Studies on the Phase I and II Metabolism and the Toxicological Analysis of the Alkaloids of the Herbal Drug of Abuse *Mitragyna speciosa* Korth. *(Kratom)* using Gas Chromatography-Mass Spectrometry and Liquid Chromatography Coupled to Low- and High-Resolution Linear Ion Trap Mass Spectrometry

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Meinen Eltern und
meiner Schwester Miriam

Nur die Sache ist verloren,

die man selber aufgibt.

Gotthold Ephraim Lessing (1729-1781)

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1 GENERAL PART

1.1 INTRODUCTION

1.1.1 *Mitragyna speciosa* (Rubiaceae)

More than ten species of *Mitragyna* plants have been found in swampy territories in the tropical and sub-tropical regions of Asia and Africa. *Mitragyna speciosa* Korth. is a member of the Rubiaceae (coffee) family, and is indigenous to Thailand and other Southeast Asian countries. *Kratom* is the original, common name used in Thailand.[1] The genus was given its name *Mitragyna* by Pieter Willem Korthals, official botanist with the Dutch East India Service from 1831 to 1836, because the shape of the stigma in this species resembled the shape of a bishop´s mitre. *Mitragyna speciosa* Korth. is characterized by a globular flowering head, bearing up to 120 florets each.[2]

1.1.2 Alkaloids of *Mitragyna speciosa*

The main alkaloids of *Kratom* are mitragynine (MG), paynantheine (PAY) and speciogynine (SG), whereof the first two are unique to *Mitragyna speciosa*. Depending on the plant species and/or plant part various alkaloids in varying concentrations could be identified. In summary, MG was found to be the main alkaloid in the leaves of adult plants of the Malaysian plant (over 10%) as well as of the Thai plant (over 60%). PAY and the MG diastereomer SG were the second abundant and the MG diastereomer speciociliatine (SC) the third abundant alkaloids in both plants.[3] In 1994 Ponglux *et al.*[4] could detected a new minor alkaloid (2%) in the leaves of *Mitragyna speciosa*, so called 7-hydroxy-mitragynine (7-OH-MG). Shellard *et al*.[5] described the distribution and probable biogenetic route of *M. speciosa* alkaloids in young and mature Thai leaves. The alkaloidal pattern is different in the young plants from that in the older trees. In leaves of young Thai plants SG is the main alkaloid, besides MG, PAY, SC, the MG diastereomer mitraciliatine (MC) and the PAY diastereomer iso-paynantheine (ISO-PAY). While in the fruits of Malaysian plants SC is the main alkaloid, besides MG, PAY and their diastereomers.[6] In Fig. 1, the chemical structures of MG and PAY and their diastereomers are depicted.

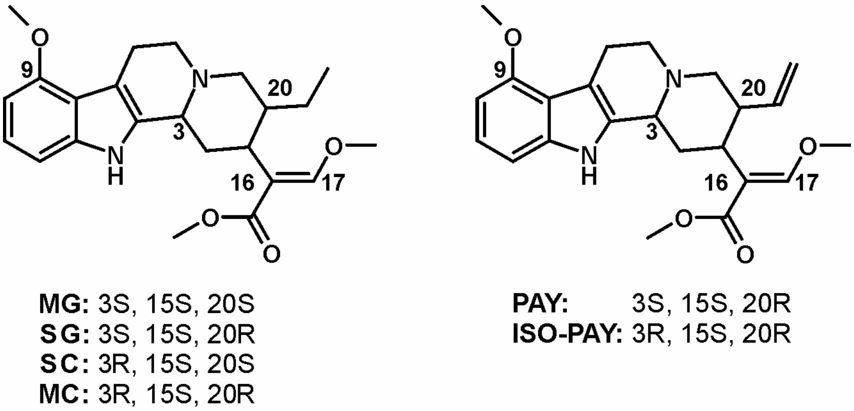

Fig. 1: Chemical structures of MG and PAY and their diastereomers SG, SC, MC, and ISO-PAY.

