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Advancements in the chemical structures of Ergot acyl glycerides by high performances liquid chromatography coupled with high resolution mass spectrometry

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ABSTRACT

A comprehensive picture of the chemical composition and structure of the acyl-glycerides from ergot (fungi of the genus *Claviceps*) sclerotia was carried out using liquid chromatography coupled with high resolution mass spectrometry (HPLC-ESI-Q-ToF) following microwave solvent extraction.

The method allowed us to study the chemical structures of > 70 compounds being most of these reported and characterized here for the first time in ergot sclerotia. Diglycerides, triglycerides and estolides were proper preliminary separated using reverse phase chromatography and the unambiguous chemical structure of each species was characterized using high resolution tandem mass spectrometry.

Our data and results could be useful for anyone interested in detecting ergot contaminations in cereals and deriving products, as alternative to the identification of alkaloids, in better understanding the behavior of ergot fungi in biological contest and in evaluating the potential uses of ergot estolides in industrial applications.

1. Introduction

The ergot, genus *Claviceps*, comprises fungi living in association with plants, especially *Gramineae*. Ergot has been known for centuries and the alkaloids produced by this fungi are commonly used in pharmaceutical preparations [1,2].

One of the peculiar characteristic of ergot composition is the presence of a relative high amount of estolides in their lipid composition [3]. Usually, the predominant species in the lipid composition of plants and animals, are glycerol esters consisting in a different number of fatty acids (FAs) esterified to the glycerol backbone: monoglycerides (MAGs), diglycerides (DAGs), and triglycerides (TAGs) [3,4]. Estolides are acyl glycerides containing more than three acyl groups, and have been reported in the lipid composition of plant seed oils and oil from ergot fungus [5–7].

In organisms that produce TAG containing fatty acids with hydroxyl groups, such as ergot fungi, secondary acylation, where additional fatty acids are esterified to the hydroxyl moieties, can result in the formation of estolides [8–10].

As far as we know, several studies on the characterization of the lipid composition of Ergot are reported in the literature but only a few described the characterization of estolides by using thin layer chromatography, gas chromatography and Matrix-assisted laser desorption/ionization mass spectrometry [5,6,8,11,12]. Even the use of these analytical methods allowed the different classes of acyl glycerides to be identified, there was several limitations related to the absence of proper separation of the various acyl glycerides species. For this reason, studying the composition of ergot lipids is still an open issue and an important opportunity for obtaining the identification and structural information of all lipid constituents.

Since analytical strategies based on the use of liquid chromatography approaches are the methods of choice for the analysis and characterization of complex lipids [13], in this paper we present the first comprehensive screening study of the chemical composition of lipids from ergot sclerotia using microwave-assisted solvent extraction followed by high resolution tandem mass spectrometry coupled with high performances liquid chromatography (HPLC-ESI-Q-ToF) analysis.

The combination of liquid chromatography together with the use of high resolution mass spectrometer allowed us to avoid the problems related to most of the isobaric isomers and to characterize the chemical structure of > 70 different compounds.

In addition, the use of microwave, that has been already used for the extraction of acyl glycerides from different matrix [14–18], was successfully applied for the first time in the extraction of the lipids from

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ergot sclerotia. The proposed analytical approach allowed us to perform a fast screening of the lipid profile of a sample by liquid chromatography after a simple and fast sample pretreatment based on the proposed microwave approach.

The analytical approach proposed in this work could be used not only for the chemical characterization of ergot acyl glycerides but also for the recognizing of this contaminant in food samples as fast alternative method to the analysis of alkaloids. Consumption of infested grain can lead to poisonings and cause strong disease. For this reason, infestation of food and grain with ergots is a serious problem [2].

Another important aspect related to the characterization of ergot is the high amount of estolides present in the lipid components. Estolides show many features that make them suitable for several applications and in many formulations, such as plasticizers, hydraulic fluids, coatings, food emulsifiers, cosmetics, pigment dispersants [19–25]. The number of papers [7,26–29] on the analytical characterization of synthetic and natural estolides, demonstrates further the high interest in this subject.

Moreover, latest application showed also the importance of the use of ergot estolides to evaluate the presence of cereals and cereals derivates in archaeological samples [30].

2. Experimental

2.1. Chemicals

For the extraction procedure, chloroform and hexane were purchased from Fluka (HPLC-MS grade, USA). *Iso*-propanol, water and methanol (HPLC-MS grade; Fluka) were used as eluents for HPLC-MS analyses.

2.2. Sample pre-treatment

Ergot (fungi of the genus *Claviceps*) sclerotia, collected from rye fields in the area of Muenster (Germany), were ground using a ball mill Mini-Mill Pulverisette 23 with zirconium oxide (ZrO_2) grinding bowl and balls (Fritsch GmbH, Germany).

0.5 mg of ergot sclerotia were subjected to extraction assisted by Milestone microwaves Ethos One (power 600 W) with 300 μL of a chloroform-hexane (3:2) mixture at 80 °C for 25 min. The extracts were dried under a nitrogen stream, diluted with 600 μL of methanol:isopropanol (90:10) and filtered on a 0.45 μm PTFE filter (Grace Davison Discovery Sciences, U.S.) just before injection [31].

