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Determination of atractyloside in *Callilepis laureola* using solid-phase extraction and liquid chromatography–atmospheric pressure ionisation mass spectrometry

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Abstract

A selective analytical method based on high-performance liquid chromatography, combined with atmospheric pressure ionisation mass spectrometry, was developed for the detection of atractyloside. The analysis was performed on an Xterra Phenyl column utilising a gradient elution profile and a mobile phase consisting of 10 mM aqueous ammonium acetate buffer–methanol–acetonitrile. The calibration curve of the method (1 ng/ml–160 μg/ml) was best described by a second order polynomial function (r^2 = 0.998) but displayed good linearity in the range of 100 ng/ml–1 μg/ml (r^2 = 0.999). The limit of detection for the atractyloside standard was determined and found to be 100 pg/ml and the limit of quantification of atractyloside in tuber matrix was found to be 250 pg/ml. The relative standard deviation of the method was on average below 5% (n = 8). The method was successfully applied to the analysis of *Callilepis laureola* tubers and unknown powdered samples for the presence of atractyloside.

Keywords: Atractyloside; *Callilepis laureola*

1. Introduction

Callilepis laureola DC (Asteraceae) is an attractive suffrutex of approximately 60 cm in height with erect, leafy annual branches arising from a permanent woody base [1,2]. The tuberous root of the plant is used in traditional medicine by the Zulu and Xhosa people of South Africa [1,3,4] and it is well known by the Zulu name *impila*, which means 'health' [1]. However, *C. laureola* has also been implicated in the death of numerous patients who used medication prepared from *impila* [1–8] in which atractyloside (Fig. 1), the dipotassium salt of a sulfonated kaurene glycoside, was identified as the toxic principle [9,10]. This compound, together with carboxyatractyloside (Fig. 1), was also identified as the toxic principle of *Atractylis gummifera* L. (Asteraceae), a plant linked to human fatalities in Mediterranean countries since ancient times [11–14]. *A. gummifera* is a traditional medicine and external use as a caustic on abscesses and boils has been reported. For attempted homicidal purposes, *A. gummifera* root was criminally added to couscous [12]. Atractyloside has a strychnine-like action, producing convulsions of a hypoglycemic type [15]. 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 [13,16].

Despite the importance of atractyloside in toxicology and biochemical experiments, limited methods have been described for the analysis of this compound. In the past, *impila* poisoning in South Africa was only determined by post mortem examinations (centrilobular necrosis of the liver). Atractyloside can be detected by an enzyme immunoassay procedure [17], but there are reservations about the quantitative accuracy of results obtained by this procedure [13].

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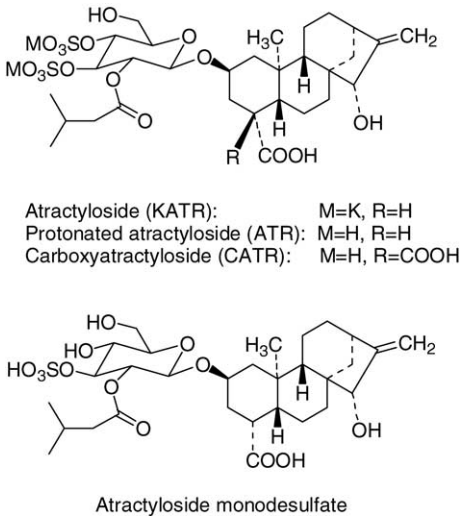

Fig. 1. Structures of atractyloside (KATR), its protonated (ATR) and desulfated forms, and carboxyatractyloside (CATR).

A TLC spot test was recently developed as a preliminary screen for the presence of atractyloside in the gastric contents and urine [7], but this test is non-specific and therefore of little use in forensic toxicology. A GC–MS method was described for the analysis of atractyloside in *impila* tuber and the gastric washing of a poisoned patient [18]. However, this method is cumbersome as it requires the derivatisation of atractyloside with trimethylsilylimidazole. A HPLC screening method for atractyloside and carboxyatractyloside using a light-scattering detector [19] is also not very useful in a forensic laboratory, as this detector is non-specific and the unambiguous identification of atractyloside in a mixture of compounds would not be possible. In an authoritative review [20], 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.

