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Identification of atractyloside by LC–ESI–MS in alleged herbal poisonings

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Abstract

An LC–MS screening method was developed to detect the presence of atractyloside (ATR), the toxic principle of a commonly used medicinal plant in South Africa, *Callilepis laureola*, in biological matrices such as body fluids and human viscera.

Keywords: *Callilepis laureola*; Asteraceae; Atractyloside; Carboxyatractyloside; HPLC; Mass spectrometry; LC–MS; Poisoning; Forensic chemistry

1. Introduction

South Africa is a country rich in plant and cultural diversity. The region has some 30,000 species of flowering plants, accounting for almost 10% of the world's higher plants. Many cultural groups use a wide variety of plants in their daily lives for food, water, shelter, fuel and medicine [1]. It is estimated that 60–80% of the South African population use traditional medicines, usually in various combinations [2,3]. Cases of acute or systematic poisoning with traditional medicines are reported from time to time, some resulting in severe organ damage [4–7] or even mortality [2,8,9]. The detection of toxins of botanical origin is problematic as has been highlighted in a recently published case report [10]. Sensitive and selective detection methods are not readily available and often require very expensive equipment and highly skilled staff. Approximately 60% of the cases submitted to the Forensic Chemistry Laboratory of Johannesburg, South Africa (FCL JHB), are of an unknown nature—the cause of death is unknown and/or no case history is submitted with the forensic exhibits. The situation is worsened by the reluctance of people to admit using herbal remedies. The opposite is also true—many samples are submitted with a case history stating ‘‘alleged *muti* (herbal) use/poisoning’’, but many of these cases turn out to be positive for commercial pharmaceuticals and/or pesticides, with no toxins of botanical origin being detected. The lack of suitable analytical procedures for the detection of herbal toxins can also lead to a distorted picture in regard to the use of herbal remedies and their role as the cause of death.

Of the 30,000 species of higher plants in South Africa, approximately 3000 species are used as medicines, and of these, some 350 species are commonly used and traded medicinal plants [3,11]. One of these more commonly used plants is *Callilepis laureola* DC. (Asteraceae), also called ‘‘Ox-eye daisy’’ or ‘‘Wildemagriet’’. *C. laureola* is an attractive suffrutex of approximately 60 cm in height with erect, leafy annual branches arising from a permanent woody base [6,9]. The tuberous root of the plant is used in traditional medicine by the Zulu and Xhosa people of

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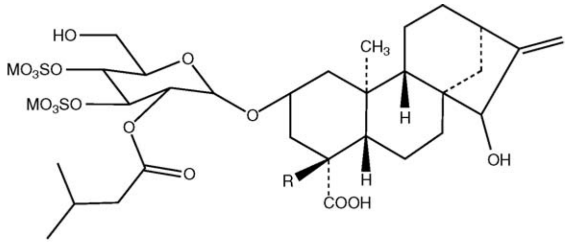
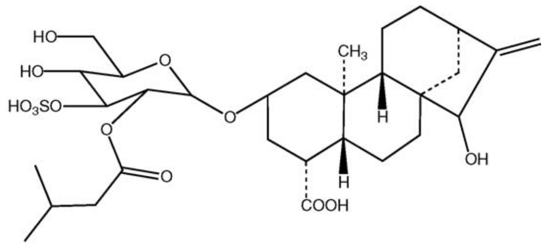


Atractyloside (KATR) : M = K, R = H

Protonated Atractylosdie (ATR) : M = H, R = H

Carboxyatractyloside (CATR) : M = H, R = COOH



Atractyloside monodesulfate

Fig. 1. Structures of atractyloside (KATR), its protonated (ATR) and desulphated forms, and carboxyatractyloside (CATR).

South Africa [2,6,11,12] and it is well known by the Zulu name *impila*, which means 'health' [6]. The applications of *impila* range from alleviating stomach complaints, tapeworm infestations, as a vermifuge, for purgative applications, as a cough expectorant, for the treatment of whooping cough, to enemas and as an emetic. It is also used in spiritual context to ward-off evil spirits [11,13]. However, *C. laureola*, which contains atractyloside (ATR) and carboxyatractyloside (CATR; Fig. 1) [14–16] as toxic principles, has also been implicated in the death of numerous patients who used medication prepared from *impila* [2,4,6,7,9,12,17,18]. Atractyloside has a strychnine-like action and gives rise to hypoglycaemic type convulsions [19], which may be accompanied with gastrointestinal symptoms, confusion, stupor and coma [20]. It acts as a specific inhibitor of oxidative phosphorylation by blocking ADP transport at the mitochondrial membrane and as such it is often used as a biochemical tool [21,22].

In a previous paper [14], the absence of good analytical methods for the detection of atractyloside, especially for forensic application, was described. Most of these methods failed due to the lack of quantitative accuracy of results [23], non-specific response [17,24], or was time-consuming, cumbersome and failed to detect the intact atractyloside molecule [25]. In an authoritative review [26], the authors commented on the absence of general screening methods in forensic toxicology for poisonous plants and mentioned, in particular, the lack of chromatographic procedures for atractyloside and carboxyatractyloside.

Recently we have developed a screening method utilising reversed-phase high performance liquid chromatography as separation technique, coupled to an atmospheric pressure ionisation mass spectrometer for the detection of atractyloside in *C. laureola* tuberous roots [14]. However, this method failed to detect the presence of atractyloside in biological matrices like body fluids and human viscera. Herewith, we want to report on the forensic application of a modified HPLC–MS method [14] for the detection of atractyloside and related compounds in biological samples (especially viscera samples).