2.3. HPLC-ESI-Q-ToF instrument and chromatographic operating conditions

The analyses were carried out on a 1200 Infinity HPLC coupled by a Jet Stream ESI interface with a Quadrupole-Time of Flight tandem mass spectrometer 6530 Infinity Q-ToF detector (Agilent Technologies, USA). The chromatographic separation was performed on an Agilent Poroshell 120 EC-C18 column (3.0 mm × 50 mm, 2.7 μm) with a Zorbax eclipse plus C-18 guard column (4.6 mm × 12.5 mm, 5 μm) [18,31–34].

The injection volume was 2 μL and the column temperatures was 45 °C. Separation was obtained by using a gradient of methanol/water 85:15 (eluent A) and isopropanol (eluent B), programmed as follows: 90% A for 5 min, followed by a linear gradient to 90% B in 30 min, then 10 min at 90% B. Re-equilibration time for each analysis was 10 min. The chromatographic runs were performed at a flow rate of 0.3 mL/min.

The Jet Stream ESI operating conditions were: drying gas (N_2, purity > 98%): 350 °C at 10 L/min; capillary voltage 4.5 kV; nebulizer gas 35 psi; sheath gas (N_2, purity > 98%): 375 °C at 11 L/min. High resolution mass spectra were acquired in the range 100–3200 *m/z* in positive mode. MS/MS spectra were acquired in the range 100–3200 *m/z*. The fragmentor was kept at 200 V, nozzle voltage 1000 V, skimmer 65 V, octapole RF 750 V. The voltage applied to the collision cell to induce CID for the MS/MS experiments was 50 V. The collision gas was N_2 (purity 99.999%) [35].

Data were collected in full Scan with a MS scan rate of 1.03 spectra/s and MS/MS scan rate of 1.05 spectra/s; only one precursor was acquired per cycle (relative threshold 0.010%), active exclusion after 3 spectra and 0.50 min (selection by abundance only). The mass axis was calibrated daily using the commercial Agilent tuning mix HP0321.

Diglycerides, triglycerides and estolides are named according to the following abbreviations used for the acyl substituents: G: gadoleic acid ($C_{20:1}$) A: arachidic acid ($C_{20:0}$), Ln: linolenic acid ($C_{18:3}$), L: linoleic acid ($C_{18:2}$), O: oleic acid ($C_{18:1}$), S: stearic acid ($C_{18:0}$), P: palmitic acid ($C_{16:0}$), Po: palmitoleic acid ($C_{16:1}$), La: lauric acid ($C_{12:0}$), Rn: ricinoleic acid ($C_{18:1,1OH}$).

3. Results and discussion

The chromatographic profile of ergot lipids was characterized by a very high complexity due to the presence of > 70 molecular species linkable to four different acyl glyceride classes: diglycerides, triglycerides esterified with ricinoleic acid, unsaturated triglycerides, and estolides. The use of the extraction mixture chloroform: hexane (3:2) allowed us to extract only the lipid portion of Ergot due to the insolubility of the polysaccharides, proteins, and lignocellulose in such solvents (full scan reported in Fig. S.1).

Fig. 1 shows the HPLC-MS chromatogram of the lipid fraction of ergot and Tables 1–4 list all the identified species with their relative mass spectra fragmentation pattern.

Considering the absence of a database of ergot lipid constituents, the identification of each molecular species was performed by manual interpretation of all the mass spectra.

The high resolution tandem mass spectrometry experiments were performed using sodiate adducts: based on the observation of fragmentation as a function of collision energy, the relative ease of generating DAG ions from $[M + X]^+$ adducts follows the order $[M + Na]^+ < [M + Ag]^+ < [M + Li]^+ < [M + NH_4]^+$: sodium adducts of TAGs were demonstrated to be useful for the quantitation of positional isomers by low-energy collision-induced dissociation in tandem mass spectrometry, and offered better quantitative performance while not requiring the addition of a salt to the mobile phase [4,32,36]. Moreover, the conversion of all the molecular species in sodiate adducts were quantitative in our chromatographic conditions [4].

In order to define the best condition for the induced collision cell (CID), several fragmentation energies were tested, and the optimal performances were obtained with an energy of 50 V.

All the proposed structures in this work were characterized by a difference in ppm between the theoretical and the experimental masses equal or lower than 10 ppm.

In the following sub-sections, the different classes of lipid compounds are discussed separately. For each class of compounds, the relative abundances of the acyl glycerides were calculated on the integrated chromatographic areas and normalized to 100%, as normal practice in the literature for the analysis of oils [37–42].

3.1. Diglycerides

Fig. 2 shows the distribution of all the diglycerides detected in ergot sclerotia, while Table 1 lists all the obtained product ions of the identified species. The fragmentation patterns of all the diglycerides were characterized by the presence of the molecular ion $[M + Na]^+$ and of other two main ions deriving from the cleavage of the two acyl-glycerides (Ac) esterified in the glycerol backbone ($[M\text{-}Ac_1 + Na]^+$ and $M\text{-}Ac_2 + Na]^+$).

The diglyceride fraction of ergot was mainly characterized by the presence of DAGs containing ricinoleic acid as acyl substituent as main compounds: RnPo (*m/z* 631.497, $[M + Na]^+$), RnL (*m/z* 657.505, $[M + Na]^+$), RnO (*m/z* 659.523, $[M + Na]^+$), and RnP (*m/z* 633.486,