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Purification of fungal secondary metabolites by centrifugal partition chromatography

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List of abbreviations

AF – aflatoxin
AFB – aflatoxin B
AFG – aflatoxin G
AFM – aflatoxin M
CBS – Fungal Biodiversity Centre number
CCC – Countercurrent Chromatography
CI-MS – Chemical Ionization Mass Spectrometry
CPA – cyclopiazonic acid
CPC – Centrifugal Partition Chromatography
EU – European Union
ErgM – Ergometrine
FLD – Fluorescent Light Detector
HESI – Heated ElectroSpary Interface
HPLC-UV – High-Performance Liquid Chromatography with Ultraviolet detection
HSCCC – High-Speed Countercurrent Chromatography
IAC – Ion Affinity Column
ITS – internal transcribed spacer
LLC – Liquid-liquid Chromatography
LSD – Lysergic acid diethylamide
MEA – malt extract agar medium
MS/MS – Tandem Mass Spectrometry
MTBE – *tert*Buthyl-methylether
NRRL – Agricultural Research Service Culture Collection number
OHRMS – Orbitrap High-Resolution Mass Spectrometry
OTA – ochratoxin A
PDB – Potato Dextrose Broth
STC – sterigmatocystin
TFA – Trifluoroacetic acid
TIC – Total Ion Chromatogram
TLC – Thin Layer Chromatography
TOF – Time of Flight detection
SZMC – Szeged Microbiology Collection
YEB – yeast extract broth
YES – yeast extract sucrose medium

1. Introduction

Secondary metabolites are not vital but play an important role in the life of a microbe, fungus. These compounds are produced in the ideal state of growth, and also can be broken down by the organism.

Aspergillus species produce a wide variety of these secondary metabolites. Probably the most examined of these is the group of aflatoxins (AFs). These chemicals were named after *Aspergillus flavus*, the fungi that were isolated from. Their mutagenic, teratogenic, and carcinogenic properties were revealed, hence strict limit levels were imposed in the EU. These restrictions imply the regular quality assurance of food and feed and toxicological studies in Europe, therefore the demand for pure AFs as reference material is high. Numerous publications exist on the isolation and purification of AFs, including thin layer chromatography (TLC), chromatographic methods including silica gel and alumina, and HPLC separations as well. But the application of novel liquid-liquid chromatographic techniques could result in faster and cheaper production of these toxins.

Ergometrine (ErgM) is produced by mainly *Claviceps* species. Most *Claviceps* species can be found in equatorial climates, but *Claviceps purpurea* infects crops and grasses in Mediterranean and temperate climates as well. ErgM and its variants are used in medicine, therefore the demand for the pure form by the pharmacological industry is high. It can be synthesized, but the isolation and purification can also be carried out from the overwintering sclerotium of the fungus, utilizing liquid-liquid chromatography as the separation technique.

Centrifugal partition chromatography (CPC) belongs to the preparative separation techniques, where two, immiscible liquid phases are used to partition the different components of a mixture. The differences in the partition coefficients of the compounds result in separation during the elution. The stationary phase is immobilized by a constant amount of centrifugal force, while the mobile phase is pumped through it, resulting in droplets with high specific surface area, achieving better distribution. Since the two-phase can be assembled from several solvents, this separation technique offers a wide variability, fast and robust separations, with high efficacy.

In this work the method development for the purification of AFs and ErgM by CPC is described in detail, as well as the scale-up of the separation of AFs.

1.1. The Aspergillus genus

The *Aspergillus* genus is a diverse group of filamentous fungi in the family of *Aspergillaceae*, in the order of *Eurotiales*, class of *Eurotiomycetes*, and the division of *Ascomycota* [1].

The species *Aspergillus* was first described by Pier Antonio Micheli in 1729 [2]. The shape of the fungi under the microscope reminded the Italian priest and biologist of a holy water sprinkler (*aspergillum*) and named the newly discovered species *Aspergillus*. Their prevalence in the natural environment, their ease of cultivation on laboratory media, and the economic importance of several of its species ensured that many mycologists and industrial microbiologists were attracted to their study. Furthermore, this common mold is involved in many industrial processes as producers of enzymes (e.g. amylases) [3] and commodity chemicals (e.g. citric acid) [4] as well as fermented foodstuff (e.g. soy sauce). The genus has more than 800 recognized species worldwide, which is an important group of filamentous ascomycete species [4]. The species can be found in soil [5,6], food and feed [7-21] such as tomatoes [9], peppers [10], apples [11], grapes [12, 13], beans [14], almonds [15], peanuts [16, 17], maize [18, 19], rice [20] and wheat [21]. Some species were also isolated from clinical sources including keratitis [22] and several pulmonary diseases [23, 24] as well.

1.2. Extrolites of Aspergilli

Isolates of *Aspergillus* species produce a wide range of secondary metabolites that is often specific or characteristic of the different groups of *Aspergillus* (***Table 1***).

Table 1. *List of extrolites produced by Aspergillus species*

Extrolite	Section	Species	[a]Ref.
kojic acid	*Flavi*	Not specified	25
		A. nomius	40
		A. parasiticus	40
		A. pseudotamarii	43
		A. pseudonomius	43
penicillic acid	*Circumdati*	Not specified	26
	Flavi	*A. minisclerotigenes*	40
[b]OTA	*Flavi*	Not specified	26
	Circumdati	Not specified	26
	Nigiri	Not specified	26
aflatoxins	*Flavi*	Not specified	32, 33
		A. flavus	36
		A. minisclerotigenes	39
		A. nomius	39
		A. parasiticus	39, 42
		A. pseudonomius	43
aflatoxin Bs	*Flavi*	*A. flavus*	37
	Flavi	*A. pseudotamarii*	43
	Nidulantes	Not specified	32, 33
	Ochraceorosei	Not specified	32, 33
[c]STC	*Nidulantes*	*A. amoenus*	34
		A. minisclerotigenes	39
	Flavi	*A. nomius*	39
		A. parasiticus	39
	Nidulantes	*A. nidulans*	41
wentilactone A, B	*Cremei*	*A. dimorphicus*	35
[d]CPA	*Flavi*	*A. flavus*	38
		A. minisclerotigenes	40
		A. pseudotamarii	43
averufin citreoisocoumarin cordycepin gerfelin penicillin G	*Nidulantes*	*A. nidulans*	41
aspergillic acid	*Flavi*	*A. nomius*	40
		A. parasiticus	40
		A. pseudonomius	43
paspalline	*Flavi*	*A. nomius*	40
		A. pseudotamarii	43
parasitenone	*Flavi*	*A. parasiticus*	40

[a]Reference number
[b]Ochratoxin A
[c]Sterigmatocystin
[d]Cyclopyazonic acid

As an example, kojic acid (**1**) is produced by nearly all species of section *Flavi* [25], while most members of section *Circumdati* produce penicillic acid (**2**) [26]. On the other

hand, ochratoxin A (OTA) (**3**) is synthetized by sections *Flavi*, *Circumdati*, and *Nigiri* as well (***Figure 1***) [26].

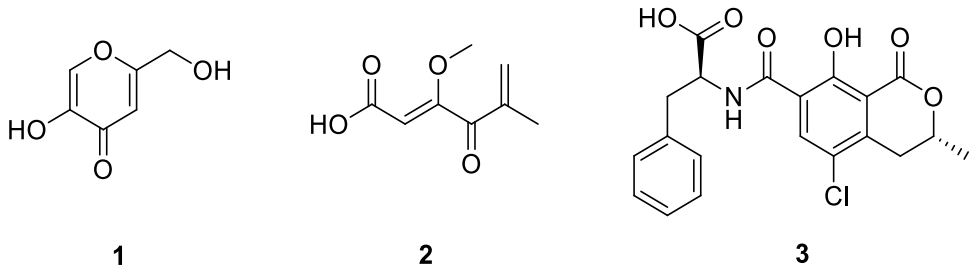


Figure 1. *Structures of kojic acid (**1**), penicillic acid (**2**), and ochratoxin A (**3**).*

Aspergillus species can produce different toxic secondary metabolites [27-32], such as ochratoxins [27], gliotoxin [28], fumagillin [29], helvolic acid [30], fumonisins, several terpenes and alkaloids [31], as well as aflatoxins (AFs) [32]. AFs can be produced by many *Aspergillus* species especially in three sections: *Flavi*, *Nidulantes*, and *Ochraceorosei* [33].

It was described that *A. amoenu*s (NRRL 236) that is isolated from *Berberis sp*. fruit, is able to produce 31 µg/ml sterigmatocystin (STC) on yeast extract broth (YEB) [34]. *A. dimorphicus* was proven to produce wentilactones (A and B), which is a class of antitumor agents. Production of these compounds is highly dependent on the pH and the temperature of the culture [35]. From section *Flavi*, *A. falvus* (UTHSC 06-3872), was reported to produce AFs on yeast-extract-sucrose (YES) media [36]. *A. flavus* (NRRL 3357) was able to produce AFBs [37], while a nonaflatoxigenic strain of *A. flavus* was also able to produce cyclopyazonic acid (CPA) (***Figure 2***) that is a toxic metabolite belonging to the ergoline alkaloid family of alkaloids [38].

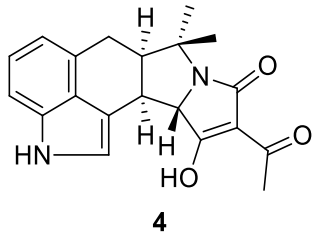


Figure 2. *Structure of CPA (**4**)*

A. minisclerotigenes (CBS 117635), *A. nomius* (NRRL 25585), and *A. parasiticus* (NRRL 2999) were able to produce all AFs and STC on several culture media [39]. *A. minisclerogtigenes* was also proven to produce penicillic acid (**2**) and CPA (**4**) as well [40]. When cultivated on malt-extract-agar (MEA) *A. nidulans* is known to produce many

extrolites [41] including averufin (**5**), citreoisocoumarin (**6**), cordycepin (**7**), gerfelin (**8**), penicillin G (**9**), STC, and several others (***Figure 3***).

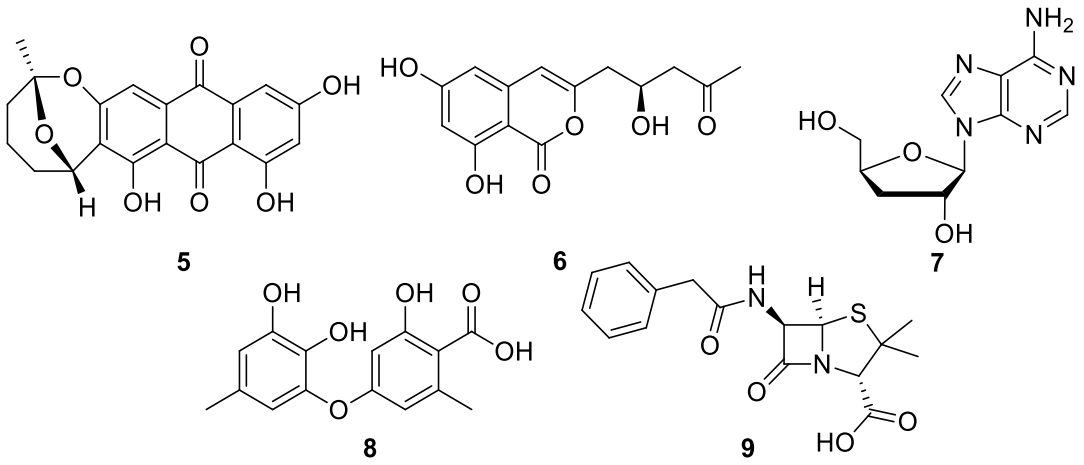


Figure 3. *Chemical structures of averufin (**5**), citreoisocoumarin (**6**), cordycepin (**7**), gerfelin (**8**) and penicillin G (**9**)*

Besides the four main AFs, *A. nomius* (NRRL 13137) is known to produce aspergillic acid (**10**), kojic acid (**1**), and paspaline (**12**), as an example [40]. Aflatoxin production of *A. parasiticus* (NRRL 3145) was also proven when the filamentous fungus was grown on YES media [42], but it can produce several other secondary metabolites such as kojic acid (**1**), aspergillic acid (**10**), parasitenone (**11**) (***Figure 4***) and many more others [40].

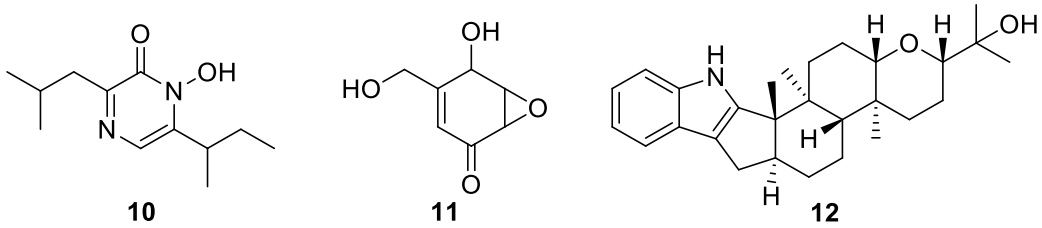


Figure 4. *Structures of aspergillic acid (**10**), parasitenone (**11**) and paspaline (**12**)*

A. pseudotamarii produces mainly AFB_1 and AFB_2 and it also can produce kojic acid (**1**), CPA (**4**), paspaline (**12**), and several other alkaloid types of toxic metabolites [43]. In the section *Flavi*, *A. pseudonomius*, just as *A. nomius*, it is able to produce all four main AFs, aspergillic and kojic acids (**10** and **1,** respectively) [43].

1.3. Aflatoxins

Aflatoxins (AFs) are mycotoxins produced as secondary metabolites mainly by two *Aspergillus* species, namely *A. parasiticus* and *A. flavus*, but there are more producers,

such as *A. minisclerotigenes* and *A. nomius* as well as many others (all of the producer strains are listed in ***Table 2***) [44, 45].

Table 2. *Complete list of AF producer strains [43,44]*

Section	Species	[a]ID	Produced AFs	GenBank number	
				[b]ITS	[c]CaM
Flavi	*A. arachidicola*	[d]CBS 117610	AFBs, Gs	MF668184	EF202049
	A. bombycis	[e]NRRL 26010	AFBs, Gs	AF104444	AY017594
	A. flavus	NRRL 1957	AFBs	AF027863	EF661508
	A. minisclerotigenes	CBS 117635	AFBs, Gs	EF409239	MG518009
	A. nomius	NRRL 13137	AFBs, Gs	AF027860	EF661531
	A. novoparasiticus	CBS 126849	AFBs, Gs	MG662397	MG518055
	A. parasiticus	NRRL 502	AFBs, Gs	AF027862	EF661516
	A. parvisclerotigenus	CBS 121.62	AFBs, Gs	EF409240	MG518089
	A. pseudocaelatus	CBS 117616	AFBs, Gs	EF409242	MG517995
	A. pseudonomius	NRRL 3353	AFBs, Gs	AF338643	EF661529
	A. pseudotamarii	CBS 766.97	AFB_1	AF272574	EF661521
	A. togoensis	CBS 272.89	AFB_1	AJ874113	FJ491489
	A. transmontanensis	CBS 130015	AFBs, Gs	JF412774	HM803020
	A. mottae	CBS 130016	AFBs, Gs	JF412767	MG518058
	A. sergii	CBS 130017	AFBs, Gs	JF412769	MG518059
Ochraceorosei	*A. ochraceoroseus*	NRRL 28622	AFBs	EF661224	EF661137
	A. rambellii	CBS 101887	AFBs	AJ874116	JN121416
Nidulantes	*A. astellatus*	CBS 134.55	AFB_1	HF545007	DQ114131
	A. olivicola	NRRL 212	AFB_1	KT359601	KT359605
	A. venezuelensis	CBS 868.97	AFB_1	AJ874119	EU443977

[a]Identification number
[b]Internal Transcribed Spacer
[c]Clamodulin, Calcium-modulated protein
[d]Fungal Biodiversity Centre number
[e]Agricultural Research Service Culture Collection number

Four main AFs, B_1, G_1, B_2, and G_2 exist (***Figure 5***), all of which are toxic to both humans and animals [39, 42, 44]. Consuming food contaminated with AFs could lead to serious health problems, including acute hepatic necrosis, acute liver failure, and liver cancer [45, 46]. Among these compounds, AFB_1 is the most toxic; it is associated with teratogenic, mutagenic, and carcinogenic effects [47] and may cause lesions mainly in the liver [48], but also in other exposed organs systems [49]. Because of the dangerous nature of AFs, very low limits (on the order of parts per billion) for these compounds in various food products and commodities are set in the European Union and around the world. The maximum residual limits for total AF contamination range from 4 to 10 μg/kg [50] but other limits are differentiated due to the nature of the food products. In most cereals, the limit for AFB1 is 2 μg/kg, while the total AF amount can be up to 4 μg/kg, while in peanuts selected for further use (not direct consumption) the upper limit of AFB1 is 8 μg/kg, and the maximum concentration of total AFs is 15 μg/kg [51].

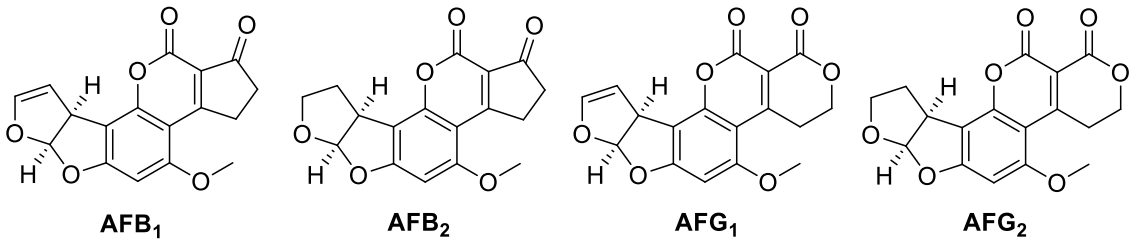


Figure 5. *Structures of the four main aflatoxins*

Numerous methods for determining AF concentrations using various matrices have been proposed previously [52-54]. However, HPLC-UV has also been used to determine AF concentrations in food products [55], the most popular technique is based on the use of a fluorescence detector (FLD), because it is more sensitive and specific than the widely applied HPLC-UV technique. In this detection, the analyte is excited by one wavelength of UV light, and the light emitted by the compound is measured on another wavelength. The most common excitation and emission wavelengths for AFs are 360 nm, 435 nm [56], 365 nm, 440 nm [57], 365 nm, 425 nm [58], 365 nm, 450 nm [59] respectively. If an enhanced sensitivity is needed, fluorophores derived from the AFs are used for the measurement, therefore an additional derivatization step must be carried out [60-63]. For this reaction, trifluoroacetic acid (TFA) is the most commonly used reagent [64]. Furthermore, HPLC coupled with mass spectrometry (MS) is used generally for the AF determination [65-73], including HPLC-MS/MS [66], HPLC-TOF [67, 68] and HPLC-HESI-Orbitrap MS [69-72]. Presently, ionization with a HESI probe and detection with an Orbitrap MS detector is the most sensitive of these previously proposed methods [71]. Separations can be achieved in both isocratic [73-75] and gradient elution modes [76-78].

1.3.1. Purification of aflatoxins

The isolation and preparation of AFs were first reported by Sargenat et al. in 1961 [79]. Toxic extracts of Brazilian groundnuts were purified via column chromatography on alumina, yielding a nearly colorless crystalline material. This material was first referred to as AFs G and B by Nesbitt et al. in 1962, who purified the hot methanol extract of *A. flavus* on silica gel and further purified the AF mixture with a counter-current distribution to separate B and G groups of AFs [80]. One year later, the four main AFs (AFG_1, AFG_2, AFB_1, and AFB_2) were described for the first time and separated on silica gel with purities above 90%; moreover, the unsaturation of the furan ring in AFG_1 and

AFB_1 and the saturation of the ring in AFG_2 and AFB_2 were reported [81]. Later, Stubblefield et al. separated the crude mixture of AFs extracted from wheat and rice using a process of successive separations with a silica gel mesh as the stationary phase and chloroform/ethanol (99:1, v/v%), and chloroform/acetone/ethanol (97.25:2:0.75, v/v/v%) as the eluents. The purity of each obtained AF was above 90% [82]. De Jesus et al. successfully purified three of the four AFs (AFB_1, AFB_2, and AFG_1) from the solid culture of *A. flavus* extracted from both the media and the mycelia utilizing consecutive normal-phase chromatography steps with different ratios of chloroform and acetone as eluents. 1.09 g of AFB_1, 360 mg of AFB_2, and 2.1 of AFG_1 were obtained from 21 g of crude broth and 9.7 g crude mycelial extract [83].

In 1977, high-pressure liquid chromatography (HPLC) was used to have AFM_1, AFB_1 and AFG_1 purified, from the mixtures of AFM_1-M_2, AFB_1-B_2, and AFG_1-G_2 [84]. In one chromatographic run 80 mg AFB_1, AFG_1, and 9-10 mg of AFM_1 could be obtained including at least two reverse phase chromatographic runs for each compound. From a 100 g of contaminated and grained peanut sample, 500 µl of cleaned-up aflatoxin extract was injected into a preparative HPLC column [85]. It was demonstrated that even though having multiple clean-up steps, the separation of these toxins can turn out very difficult. The four main aflatoxins were separated from each other and the impurities and the components were identified by chemical ionization mass spectrometry (CI-MS).

For the analytical sample preparations, extraction methods are needed. Because determinations of AFs from various matrices are performed, a large variety of extraction solvents and methods are used [86-96]. AFs are generally soluble in polar solvents such as methanol, acetone, or acetonitrile [86], thus, the extraction of aflatoxins involves the use of these organic solvents mixed in different proportions with small amounts of water [87, 88]. Several studies exploring the extraction efficiency of different organic-aqueous solvents have been carried out on the commonly contaminated matrices [89-91] and different results have been reported. Aflatoxin determination based on immunoassay technique requires extraction using a mixture of methanol-water (80/20, v/v%) [90, 92] because methanol has a less negative effect on antibodies compared to other organic solvents such as acetone and acetonitrile.

A mixture of methanol and water (55/45, v/v%) was used to gain AFs from peanuts [93], rice (80/20, v/v%) [94], and corn (60/40, v/v%) [95], while a mixture of acetone and

water (70/30 v/v%) was utilized in order to extract them from cottonseed products without any fatty contaminants [96].

Small scale analytical separations or so-called solid-phase extractions (SPEs) exist for the sample preparation of Afs to decrease the matrix effect and enhance the intensity of the corresponding analytical technique [97-103]. For the early analysis of AFs, early SPE applications focused on silica-gel clean-ups [81, 97, 98]. These early separations were unsatisfactory in terms of purity, therefore consecutive use of SPE separations were often carried out [98]. As an example, an aminopropyl column was coupled with a C18 phase column, and AFs were eluted with methanol [93, 99].

Basic aluminium oxide can also be utilized as an absorbent [100]. Samples were extracted with methanol/water 80/20 (v/v%), the diluted aliquot of samples were applied to sample cartridges filled with basic aluminium oxide and the desired aflatoxins were eluted by gravity.

C18 or so-called reverse-phased silica cartridges are also a useful tool. Multiple mycotoxins including all AFs can be recovered from beer samples, by direct loading the drink onto the column [101]. AFB_1 and OTA can be successfully separated from the matrix of licorice roots and fritillary bulbs [102], while AFs from pistachio nut-extracts can also be gained [103].

Immunoaffinity columns (IACs) became the most important and widely used clean-up tools for aflatoxin analysis. Immunoaffinity columns achieve clean-up based on the specificity of binding between antibodies and aflatoxins. The high affinity and specificity of antibodies for aflatoxins have been the cornerstone in the development of the various immunoaffinity columns. Owing to advances in the production of monoclonal antibodies with specificity and affinity, immobilization of antibodies on supporting beads, and well-established sample preparation protocols, IACs are applicable to a wide array of food matrices for aflatoxin analysis [104] Immunoaffinity column clean-up offers the extraction of aflatoxins from various food matrices [105, 106] with simple aqueous mixtures (e.g., methanol/water), making the final extracts compatible with TLC [107-109], fluorometry, HPLC-FLD [110, 111] and HPLC-MS [112-115] for both single- and multimycotoxin determination without additional sample treatment.

The stability of AFs is highly dependent on various parameters of a solution (temperature [116, 117], solvent [117-119], and time [116-118]). The examination of the thermal stability of dried AF standards was carried out in two temperatures (150°C and

180°C) [116]. The degradation of the toxins was measured every 15 minutes for 2 hours. It was concluded that only 30 minutes is enough to completely decompose Afs at 150°C. At 180°C, this effect of thermal instability degraded the standards even quicker.

Organic content dependency was tested with AF standard solutions [117]. AFs were dissolved in solvents containing different amounts (0, 20, 40, 60, 80, and 100%) of two organic solvents (methanol and acetonitrile) in water. A significant decrease in AFG_1 and AFG_2 was observed in all cases where the solutions contained water at room temperature. However, when AFG_1 and AFG_2 were prepared in 100% organic solvent (acetonitrile or methanol), the concentrations did not differ from their corresponding control standards. At 5°C, a significant decrease in concentration was realized for AFGs when no organic solvent was added; however, their decrease was only 8% of the original concentration in both cases. No significant decrease in AFBs was observed for any of the solutions maintained at 5°C. At room temperature, the rate of loss was greatest during the first 10 h, seemingly reaching an asymptote at 24 h. After 10 h, the peak areas for AFG_1, AFG_2, AFB_1, and AFB_2 decreased by 64.2, 53.1, 26.0, and 20.0%, respectively, while the total percentage loss at 24 h for the same AFs was 83.6, 74.5, 40.9, and 31.3%, respectively.

