DETERMINATION OF ARISTOLOCHIC ACID IN TRADITIONAL CHINESE MEDICINES AND DIETARY SUPPLEMENTS

Rick A. Flurer, Ph.D., Mary B. Jones, Nohora Vela, Ph.D., Laura A. Ciolino, Ph.D.
and Karen A. Wolnik
USFDA Forensic Chemistry Center, Cincinnati, Ohio

INTRODUCTION

Aristolochic acid is a known nephrotoxin and potential carcinogen that can be found in *Aristolochia* species (spp.), *Bragantia* spp. or *Asarum* spp. The *Aristolochia* spp. are most often found in traditional Chinese medicines. Because of the similarity of Chinese names for several herbs and because of the Chinese tradition of the interchangeability of similarly named herbs, the possibility exists for the inadvertent substitution of innocuous herbs with *Aristolochiu* spp. not only in traditional medicines but also in botanical-containing dietary supplements. Two recent cases of nephropathy associated with the use of Chinese botanical preparations, reported in the United Kingdom in July 1999, led to heightened concern about the presence of aristolochic acid in botanical products used in the US. A procedure based on an extraction method used by German regulators for the determination of aristolochic acid in botanical products (1) has been developed and applied to a variety of botanicals and botanical-containing dietary supplements. Aristolochic acid is extracted from the sample matrix with aqueous methanol/formic acid. The concentration of aristolochic acid in the extract is determined by gradient HPLC with detection at 390 nm and presence confirmed by LC/MS using either an ion trapping mass spectrometer or a triple quadrupole mass spectrometer.

EXPERIMENTAL

1. Sample preparation

Samples of solid plant-like material should be ground to a fine powder composite using a Krups model 408 coffee grinder. Composites of tablets, capsules, and free-flowing powders are prepared with manual grinding in a glass mortar and pestle.

2. Extraction

Solvents used for the extraction are HPLC grade methanol and ACS reagent grade formic acid obtained from Fisher Scientific, (Pittsburgh, PA) and Sigma Chemical Co. (St. Louis, MO), respectively.

Weigh 2 g. of ground sample into a disposable HDPE bottle and add 50 mL of a mixture containing 80% methanol and 20% of 10% formic acid in water. Shake for 30 min at 500 rpm (Innova Model 2100, benchtop platform shaker, manufactured by New Brunswick Scientific and distributed by Fisher Scientific) and then centrifuge for 4 minutes at 4000 rpm. The supematant is used for determination of aristolochic acid.

3. Determination of Aristolochic Acid

3.1. Standard Preparation

Reference Standard: Aristolochic acid (Sigma, lot 36H13111, labeled to contain a mixture of aristolochic acids: 69% aristolochic acid I and 19% aristolochic acid II), used "as is". Quantitation is based on aristolochic acid I.

Aristolochic acid stock standard with a concentration equivalent to 69 µg/mL aristolochic acid I is prepared by diluting a quantity of reference standard in an appropriate volume of acetonitrile and sonicating approximately 10 minutes. Calibration standards are prepared by further dilution of stock standard with extraction solvent. Initially a 6 point calibration curve is prepared with standard concentrations ranging from 0.048 to 69 µg/mL. The slope and intercept of the calibration curve are verified each day prior to analysis of samples by running 3 standard solutions (equivalent to 0.048, 14 and 69 µg/mL aristolochic acid I) and verified again immediately after sample analyses are completed.

3.2. Liquid Chromatographic Quantitation of Aristolochic Acid I with UV/vis Detection

All solutions (blanks, standards, sample extracts) are filtered (0.2 µm PTFE) prior to separation by liquid chromatography with UV/vis detection at 390 nm using a liquid chromatograph equipped with a diode array detector. The separation is performed on a 3.0 mm x 25 cm Zorbax SB-Cl8 column at a mobile phase flow rate of 0.5 ml/min. The mobile phase consists of an acetonitrile/water gradient; the water and acetonitrile fractions each contain 0.1 % (v/v) trifluoroacetic acid to suppress ionization of the carboxylic acid function on aristolochic acid. The heated column compartment is set to 40° C. The mobile phase gradient is programmed as follows:

Time (min)	Water (%)	Acetonitrile (%)
0	80	20
25	30	70
30	0	100

The post time = 10 min. and the injection volume = 25 µL.
The retention time of aristolochic acid I is approximately 21 minutes.

3.3. Sample treatment for LC/MS Confirmation

Sample treatment for LCNS confirmation is based on the aristolochic acid level observed by LC/UV. Depending on the concentration found the solution is either diluted with 1: 1 acetonitrile/water or preconcentrated, and then injected. The target level for aristolochic acid I injected is 2 to 15 ng. For samples that test negative for aristolochic acid by LC/UV, the aqueous methanol/formic acid extract is concentrated to improve the limit of detection and to reduce the injection solvent strength to allow injection of large volumes without compromising the chromatographic separation efficiency. Three milliliters of extract are taken to dryness at 90" C under dry nitrogen and the residue is reconstituted in 0.5 ml of 1: 1 acetonitrile/water with 2 min of vortex mixing. The resulting solution is then filtered (0.2 µm PTFE) and injected.

Liquid samples that are apparent suspensions should be diluted with 1 :1 acetonitrile/water and filtered (0.2 pm PTFE) prior to injection. The dilution depends on the level of aristolochic found by LC/UV; however, a minimum dilution of 1: 1 should be used.

3.4. Liquid Chromatographic/Mass Spectrometric Confirmation of the Presence of Aristolochic Acid I Using an Ion Trapping Mass Spectrometer

Blanks, liquid samples, sample extracts and standards are analyzed by LC/MS on a Finnigan MAT LCQ mass spectrometer equipped with the APCI interface and a Hewlett-Packard 1100 liquid chromatograph. The separation is done on a 3.0 mm x 150 mm Zorbax SB-Cl8 column at a flow rate of 0.5 ml/min using gradient elution. The heated column compartment is maintained at 40" C. The mobile phase consists of the following components:

Part A – 0.1% (v/v) formic acid and 0.1% (w/v) ammonium acetate in DI water
Part B – 0.1% (v/v) formic acid and 0.1% (w/v) ammonium acetate in methanol

The mobile phase gradient is programmed as follows:

Time (min)	Part A (%)	Part B (%)
0	80	20
20	0	100

The post time = 10 min. and the injection volume = 30 µL. The mass spectrometer parameters are set as follows for an MS^3 experiment to detect the presence of aristolochic acid I:

Parent m/z	Isolation Width (m/z)	Relative Collision Energy(%)
359.0 ($[M+NH_4^+]^+$)	2.0	30
298.0	2.0	35

Full scan of product ions from m/z 80 to m/z 370
Maximum injection time: 500 ms
Vaporizer temperature: 450° C
Heated capillary temperature: 150° C
Sheath gas setting: 70
Aux gas setting: 20
Discharge current: 5 µA
Divert valve: the first 6 min. of run are diverted to waste
Wideband activation: ON
The tube lens and heated capillary voltages were tuned automatically in ESI mode by infusion for maximum transmission of the aristolochic acid I $[M+NH_4^+]^+$ ion.