Herewith, we want to report a HPLC-MS method that was developed for the detection of atractyloside using a single quadrupole mass spectrometer. The method was also adapted for the detection of atractyloside in *C. laureola* tubers and unknown powdered samples.

2. Experimental

2.1. Materials 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. Sulfuric 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, 205 nm UV cutoff) were obtained from Romil, England. HPLC grade water (20 MΩ cm^{-1}) 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, 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 and NH_3-PBS were prepared as described above, but the pH was adjusted to 4.5 with hydrochloric acid and to10.0 with ammonia, respectively.

Oasis HLB solid phase extraction (SPE) cartridges (3 ml with 60 mg of sorbent) were obtained from Waters Corporation, Milford, USA. A 12-port vacuum manifold was used for all extractions.

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

Tubers of *C. laureola* were obtained from the local traditional healer's market in Johannesburg. A second, fresh sample came from the muti market in Durban, South Africa. The unknown powdered samples were submitted to the Forensic Chemistry Laboratory as part of Police Forensic exhibits linked to ten cases submitted to the Laboratory for toxicological screening and possible identification. The tubers were gently cleaned with a brush prior to extraction to remove soil and bulk surface contaminants and were then grated by hand and thoroughly mixed to provide a homogeneous sample. Five 1 g aliquots of the ground tuber were placed in separate 50 ml polypropylene screw-top centrifuge sample tubes. Thirty millilitres of the three PBS buffer mixtures (phosphate, HCl and ammonia), de-ionised water and methanol, respectively, were added to the sample tubes. Samples were 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. Five SPE cartridges were 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 per liter methanol), 3 ml basic methanol (10 ml ammonia per liter 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.

The powdered samples, of unknown origin, were extracted as described above, but by using the standard Waters SPE

Table 1
Waters Xterra Phenyl 300 mm × 2.1 mm, 3 μm (2 × 150 mm columns in series) with guard column

Variable	Optimised conditions
Column	Waters Xterra Phenyl 300 mm × 2.1 mm 3 um with guard column (2 mm × 150 mm columns in series)
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
	At 2 min: replace methanol with acetonitrile
	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	0.2 ml/min
Degassing	In-line (normal mode)
Sample temperature (°C)	5 ± 1

method (PBS buffer) [21] and adjusting the pH of the buffer to 4.5 with phosphoric acid.

2.3. LC–MS experiments

2.3.1. LC–MS 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. The ESCi interface was only used in the negative-ion electrospray mode. Various reversed phase HPLC columns from Phenomenex (Luna C18, Max-RP, Polar-RP), Thermo Hypersil-Keystone (Hypercarb, Duet), and Waters (Xterra RP-C18, MS-C18 and Phenyl) were evaluated. All the column dimensions were 250 mm × 2.1 mm except for the Xterra Phenyl column that was 150 mm × 2.1 mm. The built-in syringe pump was used for all flow-injection and optimisation experiments on the ZQ detector. 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. 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 detection of atractyloside by LC–MS are summarised in Table 1. The HPLC column was kept at 40 °C for all experiments.

Preliminary MS optimisation was performed using an APCI interface in ESI(+), ESI(−), APCI(+) and APCI(−) modes. High purity nitrogen gas (Air Products) was used as nebulising and carrier gas at a flow rate of 450 l/h while 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 at *m/z* 725 and the main fragment ion at *m/z* 645.

2.3.2. Flow-injection analysis experiments

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.

