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Kratom: The analytical challenge of an emerging herbal drug

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ABSTRACT

Mitragyna speciosa or kratom is emerging worldwide as a "legal" herbal drug of abuse. An increasing number of papers is appearing in the scientific literature regarding its pharmacological profile and the analysis of its chemical constituents, mainly represented by alkaloids. However, its detection and identification are not straightforward as the plant material is not particularly distinctive. Hyphenated techniques are generally preferred for the identification and quantification of these compounds, especially the main purported psychoactive substances, mitragynine (MG) and 7-hydroxymitragynine (7-OH-MG), in raw and commercial products. Considering the vast popularity of this recreational drug and the growing concern about its safety, the analysis of alkaloids in biological specimens is also of great importance for forensic and toxicological laboratories. The review addresses the analytical aspects of kratom spanning the extraction techniques used to isolate the alkaloids, the qualitative and quantitative analytical methods and the strategies for the distinction of the naturally occurring isomers.

1. Introduction

Mitragyna speciosa (Korth.) Havil., better known as kratom, is a tropical tree indigenous of Thailand, Borneo, Malaysia, Myanmar, Sumatra, New Guinea, and the Philippines [1]. The genus Mitragyna comprises ten species with four occurring in Africa (*M. inermis, M. ledermannii, M. rubrostipulata* and *M. stipulosa*) and six in South and South-East Asia, between India and New Guinea (*M. speciosa, M. tubulosa, M. rotundifolia, M. parvifolia, M. hirsuta* and *M. diversifolia*). The species *M. speciosa* was first described by the Dutch botanist Pieter Willem Korthals in 1839 (*M. speciosa* (Korth.)) [2] and eventually given its final name and classification in 1897 by George Darby Haviland (*M. speciosa* (Korth.) Havil.) [3].

From a botanical point of view, the kratom tree has dark glossy green and elliptical petiolate leaves, which present 12–17 pairs of veins [1]. The latter are generally used to distinguish three kinds of kratom: *red veined kratom, green veined kratom* and a third kind with two small teeth-like formations next to the apex of the leaf [4]. The green-vein type is also reported in the literature as *white-vein kratom* [5]. The colour of the veins affects the potency of the product ascribing a higher effect to the green-vein kratom [5]. On the other hand, other studies report the vein colour also as a sign of the leaf age with the red-vein type being younger and more potent than the green-vein type [6].

In the last 150 years kratom use has been documented in Southeast Asia and reported to give both a stimulant effect to stave off fatigue by chewing fresh leaves and an analgesic and relaxing effect by brewing the leaves into a tea [7]. It is also used to manage opium withdrawal, as well as an antispasmodic, muscle-relaxant, and antidiarrheal agent [8]. From a legal point of view, the use of kratom is prohibited in Malaysia under the Poisons Act of 1952 and Thailand lifted the ban on the use, production, and possession of kratom in 2018 for medicinal purposes, although its use remains widely spread in local communities [9,10]. Over the years, kratom has been regulated as a legal herbal product under US FDA and DEA policies, although a few US states have banned the local sale and possession of kratom [11]. The DEA tried to ban kratom by instituting an emergency scheduling of its active ingredients in 2016 [12] but had to withdraw its notice of intent as it caused a strong adverse public reaction [13]. In 2021 the WHO started a pre-review process of kratom, which ended up with the recommendation that even though the contribution of kratom use to the reported kratom-related fatalities is unclear, the scientific data was not sufficient to recommend a critical review, thus it should be taken under surveillance due to the opioid-like activity of some of its constituents [14]. Even though there is insufficient evidence on both therapeutic and abuse potential [14], at present kratom remains a self-medication in some countries, but a much wider diffusion

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is expected in the next years [15]. As the interest in kratom has increased in the last ten years, not only for therapeutic purposes but also as a recreational drug, the scientific research, especially in Thailand and Malaysia, has made great progresses [16]. Consequently, the number of publications concerning several aspects of this herbal drug has exponentially grown, especially in the last five years in which it has even tripled (Scopus results for "kratom").

The aim of this review is to collect all the scientific contributions to the chemical analysis of kratom and its bioactive constituents and to highlight the tricky aspects and challenges especially of kratom alkaloids analysis. To the best of the authors' knowledge, no other review has been published on this topic since analytical research on kratom started. The pharmacological properties of kratom will not be reviewed in the present work as they are beyond the scope and the literature teems with this type of contributions.

2. Chemical composition of kratom

The claimed opioid-like activity of kratom is to be ascribed to the pool of alkaloids present in the leaves, of which mitragynine (MG) and 7-hydroxymitragynine (7-OH-MG) have caught the largest interest amongst the scientific community [17,18]. To get an idea, 7-OH-MG has an approximately five-fold higher affinity at the μ-opioid receptor compared to MG [19]. Moreover, 7-OH-MG exhibited a potency 40 times higher than MG and 10 times higher than morphine in antinociception assays [20,21], whereas MG demonstrated a lower nociceptive response compared to morphine [22]. Besides MG and 7-OH-MG, kratom is reported to contain variable levels of more than 40 known alkaloids [23], but also other secondary metabolites like dietary flavonoids ((-)-epicatechin, apigenin, quercetin and their glycoside derivatives, rutin, isoquercitrin, hyperoside, kaempferol) and other phenolic compounds (caffeic acid and chlorogenic acid), saponins (sitosterol, stigmasterol, and daucosterol), triterpenoids (ursolic acid and oleanoic acid), triterpenoid saponins (quinovic acid 3-O-β-D-quinovopyranoside and quinovic acid 3-O-β-D-glucopyranoside), monoaryl glycosides (1-O-feruloyl-β-Dglucopyranoside and benzyl-β-D-glucopyranoside), cyclohexenone glycosides (3-oxo-α-ionyl-O-β-D-glucopyranoside and (6*S*,9*R*) roseoside), and secoiridoid glycosides (vogeloside and epivogeloside) have also been isolated or identified in kratom [1,24–27].

Considering their pharmacological activity alkaloids have raised greater attention compared to the other compounds. The total alkaloid content in dried leaves varies from 0.5% to 1.5%, with the most abundant compounds represented by indoles, mainly of the corynanthe type, including oxindole derivatives, which can be found in tetra- or pentacyclic rings [23]. Structural variability generally encountered for indole and oxindole alkaloids is due to an additional hydroxy or methoxy group at C-9, unsaturation at C-3, C-5, or C-18, hydroxylation at C-7, and various configurations at C-3, C-7, and/or C-20 [23].

MG (Fig. 1) is the major alkaloid in the kratom plant reaching levels as high as 66% of the total alkaloid content in Thai varieties and presents the corynanthe-type skeleton with a tetracyclic tetrahydro-β-carboline, consisting of four cycles with a methoxyl substitution at C-9, an ethyl group at C-20, and a β-methoxy acrylate moiety at C-15 [28]. Malaysian varieties are reported to contain lower concentrations of MG (up to 12% of total alkaloids) [29]. Initially isolated by Field in 1921 [30], its structure was definitely elucidated only in 1965 [31] with the confirmation of the absolute stereochemistry by X-ray crystallographic analysis [32]. The content of MG in kratom leaves varies upon plant age, tissue and origin, but also genetic and morphogenetic factors, plant defence system against pathogenic attacks, and environmental factors, including light, UV exposure, moisture, temperature, soil microorganisms, soil fertility, salinity, storage conditions [7,33].

The second major alkaloid is paynantheine (PAY), which accounts for 10% of the total alkaloid content, followed by speciogynine (SG) (7%), 7-OH-MG (2%), speciociliatine (SC) (1%), mitragynaline, mitragynalinic acid, corynantheidaline, and corynantheidalinic acid (Fig. 1) [20,34–36]. In particular, 7-OH-MG is a

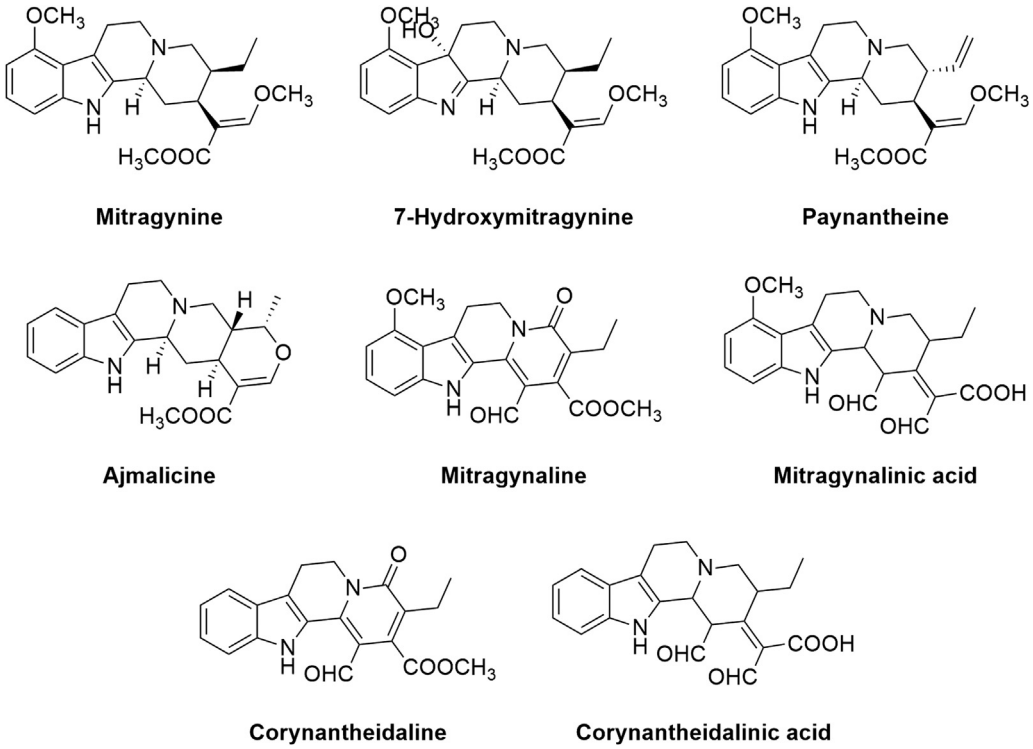

Fig. 1. Main indole kratom alkaloids. Chemical structure of the mail indole kratom alkaloids.

