acetonitrile (0.7 mL/min for 20 min). This procedure is necessary, especially when working with real matrices, to remove highly retained lipophilic matter which doesn't elute in aqueous mobile phase, but could affect column modification. Retention intra-day repeatibility was always within 3% for the same eluent preparation and inter-day reproducibility for different preparations was always within 6%.

Our results fit the model according to which the ion-interaction reagent contained in the mobile phase is bound onto the surface of the stationary phase through adsorption and electrostatic forces, giving rise to an electrical double layer. The interaction properties of the original reverse phase packing material are therefore modified and the modified surface is able to simultaneously retain cationic and anionic species.[11-14]

Sample Preparation

The mushrooms investigated were collected in October in Piedmont (North Italy) and analyzed just within three days after sampling. Alternatively the mushrooms can be frozen soon after picking and cleaning and preserved until the analysis, when they are defrosted and treated as described for the fresh ones.

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Different extraction procedures were experimented and compared. The aim was to obtain the maximum recovery yield, taking into account that conditions for the decarboxilation reactions to take place (which transform ibotenic acid into muscimol) must be avoided as much as possible.

The extraction procedures experimented were:

a) solid phase extraction (SPE) employing an anionic-exchange column (QMA Waters) for ibotenic acid and a SPE-C18 for muscimol.

b) liquid-liquid extraction, employing and comparing different extracting solvents mixtures, namely ethanol-water, methanol-water, ethanol-methanol-water in different volume ratios.

c) liquid-liquid extraction, after removal of fats by Soxhlet extractions, respectively performed with chloroform, diethylether and hexane.

d) pounding, sonication and water extraction.

Solid phase extraction (method a) and liquid extraction (method b) led to very low selectivity for the studied analytes and to a consequently heavy matrix effect in the chromatographic analysis. Soxhlet fat extraction also led to a partial extraction of the analytes. The procedure d) was therefore applied for the pretreatment of the sample, also taking into account that the conditions at minimum affect the speciation and at maximum avoid the contamination of the sample. The procedure is described in detail, as follows.

Procedure Followed

The mushrooms were cleaned and the heads and stems were separately pounded in a mortar. After 15 min of sonication treatment, the juice was separated from the pulp and resulted to be about 90% of the fresh weight. In order to improve the recovery of the analytes the pulp was washed twice with ultrapure water. The juice was diluted with ultrapure water and filtered through a 0.22 μm nylon membrane before the injection in the HPLC apparatus. The optimum final dilution prior to the HPLC analysis was 1/1300 v/v.

RESULTS AND DISCUSSION

As mentioned, no example can be found in literature of the simultaneous separation of muscimol and ibotenic acid.

By considering the molecular structure of the analytes (see Fig.1) and their different hydrophilicities, the ion-interaction chromatography seemed to be particularly suitable for their separation.

On the basis of previous results,[11-13] it was pointed out that the good applicability of the method greatly depends on its versatility and the dependance of retention on many variables which must be optimized. Our aim was the development of a method which offers the maximum sensitivity for the analytes, together with the minimum interference effect by the several other components of the matrix.

Method Development

The ion-interaction chromatographic method was optimized as concerns the pH of the mobile phase and the ion-interaction reagent chemical properties. The use of different pH values of the mobile phase showed no significant influence, as concerns both retention and sensitivity, in the pH range between 3.0 and 8.0; a pH value of 6.4 was chosen as more similar to the natural tissue.

As concerns the choice of the ion-interaction reagent, the comparison of salicylate and o-phosphate as counter anions showed that better sensitivities could be gained with o-phosphate. The use of alkylammonium o-phosphate

