Full Experimental Details and Additional Experimental Data

Article Title: Detection of Opium Alkaloids in a Cypriot Base-Ring Juglet

Experimental

Solvents and Standards

HPLC grade and HPLC-MS grade water and methanol, and HPLC grade acetonitrile and dichloromethane (DCM), were purchased from VWR Chemicals. HPLC grade hexane was purchased from Fisher Scientific. Papaverine hydrochloride (solid) and morphine solution (100 μg/mL in methanol) were purchased from Sigma Aldrich. Thebaine solution (1 mg/mL in methanol) was purchased from Thames Restek UK Ltd. A generic co-codamol tablet (30 mg codeine, 500 mg paracetamol) was obtained and a solution containing 1 mg/mL codeine (and consequently 16.7 mg/mL paracetamol) in methanol was prepared from it. A stock solution of papaverine (1 mg/mL) was made up in methanol and the solutions were stored at 5 °C (papaverine and codeine) or -80 °C (morphine and thebaine, due to the need to keep these compounds locked away). Working solutions were made by dilution of the stock solutions.

Solutions of papaverine and thebaine were prepared for quantitation purposes. Papaverine solutions in the range 1 ng/mL to 3000 ng/mL (giving masses of 5 pg –15 ng injected into the HPLC), and thebaine solutions in the range 1 ng/mL – 500 ng/mL (5 pg – 2.5 ng injected), were prepared.

Artificial Ageing

Poppyseed oil samples (100 μL each) were artificially aged by heating, sealed in glass Kilner® jars under various conditions, at 60 °C in a GC oven, as described in Table S-1. Samples were aged either at 100 % relative humidity (achieved by placing a small vial of deionised water in the jar) or at ambient humidity; in a thin layer on a glass slide or as a pool in a 1 mL vial; with or without approx. 40 – 50 mg of ceramic powder (to simulate the juglet in which the archaeological material had been discovered). The ceramic powder was prepared by crushing a portion of open-fired pot and heating the powder at 450 °C for 6 h to destroy organic matter. Samples were extracted after varying amounts of time in the oven: 17 days, 8.5 months, 10.5 months and 11 months.

Table S-1. Treatment of artificially aged samples of poppyseed oil. A tick or other comment indicates preparation of a sample. Boxes left blank indicate no such sample was prepared.

Heating time	High Humidity				Ambient Humidity			
	Thin layer, with ceramic	Thin layer, without ceramic	Pool, with ceramic	Pool, without ceramic	Thin layer, with ceramic	Thin layer, without ceramic	Pool, with ceramic	Pool, without ceramic
17 days								✓
8.5 months		lost		✓		✓		✓
10.5 months	✓	lost	✓	✓	✓	✓	✓	✓
11 months					✓	✓	✓	✓

Extraction and Purification of Lipids and Alkaloids

To prevent contamination during sample preparation nitrile gloves were worn at all times, all glassware and tools were washed in solvent and all reagents were Analar or HPLC grade (99.5% pure or better). Procedural blanks were prepared and analysed alongside all samples to monitor for laboratory contamination.

Lipids were extracted by adding 500 µL of DCM to the sample followed by 2 x 10 minutes ultrasonication at ambient temperature in an ultrasonic water bath. After centrifuging for 10 min at 3000 rpm the supernatants were decanted to clean vials. The solvent was evaporated under a gentle stream of dry N_2 while heating at 40 °C and samples were derivatised by heating at 70 °C in closed vials with 50 µL N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane prior to analysis by GC-EI-MS.

A transesterified (methanolysed) sub-sample was prepared using Meth-Prep II (a 0.2 N methanolic solution of m-(trifluoromethyl)phenyltrimethylammonium hydroxide (TMTFTH) in methanol from Alltech) by adding 500µl of a 1:2 (v:v) mixture of Meth-Prep II in benzene followed by warming on a heating block for 1 hour at 60 °C prior to high temperature injection into the GC-EI-MS. By this method, transesterification of acylglyerols and methylation of the free and liberated fatty acids is achieved in the instrument inlet.