2. Material studied, methods, techniques

2.1. Standards and reagents

Atractyloside (99% by TLC), potassium chloride, sodium chloride, potassium dihydrogen phosphate and sodium monohydrogen phosphate (>99.5%) were obtained from Sigma Chemical Company, USA. Ammonium acetate (98%) (NH_4OAc) and acetic acid (99%) were obtained from BDH Laboratory Supplies, England. Sulphuric acid (Suprapur 96%), hydrochloric acid (Suprapur 96%), phosphoric acid (Suprapur 96%), formic acid (98–100%) and ammonia (GR 25%) were obtained from Merck, Darmstadt, Germany. Acetonitrile (gradient quality, 200 nm UV cutoff) and methanol (gradient quality, 215 nm UV cutoff) were obtained from Romil, England. HPLC grade water (20 MΩ/cm) was obtained from a Milli-Q/reversed osmosis system (Millipore, USA). The phosphate-buffered saline (PBS) solution was prepared according to the prescribed method supplied by Waters Corporation, Milford, USA [21]. The anhydrous salts of KCl (0.20 g), NaCl (8.00 g), KH_2PO_4 (0.20 g) and Na_2HPO_4 (1.15 g) were dissolved in 1 l deionised water and the pH adjusted to 7.0 with 10% phosphoric acid. HCl–PBS was prepared as described above, but the pH was adjusted to 4.5 with hydrochloric acid. Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation. A 12-port vacuum manifold was used for all extractions.

2.2. Instruments and conditions

A Waters 2695 separations module (SM) equipped with a 996 photodiode array (PDA) detector and a Z spray mass selective detector (ZQ) (Micromass, UK) [multi-mode ESCi, maximum mass 4000 *m/z*, ESCi (±) mode] was used. Preliminary MS optimisation was performed using an ESCi interface in ESI (+), ESI (−), APCI (+) and APCI (−) modes. Eluent composition and the chemical properties of ATR limit the use of the ESCi interface to negative-ion electrospray, and this mode was used in all experiments. The built-in syringe pump was used for all flow-injection experiments on the ZQ detector.

Table 1
Optimised HPLC conditions for the analysis of atractyloside

Variable	Optimised conditions
Column	Waters Xterra Phenyl 300 mm × 2.1 mm, 3 μm (2 × 150 mm columns in series) with guard column
Column temperature (°C)	40 ± 1
Mobile phase components	Methanol Acetonitrile Buffer (10 mM aqueous NH_4OAc, pH 4.5, with acetic acid)
Gradient profile	Initial conditions of 90:10 buffer:methanol for 2 min Linear gradient to 10:90 buffer:acetonitrile for 18 min Isocratic conditions of 10:90 buffer:acetonitrile for 5 min Return to initial conditions within 5 min Equilibrate for 5 min
Mobile phase flow rate (ml/min)	0.2
Degassing	In-line (normal mode)
Sample temperature (°C)	5 ± 1

Various reversed-phase HPLC columns were evaluated, but the Waters Xterra Phenyl (150 mm × 2.1 mm, two columns in series) consistently produced the best results [14]. For optimal chromatography, the following conditions were used: a linear gradient mobile phase consisting of buffer (25 mM NH_4OAc, pH 6.7); acetonitrile, starting at 90% buffer; 10% acetonitrile and ending at 10% buffer; 90% acetonitrile after 20 min. The column was allowed to flush at the high organic mobile phase for 5 min where after the mobile phase composition was changed to initial conditions and allowed to equilibrate for 10 min. The flow rate was 0.2 ml/min without splitting and the HPLC column was kept at 40 °C for all experiments.

However, these conditions were not optimal for the mass spectral detection of the atractyloside standard or the separation and detection of atractyloside in a biological matrix. The best-compromised conditions for the chromatography of atractyloside by HPLC–MS are summarised in Table 1 [14] and used in all the experimental work. High purity nitrogen gas (Air Products) was used as nebulising and carrier gas at a flow rate of 450 l/h whilst cone gas was utilised at a flow rate of 30 l/h. In later experiments, the cone gas was found to reduce the efficiency of the ionisation of atractyloside and was deactivated. The optimised MS conditions are collated in Table 2. Optimisation experiments were done in scan mode (*m*/*z* 200–900) and quantitative work was done in single ion monitoring (SIM) mode with a dwell time of 0.6 s and a span of 0.4 Da. The ions used for SIM experiments were the deprotonated molecule ion *m*/*z* 725 and the main fragment ion *m*/*z* 645.

Flow-injection analysis (FIA) experiments were initially done by infusing 10 μl/min of 10 μg/ml atractyloside standard into the HPLC mobile phase of 0.2 ml/min 50:50 buffer:methanol. However, in the presence of methanol, atractyloside was poorly ionised and in later experiments, the mobile phase was changed to 50:50 buffer (10 mM ammonium acetate, pH 4.5, with acetic acid):acetonitrile.

2.3. Determination of LOD, LOQ and calibration range

The limit of detection (LOD) was determined experimentally, and was taken as the concentration that produced a detector signal that could be clearly distinguished from the baseline noise (>3 times baseline noise). The limit of quantification (LOQ) was taken as the concentration that produced a detector signal >10 times greater than the LOD signal. The practical limit of quantification was determined by spiking old *impila* tuber (void of atractyloside) and extracting the spiked sample as described. A calibration curve was prepared by covering the 1 ng/ml–160 μg/ml concentration range. The precision of the instrumental method was determined by multiple injections ($n = 10$) of the atractyloside standard at three concentration levels covering the analytical range of the method.

Table 2
Optimised MS conditions for the determination of atractyloside

Variable	Optimised conditions	
	ATR	ATR–SO_3
Mode and polarity	ESI negative	ESI negative
Capillary voltage (kV)	3.00	3.50
Cone voltage (V)	27	65
Desolvation temperature (°C)	400	450
Extractor voltage (V)	1.0	1.0
RF lens (V)	1.0	3.5
Source temperature (°C)	120	120
Cone gas flow (l/h)	0	0
Desolvation gas flow (l/h)	450	450
Ion energy	0.5	0.5
Multiplier (V)	−650	−650
Resolution (LMR and HMR)	15.8	15.8

2.4. Extraction of *C. laureola* tubers and unknown powdered samples

The SPE extraction of pure atractyloside standard has been described in our previous paper [14]. It was found that ATR could be successfully retained and eluted from the Oasis HLB cartridges with any of the PBS buffers evaluated as well as water. However, extraction of atractyloside from the tuber produced mixed results as ATR yields were inconsistent and some of the related compounds were not successfully retained. The PBS–HCl yielded the most atractyloside; however, PBS–NH_3 produced the cleanest extracts but failed to extract all the compounds of interest (CATR was not detected in this extract). The PBS–HCl buffer was selected as the buffer of choice for all further extractions as it extracted all the compounds of interest.