The stability of AFB_1 and AFG_1 was tested in several solvents (chloroform, acetone, methanol, ethyl acetate, water and mixtures of chloroform/acetone = 1/1 (v/v%), chloroform/methanol = 1/1 (v/v%) and methanol/acetone = 1/1 (v/v%)) at 4°C [118]. High degrees of stability were obtained with most of the different organic solvents, but the highest stability was observed in the case of chloroform.

The stability of AFB_1 was examined in four different apolar organic solvents, such as benzene, toluene, cyclohexane, and heptane [119]. Standard solutions of AFB_1 (5 mg/ml) were stored in the dark at room temperature for 6 months. The absorbance of the solutions was measured monthly. The greatest decrease of AFB_1 content was that of cyclohexane (16.2%), followed by toluene (10.3%), heptane (9.4%), and benzene (9.0%).

1.4. The Claviceps genus

The *Claviceps* genus belongs into the division of *Ascomycetes*, in the class of *Sordariomycetes* and the order of *Hypocreales*. The genus is in the family of *Clavicipitaceae* [120]. There are approximately 38 species in the genus, most of which can be found in tropical climates [120].

The overwintering structure of the sclerotia contains more than a hundred biologically active compounds, mostly alkaloids, in which there are toxic ones (lysergic-acid-diethylamide, ergocryptine, ergocristine in high doses) as well [121]. Consumption of this sclerotia can lead to various negative effects in the human body, such as hallucinations, nerve cramps, miscarriages, and when consumed in high doses, to death [122]. Filamentous fungi in the genus *Claviceps* are mostly parasitic fungi. *C. purpurea*, *C. fusiformis*, *C. paspali*, and *C. africana* are the ecologically most important ones among them. *C. purpurea* parasites grass and cereals, while the other ones can be isolated from millet, rye, and sorghum as well [123].

The genus has historical significance as well, it was the main cause of ergotism in the Middle Ages [124]. When cereals contaminated with the fungus were harvested and milled into flour, the ergot alkaloids were baked into the final products. Consuming these contaminated products leads to serious hallucinations and severe burning sensations in the limbs. The common name for ergotism is St. Anthony's fire. The consumption of these contaminated products has led to the death of thousands of people. The persecution of witches associated with ergotism was a significant problem in the Middle Ages, and according to some research, more than 40,000 deaths were linked to ergotism in 943 [125].

Nowadays ergot alkaloids play an important role in the pharmaceutical industry. Ergot alkaloids isolated from *Claviceps* are one of the most important active ingredients driven from a natural source. They also cover a wide range of therapeutic use, such as the alleviation of atonic bleeding after giving birth, relief of migraines, treatment of orthostatic circulatory disorders, reduction of high blood pressure, treatment of hyperprolactinemia, and Parkinson's disease. The pharmacological relevance of *Claviceps* can be related to compounds like lysergic-acid-diethylamide (LSD), ergometrine (ErgM), ergotamine, ergocristine, and ergocryptine [122, 126].

1.4.1. Claviceps purpurea

1.4.1.1. Importance

Claviceps purpurea is a significant species, with widespread application in various fields of science. It is used in fields like molecular biology, microbiology, biotechnology, and biochemistry as well [127]. It is widely utilized as a model organism, metabolite production, and pathogenic behavior is well studied [123, 128]. The pharmaceutical use of the fungus is very significant, the obtained alkaloids, especially ergot alkaloids are widely used in gynecology and obstetrics, or the treatment of hypotony and migraines. [129]. In the past, it was used as an anti-schizophrenic agent in psychotherapeutics, and it was also prescribed in therapy for children with autism [126]. On the other hand, several research was shut down, when the risk of chromosomal and genetic diseases arose [122,126].

1.4.2. Extrolites of Claviceps

In the overwintering sclerotium of *C. purpurea* hundreds of biologically active compounds can be found beside the stored essential nutrients [130]. From these produced secondary metabolites, the most important ones are the group of alkaloids, especially ergot alkaloids. Besides these alkaloids, the sclerotium contains other, structurally different compounds (***Figure 6***), such as sulphur-containing histidine derivatives (ergothioneine, **28**), unusual fatty acids, indole diterpenes (paxilline, **29**), anthraquinone-carboxylic acids (anthraquinone-2-carboxylic acid, **30**), and pigments called ergochromes (secalonic acid, **31**) [131].

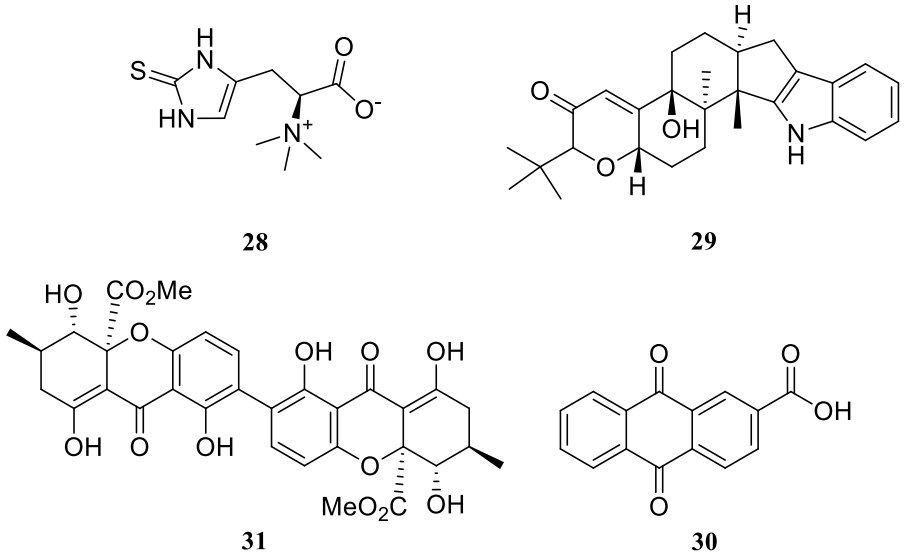


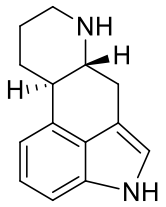
Figure 6. *Structure of compounds found in the sclerotium of C. purpurea*

1.4.3. Ergot alkaloids

Alkaloids are mostly heterocyclic compounds that contain nitrogen as a heteroatom. These compounds can be found mostly in plants, but certain types are originated from animals as well [132]. Besides carbon, hydrogen, and oxygen, they might contain sulphur, chlorine, bromine, and phosphorus as well. Alkaloids can be used in agriculture, in several insecticides, as psychoactive agents, or in the food industry as well. Most alkaloids are insoluble in water but freely soluble in organic solvents such as diethyl-ether, ethyl-acetate, and chloroform. Most alkaloids act as a weak base, but some are amphoteric, like theobromine and theophylline. Freely soluble salts are formed when they get in contact with an acid.

The group of alkaloids can be characterized by structural diversity. They can be separated into true alkaloids, protoalkaloids, polyamine alkaloids, peptide and cyclopeptide alkaloids, and pseudo- (not real) alkaloids. [133]. True alkaloids are driven from amino acids. They contain a heterocyclic ring with a nitrogen atom. These compounds are highly reactive and biologically active, even in small doses. Most of them are white powder with a sour taste, but in this group belongs nicotine with its' brown color and viscous liquid state, as an exception [134]. L-ornithine, L-lysine, L-phenylalanine, L-tyrosine, L-tryptophan, and L-histidine are among their primary precursors. True alkaloids include the important group of ergot alkaloids.

Until now, more than 80 ergot alkaloids were isolated, primarily from the genus *Claviceps*, but they can be found in organisms belonging to other taxons as well [135, 136]. Ergot alkaloids belong into the class of indole-alkaloids, their basic structure is a tetracyclic ergoline (**41**) (***Figure 7***), which is a partially hydrogenated indole-quinoline.

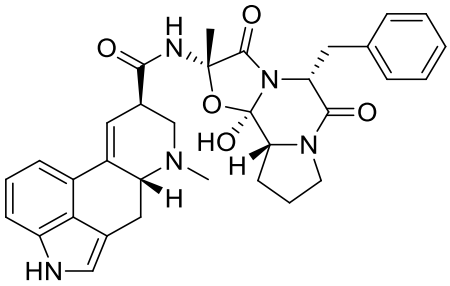


41

Figure 7. *Chemical structure of ergoline*

Ergot alkaloids can be separated into three main groups according to their structure. There are ergopeptides or ergopeptines, clavines, and lysergic-acid amides. Their name can be created according to the botanical name of the host plant (ergosecalin from *Secale spp.*), from the special circumstance of their discovery (ergocryptine from the Greek *cryptos*, as mysterious), a family relation to the discoverer's family (ergocristine from the name of Cristine Stoll, daughter of the discoverer, Artur Stoll), or the name can relate to the special pharmacological effect of the compound (ErgM, according to its effect on the uterus (the uterus is *endometrium uteri* in Latin)).

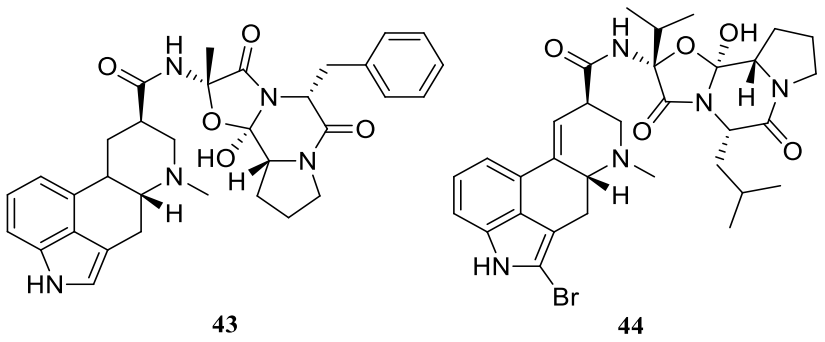
Ergot alkaloids contain several chiral centers that are different in configuration, but the *R*-chirality of the 5^{th} carbon atom is stagnant. This reflects the origin from L-tryptophan (the amino acid is the precursor of the indole ring) [135]. The product of the strong alkaline hydrolysis of ergot alkaloids is either lysergic acid or its C8-epimer, isolysergic acid. Ergopeptines are tetrapeptides that are created biosynthetically, and the first member of the peptide chain is always a lysergic acid. The following three amino acids can vary that is why there are so many derivatives of these compounds. As an example, ergotamine (**42**) contains lysergic acid and L-proline in the peptide chain (***Figure 8***).



42

8. Figure *Structure of ergotamine*

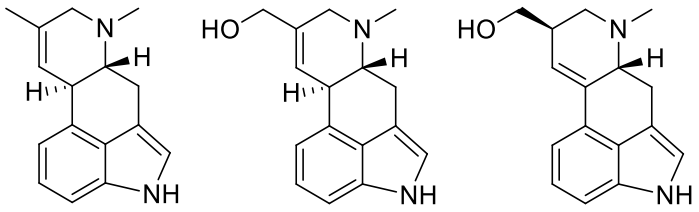
A unique structural property, that cannot be found in other natural molecules is the cyclol moiety of the molecule. This specific part comes from the reaction of the α-hydroxyamino acid and the carboxyl group of the proline in the chain. Other molecules in the tripeptide chains of ergopeptines usually can be L-alanine, L-phenylalanine, L-valine, L-leucine, L-isoleucine, and 2-aminobutyric acid. Because of the non-polar side chains of the amino acids, these compounds are highly insoluble in water [130]. Ergotamine is the only ergopeptine used in the pharmaceutical industry as an active ingredient [135], but its semi-synthetic derivatives are widely applied, such as dihydroergotamine (**43**) and bromocriptine (**44**) (***Figure 9***).



43 **44**

Figure 9. *Structures of dihydroergotamine (**43**) and bromocriptine (**44**)*

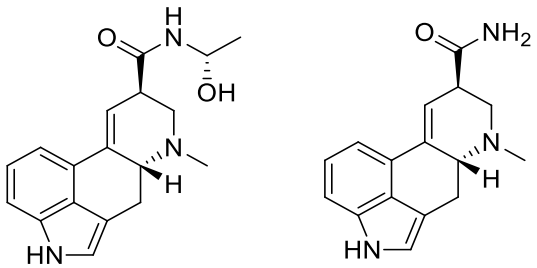
Clavines are derivatives of 6,8-dimethylergoline, structurally related to the natural ergoline, like agroclavine (**45**), elimoclavine (**46**), and lysergol (**47**) (***Figure 10***). Despite there being at least 35 compounds of this group isolated and characterized, none is used in the pharmaceutical industry.



45 **46** **47**

Figure 10. *Chemical structures of agroclavine (**45**), elimoclavine (**46**) and lysergol (**47**)*

Lysergic acid amides can be divided into two groups, since the amidation of the carboxyl group of lysergic acid results in two types of products. The two groups are the simple non-peptide amides, that have a short carbon chain, and the peptide amides that generally form tripeptides. In the overwintering sclerotium usually ErgM (also known as ergonovine or ergobasine) (**50**) (***Figure 12***), lysergic acid hydroxyethylamide (LSH) (**48**) and ergine (**49**) (***Figure 11***) can be found as non-peptide amides.



48 **49**

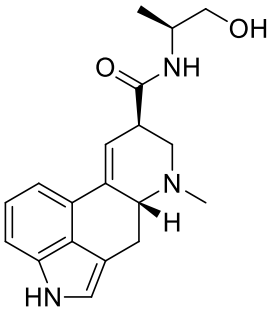
Figure 11. *Chemical structures of LSH (**48**) and ergine (**49**)*

Lysergic acid derivatives are pharmacologically very active [135]. ErgM and its derivatives, such as methyl-ergotamine and dihydroergotamine. Lysergic acid amides are being investigated since 1938 when lysergic acid diethylamide (as known as LSD) was first synthetized by Albert Hofmann from ergotamine [122]. The effects on the human body of this compound were first described by him after an accidental consumption. LSD was applied as a psychiatric and psychotherapeutic drug in the treatment of alcoholism and schizophrenia in the 1950s [136]. The use of the compound as a pharmaceutical agent and experiments on humans were terminated in the USA in the 1970s.

1.5. Ergometrine

ErgM (ergonovine, ergobasine, D-lysergic acid-β-propanolamide) is used as a medicine since its discovery in 1935, by Harold Ward Dudley [135]. It is a naturally occurring ergot alkaloid, that is produced by the filamentous fungi of the genus *Claviceps* [123]. The compound belongs to the non-peptide amide group of alkaloids. It is a

monocarboxylic acid amide, that has one of its hydrogen atoms of the amide group substituted with a 1-hydroxy-2-propyl group in *S*-configuration (***Figure 12***).



50

Figure 12*. Structure of ErgM (**50**)*

The compound is highly insoluble in apolar solvents (hexane, chloroform), but can be dissolved in polar solvents or mixtures, such as water and lower alcohols. It might decompose at higher temperatures, and humidity or by light [135].

1.5.1. Pharmacology

ErgM has its relevancy in the pharmaceutical industry, but due to its instability, more stable synthetic derivatives, like methylErgM and ErgM-maleate are used much more frequently in alkaloid-type medicine. It is used to prevent and treat postpartum bleeding and to stimulate uterine involution in veterinary medicine and humans as well [130, 135]. The compound directly stimulates the muscular tissue of the uterus, increases the strength and frequency of contractions, and also causes cervical contractions.

The regular daily dose *per os* is around 1.5 mg to 1.8 mg. (about 0.03 mg/kilogram of body weight). Oral bioavailability is regularly around 80%, which can be affected by water consumption. When it is applied *per os*, the elimination half-life is quite fast, it only takes about 2 hours. The intravenous application can elongate this half-life to 2.57 hours, and it takes about 32 hours to get it fully eliminated from the body [137].

1.5.2. Determination of ergometrine

Since ErgM is a mycotoxin and registered as a number one drug precursor in the European Union (EU), monitoring the compound in food and feed is crucial, although in the current regulation no limits are established for ergot alkaloids (2006/1881/EC) [140]. Several methods have been proposed for the determination of ErgM, including HPLC-

FLD [141-147], HPLC-MS/MS, [148-150], HPLC-TOF [151], ion trap mass detector (HPLC-ITMS) [152], and HPLC-Orbitrap MS [153] techniques.

The most common technique is fluorescent detection because it is more sensitive and specific than the widely applied HPLC-UV technique. For ErgM a specific pair of wavelengths cannot be found in the literature. 214 nm, 418 nm [154]; 330 nm, 415 nm [155, 156]; 315 nm, 415 nm [157]; 240 nm, 410 nm [158]; 327 nm, and 398 nm [159] were used as excitation and emission wavelengths, respectively.

Separation can be achieved with both isocratic and gradient mobile phases. Generally, alkaline conditions are preferred for the separation of not just ErgM, but most ergot alkaloids. Addition of ammonia, or any ammonium salt (NH_4OH, NH_4HCO_3, $(NH_4)_2CO_3$ or triethylamine) to the eluents are common, to stabilize both the desired alkaloid and its epimer from (in the case of ErgM, it is ergometrinine) [160].

Regarding the mass spectrometric detection of ErgM, the most commonly used technique is the triple quadrupole mass detection with electrospray ionization (ESI), because that is the most suitable for most routine analyses [148-150]. Because of the alkaline conditions, mostly the protonated molecule ion [$M+H^+$] and the ammonium ion adducts [$M+NH_4^+$] are detected [150].

1.5.3. Purification of ergometrine

Regarding the purification of ErgM, there are a few available papers in connection with this topic [160-162].

In the original article of Dudley [160] from 1932, defatted ergot powder was extracted with hot and dilute sulphuric acid, then it was filtered and treated with base. The alkaline solution was then concentrated to a small volume under reduced pressure and alcohol was added. This alcoholic concentrate was then extracted with chloroform twice, and the organic phase was washed with dilute sulphuric acid. After the solution had been rendered to alkaline to ensure its freedom from contamination. This organic phase was evaporated to dryness and a dark resinous residue was obtained in which crystals were observed. Chloroform was cautiously added until a solution with the observed crystals being undissolved was created. The crystals were filtered and washed. From 10 kg of defatted ergot powder, only 820 mg of pure crystals were obtained. Later these crystals were proven as ErgM.

In 1957, a patent was submitted for the preparation of ErgM and the epimer form, ergometrinine. According to the invention, the alkaloid content is dependent on the zinc and iron ions in the nutrient solution. In the patent, alkaloids are both isolated from the dried mycelia and the culture media as well. In both cases in general, the fatty acid constituents were first extracted, which leaves the desired alkaloids undissolved. The residue is then made alkaline with Na_2CO_3. This liberates the alkaloids from their salt form. This residue then was extracted with an acidic solution (tartaric acid) and the aqueous extract was purified by shaking with an organic solvent. Then the entire volume of the aqueous solution was made alkaline by the addition of $NaHCO_3$, and the liberated alkaloids were washed out with an organic solvent. The solution was then concentrated and purified either by silica gel or alumina to obtain pure fractions of ergot alkaloids.
As an example, 260 g of dried mycelium was defatted with 2.6 liters of petroleum ether two times. The defatted material was made into a paste with 260 ml of 5% aqueous Na_2CO_3, then extracted with 1.3 l of petroleum ether twice. Ergot alkaloids were shaken out from the solution with 1.3 l and 0.65 l of 1% tartaric acid solution in water. These combined aqueous phases were then extracted with 300 ml of diethyl ether twice. Combined organic layers were dried over Na_2SO_4 and were evaporated to dryness. This method resulted in 310 mg of crude alkaloid extract. The obtained crude mixture was then purified by chromatography on alumina, using chloroform as the eluent. The separation resulted in 133 mg of ErgM, as polyhedral crystals [161].

In another patent for a procedure to isolate a whole spectrum of ergot alkaloids was submitted in 1980. This is achieved through the addition of 4-5% (by weight) of absorbent clay to the culture media and stirring for about 30 mins. After filtration and drying, this absorbent clay is placed in a lightly alkaline solution of aqueous ammonia and extracted with an apolar organic solvent such as ethyl-acetate or some halogenated hydrocarbon. This organic phase is then treated with a weakly acidic aqueous solution to eliminate the non-basic residue. The aqueous solution containing the total alkaloid content is then made basic (pH 8 – 9.5) and the liberated ergot alkaloids are extracted with an organic solvent, preferably ethyl-acetate. In this patent, there is no information about the yields and the purities of the final product, nor any chromatographic information [162].

1.6. Liquid-liquid chromatography (LLC)

Liquid-liquid chromatography is based on a two-phase solvent system. One phase is immobilized by a strong force field (stationary phase) and the other one (mobile phase) is pumped through it. If several compounds are solved in one or both phases, they can be separated due to their different partition in the two phases.

The basics of liquid-liquid chromatography were stated by Yoichiro Ito, who first constructed an apparatus designed for the differentiation of particles in a suspension or solutes in solution in a solvent system exposed to a centrifugal field [163]. From that point, the development of instruments was led in two directions.

The first way is today known as countercurrent chromatography (CCC), which is a hydrodynamic construction. Countercurrent chromatographs are consisting of coiled tubes that are performing planetary motion and therefore the force field is variable. There is a main axis, which is stabilized, and the rolled-up coil circulates around it (***Figure 13***). Another, more developed, and modern version of this technique is called high-speed countercurrent chromatography (HSCCC). This technique is a widely accepted separation technique, that is used for the separation and purification of various synthetic and natural products [164, 165].

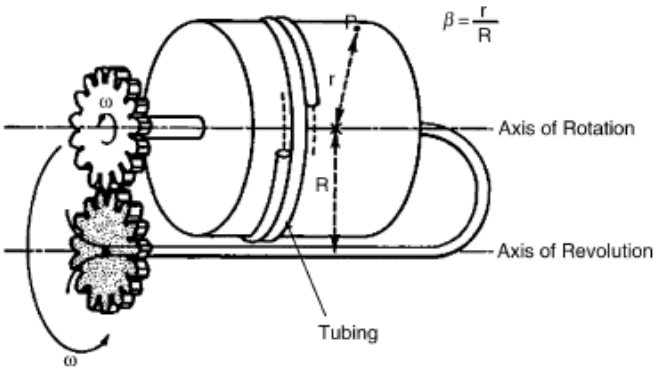


Figure 13. *The basic construction of a CCC instrument [163]*

The hydrostatic version of liquid-liquid chromatography is called centrifugal partition chromatography (CPC). This technique uses a constant gravitational field produced by a single-axis rotation mechanism. The instrument consists of discs, that have partition cells carved into them and these discs rotate around the fixed axis (***Figure 14***).

Figure 14. *Structure of a CPC disc [163]*

1.7. Centrifugal Partition Chromatography (CPC)

Centrifugal partition chromatography (CPC) is an extensively studied technique, but it is less well-known and not as commonly used as solid-liquid chromatography. In CPC, a solvent from a biphasic solvent system is immobilized by a centrifugal force in a stacked and interconnected rotating-discs (the stationary phase), while another phase (the mobile phase) is pumped through this channel and the components injected in a mixture into the mobile phase are eluted from the column according to their partition coefficients [166]. However, for the successful CPC separation firstly the biphasic solvent system should be selected such that the sample components have different partition coefficients in a range of 0.5–2.0 in each phase [167]. CPC further allows rapid and inexpensive method development, higher throughput, higher yields, and reduced costs compared to typical preparative HPLC techniques [168]. Based on these unique advantages and the high resolution offered by CPC, this technique has been applied for the separation of numerous bioactive natural products including sesamin, catharanthine, and vindoline [169-173] as well as certain mycotoxins [174-178].

1.8. Fungal secondary metabolites purified by CPC

Fumonisin B_1 was separated from the crude extract of *Fusarium verticillioides* by CPC in a single 70-min run with a purity above 98% and a total yield of 68% [174]. B-type fumonisins have also been purified by several consecutive separation steps with pentane/*tert*-butyl methyl ether/butan-1-ol/ethanol/1% formic acid in water and pentane/butan-1-ol/ethanol/1% formic acid in water as the solvent systems. As a result, a total of 500 mg of fumonisin B_1, B_2, and B_3 was obtained with a purity above 98% from 1 kg of maize culture [175]. In another study, nivalenol and fusarenon-X were purified by passing a crude acetonitrile extract from *Fusarium graminearum* through silica gel then running two consecutive CPC separations: the first with water as the stationary phase

and butanol as the mobile phase and the second with a chloroform/methanol/water (65/65/40, v/v/v%) solvent system. From 1 kg of pressed barley culture, 340 mg nivalenol and 600 mg fusarenon-X were obtained with yields of 44% and 68%, respectively [176]. Another liquid-liquid chromatographic separation with a 1/1 ethyl-acetate/water solvent system (by volume) was used to purify deoxynivalenol from a rice culture of *F. graminearum*, resulting in 95% purity after several consecutive runs [177]. Similarly, a 7.5/2.5/10 hexane/ethyl-acetate/pH 4 acetic acid solution (by volume) was used as the solvent system to purify the mycotoxin patulin produced by *Penicillium expansum* that was cultivated in sterilized apple juice and apple cider. The compound was purified with a maximum purity of 98.6% and a recovery of 86.2% [178]. In a recent study, fungal anthraquinones were purified by CPC [179]. These fungal pigments, that can treat microbial infections and malignant diseases, were extracted by methanol from the fungal bodies of *Cortinarius sanguineus*. The two-phase system, chloroform/ethyl acetate/methanol/water/acetic acid 3:1:3:2:1 (v/v/v/v/v%) was obtained to fractionate the extract in ascendent mode. For the analytical scale separations, a 55 ml rotor and 1.25 ml/min flow rate were utilized, and 50 mg of crude extract was separated, while scale-up studies of this separation were carried out including a 1000 ml rotor, and a flow rate of 20 ml/min, separating 1.0 g of crude material. Six pigments were separated with good selectivity, and the separation was scaled-up without compromising this achieved selectivity. There is no information about the recovery and the amounts of the gained pigments.