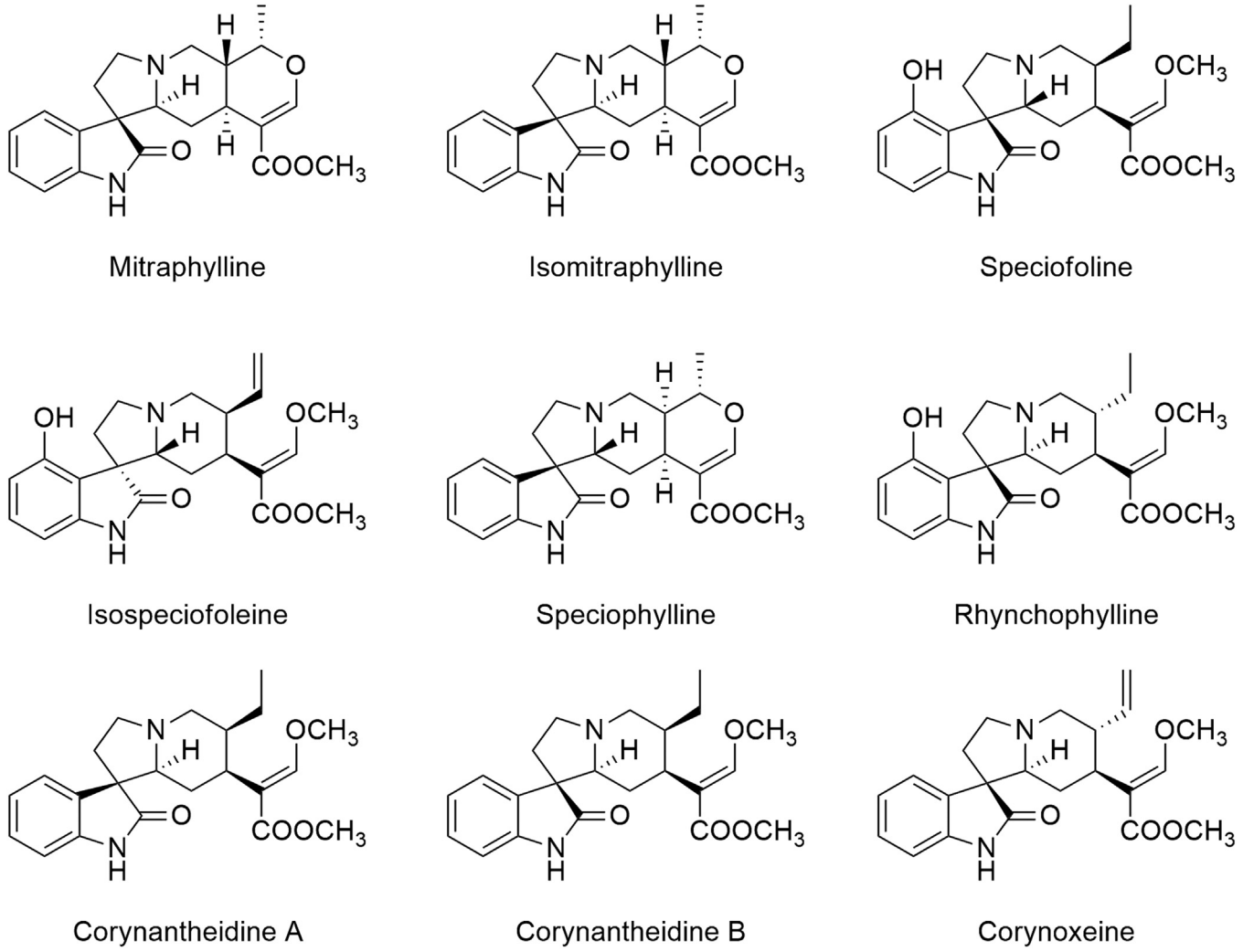

Fig. 2. Main oxindole kratom alkaloids. Chemical structure of the mail oxindole kratom alkaloids.

metabolite of MG formed by the oxidation of the 2–3 indole double bond. Kruegel demonstrated that a photooxidation reaction could take place either through irradiation with visible light in the presence of rose Bengal, under air or pure O_2 atmosphere (58% yield by NMR) or at room temperature under the sunlight without the addition of an external reducing agent, albeit in low yield (8% by NMR) [19]. This suggested that such conversion could occur also *in vivo* in the plant or, more likely, in dry leaf material that has been exposed to the sunlight for prolonged time. Probably, the presence of strongly coloured phytochemicals, such as porphyrins, could intervene in the reaction as a substitute for rose Bengal [19].

Besides the aforementioned indole alkaloids, other relevant kratom alkaloids are those belonging to the oxindole class bearing an N–C=O group, such as mitraphylline and isomitraphylline, speciofoline, isospeciofoleine, speciophylline, corynoxine A (CORY A) and corynoxine B (CORY B), and rhynchophylline (Fig. 2) [37].

3. Alkaloids stereochemistry

The presence of three stereogenic centres and an *E-Z* isomerism in the double bond on the methoxy acrylate group in the structure of MG lead to the possible existence of sixteen different stereoisomers. However, only four stereoisomers are reported to occur naturally in *M. speciosa*, all with the *E* stereochemistry at the double bond on the C-15 methoxy acrylate group: MG (3*S*,15*S*,20*S*), SC (3*R*,15*S*,20*S*), SG (3*S*,15*S*,20*R*), and mitraciliatine (MC) (3*R*,15*S*,20*R*) (Fig. 3) [1,24]. SC and SG together account for an average of 8–9% of the total alkaloid content in kratom leaves, while MC is the least abundant with less than 1% of the total alkaloid content [24].

Similarly, 7-OH-MG possesses four stereogenic centers and an *E-Z* isomerism at the double bond on the methoxy acrylate group, which can generate sixteen potential stereoisomers, but only three are found naturally in *M. speciosa*: 7-OH-MG (3*S*,7*S*,15*S*,20*S*), 7-hydroxyspeciociliatine (7-OH-SC) (3*R*,7*R*,15*S*,20*S*), and 7-hydroxymitraciliatine (7-OH-MC) (3*R*,7*R*,15*S*,20*R*), all presenting the *S* configuration and the *E* stereochemistry at the double bond on the C-15 methoxy acrylate group (Fig. 3) [24].

The second major indole alkaloid PAY has three stereogenic centers at C-3, C-15 and C-20 and an *E-Z* isomerism at the double bond on the methoxy acrylate group. Although eight stereoisomers can exist, only three have been identified so far: PAY (3*S*,15*S*,20*R*), iso-PAY (3*R*,15*S*,20*R*) and epiallo-iso-PAY (3*R*,15*S*,20*S*), all preserving the *S* configuration at C-15 and the *E* stereochemistry at the double bond on the C-15 methoxy acrylate group (Fig. 3) [23,38,39].