1.1.3 Pharmacology and Toxicology

Kratom is a well established psychoactive herbal drug. The leaves of this plant have been used in Thailand and other Southeast Asian countries for its opium-like effect and its coca-like ability to combat fatigue of and enhance tolerance to hard workers under a scorching sun.[7] In addition, it has been used as a traditional medicine for common illnesses such as coughing, diarrhea, muscle pain, hypertension and to cure morphine addicts.[8,9]

Pharmacological *in vivo* and *in vitro* investigations revealed that the main alkaloid MG shows anti-nociceptive effects on supraspinal opioid receptors, mainly of *μ*- and *δ*-subtypes.[10-13] The *μ*-receptor mediates analgesia, euphoria, and respiratory depression, which accounts for the analgesia activity of MG, as well as its amelioration of opiate withdrawal symptoms.[14] MG has also been postulated to be involved in the activation of descending noradrenergic and serotonergic pathways in the spinal cord.[10] In addition, animal studies suggest that MG has agonist activity at α_2-adrenergic receptors, perhaps contributing to its effect in mitigating opioid withdrawal symptoms.[15,16] However, the analgesic effect of MG is less potent than that of the crude extract of *Mitragyna speciosa*. The opium-like effect of this plant cannot be fully explained by that of MG. These findings suggest that other constituents of *M. speciosa* have very potent analgesic effects.[17] Pharmacological studies revealed, that 7-OH-MG has an opioid agonist property on *μ*- and/or *κ*-opioid receptors.[7] Matsumoto *et al.*[7] demonstrated via tail-flick and hot-plate tests on mices, that the anti-nociceptive effect of 7-OH-MG may be more potent than morphine, even after oral administration. Furthermore, the inhibitory effect of naloxone on anti-nociception induced by 7-OH-MG in mice indicates the involvement of opioid receptor systems in its action. It appears that MG and 7-OH-MG may be the active principle of the herb used in traditional medicine.[18]

The clinical effects of *Kratom* are dose-dependent and consist of stimulant effects at lower doses and opiate effects at higher doses in humans.[7,9] These effects have also been witnessed in animal models.[19] However, there is little information about the *Kratom* toxicity and the dose required in humans to produce stimulation, analgesia, and toxicity.[7,9,14] In animal models, MG has been shown to cause less respiratory depression than other narcotics.[20] Nevertheless, users who combine *Kratom* with central nervous system depressants may experience respiratory depression.[1] In this case, the opioid antagonist naloxone should be considered in addition to supportive care.[14]

Kratom users typically chew fresh leaves or make a tea from dried leaves, but some users smoke the dried leaves. As *Kratom* is widely misused as herbal drug of abuse, since a few years also in the Western countries, it is scheduled in Thailand, Australia and in the meantime also in some EU countries, but not yet in Germany and the United States of America. The wide availability of *Kratom* via the internet reflects extensive demand for this product. Additionally, a new herbal blend has recently appeared on the drugs of abuse market, named *Krypton*, a mixture of *Kratom* and the opioid agonist *O*-demethyl-tramadol (ODT).[1,14,21] ODT is more potent than the parent substances tramadol and is not yet scheduled in Germany.

1.1.4 Metabolism

In 1974, Zarembo *et al.*[22] studied the metabolism of MG by microbial transformation using *Helminthosporum* sp. However, the authors could not exclude that the two isolated metabolites - pseudoindoxyl MG and hydroxy pseudoindoxyl MG - were formed simply by air oxidation during the fermentation. Beckett *et al.*[23] investigated the metabolism of corynantheidine and 9-methoxycorynantheidine-type alkaloids using liver microsomes of rats, guinea pigs, and rabbits. MG, SG, and SC, some of the tested corynantheidine alkaloids, were metabolized only in rabbit liver microsomes by *O*-demethylation of the methoxy group in position 17. Whereas MC, one of the MG diastereomers, was metabolized by rabbit, rat, and guinea-pig liver microsomes by a different process, that yield unidentified products. *O*-Dealkylation of the enol ether group occurs more readily in planar than in non-planar isomers.

So far, no further information about the metabolism of the herbal drug *Kratom* is available in the literature. However, for developing toxicological screening procedures, especially in urine, it is a prerequisite to know the metabolism of the compounds in question, especially if they are excreted in urine primarily or even exclusively in form of metabolites. Furthermore, data on the metabolism are needed for toxicological risk assessment, because the metabolites may play a major role in the toxicity of a drug.

