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Analysis of carbazole alkaloids in *Murraya koenigii* by means of high performance liquid chromatography coupled to Tandem mass spectrometry with a predictive multi experiment approach

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ABSTRACT

Murraya koenigii (*M. koenigii*) is recognized as one of the most significant Indian medicinal plants, due to its therapeutic properties. In fact, various biological activities, including anti-inflammatory and antifungal, are attributed to carbazole alkaloids (CAs) and their metabolites, but it is still difficult to achieve a full classification of these compounds because of their heterogeneous structures. For this reason, the development of new strategies for their identification is necessary. In this work, a reliable method, including the simultaneous quantification of three main CAs of Murraya (Mahanimbine, Koenimbine and Koenigicine) and a putative identification for other compounds belonging to the huge family of CAs, was developed by means of HPLC-MS/MS. Also, an efficient extraction procedure followed by a suitable clean-up step was presented, in order to obtain reliable recoveries (resulted from 60 to 85% for all the analytes). The analyses were performed by using predictive multi experiment approach based on information-dependent acquisition (IDA), coupling multiple reaction monitoring (MRM) and precursor ion (PI) as survey scans, and enhanced product ion scan (EPI) as dependent scan. Competitive Fragmentation Modeling-ID (CFM-ID) was used to predict MS/MS spectra from the chemical structures of the compounds in order to create a suitable MRM inclusion list. The obtained results showed that this method can simultaneously provide quantitative information for the target analytes (Mahanimbine (7.22–5.62 mg/kg), Koenimbine (1.26–1.62 mg/kg) and Koenigicine (0.44–1.77 mg/kg)) and a putative identification for several compounds belonging to different classes of CAs which are not included in the target list, thanks to PI survey scan.

1. Introduction

M. koenigii is a tropical plant belonging to the *Rutaceae* family, studied for its different pharmacological activities due to a high content of Carbazole alkaloids (CAs) [1]. This is a class of tricyclic hetero-aromatic compounds, with two benzene rings fused onto a pyrrole ring as core structure [2]. The term carbazole refers to their basic skeleton, which is denoted by A, B and C [3] (Fig. 1)

CAs show various biological activities such as anticancer [2,4], anti-inflammatory and antimicrobial activities against several pathogens both *in vivo* and *in vitro* [5,6]. Furthermore, it is also known that CAs are characterized by antioxidant, radical scavenging [7] antiplasmodial, antiparasitic [8], and antiviral effects [9].

As regards analytical methods for CAs, there are few studies conducted with gas chromatography-mass spectrometry (GC-MS) [10] or liquid chromatography (LC) coupled with different detectors, such as ultraviolet-visible (UV-vis) [11] and photodiode array detector (PDA) [12,13]. The most reported approaches are by nuclear magnetic resonance (NMR) and high resolution mass spectrometry (HRMS) [5,14–18]. Triple quadrupole mass spectrometry (QqQ), which is usually used for quantification, is not prone for the screening for unknown structures [19]; in fact, the QqQ is usually limited by the need for reference standards in terms of identification of compounds. On the other hand, the QqQ coupled with Linear Ion Trap (QqQ-LIT) hybrid system can provide different acquisition strategies useful for structural analysis. For example, through the information-dependent acquisition (IDA) it is possible to design an experimental set-up that allows a semi-targeted analysis providing interesting information on specific classes of compounds even without analytical standards. The IDA experiment usually uses a survey scan and a dependent scan triggered by the survey scan(s). The EPI spectra, can provide additional information on the chemical structure in order to confirm known analytes or to suppose the structure of unknown ones [19,20]

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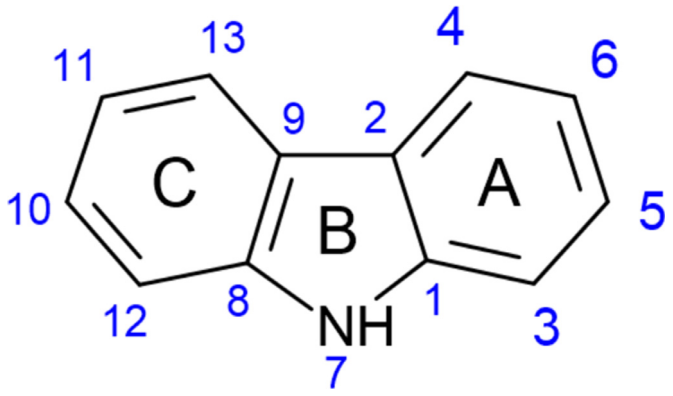

Fig 1. Carbazole alkaloid's structure.

In this work, a sensitive and reliable HPLC-QqQ-LIT method, was developed, performing targeted and semi-untargeted analysis, including the simultaneous quantification of three of the main CAs of Murraya (Mahanimbine, Koenimbine and Koenigicine) and putative identification of different compounds belonging to the CAs family. The development of quantitative method for the target analytes, allowed to set-up a suitable extraction and clean-up method also for testing the proposed semi-untargeted approach; so, the quantitative analysis of the three CAs was pivotal for the possibility a putative identification of the compounds with similar structures. MRM acquisition mode was used both for the quantification of the target compounds and as survey scan in the IDA-EPI experiment. The MRM transitions were generated both by tuning the instrumental parameters on three available standards, and by *in silico* experiments using the Competitive Fragmentation Modeling for Metabolite Identification (CFM-ID) [21,22] to create the inclusion list. A further survey scan by precursor ion (PI) was also used in order to increase the information used by the IDA criteria, allowing to start enhanced product ion (EPI) dependent experiments based on the results obtained both from MRM and PI scans, for the detection of compounds with a common moiety among the different CAs. In literature, this approach has never been used for the identification of this class of compounds in this matrix and it could represent a useful tool in detecting unpredicted compounds belonging to CAs family.

2. Materials and methods

2.1. Chemicals

The dried leaves of *M. koenigii* were purchased by local retail. The standards of Mahanimbine, Koenigicine and Koenimbine were purchased from Sigma-Aldrich (St. Louis, USA). The stock solutions were prepared by appropriate dilution in methanol and stored at −20 °C. Toluene-2 (Tol-2), 2-Propanol (ISOP), acetone (ACT), water (H_2O), formic acid (HCOOH), acetic acid (CH_3COOH), methanol (MeOH) and acetonitrile (ACN), all UPLC grade, were purchased from VWR (Radnor, USA).

2.2. Sample preparation

Ground dried leaves were weighted (10 mg) and added with 1 mL of MeOH in an eppendorf, then vortexed for 3 min. The sample was homogenized by Precellys® Evolution homogenizer (Bertin Technologies SAS, Montigny-le-Bretonneux, France) with three 10 s cycles at 5000 rpm at room temperature and centrifuged at 4 °C for 20 min at 10,000 rpm. The supernatant was diluted 1:1000 ratio with a solution of H_2O: MeOH 50:50, vortexed and processed by SPE for clean-up.