4. Alkaloids extraction

Regardless of the technique employed, the alkaloids extraction yield is affected by several factors, such as temperature and pH. According to Basiliere and Kerrigan, MG and its diasteromers (SC

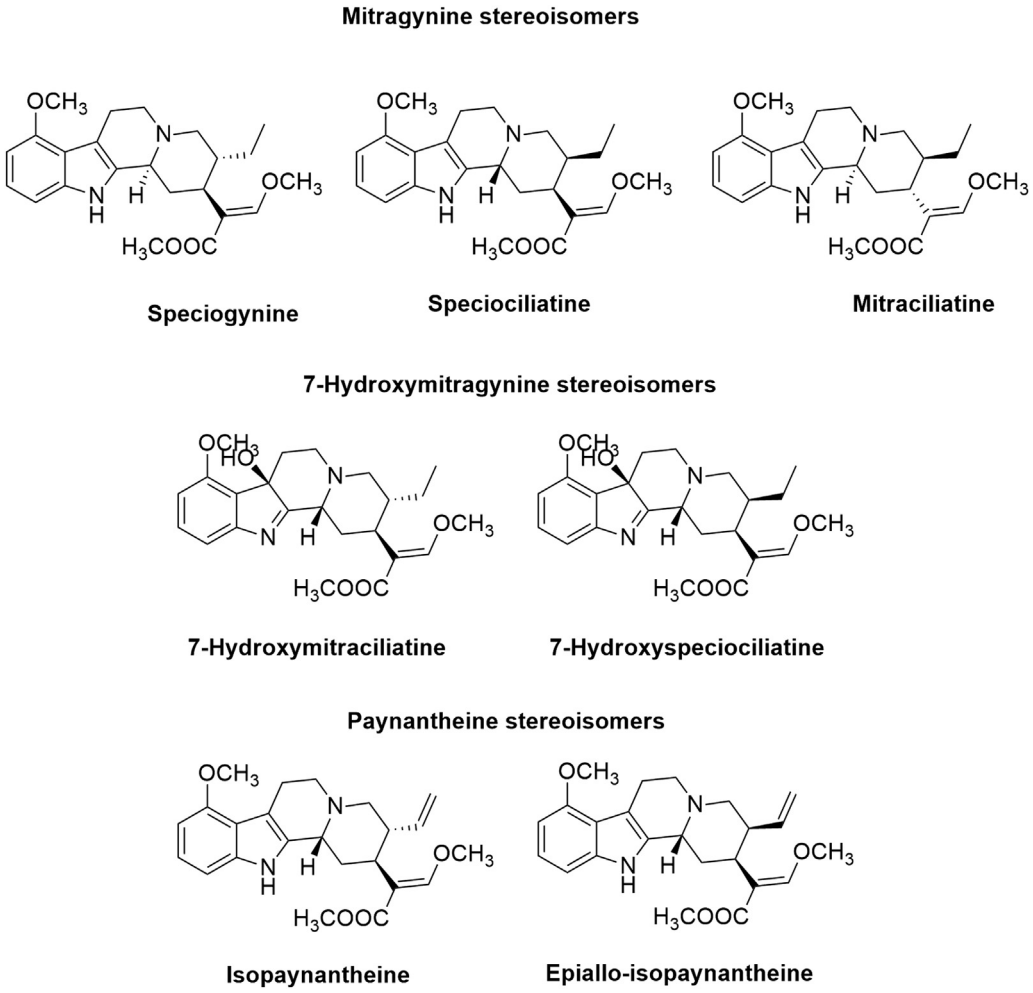

Fig. 3. Naturally occurring stereoisomers of MG, 7-OH-MG and PAY. Chemical structure of the naturally occurring stereoisomers of the main indole alkaloids MG, 7-OH-MG and PAY.

and SG) and PAY demonstrated higher stability compared to 7-OH-MG, which was the least stable alkaloid exhibiting a significant loss after 8 h (at any pH) at 40 °C [40]. However, the authors reported that MG undergoes chemical hydrolysis in alkaline conditions at the methyl ester group to produce the metabolite 16-carboxymitragynine [40]. Moreover, all alkaloids tested were acid labile (pH 2, 80 °C) [40]. As a result, extraction methodologies involving sample heating (e.g. Soxhlet) may dramatically affect the extraction yield.

4.1. Plant material

Numerous methods have been developed for the extraction of kratom alkaloids with particular attention to MG and 7-OH-MG. The most widely employed protocols are collected in Table 1 with relative sample matrix and analytical technique used. Alkaloids are generally soluble in acidic water or organic solvents, such as chloroform ($CHCl_3$), methanol (MeOH) or dimethylsulfoxide (DMSO), while their salts are more likely to be extracted in aqueous solvents at neutral pH [41]. Indeed, a typical extraction of kratom alkaloids involves alcoholic solvents, such as MeOH [42,43] and ethanol (EtOH) [44], but also their aqueous mixtures [45,46], added in a classical 10:1 ratio with the plant material [47]. However, based on the extraction methodology and subsequent analytical technique used, the sample to solvent ratio can vary from 1:5 to 1:1000 for fresh and dried leaves [37,44–46,48–52]. Although they cannot be properly defined as plant material, commercial products have also been reported as analysed samples for the determination of the alkaloid content and herein included as plant material. In this kind of samples, the sample to solvent ratios applied are equally variable as for the raw plant material (from 1:2 to 1:100) [37,42,46,49,52–55].

The most frequently employed techniques are maceration in MeOH [42,43,46,56] and Soxhlet extraction with yields in alkaloid fraction ranging from 0.80 mg/g to 16 mg/g [57–59]. Even though these extraction procedures are simple and relatively unexpensive, they are time- (up to seven days) and solvent consuming, which is also translated in high risk for human and environmental health [60]. In order to increase the alkaloid extraction yield, an acid-base extraction is often performed [23,38,47,58,59,61,62]: the plant material is suspended in acidic water (pH 2–3), which converts alkaloids into their water-soluble salt form, and washed with an organic solvent (e.g. petroleum ether, ethyl acetate (EtOAc), hexane, etc.). Then, the pH is brought to about 9 and an alkaloid-rich fraction is recovered with an organic solvent, typically dichloromethane (CH_2Cl_2) or EtOAc. Exploiting this additional step, Flores-Bocanegra et al. were able to recover an alkaloid-rich fraction of 37.5 mg/g from which nineteen alkaloids were isolated and characterized [23].

Less harmful techniques have also been explored, including ultrasound assisted extraction (UAE) of $CHCl_3$/MeOH mixture (1:4, *v/v*) [45,63], generally improving the extraction yield of the alka-

Table 1
Analytical strategies employed for the analysis of kratom alkaloids in plant material and derived products.

Analytical method	Matrix	Extraction methodology	Sample/solvent ratio (g/mL or mL/mL)	Identified compounds	LLOQ	Ref.
UHPLC-HR-ESI-MS (LTQ Orbitrap XL)	Plant material	Maceration in $CHCl_3$/MeOH (1:1, *v/v*) and 10% aqueous KOH, 24 h, r.t., acid-base treatment and extraction with $CHCl_3$	1:0.125	MG, SC, SG, MC, PAY, iso-PAY, epiallo-iso-PAY, MG-*N*(4)-oxide, SC-*N*(4)-oxide, iso-PAY-*N*(4)-oxide, epiallo-iso-PAY-*N*(4)-oxide, speciofoline, isorotundifoleine, isospeciofoleine, CORY A, CORY B, 3-epirhynchophylline, 3-epi-CORY B, and corynoxeine	Qualitative method	[23]
UHPLC-HRMS (Q Exactive Plus Hybrid Quadrupole-Orbitrap)	Plant material and commercial products	Maceration in MeOH, 24 h, 20 °C, 150 rpm	1:100	7-OH-MG, PAY, MG, SG, SC, MC, ajmalicine, iso-PAY, isomitraphylline, isospeciofoleine, speciofoline, CORY A, CORY B, and rhynchophylline	9.77 ng/mL	[37]
GC-MS	Leaves	Extraction with MeOH, 24 h, four times. Drying, acid-base treatment and extraction with EtOAc	1:20	MG, SC, SG, PAY, iso-PAY, CORY A, CORY B	Qualitative method	[38]
UHPLC-MS-DAD	Leaves	Extraction with MeOH, 24 h, four times. Drying, acid-base treatment and extraction with EtOAc	1:20	MG, SC, SG, PAY, iso-PAY, CORY A, CORY B	Qualitative method	[38]
SFC-DAD	Leaves	Extraction with MeOH, 24 h, four times. Drying, acid-base treatment and extraction with EtOAc	1:20	MG, SC, SG, PAY, iso-PAY, CORY A, CORY B	Qualitative method	[38]
DART-HRMS	Commercial products	Maceration in MeOH overnight	1:10	MG	5 μg/mL	[42]
UHPLC-HRMS (Q-ToF[a])	Dry leaves	Extraction with EtOH (95 % *v/v*), three times over three days	1:5	MG, 7-OH-MG, PAY, SG, SC, corynantheidine, CORY A, CORY B, isocorynantheidine, mitraphylline	1 ng/mL	[44]
LC-UV-MS	Leaves, powder, resin	UAE in 80% aqueous MeOH, 1 h, storage overnight	1:200 – 1:1000	MG, 7-OH-MG	1 μg/mL (MG), 1 ng/mL (7-OH-MG)	[45]
HPLC-UV	Plant material and commercial products	Extraction with MeOH	1:100 (dry material) 1:2 (liquids)	MG, 7-OH-MG	0.6 μg/mL	[46]
LC-DAD-MS	Leaves	Comparison of: 1. Maceration (r.t, 3 h). 2. Oil bath (50°C, 3 h) 3. UAE (r.t., 1 h) 4. MAE (110 °C, 60 W, 1 h) 5. SFE-CO_2 (65 °C, 300 bar, 12 kg/h CO_2 flow, 45 min). Solvents used: MeOH, EtOH, MeOH:H_2O 1:1 (*v/v*), or EtOH:H_2O 5:95 (*v/v*, pH 3).Acid-base treatment and extraction with CH_2Cl_2.	1:10	MG and other four non specified alkaloids	Qualitative method	[47]
HPLC-HRMS (Q-ToF)	Fresh and dried leaves	Boiling the fresh leaves in water and partitioning with dichloromethane, 0.5 h	1:20	MG, PAY, SG	31.25 μg/mL (MG), 15.62 μg/mL (PAY, SG)	[48]