Unextracted samples for direct analysis by pyrolysis-GC-EI-MS (pyGC-EI-MS) were placed in quartz tubes and excess trimethylphenylammonium hydroxide added prior to analysis.

For alkaloid extraction, DCM was added to samples when necessary to solubilise/suspend the sample. Alkaloids were extracted by adding 4 mL HCl (0.1 M) to the sample, vortex mixing for a few seconds followed by ultrasonicating for 15 min in an ultrasonic water bath at ambient conditions. 1 mL hexane was added, followed by vortex mixing for a few seconds and centrifugation to separate the layers, and the organic (DCM and hexane) layer was removed. The aqueous layer was subjected to solid-phase extraction (SPE).

SPE was carried out using Strata C18-E (50 mg/1 mL) cartridges (Phenomenex). Each cartridge was conditioned with 1.5 mL methanol followed by 1.5 mL water. After loading the aqueous sample the cartridge was washed with 1 mL water and the alkaloids eluted with 1 mL methanol. The alkaloid-containing extract was dried under a stream of N_2 and redissolved in water:acetonitrile (9:1, v/v) for analysis by HPLC-MS. The volume of solvent used was the same as that of the original oil sample, while archaeological samples were redissolved in 100 μL solvent.

Extraction blanks were generated alongside sample extractions, by adding all of the reagents (without sample) to a sample tube and subjecting this to the full extraction and purification process.

GC-EI-MS Analysis

The DCM extract was analysed using two different instruments and methods, the first to achieve the best separation of fatty acids and their degradation products and the second to allow detection of acylgylcerols:

1) Using an Agilent 6890N gas chromatograph (GC) coupled to an Agilent 5973N mass spectrometer (MS) and fitted with an Agilent HP5-MS, 30 m × 0.25 mm column with a film thickness of 0.25 μm. A 1 m × 0.32 mm pre-column was used and the samples were injected in splitless mode at 250 °C. The carrier gas was helium in constant flow mode at 1.5 mL per minute. After a one minute isothermal hold at 50 °C, the oven was temperature programmed to 325 °C at 10 °C per minute with the final temperature held for 15 minutes. The MS interface temperature was 280 °C. Acquisition was in scan mode (*m/z* 50–600 over 1 s) after a solvent delay of seven min.

2) Using an Agilent 6890N GC coupled to an Agilent 5975C MS. Samples of 1 μL were injected at 10.74 psi in on-column mode with the inlet temperature programmed to follow the oven cycle. An SGE HT-5, 12 m x 0.1 mm, 0.1 μm film thickness, column fitted with 1 m x 0.53 mm retention gap was used. The carrier gas was helium in constant flow mode at 1.0 mL/min. After a 2 min isothermal hold at 40 °C the oven was temperature programmed to 350 °C at 10 °C/min with the final temperature held for 12 min. The MS interface temperature was 330 °C. Acquisition was in scan mode (45-700 amu/sec) after a solvent delay of seven min.

The sample prepared using Meth-Prep II was analysed using an Agilent 6890N GC coupled to an Agilent 5973N MS. Samples of 1 μL were injected at 7.6 psi in splitless mode at 300 °C. An HP-5MS, 30 m x 0.25 mm, 0.25 μm film thickness, column fitted with 1m x 0.32 mm retention gap was used. The carrier gas was helium in constant flow mode at 1.0 mL/min. After a 2 min isothermal hold at 50 °C the oven was temperature programmed to 100 °C at 10 °C/min, then to 190 °C at 4 °C/min, then to 280 °C at 25 °C/min, with the final temperature held for 30 min. The MS interface temperature was 280 °C. Acquisition was in scan mode (45-700 amu/sec) after a solvent delay of nine min.