Finely ground tuber (1 g) was placed in a 50 ml polypropylene screw-top centrifuge sample tube, followed by the addition of 30 ml of the PBS–HCL buffer. The sample was mixed on a Heidolph rotating sample mixer for 30 min, and the supernatant recovered by centrifugation at 4000 rpm using a Hermle Z 300 centrifuge for 15 min. The SPE cartridge was prepared by washing consecutively with 6 ml of methanol, water and the extracting buffer used. The supernatants were applied to the SPE cartridges by hand and drawn through the cartridges at approximately 1 ml/min by applying a mild vacuum.

The cartridges were washed with 3 ml extracting buffer and dried under vacuum for 10 min. They were then eluted with 3 ml methanol, 3 ml acidic methanol (10 ml formic acid/l methanol), 3 ml basic methanol (10 ml ammonia/l methanol) and 3 ml acetonitrile and the eluates of each cartridge combined into one collection tube. The collection tubes were placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of a 50:50 methanol:water mixture.

2.5. Extraction of viscera samples

As the cause of death in most of the forensic toxicology cases submitted to the Forensic Chemistry Laboratory of Johannesburg is unknown, a general extraction procedure is usually followed using SPE and the standard PBS buffer. To enhance the extraction of ATR and related compounds, the PBS buffer was replaced with PBS–HCL buffer. An aliquot of each liquefied viscera sample submitted – stomach (including contents), liver, kidney, blood and bile – (5 g or 5 ml each) was mixed with 20 ml of PBS–HCL buffer solution and shaken for 30 min on the rotating sample mixer. After centrifugation for 15 min (4500 rpm), the aqueous phase was passed through an Oasis HLB cartridge and the absorbed compounds stripped from the solid phase material with 3 ml methanol, 3 ml acidic methanol, 3 ml basic methanol and 3 ml acetonitrile. The eluates of each cartridge were combined into one collection tube, placed into a Zymark Turbovap and evaporated at 40 °C under a regulated stream of nitrogen gas. The dry residues were reconstituted with 1 ml of a 50:50 methanol:water mixture.

3. Results and discussion

3.1. Detection of atractyloside and carboxyatractyloside

C. laureola contains two highly toxic compounds. Atractyloside (KATR; Fig. 1), first isolated by M. Lefranc in 1868 [27], has an empirical formula of $C_{30}H_{44}K_2O_{16}S_2$ and a molecular mass of 802.13 [protonated form ATR (Fig. 1): $C_{30}H_{46}O_{16}S_2$, molecular mass 726.22]. Carboxyatractyloside (Fig. 1) was first isolated and reported by Piozzi et al. [28] and has an empirical formula of $C_{31}H_{46}O_{18}S_2$ and a molecular mass of 770.83.

Atractyloside and carboxyatractyloside do not have strong chromophoric properties and can therefore easily be missed in a general screening process based on UV or photodiode array detection. A published GC–MS method for the detection of atractyloside is not able to detect the intact molecule and required derivatisation to detect the fragments of atractyloside [25]. An attempt was made to analyse the underivatised molecule by LC–MS (EI) using a Waters Thermabeam detector previously described [14], but resulted in the total fragmentation of the molecule. A gentler ionisation technique (ESI) was successfully used to detect atractyloside [14] after chromatographic separation. It was found that atractyloside is only detected in negative electrospray mode and all other detection modes, including the PDA detector, were deactivated in subsequent experimental work.

Under the normal reversed-phase chromatographic conditions, the acidic form of atractyloside (ATR) is virtually unretained on all the reversed-phase columns evaluated, except for the Waters Xterra Phenyl and Hypersil Hypercarb columns. The Hypercarb column showed excessive retention, as ATR is strongly attracted to the graphite surface and displayed pronounced tailing. Retention on the Waters Xterra Phenyl column (150 mm × 2.1 mm, 5 μm) depended on the buffer concentration and pH. This column outperformed the other HPLC columns evaluated and was used for all experiments. The best chromatographic effects were observed using an eluent containing ammonium acetate buffer (25 mM at pH 6.7) and acetonitrile. These conditions, however, were not conducive to the MS detection of ATR due to ionisation suppression. By lowering the buffer concentration to 10 mM and adjusting the pH to 4.5 with acetic acid, stable chromatography and ionisation were observed for ATR.

To obtain optimal MS conditions, flow-injection analysis experiments were performed. The results obtained from FIA experiments differed, as expected, from those obtained by injecting the atractyloside standard with the 2695 SM. Therefore, only those results that had an impact on the LC–MS separation and detection of ATR were further investigated and discussed. It was found that a cone voltage (CV) of 27 V was optimal for the detection of the unfragmented ATR [ATR–H]$^-$ (*m/z* 725). Increasing the cone voltage to 65 V resulted in the fragmentation of ATR and the formation of a desulphated species [ATR–SO_3–H]$^-$ (*m/z* 645). Under chromatographic conditions, at a low cone voltage (27 V), the full scan spectrum of ATR (Fig. 2) was dominated by the deprotonated ion [ATR–H]$^-$ (*m/z* 725). Other ions observed were [ATR–2H + Na]$^-$ at *m/z* 747, a fragment due to the loss of SO_3 [ATR–SO_3–H]$^-$ at *m/z* 645, as well as two doubly charged ions, [ATR–2H]$^{2-}$ and [ATR–SO_3–2H]$^{2-}$, at *m/z* 362 and 322, respectively.