1.1.5 Isolation and Identity Confirmation of *Kratom* Alkaloids

1.1.5.1 Isolation of the Diastereomers of MG and PAY

MG was delivered from the Department of Forensic Medicine, Johannes Gutenberg University, Mainz, Germany, where it was isolated from *Kratom* leaves obtained from head&nature (Regensburg, Germany).[24] SG, SC, MC, PAY, and ISO-PAY were isolated and purified according to Ponglux *et al*.[4] and Klein *et al*.[24] Briefly, the dried, shredded leaves (123 g) were extracted with 2.1 l of methanol (MeOH) using Soxhlet extraction. The residue was dissolved in 500 ml of 10% aqueous acetic acid solution. The aqueous layer was washed with 4 x 200 ml of n-hexane, separated and basified with 10 M NaOH (pH 8-9) at 0 ºC and extracted with 4 x 170 ml of chloroform ($CHCl_3$).The organic layer was evaporated and purified by silicon dioxide (SiO_2) column chromatography (3 cm x 21 cm, particles of 50-200 µm) using the following mixtures: $CHCl_3$/ethylacetate (EtAc) (9:1; 500 ml; fraction *A* (*A1-10*)); $CHCl_3$/EtAc (4:1; 150 ml; *A11-13*), $CHCl_3$/EtAc (1:1; 150 ml; *A14-16*), EtAc (150 ml; *A17-19*), EtAc/MeOH (19:1; 150 ml; *A20-22*), EtAc/MeOH (4:1; 150 ml; *A23-25*), and dichloromethane (CH_2Cl_2) (250 ml; *A26-30*). The fraction (*A*) volume of the first SiO_2 column chromatography was 50 ml.

PAY/SG: The fraction *A15* of the first column chromatography of the crude base was subjected again to SiO_2 column chromatography (1 cm x 21 cm, particles of 35-70 µm) using $CHCl_3$/EtAc (4:1; 150 ml; *B1-15*), $CHCl_3$/EtAc (1:1; 250 ml; *B16-40*), and MeOH (100 ml; *B41-50*). The fraction volume (*B*) of the second column chromatography was 10 ml. The **PAY *(B5-9)*** and **SG *(B41-50)*** containing fractions were evaporated to dryness.

The fractions *A16-26* of the first column chromatography were subjected again to SiO_2 column chromatography (3 cm x 24 cm, particles of 50-200 µm) using $CHCl_3$/EtAc (9:1; 500 ml; *C1-10*), $CHCl_3$/EtAc (4:1; 150 ml; *C11-13*), $CHCl_3$/EtAc (1:1; 150 ml; *C14-16*), EtAc (150 ml; *C17-19*), EtAc/MeOH (19:1; 150 ml; *C20-22*), EtAc/MeOH (4:1; 150 ml; *C23-25*), CH_2Cl_2 (250 ml; *C26-30*), MeOH (100 ml; *C31-32*), MeOH/Water (H_2O) (1:1; 100 ml; C*33-34*). The fraction (*C*) of the third SiO_2 column chromatography was 50 ml.

SC: The fractions of *C16-20* of the third column chromatography were subjected again to SiO_2 column chromatography (1 cm x 28 cm, particles of 35-70 µm) using $CHCl_3$/EtAc (4:1; 150 ml; *D1-15*), $CHCl_3$/EtAc (1:1; 130 ml; *D16-28*), and MeOH (100 ml; *D29-38*). The fraction volume (*D*) of the fourth SiO_2 column chromatography was 10 ml. The **SC *(D7-11 and D17-24)*** containing fractions were evaporated to dryness.

ISO-PAY: The fractions of *C22* and *C31* of the third column chromatography were subjected again to SiO_2 column chromatography (1 cm x 28 cm, particles of 35-70 µm) using $CHCl_3$/EtAc (4:1; 100 ml; *E1-10*), $CHCl_3$/EtAc (1:1; 100 ml; *E11-20*), EtAc (100 ml; *E21-30*), EtAc/MeOH (1:1; 100 ml; *E31-40*), MeOH (50 ml; *E41-45*), MeOH/H_2O (1:1; 50 ml; *E46-50*). The fraction volume (*E*) of the fifth SiO_2 column chromatography was 10 ml. The **ISO-PAY *(E27-29)*** containing fractions were evaporated to dryness.

MC: The fractions of *C32* of the third column chromatography were subjected again to SiO_2 column chromatography (1 cm x 27 cm, particles of 35-70 µm) using $CHCl_3$/EtAc (4:1; 100 ml; *F1-10*), $CHCl_3$/EtAc (1:1; 100 ml; *F11-20*), EtAc (100 ml; *F21-30*), EtAc/MeOH (2:1; 60 ml; *F31-36*), EtAc/MeOH (1:1; 70 ml; *F37-43*), and MeOH/H_2O (1:1; 50 ml; *F44-48*). The fraction volume (*F*) of the sixth SiO_2 column chromatography was 10 ml. The **MC *(F29-30)*** containing fractions were evaporated to dryness.