(*continued on next page*)

Table 1 (*continued*)

Analytical method	Matrix	Extraction methodology	Sample/solvent ratio (g/mL or mL/mL)	Identified compounds	LLOQ	Ref.
HPLC-UV	Plant material and commercial products	Comparison of: 1 Water extraction, 4 h 2 MeOH extraction, 10 days Acid-base treatment and extraction with $CHCl_3$	1:3 (water extracts) 1:10 (MeOH extracts)	MG	0.5 µg/mL	[49]
GC-MS	Dried leaves	UAE in $CHCl_3$:MeOH (1:4, *v/v*), 30 °C, 1 h	1:6.25	MG	50 µg/mL	[50]
DART-HRMS	Plant material	No extraction	-	MG	5 µg/mL	[51]
IMS	Powder, liquid, capsule, ground leaves	Extraction with MeOH, 1 min	1:60 (liquids) 1:200 (powder) 1:20 (capsules)	MG	0.1 µg/mL	[52]
HPLC-MS/MS (ion trap)	Powder, liquid, capsule, ground leaves	UAE in MeOH, 20 min	1:100	MG	6 ng/mL	[52]
DART-TD-MS	Capsules, bulk powder, and bulk plant material	Vortexing in $H_2O/CHCl_3$ (1:1, *v/v*)	1:40	MG	LOD 200 ng	[53]
portable handheld MS	Capsules, bulk powder, and bulk plant material	Vortexing in $H_2O/CHCl_3$ (1:1, *v/v*)	1:40	MG	LOD 80 ng	[53]
IMS	Capsules, bulk powder, and bulk plant material	Vortexing in $H_2O/CHCl_3$ (1:1, *v/v*)	1:40	MG	LOD 65 ng	[53]
portable FTIR spectroscopy (FT-IR)	Capsules, bulk powder, and bulk plant material	Vortexing in $H_2O/CHCl_3$ (1:1, *v/v*)	1:40	MG	ND	[53]
IA (ELISA)	Cocktail samples and leaves	Boiling in H_2O, 2 h (leaves) Basification and partitioning with $CHCl_3$, five times (all samples)	1:5 (leaves)	MG, PAY, SG	32.9 µg/mL	[54]
DART-MS	e-liquids, resins, and powders	Sonication in MeOH (resins and powders)	1:100	MG, 7-OH-MG	Qualitative method	[55]
LC-MS and LC-HRMS	Leaves	Extraction with Soxhlet in MeOH. Acid-base treatment and extraction with $CHCl_3$	1:17	PAY, SG	Purification method	[58,59]
HPLC-MS/MS (QqQ[b]) with HILIC	Leaves	Acid-base treatment and extraction with hexane and CH_2Cl_2	1:60	MG	Purification method	[61]
UHPLC-MS/MS (Q-ToF)	Leaves	Sonication in MeOH, 30 min, and centrifugation, three times. Acid-base treatment and extraction with EtOAc.	1:200	MG, 7-OH-MG, CORY A, CORY B, 7β-OH-7*H*-MC, SG, PAY, SC, iso-PAY, isospeciofoline, isospeciofoleine, isorotundifoline,	Qualitative method	[62]
TLC	Leaves	Sonication in EtOH, 30 min	1:5	MG	LOD 1 µg/mL	[92]
GC-MS	Dried plant material and resins	UAE in MeOH, 20 min	1:20	MG	Qualitative method	[93]
IA (immunochromatographic strip)	Cocktail samples and leaves	Extraction with $CHCl_3$ five times (cocktail samples) Sonication in MeOH, 30 min, five times (leaves)	1:5 (cocktail samples) 1:50 (leaves)	MG	LOD 0.6 mg/mL (cocktail samples) LOD 1 mg/mL (leaves)	[94]

[a] Q-ToF: Quadrupole-Time of Flight.
[b] QqQ: triple quadrupole.

loid fraction, which results comparable to that obtained with the acid-base treatment but avoiding time-consuming and laborious procedures [47,64]. Innovative technologies besides UAE have also been used as alternative extraction methods, such as microwave assisted extraction (MAE), supercritical fluid extraction (SFE-CO_2), and accelerated solvent extraction (ASE) with the advantage of being less solvent and time consuming [47,50,65]. For example, Orio et al. compared the extraction yield of the alkaloid fraction from plant material obtained with different techniques including classic maceration, oil bath, UAE, MAE and SFE-CO_2 with MAE affording the highest yield (16.6 mg/g) [47]. Notably, the SFE-CO_2 yielded the smallest alkaloid fraction (below 1 mg/g) [47]. Goh et al. explored the use of ASE with different solvents (water, MeOH, EtOH and EtOAc) for the extraction of MG: with less than 100 mL of solvent in 5 min the extraction yield ranged from 53 to 291 mg/g, the highest value obtained using MeOH [65].

The growing interest towards kratom alkaloids has prompted the research to the isolation of these phytoconstituents from the plant by means of various chromatographic techniques, although encompassing some difficulties regarding the purity of the isolated compounds. This issue is due to the arduous separation of isomeric alkaloids, such as MG stereoisomers. Nonetheless, the literature reports some successful isolation of MG [65], SG and SC [66] with high purity (>98%), which is a key requirement for pharmacological testing.

4.2. Biological samples

Extraction of kratom alkaloids from biological samples is more complicated as each specimen needs a proper pre-treatment. So far, the literature reports analysis of kratom alkaloids mainly in urine and to a lesser extent in blood, vitreous, liver, bile, and gastric content, most of them taken from post-mortem specimens. While blood samples only need to be deproteinized to be analysed, urine samples generally undergo acidification and enzymatic digestion in a first step as the metabolites of the analytes are mainly present as their conjugated forms. Another typical biological specimen analysed for toxicological purposes is hair, which needs to undergo a series of pre-treatment steps including washing, drying, and pulverization. Then a proper extraction process is carried out for all specimens and generally involves the use of an organic solvent, such as acetonitrile (ACN), MeOH, EtOAc, methyl *tert*-butyl ether (MTBE) or mixtures of organic solvents. Table 2 summarizes the most common extraction and sample pre-treatment methods applied to each biological specimen. Typical sample to solvent ratios range from 1:1 to 1:8 for blood [67–72] and from 1:1 to 1:25 for urine [70,72–76]. Hair generally requires concentrated extracts (1:1.5) [77], although Carlier et al. were able to quantify MG in hair with a 1:40 sample to solvent ratio obtaining good sensitivity (limit of quantification (LOQ) 0.5 ng/mL) in spite of a low recovery (49% for MG) in a screening method for the determination of thirty-four toxic principles of plant origin [78].

Due to the low concentration of the analytes generally found in biological specimens, choosing a suitable sample preparation step prior to the analysis is extremely important. Solid-liquid extraction (SPE) seems the most promising sample preparation procedure as it reduces potential interferences from the matrix, providing cleaner chromatograms and leading to improved sensitivity, precision, and accuracy, besides requiring short total analysis time and low sample volumes [69,73,74,79,80]. The SPE procedure involves the loading of the extract onto properly conditioned cartridges and elution with either MeOH, MTBE or mixtures of organic solvents, such as *n*-butyl chloride (*n*-BuCl):EtOAc and dichloromethane/isopropanol/ammonium hydroxide (CH_2Cl_2/*i*PrOH/NH_4OH). The cartridges employed include simple reversed-phase C18 stationary phases [81,82] and the strong mixed-mode cation exchange sorbents for selective extraction of basic compounds [58,59,78,83,84]. Nonetheless, extraction efficiencies, which range from 73% [83] to 96% for MG [73] and from 63% to 96% for the other alkaloids [73] in urine, are comparable to those obtained with standard liquid-liquid extraction, which are in the range 77.8–100% for the same biological specimens [61,75,76]. Notwithstanding these satisfactory extraction efficiencies, lower values can be expected for 7-OH-MG due to its physicochemical properties, which confer a marked instability, thus requiring the selection of the appropriate internal standard [69].

Interestingly, only one literature record deals with oral fluids analysis and applied a recently developed sample collection technique called dried oral fluid spots (DOFs), which consists of spotting a very low volume of oral fluid on a filter paper and letting it dry before storage [85]. In the same work, the authors applied a combination of SPE and salting-out assisted liquid-liquid extraction (SALLE) obtaining an average 55% recovery for MG [85]. The SALLE technique consists of the addition of salt and an organic solvent, e.g. ACN, to simultaneously allow protein precipitation and separation of the analytes of interest from the water-miscible organic solvent [86]. Compared to the simple protein precipitation, the SALLE technique yields cleaner extracts due to a real phase separation [86].