As mentioned before, ATR was only observed in the ESI negative mode, and was very susceptible to changes in the eluent composition, MS parameters and nebulisation process. In FIA experiments, a 100 μg/ml atractyloside standard produced poor detector response on the ZQ detector. The use of a 1000 μg/ml atractyloside standard for experimental work led to excessive contamination of the MS inlet. Deactivation of the cone gas resulted in a dramatic improvement in detection and allowed the use of a 10 μg/ml standard. The eluent composition also affected the ionisation of ATR. In FIA experiments, methanol:buf-

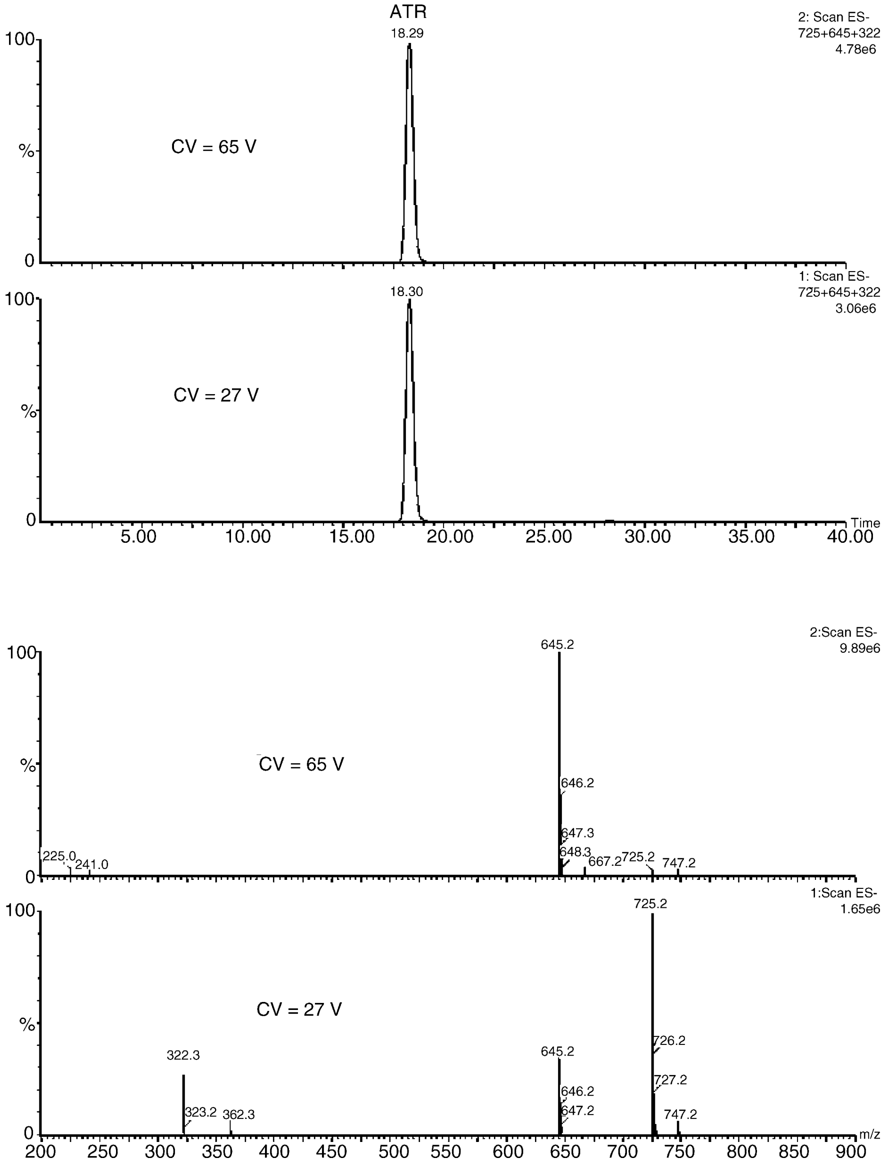


Fig. 2. Typical ESI–chromatogram of a 10 μg/ml ATR standard obtained under optimised chromatographic (Table 1) and mass spectral (Table 2) conditions. The cone voltage was alternated between 27 V and 65 V.

Table 3
Evaluation of R.S.D. values in various matrices for ATR analysis

Sample	Concentration	*N*	%R.S.D. on peak area of ATR
ATR standard	10 ng/ml	10	2.9
	1 μg/ml	10	2.2
	100 μg/ml	10	3.8
Fresh tuber	N/A	8	3.5
Unknown powder #1294	N/A	8	5.3
Viscera samples of case #283 (averaged)	N/A	8	5.9

fer was used as eluent, but the presence of methanol in the eluent suppressed the ionisation of ATR and acetonitrile was used in subsequent experiments. This change in chromatographic conditions not only enhanced the detection of ATR in LC–MS analysis, but also improved peak shape and shortened, as expected, the retention time of ATR. However, the presence of acetonitrile in the initial mobile phase resulted in co-elution of the various com-