Aristolochic acid I is confirmed by the presence of a chromatographic peak at the retention time of standard aristolochic acid I that results in product ions at m/z 251, m/z 252 and m/z 268 with relative abundances similar to those obtained for the standard.

3.5. Liquid Chromatographic/Mass Spectrometric Confirmation of the Presence of Aristolochic Acid I Using a Triple-Quadrupole Mass Spectrometer

Blanks, sample extracts and standards are analyzed by LC/MS on a Firmigan MAT TSQ 700 mass spectrometer equipped with a Finnigan MAT ESI interface and a Hewlett-Packard 1090 liquid chromatograph. The separation is done on a 2.1 mm x 5 cm Zorbax SB-C 18 column at a flow rate of 0.2 ml/min using gradient elution. The heated column oven is maintained at 40" C. The mobile phase consists of the following components:

Part A – 5 % methanol, 0.1 % (v/v) formic acid and 10 mM ammonium acetate in DI water
Part B – 1: 1 acetonitrile/methanol containing 0.1 % (v/v) formic acid and 10 mM ammonium acetate

The mobile phase gradient was programmed as follows:

Time (min)	Part A (%)	Part B (%)
0	70	30
13	30	70
15	0	100

The post time = 5 min. and the injection volume = 25 µL. The mass spectrometer parameters were set as follows for a selected reaction monitoring experiment for the presence of aristolochic acid I:

Precursor ion: m/z 359.1 ($[M+NH_4^+]$ of aristolochic acid I)
Product ions monitored: m/z 265.0, m/z 281 .0, m/z 296.0
Collision energy (lab frame, eV): 33
Collision cell pressure (Ar): ca. 1.9 mTorr
Dwell time (ms): 200 per channel
SIMwidth: ± 0.2 amu
Settim (ms): 10
Multiplier voltage (V): 1300
Divert valve: the first 5min. of run are diverted to waste
Tube lens voltage (V): 25, set to maximize the transmission of the aristolochic acid ammonium adduct ion

Aristolochic acid I is confirmed by the presence of a chromatographic peak at the retention time of standard aristolochic acid I that produces peak area ratios for the specified product ions which match the ratios obtained for the standard within ± 20% absolute.

4. Application to Samples

Analyze samples in duplicate. Extract solid samples, centrifuge, filter and analyze by LC/UV. Dilute liquid samples with an equal volume of extraction solvent, filter and analyze by LC/UV. The presence or absence of aristolochic acid in the sample must be confirmed by LC/MS. Depending on the results obtained by LC/UV, spike the ground sample or the liquid sample prior to filtration at an appropriate level and repeat the extraction. For samples that test negative for aristolochic acid by LC/UV, spike at 0.5 ug/g, extract and analyze by LC/MS. For samples that test positive for aristolochic acid, spike at a level between 50% and 100% of the concentration found in the sample. Analyze all method blanks by LC/MS. The necessity of repeating analyses or preconcentrating the sample prior to LC/UV or to a greater extent prior to LC/MS analysis has to be determined on a sample by sample basis.

RESULTS AND DISCUSSION

Due to the concerns about its toxicity, the emphasis of this procedure is on detection and confirmation of the presence of aristolochic acid with somewhat less emphasis placed on precise quantification of the level present. It was found that preparation of a finely ground composite was critical to extracting the aristolochic acid present. For products that consisted of large pieces of heterogeneous botanical material, it was necessary to reduce the size of the pieces prior to final grinding.

The wide variety and complexity of sample matrices encountered in dietary supplements makes it necessary to verify method performance for each sample analyzed using the controls described. For LC/MS, based on repeated injections of a low-level aristolochic acid standard, the estimated detection level of aristolochic acid I which meets the criteria for confirmation is equivalent to 0.5 µg/g in the sample. However, actual sample detection limits may vary with the sample matrix, so minimum detectable levels should be established for each sample that is negative for aristolochic acid by detection and confirmation of a low-level aristolochic acid spike. Example total ion chromatograms

and mass spectra obtained by ion trap mass spectrometry for an aristolochic acid standard and aristolochic acid containing sample are shown in Figures 1 and 2, respectively. Figures 3 and 4 illustrate the results obtained using a triple quadrupole mass spectrometer for a standard and sample, respectively.

The quantitation limit for the HPLCAJV method was calculated as the concentration equivalent to 10 times the standard deviation obtained for repeated injections of a 0.08 μg/mL aristolochic acid I standard. The quantitation limit is equivalent to 1.7 μg/g in solid samples and 0.14 μg/mL in liquid samples. Response at 390 nm is linear (correlation coefficient of 0.9999 for single injections of the 6 standards used to generate the calibration curve) up to 1725 ng aristolochic acid I injected. A chromatogram and uv spectrum of an aristolochic acid standard is shown in Figure 5. Example sample chromatograms for a liquid and solid are shown in Figure 6.

For samples analyzed to date which contain aristolochic acid, duplicate results for LC/UV are in good agreement and spike recoveries range from 89% to 99%. In one case, an interfering peak prevented detection and quantitation by LC/UV but aristolochic acid I was detected and confirmed by LC/MS in the sample. Because the levels of aristolochic acid present in samples can range from not detected to 1500 μg/g or more, it is important to incorporate controls to prevent carry over from samples with high levels of aristolochic acid and document the absence of contamination by frequent analysis of blanks.

REFERENCES

1. Personal communication from Dr. Michael Wierer, Landesinstitut für den Öffentlichen Gesundheitdienst NRW, Münster, Germany.