In a more in-depth evaluation of the appropriate extraction technique, it is of utmost importance to consider that not all alkaloids are stable in any conditions and this has relevant implications in the pre-analytical steps. Indeed, MG was found to be stable in plasma at room temperature for 6 h [80] and in refrigerated urine for two weeks [87]. Moreover, potassium oxalate/sodium fluoride is reported to preserve blood concentrations of MG up to 7 days at room temperature and up to 30 days when refrigerated or frozen, but a decrease in concentration greater than 20% has been reported from 30 to 90 days at any temperature [68]. As regards its metabolite 7-OH-MG, its concentrations in heparinized rat plasma are maintained constant for 12 h at room temperature and for 30 days at 20 °C [71]. Additionally, 7-OH-MG decreased by 27% after 2 h in simulated gastric fluids (pH 1.2), of which 23% accounted for its conversion into MG, while it decreased by 6% in simulated intestinal fluids (pH 6.8) [88]. As for the analysis of the metabolites in urine, both phase I and phase II derivatives should be extracted. An enzymatic deconjugation is generally performed for phase II metabolites but sulphated species are reported to be resistant to enzymatic hydrolysis [76,89]. Therefore, chemical hydrolysis could be a valuable alternative, although some metabolites (e.g. 7-OH-MG and 9-*O*-desmethylmitragynine) rapidly decrease in acidic conditions [74]. The use of alkaline conditions may preserve some species, while some others are both acid and alkaline labile [40,88,90].

These considerations should be taken into account when delays occur from the time of sample collection to that of sample pre-treatment and extraction or re-analysis for inter-laboratory results validation. Moreover, given the thermal and pH lability of basically all kratom alkaloids, results may be compromised by the use of strongly acidic or alkaline conditions for the extraction as well as high temperatures during deconjugation of urine metabolites and evaporation steps [91].

5. Alkaloids analysis

Several methods have been developed, and often validated, for the identification and quantification of kratom alkaloids in various matrices. Being an emerging drug of abuse, numerous methods have been developed for the qualitative and quantitative determination of the known presumed psychoactive alkaloids MG and 7-OH-MG for forensic purposes in biological fluids, such as urine, serum and post-mortem tissues (from Scopus search “kratom” AND

Table 2
Analytical strategies employed for the analysis of kratom alkaloids in biological specimens.

Analytical method	Matrix	Extraction methodology	Sample/solvent ratio (g/mL or mL/mL)	Identified compounds	LLOQ	Ref.
LC-MS and LC-HRMS	Rat urine	Acidification, enzymatic digestion and SPE with MeOH/aqueous ammonia (98:2, *v/v*)	1:1	SG and its metabolites PAY and its metabolites	Qualitative method	[58,59]
HPLC-MS/MS (QqQ[a]) with HILIC	Human urine	Extraction with MTBE	1:1.5	MG	0.1 ng/mL	[61]
UHPLC-MS/MS (QqQ)	Plasma	Protein precipitation	1:8	corynantheidine	1 ng/mL	[67]
UHPLC- MS/MS (QqQ)	Blood	Basification, extraction with 70:30 (*v/v*) *n*-BuCl:EtOAc	1:8	MG	5 ng/mL	[68]
HPLC-HRMS (Q-ToF[b])	Post-mortem blood and liver	Protein precipitation, acidification, SPE with hexane, EtOAc and MeOH.	1:2	MG, 7-OH-MG, SC, SG, and PAY	0.5 ng/mL (MG), 1 ng/mL (PAY), 2 ng/mL (7-OH-MG, SG, and SC)	[69]
GC-MS SIM[c]	Post-mortem blood, vitreous, liver, urine and gastric	Buffering at pH 6, SPE with CH_2Cl_2/*i*PrOH/NH_4OH, 78/20/2, *v/v/v*)	1:2	MG	0.05 µg/mL	[70]
UHPLC-MS/MS (QqQ) with HILIC column	Rat plasma	Extraction with MTBE	1:5	MG	1 ng/mL	[71]
LC-MS/MS (QTRAP)	Post-mortem urine, blood, tissue	Hydrolysis, protein precipitation, basification and extraction with *n*-BuCl	1:3	MG	1 ng/mL	[72]
HPLC-HRMS (Q-ToF)	Urine	Acidification, SPE with hexane, EtOAc and MeOH	1:1	MG, 7-OH-MG, SC, SG, and PAY	0.5 ng/mL (MG, SG, SC), 1 ng/mL (7-OH-MG, PAY)	[73,74]
HPLC-DAD	urine	Bar adsorptive microextraction, 4 h, 1300 rpm, pH 5.5, sonication MeOH/ACN (1:1, *v/v*), 10 min	1:25	MG	0.33 ng/mL	[75]
UHPLC-MS/MS (Q-LIT[d]) and HPLC-MS/MS (QqQ)	Urine	Hydrolysis, basification, and extraction MTBE	1:15	MG	1 ng/mL	[76]
HPLC- MS/MS (QqQ)	Hair	Washing, extraction with H_2O:MeOH (1:1, *v/v*), 4 h, then SPE with MeOH	1:1.5	MG, 7-OH-MG	4 pg/mg (MG), 30 pg/mg (7-OH-MG)	[77]
UHPLC-MS/MS	Whole blood, urine, bile, hair	SPE with MeOH (liquid samples). Sonication in a methanolic solution, 2 h, and SPE with MeOH (hair)	1:1.5 (liquid samples) 1:40 (hair)	MG	0.5 ng/mL	[78]
HPLC-HRMS (LIT)	Rat and human urine	Enzymatic digestion and SPE with MeOH/aqueous ammonia (98:2, *v/v*)	1:1	SC and its metabolites MC and its metabolite Iso-PAY and its metabolites	Qualitative method	[81,82]
LC-MS/MS	urine	Enzymatic digestion and SPE with 10% MeOH in EtOAc and MeOH (for the acidic fraction) or with EtOAc/*i*PrOH/20% NH_4OH (84:14:2, *v/v/v*) (for the basic fraction).	1:3	MG	5 ng/mL	[83]
GC-MS	Rat and human urine	Acidification, enzymatic digestion, SPE with MeOH/aqueous ammonia (98:2, *v/v*), derivatization	1:0.33	MG and three metabolites, two PAY metabolites, one SG metabolite, and two SC metabolites	LOD 100 ng/mL	[84]
LC-MS/MS (QqQ)	Oral fluid	Sonication of DOFs with 50:50 (*v/v*) MeOH:ACN, SPE with ACN and SALLE	1:20	MG	0.2 ng/mL	[85]
LC-MS/MS (QTRAP)	Blood and urine	Protein precipitation	1:2.7	MG	5 ng/mL	[95]
LC-MS/MS (QqQ)	Post-mortem whole blood and urine	Basification, extraction with 70:30 *n*-BuCl:EtOAc and acidification	1:6	MG, 7-OH-MG	2.5 ng/mL	[96]
IA (electrochemical immunosensor)	Urine	Dilution in PBS	1:1	MG	0.06 µg/mL	[97]
LC-HRMS/MS (Q-Exactive Focus Orbitrap)	Post-mortem and ante-mortem whole blood	Protein precipitation, acidification, extraction in MeOH, drying, 60 °C, 3 min	1:2	MG	2 ng/mL	[99]
LC-MS/MS (QqQ)	Wastewaters	Acidification upon collection	–	MG, 7-OH-MG	15.2 ng/mL (7-OH-MG), 149.3 ng/mL (MG)	[100]

[a] QqQ: triple quadrupole.
[b] Q-ToF: quadrupole-Time of Flight.
[c] SIM: selected ion monitoring.
[d] LIT: linear ion trap.

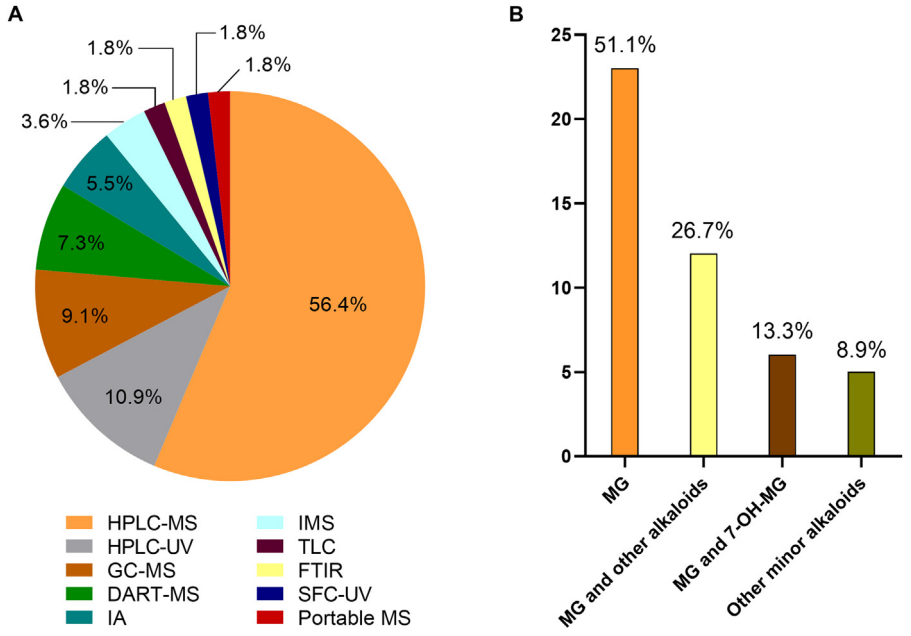

Fig. 4. Analytical techniques and alkaloids analysed in plant and biological samples. Percentage of articles published on the analysis of kratom alkaloids with one or multiple specific techniques (**A**); alkaloids analysed in the same papers (total number of papers investigated 46) (**B**).