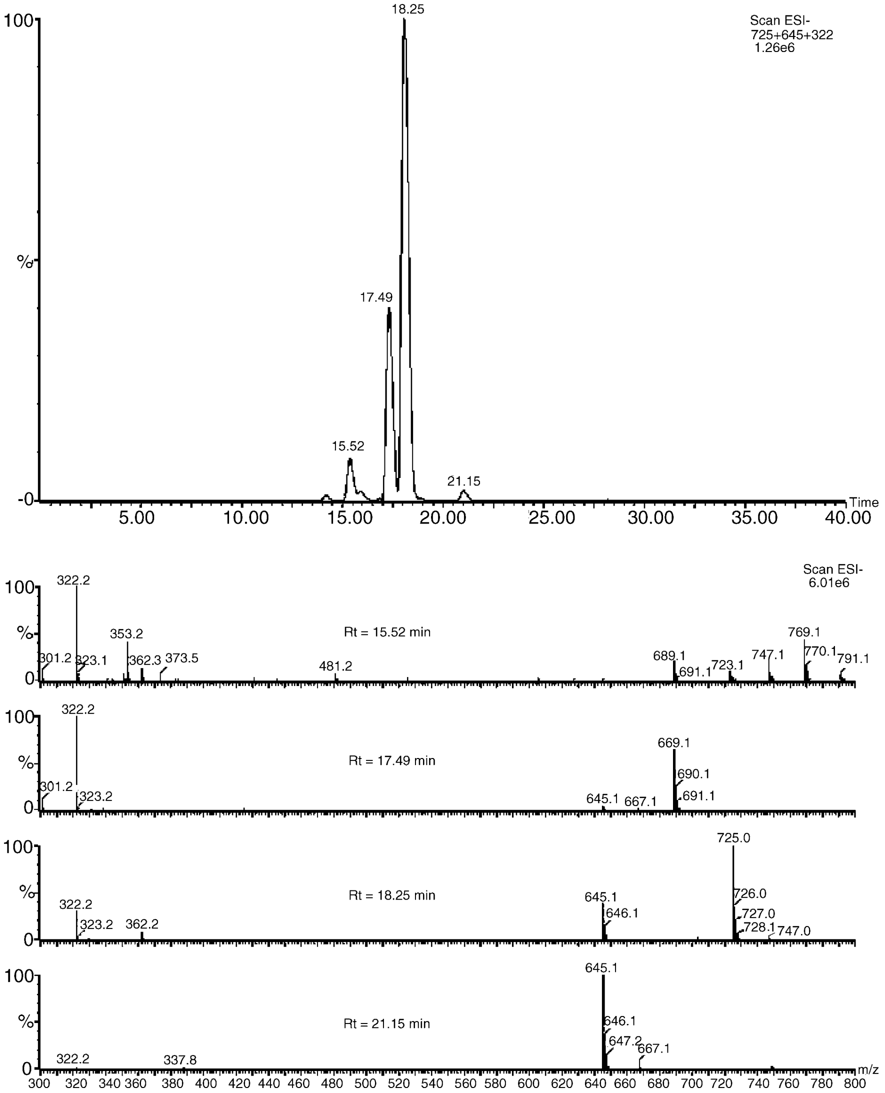


Fig. 3. Chromatogram and spectra of the main compounds detected in the fresh tuber extract. The injection volume was 10 μl and the HPLC–MS conditions are summarised in Tables 1 and 2.

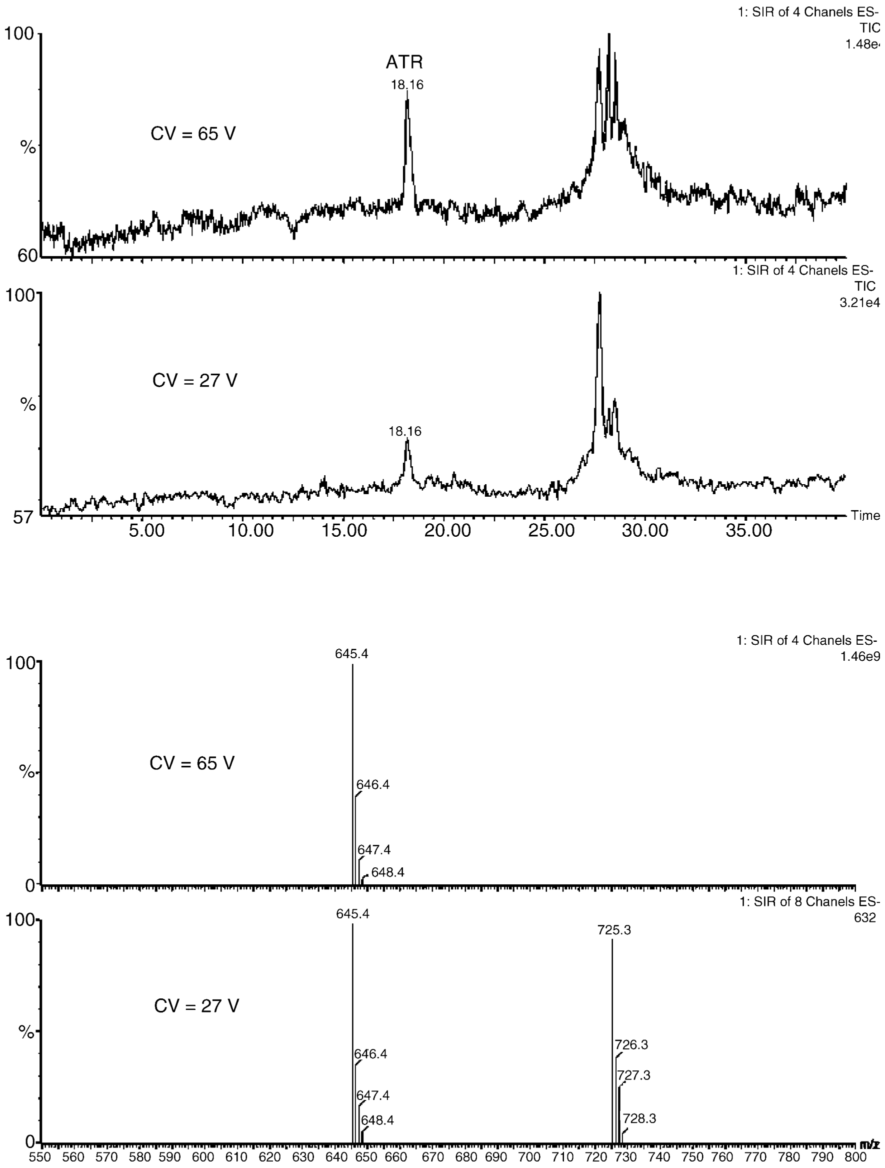


Fig. 4. Chromatogram and spectra of the analysis of an unknown powdered sample of forensic case #1294. The injection volume was 10 μl and the HPLC conditions are summarised in Table 1. The MS–SIM conditions are as described in the text.

pounds detected in the tuber extracts. A starting mobile phase of buffer:methanol, followed by a replacement of the methanol with acetonitrile after 2 min, restored the resolution needed without any compromise in the ionisation of ATR.