Pyrolysis of unextracted samples was undertaken using a CDS Pyroprobe 1000. The method parameters were optimised by experiment with standards over a range of different pyrolysis

temperatures, flow rates, split ratios and interface temperatures. Further analysis was undertaken on another instrument to facilitate cold-trapping (see acknowledgements). The details of this method development are outside the scope of this paper. The optimised method used a probe temperature of 350 °C (15 s) and interface temperature of 350 °C. Pyrolysis products were introduced to the GC in split (200:1) mode at 300 °C. After a two minute isothermal hold at 40 °C, the oven was temperature programmed to 300 °C at 6 °C per minute with the final temperature held for seven minutes. The MS interface temperature was 280 °C. Acquisition was in scan mode (*m/z* 50–550 over 1 s).

In all methods system control and data collection/manipulation were achieved using G1701DA Chemstation (G1701DA) software. Mass spectral data were interpreted manually with the aid of the NIST/EPA/NIH Mass Spectral Library version 2.0 and comparison with published data.

HPLC-ESI-MS Analysis

HPLC-ESI-MS analysis was carried out with a Dionex UltiMate 3000 HPLC fitted with a Dionex Acclaim 120 C18 column (3 μm, 120 Å, 2.1 x 150 mm) and Phenomenex SecurityGuard system with a C18 (4 x 2.00 mm) cartridge, coupled to a Bruker HCTultra ETD II ion trap MS, a Bruker solariX XR 9.4T Fourier transform ion cyclotron resonance (FTICR) MS or an Applied Biosystems/MDS Sciex API 3000 triple quadrupole MS. All mass spectrometers were operated in positive ion mode with electrospray ionisation (ESI, the triple quadrupole fitted with Sciex's TurboIonSpray source). The mobile phase was composed of water (solvent A) and acetonitrile (solvent B). The mobile phase flow rate was 220 μL/min and the gradient was as follows: 10 % B initially, increasing linearly to 90 % B over 5 min. A further increase to 95 % B took place over 0.1 min followed by holding for 2 min. B was then returned to 10 % over 0.1 min and held for 5 min to allow column equilibration. The column compartment was held at 40 °C and the injection volume was 5 μL.

On the ion trap and FTICR mass spectrometers, extracted ion chromatograms for each analyte were produced. On the triple quadrupole MS, selected reaction monitoring (SRM) was used in order to obtain the lowest limits of detection possible, and to allow quantitation. Suitable transitions were identified from the literature[1–7] and the instrument parameters were optimised by direct infusion of a solution of 10 ng/mL of each analyte in methanol. Table S-2 shows the transitions and optimised parameters for each analyte. The ion trap was used for chromatographic method development experiments before moving onto the triple quadrupole in order to gain optimal sensitivity, and the FTICR was used to obtain full scan data with high mass accuracy in order to try to detect compounds for which standards could not be obtained. External calibration of the FTICR instrument was carried out using sodium formate cluster ions to ensure a 1 ppm mass accuracy. Extracted ion chromatograms were produced from the full scan data, with *m/z* values for $[M+H]^+$ extracted to 3 decimal places ± 0.001.

Table S-2. SRM transitions for analysis of the alkaloids, together with optimised triple quadrupole instrument parameters.

Analyte	**Transition / *m/z***	**Declustering Potential / V**	**Focusing Potential / V**	**Entrance Potential / V**	**Collision Energy / eV**	**Collision Cell Exit Potential / V**
Morphine[1,3,6]	286 → 165	80	80	15	50	12
Codeine[1,3–7]	300 → 215	35	200	15	35	15
Thebaine[1]	312 → 281	22	150	10	17	22
Papaverine[1–3]	340 → 202	45	280	15	36	15

X-radiography

Radiographs were taken using a Siefert Isovolt DS1 X-ray tube held within a lead enclosure and Kodak Industrex MX and AA films. Voltages of between 70 and 90 kV were used with exposures of around 5 mA minutes. Lead screens (0.125 mm) were used at both the back and front of the films. The films were subsequently scanned using an Agfa RadView digitizer with a 50 micron pixel size and 12-bit resolution in order to allow digital manipulation and enhancement of the images. The image shown here (Figure 1c) has been slightly enhanced with an 'unsharp mask' to emphasize edges and greyscale levels have been adjusted but features are also detectable on the unenhanced films.