"analysis" and "mitragynine" AND "analysis"). An additional source of analytical strategies for the determination of kratom alkaloids can be found in the 44th report of the Expert Committee on Drug Dependence (ECDD) of the WHO [14].

As shown in Fig. 4A, the most widely employed technique for the analysis of kratom alkaloids is high-performance liquid chromatography coupled to a mass spectrometric detector (HPLC-MS, 56.4% of the total 46 articles reviewed and included in Tables 1 and 2), followed by the same chromatographic system with a UV (or diode array (DAD)) detector (10.9%) and gas chromatography coupled to mass spectrometry (GC-MS, 9.1%). HPLC-MS apparently represents the most valuable choice for kratom alkaloids analysis, due to the superior sensitivity and resolution performances, which account for the higher number of papers reporting this type of analysis. However, MS alone fails in distinguishing alkaloids diastereoisomers, thus making the retention time the only actual discriminating factor. Other techniques include immunoassays (IA, 7.3%), direct analysis in real time coupled to mass spectrometry (DART-MS, 5.5%) and ion mobility mass spectrometry (IMS, 3.6%), all characterized by a lower sensitivity compared to HPLC-MS but with the opportunity be miniaturized for preliminary assays. Very few works described the use of thin layer chromatography (TLC, 1.8%) and Fourier-Transform Infrared spectroscopy (FTIR, 1.8%), which are far less sensitive than the abovementioned techniques. Although very little investigated, supercritical fluid chromatography coupled to UV detector (SFC-UV, 1.8%) deserves to be discussed as it is devoid of some limitations characteristic of the HPLC system. A few papers compared two or more techniques for the analysis of kratom alkaloids outlining the differences between them with advantages and drawbacks of each one. For example, Wang et al. compared HPLC-UV, HPLC-MS and GC-MS and evaluated the performance of the analytical techniques in terms of diasteromers resolution, but did not quantify the analytes [38]. Fuenffinger et al. evaluated the ability of IMS to determine MG in kratom products compared to HPLC coupled to tandem MS (HPLC-MS/MS) [52]. Lastly, an interesting work was presented by Voelker et al. where four portable devices, including portable handheld MS, FTIR, DART and IMS, were compared for MG testing in suspected kratom products [53]. Portable devices surely represent a valuable tool for preliminary assessment of the presence of kratom constituents in raw and manufactured products. The authors drew up a critical overview of the advantages and drawbacks of these devices, which will be helpful for future forensic implementation. All these analytical platforms and combinations will be described in the next paragraphs.

The majority of the works deal with the exclusive analysis of MG as it is the most abundant compound in plant material and is also detected in biological fluids even after its metabolic transformation (Fig. 4B). It is noteworthy that a large number of papers describe the simultaneous analysis of MG and other alkaloids, including 7-OH-MG, PAY, SG, SC, MC and other (26.7%) up to fourteen compounds in plant and commercial products with good sensitivity (LLOQ 9.77 ng/mL) [37]. Analytical methods, especially when biological specimens are involved, are often developed for the evaluation of MG and its active metabolite 7-OH-MG concentrations (13.3% of the works reviewed). To a lesser extent (8.9%), other alkaloids different from MG and 7-OH-MG are analysed in plant material and biological samples. This kind of studies are generally carried out to investigate the pharmacokinetics of a specific alkaloid as shown in the works by Philipp et al. on SG, MC, SC, PAY, and iso-PAY metabolism [58,59,81,82] and the work by King et al. on corynantheidine [67].

Particular concern lies around the stability of kratom alkaloids, which can dramatically affect the analytical results. Although many bioanalytical methods are validated for stability showing MG as being relatively stable in processed samples (after sample pretreatment) and during freeze-thaw cycles [49,73,80], instrumental systems like MS can mislead the interpretation of the results. For example, 7-OH-MG is particularly susceptible to form adducts when using electrospray ionization, generating the M+18 product at *m/z* 433 [45]. This issue can be overcome by using ammonium acetate in the mobile phase rather than formic acid or ammonium formate [73]. As mentioned above, MG is chemically hydrolysed at the methyl ester group in alkaline conditions and the metabolite 16-carboxymitragynine can be identified [40]. A similar MS fragmentation pattern has been described by Houghton et al. for 3-

dehydromitragynine found in fresh leaves and derived from the dehydrogenation of the quinolizideine moiety [35]. Although much work has been done on the analysis of kratom alkaloids, further effort is needed to deeply investigate both their stability from sample collection to analysis on a case-by-case basis and their degradation pattern.

5.1. Plant material

With fresh leaves available, a microscopic analysis can help to identify characteristic traits in the powdered samples that cannot be distinguished in dried leaves due to the difficulty in removing chlorophyll completely [92]. However, microscopic analysis alone cannot be considered a reliable method to identify kratom plant material especially for the high similarities across all *Mitragyna* species. Particularly for *M. speciosa*, the detection of MG is key to confirm the identity of the species.

Given the toxicological relevance of both MG and 7-OH-MG, it is important to work with easy, efficient and cost-effective analytical methods to identify and quantify these purported psychoactive compounds, especially when they are present within a complex mixture of other substances. Indeed, most case reports deals with intoxication by more than one abuse substance. Table 1 summarizes all methods published in the literature for the analysis of MG, 7-OH-MG and other kratom alkaloids extracted from plants and derived products.

Chromatographic methods seem the most popular choice for the separation of kratom alkaloids, while mass spectrometry (MS) is the most largely employed detection system [23,37,44,45,47,48,55,62], with the high-resolution platforms (HRMS) being the system of choice, probably due to the higher sensitivity and accuracy reached compared to UV (or DAD) in quantitative methods [46,49].

Amongst all chromatographic methods, various techniques have been reported to have satisfactory performances in terms of sensitivity, selectivity and specificity. In particular, to give an example, the determined lower limit of quantification (LLOQ) for MG ranges from 1 ng/mL to 31.25 µg/mL respectively in plant derived samples. In order to provide an idea of the sensitivity achieved, some methods applied to plant material report only the limit of detection (LOD), which overall ranges from 0.5 ng/mL to 1 mg/mL, and are generally used as preliminary screening methods. A good example is the work by Kowalczuk et al. [92], which deals with the analysis of MG by thin layer chromatography (TLC) carried out on silica gel plates with the use of a UV lamp as detector set at 254 nm. The method showed good separation and specificity for MG, thus proving to be more time saving and cost-effective compared to other more sensitive techniques like high performance liquid chromatography (HPLC) [92].

However, a higher sensitivity is required when dealing with toxicological analyses that must provide accurate results also for trace amounts of the analytes. At this regard, HPLC, and its ultra-high variant (UHPLC), seems the technique of choice that meets all the requirements for an accurate and precise analysis. Notwithstanding the ease of use, selectivity and relatively low maintenance costs, gas chromatography (GC) showed some analytical limitations, such as the lack of resolution of alkaloid mixtures due to the high temperature required for the elution of these analytes, which are not compatible with the upper temperature limits of GC columns [38]. Moreover, diastereomers of MG could be neither resolved by GC couple to mass spectrometry detector (GC-MS) nor be distinguished by their MS fragmentation spectra [38]. For these reasons, a derivatization step is required before the analysis [84]. Additionally, the LODs achieved are either not comparable to those obtained with HPLC methods [50,84] or are not even reported as the method was used for qualitative purposes only [93].

Wang et al. compared three chromatographic techniques including HPLC-MS-DAD, SFC-DAD and GC-MS for the determination of the indole alkaloids MG, SG, SC, PAY, 3-iso-PAY, and the oxindole alkaloids CORY A and CORY B [38]. Besides what already discussed for GC methods, SFC provided results as satisfactory as those obtained by HPLC in terms of resolution of MG diasteromers, in particular SG and SC [38]. Compared to HPLC, analysis of alkaloids by SFC is less explored and not well established. However, HPLC analysis is slow, shows differences in the elution order according to the pH of the mobile phase used, and distinction between stereoisomers cannot be accomplished by the comparison of the MS fragmentation patterns. On the other hand, SFC is faster, can be interfaced to the same detectors used for HPLC and provides better diasteromers resolution [38]. Given the isospectral properties of diasteromers using either UV or MS detectors, retention time is the key factor to distinguish each species in a complex alkaloid mixture. A comparison of the chromatographic separation of a standard mixture and an alkaloid extract between HPLC-UV, SFC-UV and GC-MS is reported in Fig. 5.