2.4. Evaluation of the analytical procedure

The limit of detection (LOD) for the standard was determined experimentally, and was taken as the concentration of the standard that produced a detector signal that could be clearly distinguished from the baseline noise (±3 times baseline noise). The limit of quantitation (LOQ) was taken as the concentration of the standard that produced a detector

Table 2
Optimised MS conditions for the determination of atractyloside (ATR)

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

signal ±10 times greater than the LOD signal. The practical limit of quantification was determined by spiking old *impila* tuber and extracting the spiked sample as described. A calibration curve was prepared covering the 1 ng/ml–160 ug/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.

3. Results and discussion

3.1. Chromatographic evaluation of atractyloside

Atractyloside (KATR, Fig. 1), first isolated by M. Lefranc in 1868 [14], 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]. The compound contains multiple polar groups and is soluble in water but has relatively poor solubility in organic solvents. Under the normal reversed-phase chromatographic conditions, the acidic form of atractyloside (ATR) is virtually unretained on all the Phenomenex, Waters and Hypersil-Keystone columns we tested, 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.

ATR was poorly detected on the UV (PDA) detector due to the chromophoric properties of the compound, and this detection mode was discontinued in subsequent experiments.

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. ATR was only observed in the ESI negative (ESI^-) mode and all other detection modes were deactivated. It was found that a cone voltage 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 fragmen-