2.3. Solid phase extraction clean-up

Clean-up step was performed by using a Strata C18-E cartridge (330 mg, 1 mL) from Phenomenex (Torrance, CA, USA). Initially, the cartridges were conditioned with 1 mL of MeOH and subsequently equilibrated with 1 mL of H_2O: MeOH 50:50 solution. Then, the sample was loaded into the cartridge. For the washing step, 1 mL of H_2O: MeOH 50:50 solution was used. Finally, the analytes were eluted with 500 μL of MeOH; and collected for LC-MS/MS analysis.

2.4. High performance liquid chromatography method

The chromatographic separation was performed by an UHPLC system Nexera XR from Shimadzu (Shimadzu, Tokyo, Japan); the column was an ACE Excel 2 C18-PFP (10 cm × 2.1 mm id) from ACE (Advanced Chromatography Technologies, Aberdeen, UK) packed with particles of 2 μm, equipped with a security guard column. The mobile phases consisted of water containing 0.1% HCOOH (A) and ACN (B). The elution of the analytes was carried out with the following gradient: the initial condition with phase B at 40% was maintained for 1 min, then brought to 100%in 8 min, and kept for 3 min, the initial conditions (40% B) were restored in 2 min, and held for 2 min to equilibrate the column.

2.5. Tandem mass acquisition

MS analyses were performed onto a 4500 QTRAP hybrid QqQ-LIT mass spectrometer (Sciex, Framingham, MA, USA), equipped with a TurboV source operating in electrospray ionization positive mode (ESI+). Quantitative analysis was performed by means of MRM acquisition mode for Mahanimbine, Koenigicine and Koenimbine. Semi-untargeted analyses were performed in MRM/PI-IDA-EPI in a single run: the MRM and PI experiments were used as survey scans for the IDA, which was employed to trigger the EPI scans.

2.5.1. In silico *experiments*

CFM-ID was used to predict all the MRM transitions related to compounds belonging to CAs family, without using analytical standards; furthermore the predicted fragmentation spectra were matched with the information reported in the literature [22]. The chemical structure of each compound was converted in SMILES [23] or in InChI format [24]. For the spectra prediction, it was used a fragmentation algorithm based on two approaches: combinatorial fragmentation [21,22,25] and rule-based fragmentation. Once the *in silico* fragmentation step was completed, three MS/MS spectra were generated at 10, 20 and 40 V [21]. For each predicted fragment, the following information were obtained: the m/z values, the relative abundance of each fragment ion at each energy level and their chemical structure (expressed as InChI or SMILES strings). The fragments generated by CFM-ID were selected according to their intensity and added to the MRM survey scan. The source parameters were set based on the target analytes, as described below, while the collision energy (CE) values were calculated by CFM-ID.

2.5.2. MRM/PI-IDA-EPI acquisition mode

MRM-IDA-EPI mode was used for quantitative analysis of Mahanimbine, Koenigicine and Koenimbine and putative identification of predicted MRM transitions obtained by CFM-ID; the instrumental parameters, such as declustering potential (DP), entrance potential (EP), were tuned by infusion of each analytical standard diluted in methanolic solution (10 ng mL^{-1}) at a flow rate of 10 μL min^{-1}. Also source parameters were tuned: a capillary voltage was set at 5500 V, nebulizer gas (GS1) and turbo gas (GS2) both at 40 psi, and source temperature 500 °C.

For the inclusion list, 24 MRM transitions were used as survey experiment, obtained both from tuning of standards and *in silico* tests. For each precursor, two MRM transitions were chosen. A PI-IDA-EPI acquisition mode was also set up to identify the compounds not presented in the inclusion list; for the PI survey scan the fragment ion at m/z 210.0

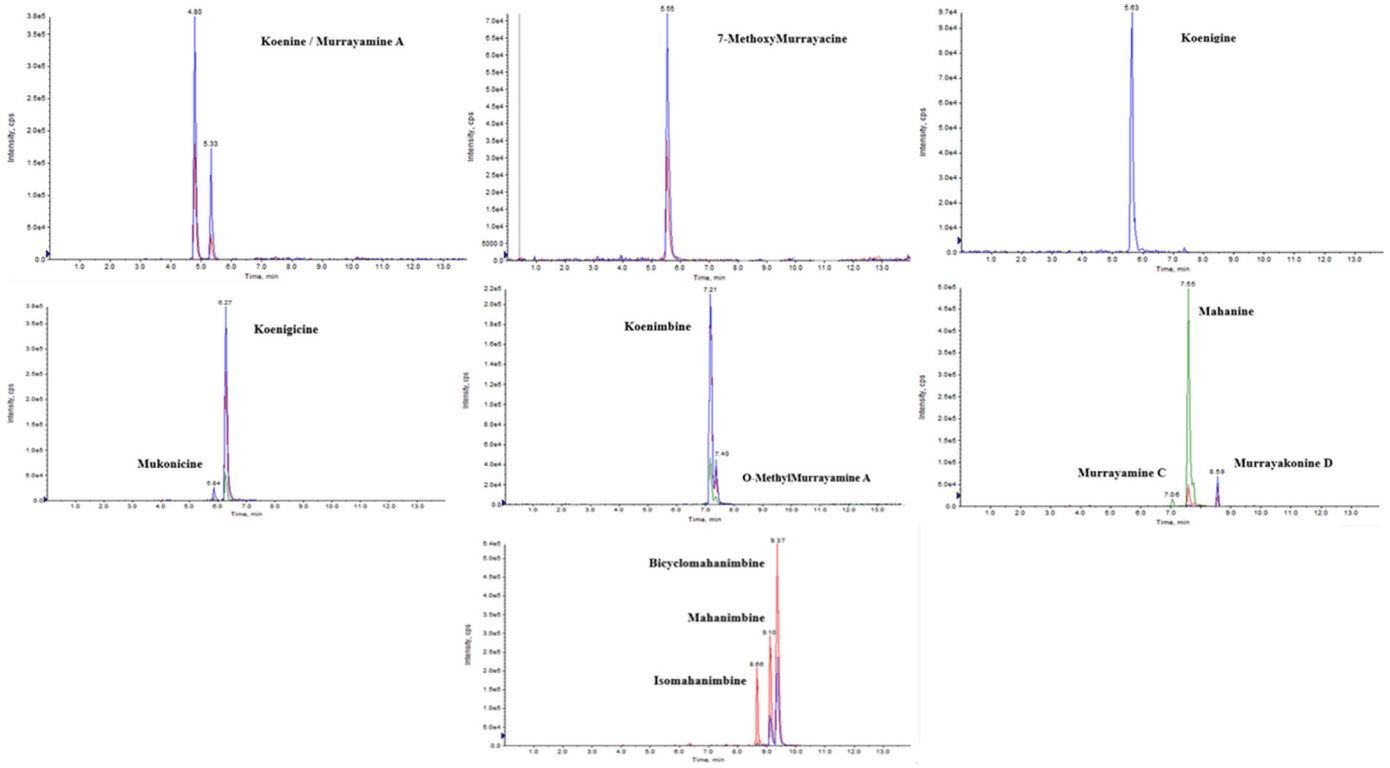

Fig 2. Chromatographic profiles obtained with the MRM method expressed as extracted ion currents (XICs) of *Murraya Koenigii* leaves.

was used, while Q_1 was set in the mass range between 250 and 1000 Da. The acquisition mode was performed using the following values for DP, EP, CE and CXP: 95 V, 9 V, 40 V, 8 V, respectively. The data obtained from PI scans for m/z 210.0 were then used to trigger. For IDA criteria, the minimum intensity threshold of 500 counts per second (cps) was set without exclusion after dynamic background subtraction of the survey scan.