As given below, gas chromatography-mass spectrometry (GC-MS) in the electron ionization (EI) mode was used for the detection of all MG and PAY diastereomers in the eluate fractions.

1.1.5.2 Identity and Purity Confirmation of the Isolated Alkaloids

During the isolation process identity check was performed by GC-MS. Therefore, 10 µl of each respective fraction was diluted with MeOH (ratio 1:10) and analyzed using a Trace GC Ultra gas chromatograph combined with a DSQ II MS (Thermo Fisher Scientific, TF, Dreieich, Germany) with Xcalibur 1.4 software. The GC conditions were as follows: splitless injection mode; column, TF TR-5MS capillary (15 m x 0.25 mm I.D.), cross linked silphenylene-based phase, 250-nm film thickness; injection port temperature, 280 °C; carrier gas, helium; flow-rate, 1.2 ml/min; column temperature, programmed from 80 to 310 °C at 30 °C/min, initial time 2 min, final time 5 min. The MS conditions were as follows: full scan (FS) mode, *m/z* 50-650 u; EI mode, ionization energy, 70 eV; ion source temperature, 240 °C; capillary direct interface heated at 280 °C. The isolated and purified alkaloids were identified by comparison of the underlying mass spectra with the published reference spectra.[25]

Further structure and purity confirmation was proved by liquid chromatography-high resolution mass spectrometry (LC-HRMS), ^{1}H-NMR, and ^{13}C-NMR. Therefore, 10 µl of each isolated alkaloid was diluted with MeOH (ratio 1:1000) and analyzed by LC-HRMS with an Orbitrap (OT) analyzer providing the empirical formula of the corresponding molecular ion and fragments. The samples were analyzed by a TF Accela LC system consisting of a degasser, a quaternary pump and an autosampler coupled to a TF LTQ Velos Orbitrap MS system equipped with a heated electrospray ionization (ESI) source. The LC conditions were as follows: TF Hypersil Gold C18 column (100 x 2.1 mm, 1.9 µm); column temperature, 35 °C; mobile phase A consisted of 10 mmol/l aqueous ammonium formate buffer containing 0.1% (v/v) formic acid (final pH 3), mobile phase B consisted of acetonitrile containing 0.1% (v/v) formic acid; solvent gradient for metabolism studies: 0.00-5.00 min: 98% mobile phase A, flow rate 0.5 ml/min; 5.00-35.00 min: continuous increase of mobile phase B to 24%, flow rate 0.5 ml/min; 35.01-42.00 min continuous increase of mobile phase A to 98%, flow rate 0.4 ml/min; 42.00-58.00 min continuous increase of mobile phase

B to 80%, flow rate 0.4 ml/min; 59.00-67.00 min re-equilibration to start conditions, flow rate 0.4 ml/min. The total run time was 67 min.

The MS conditions for the OT were as follows: source heater temperature, 250 °C, positive ionization mode; sheath nitrogen gas flow rate of 20 AU; auxiliary gas, 20 AU; source voltage, 3.0 kV; ion transfer capillary temperature, 300 °C; S-lens RF level, 60%. CID-MS/MS experiments were performed on following precursor ions selected from MS^1: 399.22838 *m/z* and 397.21273 *m/z* with a resolution of 15.000. Additionally, FS was performed from 100-800 *m/z* with a resolution of 60.000. MS^3 and MS^4 were performed in the IDA mode: MS^3 on the most and second most intense signal from the MS^2 and MS^4 on the most and second most intense signal from MS^3. Normalized collision energies were 35 % in MS^2, 40% in MS^3, 45% in MS^4 respectively. Other settings were as follows: minimum signal threshold: MS^3: 125 counts with a resolution of 7.500, MS^4: 75 counts with a resolution of 7.500, isolation width, 1.5 u; activation Q, 0.25; activation time, 16 ms; dynamic exclusion mode, repeat counts 2, repeat duration 5 s, exclusion list 50, exclusion duration 5 s.

The elemental composition of all diastereomers of MG and PAY could be confirmed by HRMS. The measured (m) and calculated (c) protonated molecular ions in the MS^1 spectra, the empirical formula, and the mass errors in ppm of all diastereomers of MG and PAY are given in Table 1.