Results and Discussion - Additional Figures

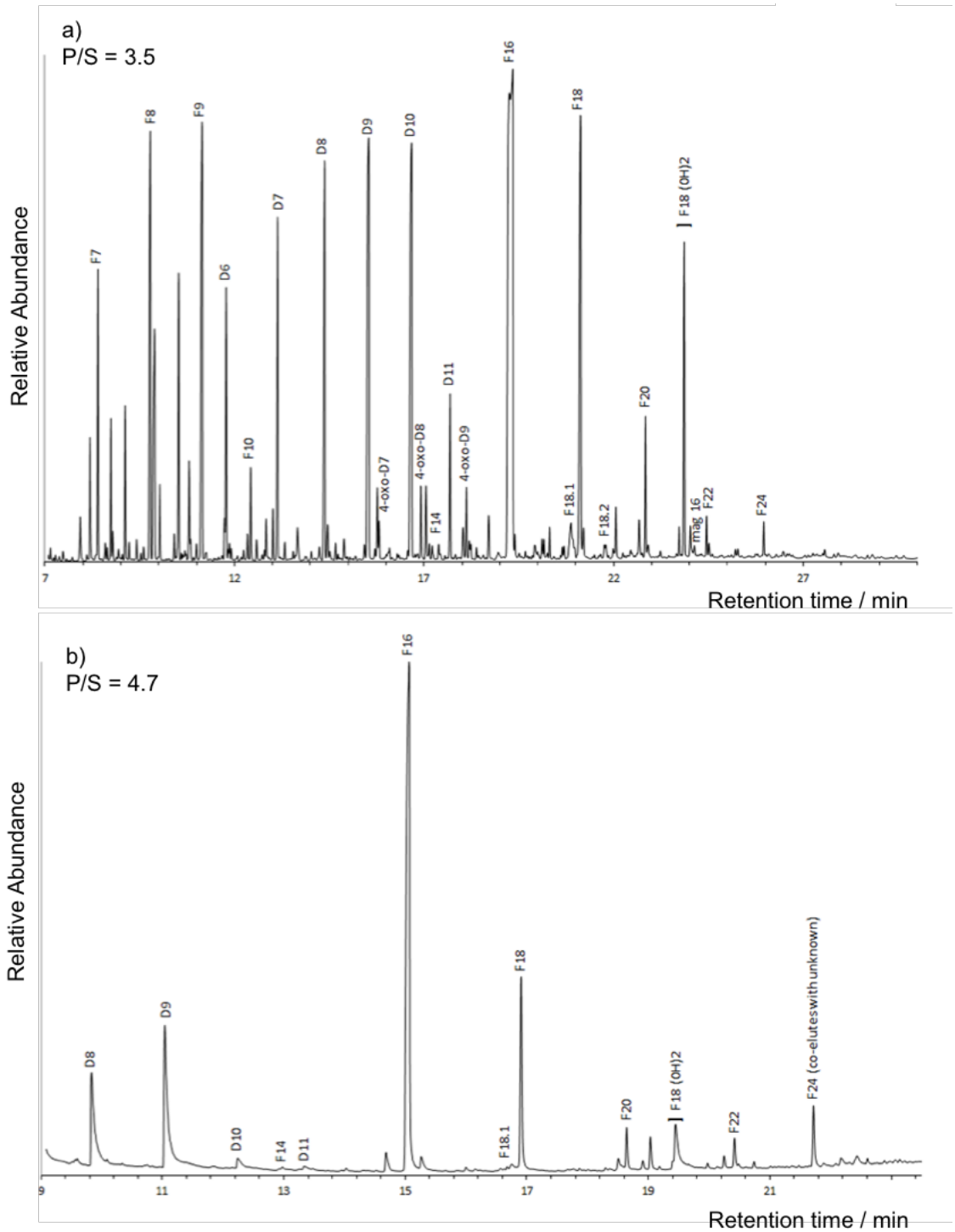

Figure S-1. Partial GC-EI-MS total ion chromatograms obtained showing lipid profiles obtained from the residue in the juglet. (a) The DCM extract (TMS derivatives); (b) the methanolysed extract (methyl ester derivatives). Peaks are labelled as: Fn – fatty acid with carbon chain length n; Dn – dicarboxylic acid with carbon chain length n; F18.n – C18 fatty acid with n double bonds; F18(OH)2 – dihydroxy C18 fatty acid; 4-oxo-Dn – 4-oxo-dicarboxylic acids with carbon chain length n; mag16 – monopalmitin. P/S ratios are based on peak areas of F16 and F18.