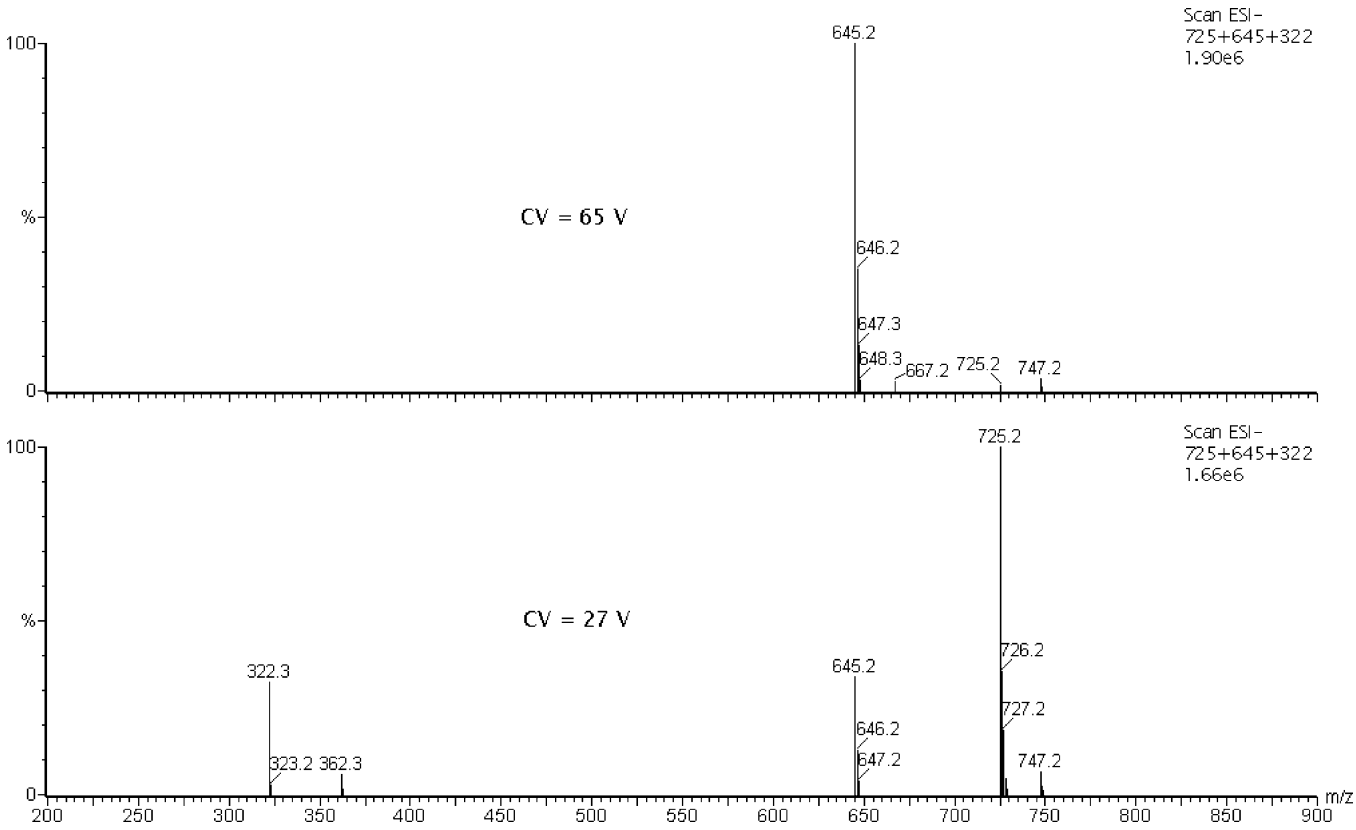

Fig. 2. Typical ESI-spectrum of a 10 μg/ml ATR standard obtained under optimized chromatographic (Table 1) and mass spectral (Table 2) conditions. The cone voltage was alternated between 27 and 65 V.

tation of ATR and the formation of a desulfated 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:buffer 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 compounds detected in the tuber extracts. A starting mobile phase of buffer:methanol, followed by a replacement of the methanol with acetonitrile after two minutes, restored the resolution needed without any compromise in the ionisation of ATR.

3.2. Evaluation of the analytical method

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 ug/ml range, was best described by a second order polynomial function (r^2 = 0.998). For the range 100–1000 ng/ml, the calibration curve was linear with a coefficient of determination of 0.999.

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), while the R.S.D.'s in the biological matrices increased to 3.5% (*n* = 8) in the fresh *impila* tuber and 5.2% in the unknown powdered sample # 207.

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 # 207	N/A	8	5.2

3.3. Solid phase extraction of ATR, C. laureola tuber and powdered herbal samples

Initial SPE experiments with an atractyloside standard indicated that the compound could be successfully retained and eluted from the Oasis HLB cartridges with any of the PBS buffers as well as water. However, extraction of atractyloside from the tuber produced mixed results. Of the five extraction solvents evaluated (PBS-Phosphate, PBS-HCl, PBS-NH_3, MeOH and H_2O), the MeOH extracted the least atractyloside, followed by H_2O, PBS-Phosphate and PBS-NH_3. Although the PBS-HCl yielded the most atractyloside, the PBS-NH_3 produced the cleanest extract 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.

Unknown powdered samples are regularly sent to our laboratory as part of forensic investigations. These samples are usually of botanical origin, sometimes fortified with metal salts to create a "stronger muti", or might even contain human or animal remains. Ten cases involving unknown powdered samples were previously extracted according to our systematic toxicological screening process and found to contain no toxic substances. As the original samples were unavailable, the original extracts of the powdered samples were used.

3.4. Analysis of C. laureola tuber and unknown powdered samples

The extract of the *impila* tuber obtained from the local traditional healer's market was analysed with the method described above. The masses of interest, namely 725, 645 and 322 were monitored simultaneously. In initial experiments with a 150 mm column, it was clear that co-elution prevented obtaining a clean spectrum for each compound eluted. The column length was doubled (two columns used in series) and the mobile phase changed to afford better separation without drastically altering the retention times of those compounds detected (see Section 3.1). The optimised HPLC conditions are summarised in Table 1 and the optimised MS conditions are listed in Table 2. These conditions were found to give near-baseline resolution of the compounds detected. The *impila* tuber extract was re-analysed with the optimised HPLC conditions (Fig. 3). From the results, it was clear that ATR was not detected, but that the main constituent was a monodesulfated derivative of carboxyatractyloside (R_t 17.44). Also detected was the monodesulfated derivative of atractyloside (R_t 21.16). The latter compound can also be produced by in-source collision-induced dissociation (ISCID) of atractyloside. The absence of atractyloside was

