Analysis of enzymatically treated tannins – method development challenges

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INTRODUCTION:

Fungi are able to release a spectrum of intracellular and extracellular enzymes that help them with the digestion of the present substrate and allow them to grow. The selection of a „qualified“ fungal strain with a suitable enzymatic „palette“ is therefore very important to point the biodegradation to a desired pathway. Furthermore, the optimal cultivation conditions (e.g. time, temperature, duration, pH value, air flow, light conditions, nutritions – direct energy sources and essential elements) have to be taken into account because they affect the fungal growth, production of those enzymes and the nature of the final hydrolytical residues. However, as the fungi are living organisms, they might have varying demands and despite their fulfilment, they might behave not expectable.

Method development is therefore challenging in the first step during hydrolytical treatment setting and controlling, and then, in the second step, when an analytical technique is being developed and/or optimized to: i) follow the process in terms of enzymatic activity, and to ii) characterize the chemical changes of hydrolytical products at a particular moment, as well as over the whole set time period.

Tannins are complex polyphenolic compounds found in variety of higher plants playing the defense role against pests. Their chemical composition is dependent on the species and the way how they are obtained. Tannins are typically extracted from the tree bark and heartwood, where they represent a higher percentage of the mass compared to other tree parts.

The available analytical assays, when it comes to the compounds of natural origin, have their main limitations in the: 1) selectivity and specifity, when a mixture of more compounds and/or very similar compounds being hardly separable from each other is present; 2) accuracy within the desired range; 3) availability of relevant external standards with similar structure for the method calibration; 4) price of such chemically well-defined, pure standards.

Within this study several method were applied and adapted for selected fungal strains. Some still need further adjustments or even establishment of totally new method. The crucial aspects and settings that needed to be considered during the method development/optimization are listed below.

GROWTH PARAMETERS:

1) Cultivation + Biomass production
   → *fungi selection; nutritions; duration*
2) Ergosterol extraction
   → *fresh/dried mycelium?; saponification?; solvent for phase separation (liquid-liquid); separation efficiency; solvent evaporation*
3) Ergosterol detection by high-performance liquid chromatography (HPLC) (Fig. 1)
   → *mobile phase selection; solvent volume; analysing time; injection volume*