The EPI-dependent acquisition, triggered by MRM and PI, was performed at a scan speed of 10,000 Da/s with a m/z range between 50 and 500; the LIT fill time was set to 25 ms. For the acquisition of the collision induced dissociation spectra (CID) with nitrogen, used as collision gas, was set at high level and a CE at 45 V with a spread of ±10 V, according to standard tuning and *in silico* experiments. For the DP, EP and CXP parameters were 75 V, 10 V and 10 V, respectively. The mass tolerance was set to 250 ppm and resolution of Q_1 was set to unit. Dependent MS/MS spectra for compounds were confirmed by matching them with the literature or with the CFM-ID ones. A LC-MS/MS chromatogram of sample is reported in Fig. 2.

The selected ions, together with the main HPLC-MS/MS parameters are shown in Table 1. Data management was performed with Analyst 1.7.2 software and quantitation with Multiquant 3.0.3 software form Sciex.

2.6. Method validation

As concerns the quantitative analysis, the proposed method was validated on the three target analytes according to the FDA guidelines [26], considering the following parameters: limit of detection (LOD), limit of quantification (LOQ), linearity, precision and accuracy. For the linear dynamic range, a calibration curve consisting of 9 points was built and each point was performed in triplicate. The linearity was checked between LOQs and 50 ng mL^{-1} for all analytes. The LODs and LOQs were estimated at a signal-to-noise (S/N) ratio of 3 and 10, respectively. Accuracy was determined by spiking the analytes in matrices in quintuplicate for 4 different levels of concentration after the dilution and calculated by blank subtraction method. For precision determination intraday RSDs were considered one-day measures of 5 sample replicates at 4 levels of concentration (intra-day precision or repeatability), whereas for inter-day RSDs, samples were analyzed for three consecutive days and each day twice.

3. Results and discussion

3.1. Extraction set-up

The development of a suitable extraction method was required to obtain effective and reproducible recoveries, together with a good selectivity. To this aim, different solvents with a wide range of polarities, such as Tol-2, ACT, ISOP, ACN and MeOH were tested by ultrasound-assisted extraction; the latter showed the best result about recovery yield was obtained for MeOH, as shown in Fig. S1.

Subsequently, the samples were processed by the comparison of the ultrasound-assisted extraction (Experiment 1) and the extraction performed with the Precellys homogenizer (Experiment 2). As shown in Fig. S2, the homogenizer-assisted procedure provided higher recoveries for the selected analytes as the homogenization with the ceramic beads facilitated the release of the analytes into the solvent and also resulted in a faster extraction compared to the ultrasound. For these reasons the “Precellys” procedure was chosen.

3.2. Solid phase extraction set up

In order to clean-up the extract and minimize the matrix effect, an efficient SPE procedure was developed providing good recoveries and low matrix effect (ion suppression) for target analytes. For this purpose, two different SPE cartridges were tested (Fig. S3(A,B)): a silica based Strata C18-E (55 µm particle size) and a polymeric Strata X (33 µm particle size). As the first step the loading conditions were evaluated to maximize the analyte retention; for this purpose different H_2O:MeOH ratios for the loading solution were tested: 90:10, 80:20, 70:30, 60:40,

Table 1
MS/MS parameters of the selected analytes for the MRM acquisition, m/z value of parent compounds in first quadrupole (Q1) was reported as [M-H]-; also, m/z values of the fragment in the third quadrupole (Q3) are reported.

ID	R_T (min)	Q_1(Da)	DP(V)	EP(V)	Q_3(Da)	CE(V)	CXP(V)
Koenine	4.85	280.1	65	8	196.1	40	8
					220.0	40	8
Murrayamine A	5.41	280.1	65	8	196.1	40	8
					220.0	40	8
7-Methoxymurrayacine	5.55	308.1	15	8	280.1	20	9
					210.1	40	9
Koenigine	5.62	310.1	65	8	278.1	40	9
					208.1	40	9
Mukonicine	5.84	324.4	75	6	309.1	28	12
					238.1	30	8
Koenigicine*	6.27	324.4	75	6	309.1	28	12
					238.1	30	8
Koenimbine*	7.21	294.1	85	8	236.1	48	9
					208.1	55	7
O-Methylmurrayamine A	7.40	294.1	85	8	236.1	48	9
					208.1	55	7
Mahanine	7.55	348.2	65	8	226.1	20	9
					123.1	20	8
Isomahanine	8.66	332.2	35	8	234.1	53	9
					210.1	26	9
Mahanimbine*	9.10	332.2	35	8	234.1	53	9
					210.1	26	9
Bicyclomahanimbine	9.34	332.2	35	8	234.1	53	9
					210.1	26	9

50:50, 40:60 v/v. The performances for each analyte were evaluated by comparing the area of the single analyte to the area of a reference mixture with known concentration (500 ng/mL). Among the different conditions, the solution with H_2O: MeOH 50:50 ratio allowed the best results in terms of recovery for the analytes.

In the next step the elution was tuned considering ACN, MeOH and ISOP. Three fractions of 500 μL were eluted for each solvent in order to evaluate the recoveries and selectivity of eluent solution. As concerns the tests carried out on C18 cartridges, good recoveries were already obtained with a single elution each solvent For the Strata X a higher amount of eluent was needed, so C18-E cartridges were preferred, with 500 μL of 100% MeOH as eluent phase.

Finally, the washing phase was checked with different combinations of H_2O and MeOH; the solution with H_2O: MeOH 50:50 resulted in the best option for recoveries and selectivity.

3.3. High performance liquid chromatography method development

The chromatographic run was initially set-up to provide a good separation for Koenigicine, Koenimbine, Mahanimbine. During the initial LC set-up, the mobile phases taken into consideration were (A) H2O 0.1% HCOOH and (B) 50:50 MeOH: ACN 5 mM HCOOH. Different chromatographic columns were also tested: Kinetex 2.6 µm XB-C18 column (100 × 2.1 mm) and Kinetex 2.6 µm PFP column (75 × 30 mm). Phase B was subsequently replaced with ACN, providing better resolution of the peaks. Initially, however, the peaks of Koenigicine and Koenimbine were not well separated. For this reason, a column combining the C18 and PFP stationary phase, was tested (ACE Excel 2). This chromatographic conditions turned out to be the best compromise to obtain a good selectivity and efficiency of the system.