3.2. LOD, LOQ and analytical range

The LOD was determined by using various dilutions of the atractyloside standard. The LOD of ATR in SIM mode, using the main fragmentation ion (*m/z* 645), was found to be 100 pg/ml and the unfragmented ion (*m/z* 725) was used as confirmation. The calibration curve covering the 1 ng/ml–160 μg/ml range was best described by a second-order polynomial function ($r^2 = 0.998$). For the range 100–10,000 ng/ml, the calibration curve was linear with a coefficient of determination of 0.999. The inclusion of an internal standard may overcome the problem in linearity, but a suitable internal standard could not be commercially obtained.

The precision of the method was determined and the results are summarised in Table 3. The R.S.D.s of the peak areas for the standards were, on average, below 3% ($n = 10$), whilst the R.S.D.s in the biological matrices increased to 3.5% ($n = 8$) in the fresh *impila* tuber, 5.2% in the unknown powdered sample #1294 and

Table 4
Analysis of forensic samples using the optimised MS conditions for the determination of ATR, CATR and their monodesulphated derivatives

	Scan function 1[a]								Scan function 2[b]							
	CATR		CATR–SO_3		ATR		ATR–SO_3		CATR		CATR–SO_3		ATR		ATR–SO_3	
	FS%	RS%	FS%	RS%	FS%	RS%	FS%	RS%	FS%	RS%	FS%	RS%	FS%	RS%	FS%	RS%
Stomach	91.6	98.6	99.5	100	98.3	99.5	99.8	100	85.4	DNM	98.3	100	97.4	99.8	99.7	100
Liver	97.5	98.5	95.8	100	ND	ND	94.9	99.9	95.8	DNM	99.3	100	ND	ND	99.8	99.9
Kidney	97.3	DNM	94.0	100	ND	ND	94.8	100	97.2	DNM	99.6	100	ND	ND	96.9	99.6
Bile	49.9	94.6	97.6	98.9	ND	ND	97.5	97.5	94.7	DNM	98.3	DNM	ND	ND	96.7	100
Blood	96.2	97.1	96.8	98.5	92.1	DNM	92.9	99.9	94.8	DNM	95.8	100	95.0	99.8	97.0	99.9

FS% and RS% refers to the forward search% and reverse search% done against the compounds detected in the *impila* tuber; DNM, did not match.

[a] 16 masses CV = 27 V.

[b] 16 masses CV = 65 V.

5.9% (averaged) ($n = 8$) for the viscera samples of case #283.

3.3. Solid phase extraction and analysis of C. laureola tuber

Initial SPE experiments [14] indicated that atractyloside, and a variety of compounds naturally occurring in *impila* tubers, could be successfully retained and eluted from the Oasis HLB cartridges using a PBS–HCl buffer. Fresh *C. laureola* tubers were obtained and sample preparation was done as described. The extract was analysed with the optimised HPLC and MS method (Fig. 3). Atractyloside (R_t 18.25) was detected as the major component in the tuber extract. The other components detected were carboxyatractyloside (R_t 15.52) and the monodesulphated derivatives of atractyloside (R_t 21.15) and carboxyatractyloside (R_t 17.49). Although desulphation could be chemically induced, it is likely that in the plant material, atractyloside and carboxyatractyloside lose one sulphate group with ageing as suggested in previous research [14].

3.4. Solid phase extraction and analysis of viscera samples

Due to the lack of a suitable chromatographic separation and mass spectral detection technique for ATR and CATR, extracted viscera were not screened in the Forensic Chemistry Laboratory of Johannesburg for the presence of these two compounds prior to the development of the method described. A selection of 70 cases, previously analysed and found to be negative (no toxic substances detected), were analysed with the full scan method. The results were disappointing as no ATR, CATR or any of the monodesulphated compounds could be detected.

The samples were re-analysed with the more sensitive SIM method by using the main fragmentation ion (m/z 645).

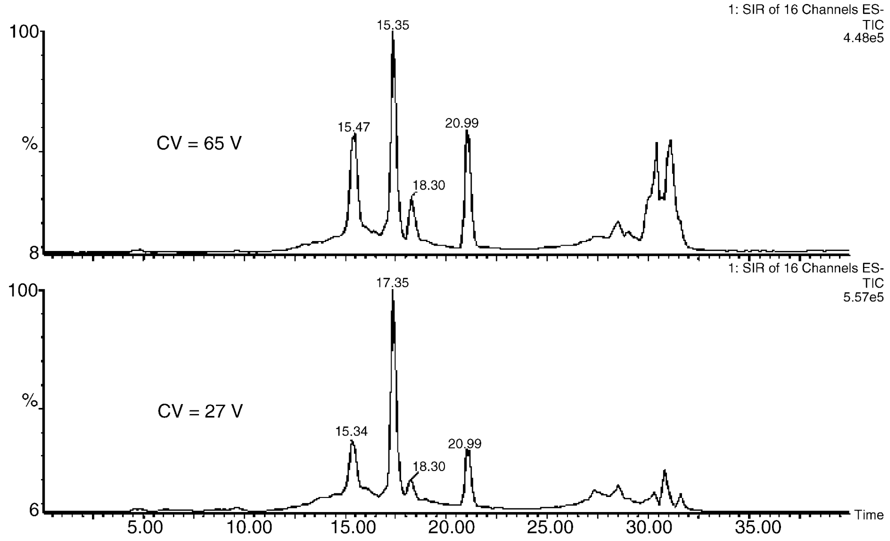


Fig. 5. Chromatogram of the analysis of the stomach and its contents of forensic case #283. The injection volume was 10 μl and the HPLC conditions are summarised in Table 1. The MS–SIM conditions are as described in the text.