1.9. Scale-up procedures in the liquid-liquid chromatography

Scaling-up in preparative chromatography can be carried out by applying different models for the calculations. One of the models is called the equilibrium dispersive model [180]. According to this model, the chromatograms for larger columns can be predicted by only calculating or determining the absorption isotherms of the compounds on a smaller (analytical) column. Another approach for scaling up a separation is to maximize the loading on the preparative column while maintaining the same resolution [181]. Using this loading maximization method, the separation of chiral isomers of naringenin was tested on a small, analytical CPC column. The column showed great loading behavior and it would produce a maximum of 500 mg of pure products daily. The maximum produced amount was calculated on a bigger column, however, when the optimal conditions were

applied, and runs were carried out with increasing amounts to purify, separation of the chiral isomers resulted in overlapping.

Linear scale-up in LLC, especially in centrifugal partition chromatography (CPC) can be achieved easily. In this chromatographic technique, the volume of the column (V_c) is the factor that is increasing. As the V_c increases, so does the diameter of the column, and the centrifugal force. In order to achieve the same resolution on a larger column the same amount of centrifugal force must be applied. One of the approaches to ensure that the resolution remains the same during the scaling-up procedure is to calculate the centrifugal forces and adjust the rotational speed of the larger column to the smaller one [182]. Sutherland et al. worked on a scale-up of a protein separation. A laboratory-scale separation was developed on a 500 ml CPC instrument with the condition of 10 ml/min flow and 2000 rpm rotor speed, which means 224 *'g'*. Linear scale-up was carried out to a pilot-scale 6.25 l column. Eluent flow, fraction volume, and loading volume were linearly increased (multiplied by 12.5), meanwhile, the rotor speed was decreased to 1293 rpm, to ensure the same 224 *'g'* force field. As a result, 40 g/day throughput was achieved. Another scaling-up approach can be to apply the "fee space between peaks" method [183]. This scale-up procedure is based on the experimentally measured fee-space ΔV between solute peaks within the same liquid-liquid system on a small and a large preparative column as well. Once the separation is developed the maximum loading capacity must be determined on the small column. For the scale-up, one small quantity injection on the larger column is enough to determine the maximum loading capacity, and productivity. Applying this methodology, Bouju et al. scaled up the purification of carnosol [184]. With their calculations, the production of carnosol from 3.1 mg/h on a 35 ml rotor, was increased to 49.9 mg/h on an 812 ml rotor. It was also suggested that the rotational speed of every rotor should be maximized, and the flow rate has to be adjusted accordingly. It was demonstrated that the "free space between peaks" method provides a simple way to predict the maximum load on any CPC rotor.

2. Objectives

This work aimed to determine AF and ErgM production of isolated endophytic fungi and to develop novel liquid-liquid purification methods for the compounds. Furthermore, to carry out effective scale-up of the separations, and also to verify the quality and quantity of the achieved final products.

Regarding AF purification the main objectives are,

- Screening for an AF producer *Aspergillus* strain,
- Large scale cultivation of the selected strain,
- Effective, large-scale extraction of the produced AFs,
- CPC method development for the separation of AFs, possibly to separate them in one run,
- Scale-up of the separation, to achieve maximum yield,
- Verify the quality and purity of the final products.

In the case of ergometrine purification, the main objectives are,

- Screening for an ergometrine producer *Claviceps* strain,
- Large scale cultivation of the selected strain,
- Effective, large-scale extraction of the produced ErgM,
- CPC method development for the separation of ErgM,
- Verify the quality and purity of the final product.

3. Results and discussion

3.1. Purification of aflatoxins

3.1.1. AF production of different Aspergillus species

On 11 different culture media, 10 *Aspergillus* species and 13 isolates were cultivated in order to have their AF and STC production capability and AF and STC content determined. STC content of the cultures was also examined because sterigmatocystin is a precursor in the biosynthesis of AFB_1 and AFG_1 [185], therefore the compound is the focus of our interest as well.

All species were cultivated both on solid and liquid media in three parallels. After the successful cultivation and extraction of the desired mycotoxins, the content of the prepared samples was measured by HPLC-MS/MS technique. All data regarding the produced amounts of AFs and STC is listed in ***Table 11***, in the supplementary information (chapter 9.).

3.1.1.1. AF production on solid media

From the cultivated 13 fungi AF production was observed in 9 cases (***Figure 15***). *A. parasiticus* strains (Szeged Microbiology Collection (SZMC) 2473, SZMC 22727 and SZMC 22728) and *A. nomius* (SZMC 22631) produced all AFs in high amount on most of the utilized solid culture media (≥ 10 mg/kg). *A. flavus* (SZMC 12576), *A. minisclerotigenes* (SZMC 22438), *A. pseudotamarii* (SZMC 2018 and SZMC 3055) strains produced only ABs in high or moderate yields (between 70 mg/kb and 10 mg/kg), and *A. pseudonomius* (SZMC 22273) produced AFB_1 and AFG_1 in low yields (between 0.03 mg/kg and 0.3 mg/kg). All the others (*A. dimorphicus* (SZMC 2024), *A. nidulans* (SZMC 26961), *A. amoenus* (SZMC 20877), and *A. tabacinus* (SZMC 23543)) produced only STC in moderate or low yields (all below 65 mg/kg).

Regarding culture media, it can be concluded that wheat (M4), barley (M5), and rice (M6) are the best for AF production. Cultivation on corn (M1) and maize (M2) resulted in moderate yields, and AF content of cultures on corn cob (M3) was the lowest, or in some cases, no AFs were detected.

Ratios of the produced AFs differ within the three *A. parasiticus* species. While AFBs are produced in higher amounts by SZMC 2473 on all solid culture media, SZMC 22727 and SZMC 22728 produced more AFG_1 on M4 and M5. These differences between the

three fungi might be observed because they are three different isolates. SZMC 2473 was isolated originally in Japan, while SZMC 22727 and SZMC 22728 were isolated from indoor air in Croatia. On the other hand, *A. nidulans* produced AFGs in greater amounts on all solid culture media.

Fungi that produced AFs in high or moderate yields produced STC in negligible amounts. This might be the result of converting most of the existing STC into AFs during biosynthesis.

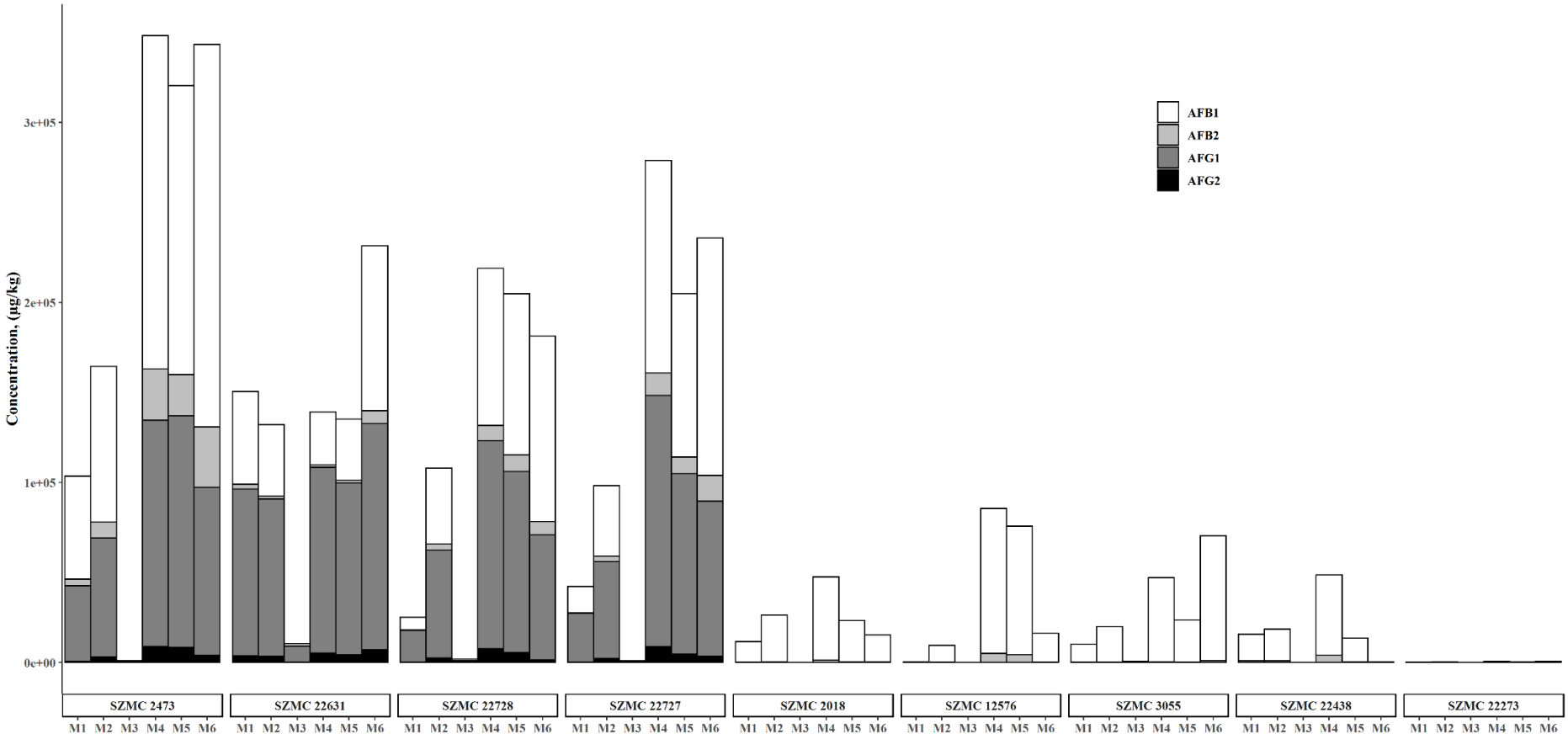


Figure 15. *AF and STC production of SZMC 12576, SZMC 2473, SZMC 22727, SZMC 22728, SZMC 22631, SZMC 22273, SZMC 22438, SZMC 2018 and SZMC 3055 on solid culture media. The values are in µg/kg units.*

3.1.1.2. AF production in liquid media

The same 13 fungi were cultivated on liquid media as well. It can be noticed that the same four organisms (SZMC 2473, SZMC 22727, SZMC 22728, and SZMC 22631) produced all four main AFs in high yields (all above 1600 µg/l), as on solid medium, and the same ones (SZMC 12576, SZMC 22438, SZMC 2018 and SZMC 3055) produced only AFBs in moderate yields as well (between 18 µg/l and 10000 µg/l), and *A. pseudonomius* (SZMC 22273) only produced a small amount of AFG_1 on M11 (30.8 µg/l) (***Figure 16***).

A. parasiticus (SZMC 2473) produced the most AFs in these cases again. The highest amount was produced when the organism was grown on bio vegetable cocktail (M11), lower amounts were produced on potato dextrose broth (M10) and on complex malt broth (M9). It can also be noticed that the ratios between the AFBs and AFGs change when the organism is cultivated on PDB (M10). It is also noticeable, that only two fungi produced all AFs but no STC on synthetic vitamin broth (M8) (*A. parasiticus* (SZMC 2473) and *A. nomius* (SZMC 22631)). Regarding the three *A. parasiticus* species, it can be noted that all of them produced the four main AFs in the same ratios on the same medium, but on the other hand, *A. nomius* produced AFBs in higher amounts on complex malt broth (M9). *A. nomius* also produced more AFGs, together with *A. parasiticus* (SZMC 22728), than the other two examined *A. parasiticus* strains (SZMC 2473 and SZMC 22727).

In conclusion, it can be noted that from the examined fungi *A. parasiticus* (SZMC 2473) produced all AFs in the highest amount, but the other two isolates (SZMC 22727 and SZMC 22728) produced the toxins in relatively high amount, just as *A. nomius* (SZMC 22631) did. From the solid culture media, the best ones for AF production are wheat (M4), barely (M5) and rice (M6), because these ones indicate the production the most. For obtaining AFs from a liquid culture media, bio vegetable cocktail (M11), PDB (M10), and complex malt broth (M9) are the best. For further experiments complex malt broth (M9) was selected for cost-effectiveness, easy availability, and composition uniformity reasons.

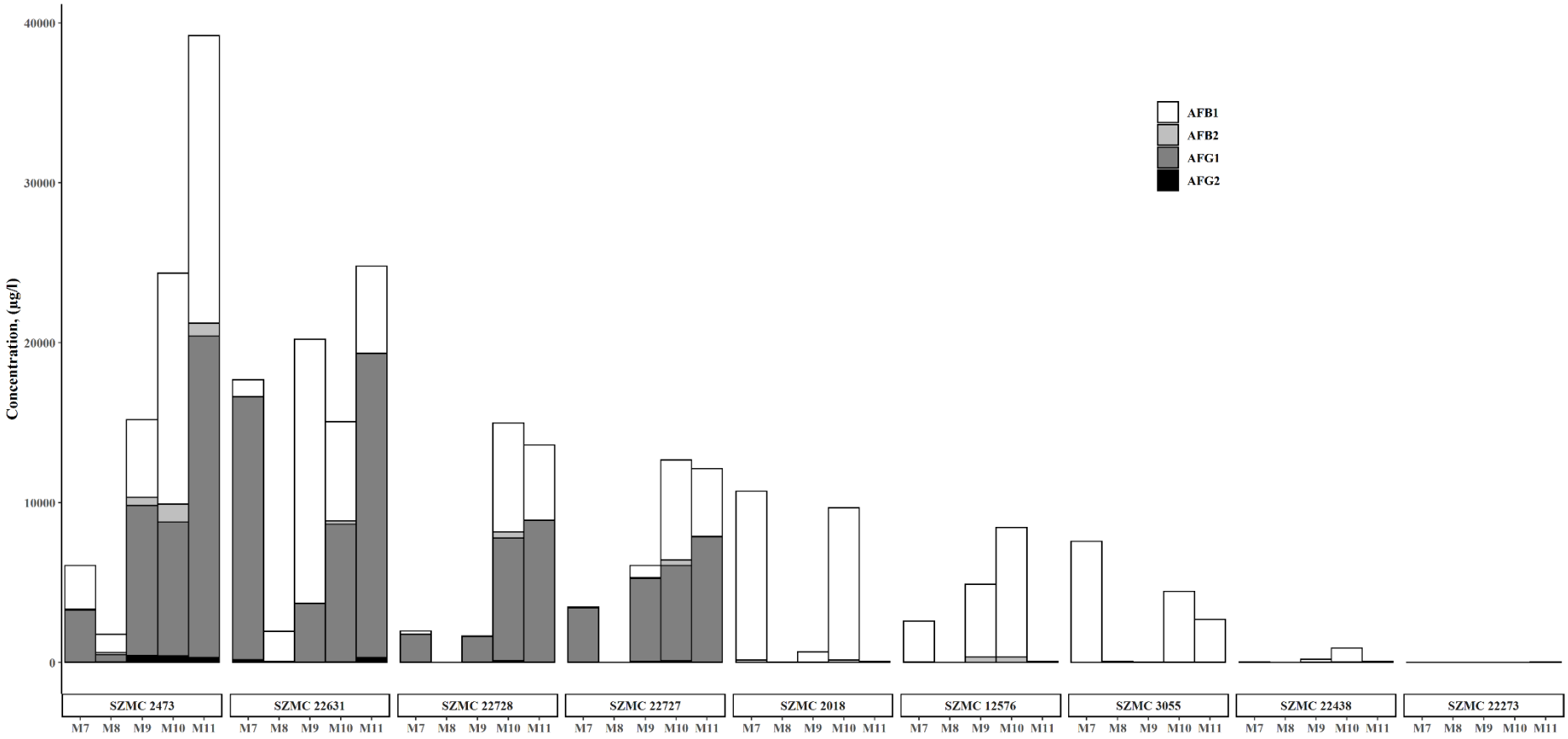


Figure 16. *AF and STC production of SZMC 12576, SZMC 2473, SZMC 22727, SZMC 22728, SZMC 22631, SZMC 22273, SZMC 22438, SZMC 2018 and SZMC 3055 on liquid culture media. The values are in µg/l units.*

3.1.2. Large scale cultivation and extraction

A. parasiticus strain SZMC 2473, an ex-type strain of the species [43], was selected for cultivation because it has been revealed to produce both AFGs and AFBs in high amounts. After seven days of cultivation, the AFs were extracted in a three-step process using firstly dichloromethane, followed by a hexane/methanol/water ternary system [186] to remove the fat and other non-polar components produced by the fungus. At this stage, AFs were distributed into the aqueous phase, which was partitioned again with dichloromethane. HPLC-UV analysis (***Figure 17***) revealed that the *A. parasiticus* produced AFG_1 (47.5%, R_t = 9.564 min) and AFB_1 (42.6%, R_t = 12.560 min) as the major products and AFG_2 (3.0%, R_t = 8.109 min) and AFB_2 (4.2%, R_t = 10.523 min) in smaller quantities; the total impurity content was 2.7% (R_t = 7.255 min and R_t = 7.493 min).

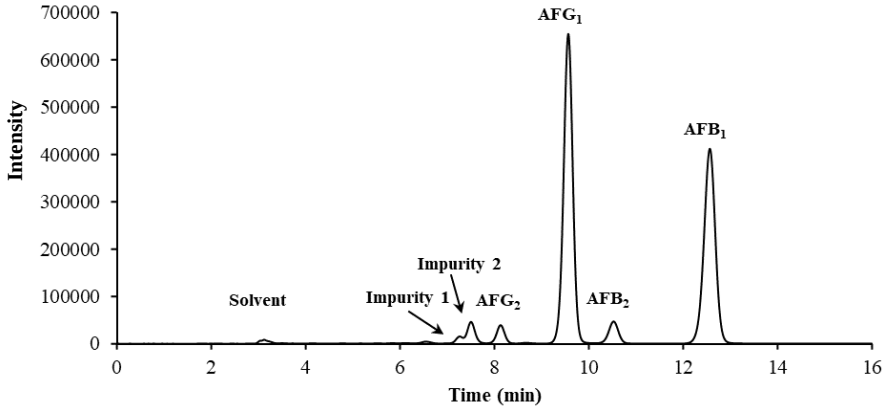


Figure 17. *HPLC-UV chromatogram of the crude extract at λ = 365 nm.*

3.1.3. Selection of solvent systems

For the separation of AFs by liquid-liquid chromatography, several ternary systems, each comprising a "bridge" solvent was used in combination with more polar and less polar partitioning solvents, were created in accordance with the "best solvent" approach [187]. Here, one protic and two aprotic solvents were selected as the best solvents. To form a non-aqueous ternary system, chloroform was used as the best solvent and was paired with hexane and acetonitrile; to form an aqueous ternary system, acetone was used with either hexane and heptane or toluene and water (***Table 3***). Furthermore, the protic best solvent was acetic acid, which was used in combination with diethyl ether, chloroform, and toluene as the non-polar solvent and water as the polar solvent to form a ternary system (***Table 4***). Thus, a total of 63 biphasic systems based on these seven compositions were investigated.

Table 3. *Tested ternary systems and the corresponding P values.*

	Solvent system	Volume ratio	P_{Imp1}	P_{Imp2}	P_{AFG_2}	P_{AFG_1}	P_{AFB_2}	P_{AFB_1}
1	hexane/chloroform /acetonitrile	55/5.5/39.5	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
2		55/3.8/41.2	0.15	0.13	0.14	0.15	0.14	0.15
3		77.7/3.2/19.1	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
4		77.7/5/17.3	0.19	<0.10	<0.10	<0.10	<0.10	<0.10
5		42/9/49	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
6		34/8/58	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
7		55/7/38	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
8		63/10/27	<0.10	<0.10	<0.10	<0.10	0.11	<0.10
9		62/9/29	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
10	hexane/acetone/water	36/39/25	0.14	<0.10	<0.10	<0.10	0.14	<0.10
11		10/50/40	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
12		9/39/52	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
13		15/60/25	0.14	0.13	0.19	0.24	0.26	0.29
14		56/24/20	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
15		66/24/10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
16		50/40/10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
17		30/60/10	<0.10	<0.10	<0.10	0.11	0.13	0.15
18		40/50/10	0.16	0.14	0.23	0.24	0.29	0.32
19		32/63/5	0.13	0.13	0.19	0.21	0.24	0.26
20		23/77/10	0.53	0.61	0.62	0.64	0.66	0.68
21		20/70/10	<0.10	<0.10	0.14	0.14	0.18	0.20
22	heptane/acetone/water	44/29/27	0.17	0.15	0.16	0.19	0.18	0.20
23		8/65/27	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
24		27/57/16	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
25		30/60/10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
26		40/20/40	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
27		20/10/70[a]	-	-	-	-	-	-
28		55/25/20	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
29		80.5/9.5/10	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
30		23/57/20	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
31		22/38/40	<0.10	<0.10	<0.10	<0.10	<0.10	<0.10
32	toluene/acetone/water	39/26/35	1.81	2.98	5.77	9.80	11.28	22.67
33		14/26/60	2.16	3.42	6.26	9.11	11.58	18.53
34		10/40/50	8.33	38.64	6.46	4.11	2.66	58.49
35		14/51/35	6.59	3.26	2.80	3.58	3.39	5.14
36		29/55/16	1.39	1.83	2.86	3.86	5.00	5.03
37		30/10/60	0.87	2.04	4.21	9.03	22.75	19.06
38		6/74/20[b]	-	-	-	-	-	-
39		18/12/70	0.86	1.72	2.27	6.16	13.11	12.32
40		12/67/21	1.21	1.26	1.40	1.51	1.53	1.60
41		17/67/16	1.08	1.11	1.16	1.21	1.25	1.25
42		8/67/25	1.21	1.26	1.43	1.63	1.67	1.75
43		70/10/20	0.99	1.78	2.90	5.38	9.92	9.42
44		35/55/10	1.36	1.64	2.46	3.24	4.13	4.00
45		15/55/30	1.38	1.58	2.19	2.83	2.84	3.36
46		6/55/39	1.40	1.62	2.20	2.84	3.02	3.41

[a]Could not dissolve the sample as it was too polar.
[b]Did not form a biphasic system

3.1.3.1. Chloroform and acetone as best solvents

The results with the ternary systems containing chloroform as the best solvent showed that the P value was below 0.1 in most systems, indicating that the examined components remained mainly in the lower, chloroform-rich phase (***Table 3***). The two-phase region in the ternary diagram shows that it exists only in a narrow range of chloroform contents (1–12%); therefore, these tested ternary systems covered almost the entire two-phase region of the system [166]. Hence, it can be concluded that none of the examined components could have been transferred to the upper phase; therefore, this ternary system cannot be applied for the CPC separation of AFs.

The application of the next set of ternary systems based on acetone led to similar observations. In the systems containing hexane and heptane, most P values were below 0.1, indicating that the components remained in the aqueous (lower) phase (***Table 3***). Examining the hexane/acetone/water ternary systems, in certain cases, the P values were closer to one, indicating the successful partitioning of the AFs.

Because none of the previous two ternary systems based on chloroform or acetone fulfilled the requirements of the CPC application, an organic phase with higher polarity was needed, leading to the use of toluene. Using toluene as the third solvent the P values were higher than one (***Table 3***), indicating that the AFs were mainly concentrated in the upper phase. In the biphasic systems containing more water than toluene, the AFs and the impurities were all transferred completely into the upper phase (***Table 3***). Furthermore, in the ternary system containing around 65% acetone, the P values varied within a narrow range around one and stagnated around it (***Table 3***); this was likely due to the shallower slopes of the tie lines in this ternary system [176]. When the amount of toluene was 70%, the AFs are concentrated mostly in the upper phase and the P values were within the acceptable range (0.9–9.9).

Furthermore, based on the quotients (α values) of the ordered P values, the system composed of 70/10/20 toluene/acetone/water was able to separate the impurities from the AFs. Furthermore, using this system, AFG_1 and AFG_2 could be purified separately although the coelution of AFB_1 and AFB_2 could be expected during the CPC separation due to low α value (1.05) between these components (***Figure 18***).

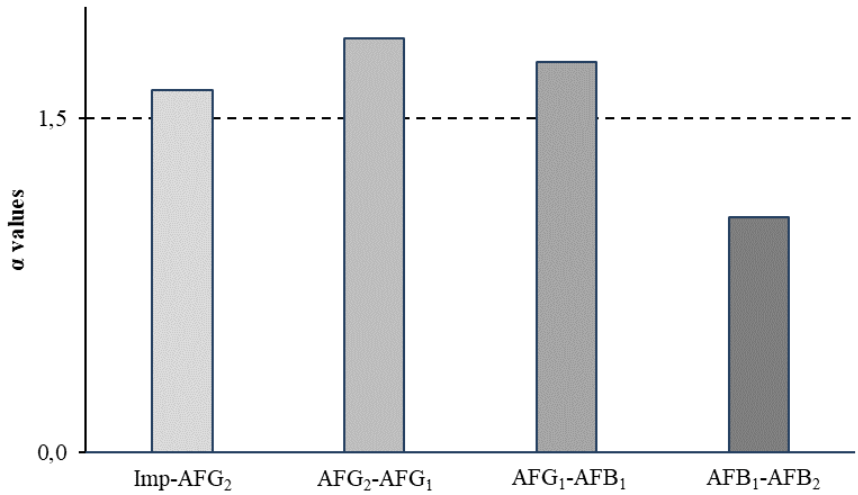


Figure 18. *Separation factors of the components partitioned in the ternary system of 70/10/20 toluene/acetone/water. Imp/ impurity; AFG_1, AFG_2, AFB_1 and AFB_2/ Aflatoxins G_1, G_2, B_1, B_2.*

3.1.3.2. Acetic acid as the best solvent

Acetic acid was also tested as the best solvent because it has been shown to dissolve the components very well [188, 189]. In addition to the acetic acid and water, diethyl ether, chloroform, and toluene were tested as the non-polar component (***Table 4***).