3.4. Mass Tandem method development

An MS/MS method was developed and applied for simultaneous quantification, screening and putative identification of CAs in vegetal matrices. The samples were analyzed by MRM/PI-IDA-EPI. The MRM acquisition mode provided the quantitative analysis for Koenigicine, Koenimbine, and Mahanimbine, and, coupled with IDA-EPI experiment, allowed also the putative identification of 14 CAs thanks to MRM inclusion list generated by CFM-ID. Furthermore, PI-IDA-EPI experiment allowed to detect 8 compounds not included in the MRM list, thanks to the presence of common fragment 210.0 m/z.

3.4.1. In silico *experiment development*

In order to detect as more as possible compounds in the plant extract, a fragment prediction by CFM-ID of CAs was performed (Table S1). Based on the mass and structure, it was possible to predict the precursor ion and a product ion for several molecules belonging to CAs by means of CFM-ID; this tool is able to accurately predict MS/MS spectra from chemical structures and to allow putative identification via MS/MS spectral matching. 119 analytes, belonging to CAs class (Table S2), were processed with CFM-ID and used to build a database. The transitions of each analyte, according to their relative abundance and the score indicating the degree of probability, were selected and then added to the MRM inclusion list. Among the 119 selected analytes, the following molecular ions produced a significant signal: m/z 280.1, m/z 308.0, m/z 310.1, m/z 348.2. Moreover, for these ions different chromatographic peaks were generated, probably due to the presence of isomeric forms. For example, for the transition 280.1→196.1/220.1 m/z, calculated for Koenine, two peaks with different retention time (R_T) 4.85 and 5.33 min were generated.

3.4.2. MRM-IDA-EPI development

The method was developed starting by testing a mixture containing three standards at a concentration of 25 ng/mL and their MRM transitions were initially used as a survey scan, in order to develop MRM-IDA-EPI. Different tests were carried out on plants extract to establish the most suitable MS parameters, starting from CE value, to obtain the best fragmentation. In this regard, different CE values 35, 45, 55, 65 V and a stable collision energy spread (CES) ±10 V were tested. The CE value 45 V ensured the best fragmentation spectra that allowed to acquire characteristic fragments as base fragment peak and at the same time to maintain detected the precursor ion peak in the spectra. The value of DP was established at 75 V, which is given by the average of the different DPs of all analytes.

Subsequently, an MRM scan was performed comprising the MRM transitions of the standards and theoretical transitions from CFM-ID,

coupled to EPI experiments with a scan range m/z 50–500 Da. The IDA threshold was set at 500 cps, above which the fragmentation patterns of each analyte were collected by the EPI from the 6 most intense peaks.

The EPI experiments allowed the fragmentation of the precursor ion selected in Q_1 and the generation of characteristic fragmentation patterns for each analyte; the results obtained were compared with the data present in the literature and with the secondary data reported in the database, created using CFM-ID. The reliability of the method was then tested both on the analytical standards and real samples.

As is showed in Fig. S4, the EPI spectrum of the Mahanimbine standard (R_T 9.10) was compared to the MS/MS spectra reported in the literature for the fragments m/z 276.0, 248.2, 210.1, 250.0 and 135.0 [28,29]. The mass fragment m/z 248.2 indicated the presence of a 2,2-substituted pyran system. Furthermore, the presence of the fragment m/z 123.0 indicated the opening of the ring with consequent loss of [M+H-CH_2=C(CH_3)-CH_2CH_2CH=C(CH_3)$_2$], the ion m/z 332.2 contained two intense peaks at m/z 182.0, indicated the 3-methylcarbazole, and the fragment m/z 167.0, corresponding to carbazole, and the loss of a methyl group (15.02 Da) [30].

The acquired EPI spectrum for Koenimbine (RT 7.21 min) was compared with the predicted spectra present in the MS/MS literatures. Fragment 279.0 has been compared in the literature [28]. The MS/MS spectrum contained an intense ionic peak at m/z 264.1 which corresponds to girinimbine and loss of a group [M+H-O-CH3]. The structural analogy with girinimbine allows us to compare the following fragments m/z: 249.1; 235.9 [31] and 167.0; 131.1; 91.2 [30]. In this way screening method and identification workflow was validated on reference standard and samples.

3.4.3. PI-IDA-EPI development

Analogous forms of CAs, not present in the target list, were identified using the PI in semi-untargeted mode according to Oliva et al. [32]. In this work, PI was used as survey scan for CAs not predicted by CFM-ID, and therefore included in the MRM list, but having a similar fragmentation pattern with at least one identical portion. In the fragmentation spectra obtained by MRM-IDA-EPI, the m/z 210.0 fragment was common for the detected compounds; it was reported in some studies, in which CAs were isolated, in particular the Pyrano [3,2-a] CAs [28,33–35]. For this reason, based on the study of the predicted fragmentation pattern for CAs in the literature [36], it was hypothesized that m/z 210.0 fragment derives from the cleavage of the pyranic ring and rearrangement of the molecule. So it could be considered as common fragment to the whole class of Pyrano CAs, including the open and cyclized forms, which constitutes the most abundant class of compounds isolated from *M. koenigii* leaves. For this aim, the PI scan was used to investigate the structures of compounds that share the precursor ion m/z 210.0, so it was possible to screen unknown analytes, not included in the MRM method, but structurally related. The test was carried out directly on the matrix and the identification of the standard analytes and those present in the *in silico* MRM method was performed on the R_T. Different values of CE were also assessed to maximize the signal of the fragment ion selected for the precursor ion scanning experiments; in particular, CE value of 40 V was selected. CE value led to a decrease of the analytes signal, in favor of a more extensive fragmentation. For DP, EP and CES the same values of the MRM-IDA-EPI method were used.

3.5. Validation of quantitative analysis

Good correlations for all compounds were obtained over the linear dynamic range (0.04–50 μg mL^{-1}), and the correlation coefficients (r^2) were between 0.9993 and 0.9999. Accuracy tests showed that analytical method was in according with FDA parameters. RSD values ranged from 1 to 8% in intra-day tests and were always lower than 10%, indicating good intra-study variation. Also, the RSD value for inter-day reproducibility within the laboratory is less than 15%. All validation parameters, including LOD and LOQ for each compound, are reported in Tables S2 and S3.

Table 2
CAs identified and quantified with the targeted method in *Murraya Koenigii* leaves A e B. Data are reported as mg/kg.

Analytes	*Murraya Koenigii* A	*Murraya Koenigii* B
Koenigicine	0.44	1,77
Koenimbine	1,26	1,62
Mahanimbine	7,22	5,62

4. Real samples

4.1. Targeted analysis

Targeted MRM analysis was used for the quantification of Koenigicine, Koenimbine and Mahanimbine in 2 different samples of *M. koenigii* (A and B). For this purpose, *M. koenigii* leaves were chosen as the matrix for the quantitative analysis of compounds, since most of the biological activities of this plant are attributed to them [37–40]. The compound most present in *M. koenigii* is Mahanimbine, which represents the main CA in both samples, in accordance with the data in the literature [1,12]. Koenigicine and Koenimbine were found in lower amounts as shown in the Table 2.