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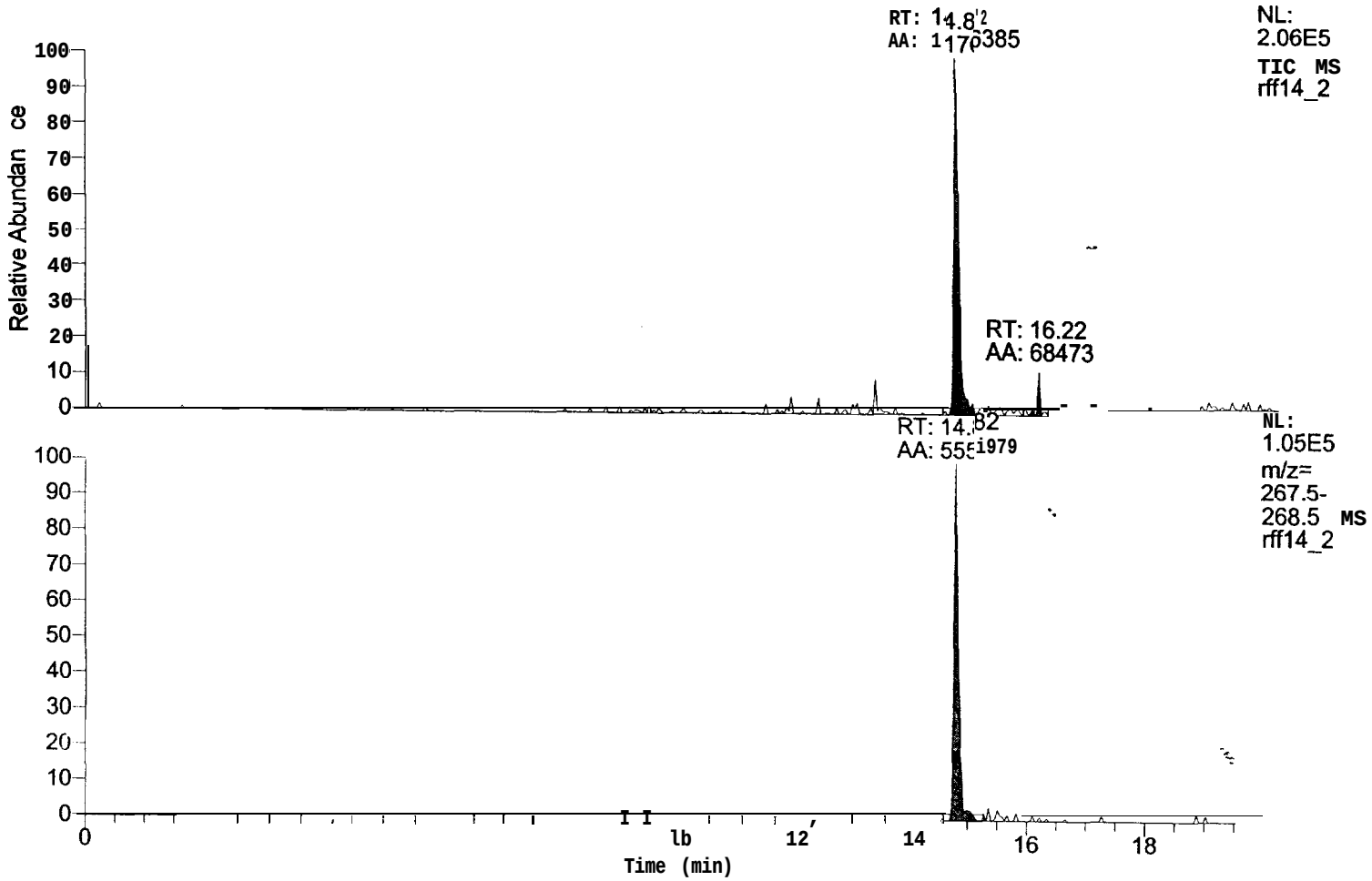

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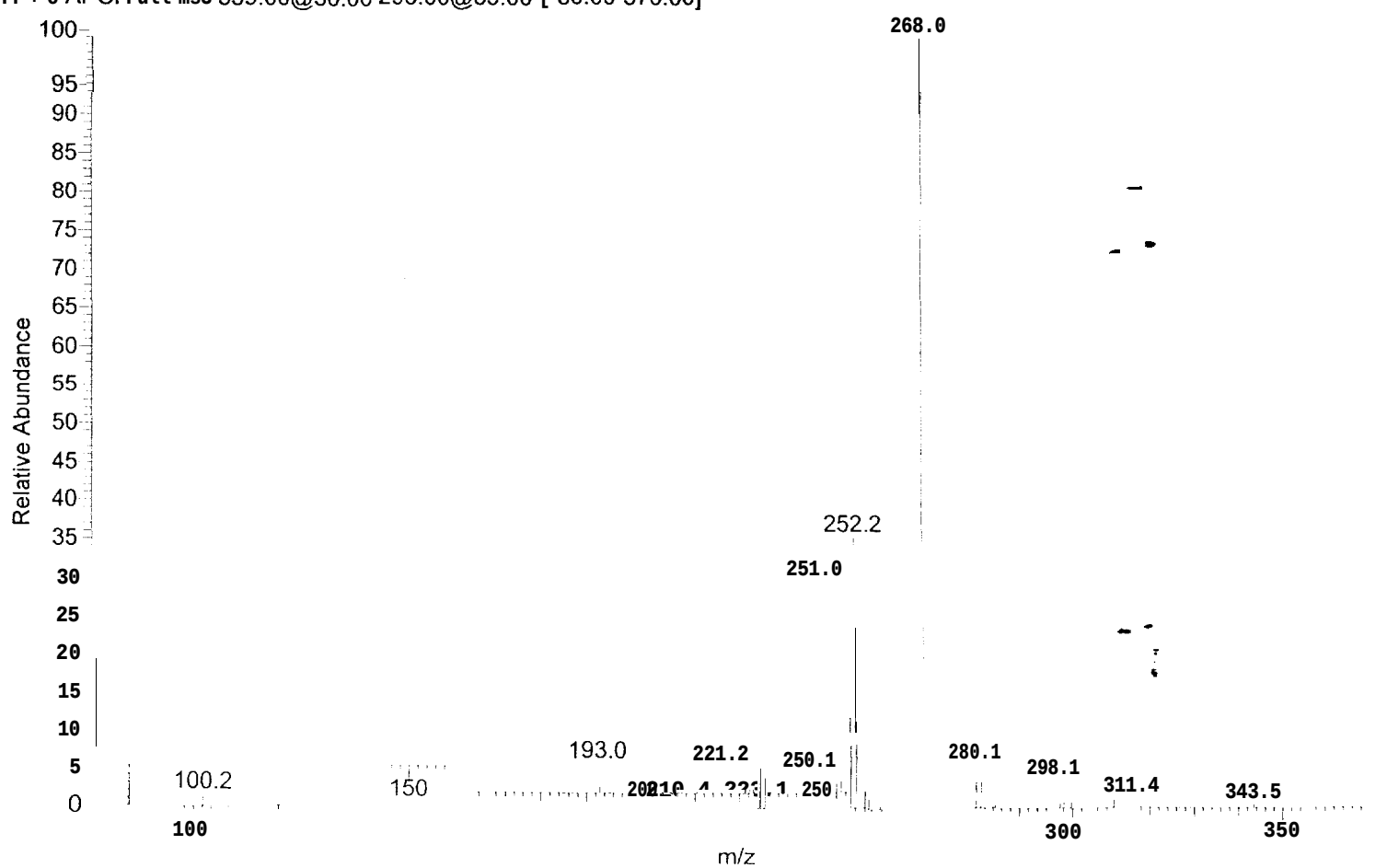