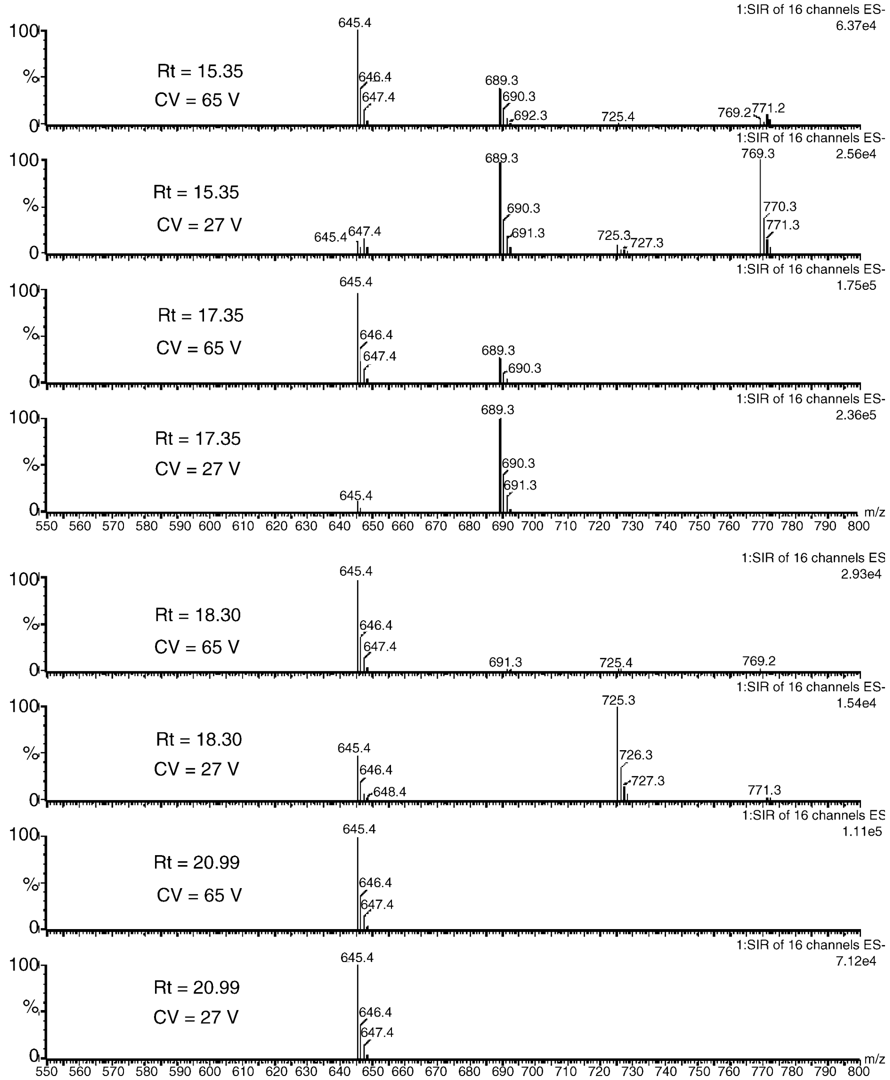


Fig. 6. Spectra obtained from the analysis of the stomach and its contents of forensic case #283. The injection volume was 10 μl and the HPLC conditions are summarised in Table 1. The MS–SIM conditions are as described in the text.

The results indicated that 65% of the cases contained compounds with the *m*/*z* 645 mass ion. The samples were re-analysed with a dual-function method using both the SIM masses (*m*/*z* 725 and 645). The new set of results indicated that only 45% of the cases contained compounds with both mass ions, the correct mass ion ratios and within the retention time window.

3.5. Optimisation of mass spectral conditions for trace level analysis

If SIR results must be presented and defended in a court of law, it is the accepted norm to use three to four unique mass ions to monitor the compound on interest. It is also preferable to use collision-induced dissociation, as mass ions produced under those conditions can serve as a confirmation of the initial results. Due to the fact that the results were not sufficiently conclusive, a new MS method was developed utilising the two scan functions at different cone voltages (27 V and 65 V) but also monitoring the four mass ions in each of the isotope clusters at *m*/*z* 725 and 645. The 70 cases were re-analysed with the new method resulting in 30% producing positive results, of which 6% conclusively proved the presence of ATR and/or ATR–SO_3. The results from one of the positive cases (unknown powdered sample of case #1294) are depicted in Fig. 4 and clearly show the typical isotope clusters at *m*/*z* 725 and 645 within the expected retention time window.

The newly developed method was expanded even further to enable the detection of CATR and CATR–SO_3 by also