4.2. Semi-untargeted analysis

The MRM-IDA-EPI was used for a putative identification of CAs, whose peaks were detected by means of *in silico* MRM transitions; the results are shown in Table 3.

A similar strategy was already successfully used for other analytes in different matrices [41]. Among the several findings, the MRM transitions obtained from Koenigicine gave two chromatographic peaks at R_T 5.84 and 6.27. While the peak at R_T 6.27 corresponded to Koenigicine, as confirmed through the comparison with the analytical standard, the peak at R_T 5.84 was further investigated. The acquired EPI spectrum for Koenigicine was compared with fragments in a study reported in the literature [28] and with the predicted MS/MS spectrum for Koenigicine by CFM-ID. The following m/z fragments were confirmed by the literature: m/z 309.0 is correlated with [M+H-CH_3] $^+$ to the loss of a methyl group, m/z 293.0 is due to the loss of 31 Da [M+H-O-CH_3]$^+$ and the m/z 282.0 fragment due to the opening of the pyranic ring and loss of the group [M+H-C(CH_3)$_2$]$^+$ [28]. The ion at m/z 167.0 corresponds to carbazole. The following fragments were also confirmed by the analysis of the MS/MS spectrum predicted by CFM-ID: m/z 309.0 and m/z 282.0, already confirmed by the literature [28]. m/z 278.0, which derived from the loss of a methoxy group and methyl belonging to the methoxy group or methyl in C-3 position; m/z 268.0, which was formed by the opening of the pyranic ring and a loss of 56 Da [M+H-CH_2=C(CH_3)$_2$]$^+$. The other fragments compared with the fragmentation pattern predicted by CFM-ID were m/z 238.0 and m/z 210.0. The peak at R_T 5.84 was hypothesized to belong to Mukonicine, an isomer of Koenigicine, which differs for the position of a methoxyl group. The methoxy group is found in Mukonicine in position 10, with respect to position 9 occupied in Koenigicine. Mukonicine was isolated from *M. koenigii* leaves [42,43]. From the analysis of the EPI spectrum, which was compared with the MS/MS spectrum predicted by CFM-ID, a fragmentation pattern similar to that of Koenigicine was evident, and the following fragments were confirmed: m/z 309.0, 278.2, 266.0, 250.1, 238.0, 210.1. For the correspondence of the fragments, it was possible to hypothesize that this compound was probably Mukonicine.

From the optimized MRM transition for Mahanimbine, three peaks at R_T 8.66, 9.10 and 9.34 min were shown. The peak at R_T 9.10 was identified as Mahanimbine, while it was hypothesized that the peak at

Table 3
Results obtained from the *Murraya Koenigii* sample by means of the proposed MRM-IDA-EPI acquisition method.

R_T (min)	m/z	Main fragments	Compound ID
4.85	280.1	265.1; 236.0; 224.0; 210.0; 167.1;	Koenine/Murrayamine A
5.41	280.1	265.1; 238.0; 236.0; 224.2; 210.1; 196.0;	Koenine/Murrayamine A
5.55	308.0	292.0; 278.0; 250.1; 222.1;	7-MethoxyMurrayacine
5.62	310.1	295.1; 278.1; 254.0; 252.0; 250.0; 234.0; 224.0; 220.0; 196.0;	Koenigine
5.84	324.1	309.0; 278.2; 266.0; 250.1; 238.0; 210.1;	Mukonicine
6.27	324.1	309.0, 293.0; 282.0; 278.0; 268.0; 238.0; 210.0; 167.0;	Koenigicine
7.06	348.2	332.0; 330.1; 276.1; 264.0; 210.0; 194.1; 81.0;	Murrayamine C
7.21	294.1	279.0; 264.1; 249.1; 235.9; 167.0; 131.1; 91.2;	Koenimbine
7.40	294.1	279.0; 266.0; 236.0; 220.1; 208.1; 196.0; 184.2;	O-Methylmurrayamine A
7.55	348.2	333.0; 292.1; 279.0; 266.0; 264.1; 236.1; 224.1; 212.1; 167.0; 123.1;	Mahanine
8.58	348.2	330.2; 196.0; 182.1; 169.1	Murrayakonine D
8.66	332.2	276.0; 250.0; 248.2; 210.1; 182.0; 167.0; 135.0; 123.1;	Isomahanimbine
9.10	332.2	276.0; 250.0; 248.2; 210.1; 182.0; 167.0; 135.0; 123.1;	Mahanimbine
9.34	332.2	317.1; 288.0; 248.0; 210.0; 194.0; 182.0; 167.0; 135.2;12.1; 109.2; 107.2;	Bicyclomahanimbine

8.66 can be identified as Isomahanimbine, an isomer of Mahanimbine, isolated by Joshi from the leaves of *M. koenigii*, which pyranic ring is linked in position C-7 and C-8 of the benzene ring [44]. The EPI scan of this compound did not show particular differences in the fragmentation pattern of Mahanimbine, for this reason it was considered as its isomer. Instead, the peak at R_T 9.34 may be a cyclized form of the mahanimbine, as it is more apolar and therefore it was eluted later. In fact, the geranyl monoterprene unit in position C-1 on the carbazole nucleus can undergo to several cyclizations, leading to isomeric form with a C-23 skeleton [4]. According to these, the peak at 9.37 was associated to cyclic compounds, such as Cyclomahanimbine or Bicyclomahanimbine. The EPI spectrum obtained for these analytes was matched to the spectra predicted by CFM-ID 3.0. The following m/z fragments were confirmed for both of them: 317.1, 288.0, 248.0, 210.0, 194.0, 182.0, 167.0, 135.2. The EPI spectrum acquired for the peak also presented the m/z fragments: 123.1, 109.2, 107.2; they were found only in the MS/MS spectrum predicted for Bicyclomahanimbine.

From the Koenimbine tuned transition, two more intense peaks at R_T 7.21 and 7.40 min were observed. The peak to 7.20 min belongs to the Koenimbine standard. It was suggested that the peak at R_T 7.40 min could be identified with two possible Koenimbine isomers: O-Methylmurrayamine-A and Mupamine. The O-Methylmurrayamine A is a 9-methoxy-3,3,5-trimethyl-11H-pyrano [3,2-a] carbazole and was isolated from the leaves of *M. koenigii* [45]. In Mupamine the methoxy group is in position 10, but this compound has not yet been isolated from this plant [3,27]. It is therefore more probable that the isomer present is O-Methylmurrayamine-A. The EPI spectrum acquired for the peak at R_T 7.32 was matched with the MS/MS spectrum predicted by CFM-ID. The following m/z fragments were confirmed: 279.0, 266.0, 236.0, 220.1, 208.1, 196.0, 184.2. It was possible to hypothesize that the compound identified is O-Methylmurrayamine A.