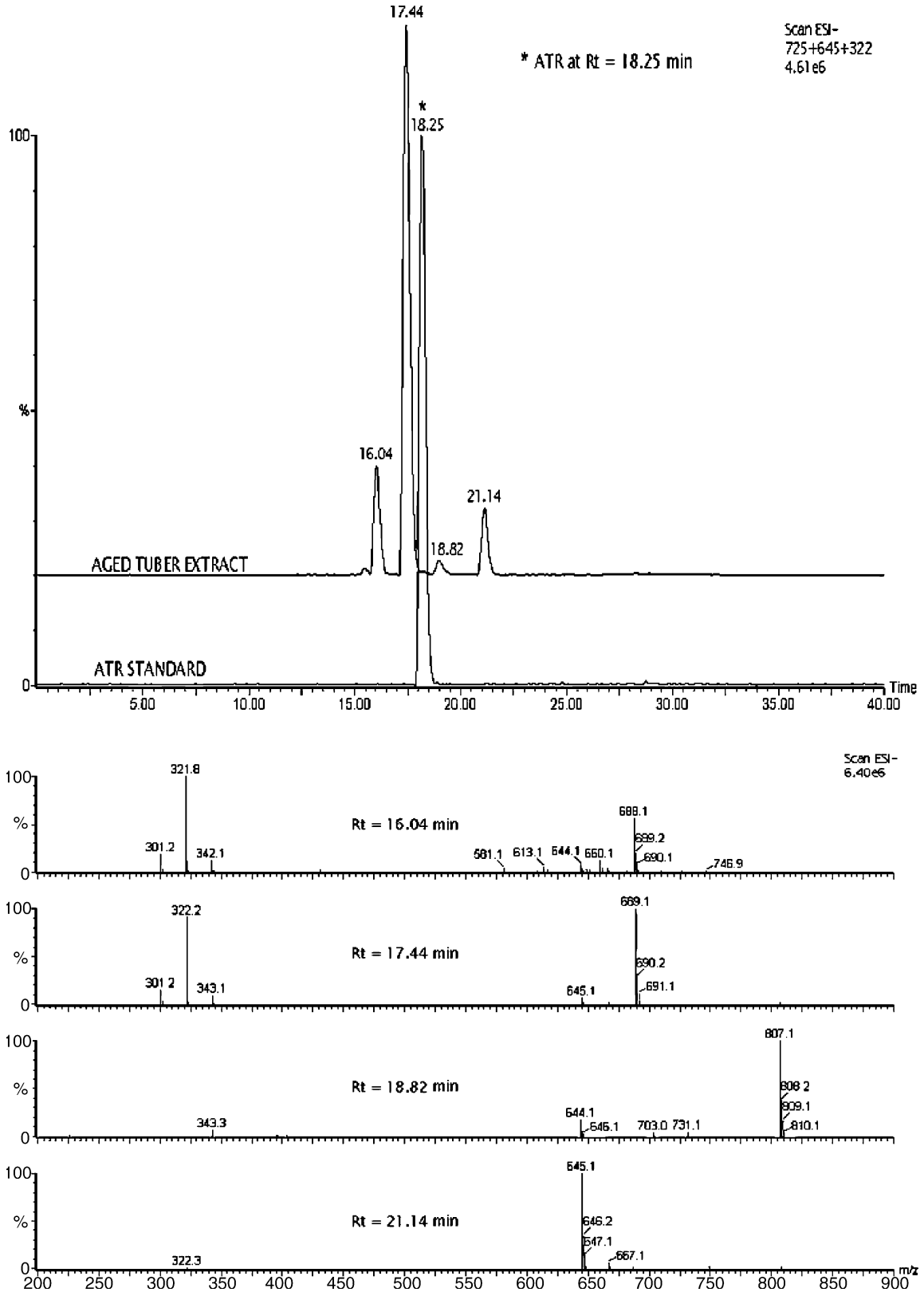

Fig. 3. Chromatogram and spectra of the main compounds detected in the aged tuber extract. The injection volume was 10 μl and the HPLC-MS conditions are summarized in Tables 1 and 2. Overlaid the chromatogram of a 10 μg/ml ATR standard obtained under optimised chromatographic (Table 1) and mass spectral (Table 2) conditions.