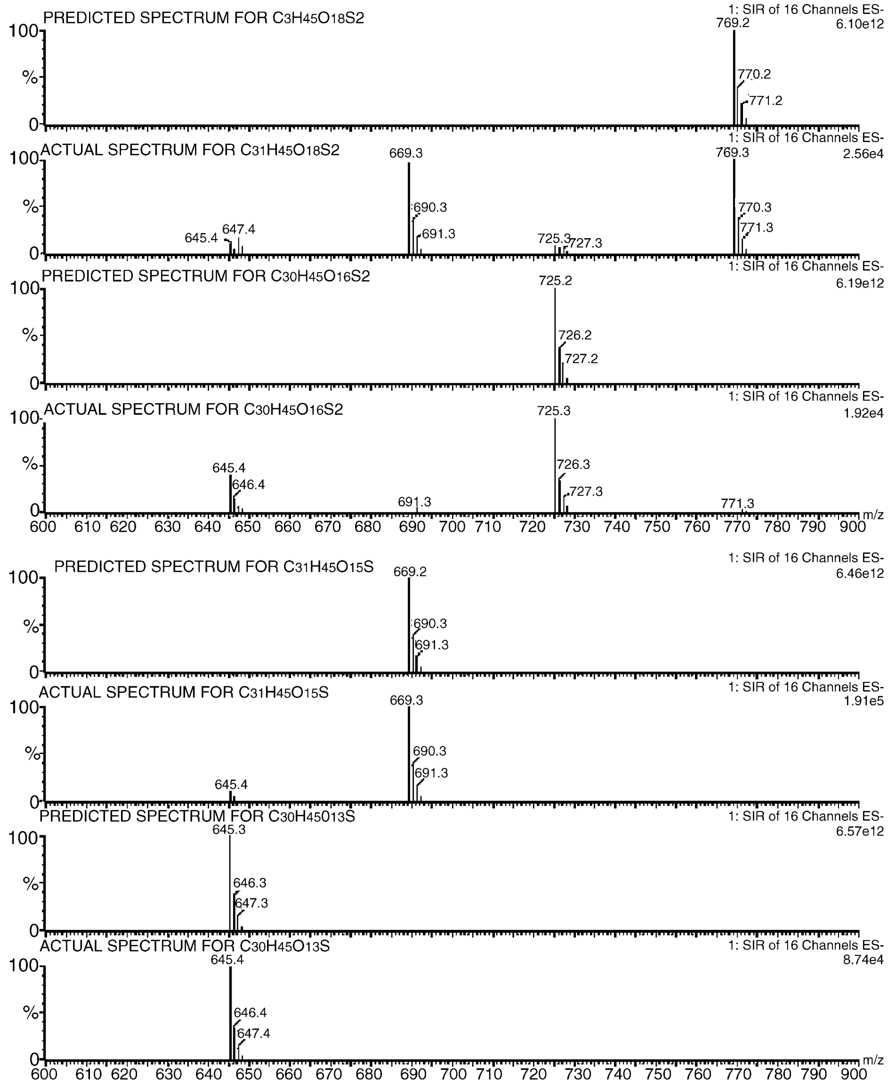


Fig. 7. Comparison of the calculated and actual isotope clusters of ATR, CATR, ATR–SO_3 and CATR–SO_3 as observed in the analysis of the stomach and its contents of forensic case #283.

monitoring the four mass ions in each of the isotope clusters at *m*/*z* 769 and 689, respectively. This adaptation of the MS method did not improve the results of the 70 "historic" cases evaluated as no CATR or CATR–SO_3 could be detected. Although the new MS method is very specific and versatile in detecting the main toxic principles of *impila* and their monodesulphated derivatives, it showed a decrease in sensitivity due the long cycling times necessary to monitor the 16 masses in each of the scan functions.

A recently received forensic case (#283) comprising of various viscera samples, namely stomach and its contents, liver, kidney, bile and blood, was investigated. The samples were extracted with the PBS–HCL method and analysed with the new MS method. All the forensic exhibits submitted for analysis were found to contain ATR, ATR–SO_3, CATR and CATR–SO_3. The detected compounds were matched using the forward search (pure compound evaluation) and reverse search (impure compound evaluation) search routines of MassLynx software (Table 4). The results obtained from the analysis of the stomach and its contents (Figs. 5 and 6) clearly indicate the excessive use of herbal preparation containing *impila* tuber or the direct ingestion of the tuber.

As a final confirmation of the identities assigned to the four compounds detected in the viscera samples, the proposed empirical formulas of $[CATR–H]^-$ ($C_{31}H_{45}O_{18}S_2$), $[CATR–SO_3–H]^-$ ($C_{31}H_{45}O_{15}S$), $[ATR–H]^-$ ($C_{30}H_{45}O_{16}S_2$) and $[ATR–SO_3–H]^-$ ($C_{30}H_{45}O_{13}S$) were manipulated using the isotope calculator function on the MassLynx software.

The calculated isotope ratios were a near perfect match with $[M\text{-H}]^-$ isotope clusters of the compounds detected in the stomach (Fig. 7).

4. Conclusion

The analysis of atractyloside in plant material has always been problematic. The detection and positive identification of intact ATR and CATR in human viscera has in the past been a major challenge and is reported here for the first time. From the results of this study, it seems that atractyloside poisoning is not as infrequent as commonly reported, because the lack of screening procedures for atractyloside and carboxyatractyloside resulted in many forensic cases not being linked to exposure to or abuse of *impila* or products derived from the *impila* tuber.

The LC–ESI–MS method described here allows for the analysis of atractyloside and carboxyatractyloside in *C. laureola*, unknown powder samples and human viscera, and is capable of detecting various other compounds naturally occurring in the tuber. With the proposed method, linearity was observed in the range of 100–10,000 ng/ml but the working range covered the 1 ng/ml–160 μg/ml concentration range with R.S.D. values <3% ($n = 10$). The method facilitated the quantification of ATR in fresh *impila* tuber (8.8 ± 0.4 μg/g; $n = 5$), an unknown powdered exhibit of case #1294 (10.1 ± 0.6 ng/g; $n = 5$) and the viscera samples of case #283 (280.1 ± 16.5 ng/g (averaged); $n = 8$). Due to the simplicity of the method, it is ideally suited as a general screening method of samples submitted for forensic analysis for the presence of atractyloside and carboxyatractyloside, and can be used to confirm the identity of tubers collected for medicinal use and to determine the ATR levels of collected plant material.

The method described is robust and can be adjusted to do full or SIR MS scans, therefore ideally suited for either high concentration or trace level analysis of ATR and CATR in various matrices. By using a dual MS scan function approach, the compounds of interest can be detected and confirmed using a single quadrupole mass spectrometer without any derivatisation or lengthy sample preparation steps. This method would be useful to any laboratory tasked with the screening of biological samples for the presence of atractyloside, carboxyatractyloside or their monodesulphated derivatives. This method could be improved if the analysis is done on a triple quadrupole instrument and using an MRM analysis procedure. This would improve the sensitivity and selectivity of the method as multiple transitions could be monitored simultaneously. The use of parent, daughter and neutral-loss scan function could also be utilised to make this method more specific. This was unfortunately not possible due to the absence of LC–MS–MS instrumentation at the Laboratory.

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