The MS/MS spectrum for Koenine was predicted by CFM-ID. For the more intense fragments m/z 220.1 and m/z 196.1, which were added to the MRM method, the following peaks at R_T 4.85 and 5.41 were present in chromatographic run. Due to no differences in the fragmentation pattern, it was hypothesized that the peaks may belong to two isomers: Koenine and Murrayamine A. Koenine was isolated from *M. koenigii* leaves, while Murrayamine A was isolated from *Murraya euchrestifolia* leaves and *M. koenigii* roots [3] . From the analysis and comparison with the MS/MS spectrum predicted by CFM-ID, as shown in Fig. 3 the following m/z fragments were confirmed for koenine: 265.1, 236.0, 224.0, 210.0, 167.1. The confirmed m/z fragments for Murrayamine A were: 265.1, 238.0, 236.0, 224.2, 210.0, 196.0.

The koenigine fragmentation pattern was predicted by CFM-ID, and the obtained spectrum was used for the comparison in EPI experiment. The more intense fragments m/z 278.1 and m/z 208.1 were added to the MRM method and the response signal generated a peak at R_T 5.63 min. The EPI spectrum was also acquired, and the following m/z fragments were observed: 278.1, 252.0, 250.0, 234.0, 224.0, 222.0, 196.1. Their presence confirmed the hypothesis that the compound was Koenigine. From the analysis of the EPI spectra acquired for Konine, Koenigine, Koenigicine and Koenimbine it was possible to notice how these molecules have a similar pattern of fragmentation. In fact, these compounds are structurally pyran [3,2-a] carbazole, which differ in the oxygen substitution pattern at positions 6 and 7. For example the following m/z fragments: 309.1 for Koenigicine, 295.1 for Koenigine, 279.1 for Koenimbine and 265.1 for Koenine are formed by a rearrangement of the molecule due to the loss of 15 Da [M+H-CH_3]. This kind of fragmentation was confirmed through the proposed study for the identification of Clausamine H [46]. Instead, the m/z fragments: 268.0 for Koenigicine; 254.0 for Koenigine; 238.0 for Koenimbine and 224.1 for Koenine formed for a loss of 56 Da. The common fragmentation mechanism of the analytes could be due to the loss of the following fragment [M+H-CH_2=C(CH_3)$_2$]. It was shown in this study conducted on carbazole derivatives containing a pyranic ring that could lose the carbon of the isopropyl group during fragmentation the pyranic ring. Subsequently, the cleavage of the bond adjacent to the isopropyl carbon occurs [47]. The same loss [M+H-CH_2=C(CH_3)$_2$] occurs during the fragmentation of Clausamine H, which structurally has only the prenyl chain, since the adjacent hydroxyl group which leads to the formation of the pyranic ring is not positioned [46]. The common mechanism of compound fragmentation could confirm the hypothesis that the peaks obtained at R_T 4.85, 5.41 and 5.61 min are the proposed analytes.

Mahanine was isolated by Narasimhan et al. from the leaves of *M. koenigii* [3]. The fragments m/z 226.1; m/z 224.1; m/z 123.1 obtained by CFM-ID, were calculated for Mahanine. The response signal for the following fragments showed the most intense peak to 7.55 min. The acquired EPI spectrum was matched with the Mahanine spectrum present in the literature [29]. The following m/z fragments were confirmed: 279.0, 266.0. The presence of the fragment m/z 333.0 is probably due to the loss of the methyl group -CH_3 in position C-3. The presence of the fragment m/z 123.0 indicates the opening of the ring with consequent loss of [M+H-CH_2=C(CH_3)-CH_2CH_2CH=C(CH_3)$_2$]. The fragment at m/z 167.0 confirmed the presence of a compound with a carbazole nucleus [30]. Furthermore, from the comparison with the MS/MS spectrum obtained by CFM-ID, many fragments of the most intense were confirmed: m/z 292.1, 266.1, 264.1, 236.1, 224.1, 212.1. From the analysis of the data, it is possible to hypothesize that the compound is Mahanine.

The PI-IDA-EPI scan mode was used to identify analogues of CAs, not included in the MRM list, but with similar fragmentation pattern. The analytes, already present in the MRM list, were also confirmed with this approach.

By means of PI-IDA-EPI with the common ion precursor m/z 210.1, the ions at m/z: 258.0, 264.1, 334.2, 346.2, 364.2, 376.1, 393.1 were observed, which corresponding probably to the mass 6,7-Dimethoxy-1-hydroxy-3-methylcarbazole, Girinimbine, Mahanimbinol, Murrayamine