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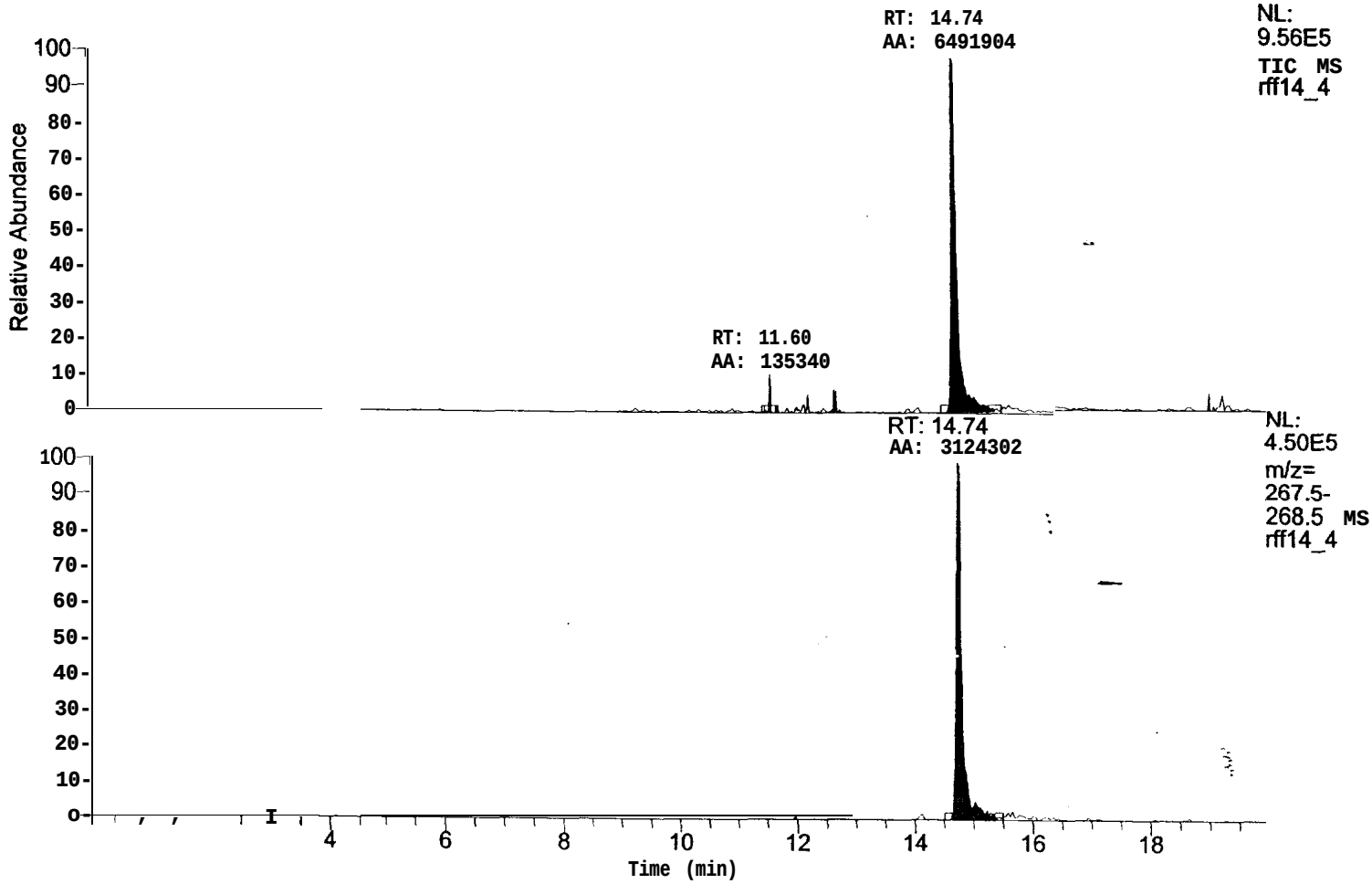