Tab. 1: Accurate masses of the measured [m] and calculated [c] protonated molecular ions in the MS^1, their empirical formula, and the mass errors in ppm of all diastereomers of MG and PAY.

Alkaloid	**Protonated molecular ion (m) [*m/z*]**	**Protonated molecular ion (c) [*m/z*]**	**Empirical Formula**	**Mass errors [ppm]**
Mitragynine	399.22754	399.22838	$C_{23}H_{31}O_4N_2$	- 0.736
Speciogynine	399.22711	399.22838	$C_{23}H_{31}O_4N_2$	+ 0.176
Speciociliatine	399.22708	399.22838	$C_{23}H_{31}O_4N_2$	- 3.262
Mitraciliatine	399.22751	399.22838	$C_{23}H_{31}O_4N_2$	- 2.185
Paynantheine	397.21173	397.21273	$C_{23}H_{29}O_4N_2$	- 2.523
Iso-Paynantheine	397.21152	397.21273	$C_{23}H_{29}O_4N_2$	- 3.052

For structure confirmation by nuclear magnetic resonance (NMR) spectroscopy, a solution of each alkaloid (4 mg/0.7 ml) was prepared in $CDCl_3$ or acetone-d_6. ^{1}H-NMR (500 MHz) and ^{13}C-NMR (125 MHz) together with 2D NMR ^{1}H-^{1}H correlated spectroscopy (COSY), nuclear Overhauser and exchange spectroscopy (NOESY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) were recorded on a Bruker DRX 500 (Bruker, Rheinstetten, Germany) at 300 K. The chemical shifts were given in δ values (ppm) relative to $CHCl_3$ at δ_H 7.24 or $CDCl_3$ at δ_C 77.00 and for acetone at δ_H 2.05 and acetone-d_6 at δ_C 29.80. The ^{1}H-NMR and ^{13}C-NMR data of all diastereomers of MG and PAY are depicted in Table 2 and 3.

Tab. 2: ^{1}H NMR data of Mitragynine (MG), Speciogynine (SG), Speciociliatine (SC), Mitraciliatine (MC), Paynantheine (PAY), and Iso-Paynantheine (ISO-PAY).

	MG[a]	SG[b]	SC[a]	MC[a]	PAY[a]	ISO-PAY[a]
3	3.14 *brd* (11)	3.57 *m*	4.35 *brs*	4.91 *brs*	3.27 *brd* (11)	5.00 *m*
5	2.89 *m* 2.51 *m*	n.d.	3.59 *m* (2H)	3.53 *m* (2H)	3.06 *m* 2.55 *m*	3.59 *m* (2H)
6	3.10 *m* 2.51 *m*	3.41 3.26	3.26 *m* 3.00 *m*	3.27 *m* (2H)	3.17 *m* 2.99 *m*	3.32 *m* 3.22 *m*
10	6.43 *brd* (8)	6.49 *brd* (8)	6.36 *brd* (8)	6.50 *brd* (8)	6.44 *brd* (8)	6.51 *brd* (8)
11	6.97 *dd* (8/8)	7.00 *dd* (8/8)	6.88 *dd* (8/8)	7.11 *dd* (8/8)	6.98 *dd* (8/8)	7.13 *dd* (8/8)
12	6.88 *brd* (8)	6.93 *brd* (8)	6.90 *brd* (8)	7.06 *brd* (8)	6.86 *brd* (8)	7.07 *brd* (8)
14	2.49 *m* 1.78 *m*	2.43 *m* (2H)	2.41 *m* 1.96 *m*	2.75 *m* 2.22 *d* (14)	2.10 *m* 1.94 *m*	2.76 *dd* (15/12.5) 2.29 *brd* (15)
15	3.02 *ddd* (14/4/4)	2.98 *m*	2.59 *ddd* (14/4/4)	2.32 *brdd* (12/12)	2.74 *ddd* (12/12/3)	2.49 *ddd* (12/12/3)
17	7.41 *s*	7.52 *brs*	7.37 *brs*	7.31 *s*	7.31 *s*	7.28 *s*
18	0.85 *t* (7.5;3H)	0.86 *t* (7.5;3H)	0.83 t (7.5;3H)	0.73 t (7.5;3H)	4.98 *dd* (17.5/2) 4.93 *dd* (10.5/2)	5.02 *brd* (17.5) 4.98 *brd* (10.5)
19	1.77 *m* 1.18 *m*	1.48 *m* 1.17 *m*	1.43 *m* 1.20 *m*	1.37 *m* 0.87 *m*	5.56 *m*	5.25 *m*
20	1.60 *m*	2.63 *m*	1.78 *m*	2.59 *m*	3.03 *m*	3.36 *m*
21	2.99 *dd* (12/2.5) 2.43 *dd* (12/3)	3.75 *brd* (11.5) 3.14 *m*	3.21 *m* 2.72 *m*	3.20 *brd* 2.79 *dd*	3.01 *m* 2.27 *m*	3.15 *dd* (12/4) 3.00 *dd* (12/12)
9-OCH_3	3.86 *s* (3H)	3.89 *s* (3H)	3.82 *s* (3H)	3.88 *s* (3H)	3.85 *s* (3H)	3.89 *s* (3H)
17-OCH_3	3.71 *s* (3H)	3.85 *s* (3H)	3.75 *s* (3H)	3.79 *s* (3H)	3.76 *s* (3H)	3.79 *brs* (3H)
$COOCH_3$	3.69 *s* (3H)	3.67 *s* (3H)	3.60 *s* (3H)	3.66 *s* (3H)	3.67 *s* (3H)	3.66 *s* (3H)
NH	7.65 *brs*	10.12 *brs*	9.12 *brs*	8.78 *brs*	7.66 *brs*	8.79 *brs*