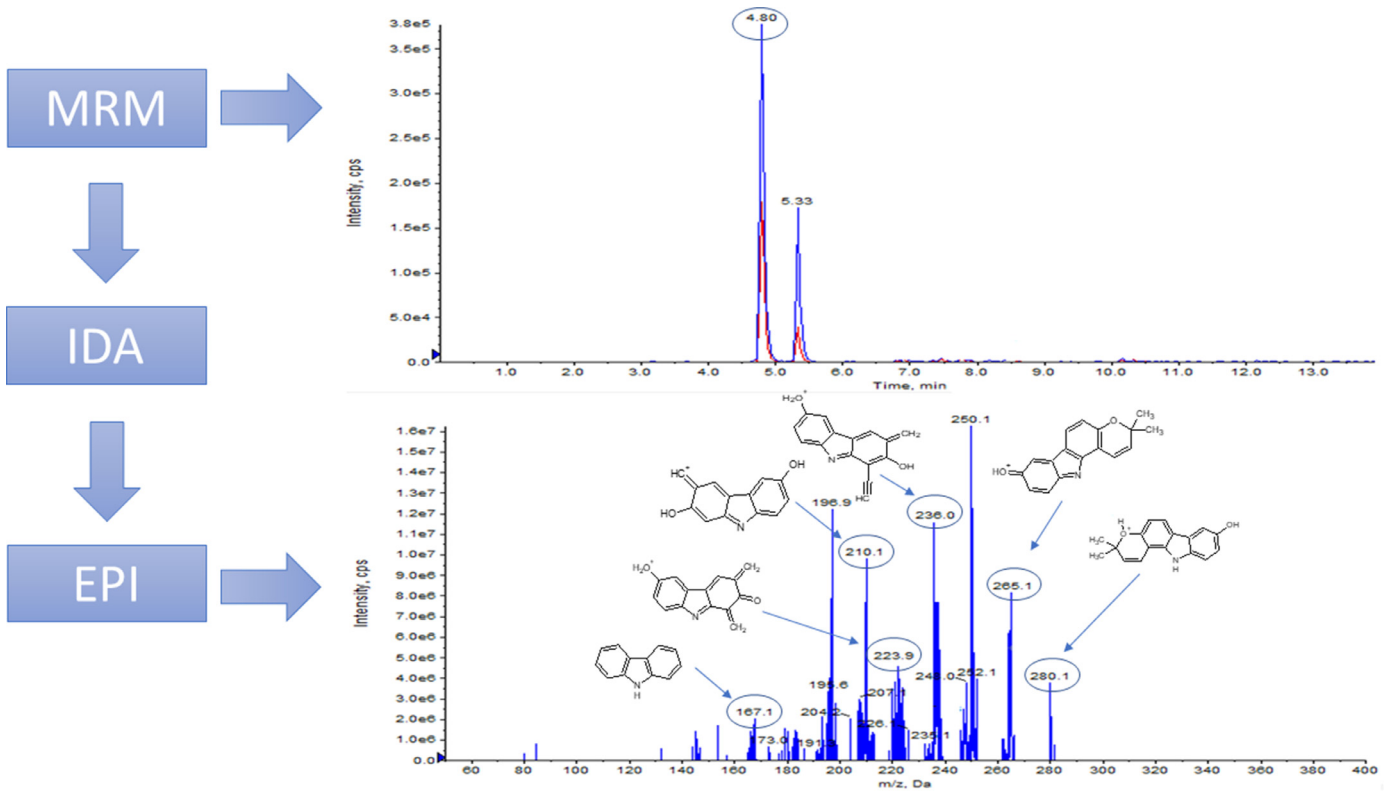

Fig 3. Mass spectra of Koenine fragmentation pattern identified in MRM-IDA EPI-mode.

Table 4
Results obtained from the *Murraya Koenigii* samples by means of the proposed PI-IDA-EPI acquisition method.

RT	m/z	PI	Main fragments	Compound ID
1.51	258.0	$[M-H-210]^+$	240.0;198.0;184.2;170.0;	6,7-Dimethoxy-1-hydroxy-3-methylcarbazole
2.84	264.1	$[M-H-210]^+$	249.0; 246.0; 236.0; 222.0; 206.4;	Girinimbine
3.85	376.1	$[M-H-210]^+$	385.2; 292.1; 272.1;	Murrayakonine C
4.32	393.2	$[M-H-210]^+$	376.1; 357.2; 335.0: 210.1;	Bis-2-hydroxy-3-methylcarbazole
4.85	280.1	$[M-H-210]^+$	265.1; 236.0; 224.0; 210.0; 167.1;	Koenine/Murrayamine A
5.41	280.1	$[M-H-210]^+$	265.1; 238.0; 236.0; 224.2; 210.1; 196.0;	Koenine/Murrayamine A
5.62	310.1	$[M-H-210]^+$	295.1; 278.1; 254.0; 252.0; 250.0; 234.0; 224.0; 220.0; 196.0;	Koenigine
6.27	324.1	$[M-H-210]^+$	309.0, 293.0; 282.0; 278.0; 268.0; 238.0; 210.0; 167.0;	Koenigicine
6.75	346.2	$[M-H-210]^+$	224.0; 220.0; 210.0; 196.1; 182.0; 166.1;	Murrayamine J
7.06	348.2	$[M-H-210]^+$	332.0; 330.1; 276.1; 264.0; 210.0; 194.1; 81.0;	Murrayamine C
7.21	294.1	$[M-H-210]^+$	279.0; 264.1; 249.1; 235.9; 167.0; 131.1; 91.2;	Koenimbine
7.40	294.1	$[M-H-210]^+$	279.0; 266.0; 236.0; 220.1; 208.1; 196.0; 184.2;	O-Methylmurrayamine
7.43	334.2	$[M-H-210]^+$	318.0; 252.1; 210.0; 208.0; 196.0; 182.2; 167.1; 123.0; 82.1;	Mahanimbinol
7.55	348.2	$[M-H-210]^+$	333.0; 292.1; 279.0; 266.0; 264.1; 236.1; 224.1; 212.1; 167.0; 123.1;	Mahanine
9.10	332.2	$[M-H-210]^+$	276.0; 250.0; 248.2; 210.1; 182.0; 167.0; 135.0; 123.1;	Mahanimbine
9.34	332.2	$[M-H-210]^+$	317.1; 288.0; 248.0; 210.0; 194.0; 182.0; 167.0; 135.2;12.1; 109.2; 107.2;	Bicyclomahanimbine
9.88	364.2	$[M-H-210]^+$	334.4; 280.1; 240.1; 226.2; 123.0; 67.1	Murrayanol

J, Murrayanol, Murrayakonine C and Bis-2-hydroxy-3-methylcarbazole. Furthermore, the ions set m/z 280.1, 294.1, 310.1, 332.2, 348.1 were observed as well as in MRM experiment. The results are shown in Table 4.

For the m/z 264.1 a peak was obtained at R_T 2.84 min, which has been associated with Girinimbine. The compound was the first pyrano[3,2-a]carbazole alkaloid isolated by Chakraborty et al. in 1964 from *M. koenigii* plant [3]. The acquired EPI spectrum was compared with the data reported in the literature [31]. The loss of $[M+H-CH_3]^+$ in position C_3 or on the pyranic ring would justify the presence of the m/z 249 fragment. Fragment m/z 236.0 could result from the loss of 28 Da due to pyranic rupture of the ring and loss of the $[M+H-C_2H_4]^+$ fragment. The m/z 246.0 and the m/z 222.0 fragments derived from the loss due to rupture of the pyranic ring and loss of the isopropyl group $[M+H-C(CH_3)_2]^+$ were also confirmed. The EPI spectrum was compared with the fragmentation spectrum produced by CFM-ID 3.0 and the following fragments were compared: m/z 236.0, 222.0, 206.4. From the data reported in the literature and from the data processed with CFM-ID, it was conceivable that the compound is Girinimbine.

The EPI spectrum of the peak at R_T 3.85 was acquired and compared with the predicted MS/MS spectrum by CFM-ID. From the spectrum analysis it was assumed that it was probably Murrayakonine C. From the fragmentation pattern, the following m/z fragments were observed and compared with predicted spectra of the compound mentioned above: m/z 358.2, 292.1, 272.2. Furthermore, this compound was isolated from the leaves of *M. koenigii* [5].