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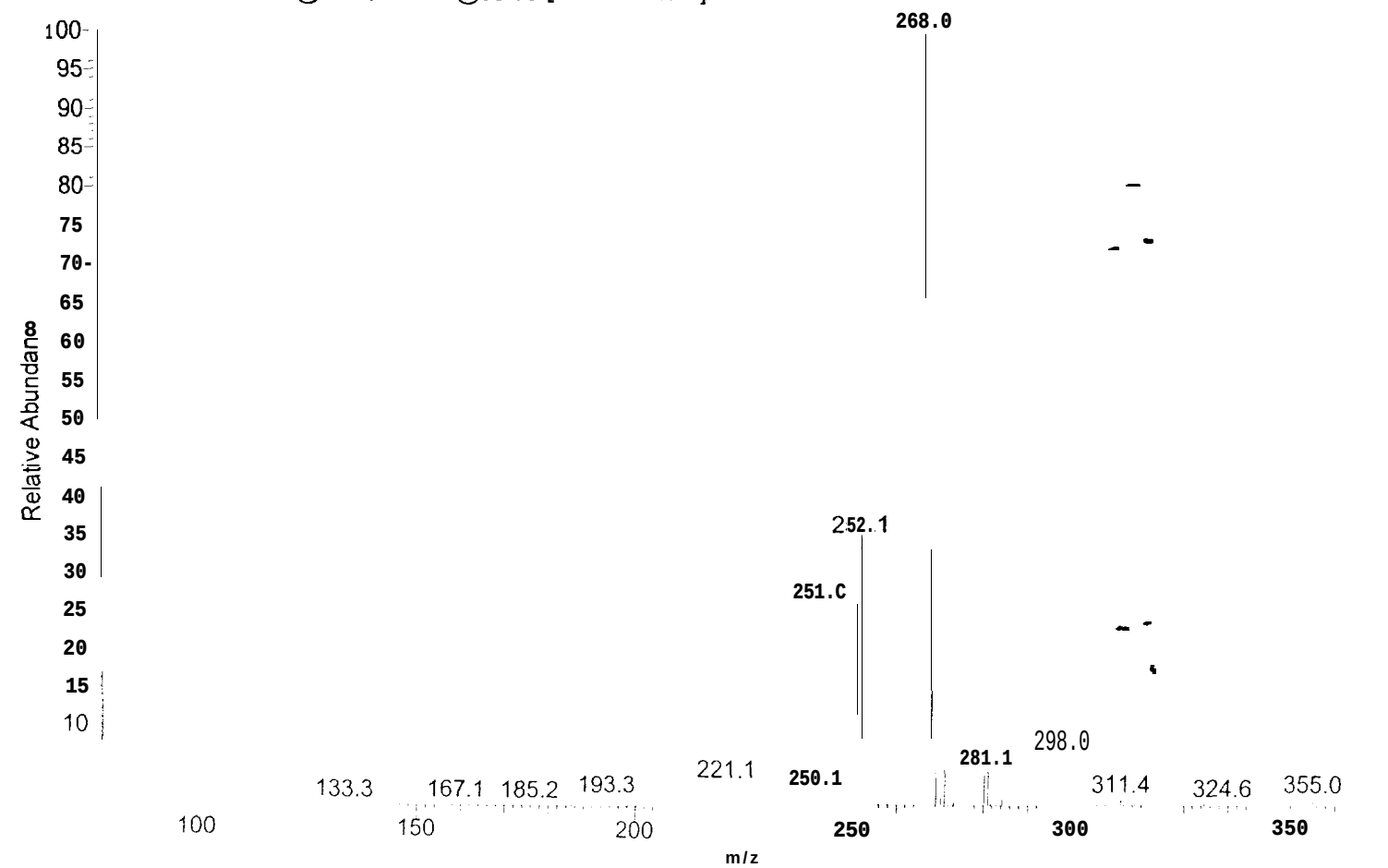

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Oper: raf
Peak: 1.0 amu
Area: 2, 3, 0
Study:
RIC: 43130040
Baseline: 80, 22

Elapse: 1 @ 5.14
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Client:
Masses: 264 > 296
Peak ID: 8, 80

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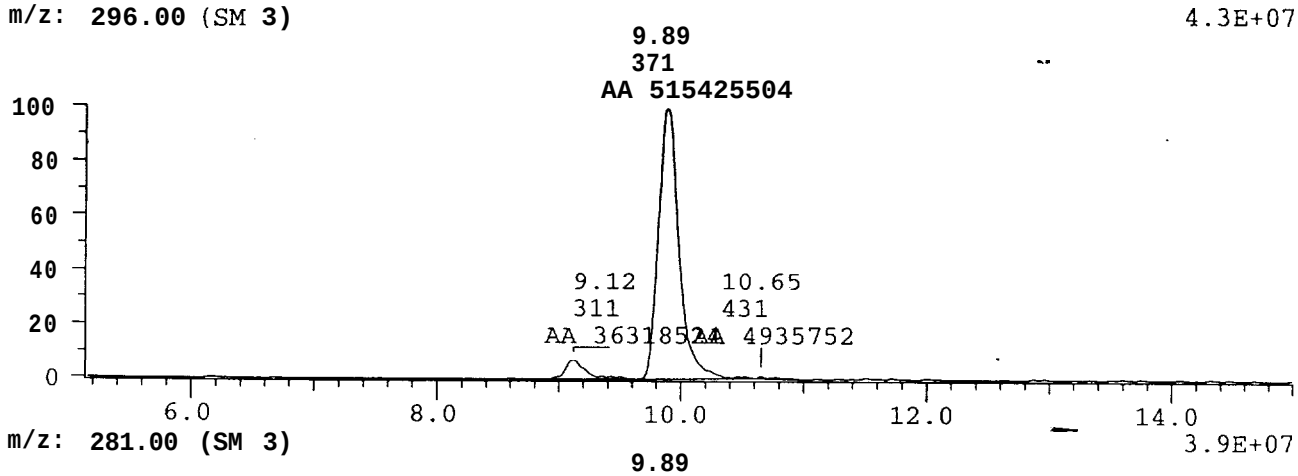

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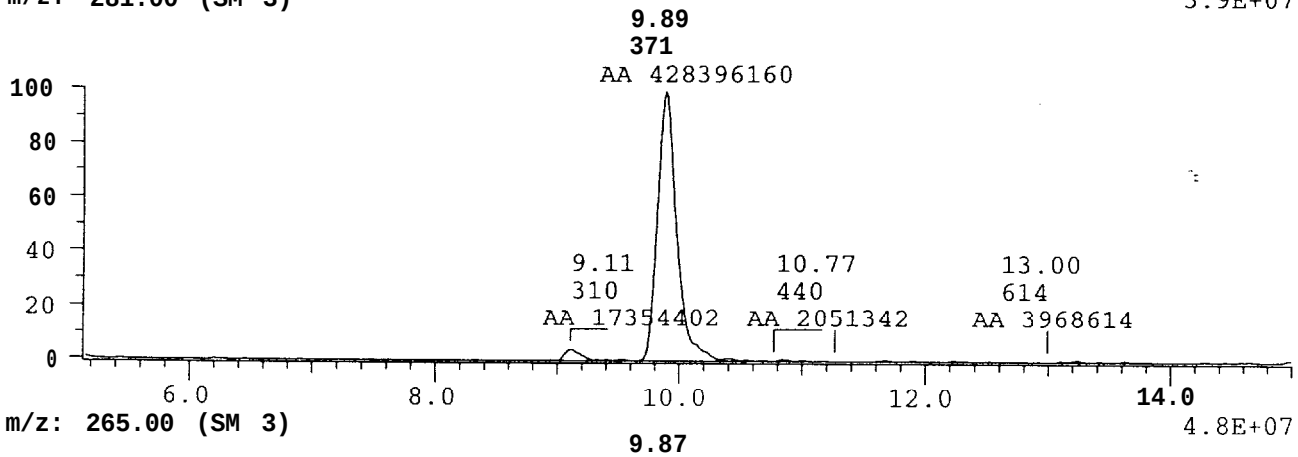