Tab. 3: ^{13}C NMR data of Mitragynine (MG), Speciogynine (SG), Speciociliatine (SC), Mitraciliatine (MC), Paynantheine (PAY), and Iso-Paynantheine (ISO-PAY).

	MG[a]	**SG**[b]	**SC**[a]	**MC**[a]	**PAY**[a]	**ISO-PAY**[a]
2	133.73	n.d	130.63	n.d	133.7**	n.d
3	61.25	61.3*	55.86	55.30	60.05	55.78
5	53.78	n.d	51.25	51.49	53.24	51.16
6	23.94	22.28	21.66	18.10	23.72	18.05
7	107.91	n.d	108.82	105.83	107.88	105.84
8	117.77	117.35	116.80	116.87	117.54	116.81
9	154.53	155.34	153.95	154.27	154.51	154.32
10	99.79	100.48	99.35	99.96	99.79	100.09
11	121.82	124.02	122.35	123.59	121.96	123.90
12	104.15	105.67	105.11	105.20	104.18	105.21
13	137.25	139.27	137.79	138.27	137.31	138.34
14	29.98	33.2*	34.28	30.51	33.44	29.33
15	39.92	36.45	35.80	33.4*	38.5*	n.d.
16	111.56	110.40	109.99	109.1**	111.5**	n.d
17	160.47	162.1	161.17	160.64	159.82	160.68
18	12.85	10.67	12.00	10.56	115.47	118.42
19	19.09	24.28	21.69	23.77	139.1*	135.44
20	40.70	37.1*	38.36	36.51	42.87	39.90
21	57.78	59.20	49.55	49.95	61.34	49.83
9-OCH$_3$	55.32	55.49	55.03	55.20	55.31	55.22
17- OCH$_3$	61.51	62.31	61.62	61.97	61.56	62.0*
COOCH$_3$	51.32	51.3*	51.34	51.25	51.29	51.52
COO	169.20	n.d	169.23	168.2**	172.2**	168.6**

n.d.: not detected

[a] measured in CDCl$_3$, [b] measured in acetone-d$_6$

*taken from the HSQC

** taken from the HMBC

2D HHCOSY, HSQC, HMBC and NOESY spectra were measured for all compounds and used for the assignments

The identity was checked by comparison of the NMR spectra with those published by Kitajima *et al.*[6] and with the help of HHCOSY and HSQC. The proton spectra recorded in different solvents at ambient temperature showed the same extensive line broadening of the aliphatic resonances as described for dihydrocorynantheine,[26] its 9-deoxy derivative. This is due to the fact that at room temperature several conformers are present in slow exchange on the NMR time scale.