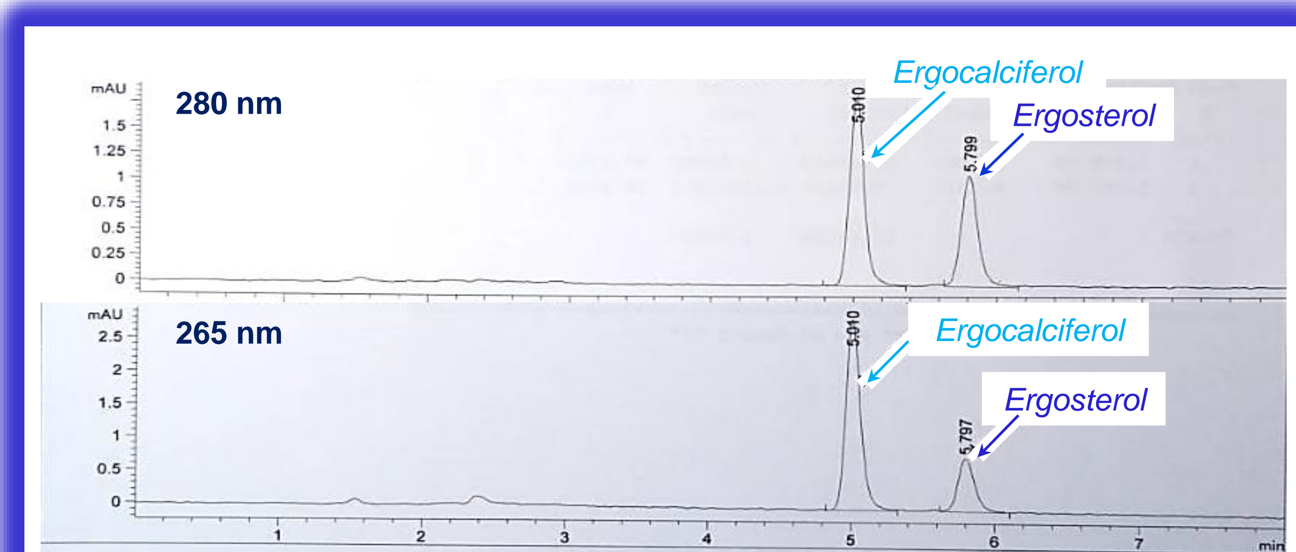

Fig. 1: HPLC-chromatograms of standard solution containing known concentration of ergosterol and ergocalciferol (at different wavelengths)

PRODUCT ANALYSIS:

4) Total phenolic content (UV-Vis absorbance) (Fig. 2)
   → *aliquot volume; optimal exposure time at room temperature*
5) Total soluble carbohydrates content (UV-Vis absorbance) (Fig. 3)
   → *aliquot volume; optimal exposure time at room temperature*
6) Procyanidin quantification (UV-Vis absorbance) – in optimization
   → *aliquot volume; optimal exposure time at room temperature;* ***expensive standard (€€€)***
7) Size-exclusion chromatography (SEC-HPLC) – under development
   → ***no available method with our detector;*** *aliquot volume; optimal exposure time at room temperature;* ***expensive column (€€€€); expensive standards with short shelf life (€€€€)***

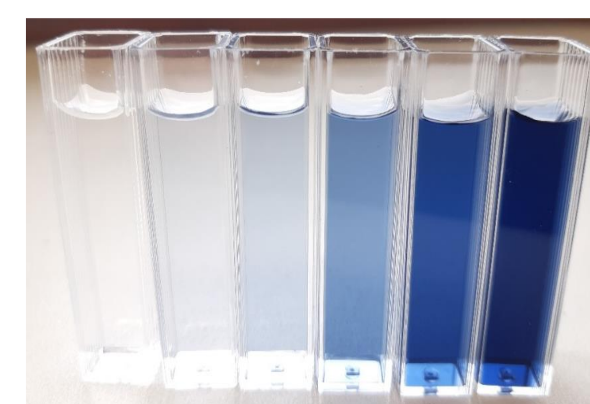

Fig. 2: Calibration solutions for the total phenolic content (measurable at 700 nm)

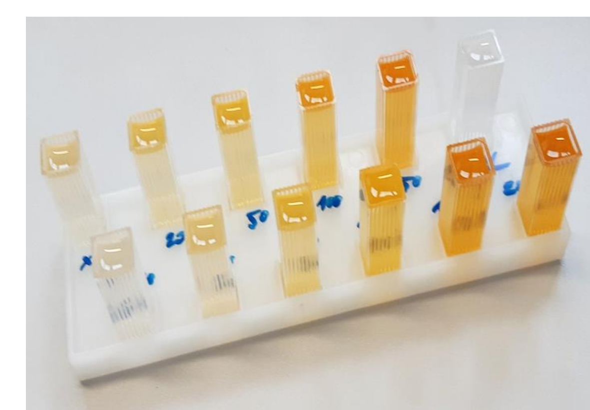

Fig. 3: Calibration solutions for the total soluble carbohydrates content (measurable at 490 nm)

ENZYMATIC ACTIVITY:

8) Tannase (UV-Vis absorbance) (Fig. 4) – in optimization
   → ***optimal exposure time at room temperature (time = main parameter in calculations);*** *aliquot volume; time-demanding solutions preparation; activity calculations;* ***low response; high usage of standards; exclusive and expensive standards (€€€)***

Fig. 4: Tannase assay – calibration showed limited range (absorbance) response at proposed wavelength 520 nm

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