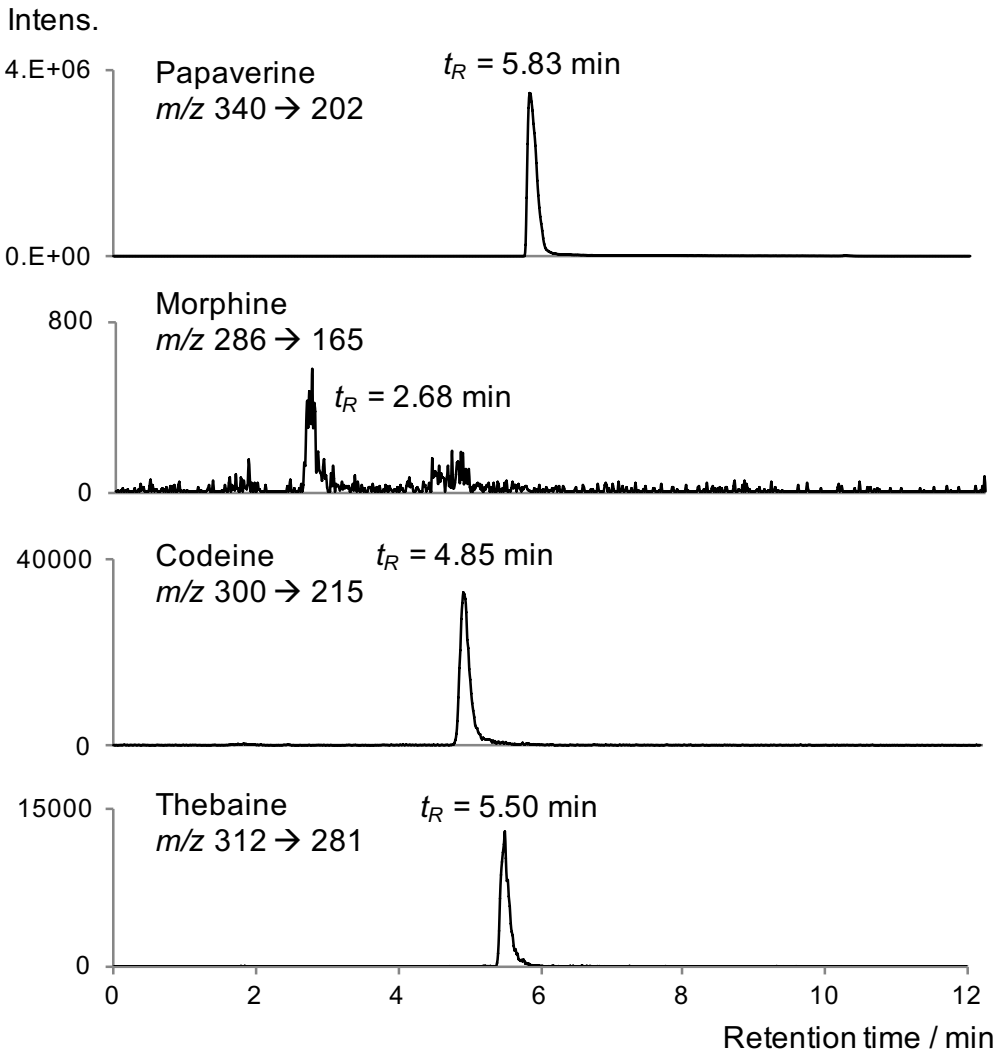

Figure S-2. SRM chromatograms for each of the four alkaloids in the extract of Fandler poppyseed oil.