Of the diethyl-ether/acetic acid/water systems, those with compositions of 30/10/60 and 45/15/40 were associated with low P values for the AFs. The P value increased when the diethyl ether/water ratio was increased (e.g. to diethyl ether contents of 60% and 75%), but the calculated α values remained below 1.5. The above-mentioned four tested compositions covered the full range of acetic acid percentages of the biphasic part of the ternary diagram [166] and due to the inappropriate partitions of the components obtained with diethyl ether, the non-polar component of the system was changed to chloroform. In these systems, the partition of the components shifted toward the lower (non-polar) phase with low P values with low acetic acid concentrations (***Table 4***). As the tie lines of these ternary systems lean to the left, increasing the volumetric ratio of acetic acid is expected to cause its concentration in the polar phase to increase proportionally. Therefore, the acetic acid concentration was increased, causing the components to shift into the upper (polar) phase (***Table 4***) while maintaining α values under the acceptable limit for most component pairs. With the system containing 45% acetic acid, the components should theoretically (based on α values) be eluted in three groups: AFB_2, AFG_1+AFG_2, and Imp_1+Imp_2+AFB_1.

Table 4. *Tested ternary systems with acetic acid as the best solvent and the corresponding P values.*

	Solvent system	Volume ratio	P_{Imp1}	P_{Imp2}	P_{AFG_2}	P_{AFG_1}	P_{AFB_2}	P_{AFB_1}
1	diethyl ether/acetic acid/water	75/5/20	0.70	0.86	1.67	1.75	3.40	3.43
2		60/20/20	0.26	0.32	0.46	0.53	0.66	4.00
3		30/10/60	0.10	0.11	0.21	0.22	0.25	0.43
4		45/15/40	<0.10	0.15	0.19	0.20	0.33	0.41
5	chloroform/acetic acid/water	26/24/50	0.42	0.37	<0.10	<0.10	<0.10	<0.10
6		36/34/30	0.47	0.93	0.18	0.16	0.11	<0.10
7		35/45/20	1.03	1.15	0.58	0.57	0.29	0.84
8	toluene/acetic acid/water	80/6/14	<0.10	<0.10	0.31	0.62	1.56	1.88
9		20/10/70	0.14	0.43	0.73	1.70	3.18	4.57
10		30/10/60	<0.10	0.4	0.63	1.36	1.73	3.33
11		20/20/60	<0.10	0.3	0.3	0.63	0.94	2.65
12		**30/24/50**	**0.04**	**0.14**	**0.18**	**0.36**	**0.54**	**1.21**
13		20/30/50	<0.10	0.12	0.16	0.28	0.56	0.92
14		40/30/30	<0.10	<0.10	<0.10	0.16	0.42	0.47
15		63/30/7	<0.10	<0.10	<0.10	0.10	0.23	0.20
16		40/42/18	<0.10	0.11	0.11	<0.10	0.23	0.22
17		20/55/25	<0.10	<0.10	<0.10	<0.10	0.31	0.21

When the non-polar solvent was changed to toluene, the ratio of ascending P values changed significantly (***Table 4***), indicating that the components tended to remain in the upper phase in these systems. The α values corresponding to these systems are shown in ***Figure 20***. Based on these calculations, the system of 20/10/70 toluene/acetic acid/water (***Table 4***, system 9.) could separate the AFs and the impurities, but the range of the P values was remarkably wide, necessitating a very long chromatographic run. When the toluene/acetic acid/water ratios were 20/20/60 (***Table 4***, system 11.) and 20/30/50 (***Table 4***, system 13.), the α values between the impurities and AFG_2 were low; similarly, when this ratio was 40/30/30 (***Table 4***, system 14.), the α value between AFB_1 and AFB_2 was not high enough (***Table 4***, ***Figure 19***). However, when the **toluene/acetic acid/water** ratio was **30/24/50** (***Table 4***, system 12.), the P values were in the most suitable range (0.04 to 1.82), allowing for a CPC run that can be completed within an acceptable time and the α values were appropriate (above 1.5 for all AFs), indicating that perfect resolution can be expected (***Table 4***, ***Figure 19***). Therefore, this system was selected as the optimal system for the final liquid-liquid chromatographic separation.

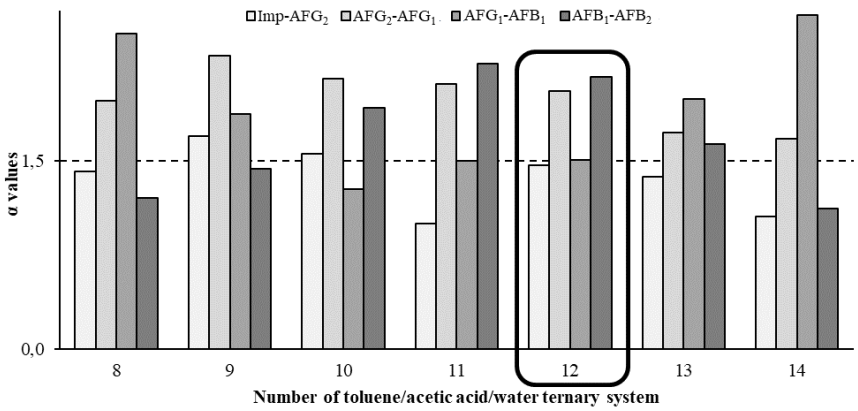


Figure 19. *Separation factors in the toluene/acetic acid/water ternary system.*

3.1.4. Optimization of the CPC method

To determine the mode (ascendant or descendant) for the liquid-liquid separation, the elution volume (V_e) was calculated in each mode, while using the optimal theoretical mobile phase/stationary phase ratio (20/80 by volume). The retention volumes for AFB_1, AFB_2, AFG_1, and AFG_2 were predicted as 159, 304, 426, and 842 ml, respectively in ascendant mode and 1605, 1045, 560, and 243 ml, respectively, in descendant mode. Hence, the lower phase was used as the stationary phase and the upper phase was used as the mobile phase in subsequent experiments, leading to a normal-phase-like separation.

To set up the instrument for the separation, the column was filled with the stationary phase at a rotor speed of 500 rpm and a flow rate of 50 ml/min. Then, the mobile phase was pumped through the system at a flow rate of 10 ml/min with an initial rotor speed of 2200 rpm, which was decreased gradually according to the observed pressure, which should be below 100 bar. Only 10 ml of the stationary phase was extruded (4/96 by volume) when the rotor speed was decreased to 2000 rpm and approximately 25 ml was extruded after reaching a stable pressure when the rotor speed was decreased further to 1800 rpm. Hence, these parameters were subsequently used for the first chromatographic run.

However, this separation process proved to be considerably longer due to the high retention of AFG_2, which was only eluted from the column after 120 min. To shorten the retention time of this slowly eluted component, the flow rate was increased to 15 ml/min in the final method. The pressure of this system was only 46 bar, which is far below the maximum limit (100 bar) and the extruded volume of the stationary phase increased to 60 ml (24 v%). 90 mg of same dried AF extract, as used for the solvent system selection, was dissolved in 4 ml of each the upper and lower phases and injected into the instrument;

the sample was dissolved well to preserve the integrity of the liquid-liquid system. The pressure was stable throughout the run, indicating the stability of the selected biphasic system. Using this method, the four AFs and the impurities were separated successfully, the AFs were eluted completely from the column while the impurities were retained in the system during the 75 min run time and, thus, were not included in the fractogram (***Figure 20A***).

After confirming that the separation was successful, the stability and repeatability of the system were evaluated by repeating the same separation process three times with fresh eluents for both the stationary and mobile phases and the same amount of crude AF sample (90 mg). The fractograms of the three consecutive runs (***Figure 20B***) show that the composition of the collected fractions during the separation are fit to each other and the relative standard deviations of the AF contents in corresponding fractions were under 10%. Thus, it was concluded that the proposed separation process is repeatable.

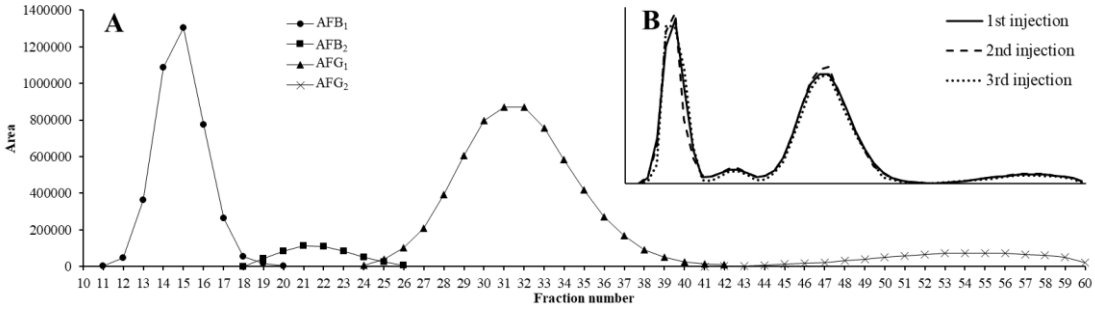


Figure 20. *Fractograms of (**A**) the optimized CPC run and (**B**) repeated CPC runs.*

60 fractions were collected in total during each run, beginning immediately after the sample injection and proceeding until the 9 ml was eluted. The compositions of the fractions were evaluated by HPLC-UV (***Figure 21***); the results showed that most fractions had purities above 90%. Fractions 11–17 were pooled to obtain the AFB_1 sample (24 mg), fractions 21-23 were pooled for the AFB_2 (2 mg), fractions 27-40 were pooled for the AFG_1 (49 mg), and fractions 44-59 were pooled for AFG_2 (6 mg). The rest of the fractions containing mixtures of more than one AF were then pooled, neutralized, and evaporated to dryness for further analyses and purification, such as AFB_2-rich fractions 18-20 and 24-26 contained 3 mg AFB_2 as well as 2 mg AFB_1 and 1 mg AFG_1.

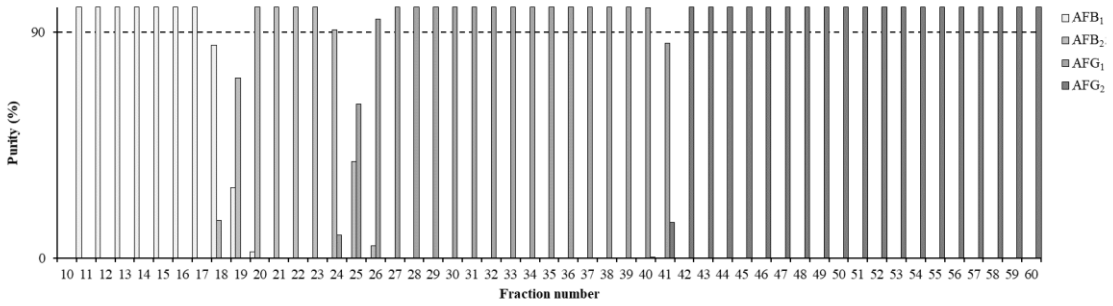


Figure 21. *Composition and purity (%) of the fractions obtained in the optimized CPC run.*

3.1.5. HPLC-UV and OHRMS to verify identity and determine purity

To verify the structures of the purified compounds, each sample was injected into an HPLC-UV and an Orbitrap-High Resolution Mass Spectrometry (OHRMS) system containing both quadrupole and orbitrap analyzers via flow injection. According to the HPLC-UV chromatograms (***Figure 22A***, ***D***, ***G***, and ***J***), none of the separated AFs contained major impurities and the retention times for all AFs were consistent with those of the reference standards. The adduct ions (the $[M+H]^+$ and $[M+Na]^+$ in all samples and $[2M+Na]^+$ in AFB_1 and AFG_1) were detected in the full-scan measurements (***Figure 22B***, ***E***, ***H***, and ***K***). Further, after the fragmentation of $[M+H]^+$ as a precursor ion, the three most intense fragment ions were selected to confirm the identities in the MS^2 evaluations (***Figure 22C***, ***F***, ***I***, and ***L***).

The MS and MS^2 spectra of the purified AFs were compared with those of the corresponding AF standards. The results, which are summarized in Table 6, show that the measured mass values were consistent with the *m/z* values of the reference compounds with a maximum deviation of only 2.55 ppm. Furthermore, the ratios between the fragment ions were only slightly different (differences were within the range of 0–5%) compared with the standards as well (***Table 5***).

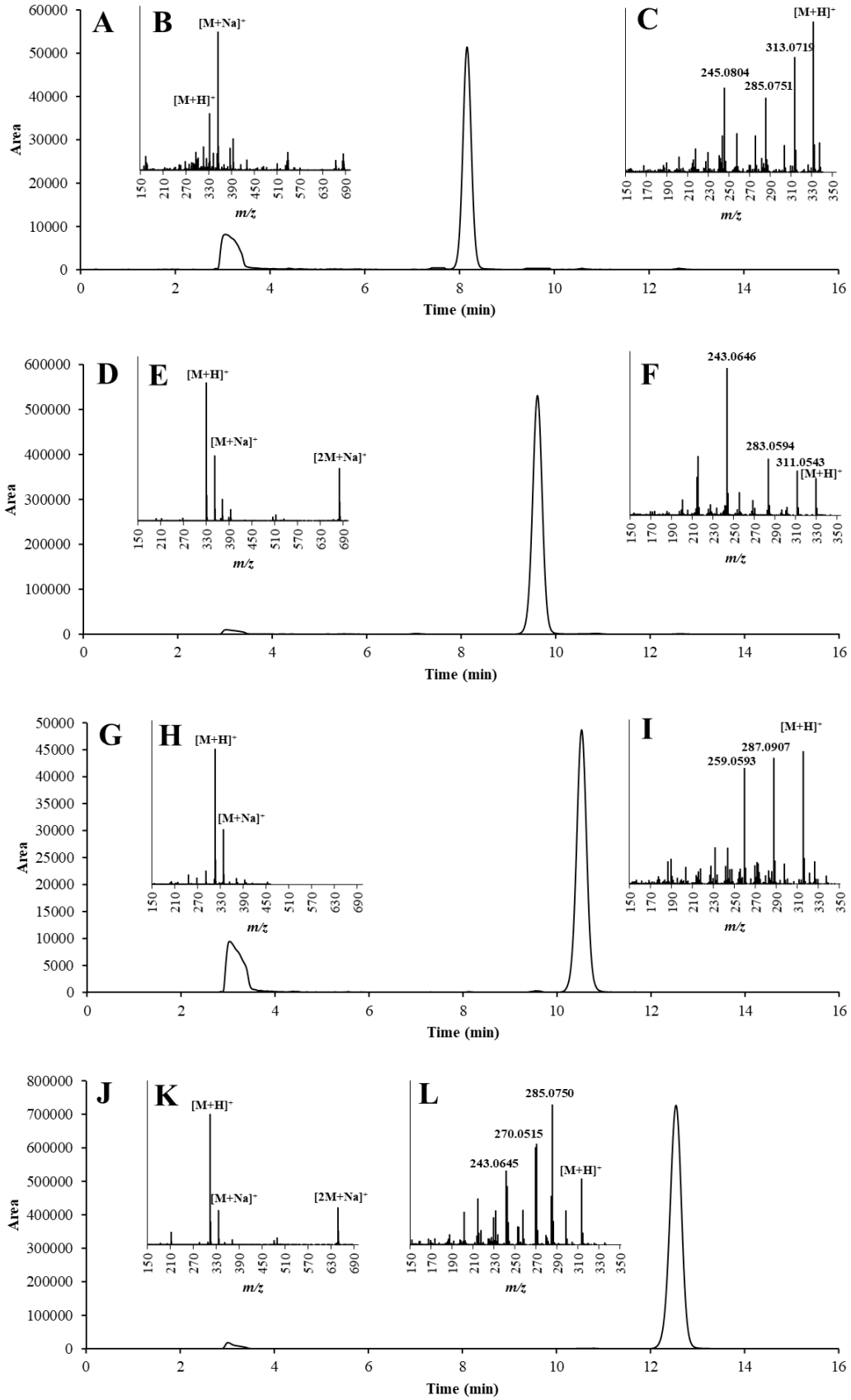


Figure 22. *HPLC-UV chromatograms of the purified AFG_2 (**A**), AFG_1 (**D**), AFB_2 (**G**), and AFB_1 (**J**); MS spectra of the purified AFG_2 (**B**), AFG_1 (**E**), AFB_2 (**H**), and AFB_1 (**K**); and MS^2 spectra of the $[M+H]^+$ adduct ions of the purified AFG_2 (**C**), AFG_1 (**F**), AFB_2 (**I**) and AFB_1 (**L**).*

Table 5. *Comparisons of the masses and intensity ratios of the detected ion forms with those of the reference standards via full-scan and MS^2 high-resolution mass spectrometry to confirm the identities of the AFs.*

		[a]Full scan			[b]MS^2		
	[c]Ref. (*m/z*)	313.0699	335.0504	647.1142	285.0750	270.0515	243.0645
AFB_1	[d]Mass dev. (ppm)	1.25	1.56	2.02	1.99	1.04	2.30
	[e]Ratio dev. (%)	0	0	0	5	3	1
	[c]Ref. (*m/z*)	315.0849	337.0669	-	287.0907	259.0593	-
AFB_2	[d]Mass dev. (ppm)	0.99	1.18	-	1.37	1.18	-
	[e]Ratio dev. (%)	0	0	-	1	2	-
	[c]Ref. (*m/z*)	329.0646	351.0464	679.1039	311.0543	283.0594	243.0646
AFG_1	[d]Mass dev. (ppm)	1.43	1.26	2.54	2.57	1.79	2.01
	[e]Ratio dev. (%)	0	0	0	1	3	4
	[c]Ref. (*m/z*)	331.0811	353.0631	-	313.0719	285.0751	245.0804
AFG_2	[d]Mass dev. (ppm)	1.17	1.66	-	2.03	1.98	2.13
	[e]Ratio dev. (%)	0	0	-	0	1	1

[a]The full-scan *m/z* values are consistent with the molecular ion adducts including $[M+H]^+$, $[M+Na]^+$ and $[2M+Na]^+$.

[b]The presented product ions originated from the fragmentation of the protonated molecular ions.

[c]The *m/z* values of the reference standards.

[d]Mass deviation of the purified compounds from the corresponding ion reference standards determined with the high-resolution mass spectrometer.

[e]Differences between the ratios of the formed ion intensities measured in the reference standards and the purified components.

To test the purity of the separated and fractionated AFs, MS was also applied due to its high sensitivity. Hence, even considering the presence of the $[M+H]^+$ molecular ions of AFs other than the purified one, the *m/z* values remained under 0.05% in all cases.

3.1.6. The yield of the entire purification procedure

From the 4.5 l of liquid culture of *A. parasiticus* SZMC 2473 strain, a total of 1351 mg of AFs was obtained by the proposed CPC method including 442 mg of AFB_1, 43 mg of AFB_2, 817 mg of AFG_1, and 100 mg of AFG_2 with purities of 98.2%, 96.3%, 98.1%, and 97.0%, respectively (Table 4). At these yields, the recovery rates from the entire procedure were 90.5%, 85.3%, 98.7%, and 96.0% for AFB_1, AFB_2, AFG_1, and AFG_2, respectively, based on the original concentrations in the crude extracts as measured by HPLC-UV. Furthermore, the overall AF recovery rate was 92.6% (***Table 6***), indicating that the applied extraction steps did not cause any remarkable loss of AFs.

Table 6. *Purification efficiencies of the AFs from A. parasiticus liquid culture in each separation step.*

		AFB_1	**AFB_2**	**AFG_1**	**AFG_2**	**Total AFs**
Crude extract (culture medium → dichloromethane)	Yield (mg)	442	40	827	105	1414
	Purity (%)	39	4.0	45.4	2.9	91.3
Second extract (dichloromethane → hexane/methanol/water)	Yield (mg)	442	40	827	105	1414
	Purity (%)	41.7	4.0	46.9	3.0	95.6
	Recovery (%)	100	100	100	100	100
Third extract (hexane/methanol/water → dichloromethane)	Yield (mg)	442	40	827	105	14141
	Purity (%)	42.6	4.2	47.5	3.0	97.3
	Recovery (%)	100	100	100	100	100
CPC separation (final product) (30/24/50 toluene/acetic acid/water)	Yield (mg)	400	34	817	100	1351
	Purity (%)	98.2	96.3	98.1	97.0	97.3
	Recovery (%)	90.5	85.3	98.7	96.0	92.6
Whole procedure	Recovery (%)	90.5	85.3	98.7	96.0	**92.6**

Note/ AFB_1, AFB_2, AFG_1, and AFG_2/ aflatoxins B_1, B_2, G_1, and G_2; Total AF/ sum of the amounts of the four detected AFs; CPC/ centrifugal partition chromatography.

3.1.7. Determining the maximum loading capacity on a 250 ml rotor

To improve the capacity of the separation, initially, the maximum load of the 250 ml column was determined using the available 10 ml sample loop of the instrument. For this purpose, increasing amounts of the crude AF extract (90 mg, 180 mg, 210 mg, 240 mg, and 270 mg) were injected into the column to be separated by the optimal conditions. In each case, the dried extracts were prepared in 10 ml mixture of upper and lower phases (1/1, v/v%) of the ternary system. However, while the extracts were soluble at the lower concentrations, the extract could not have been dissolved completely at the highest applied amount (270 mg). Therefore, the fine stepwise tune of the applicable extract amount was carried out, in 10 mg increments within the 240 mg - 270 mg range, where the maximum solubility was determined to be 250 mg of crude AF mixture (25 mg/ml).

Based on the resulted fractograms of each separation (***Figure 23***), it can be concluded that the resolution of the system remained stable and no overlapping of the neighboring peaks was noticed despite of the increasing loading concentrations. Therefore, the available highest loading capacity in the applied biphasic system with the system provided 10 ml loop injection is a maximum of 250 mg (25mg/ml).

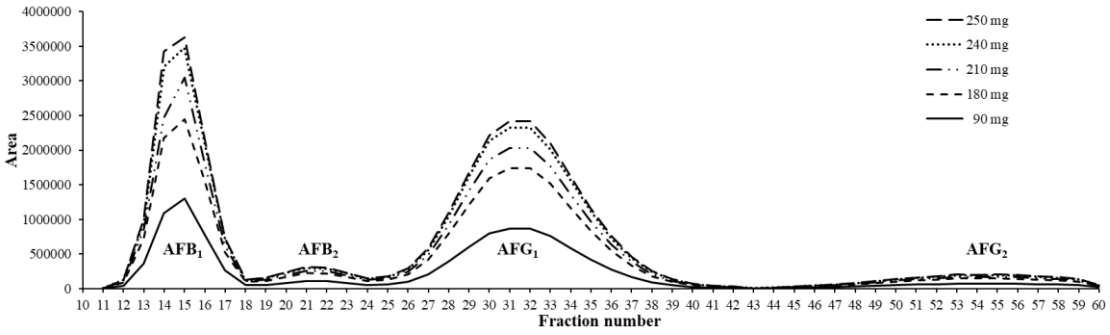


Figure 23. *Fractograms of the CPC separations with increasing sample concentration injected with 10 ml loop on a 250 ml rotor.*

Regarding the collected AFs of each run, the summarized areas of the collected AFs were represented on ***Figure 24***. It can be concluded, that as the solvent system gets more saturated with the compounds, the collected amounts are bending into a saturation curve (***Figure 24***). This also confirms, that with this injection method, the separation cannot be more enhanced in this way, the system is at its' maximum capacity.