HRMS is also a good choice of detector when the direct analysis in real time (DART) is used in place of HPLC. The little or even no sample preparation needed and the fast analysis time make this technique very attractive and have prompted many laboratories in the US to use the DART equipment not only as a rapid screening method to identify kratom products, as well as adulterants or diluents, but also as a quantitative technique for the most investigated component MG with a fair sensitivity (LLOQ 5 µg/mL) [42,51]. DART-HRMS proved also to be efficient in the distinction of *M. speciosa* from other species and between two different *M. speciosa* varieties by analysis of the leaf without requiring any extraction process [51]. A rapid screening of the presence of MG in commercial products can be also achieved by means of ion mobility mass spectrometry (IMS), which ensures fast analysis time (within minutes including sample preparation) and requires very low sample volumes (1 µL) [52]. These advantages, along with a good sensitivity (LLOQ 0.1 µg/mL) and the possibility to use it as a handheld device, make this technique a very attractive system for on-site analysis [53]. Both DART and IMS are also very convenient as preliminary screening to eliminate negative samples and reduce the number of analyses before processing with more sensitive and time-consuming laboratory techniques. Voelker et al. evaluated four portable devices, including DART-TD (thermal desorption)-MS, IMS, FTIR and handheld MS, for the detection of MG in commercial kratom products [53]. The authors concluded that both DART-MS and IMS are ideal for fast on-site preliminary screening, while FTIR needs extracted samples for analysis, thus it appears less convenient unless used as an orthogonal technique to one of the other two; on the other hand, the handheld MS showed the highest false negative rate [53].

In the context of preliminary screening methods, immunoassays (IA) are easy-to-handle diagnostic tools involving the use of monoclonal antibodies (mAb) against the analyte of interest. In the work by Limsuwanchote et al. the mAb was conjugated to colloidal gold (gold-anti-MG mAb), while MG-ovalbumin conjugate (MG-OVA) and goat anti-mouse IgG were immobilized on a strip to produce a test zone and control zone, respectively [94]. In this immunochromatographic method, MG present in cocktails and leaves competed with MG-OVA in the test zone to bind to colloidal gold-anti-MG mAb, eventually giving a colour intensity at the test zone that was inversely correlated with the MG concentration [94]. The LOD of the method was 1 mg/mL for MG by visual assessment and 0.60 mg/mL by imaging detection [94]. An issue that can be encountered with IA is the potential cross-reactivity of the developed antibody against a specific kratom alkaloid with other alkaloids present in a sample [54].

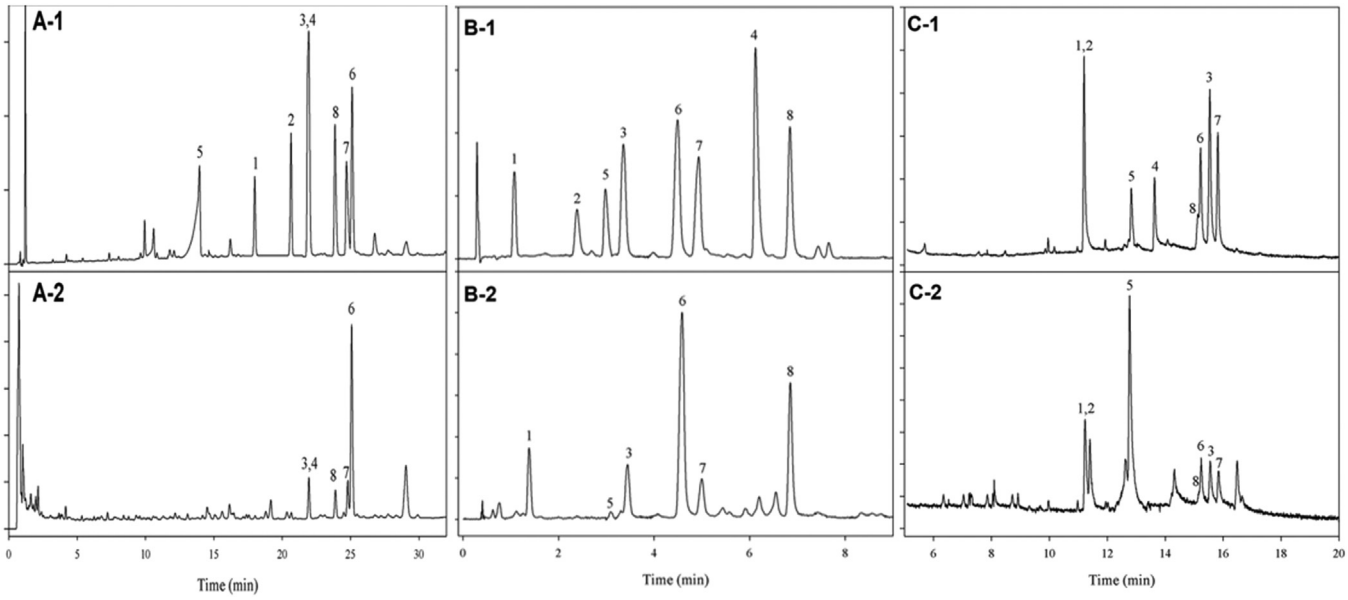

Fig. 5. Comparison of the chromatographic resolution of a standard mixture and an alkaloid extract by HPLC-UV, SFC-UV and GC-MS. Chromatograms of *M. Speciosa* (kratom) reprinted and modified from Wang et al. [38] with permission of Wiley. HPLC–DAD (220 nm): (A-1) standard mixture; (A-2) alkaloid extract; (A-3) methanol extract. SFC–DAD: (B-1) standard mixture; (B-2) alkaloid extract. GC–EI total ion: (C-1) standard mixture; (C-2) alkaloid extract. The standard mixture is composed of CORY B (1), CPRY A (2), PAY (3), 3-iso-PAY (4), MG (6), SG (7), and SC (8).

5.2. Biological samples

Unlike methods developed for plant material and derived products, analytical methods for biological samples are mainly focused on the determination of the main alleged psychoactive components MG (and its metabolites) and 7-OH-MG for toxicological purposes. All other *Mitragyna* alkaloids seem to be neglected. Very few works deal with PAY or SG as the parent compound and study their metabolism [58,59,81,82]. Interestingly, Basiliere et al. proposed SG and SC as alternative biomarkers in urine of kratom consumption instead of MG and its metabolites [74]. In particular, the concentration of SG and SC exceeded that of MG in 75% and 63% of cases, respectively, most likely because of the increased stability of these isomers compared to MG [40,74]. Lastly, King et al. developed an HPLC-HRMS method for the quantitative analysis of corynantheidine in rat plasma carrying out only a protein precipitation step before the analysis and reaching a sensitivity at the order of the ng/mL [67].

As evident from the list in Table 2, the most widely employed separation technique is HPLC coupled to HRMS in tandem mode (MS/MS or MS^2) due to its superior sensitivity in comparison to other detection platforms. Indeed, the LLOQ achieved with this kind of analytical apparatus ranges from 0.1 to 5 ng/mL for MG [70,73,78,83,95] and from 1 to 2.5 ng/mL for 7-OH-MG [75,96]. GC-MS has been also used for the analysis of MG though with lower sensitivity (LLOQs from 50 ng/mL) [70,84]. These alkaloids are generally separated on (or through) reversed-phase chromatography, although some examples of separation on HILIC columns are present in the literature [61,71]. Basiliere et al. were able to develop a highly selective HPLC-HRMS/MS method for the determination of five kratom alkaloids (MG, 7-OH-MG, SG, SC, and PAY) in urine [73] and post-mortem blood and tissues [69]. The authors succeeded in resolving the analytes on the chromatographic system (Fig. 6A) but highlighted the difficulty to distinguish the same compounds by their MS^2 spectra as they were almost identical (Fig. 6B).

The metabolic studies carried out by Philipp et al. in human urine are worth of note as the authors were able to distinguish MG metabolites from SG and SC metabolites by using a C18 column with a small particle size (1.9 µm) and a long mobile phase gradient up to over 1 h [58,79,81]. Importantly, the formation of the 9-*O*-desmethyl derivative as well as of other metabolites is common in the metabolic pathway of both MG and its diastereomers and the MS^2 pattern is identical. This can easily lead to an overestimation of one species as metabolites coming from different diastereomers but having the same precursor ion and fragmentation patterns could co-elute. Nevertheless, Philipp et al. were able to distinguish the metabolites of the different diastereomers by the peculiar differences in the MS^3 fragmentation pattern [58,79,81].

Another analytical system for the determination of MG in human urine has been proposed by Neng et al., who developed a bar adsorptive microextraction with a modified *N*-vinylpyrrolidone polymer followed by liquid desorption and subsequent analysis by HPLC-DAD (BAµE-LD/HPLC-DAD) [75]. The method reached a LOQ as low as 0.33 ng/mL and proved to be easy to perform, reliable, and sensitive [75]. Additionally, it required low sample volumes and promised to be a good alternative to detect MG in biological fluids [75].