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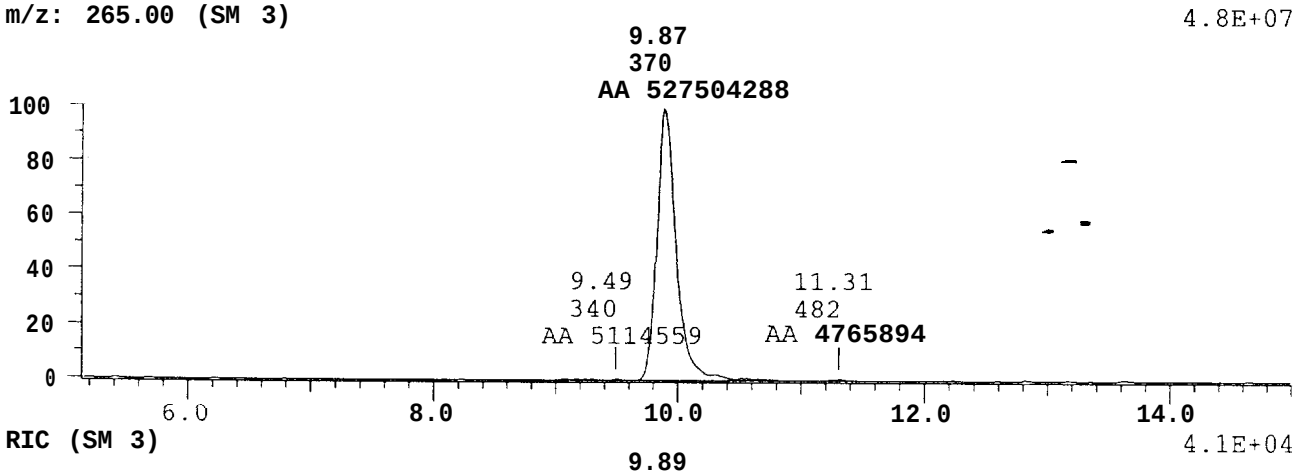

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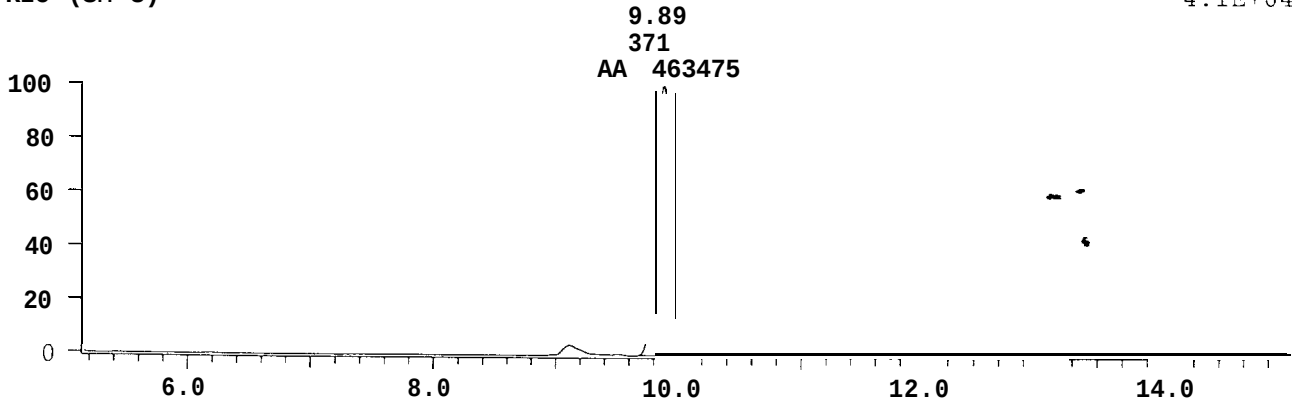

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Oper: raf
Peak: 1.0 amu RIC: 7737503
Area: 2, 3, 0 Baseline: 80, 22
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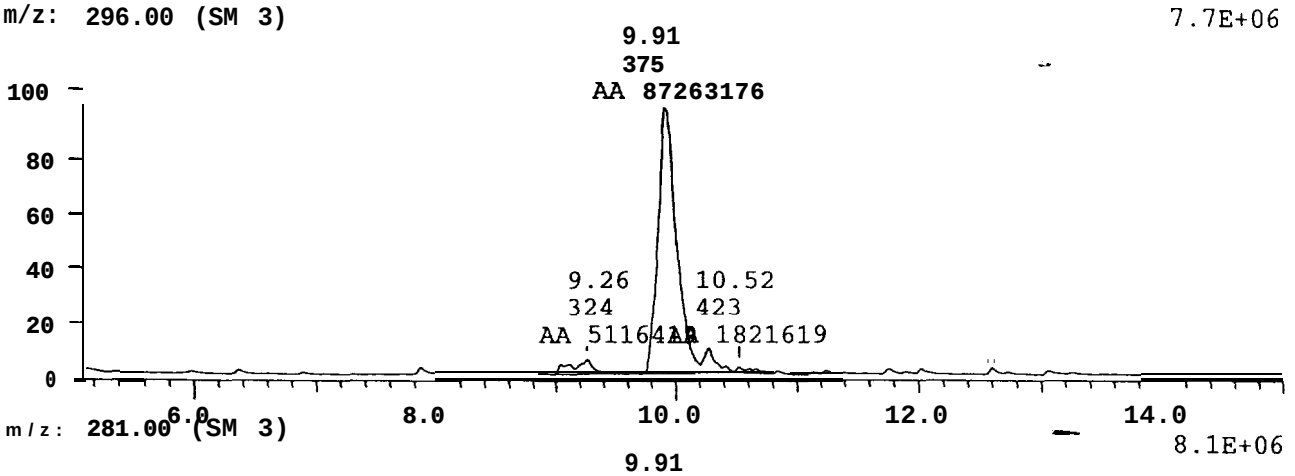
Client:
Masses : 264 > 296
Peak ID: 8, 80