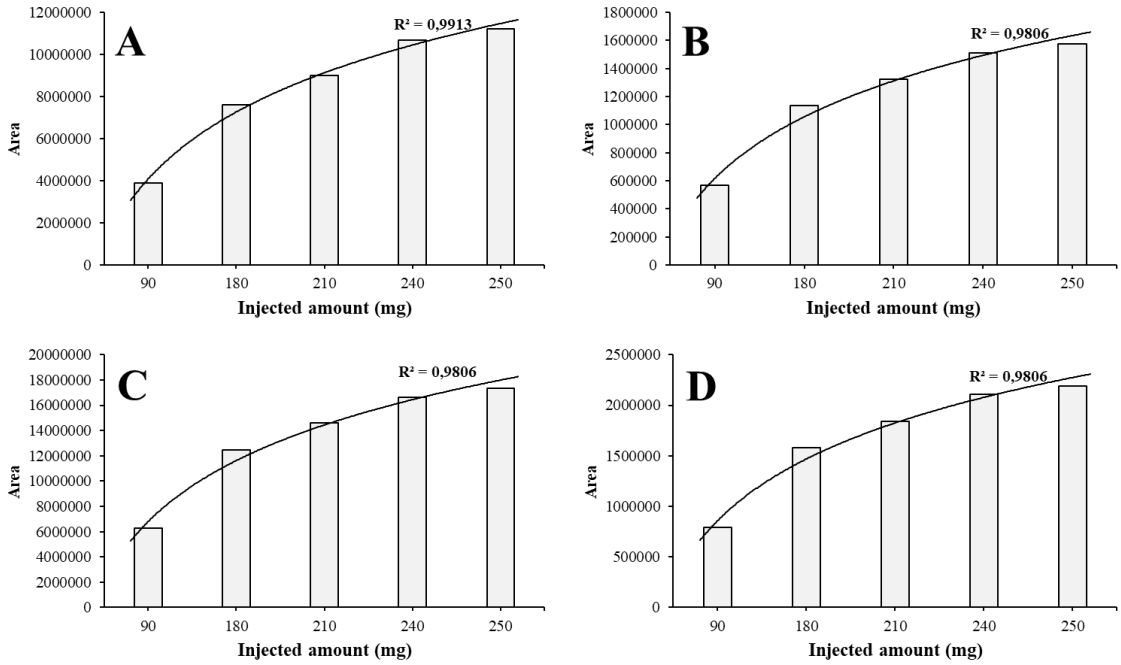


Figure 24. *Area of collected AFB_1 (**A**), AFB_2 (**B**), AFG_1 (**C**), and AFG_2 (**D**) at different injected amounts on a 250 ml rotor.*

After the maximum solubility was determined, the next step was to determine the maximum loading volume with the highest concentration to improve the capacity of the whole purification system. Injections in increments of 5 ml were carried out via the built-up pump of the system from 10 ml until 10% of V_c (25 ml), which is the usually applied value as the maximum loading volume onto a column in the case of liquid-liquid

chromatography [190]. Based on these volumes, 250 mg, 375 mg, 500 mg, and 625 mg of the crude mixture were injected into the 250 ml column, respectively. According to the resulting fractograms of the CPC runs, it can be concluded that with increasing injection volumes the resolution remained nearly the same, and the peaks were not broadened dramatically (***Figure 25***). It can also be seen that the system was stable, and the retentions of the compounds were not shifted. These phenomena and the application of the pump injection allow both the sequential application of the CPC runs and the collections of each AF into the same tubes within the consecutive separations. This sequence of the CPC separations can be set in the control software providing fully automatized continuous purification of the AFs.

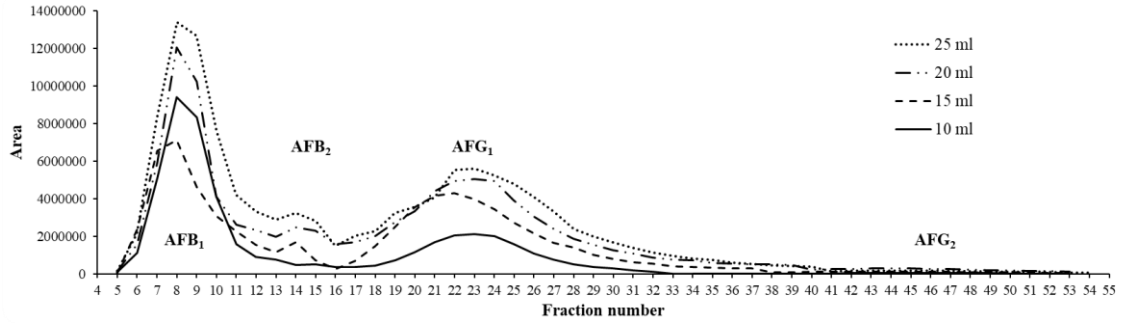


Figure 25. *Fractogram of the CPC separations with increasing injection volumes on a 250 ml rotor.*

From all injections above fractions that contained one AF with 95% of purity or more were pooled. Fractions 5-11; 13-16; 17-31 and 40-54 were combined for AFB_1, AFB_2, AFG_1, and AFG_2, respectively during each separation.

The pure (≥95%) content was summarized for each AF in each injection (***Figure 26***). It can be concluded, that with increasing injection volumes the amount of pure material increases linearly, except in the case of AFG_2 (***Figure 26D.***). When 15 ml of AF solution was injected, AFB_1 resulted in a lower amount than expected, because it coeluted with AFB2. This happened only once, and never in further experiments.

This series of experiments determine that if the injection volume could be greater, a larger amount of pure AFs could be gained. Since the maximum injection volume on a column is 10% of the V_c and 25 ml of the most concentrated possible crude extract was injected to a 250 ml column, the system was utilized most advantageously.

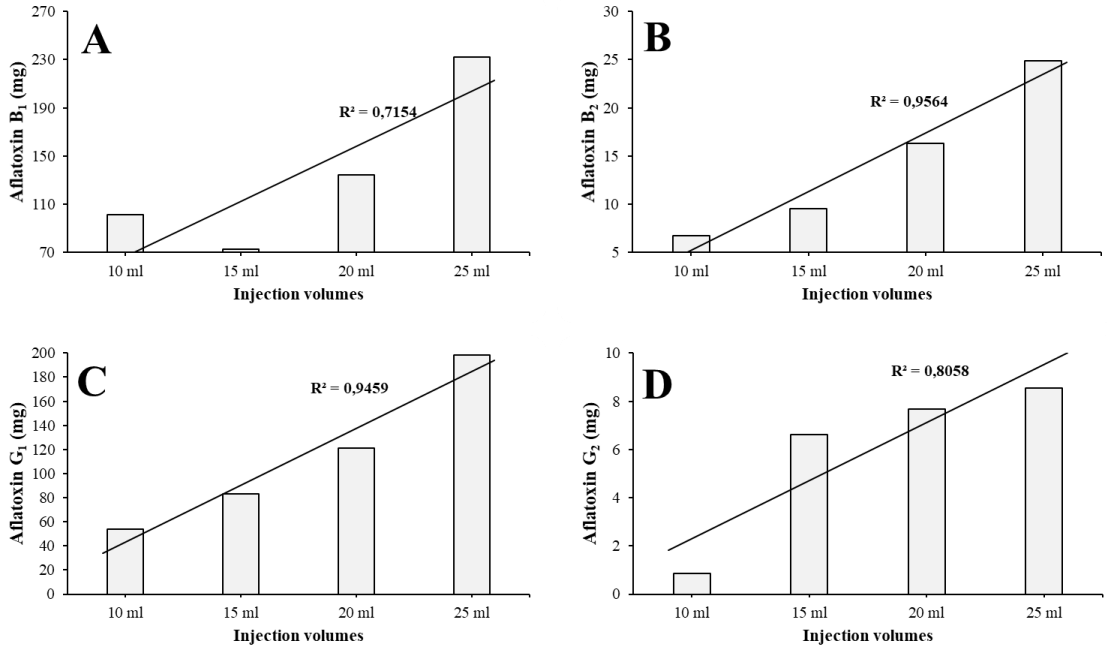


Figure 26. *Pure (≥95%) AFB_1 (**A**), AFB_2 (**B**), AFG_1 (**C**), and AFG_2 (**D**) content of each injection on a 250 ml column.*

3.1.8. Linear scale-up of the purification to a 1000 ml column

As the maximum loading capacity was reached on the 250 ml column including both the concentration and volume, the next step of our work was to scale up the entire separation to the 1000 ml rotor. During the linear scaling-up procedure, the main idea was based on the ratio of the rotor volumes to quadruplicate the injected amount and the flow rate. However, in order to keep the same resolution, the centrifugal force field had to be the same on the larger column as it was on the smaller one before. During the separation on the 250 ml column, the rotation was 1800 rpm, which creates a force field of 435 *'g'*. This centrifugal force field can be created by setting the rotation speed to 1267 rpm on the 1000 ml column.

Determination of the maximum or optimal load was carried out in the same way as it was achieved on the 250 ml rotor. Injections via pump utilizing the solution of 25 mg/ml crude extract were performed in 20 ml increments from 4% of V_c (40 ml) to 10% of V_c (100 ml), thus the injected amounts of crude AF extracts were 1 g, 1.5 g, 2 g, and 2.5 g, respectively. Furthermore, based on the criterion of the linear scale-up [191], the flow rate was quadruplicated from 15 ml/min to 60 ml/min. The extruded stationary phase was 250 ± 5 ml regarding all injections (25% of V_c), which is similar to the V_m on the smaller rotor. In each run, 90 fractions were collected.

With the application of the described linear scale-up approach, the CPC runs could be also performed successfully on the higher volume column, where the separation possessed similar parameters than it was observed in the case of 250 ml rotor (***Figure 27***). Furthermore, during the series of injections of the crude extracts in incrementing amounts, the system remained stable despite the increasing volumes of the injections (***Figure 27, 28***).

As this technique relies on a basic liquid-liquid extraction method, it can be expected that the resolution will stagnate, the following peaks will not overlap, because the distribution of each compound will not change despite the increasing volume or concentration. This phenomenon can be changed in one case if the phases become oversaturated with the compounds. Since solubility was first determined, this problem can be ignored, as it is demonstrated on ***Figure 28***.

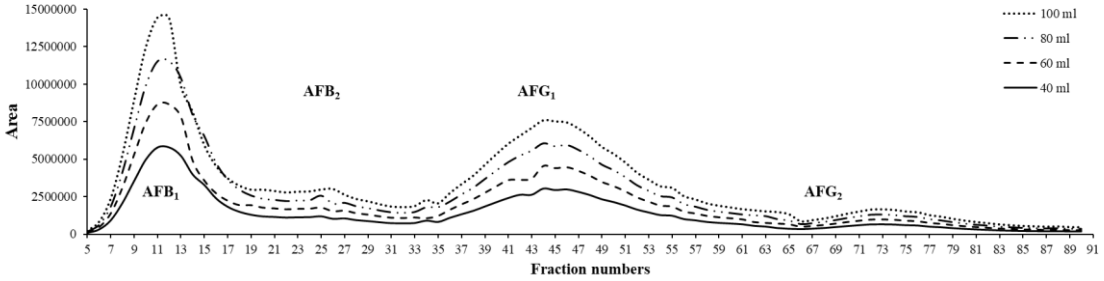


Figure 27. *Fractogram of injections with increasing injection volumes on a 1000 ml rotor.*

The fractions containing pure (≥95%) AFs were worked up and the pure AF content of each run and each compound was determined (***Figure 28***). Fractions 5-18; 20-32; 33-65 and 67-90 were combined for total AFB_1, AFB_2, AFG_1, and AFG_2, respectively at the end of each run. It can be concluded that the gained pure AF amount increases linearly as it was observed on the 250 ml column in all cases (**Figure 26**). This phenomenon could be caused by the lack of solubility of the crude extract in the two-phase system. If more analyte could be dissolved in one ml of the solutes, more material could be injected into the system, therefore more AFs can be prepared.

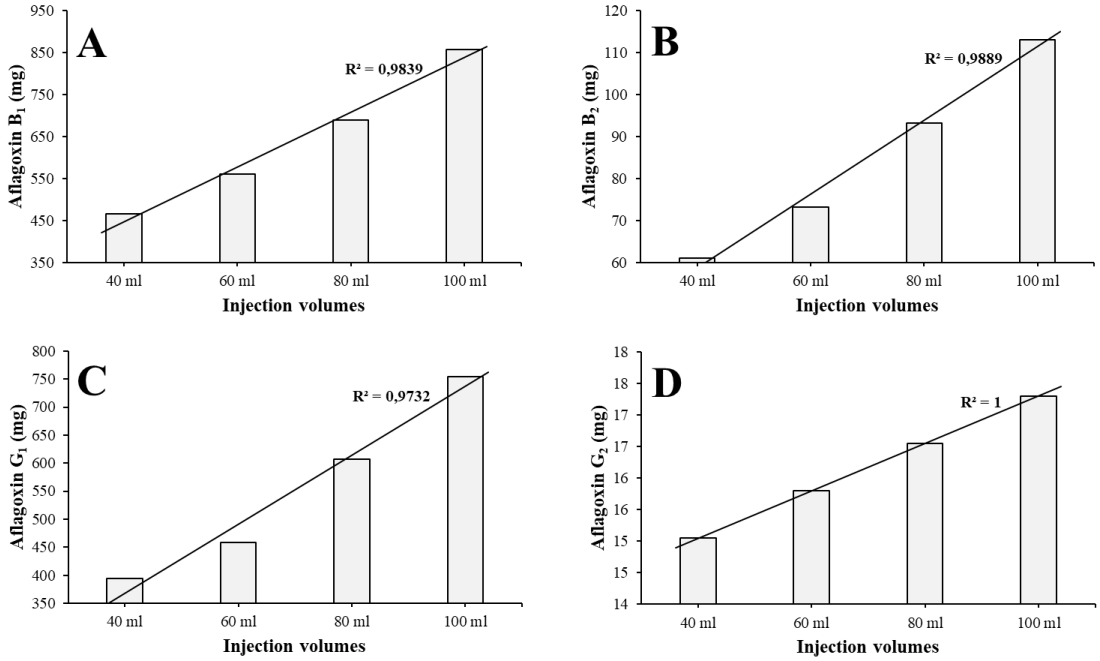


Figure 28. *Pure (≥95%) AFB_1 (**A**), AFB_2 (**B**), AFG_1 (**C**), and AFG_2 (**D**) content of each injection on a 1000 ml column.*

3.1.9. Product purity by HPLC-OHRMS

After each run, the combined pure fractions were analyzed by HPLC-OHRMS technique. Purities of the resulted compounds were calculated from the HPLC-UV spectra and were confirmed by high-resolution mass spectrometry. The total ion chromatograms (TIC) and mass spectra of the four purified AFs are shown on ***Figure 29*** and ***Figure 30,*** respectively.

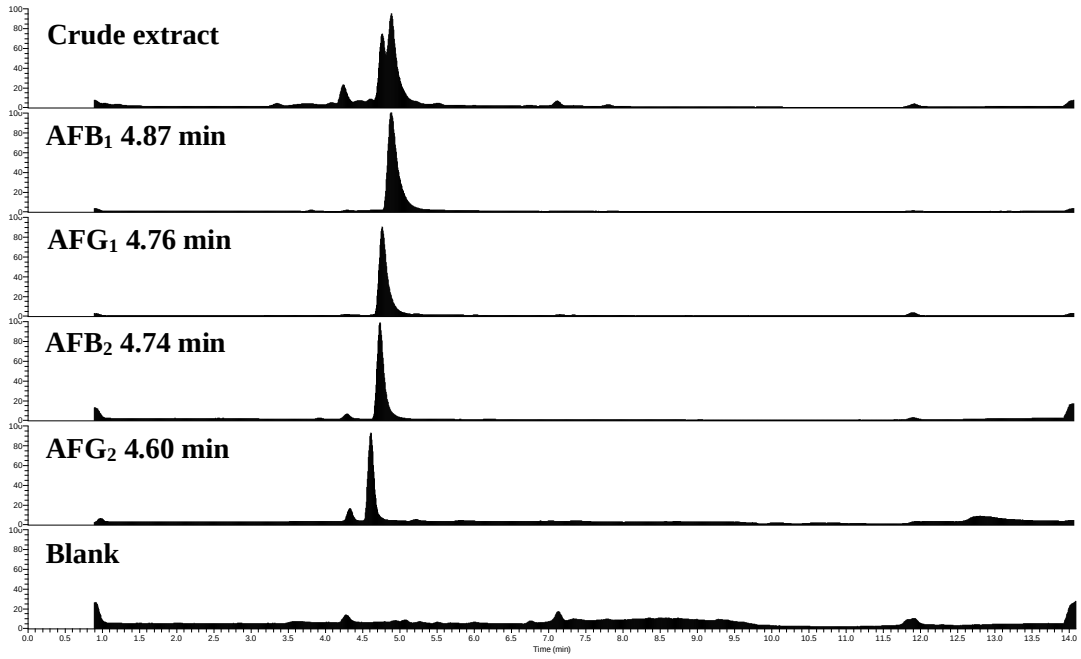


Figure 29. *TIC of the separated crude extract, the pure AFB_1, AFG_1, AFB_2, AFG_2, and a blank run.*

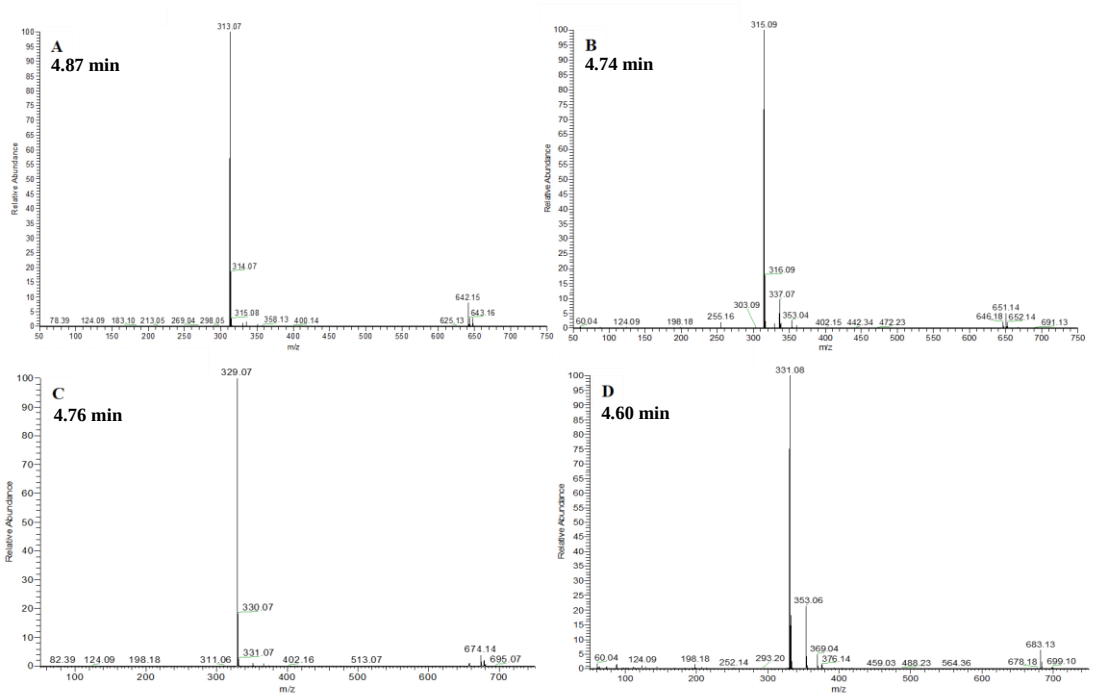


Figure 30. *Mass spectra of the purified AFB_1 (**A**), AFB_2 (**B**), AFG_1 (**C**), and AFG_2 (**D**).*

3.1.10. The yield of the entire scale-up procedure

In all the cases AFB_1 resulted in a light-yellow powder, while AFB_2, AFG_1, and AFG_2 resulted in white powders. After summarizing the yields, the purities, the recoveries, and the solvent consumption of each performed separation (***Table 7***), it can be concluded, that the recoveries during the runs, when the crude extract was injected via the loop, more or less stagnated, while more pure material could be gained. On the other hand, changing the loop injection to the pump injection causes loss in recovery (from 89,6% to 71,6%). Recovery also decreases when higher amounts of crude extracts are injected onto the 1000 ml column, regarding that the aim was to gain pure (≥95%) AFs.

Table 7. *Yields and purities of the performed separations*[a]

Injected amount	Column (ml)		AFB_1 (mg)	AFB_2 (mg)	AFG_1 (mg)	AFG_2 (mg)	Total AFs (mg)	[b]Recovery (%)	Solvent consumption (l)
90 mg (loop)	250	Yield (mg)	24	2	49	6	81	90.0	1.7
		Purity (%)	98.2	96.3	98.1	97.0	97.3		
180 mg (loop)		Yield (mg)	50	6	93	10	159	88.3	1.7
		Purity (%)	97.8	96.0	98.5	98.0	97.6		
210 mg (loop)		Yield (mg)	61	6	112	13	192	91.4	1.7
		Purity (%)	97.8	97.0	99.3	98.0	98.0		
240 mg (loop)		Yield (mg)	67	7	127	15	216	90.0	1.7
		Purity (%)	98.0	97.9	98.6	97.8	98.0		
250 mg (loop)		Yield (mg)	70	7	131	16	224	89.6	1.7
		Purity (%)	98.8	98.1	99.3	98.0	98.5		
250 mg (pump)		Yield (mg)	60	4	110	5	179	71.6	1.7
		Purity (%)	98.1	97.0	99.0	98.0	98.0		
375 mg (pump)		Yield (mg)	66	3	125	5	199	53	1.725
		Purity (%)	98.5	96.5	99.0	97.0	97.8		
500 mg (pump)		Yield (mg)	107	16	145	22	290	58	1.725
		Purity (%)	98.7	97.0	99.5	99.0	98.5		
625 mg (pump)		Yield (mg)	169	28	213	53	463	74.1	1.725
		Purity (%)	99.0	97.8	98.9	96.5	98.1		
1 g (pump)	1000	Yield (mg)	320	29	434	70	853	85.3	6.9
		Purity (%)	98.3	96.9	99.0	99.0	98.3		
1.5 g (pump)		Yield (mg)	420	45	502	100	1067	71.3	6.9
		Purity (%)	98.9	98.0	98.9	97.3	98.2		
2 g (pump)		Yield (mg)	513	72	625	194	1404	70.2	6.9
		Purity (%)	97.9	98.7	98.9	99.0	98,6		
2.5 g (pump)		Yield (mg)	602	101	840	199	1742	69.7	6.9
		Purity (%)	99.0	99.0	98.5	98.7	98.8		

[a]Purities were calculated from the injections of each compound to HPLC-UV.
[b]Recoveries are calculated from each injected amount of crude extract in each run.

3.2. Purification of ergometrine

3.2.1. HPLC-FLD method development for the detection of ergometrine

According to the literature, there is no consensus on the excitation and emission wavelengths in the case of ErgM measurements. Therefore, the HPLC-FLD method development was begun by searching for a pair of wavelengths that result in the highest integrated area of ErgM, when a constant concentration of the compound is injected into the system. In the case of ErgM, the gained areas regarding different pairs of wavelengths found in the literature are represented on Figure 36. It can be concluded that the most sensitive excitation and emission wavelengths are λ_{ex} = 245 nm and λ_{em} = 418 nm when 5 µl is injected from a 2.5 µg/ml of ErgM solution (***Figure 31***).

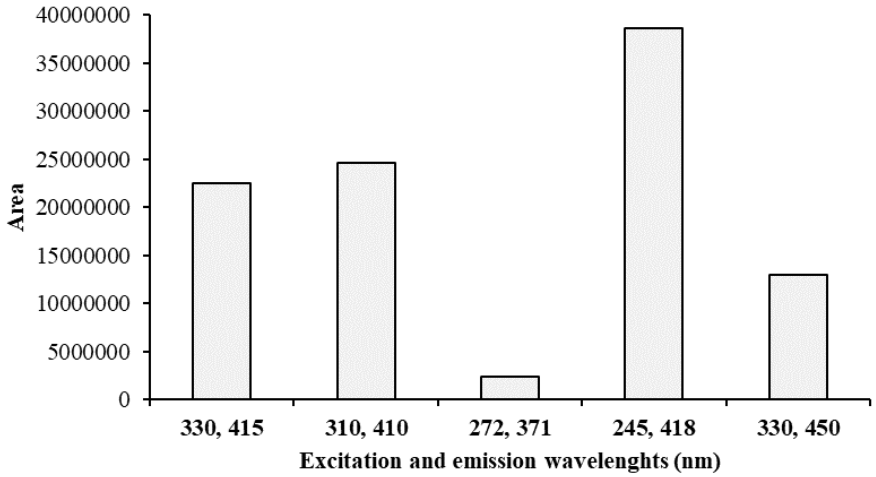


Figure 31. *Area of ErgM utilizing different excitation and emission wavelengths in an FLD detector.*

After the determination of the wavelengths, a method for the separation of ErgM was developed. It is known from the literature, that ergot alkaloids can be separated under alkaline conditions [169]. Therefore, for the developed analytical method, water and methanol were applied as eluents, which were completed with 5 mM of NH_4HCO_3 in order to maintain the alkaline pH, and a short, C18 stationary phase column was selected to achieve a quick separation. Method development was carried out in gradient elution and ErgM was separated from ergotamine and several impurities found in the standard mixture (***Figure 32***).

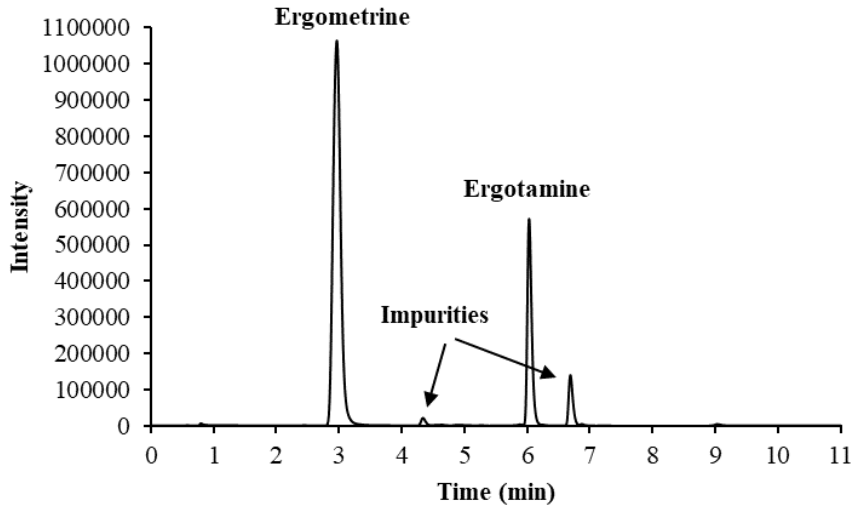


Figure 32. *Chromatogram of the ergot alkaloid mixture.*

3.2.2. Ergometrine production of isolated Claviceps purpurea

In the next phase, the determination of produced ErgM content was determined from several *C. purpurea* isolates. Fungi were previously isolated from sclerotia collected from several triticale fields around Szeged, Hungary. Altogether 16 isolates were identified according to their ITS (Internal Transcribed Spacer) region as *C. purpurea*.