IAs have also been developed for biological samples testing. Mustafa et al. developed for the first time a multiwalled carbon nanotubes/chitosan nanocomposite as modified electrode for an electrochemical immunosensor for the sensitive and rapid detection of MG [97]. In particular, the authors immobilized the MG-OVA conjugate on the electrode surface and incubated the MG containing mixture with MG primary antibody. Then, they exploited the principle of an indirect competitive ELISA: the signal in terms of redox potential generated by the reaction of goat anti-rabbit horseradish peroxidase (HRP)-modified secondary antibody with 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 was inversely proportional to the concentration of MG. The developed electrochemical immunosensor showed a sensitivity 10-fold higher than conventional ELISA (LOQ 0.06 μg/mL) [97].

When working with biological specimens, and urine in particular, it is important to detect the main metabolites along with the parent compound in order to assess kratom consumption [76]. However, analytical standards for the metabolites of kratom alkaloids are not available, therefore, kratom use in forensic toxicology specimens is currently evaluated by analysis of the par-

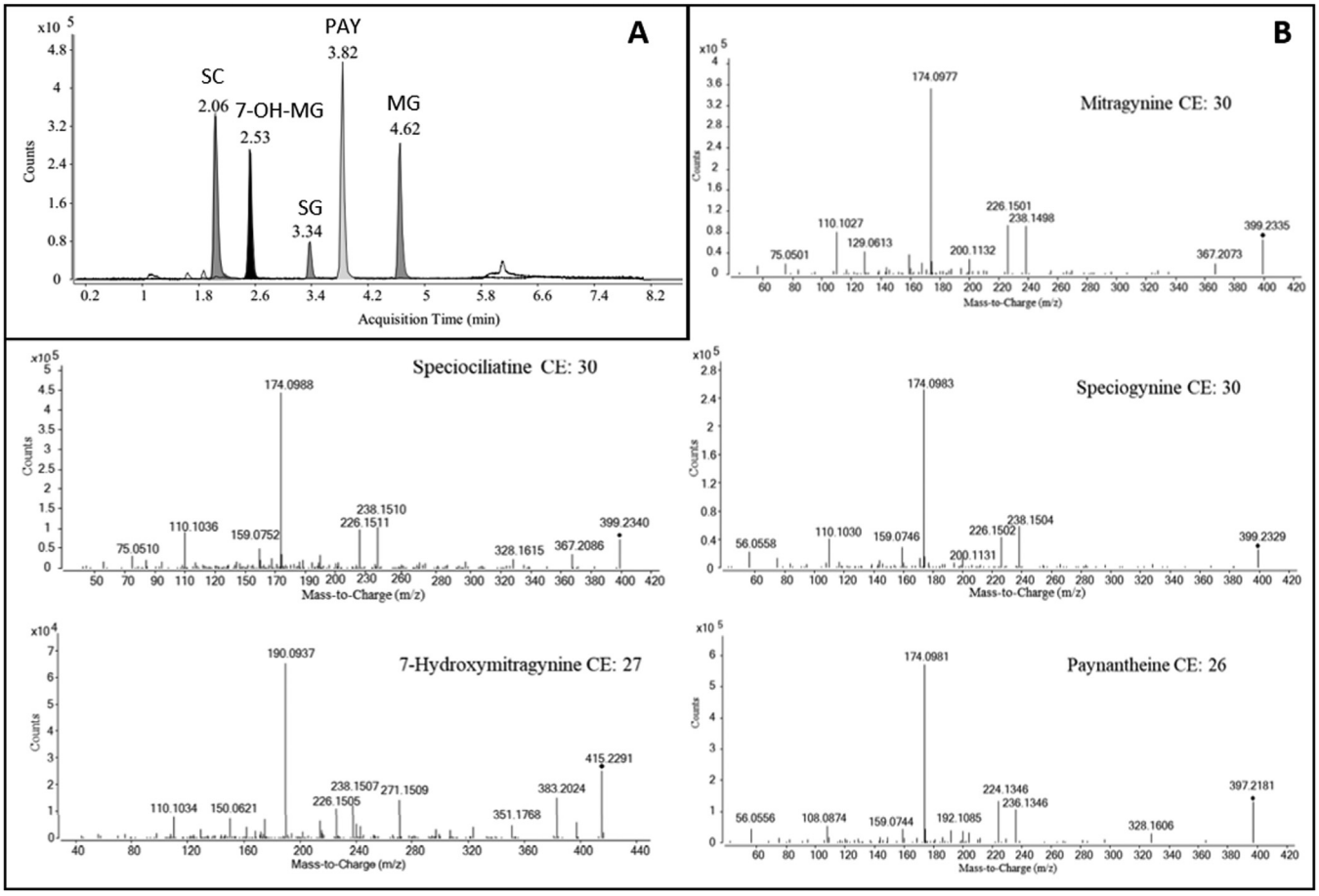

Fig. 6. HPLC-HRMS/MS analysis of five kratom alkaloids. Chromatographic separation of *Mitragyna* alkaloids in a representative blood extract (100 ng/mL). SC, 2.06 min; 7-MG-OH, 2.53 min; SG, 3.34 min; PY, 3.82 min; and MG, 4.62 min (**A**). HRMS/MS spectra of the five alkaloids (**B**). Reprinted and adapted from Basiliere et al. [69] with permission of Springer Nature.

ent drug and sometimes the related alkaloids. Moreover, analytical standards are expensive and not commercially available for all alkaloids, thus requiring the isolation of the analytes of interest directly from the plant through an extraction and purification process [68]. In the absence of the proper analytical standards, identification of MG metabolites was also achieved by use of recombinant cytochrome P450 (rCYP) enzymes to generate 9-*O*-demethylmitragynine and 16-carboxymitragynine and confirm MS and retention time [98]. On the other hand, identification and quantification of alkaloids metabolites remain confined to the research area and no trace of application of the published methods has been recorded in the literature at least for the last ten years.

Although less extensively, hair samples [77,78], oral fluids [85] and several post-mortem tissues [69,70,72,96,99] have been also included in the biological specimens analysed for kratom alkaloids quantification, in particular MG and 7-OH-MG.

Moreover, many research works addressed the identification and quantification of the major kratom alkaloids MG and 7-OH-MG along with a large number of other drugs of abuse and new psychoactive substances (over one hundred compounds in a single analysis). Although the methods were not specifically developed for the two compounds, the sensitivity achieved is particularly high and the LOQs ranged from 0.5 to 5 ng/mL [78,85,95,96,99]. Although it can be neither included in the biological specimens nor in the plant samples, the work by Bade et al. is worth of note as it describes a sensitive analytical method based on direct infusion of Australian wastewaters coupled into tandem MS (LOQs 149.3 ng/mL for MG and 15.2 ng/mL for 7-OH-MG) [100]. The samples were directly injected into the analytical apparatus without prior treatment or dilution and were found to contain over thirty new psychoactive substances [100].

6. Conclusions

Mitragyna speciosa or kratom is a plant with interesting documented pharmacological properties but that is emerging as a new drug of abuse due to the increasing number of fatalities where high concentration of MG and its active metabolite 7-OH-MG are found. As a result, although fatalities are generally characterized by a polydrug abuse and the contribution of kratom is unclear, a public debate regarding the safety profile of MG and kratom has recently emerged.

Considered the proven chemical instability in biological samples, accurate and reliable analytical methods are necessary for the quantification of these alkaloids in different matrices. The extraction methodologies, which were here reviewed, significantly affect the final quantitative results, as well as the analytical technique employed dramatically influences the sensitivity of the method. In particular, the ASE and the SPE methods resulted the most efficient sample extraction approaches for the isolation of the analytes from plant and biological specimens, respectively. As regards to the analytical platforms, the most suitable technique to achieve high sensitivity is HPLC-HRMS. Although MS fragmentation spectra cannot be distinguished for diasteroisomers of alkaloids, a good separation of the peaks in the chromatographic step will ensure reliable identification and accurate quantification assuming that analytical

standards are available. Regardless of the extraction strategy and analytical technique employed, considerable work is still needed for an in-depth investigation of the stability of kratom alkaloids both in plant material and biological fluids. Although some chemical decomposition products and human metabolites are known, the degradation pattern has not been fully elucidated for all alkaloids. Moreover, the literature lacks long-term stability studies in plants and biological fluids and a careful evaluation of the sample storage conditions needs to be undertaken on a case-by-case basis.

At present, even the most sensitive and accurate method requires laborious sample preparation and is neither easily accessible nor cost-effective. Even though kratom is increasingly become a safety emergency, it is not included in the rapid police screening tests. All the methods discussed in this review are still at the research stage and need to be validated to be applied on site.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Cinzia Citti: Conceptualization, Methodology, Formal analysis, Writing – original draft. **Aldo Laganà:** Data curation, Methodology, Validation, Writing – review & editing. **Anna Laura Capriotti:** Data curation, Methodology, Validation, Writing – review & editing. **Carmela Maria Montone:** Data curation, Methodology, Validation, Writing – review & editing. **Giuseppe Cannazza:** Conceptualization, Methodology, Formal analysis, Writing – original draft.

Data availability

No data was used for the research described in the article.

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