1.2 AIMS AND SCOPES

The Thai medicinal plant *Mitragyna speciosa* (*Kratom*) is widely misused as herbal drug. Besides this, a new herbal blend has appeared on the drugs of abuse market, named *Krypton*, a mixture of ODT and *Kratom.* Therefore, an intake of such drugs must be monitored in clinical and forensic toxicology and doping control.[27] As many psychotropic drugs are extensively metabolized, they can be detected particularly in urine only via their metabolites.[28] So far there is no information about the metabolism of *Kratom* alkaloids available, so that the aims of these presented studies were:

- Isolation of all diastereomers of mitragynine and paynantheine from Thai *Kratom* leaves

- Identification of phase I and II metabolites in rat and human urine using liquid chromatography coupled to low- and high-resolution mass spectrometry

- Development of a urine screening procedure using GC-MS

- Systematic evaluation of 120 human urine samples of *Kratom* and *Krypton* users for typical markers using GC-MS

2 PUBLICATIONS OF THE RESULTS

The results of the studies were published in the following papers:

2.1 STUDIES ON THE METABOLISM OF MITRAGYNINE, THE MAIN ALKALOID OF THE HERBAL DRUG KRATOM, IN RAT AND HUMAN URINE USING LIQUID CHROMATOGRAPHY-LINEAR ION TRAP MASS SPECTROMETRY[29]

(DOI: 10.1002/JMS.1607)

2.2 USE OF LIQUID CHROMATOGRAPHY COUPLED TO LOW- AND HIGH-RESOLUTION LINEAR ION TRAP MASS SPECTROMETRY FOR STUDYING THE METABOLISM OF PAYNANTHEINE, AN ALKALOID OF THE HERBAL DRUG *KRATOM* IN RAT AND HUMAN URINE[30]

(DOI: 10.1007/s00216-009-3239-1)

2.3 PHASE I AND II METABOLITES OF SPECIOGYNINE, A DIASTEREOMER OF THE MAIN *KRATOM* ALKALOID MITRAGYNINE, IDENTIFIED IN RAT AN HUMAN URINE BY LIQUID CHROMATOGRAPHY COUPLED TO LOW- AND HIGH-RESOLUTION LINEAR ION TRAP MASS SPECTROMETRY[31]

(DOI: 10.1002/JMS.1848)

2.4 METABOLISM STUDIES OF THE *KRATOM* ALKALOID SPECIOCILIATINE, A DIASTEREOMER OF THE MAIN ALKALOID MITRAGYNINE, IN RAT AND HUMAN URINE USING LIQUID CHROMATOGRAPHY-LINEAR ION TRAP MASS SPECTROMETRY[32]

(DOI: 10.1007/s00216-011-4660-9)

2.5 METABOLISM STUDIES OF THE *KRATOM* ALKALOIDS MITRACILIATINE AND ISOPAYNANTHEINE, DIASTEREOMERS OF THE MAIN ALKALOIDS MITRAGYNINE AND PAYNANTHEINE, IN RAT AND HUMAN URINE USING LIQUID CHROMATOGRAPHY-LINEAR ION TRAP MASS SPECTROMETRY[33]

(DOI: 10.1016/J.JCHROMB.2011.03.005)

2.6 MONITORING OF *KRATOM* OR KRYPTON INTAKE IN URINE USING GC-MS IN CLINICAL AND FORENSIC TOXICOLOGY[34]

(DOI: 10.1007/ s00216-010-4464-3)

3 CONCLUSIONS

The used LC-MS technique allowed the identification of all diastereomers of MG and PAY and their phase I and II metabolites in rat and human urine. All diastereomers of MG and PAY and their metabolites could be differentiated in the human samples from the diastereomeric MG and PAY and their metabolites considering the mass spectra and the different retention times determined after application of the single alkaloids to rats. The studies presented here showed that the diastereomers of MG and PAY were mainly metabolized by hydrolysis of the methylester in position 16 and *O*-demethylation of the 9-methoxy group. Further steps were the *O*-demethylation of the 17-methoxy group, followed, via the corresponding aldehydes, by oxidation to carboxylic acids or reduction to the correspondent alcohols, and combinations of some of these steps. As metabolic phase II reactions partial glucuronidation and sulfation were observed. The metabolism studies showed that all diastereomers of MG and PAY were extensively metabolized in rats and humans with some differences, particularly in phase II metabolism.[29-33]

The GC-MS screening procedure allowed the detection of the studied compounds in rat urine after administration of common drug abusers' doses mainly via their metabolites. Systematic evaluation of 120 human urine samples of *Kratom* and *Krypton* users clearly indicated, that the described toxicological screening procedure should be suitable to monitor *Kratom* use as well as *Krypton* use via the detection of MG, its metabolites and/or ODT.[34]

However, it should be kept in mind that the human urine samples were single samples submitted for drug testing without information on the application form (tea, resin, extract, young/old plants, species etc.), dosage, administration route, and/or sampling time.