Fungi were cultivated in three parallels for 16 days in a liquid culture media, then mycelia were filtered off and the aqueous phases were extracted with ethyl-acetate. in order to obtain the produced ErgM. ErgM content of the crude extracts was measured by the developed HPLC-FLD method.

It can be concluded that from the cultivated 16 *C. purpurea* isolates 14 produced ErgM, but mostly only small amounts could be detected. *C. purpurea* (SZMC 25562) produced ErgM in the largest quantity, 33.15 ± 6.25 μg/ml (***Figure 33***), and *C. purpurea* (SZMC 25575) produced ErgM in a concentration of 19.16 ± 0.19 μg/ml.

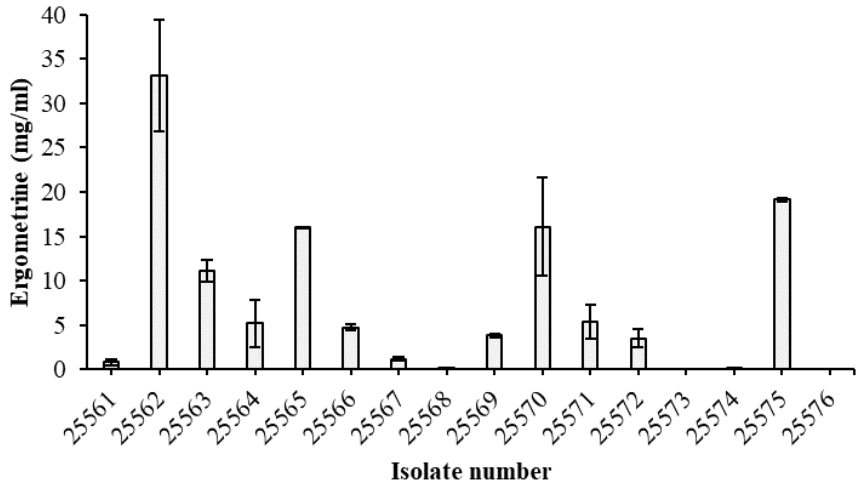


Figure 33. *ErgM production of the C. purpurea isolates.*

Further experiments were performed including the organism that produced the most ErgM since the aim was to gain the most ErgM possible.

3.2.3. Selection of the solvent systems

3.2.3.1. Producing of crude ergometrine for the solvent system selection

Large-scale cultivation (9 l) was carried out utilizing the selected organism. Grown mycelia were centrifuged and the collected culture media was extracted with ethyl-acetate resulting in 450 mg dried material after the evaporation. 5 µl of a solution containing 500 µg/ml crude mixture solved in MeOH/IPA = 1/1 (v/v%) then was injected into the HPLC-FLD system to determine the ErgM content and to detect any impurities. It can be concluded that the crude mixture contains ErgM (483 µg/ml), and several other undesirable compounds (***Figure 34***), but there is one main impurity that elutes directly in front of ErgM.

The next stage of our work was to develop such a liquid-liquid chromatographic method, in which ErgM can be separated not just from the main impurity, but from the other components of the crude mixture.

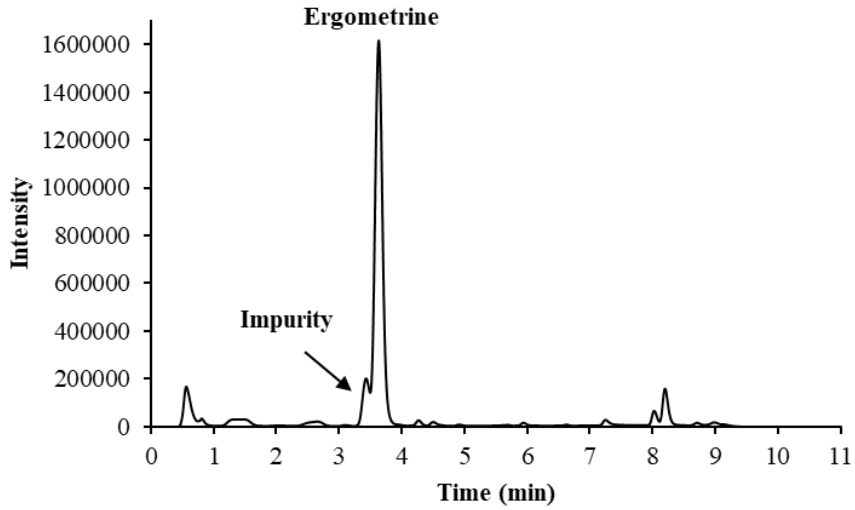


Figure 34. *HPLC-FLD chromatogram of the obtained crude extract.*

3.2.3.2. Ether/alcohol/water ternary systems

It is a known fact that ErgM can be dissolved in several, lower alcohols [191]. For a successful liquid-liquid separation, ternary systems were tested, where the distribution coefficient (P) of the desired product is around one, meanwhile the impurities have different ones. The selection of the solvent system was carried out again by the "best solvent" method using lower alcohols for this purpose. As a less polar solvent, ethers were selected, because they are freely mixable with alcohols, but form a two-phase with the selected polar solvent, water. Ethers are also a good choice because aliphatic and halogenic solvents do not solve ErgM well [191], while ethyl-acetate solves it so well, that it would have been impossible to partition it between the phases.

Based on these facts two compositions were selected from the ternary diagram of diethyl-ether/ethanol/water, which certainly form a two-phase (***Figure 41.*** in the supplementary information (chapter 9.)). These were diethyl-ether/methanol/water 40/20/40 (v/v/v%) and 30/20/50 (v/v/v%). The two compositions were applied with 3 ethers and 10 alcohols in all possible combinations (***Table 8***). The utilized ethers were diethyl-ether, diisopropyl-ether, and *t*-buthyl-methylether (MTBE) and the alcohols were methanol, ethanol, *n*-propanol, *i*-propanol, *n*-butanol, *s*-butanol, *i*-butanol, *t*-butanol, *n*-pentanol, and *i*-pentanol.

***Table 8**. The tested ternary systems for the ErgM partition*

Number		***Ether***	***Alcohol***	***Water***
40/20/40	**30/20/50**			
1	*31*	Diethyl-ether	Methanol	Water
2	*32*		Ethanol	
3	*33*		*n*Porpanol	
4	*34*		*i*Propanol	
5	*35*		*n*Buthanol	
6	*36*		*s*Buthanol	
7	*37*		*i*Buthanol	
8	*38*		*t*Buthanol	
9	*39*		*n*Penthanol	
10	*40*		*i*Penthanol	
11	*41*	Diizopropyl-ether	Methanol	Water
12	*42*		Ethanol	
13	*43*		*n*Porpanol	
14	*44*		*i*Propanol	
15	*45*		*n*Buthanol	
16	*46*		*s*Buthanol	
17	*47*		*i*Buthanol	
18	*48*		*t*Buthanol	
19	*49*		*n*Penthanol	
20	*50*		*i*Penthanol	
21	*51*	*tercier*Buthyl-methyether	Methanol	Water
22	*52*		Ethanol	
23	*53*		*n*Porpanol	
24	*54*		*i*Propanol	
25	*55*		*n*Buthanol	
26	*56*		*s*Buthanol	
27	*57*		*i*Buthanol	
28	*58*		*t*Buthanol	
29	*59*		*n*Penthanol	
30	*60*		*i*Penthanol	

From the upper and lower phases of the compiled systems, 500-500 µl was added to a small portion of the dry crude extract, to have the components distributed. After the dissolution, phases were separated and ErgM content was determined in both phases. During the evaluation, P values were calculated.

In general, in the case of compositions 40/20/40 (v/v/v%) (***Figure 35***), when diisopropyl-ether was utilized, the partition of ErgM shifted into the lower phase, since the P values were lower compared to the systems with the other two ethers. Regarding diethyl-ether, in the case of *i*-propanol (4) and *i*-pentanol (10), partition coefficients resulted around one, according to our expectations (0.99 and 1.00 respectively). In the case of MTBE, the P value became around one (0.98) in the case of system number 30, when *i*-pentanol was applied.

Regarding the aforementioned systems the **diethyl-ether/isopropanol/water 40/20/40** (v/v/v%) can be suitable for separation, not just because *i*-pentanol has a higher boiling point, making it difficult to get rid of after the separation, but it is harder to work with and would be difficult to scale-up the entire purification procedure.

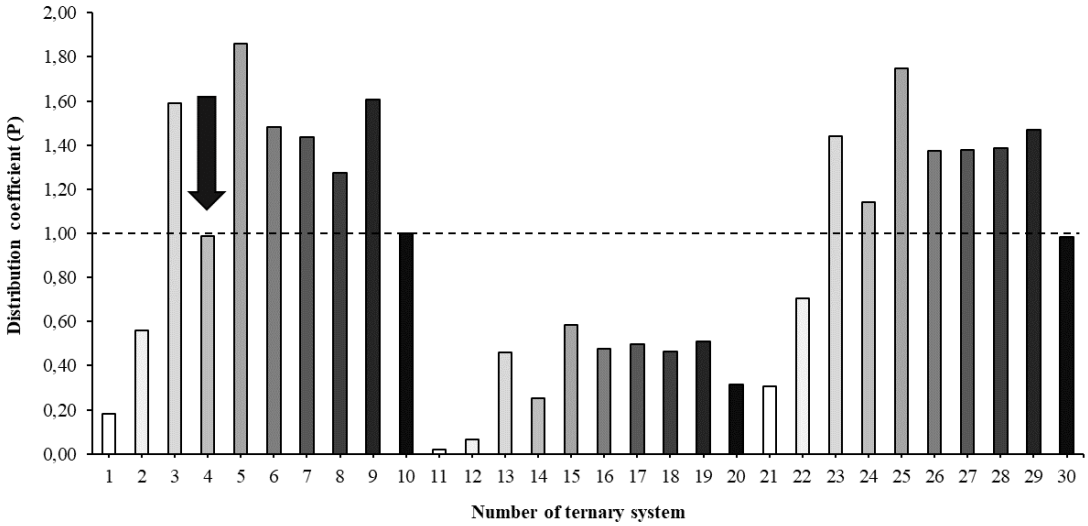


Figure 35. *Distribution coefficient of ErgM in ether/alcohol/water 40/20/40 ternary systems.*

In the case of ternary systems with the composition 30/20/50 (v/v/v%) one propanol and one pentanol-containing system became promising. It can also be concluded that diisopropyl-ether dissolves ErgM less than the other two ethers because the P values are also lower in these cases. Interestingly, while applying the previous composition, *i*-propanol and *i*-pentanol proved to be appropriate for the separation, examining the composition 30/20/50 (v/v/v%) (***Figure 36***) the normal chain isomers of the two alcohols resulted to be suitable for our experiments (systems 33 and 39). Regarding MTBE as the ether, there was no system as good as or better than 33 or 39, although the range of the P values would be promising for the separation.

Partition coefficients were also calculated in the cases of impurities, but all evaluations showed, that the P value is extremely high in all cases (P = 3000-6000). This means that the upper, organic layer dissolves the impurities very well, and the quantities of the impurities are negligible in the lower, aqueous phase. According to this finding, the separation factor (α) values are extremely high as well, determining that ErgM can be separated from the impurities in one run. This is a very advantageous fact regarding the separation because if the upper phase is selected as a mobile phase, the impurities in the extract will elute with the solvent front.

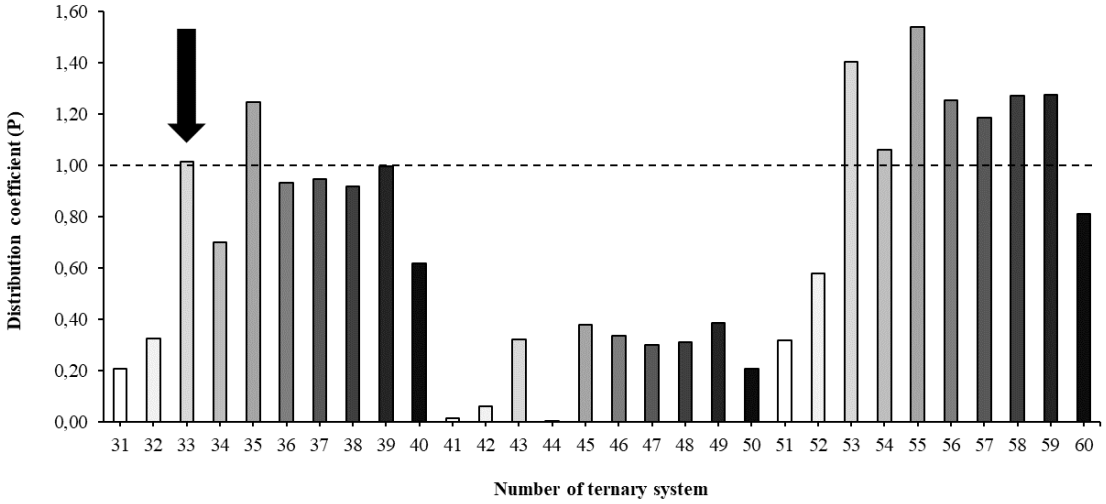


Figure 36. Distribution coefficient of ErgM in ether/alcohol/water 30/20/50 ternary systems.

Taking the findings above into consideration, **diethyl-ether/isopropanol/water 40/20/40** (v/v/v%) was finally selected for the separation. Instrumental optimization and separation were carried out utilizing this system.

3.2.4. Purification of ergometrine by CPC

Optimization of the separation was begun by the selection of the elution mode. The impurities in the crude extract were highly soluble in the upper, organic phase, thus if the upper phase was selected as the mobile phase, impurities would be eluted with the solvent front from the column, because they are nearly insoluble in the lower, stationary phase. On the other hand, when the lower, aqueous phase is selected as mobile phase, ErgM can be eluted from the column with nearly a column volume of eluent because of its partition coefficient (P = 0.99), while impurities would remain on the column, dissolved in the stationary phase. Therefore, ascendent mode was selected, as the elution mode, in which the lower phase is the stationary phase and elution is carried out with the upper, organic phase. In this case, every compound in the crude mixture can be eluted from the column, possibly allowing the consecutive injection of several batches of crude extract to purify, without the re-equilibration and refill of the column.

The rotation speed of the CPC column determines the degree of the centrifugal force acting on the two phases in the column, during flow it determines the system pressure. The rotation speed of our 250 ml rotor was determined in the next step while maintaining a constant 10 ml/min flow rate of the mobile phase. The instrument has a pressure limit of 100 bar, therefore the rotation speed has to be set in such a way that the pressure does

not exceed this limit. The suitable rotation speed turned out to be 2200 rpm that generates a 532 *'g'* gravitational field and causes a constant 76 bar of pressure.

After the equilibration of the column, for one separation, 100 mg of crude material was injected into the column, dissolved in 9 ml of 1/1 (v/v%) ratio of upper and lower phases. After three consecutive injections and separations (***Figure 37***), it can be concluded that the method repeatability is appropriate, only a small shift was observed in the elution of ErgM. The composition of the corresponding fractions fit each other, the relative standard deviation of the ErgM content in them was under 10%. The compound has a P value close to 1, so it can be expected, that ErgM elutes with a column volume of the mobile phase. According to the applied 10 ml/min eluent flow, and the collected 20 ml fractions, ErgM elutes in the 12th fraction, which equals 240 ml of mobile phase, therefore our calculations were verified by our experiments. It can be concluded that the system is stable, since the retention of the desired compound is almost the same, and the separation is repeatable.

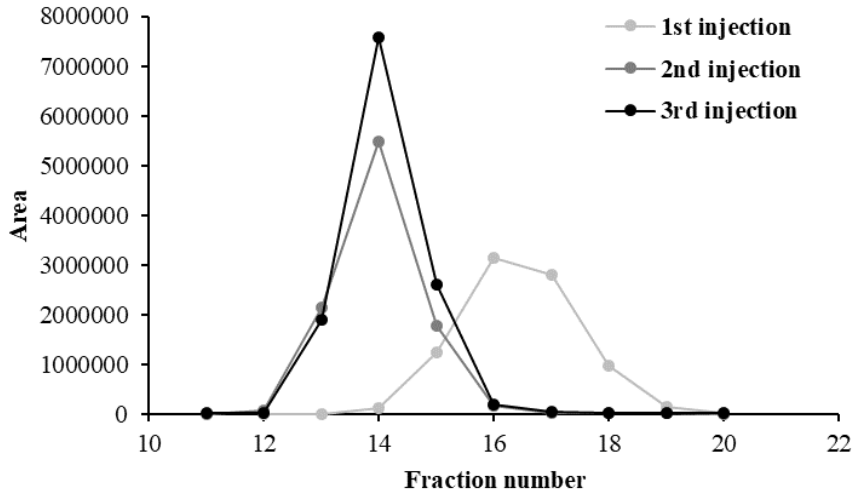


Figure 37. *Fractogram of the ErgM purification.*

Impurities are not shown on the figure, because unwanted compounds were eluted with the solvent front, and were not collected.

After the successful separations, fractions containing ErgM (13-20 in the 1st injection, and 11-18 in the 2nd and 3rd injections) were pooled and the pure ErgM content was determined.

3.2.5. HPLC-FLD and HPLC-OHRMS analyzes of the purified ergometrine

3.2.5.1. HPLC-FLD analyses

After the determination of the pure products from the three separations, **69.5 mg**, **68.3 mg**, and **70.2 mg** of ErgM (RSD=1.13%) was achieved, respectively. These results also show the stability of the system and the repeatability of the separation. The average yield of 69,3% percent can also be considered satisfactory.

Regarding the purity of ErgM, it was determined by HPLC-FLD technique (***Figure 38***). It can be concluded that there are no other impurities with the purified ErgM, it was successfully purified from major and minor impurities. Purity was above 98% in all three cases.

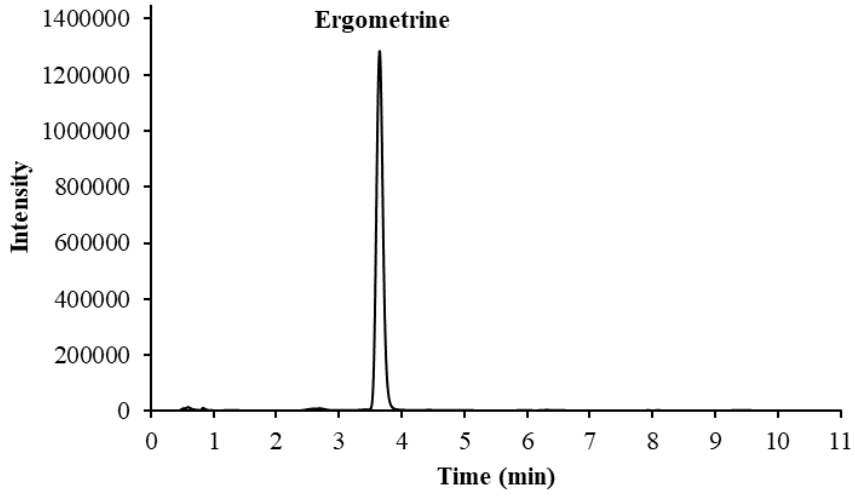


Figure 38. *HPLC-FLD chromatogram of the purified ErgM.*

3.2.5.2. HPLC-OHRMS analyses

After each run, the combined pure fractions were analyzed by HPLC-FLD and HPLC-OHRMS techniques. Purities of the resulted compounds were calculated from the HPLC-FLD spectra and were confirmed by high-resolution mass spectrometry. The total ion chromatogram (TIC) and mass spectra of the purified ErgM are shown on ***Figure 39*** and ***Figure 40,*** respectively.

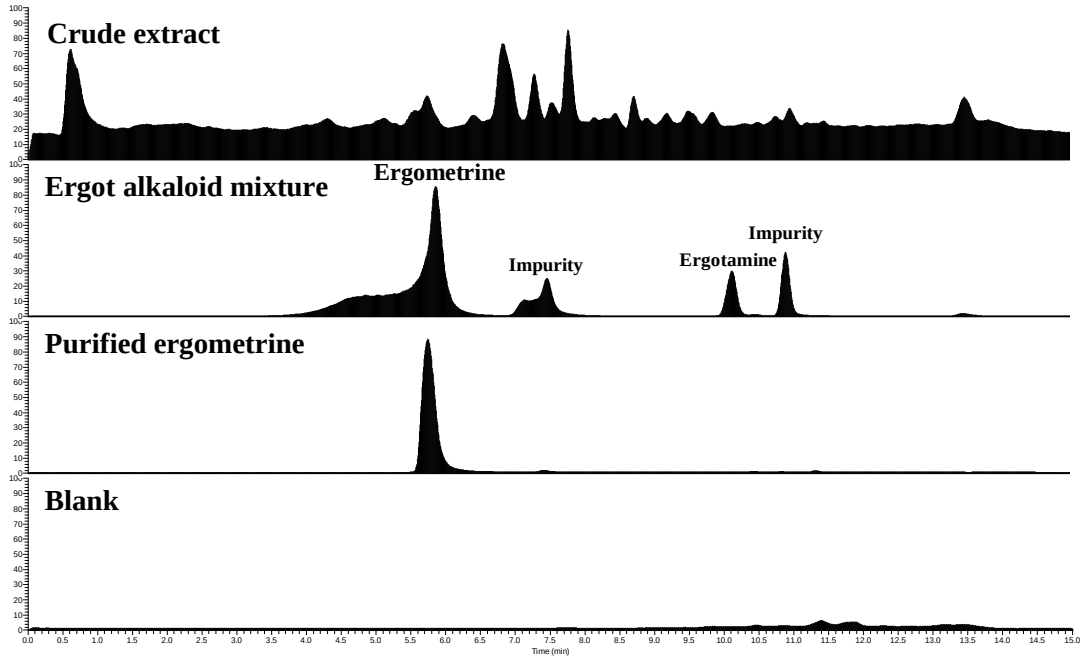


Figure 39. *TIC of the purified crude extract, the ergot alkaloid standard mixture, the purified ErgM, and a blank run.*

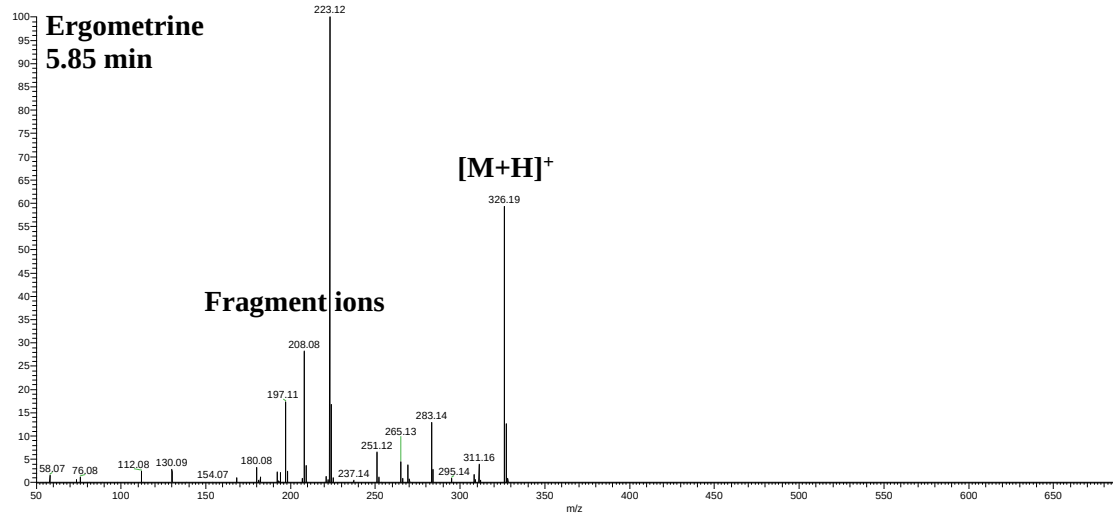


Figure 40*. Mass spectrum of the purified ErgM*

3.2.6. The yield of the entire purification procedure

In all cases, ErgM resulted in white, small crystals. After summarizing the yields, the purities, and the recoveries (***Table 9***), it can be concluded, that the developed separation yields a satisfactory amount of ErgM, which is exceptionally pure, with good recoveries.

Regarding the entire procedure, it can be concluded, that from 9 liters of fermented material (one batch of cultivation), approximately 450 mg crude material can be gained with the extraction, which consumes 6.75 liters of ethyl-acetate. With the current method, all of the extract can be purified in 5 runs, which consumes 3 liters of solvent altogether, and results in 312 mg purified ErgM.

Table 9. *Yields and purities of the performed separations*[a]

Run		Ergometrine (mg)	[b]Recovery (%)	Solvent consumption (l)
1st	Yield (mg) Purity (%)	69.5 98.6	69.5	0.6
2nd	Yield (mg) Purity (%)	68.3 99.0	68.3	0.6
3rd	Yield (mg) Purity (%)	70.2 98.9	70.2	0.6

[a]Purities were calculated from the injections of each compound to HPLC-FLD.
[b]Recoveries are calculated from each injected amount of crude extract in each run.