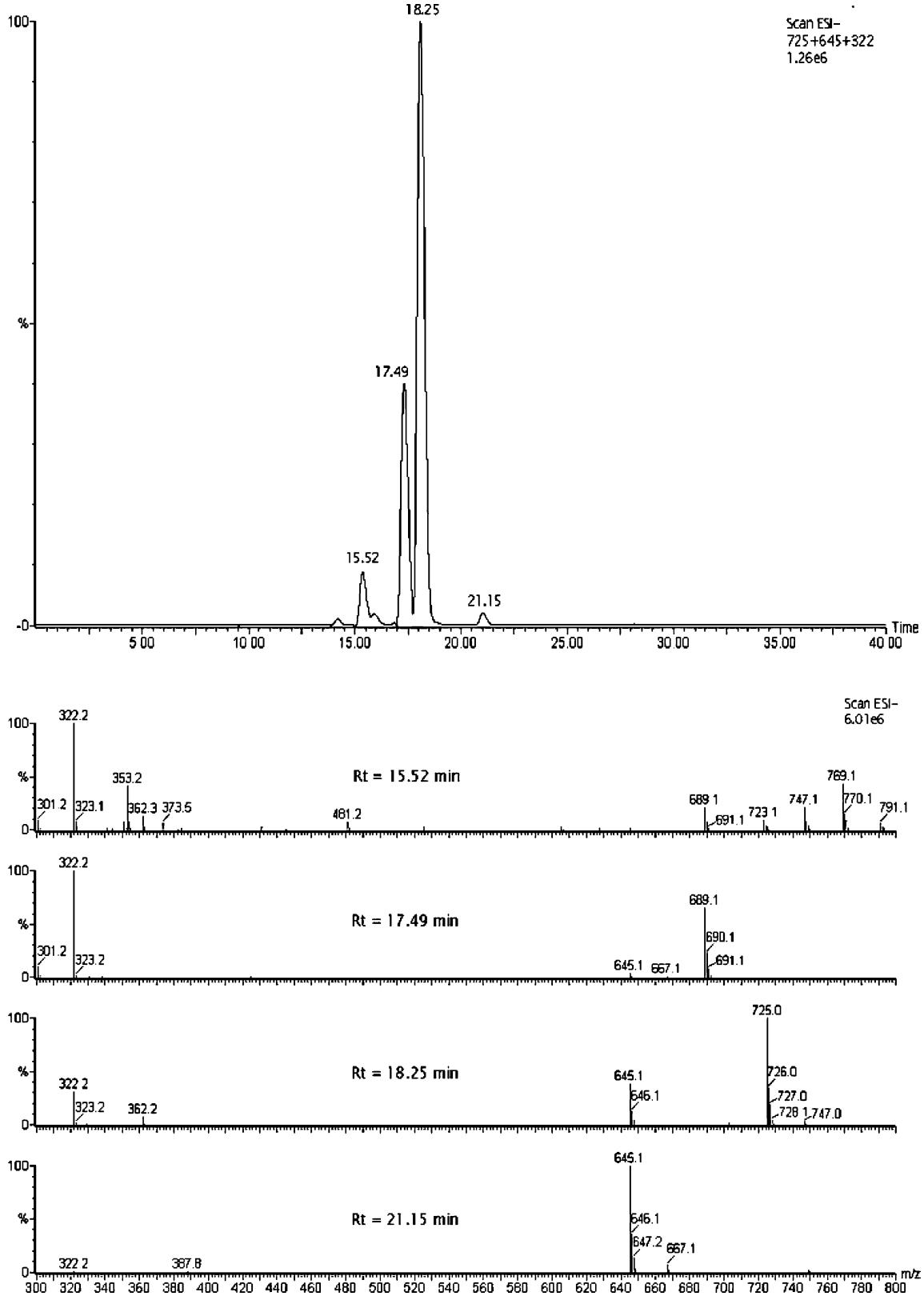

Fig. 4. Chromatogram and spectra of the main compounds detected in the fresh tuber extract. The injection volume was 10 μl and the HPLC-MS conditions are summarized in Tables 1 and 2.