The Bis-2-hydroxy-3-methylcarbazole belongs to the class of Bis-carbazoles and it was isolated from *M. koenigii* [27]. The peak at R_T

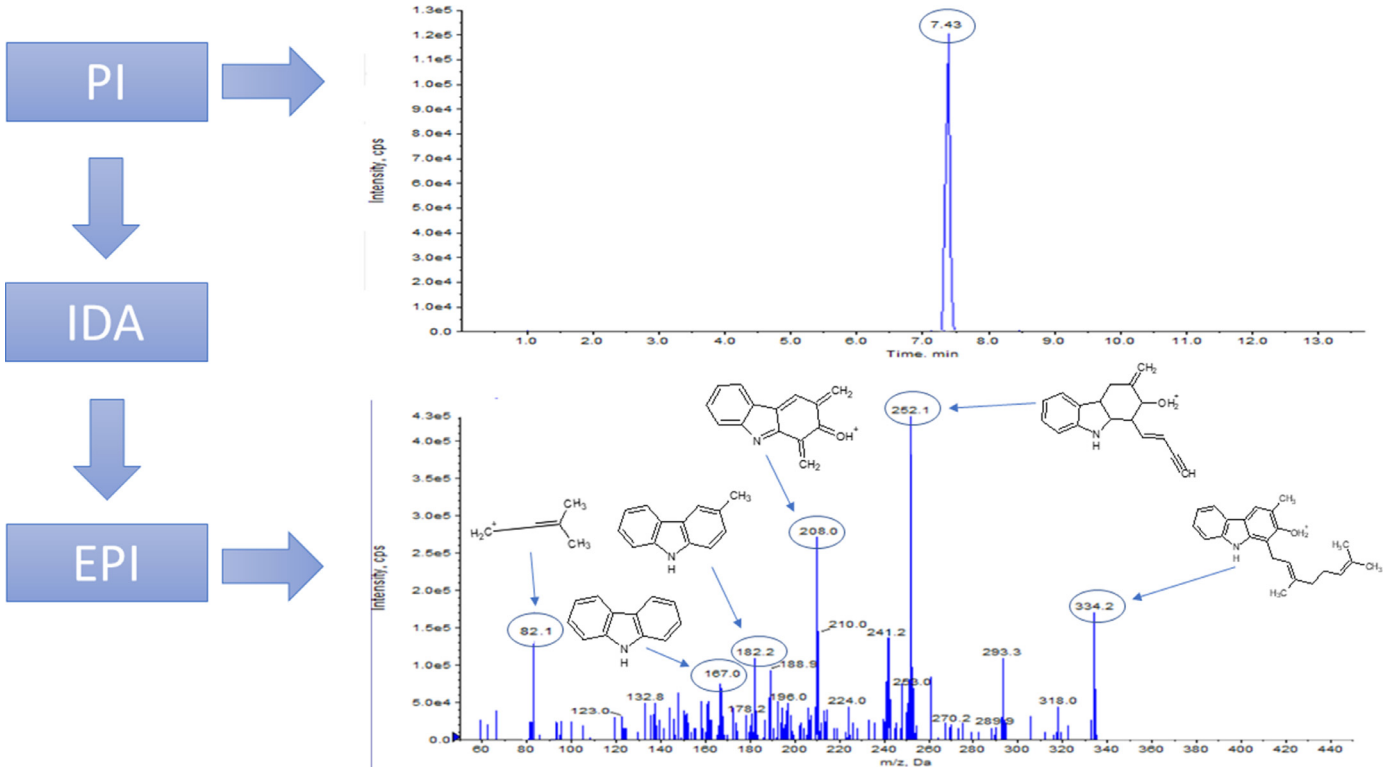

Fig 4. Mass spectra of Mahanimbinol fragmentation pattern identified in PI-IDA EPI-mode.

4.32 min corresponded to an m/z 393.2 precursor ion equal to the value of the protonated mass for Bis-2-hydroxy-3-methylcarbazole. From the analysis of the EPI spectrum, which was compared with the predicted MS/MS spectrum by CFM-ID, the following m/z fragments were observed and confirmed: 376.1, 335.0, 357.2, 210.1. From the correspondence of the fragmentation pattern, it is possible to hypothesize that the unknown compound is Bis-2-hydroxy-3-methylcarbazole.

In addition, a compound with m/z 334.2 was identified in PI scan mode. The compound showed a peak at R_T 7.43 and was identified as Mahanimbinol. This compound is considered a geranylate congener and is a precursor of carbazoles with a C-23 skeleton [3]. The spectrum of the analyte was acquired through the PI-SCAN-EPI experiment. The spectrum showed in Fig. 4 was matched with an MS/MS spectrum present in the literature [18]. The following m/z fragments were observed: 210.0, 208.0, 196.0, 167.1. These fragments were confirmed by comparison with the spectrum predicted by CFM-ID together with the m/z fragments: 318.0, 252.1, 182.2, 123.0. The intense ion peak at m/z 252.1 was formed by a loss of 82 Da. In fact, there was a peak at m/z 82.1 formed by fragmentation of the geranyl chain and formation of the fragment [M+H-CH_2-CH_2-CH=C(CH_3)$_2$]. So, it was therefore possible to hypothesize that the peak belongs to Mahanimbinol. The presence of this precursor proved circumstantial evidence that Bicyclomahanimbine, observed in MRM experiment, was also present.

5. Conclusion

CAs represent an important class of bioactive compounds due to their biological activities. For the intriguing structural characteristics of these compounds related to their structural differences, it is very important to elucidate the chemical structure and especially the most studied subclasses, such as Pyrano Carbazoles.

A HPLC-MS/MS analytical method was developed, combining targeted and semi-untargeted acquisition, which provided reliable quantitative data, by MRM acquisition, and structural information by MRM/PI-IDA-EPI. The latter approach demonstrated to be a powerful tool in identification of compounds belonging to the class of CAs.

The analysis of CAs compounds in *M. koenigii* was performed, starting from the extraction and clean-up step, which were set up according to the characteristics of the matrix. This step was essential to minimize the interfering compounds present in such complex matrices, allowing at the same time a sensitive and robust quantitative analysis on the target compounds, but also a semi-untargeted method suitable for putative identification. The MRM/PI-IDA-EPI presented approach was applied for the first time for the analysis of the CAs. allowing to investigate the content of different CAs belonging to the subclass of Pyrano Carbazole in *M. Koenigii* leaves. In particular, different compounds of the C18 series were identified: Girinimbine, Koenine, Murrayamine A, Koenigine, Koenigicine, Koenimbine and some structural isomers. The presented method also allowed the identification of CAs with a C_{23} skeleton: Mahanimbinol, Mahanine, Mahanimbine and its isomer Bicyclomahanimbine, resulting from the transformations that the monoterpenic geranyl unit can undergo.

The presented method could represent a useful tool for studying the content of CAs in different matrices, with good sensitivity, for quantification, screening and putative identification of CAs.

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Declaration of Competing Interest

All the authors declare that they have no conflict of interests, and 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

Eduardo Viteritti: Investigation, Methodology, Validation, Writing – original draft. **Eleonora Oliva:** Investigation, Validation. **Fabiola Eugelio:** Data curation. **Federico Fanti:** Conceptualization, Methodology, Resources, Writing – review & editing. **Sara Palmieri:** Data curation, Formal analysis. **Eleonora Bafile:** Conceptualization, Supervision, Funding acquisition. **Dario Compagnone:** Supervision, Funding acquisition, Writing – review & editing. **Manuel Sergi:** Conceptualization, Methodology, Project administration, Writing – review & editing.

Data availability statement

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcoa.2022.100055.

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