4. Materials and methods

4.1. Chemicals and solvents

All solvents used for extractions and preparative scale separations and all chemicals for the cultivation materials were purchased from Molar Chemicals (Halásztelek, Hungary) and were of analytical, synthetic, or laboratory-grade. The solvents used for the various solvent systems and HPLC-UV, HPLC-MS/MS, and HPLC-OHRMS measurements were of gradient grade or super gradient grade and were purchased from VWR International (Hungary). The AF standard mixture (including AFB_1, AFG_1, AFB_2, and AFG_2) was purchased from Merck GKaA, while the ergot alkaloid mixture – containing 500 µg ergotamine and an equal amount of ErgM as well – was purchased from RomerLabs Ltd. (Getzersdorf, Austria).

4.2. Fermentation materials

Potato dextrose broth, yeast extract, malt extract, glucose, and sucrose were purchased from VWR International Ltd. $NaNO_3$, $MgSO_4$ x $7H_2O$, KCl, K_2HPO_4, KH_2PO_4, $ZnSO_4$ x $7H_2O$, $FeSO_4$ x $7H_2O$, $CuSO_4$, and $(NH_4)_2SO_4$ were purchased from Molar Chemicals Hungary Ltd. (Halásztelek, Hungary). Folic acid, biotin, Ca-pantothenate, inositol, niacin, p-aminobenzoic acid, riboflavin, thiamine HCl, and pyridoxine HCl were purchased from Merck GKaA. (Darmstadt, Germany). Corn (**M1**), maize (**M2**), corn cob (**M3**), wheat, (**M4**), barley (**M5**), long grain rice (Uncle Ben's, Mars Ltd. Inc.) (**M6**), and bio vegetable cocktail (67% tomato juice, 13% carrot juice, 6% sour cabbage juice, 4% celery juice, 4% beetroot juice, 2% cucumber juice, 2% mushed bell pepper, 1% onion juice, 1% bean juice, 0.6% dill juice, 0.3% sea salt spice mixture) (Biopont Hungary Ltd.) (**M11**) were purchased from local markets in Szeged, Hungary.

One liter of Czapek-Dox minimal broth (**M7**) consisted of 2 g $NaNO_3$, 0.5g $MgSO_4$ x $7 H_2O$, 0.5 g KCl, 0.5 g K_2HPO_4, 30 g sucrose, 10 mg $ZnSO_4$ x $7 H_2O$, 10 mg $FeSO_4$ x $7 H_2O$, 3 mg $CuSO_4$ and 1000 ml H_2O. 1000 ml synthetic vitamin broth (**M8**) consisted of 5 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4$ x $7 H_2O$, 10 g glucose, 1 ml of Wickerham vitamin solution (0.2 mg folic acid, 0.2 mg biotine, 40 mg Ca-pantothenate, 200 mg inositol, 40 mg niacin, 20 mg p-aminobenzoic acid, 20 mg riboflavin, 40 mg thiamine HCl and 40 mg pyridoxine HCl for 100 ml H_2O) and 1000 ml H_2O. Complex malt broth (**M9**) consisted of 5 g Bacto malt extract, 5 g yeast extract, 5 g glucose, and 1000 ml H_2O.

1 liter of potato dextrose broth (**M10**) contained 24 g potato dextrose powder and 1000 ml H_2O.

One liter of defined *Claviceps* broth consisted of 100 g sucrose, 10 g L-asparagine monohydrate, 1 g KNO_3, 250 mg KH_2PO_4, 250 mg $MgSO_4$, 120 KCl, 100 mg yeast extract powder, 5 mg $FeSO_4$, and 5 mg $ZnSO_4$ solved in 1000 ml distilled water.

4.3. Strains and cultivation for optimization

Aspergillus strains examined in this study were isolated from different sources from all around the world and were all deposited in SZMC (www.szmc.hu). The list of the strains, their SZMC number, their other identification number, their source, and the produced known extrolites with their references can be found in ***Table 10***. For the screening of AF and STC production on different culture media, strains were cultivated on 5 g of **M3** and 10 g of **M1**, **M2**, and **M4-6,** and 50 ml of **M7**-**M11** for 7 days at 28°C in the dark. Every culture regarding AF production studies was a static culture and was repeated in three parallels.

Claviceps purpurea isolates were previously isolated from overwintering sclerotia collected from local infected triticale fields around Szeged, Hungary, and clean isolates were deposited in the SZMC (SZMC 22561-22576). For the identification of the strains, isolation of the genomic DNA from the mycelia was performed using E.Z.N.A. Fungal DNA Kit (Omega Bio-tek) according to the manufacturer's instructions. The internal transcribed spacer (ITS) region of the rDNA was amplified using the primers ITS1 and ITS4 as described previously [192]. The gathered sequences were analyzed by BLAST similarity search at the website of the National Center for Biotechnology Information () and the species were identified based on their identity values (>97%). For the screening of ErgM production, all 16 isolates were cultivated in 200 ml of defined *Claviceps* broth, at 25°C, shaken for 16 days at 120 rpm. Every culture regarding ErgM production was repeated in three parallels.

Table 10. *List of fungi used in this study.*

Fungi	[a]**SZMC**	**Other ID number**	**Source**	**Extrolites**	[b]**Ref.**
A. amoenus	20877	[c]NRRL 236	chili pepper, Hungary	[d]STC	34
A. dimorphicus	2024			Wentilactones,	35
A. flavus	12576		ceratitis, India	AFs, [e]CPA	36-38
A. minisclerotigenes	22438		cocoa beans, India	AFs, penicillic acid, CPA	39, 40
A. nidulans	26961			averufin, penicillin G, several others	41
A. nomius	22631		cheese, Hungary	AFs, kojic acid, paspalline	39, 40, 43
A. parasiticus	2473	NRRL 2999	Japan	AFs, STC, kojic acid, aspergillic acid, several others	39, 40, 42
A. parasiticus	22727		indoor air, Croatia	AFs, STC, kojic acid, aspergillic acid, several others	39, 40, 42
A. parasiticus	22728		indoor air, Croatia	AFs, STC, kojic acid, aspergillic acid, several others	39, 40, 42
A. pseudotamarii	2018	NRRL 25517		AFB_1, AFB_2, CPA, kojic acid, paspaline	43
A. pseudotamarii	3055			AFB_1, AFB_2, CPA, kojic acid, paspaline	43
A. pseudonomius	22273		corn, Serbia	AFs, aspergillic acid, kojic acid	43
A. tabacinus	23543	NRRL 4791	tobacco		

[a]Szeged Microbiology Collection
[b]Reference number
[c]Agricultural Research Service Culture Collection number
[d]Sterigmatocystin
[e]Cyclopiazonic acid

4.4. Metabolite extraction for optimalization

Aflatoxins from 30 ml of **M7**-**M11** were extracted with 15 ml dichloromethane twice. The collected organic phases were combined, were dried over anhydrous $MgSO_4$, were filtered, and were evaporated to dryness.

To the solid cultures of **M1**-**M7** 30 ml of 80% methanol was added and the samples were homogenized and extracted with a Velp OV5 UltraTurax (Velp Scientifica, Italy) for 1 minute. The samples were centrifuged at 7000 rpm for 5 mins and the supernatants were collected. These steps were repeated twice. Combined supernatants were evaporated to dryness. Dry aflatoxin samples were redissolved in 500 µl of HPLC gradient grade methanol prior to HPLC injection.

Grown cultures of *C. purpureae* were filtered off by sterile gauze, and 100 ml culture media was extracted with 50 ml and 25 ml ethyl-acetate respectively. Combined organic phases were washed with saturated aqueous NaCl solution. The organic layer was separated, dried over anhydrous Na_2CO_3 filtered, and evaporated to dryness. Dry crude extracts were redissolved in 500 µl of HPLC gradient grade methanol/isopropanol 1/1 (v/v%) prior to HPLC injection.

4.5. Cultivation for purification

Aspergillus parasiticus strain SZMC 2473 (CBS 260.67; GenBank Accession number for ITS/ MG662400) was originally isolated in Japan and is the ex-type of the species [43]. To cultivate the strain, a 500 ml liquid medium was prepared by adding 5 g Bacto malt extract, 5 g yeast extract, 5 g glucose to 1 l distilled water in a 1.2 l Roux-flask. The flask was capped with a cotton cork and sterilized in an autoclave for 30 min at 115°C. Altogether 5000 ml of culture media was prepared for one batch of cultivation. Each flask was inoculated with a 5 ml conidial suspension prepared in a sterilized saline solution and incubated in a horizontal position in the dark at 28°C for 7 days.

To cultivate the selected *Claviceps purpurea* (SZMC 25562) strain, it was inoculated by 10 pieces of agar plugs from a petri dish into 1 liter of defined *Claviceps* broth in a 2 liter Erlenmeyer flask. Altogether 9 liters of culture media were prepared for one batch of cultivation. Inoculated cultures were shaken horizontally for 16 days at 25°C.

4.6. Aflatoxin and ergometrine extraction for purification

AFs were extracted from the fermented broth in four steps. First, extraction was performed on one liter of broth with 500 ml followed by 250 ml of dichloromethane. The organic phases were combined and evaporated to water; the volume of aqueous residue was then measured. Next, methanol and hexane were added such that the water/methanol/hexane ratio was 45/50/120 (by volume). After the phases were separated, the upper phase was removed, and further extraction was performed on that phase with water/methanol (45/50 by volume). The separated water/methanol phases were then combined, and dichloromethane was added until two phases formed and extracted in two repetitions. The combined organic phases were dried over anhydrous $MgSO_4$, were filtered, and were evaporated to dryness.

Grown cultures of *C. purpurea* (SZMC 25562) were centrifuged at 10000 rpm for 10 minutes, and supernatants were collected. One liter of culture media was extracted with 500 ml and 250 ml ethyl-acetate, respectively. Combined organic phases were washed with a saturated aqueous solution of NaCl. The organic layer was separated, dried over anhydrous Na_2CO_3, filtered, and evaporated to dryness.

4.7. Testing of the solvent systems

The dried crude aflatoxin extract was dissolved in dichloromethane at a concentration of 1.8 mg/ml and split into 1 ml aliquots; the dichloromethane was then evaporated. The constituents of the solvent systems to be tested (Tables 3 and 4) were mixed in test tubes and vortexed gently for 10 s. When the phases were let to separate and 500 µl from both phases was added to the previously aliquoted and dried crude extract. After the phase-separation, 300 µl of each phase was transferred into new vials and evaporated to dryness and redissolved in 500 µl of acetic acid prior to HPLC injection.

Dried ErgM extract was redissolved in methanol/isopropanol 1/1 (v/v%) at a concentration of 500 µg/ml and was handled exactly the same way as in the case of aflatoxin extracts, with the exception of the solvent systems, and the solvent for redissolving the samples. Solvent systems in Table 8 were tested, and dry samples were redissolved in 500 µl methanol/isopropanol 1/1 (v/v%) prior to HPLC injection.

4.8. Evaluation of the separation

The partition coefficient (P) of each compound was calculated by dividing the area under the peaks detected in the upper and lower phases. The separation factor (α) for each solvent system was calculated for the neighboring eluted compound pairs by dividing the greater P value by the smallest one arranged in the ascending series. The elution volume (V_e) was calculated as follows: $V_e = V_S + P(V_C–V_S)$, where V_S is the volume of the stationary phase and V_C is the volume of the column.

4.9. Centrifugal partition chromatography

Liquid-liquid separations were carried out on a 250 ml and a 1000 ml laboratory-scale CPC column (Gilson, Saint-Ave, France). The smaller column has a maximum rotation speed of 3000 rpm, which means a maximum of 725 *'g'*. The 1 l column has a maximum rotation speed of 1500 rpm, which equals 515 *'g'*. Rotors were coupled with a PLC250 flash/prep hybrid instrument (Gilson, Saint-Ave, France) containing an UV/VIS detector, fraction collector, electronically actuated injector valve with a 10 ml sample loop, and an electronically actuated four-way two-position ascendant/descendant valve. Gilson Glider Prep (Ver. 5.1) software (Gilson, Saint-Ave, France) was used to control the instrument and acquire data.

4.9.1. Separation of AFs

Before the instrumental analysis, the selected solvent system toluene/acetic acid/water in a volumetric ratio of 30/24/50 was prepared in a 5 l separation funnel and shaken to saturate the phases. The phases were then separated and transferred into glass bottles. At the beginning of the separation on both columns, the rotor speed was set to 500 rpm, which represents 121 *'g'* on the 250 ml, and 172 *'g'* on the 1000 ml column. In the case of the smaller rotor, the stationary phase was pumped through the system for six minutes with 50 ml/min while the 1 l rotor was filled up for 12 minutes with 100 ml/min. In the case of the 250 ml column, for the equilibration and separation, the elution speed was 15 ml/min, and the rotor speed was set to 1800 rpm, which is equivalent to 435 *'g'*. The volume of the extruded stationary phase was measured before each separation on both columns using a measuring cylinder and the ratio between the volumes of phases was calculated.

Each separation lasted for 75 minutes, and 20 ml fractions were collected. In the case of the 1000 ml rotor, elution speed was quadruplicated to 60 ml/min and rotor speed was set to 1267 rpm, to achieve the same equivalent centrifugal field as on the smaller column. Separations also lasted for 75 minutes, and 20 ml fractions were collected as well. The UV detector was set to 366 nm during all separations.

For the sale up of the separation, 5 ml increments of the AF solution solved in 1/1 ratio by volume of the toluene/acetic acid/water = 30/24/50 was injected by the built-in pump of the system. Injections were carried out from 10 ml to 25 ml on the 250 ml column. On the 1000 ml column, the same methodology was carried out, except injections were started from 40 ml, and injections were carried out in 20 ml increments until 100 ml.

The pH of each fraction was neutralized by adding a saturated $NaHCO_3$ solution. The AF content in each fraction was measured by HPLC-UV. The fractions containing pure AFs (≥95%) were pooled and the solvent was evaporated. The resulting powders containing the four AFs were used for subsequent analyses.

4.9.2. Separation of ergometrine

Phases for chromatography were prepared in a 5 l separation funnel, in the ratio of diethyl-ether/isopropanol/water 40/20/40 (v/v/v%). The phases were then separated and transferred into glass bottles. Preparation of the 250 ml column was carried out in the same way as in the case of AF separation, except the rotor speed for the separation was set to 2200 rpm, which represents a 532 *'g'* centrifugal field.

Separations lasted for 45 mins and 20 ml fractions were collected. The UV detector was set to 245 nm, the same as the excitation wavelength in the FLD detector. Samples were taken from each collected fraction, and the ErgM content of each was measured by HPLC-FLD technique. Fractions containing pure (≥98%) ErgM were combined, and the solvents were evaporated. The gained white powder was furthermore analyzed.

4.10. HPLC-UV measurements

Analyses were performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a DGU-14A degasser, an LC-20AD binary pump, a SIL-20A autosampler, a CTO-10ASvp column thermostat, an SPD-10Avp UV-VIS detector, and a CBM-20A system controller. Class VP ver. 6.2 software was used for data acquisition and evaluation. The separation of AFs was performed on an injected sample volume of 5 µl

for 16 min in a Phenomenex Gemini C18, 250 mm × 4.6 mm, 5-µm column (Phenomenex, California, USA) with the mobile phase comprising water (A) and a 1/1 mixture of methanol and acetonitrile (B) combined in an A/B ratio of 60/40 by volume. The flow rate was 1 ml/min, and the column temperature was maintained at 40°C. The peaks of the AFs and the impurities were detected at λ = 365 nm.

4.11. HPLC-FLD measurements

Analyses were performed using a Shimadzu HPLC system (Shimadzu, Kyoto, Japan) equipped with a DGU-14A degasser, an LC-20AD binary pump, a SIL-20A autosampler, a CTO-10ASvp column thermostat, an RF-10A FLD detector, and a CBM-20A system controller. Class VP ver. 6.2 software was used for data acquisition and evaluation. The separation of ErgM was performed on an injected sample volume of 5 µl for 11 min in an Agilent Zorbax SB-C18, 50 mm × 4.6 mm, 3 µm column (Agilent Technologies, California, USA) with the mobile phase comprising water + 5 mM NH_4HCO_3 (A) and a methanol +5 mM NH_4HCO_3 (B). The gradient elution was the following: 0 min – 30 B%;1 min – 30 B%; 6 min – 90 B%; 8 min – 90 B%, 8.1 min – 30 B% and 11 min – 30 B%. The flow rate was 1 ml/min and the column temperature was maintained at 30°C. The peaks of ErgM and the impurities were detected at λ_{ex} = 245 nm and λ_{em} = 418 nm.

4.12. HPLC-MS/MS measurements

Qualification and quantification of AFB_1, AFB_2, AFG_1, and AFG_2 and qualification of AFM_1 and sterigmatocystin in grown *Aspergillus* culture extracts were carried out by HPLC-MS/MS technique. Liquid separation was performed on a Shimadzu HPLC system (Kyoto, Japan), equipped with a membrane degasser (DGU-20A5R), a quaternary pump (LC-20ADXR), an autosampler (SIL-20AXR), and a thermostated column compartment (CTO-10ASvp). The desired components were separated on a Gemini-NX C18 (50 mm x 2.1 mm, 3µm) column (Phenomenex, California, USA), thermostated at 25°C. The weak eluent (A) consisted of water (HPLC grade) with 0.2 % acetic acid, while the strong eluent (B) was methanol (HPLC Super Gradient Grade) with 0.2 % acetic acid. The gradient elution was performed as follows/ 0 min, 20 B%; 0.5 min, 20 B%; 10.0 min, 90 B%; 12.5 min, 90 B%; 12.6 min, 20 B% and 16.5 min, 20 B%. The flow rate was set to 350 µl/min and the injection volume was 5 µl.

Mass analyses were carried out on a TSQ Quantum Access (Thermo Fischer Scientific, Massachusetts, USA) triple-quadrupole mass spectrometer. Ionization of the components was performed using a heated electrospray interface (HESI) in positive electrospray ionization mode. The temperature of the ion transfer capillary, the vaporizer gas, the pressure of the sheath and aux gases, and the capillary voltage were 350°C, 250°C, 25, 25, and 4 kV respectively. Qualification and quantification of the components were achieved in SRM mode using Ar as collision gas. The collision gas pressure was 2.9 mTorr in the cases of AFB_1, AFB_2 AFG_1, AFG_2, and AFM_1 and 1.0 mTorr in the case of sterigmatocystin, while the collision energy was 29 eV in all cases. The optimized SRM transitions are summarized in ***Table 11***.

Table 11. *The optimized SRM transitions.*

Compound	Ret. Time (min)	Parent ion	Qualifier ion	Quantifier ion
AFG_2 $[M+H]^+$	5.85	330.9	245.0	313.0
AFM_1 $[M+H]^+$	5.90	329.0	273.0	273.0
AFG_1 $[M+H]^+$	6.23	328.8	214.7	242.9
AFB_2 $[M+H]^+$	6.54	314.9	259.0	287.0
AFB_1 $[M+H]^+$	6.91	313.0	241.0	284.9
[a]STC $[M+H]^+$	9.82	324.9	253.0	281.0

[a]Sterigmatocystin

4.13. Orbitrap-HRMS (OHRMS) analyses

The identities of the purified AFs were analyzed using a Dionex Ultimate 3000 UHPLC system (Thermo Fischer Scientific, Waltham, USA) coupled with a Q-Exacitve Focus Orbitrap mass spectrometer (Thermo Fischer Scientific, Waltham, USA) using flow injection with an isocratic eluent (20/80 water/methanol mixture by volume with 0.1% acetic acid). The capillary temperature of the heated electrospray interface (HESI) and the heater temperature were set to 250°C. The sheath gas flow rate was 30 a.u. and the auxiliary gas flow rate was 15 a.u. The capillary voltage was set to 4.5 kV. To achieve the fragmentation of the examined molecules, the HESI capillary and the auxiliary gas were heated to 350°C, the normalized collision energy in the collision cell of the instrument was set to 70 a.u. and N_2 was used as the collision gas. From the purified AF solution solved in acetic acid (100 µg/ml), 5 µl was injected for analysis.

The purified ErgM was analyzed by the same instrument as the AFs. For the flow injection analysis isocratic eluent of 30/70 water/methanol mixture by volume with 5 $mmol/dm^3$ NH_4HCO_3 was used. The capillary temperature of the HESI and the heater temperature were set to 260°C. The sheath gas flow rate was 25 a.u. and the auxiliary gas

flow rate was 15 a.u. The capillary voltage was set to 4.0 kV. To achieve the fragmentation of the examined molecules, the HESI capillary and the auxiliary gas were heated to 250°C, the normalized collision energy in the collision cell of the instrument was set to 50 a.u. and N_2 was used as the collision gas. From the purified ErgM solution solved in methanol (100 µg/ml), 5 µl was injected for analysis.

5. Summary

Secondary metabolites are produced when a microorganism reaches its ideal state of growth (idiophase). These compounds are not vital but play an important role in the life of a microbe. As an example, these molecules can be antibiotics, antifungal agents, toxins, and cell-protective compounds as well.

Numerous secondary metabolites are produced by *Aspergillus* species. A lot of them have toxic, teratogenic, mutagenic side effects when consumed by animals and humans. Probably the most examined group of toxins produced by *Aspergillus* species are aflatoxins (AFs). These toxins were discovered, when *Aspergillus* contaminated Brazilian ground nuts were fed to a large group of poultry in England in the 19th century, and the animals died. As a result of extensive research, the four main AFs were isolated. Compounds were named after their fluorescent color under the UV light, and their migration on the TLC sheet, therefore AFB_1, AFB_2, AFG_1, and AFG_2 were distinguished. As these toxins were discovered, their harmful effects were also revealed. Therefore, their purification was developed, to gain pure material to study the effect of these compounds. AFs can be purified by normal and reverse phase chromatography with different solvents and solvent combinations. All these techniques require large-scale cultivations and extractions, which is not feasible for industrial approaches.

ErgM is another secondary metabolite produced by mainly *Claviceps* species, especially *Claviceps purpurea*. It is in the family of ergot alkaloids, therefore it has alkaloid-like properties. It is used as a medicine, but due to instability reasons, synthetic variants and salts are preferred. It can be isolated from the overwintering sclerotium of the fungus, mostly by extraction and separation. *C. purpurea* can also be cultivated in liquid culture, and the desired alkaloids can be extracted and separated by normal and reverse phase chromatography as well. ErgM is a registered drug precursor in the EU, therefore monitoring of the compound would crucial, but there are no current regulations and limits to its concentration in food and feed.

Centrifugal partition chromatography (CPC) is a preparative separation technique that uses two liquid phases to distribute the desired compounds and the impurities. In this liquid-liquid chromatographic technique, one liquid phase is immobilized by a centrifugal force (stationary phase), and the other one is pumped through it (mobile phase). According to the nature of the selected two-phase solvent system ascendent and descendent modes can be utilized depending on the chosen mobile phase. In ascendent

mode, the lower phase is the stationary phase, while in descendent mode the upper phase is immobilized. Whether the stationary phase is aqueous or organic, depends on the nature of the selected two-phase solvent system. As it is presented, this chromatographic technique offers a wide variety of options, because any two-phase solvent system can be applied, if the desired compounds are soluble in the solvent system. It does not use any expensive solid chromatographic phases, and the utilized solvents can be reused.

The main objectives of this thesis are to find AF and ErgM producer endophytic fungi and gain large-scale cultures. To develop effective extractions of the produced secondary metabolites and to develop a feasible CPC separation of the desired compounds from the crude mixture. Separation of the four main AFs in one run was an important objective as well, and to achieve maximum yield, the scale-up of the separation needed to be examined. The last objective was in both cases to verify the quality, quantity, and purity of the final products.

Purification of aflatoxins:

On 11 different culture media, 10 *Aspergillus* species and 13 isolates were cultivated in order to have their AF and STC production capability and AF and STC content determined. On solid culture media, 9 fungi produced some kind of AFs. All four AFs were produced by *A. parasiticus* strains SZMC 2473, SZMC 22727 and SZMC 22728, and *A. nomius* (SZMC 22631). Regarding culture media, it can be concluded that wheat (M4), barley (M5), and rice (M6) are the best for AF production. When the same fungi were cultivated on liquid culture media, it can be noticed that the same four organisms (SZMC 2473, SZMC 22727, SZMC 22728, and SZMC 22631) produced all four main AFs in high yields. Regarding the culture media, most AFs were produced on bio vegetable cocktail (M11). For cultivation complex malt broth (M9) and PDB (M10) are also appropriate. For large-scale cultivation *A. parasiticus* (SZMC 2473) and M9 were selected.

After the large-scale cultivation, a four-step extraction was developed, to gain the produced AFs from the culture media. This crude extract was used to develop a CPC method. For the method development, the “best solvent” method was used. This means that a solvent that solves the AFs well was selected, and this solvent was paired with a more polar and a less polar solvent to form a ternary system. Initially, chloroform and acetone were selected, but pairing these solvents with hexane, heptane, and toluene as

apolar and water as polar solvents did not result in a feasible distribution. Acetic acid was then selected as best solvent and it was paired with diethyl-ether, chloroform, and toluene as apolar and water as polar solvents. The best distribution was achieved when the toluene/acetic acid/water ratio was 30/24/50 by volume.

A suitable, 75-minute-long method was developed utilizing the 250 ml column of the CPC instrument. The rotor speed was set to 2200 rpm, and the flow rate was set to 15 ml/min. 90 mg of crude AF extract was dissolved in 4 ml upper and lower phases and was injected into the instrument. All of the AFs eluted perfectly from the column, while the impurities were retained. System stability and repeatability were tested with three consecutive injections. The gained pure AFs were evaporated, and the quality and quantity were tested. From 4.5 l liquid culture, a total of 1351 mg of pure and separated AFs were gained with a purity of 97.3 % and a recovery of 92.6 %.