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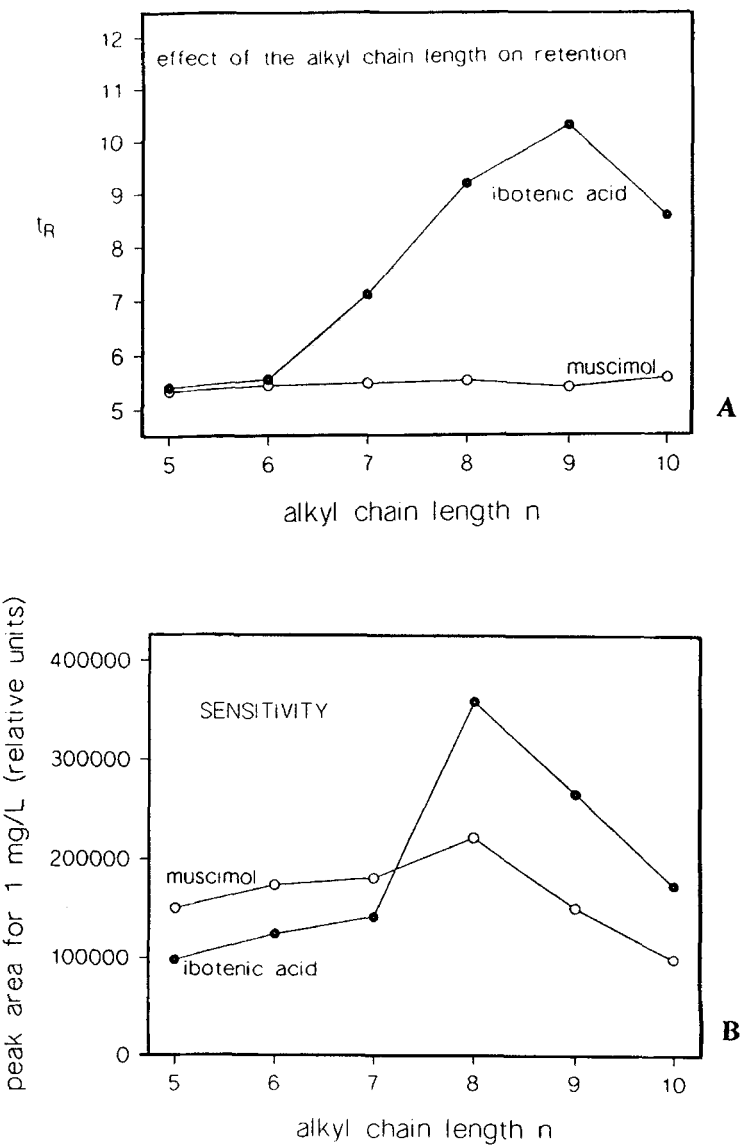

Figure 2. Plot of resolution (A) and of sensitivity (B) vs carbon chain lenght of the ion interaction reagent (aqueous 5.0 x 10^{-3} mol/L alkylammonium orthophosphate, pH 6.4) for the two hallucinogenic compounds.

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salts with the alkyl chain lenght n varying between 5 (pentylamine) and 10 (decylamine) was then compared. Fig.2A shows that the effect of n on retention is very relevant for ibotenic acid, in agreement with its more hydrophilic properties, while it can be considered as negligible for muscimol.

As a consequence, also the resolution between the two analytes is greatly affected by the alkyl chain length. Resolution R_s shows its maximum value for n=9 (R_s=3.04) and is also reasonably high for n=8 (R_s=2.28).

On the other hand, previous studies[14] have shown that the alkyl chain length of the ion-interaction reagent can also affect analyte sensitivity. The behaviour is also followed by the analytes investigated here (Fig.2B): for both analytes the best sensitivity is achieved for n=8. These results suggested the use, as mobile phase, of a 5.0 mM octylammonium o-phosphate aqueous solution at pH=6.4.

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As concerns UV detection, this was performed both at 230 and 254 nm, at which wavelengths the two analytes show significantly different absorptivity values: the comparative detection at two different wavelenghts can be helpfully employed to confirm the identification and the quantitation or to better overcome matrix interference effects.

Fig.3 shows a typical separation obtained under the optimized chromatographic conditions, with detection at λ=230 nm, of a mixture of muscimol (0.150 mg/L) and ibotenic acid (0.100 mg/L).

The calibration curves built for ibotenic acid in the concentration range between 0.05 and 1.00 mg/L, and for muscimol in the concentration range between 0.10 and 3.00 mg/L, showed a good correlation between peak area and concentration: the plots could be fitted by straight lines with correlation coefficients always higher than 0.995. From the sensitivity data and for a signal/ noise ratio = 3, detection levels as low as 30 μg/L and 18 μg/L were respectively evaluated for muscimol and ibotenic acid.

Amanita Muscaria and *Amanita Citrina* Analysis

The method was then applied on the analysis of the two extracts respectively obtained, as described in the experimental section, from heads and stems of *Amanita muscaria* and *Amanita citrina*.

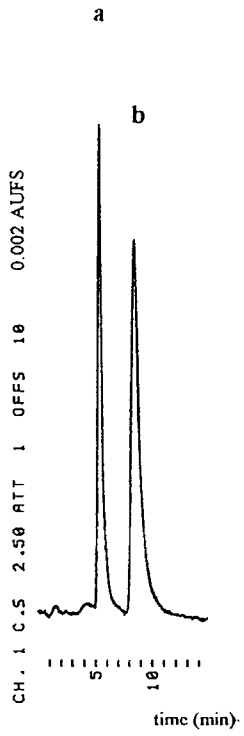

Figure 3. Chromatogram of the standard solution containing 0.150 mg/L of muscimol (a) and 0.100 mg/L of ibotenic acid (b). Experimental conditions. Phase Separation S5 ODS-2 250x4.6 mm column. Mobile phase: aqueous 5.0 x 10^{-3} mol/L octylammonium o-phosphate, pH 6.4. Flow-rate: 1.0 mL/min. Spectrophotometric detection at 230 nm.