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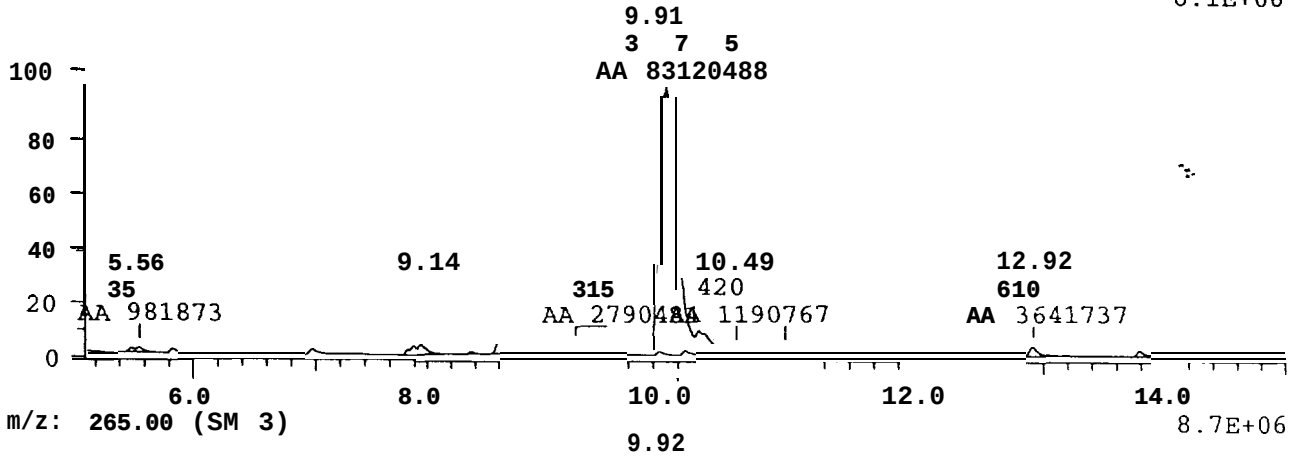

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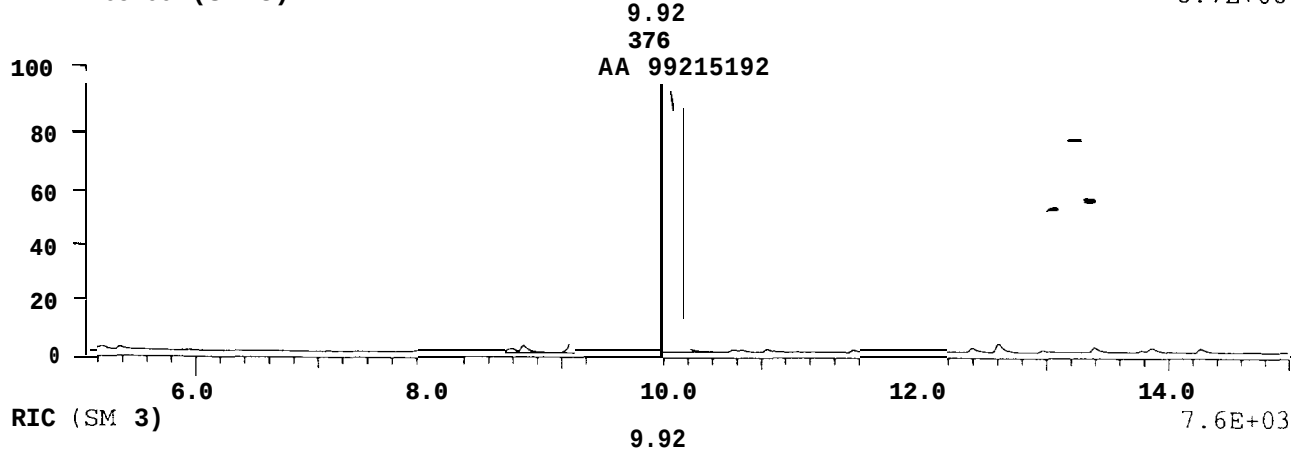

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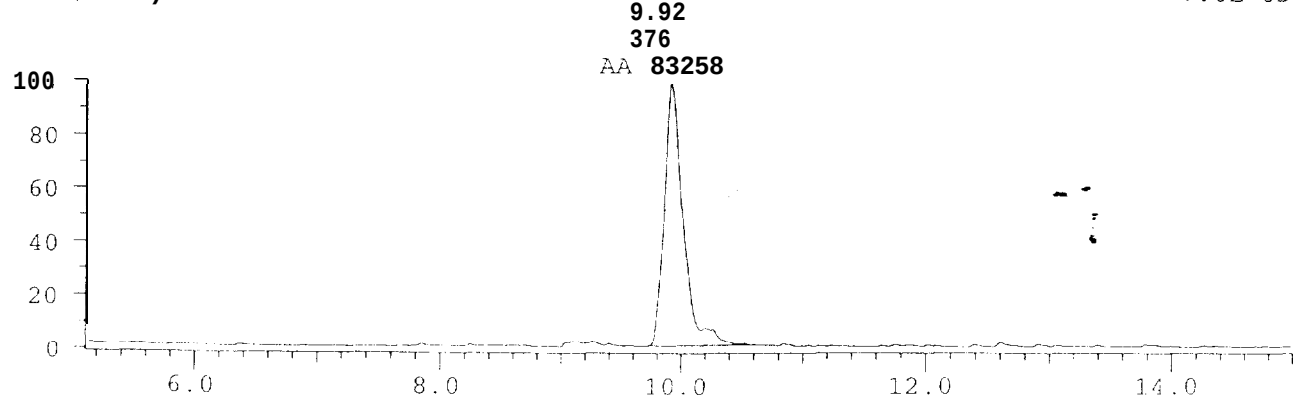