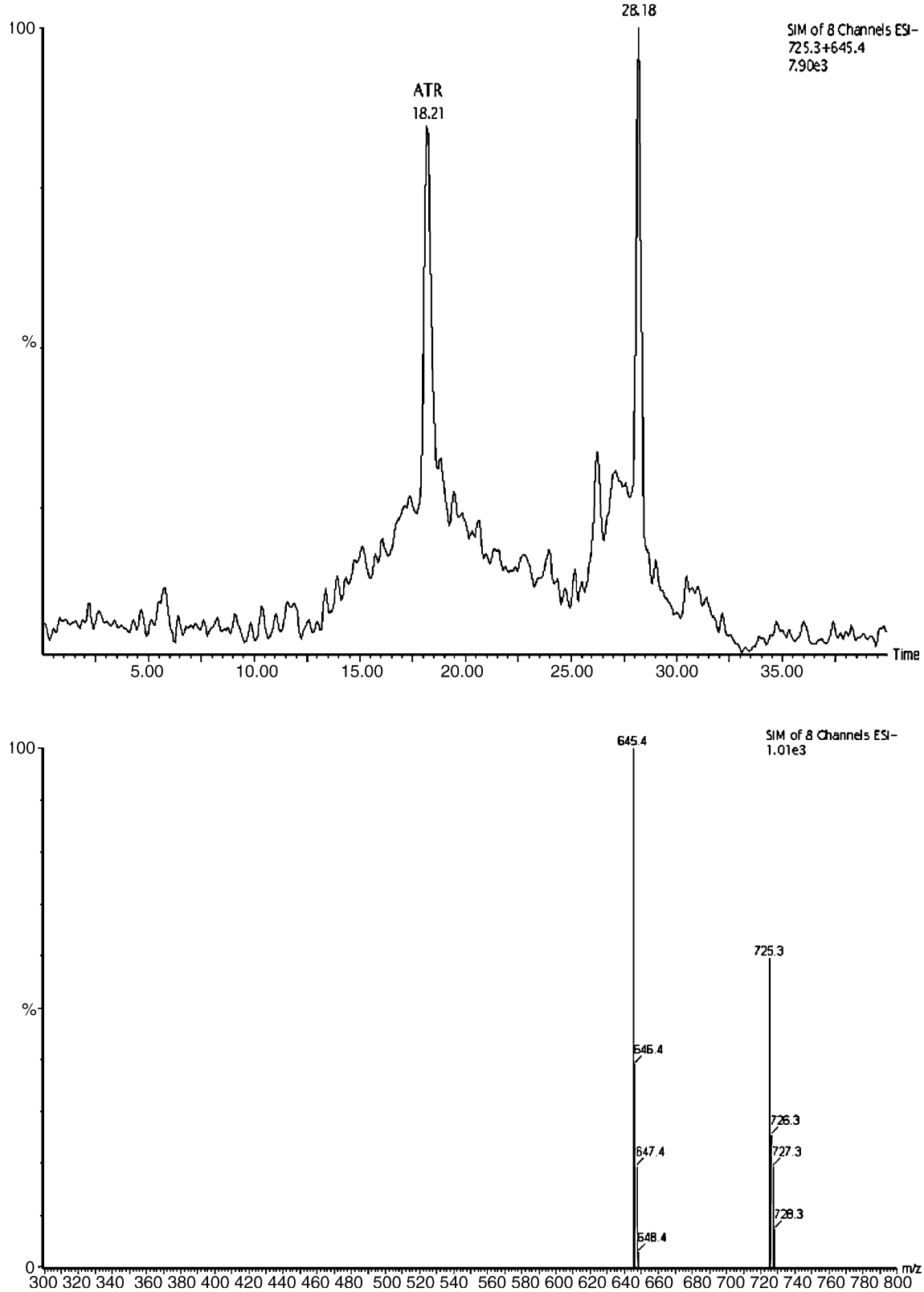

Fig. 5. Chromatogram and spectra of the analysis of the unknown powdered sample # 207. The injection volume was 10 μl and the HPLC-MS conditions are summarized in Tables 1 and 2.

Table 4
Interpretation of main fragmentation ions of atractyloside, carboxyatractyloside and derivatives

Compound	Ion 1	Ion 2	Ion 3	Ion 4	Ion 5
ATR	747 $[ATR - H + Na]^-$	725 $[ATR - H]^-$	645 $[ATR - H - SO_3]^-$	362 $[ATR\text{-}2H]^{2-}$	322 $[ATR\text{-}2H\text{-}SO_3]^{2-}$
ATR − SO_3 (CV = 65 V) formed in the source	645 $[ATR - H - SO_3]^-$	667 $[ATR - SO_3 + Na]$	322 $[ATR - 2H - SO_3]^{2-}$		
CATR	769 $[CATR - H]^-$	689 $[CATR - SO_3]^-$			
CATR − SO_3	689 $[CATR - SO_3]^-$	645 $[ATR - H - SO_3]^-$	322 $[ATR - 2H - SO_3]^{2-}$		
KATR + K (CV = 125 V) formed in the source	841 $[KATR + K]^+$	825 $[KATR + Na]^+$			

most likely caused by ageing, as these tubers were stored at room temperature for two years.

Fresh *C. laureola* tubers were obtained and sample preparation was done as discussed above. The extract was analysed with the optimised HPLC method. The results (Fig. 4) were similar to those obtained with the old tubers. Although the monodesulfated derivatives of atractyloside and carboxyatractyloside were still present, atractyloside (R_t 18.25) was detected as the major component in the tuber extract (8.8 ± 0.4 μg/g; n = 5). It is possible that, in the plant material, atractyloside and carboxyatractyloside loses one sulfate group with ageing. As tubers used in traditional medicine may be harvested and stored for extended times before utilisation, the possible degradation of atractyloside should always be taken into account in forensic cases. Therefore screening methods should be expanded to not only screen for ATR and CATR, but must also include their monodesulfated derivatives.

The 10 cases involving unknown powdered samples were analysed with the optimised method. ATR was detected in four of the cases in trace levels, but case # 207 contained sufficient ATR levels to enable quantification (10.1 ± 0.6 ng/g; n = 5) (Fig. 5). The low ATR levels may be due to degradation of the sample (assuming the whole sample was *impila* tuber), or might indicate that the powder was formulated from more than one plant source and that *impila* was but one of a few plants used to formulate the muti.

3.5. Mass spectral interpretations

During the method development and analysis of atractyloside and the tuber extracts, various intact deprotonated species and their fragments (ISCID fragments) were detected. The compounds, for which the ISCID fragments could be successfully related back to the intact deprotonated compounds, are summarised in Table 4.

4. Conclusion

The analysis of atractyloside, especially if present in plant material, has always been problematic. The HPLC-ESI-MS method described here allows for the analysis of atractyloside and carboxyatractyloside in *C. laureola* and is capable of detecting various other compounds found naturally in the tuber. With the proposed method, linearity was observed in the range of 100–1000 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) and an unknown powdered exhibit (#207) (10.1 ± 0.6 ng/g; n = 5). Due to the simplicity of the method, it is ideally suited as a general screening method for unknown powdered 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.

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