To improve the capacity of the separation, the next step was to test the maximum loading capacity of the 250 ml rotor. For this, increments of the crude AF mixture (90 mg, 180 mg, 210 mg, 240 mg, 270 mg) were dissolved and injected into the system. Because of solubility issues, it was revealed that the maximum loading capacity onto the 250 ml rotor in a 10 ml loop injection is 250 mg (25 mg/ml).

The next step was to determine the maximum loading volume of the 250 ml column. Injections in 5 ml increments from 10 ml to 25 ml (10% of V_c) were carried out. It can be concluded that the amount of pure material increases linearly, and with 25 ml injected into the column, the system was used in the most advantageous way.

As the maximum capacity of the 250 ml column was reached, the next step was to linearly scale the separation up to the 1000 ml column. This was performed in the same way as before, injections via a pump from 4 % of V_c to 10 % of V_c in increments of 20 mls were applied to the system. This series of experiments resulted, that the purified amount increases linearly therefore if more solute could be dissolved in the two-phase system, the purer material could be gained.

Purification of ergometrine

In the literature, there is no consensus on the excitation and emission wavelengths in the case of ErgM measurements by HPLC-FLD technique. Therefore, several wavelengths were examined, when a constant concentration of ErgM was injected into an HPLC instrument. It was concluded that the most sensitive excitation and emission

wavelengths are λ_{ex} = 245 nm and λ_{em} = 418 nm. After the wavelengths were selected, a reverse-phase chromatographic method was developed for the separation of ErgM.

When the HPLC-FLD method was finalized, ErgM production of *Claviceps purpurea* isolates was examined. *C. purpurea* isolates were cultivated on a liquid culture media, and their ErgM was extracted and quantified. *C. purpurea* (SZMC 25562) produced ErgM in the largest quantity, 33.15 ± 6.25 µg/ml, thus this fungus was selected for large-volume cultivation.

For the CPC separation of ErgM, a suitable solvent system had to be found. As same as before, the "best solvent" method was utilized. ErgM is highly soluble in lower alcohols, therefore ethanol was selected as the first solvent. This alcohol was paired with water as the more polar solvent, and with diethyl-ether as the less polar solvent. Two compositions (30/20/50 and 40/20/40 by volume) were selected from the ternary diagram of the diethyl-ether/ethanol/water system that definitely forms a two-phase. The distribution of ErgM was tested in 60 systems including three ethers, ten alcohols, and water in all possible combinations. From all the experiments diethyl-ether/isopropanol/water 40/20/40 (v/v/v%) was finally selected for the separation. In this solvent system, ErgM has a distribution coefficient (P) value of P = 0.99, which is ideal for the separation, indicating that the desired compound will elute with one column volume of the mobile phase.

After instrumental optimization ascendent mode was selected, therefore the lower, aqueous phase was used as stationary phase, the rotor speed was set to 2200 rpm, and the flow rate was 10 ml/min. 100 mg of crude ErgM extract was dissolved in 4.5 ml of both upper and lower phases and was injected into the system. The separation lasted for 30 minutes and ErgM eluted with the calculated column volume. This successful separation was repeated two more times, and the retention volumes did not differ, therefore it was concluded that the system was stable, and the separation is repeatable.

After the qualification and quantification of the final product by HPLC-FLD and HPLC-OHRMS techniques, each CPC run approximately 70 mg of pure (>98%) ErgM was gained. As a conclusion, a rapid, and cheap method was developed for the purification of ErgM.

6. Magyar nyelvű összefoglaló

A másodlagos metabolitokat a mikroorganizmusok akkor termelik, mikor növekedésük elér egy un. stacioner állapotot (idiofázis). Ezen vegyületek az alap életfunkciók normális működéséhez nem szükségesek, de a mikroorganizmus életében fontos szerepet játszanak. A szekunder metabolitok számos esetben rendelkeznek valamilyen bioaktivitással, lehetnek antibiotikumok, antifungális szerek, toxinok és akár a sejt védelmét szolgáló anyagok is.

Az *Aspergillus* fajok számos másodlagos metabolit termelésére képesek. Sok közülük toxikus, teratogén és mutagén mellékhatásokkal is rendelkezik, mikor az állatok vagy emberek szervezetébe kerülnek. Az egyik legszélesebb körben vizsgált, *Asperigllus* fajok által termelt toxincsoport az aflatoxinok (AFok) csoportja. A vegyületcsalád felfedezése akkor történt, mikor *Aspergillus* törzsekkel fertőzött Brazil földimogyoróval etettek angliai pulykákat a 19. században, amely azok tömeges elhullását okozta. Az esetet követő kutatás eredményeként, a kutatók sikeresen izolálták a négy fő aflatoxint. A vegyületeket az UV fényben mutatott fluoreszcenciájuk és a vékonyrétegkromatográfiás elúciós sorrendjük alapján nevezték el, mely alapján megkülönböztethetjük az aflatoxin B1-et, B2-t, G1-et és G2-t. A vegyületek felfedezésével egyidőben fény derült negatív hatásaikra is, amelyek vizsgálatához szükség volt a vegyületek kinyerésére, így a tisztításukra szolgáló különböző módszerek is kifejlesztésre kerültek.

Az ergometrin (ErgM) egy másodlagos metabolit, melyet főként a *Claviceps purpurea* faj képviselői termelnek. A vegyület az ergot alkaloidok családjába tartozik, a gyógyászatban gyakran alkalmazott vegyület, de instabilitása miatt főként szintetikus származékait és sóit használják fel. Az alapvegyület a gomba áttelelő szkleróciumából nagy mennyiségben izolálható, szerves oldószeres extrakcióval és elválasztástechnikai módszerek segítségével. A *C. purpurea* tenyészthető folyadék tápközegben is, melyből az alkaloidok szintén izolálhatók. Az ErgM, egy az Európai Unióban regisztrált drogprekurzor, ezért az élelmiszerekben és tápokban szintjének monitorozása rendkívül fontos lenne, viszont jelenleg semmilyen szabályozás nem vonatkozik a vegyület előfordulási koncentrációira.

A centrifugális megoszlásos kromatográfia (Centrifugal Partition Chromatography, CPC) egy olyan preparatív szeparációs technika, melyben két egymással nem elegyedő folyadékfázis között oszlanak meg a kinyerni kívánt komponensek és az elegy szennyezői. Ezen folyadék-folyadék kromatográfiás technikában az egyik folyadékfázist

(állófázis) egy centrifugális erő immobilizálja a forgó kolonnában, majd a másik fázist, mely a mozgófázis, egy pumpa nyomja át azon. A kiválasztott kétfázisú oldószerrendszertől függően az elválasztás során használható ún. felszálló és leszálló mód is. Felszálló, aszcendens módban az alsó fázis az állófázis, míg leszálló, deszcendens módban a felső fázis kerül immobilizálásra. Az, hogy az álló vagy a mozgó fázis a vizes vagy szerves fázis, a kiválasztott és oldószerrendszertől, azaz a fázisok sűrűségétől függ. Ahogy jelen tézisben is látható, ezen kromatográfiás technika számos új lehetőséget rejt magában, hiszen bármilyen kétfázisú oldószerrendszer alkalmazható, ha a kinyerni kívánt komponensek oldhatóak a rendszerben. A technika nem alkalmaz drága állófázisokat, és a használt oldószerek újra használhatóak.

Jelen kutatómunka során, AF és ErgM termelő gombafajokat azonosítottunk, melyeket később nagy léptékben tenyésztettünk a szekunder metabolitok nagyobb hozamának érdekében. Az izolált organizmusok által termelt másodlagos metabolitok kinyerésére optimalizáltuk az extrakciós módszereket és CPC elválasztásokat fejlesztettünk a vegyületek gyors és hatékony tisztítására, melynek megvizsgáltuk méretnövelési lehetőségeit is. A cél a négy AF és az ErgM elválasztása volt lehetőleg egy-egy kromatográfiás elválasztással. A tisztításokat követően elvégeztük a vegyületek minőségi és mennyiségi analízisét, valamint meghatároztuk a végtermékek tisztaságát.

Aflatoxinok tisztítása:

Az AF és szterigmatocisztin termelőképesség és termelési mennyiség felmérésének céljából 11 különböző táptalajon 10 *Aspergillus* faj 13 képviselőjét tenyésztettük. Szilárd táptalajon 9 gomba termelt valamilyen AF-t. Mind a négy AF-t csak az *A. parasiticus* különböző izolátumai (SZMC 2473, SZMC 22727 és SZMC 22728), illetve az *A. nomius* (SZMC 22631) termelték. A tápközegeket tekintve megállapítható, hogy AF termelés szempontjából a búza (M4), az árpa (M5) és a rizs (M6) a legmegfelelőbbek. Amikor ugyanezen gombákat folyadék tápközegben tenyésztettük, láthatóvá vált, hogy nagyobb mértékben ugyanazon négy törzs termelte mind a négy AF-t. A táptalajt tekintve kijelenthető, hogy a legnagyobb mennyiségű AF termelés a bio zöldségkoktél (M11) táptalajon volt megfigyelhető, de a gombák tenyésztésére a komplett malátás táptalaj (M9) és a burgonyadextróz tápoldat (M10) is megfelelő. Az *A. parasiticus* (SZMC 2473) nagyléptékű tenyésztésre az M9 táptalajt választottuk.

Az organizmus nagyléptékű tenyésztését követően egy négylépéses extrakciót dolgoztunk ki, a termelt AF-ek kinyerésére a tápközegből. Ezen nyers extraktumot a CPC módszer kifejlesztésére és optimalizálására használtuk. A módszerfejlesztéshez a "legjobb oldószer" módszert („*best solvent method*") alkalmaztuk, mely szerint választunk egy olyan oldószert, mely a tisztítani kívánt komponenseket a legjobban oldja, majd emellé párosítunk egy polárosabbat és egy apolárosabbat, így képezve egy három komponensből álló, azaz terner rendszert. Elsőként kloroformot és acetont választottunk legjobb oldószernek, de ezek hexánnal, heptánnal és toluollal, mint apoláros és vízzel, mint poláros oldószerekkel történő párosítása nem vezetett az AF-ek kielégítő megoszlására a fázisok között. A következőkben jégecetet párosítottunk dietil-éterrel, kloroformmal és toluollal, mint apoláros és vízzel, mint poláros oldószerekkel. Az AF-ek tekintetében a legjobb megoszlást a tolulol/ecetsav/víz = 30/24/50 térfogatszázalékos összetételű rendszer eredményezte, ezért ezt a rendszert használtuk a továbbiakban a vegyületek elválasztására.

A készülék 250 ml térfogatú oszlopát használva egy 75 perc hosszú módszert fejlesztettünk. A rotor forgási sebessége 2200 rpm-nek adódott, míg az áramlási sebességet 15 ml/percnek választottuk. 90 mg nyers AF extraktumot 4 ml alsó és 4 ml felső fázisban oldottuk, majd végrehajtottuk az elválasztást. A négy fő AF kevés átfedéssel, közel tökéletesen elválva egymástól eluálódott az oszlopról, míg a szennyezők fent maradtak azon. A rendszer stabilitását és ismételhetőségét három egymást követő injektálással teszteltük, mely tesztek eredményeként megállapítható, hogy a fejlesztett módszer ismételhető és stabil. A kinyert tiszta AF-okat bepároltuk, majd minőségük és mennyiségük ellenőrzése is megtörtént. A 4,5 l folyadékkultúrából összesen 1351 mg tiszta elválasztott AF-t nyertünk, átlagosan 97,3%-os tisztasággal és 92,6%-os visszanyeréssel.

A következőkben az elválasztás méretnövelését valósítottuk meg több lépésben. Először konstans injektált térfogat (10 ml) mellett növeltük az injektált mennyiséget (90 mg, 180 mg, 210 mg, 240 mg, 270 mg injektálása), ahol az oldhatósági problémák miatt a maximális injektálási mennyiséget 250 mg-ban határoztuk meg (25 mg/ml).

Ezután a maximális injektálási térfogat meghatározása történt meg, mely során 5 ml-es részletekben növeltük az injektált térfogatot 10 ml-től 25 ml-ig, mely az oszloptérfogat 10 %-a. Megállapítható, hogy a tiszta anyag mennyisége lineárisan növekszik az injektált térfogat növekedésével, és a 25 ml oldat injektálásával az oszlopot a lehető legjobban sikerült kihasználni.

Mivel elértük a 250 ml-es oszlop maximális kapacitását, a következő lépés az elválasztás lineáris léptéknövelése volt az 1000 ml térfogatú oszlop segítségével. Ez az előzőekben ismertetett módon történt, a készülékbe beépített pumpa segítségével 20 ml-es részletekben növeltük az injektált térfogatokat 4 %-os oszloptérfogattól 10 %-os oszloptérfogatig. A lineáris méretnövelési kísérlet eredményeként elmondható, hogy a kinyerhető tiszta AFok mennyisége is lineárisan nő, csak az oldhatóságtól függ.

Ergometrin tisztítása

Az irodalomban többféle információ is található az ErgM extinciós és emissziós hullámhosszait illetően HPLC-FLD technikával történő mérés esetén. Ezért munkánk során számos gerjesztési és kisugárzott hullámhosszat is teszteltünk konstans ErgM koncentráció injektálása mellett. Következtetésként levonható, hogy a vizsgált hullámhossz-párok közül a legmegfelelőbbnek a λ_{ex} = 245 nm és λ_{em} = 418 nm hullámhosszak bizonyultak. A hullámhosszak kiválasztása után egy fordított fázisú HPLC-FLD módszert fejlesztettünk az ergometrin elválasztására.

A módszer véglegesítése után *Claviceps purpurea* izolátumok ergometrin termelő képességét vizsgáltuk. Az izolátumokat folyadék tápközegben tenyésztettük, majd a termelt ErgM-t extrakcióval nyertük ki, és a kivonat ErgM tartalmát az előzőekben fejlesztett HPLC-FLD módszerrel kvantifikáltuk. A legtöbb célvegyületet a *C. purpurea* (SZMC 26662) termelte, 33,15 ± 6,25 μg/ml koncentrációban, ezért a nagyléptékű tenyésztéshez ezt a gombát választottuk.

Az ErgM CPC-vel történő tisztításához szintén meg kellett keresni a megfelelő oldószerrendszert, melyhez szintén a "legjobb oldószer" módszert alkalmaztuk. Az ErgM jól oldódik a kis szénatomszámú alkoholokban, ezért a legjobb oldószernek az etanolt választottuk. Ezt párba állítottuk vízzel, mint poláris és dietil-éterrel, mint apoláris oldószerrel. Két olyan összetételt (30/20/50 és 40/20/40 térfogat szerint) választottunk a dietil-éter/etanol/víz terner diagramjáról, melyek biztosan két fázist alkotnak. Az ergometrin megoszlását 60 rendszerben vizsgáltuk három éterrel, tíz alkohollal és vízzel minden lehetséges kombinációban. A kísérletek végén a dietil-éter/izopropanol/víz = 40/20/40 térfogatszázalékos összetételű rendszert választottuk az elválasztásra. Ebben a rendszerben az ErgM megoszlási hányadosa P = 0,99 volt, amely az elválasztás szempontjából megfelelő, és indikálja, hogy a tisztítani kívánt komponens oszloptérfogatnak megfelelő térfogatú mozgófázissal nyerhető ki az elegyből.

A műszeres optimalizálást követően aszcendens, felszálló módot választottunk, ahol az alsó, vizes fázis az álló fázis. A rotor forgási sebessége 2200 rpm volt, az áramlási sebesség pedig 10 ml/perc. 100 mg nyers extraktumot 4,5 ml alsó és 4,5 ml felső fázisban oldottunk, majd a készülékbe injektáltuk. Az elválasztás 30 perc hosszú volt, ahol az ergometrin a számításoknak megfelelően az oszloptérfogatnak megfelelő mobilfázis térfogatával eluálódott a rendszerből. Ezt a sikeres elválasztást még kétszer megismételtük, hogy megbizonyosodjunk a rendszer stabilitásáról és az elválasztás ismételhetőségéről. Ahogy az előzőekben, az optimalizált elválasztás stabilnak és ismételhetőnek bizonyult.

A végtermékek HPLC-FLD és HPLC-OHRMS technikákkal történő ellenőrzése után kijelenthető, hogy a három elválasztásból egyenként 70 mg körüli, legalább 98 % tisztaságú ErgM-t sikerült kinyerni. Végső megállapításként elmondható, hogy munkánk eredményeként az ErgM tisztítására sikeresen fejlesztettünk egy gyors és olcsó módszert.

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9. Supplementary information

Table 12. *ErgM production of C. purpurea isolates. The abbreviations are in the Materials and methods section.*

[a]SZMC	Ergometrine (mg/ml)	[b]SD
25561	0.84	0.3
25562	**33.15**	**6.25**
25563	11.11	1.26
25564	5.21	2.67
25565	16.03	0.02
25566	4.75	0.36
25567	1.22	0.21
25568	0.11	0.03
25569	3.86	0.19
25570	16.07	5.54
25571	5.43	1.88
25572	3.54	1.06
25573	0.00	0
25574	0.13	0.09
25575	19.16	0.19
25576	0.00	0

[a]Szeged Microbiology Collection
[b]Standard deviation

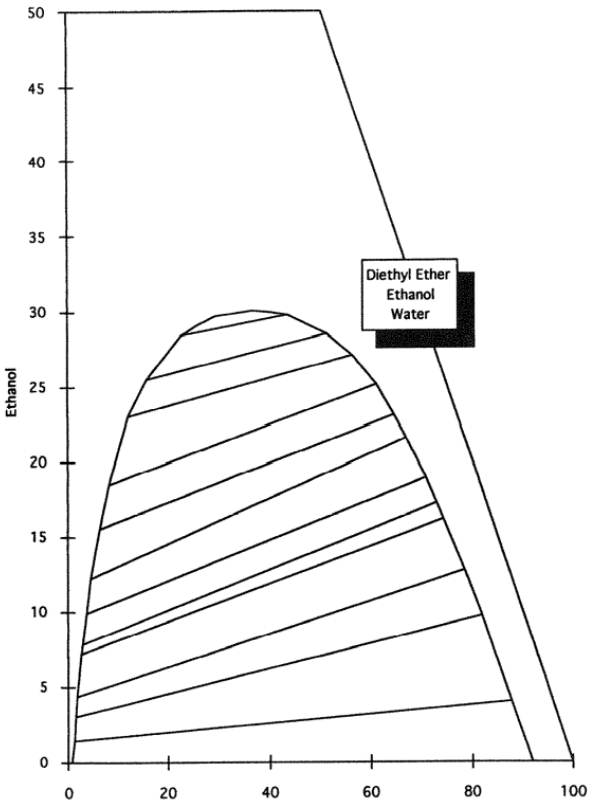

Figure 41. *Ternary diagram of diethyl-ether/ethanol/water [166].*

Table 13. *Cultivated fungi and their AF and STC production on different culture media. The abbreviations are in the Materials and methods section.*

SZMC	Fungi	Toxin	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
			µg/kg						µg/l				
12576	***A. flavus***	AFB_1	297.4	9353.8	-	80477.7	71364.9	16111.6	2544.0	-	4516.1	8082.2	42.7
		AFB_2	28.0	62.9	-	5032.0	4348.0	127.5	30.3	-	352.8	351.3	-
		AFG_1	-	-	-	-	-	-	-	-	-	-	-
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	-	21.7	-	72.6	82.9	7.3	-	-	-	-	-
2473	***A. parasiticus***	AFB_1	57226.7	86669.2	577.9	185288.3	160674.7	212562.8	2712.0	1135.5	4849.8	14449.7	17985.8
		AFB_2	3614.6	8880.1	36.0	28373.6	22726.9	33465.0	64.0	128.6	505.6	1130.2	800.1
		AFG_1	42031.9	65986.9	312.3	125764.6	128846.6	93294.6	3259.1	462.4	9391.4	8371.0	20116.2
		AFG_2	646.8	3042.5	137.7	8776.0	8292.6	3928.0	21.0	25.3	423.2	397.7	299.4
		STC	8.4	30.6	-	3.6	24.1	99.6	-	-	-	-	-
22727	***A. parasiticus***	AFB_1	14431.5	39206.6	362.6	117902.5	90774.4	131875.7	27.1	-	767.0	6233.5	4246.4
		AFB_2	338.4	3105.0	22.1	12696.6	9168.7	14223.3	6.7	-	34.7	364.2	31.2
		AFG_1	27126.0	53829.7	356.1	139606.3	100373.3	86155.6	3401.5	-	5189.5	5968.4	7840.8
		AFG_2	161.8	2101.4	10.5	8623.1	4544.3	3383.5	16.7	-	70.3	83.0	9.5
		STC	3.8	42.2	-	3.0	7.7	11.5	-	-	-	-	-
22728	***A. parasiticus***	AFB_1	7272.2	42239.1	863.9	87435.4	89484.4	103042.2	184.0	-	14.2	6811.3	4704.5
		AFB_2	141.9	3332.0	39.1	8231.6	9083.6	7429.6	10.4	-	16.1	404.8	30.1
		AFG_1	17738.2	59958.9	864.9	115677.3	100589.1	69574.2	1757.4	-	1609.5	7643.4	8856.3
		AFG_2	41.8	2396.1	26.4	7638.7	5493.6	1326.2	6.5	-	8.3	120.3	10.4
		STC	-	2.9	-	-	4.9	11.2	-	-	-	-	-
22631	***A. nomius***	AFB_1	51608.6	39897.4	1225.4	29471.0	33877.2	91584.5	1053.9	1893.1	16531.5	6209.2	5424.2
		AFB_2	2659.2	1642.2	201.1	1333.8	1640.7	7310.5	16.9	7.6	9.9	210.4	55.1
		AFG_1	92723.9	87350.4	8834.8	103167.7	95360.1	125752.3	16440.4	42.6	3670.6	8612.0	18997.8
		AFG_2	3556.3	3307.3	123.4	5222.1	4242.2	6844.7	169.2	1.2	10.7	26.6	301.4
		STC	200.7	148.3	12.0	151.7	89.3	57.2	1.8	-	-	0.7	11.7
22273	***A. pseudonomius***	AFB_1	30.3	60.1	-	119.4	58.6	331.8	-	-	-	-	-
		AFB_2	-	-	-	-	-	-	-	-	-	-	-
		AFG_1	156.1	218.3	-	335.2	149.6	385.5	-	-	-	-	30.8
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	-	-	-	-	-	-	-	-	-	-	-
22438	***A. minisclerotigenes***	AFB_1	15002.5	17623.4	-	44638.9	13215.6	300.1	18.5	-	170.6	869.9	65.9
		AFB_2	758.3	844.9	-	3945.7	260.0	-	22.4	-	14.0	25.9	-
		AFG_1	-	-	-	-	-	-	-	-	-	-	-
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	-	-	-	-	-	-	-	-	-	-	-

SZMC	Fungi	Toxin	M1	M2	M3	M4	M5	M6	M7	M8	M9	M10	M11
			µg/kg						µg/l				
2018	***A. pseudotamarii***	AFB_1	11461.1	25950.0	-	46345.5	22941.2	15034.5	10546.4	6.1	649.3	9513.7	53.7
		AFB_2	93.8	316.7	-	1075.9	280.5	231.6	153.4	-	10.6	155.7	1.8
		AFG_1	-	-	-	-	-	-	-	-	-	-	-
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	-	-	-	-	-	-	-	-	-	-	-
3055	***A. pseudotamarii***	AFB_1	9985.9	19844.6	556.1	46703.3	23417.3	69239.5	7575.7	78.5	18.9	4427.7	2675.2
		AFB_2	62.8	103.8	-	363.2	54.8	1002.7	-	-	-	21.5	11.3
		AFG_1	-	-	-	-	-	-	-	-	-	-	-
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	1.7	-	-	11.1	3.4	6.1	-	-	-	-	-
2024	***A. dimorphicus***	AFB_1	-	-	-	-	-	-	-	-	-	-	-
		AFB_2	-	-	-	-	-	-	-	-	-	-	-
		AFG_1	-	-	-	-	-	-	-	-	-	-	-
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	3.4	48.6	-	2.6	3.6	-	-	-	-	-	-
26961	***A. nidulans***	AFB_1	-	-	-	-	-	-	-	-	-	-	-
		AFB_2	-	-	-	-	-	-	-	-	-	-	-
		AFG_1	-	-	-	-	-	-	-	-	-	-	-
		AFG_2	-	-	-	-	-	-	-	-	-	-	-
		STC	49897.2	57362.5	5634.6	66863.2	39288.6	64965.0	-	-	-	-	22.3
20877	***A. amoenus***	AFB_1	-	-	Did not grow	-	-	-	-	-	-	-	-
		AFB_2	-	-		-	-	-	-	-	-	-	-
		AFG_1	-	-		-	-	-	-	-	-	-	-
		AFG_2	-	-		-	-	-	-	-	-	-	-
		STC	6119.0	20320.1		26019.7	5738.0	37394.4	-	155.4	0.4	711.7	25.0
23543	***A. tabacinus***	AFB_1	-	-	Did not grow	-	-	-	-	-	-	-	-
		AFB_2	-	-		-	-	-	-	-	-	-	-
		AFG_1	-	-		-	-	-	-	-	-	-	-
		AFG_2	-	-		-	-	-	-	-	-	-	-
		STC	32.5	29.6		2.2	-	2.8	-	0.3	-	-	-