4 SUMMARY

In the presented studies, the herbal drug *Kratom (Mitragyna speciosa)* was investigated regarding its metabolism and its toxicological analysis in rat and human urine. Depending on the plant species and plant parts the three most abundant alkaloids of *Mitragyna speciosa* are MG, PAY and the MG diastereomer SG. Further alkaloids are the MG diastereomers SC and MC and ISO-PAY the diastereomer of PAY.

The diastereomers of MG and PAY were mainly metabolized by hydrolysis of the methylester in position 16 and *O*-demethylation of the 9-methyoxy group. Further steps were the *O*-demethylation of the 17-methoxy group, followed, via the corresponding aldehydes, by oxidation to carboxylic acids or reduction to the correspondent alcohols, and combinations of these steps. As metabolic phase II reactions, partial glucuronidation and sulfation were observed. The metabolism study showed that all diastereomers of MG and PAY were extensively metabolized in rats and humans with some differences, particularly in phase II metabolism. In the case of *Kratom*, the target analytes for the toxicological analysis were the derivatized MG parent substance, the 9-*O*-demethyl metabolite of MG, the free carboxy group metabolite of MG, and the 9-*O*-demethyl-16-carboxy metabolite of MG.

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6 ABBREVIATIONS

A	fraction *A*
B	fraction *B*
C	fraction *C*
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
COSY	correlated spectroscopy
D	fraction *D*
E	fraction *E*
EI	electron ionization
ESI	electrospray ionization
EtAc	ethyl acetate
F	fraction *F*
FS	full scan
GC-MS	gas chromatography-mass spectrometry
H_2O	water
HMBC	heteronuclear multiple bond correlation
HSQC	heteronuclear single quantum coherence
ISO-PAY	iso-paynantheine
LC-HRMS	liquid chromatography-high resolution mass spectrometry
MC	mitraciliatine
MeOH	methanol
MG	mitragynine
NaOH	sodium hydroxide solution
NMR	nuclear magnetic resonance

NOESY	nuclear Overhauser effect spectroscopy
ODT	*O*-demethyl-tramadol
7-OH-MG	7-hydroxy-mitragynine
OT	Orbitrap
PAY	paynantheine
SC	speciociliatine
SG	speciogynine
SiO_2	silicon dioxide
TF	Thermo Fisher Scientific

7 ZUSAMMENFASSUNG

Im Rahmen dieser Dissertation wurden der Metabolismus und die Nachweisbarkeit der pflanzlichen Droge *Kratom (Mitragyna speciosa)* im Urin von Ratte und Mensch untersucht. Abhängig von der Pflanzenspezies und den Pflanzenteilen sind die drei Hauptalkaloide der Pflanze *Mitragyna speciosa* MG, PAY und das MG Diastereomer SG. Weitere Alkaloide sind die MG Diastereomere SC und MC sowie ISO-PAY, das das Diastereomer von PAY ist.

Alle Diastereomere von MG und PAY wurden hauptsächlich durch Hydrolyse des Methylesters in Position 16 und durch *O*-Demethylierung in Position 9 metabolisiert. Weitere Metabolismusschritte waren die *O*-Demethylierung in Position 17, gefolgt über die Zwischenstufe der Aldehyde, die entweder durch Oxidation in Carbonsäuren oder durch Reduktion in die entsprechenden Alkohole überführt wurden und Kombinationen aus diesen Schritten. Als Phase-II-Reaktionen konnten partielle Glucuronidierung oder Sulfatierung gefunden werden. Die Metabolismusstudien in Ratte und Mensch haben gezeigt, dass alle Diastereomere von MG und PAY einer intensiven Metabolisierung unterlagen, die sich hauptsächlich im Phase II Metabolismus unterschieden haben. Im Falle einer *Kratom* Einnahme sind die Zielsubstanzen im toxikologischen Nachweisverfahren die derivatisierte Muttersubstanz MG, der 9-*O*-Demethyl Metabolit von MG, der freie Carbonsäure Metabolit von MG und der 9-*O*-Demethyl-16-Carboxy Metabolit von MG.