The ion-interaction chromatographic method developed here shows advantages of selectivity towards more lipophilic components present in the matrix. This is likely due to the characteristics of the method which utilizes an aqueous mobile phase and is based on the capability of the analytes to form ion-pairs.

As expected, in agreement with botanic information[8] *Amanita citrina* did not show the presence, at least at the detection limit, of both ibotenic acid and muscimol.

As concerns *Amanita muscaria,* Fig. 4 shows, as a comparison, typical chromatograms respectively recorded at 230 nm and 254 nm for the head extract. In agreement with molar absorptivity values, sensitivity for muscimol

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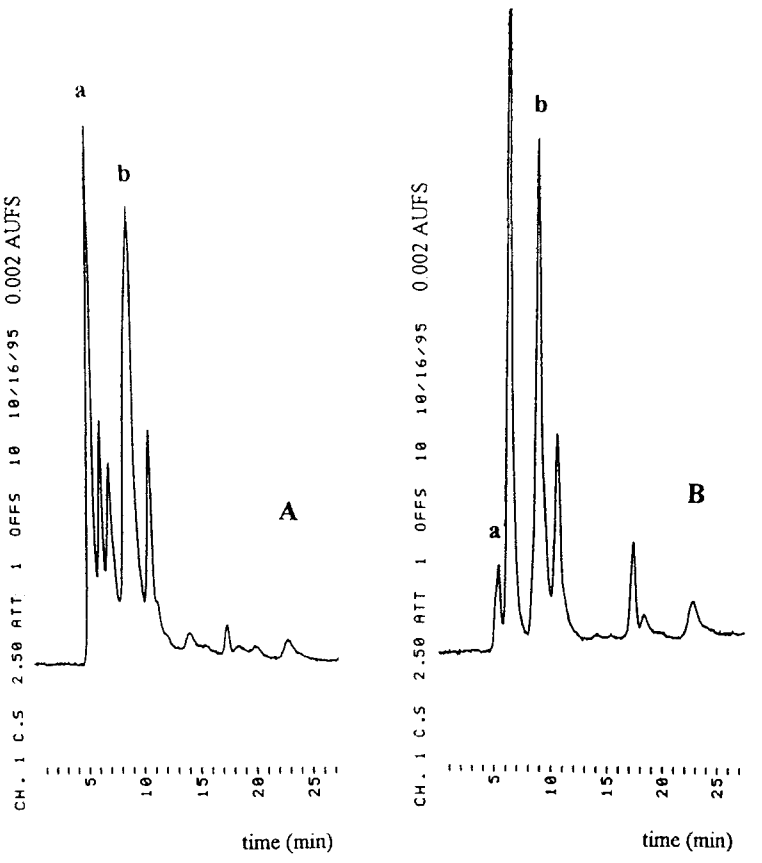

Figure 4. Chromatogram of the diluted (1/1300 w/w) juice from *A. muscaria* heads at 230 (A) and 254 nm (B). Experimental conditions as in Figure 3. a: muscimol; b: ibotenic acid.

is much lower at 254 than at 230 nm, but the interference-freedom is much higher. No significant difference can instead be observed at the two wavelenghts for ibotenic acid.

Quantitation of the two analytes was performed by a standard addition method taking into consideration the chromatographic response in terms of both peak area and peak height at the two wavelenghts. On average, the quantitative results always agree within 15%. As expected, the amount found

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for the two compounds is always higher in heads than in stems. The mean values, referred to fresh mushroom, was found to be: for muscimol 0.38±0.03 g/Kg in heads and 0.08±0.01 g/Kg in stems; for ibotenic acid 0.99±0.01 g/Kg in heads and 0.23±0.03 g/Kg in stems.

The only available literature data[9] report for heads of fresh mushroom amounts of respectively 41.8 mg/Kg of muscimol and 86.6 mg/Kg of ibotenic acid.

The higher content we found might be ascribed, besides the different geographical origin of the samples, to the treatment processes performed prior to the analysis, which in the method here proposed at maximum preserves native composition. No particular pretreatment, extraction process or derivatization reaction is in fact required, a part from the squeezing of the mushroom and the subsequent dilution and filtration of the extract. This extraction process and the absence of contaminating reagents can be of practical interest for subsequent use of the extract.

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