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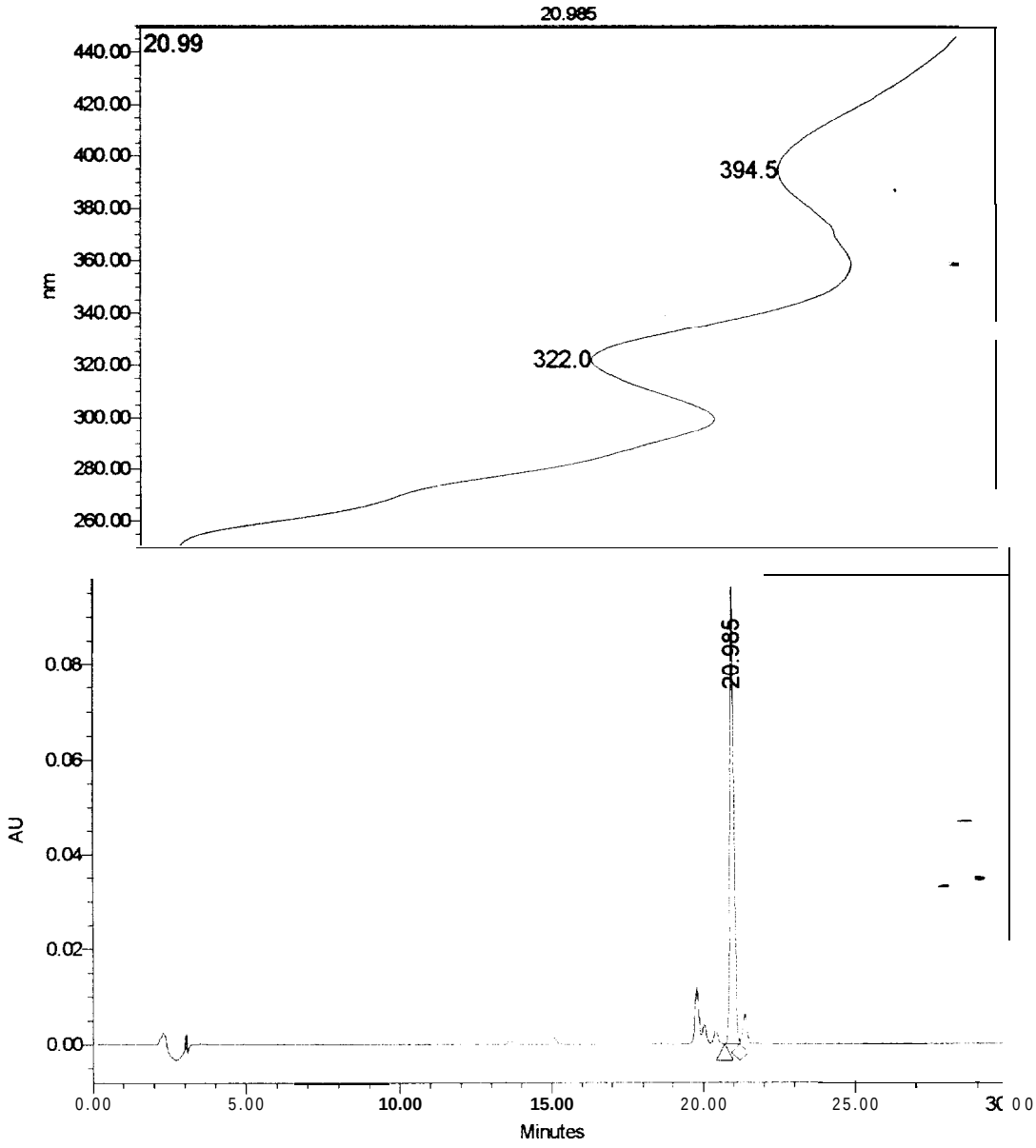
Column : Zorbax SB-C18 4.6 x 250 mm 3.0 um SN: BL 1345 w/C18 guard column
Flow 0.5mL/min Injection Volume 25 µl
Initial Pressure ~ 1260 psi Column temperature 40"

W Spectrum of Aristolochic acid standard



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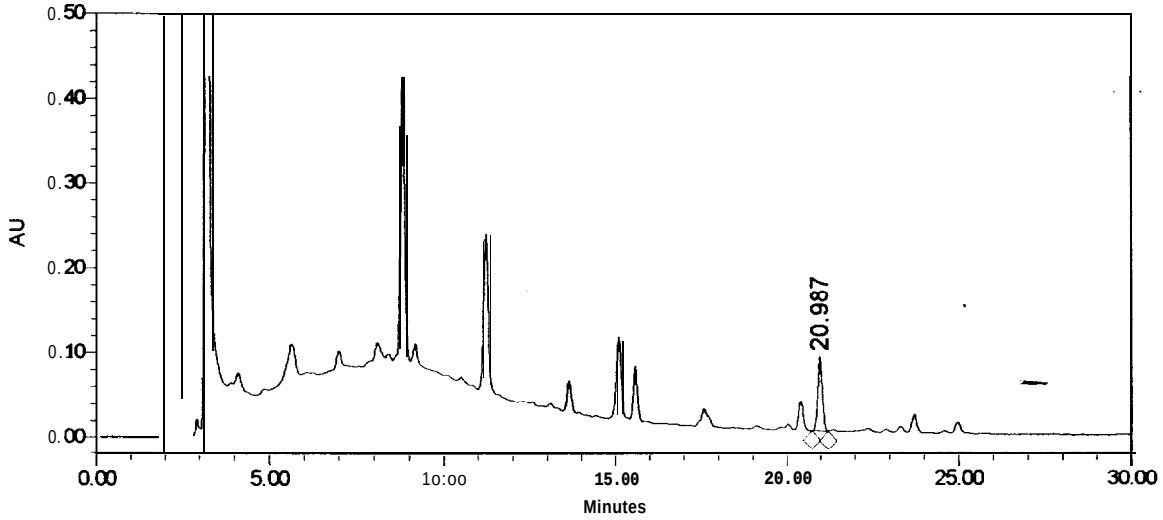
Colurm : Zorbax SB-C18 4.6 x 250 rrrn 3.0 um SN BL 1345 w/C18 guard colurm
Flow 0.5mL/min Injection Volume 25 ul Derived Channel 390 nm
Initial Pressure ~ 1260 psi Column temperature 40"

Test sample: Aristolochic acid in a liquid dosage form



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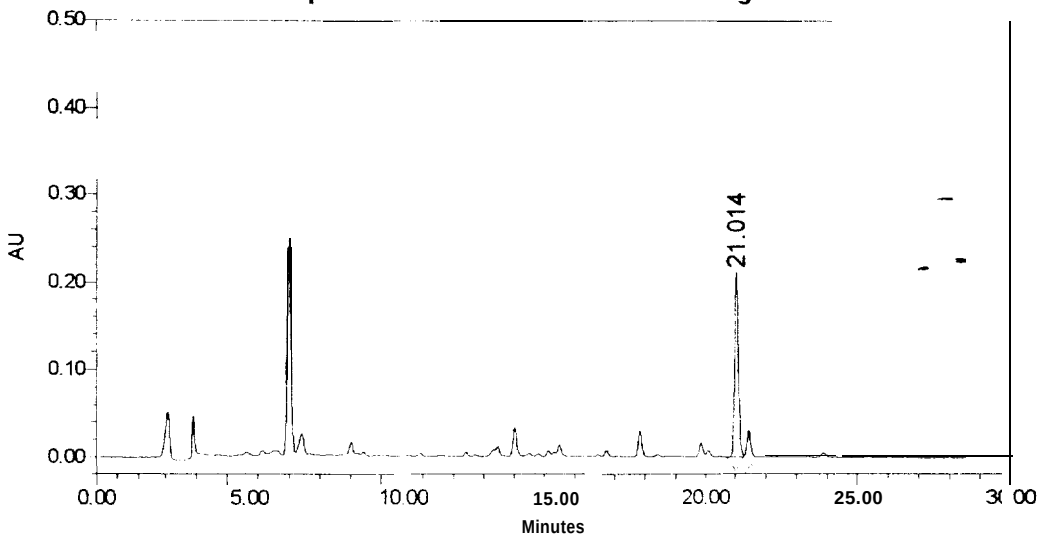

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