Table S-3. Formulae and calculated $[M+H]^+$ *m/z* values for selected opium alkaloids and breakdown products. Numbers in brackets refer to the structures shown in Figure 2.

Analyte	**Formula**	**$[M+H]^+$ *m/z***
Meconic acid (8)	$C_7H_4O_7$	201.003
Opianic acid (9)	$C_{10}H_{10}O_5$	211.060
Cotarnine (6)	$C_{12}H_{15}NO_4$	238.107
Hydrocotarnine (7)	$C_{12}H_{15}NO_3$	222.112
Morphine (1)	$C_{17}H_{19}NO_3$	286.144
Codeine (2)	$C_{18}H_{21}NO_3$	300.159
Thebaine (3)	$C_{19}H_{21}NO_3$	312.159
Papaverine (4)	$C_{20}H_{21}NO_4$	340.154
Noscapine (5)	$C_{22}H_{23}NO_7$	414.155

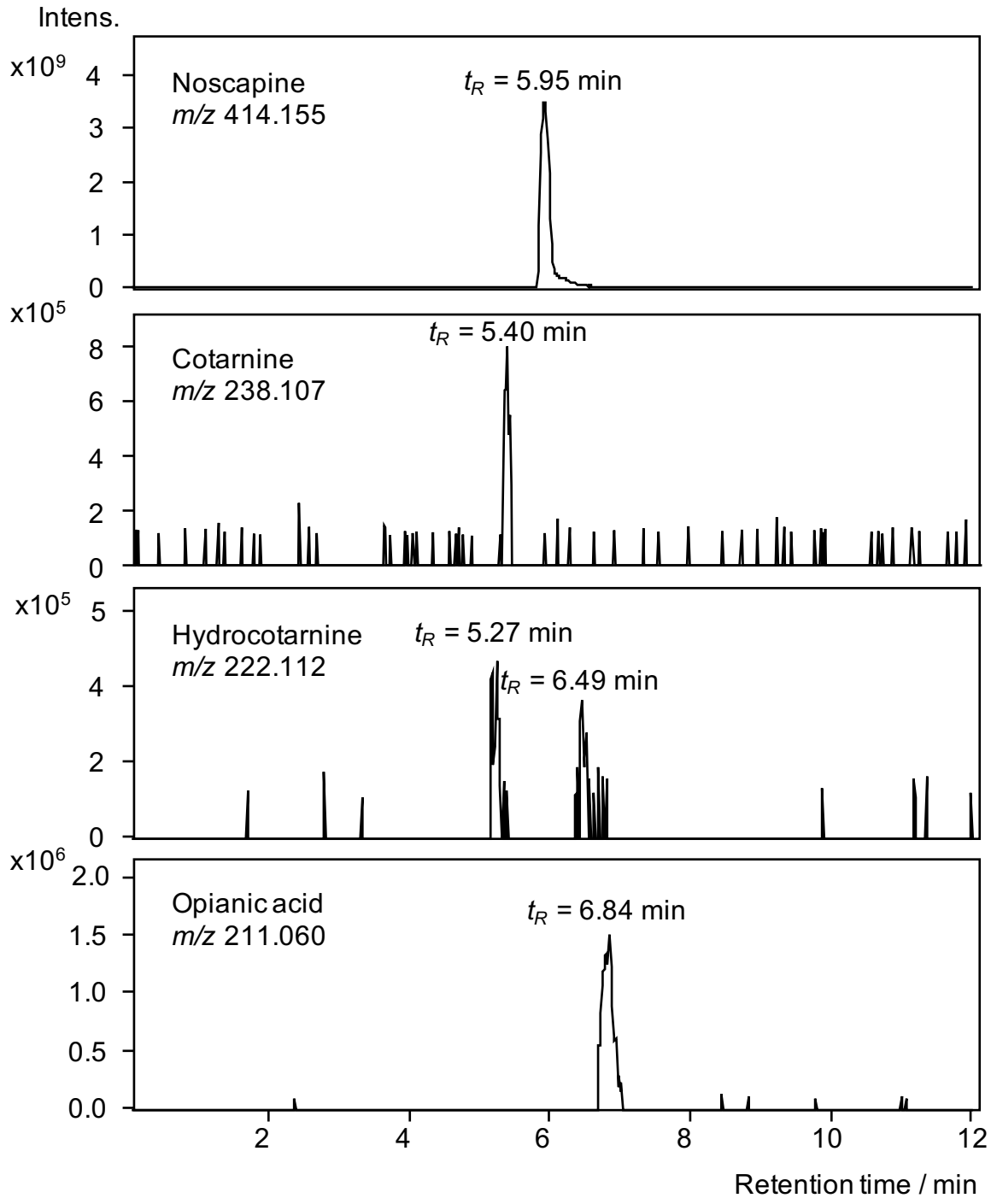

Figure S-3. EICs for noscapine, cotarnine, hydrocotarnine and opianic acid in Fandler poppyseed oil analysed using HPLC-ESI-FTICR-MS.

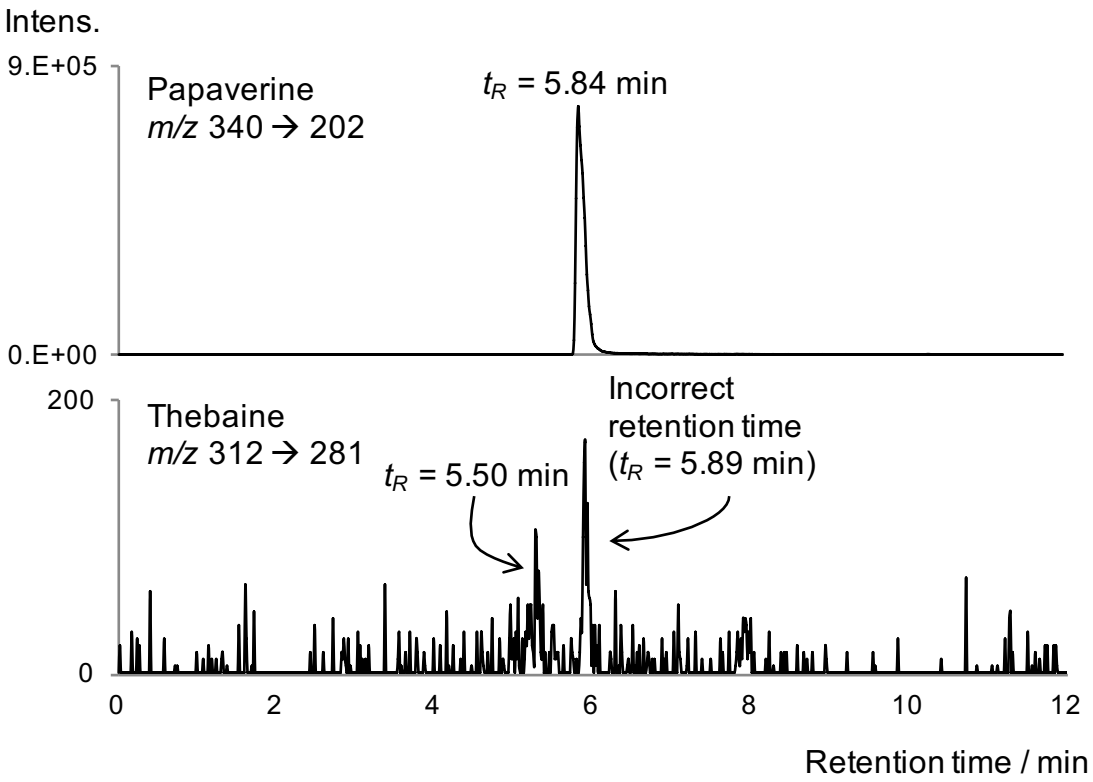

Figure S-4. SRM chromatograms of transitions for papaverine and thebaine in Fandler poppyseed oil aged for 17 days at 60 °C: dissolved in DCM before extraction.

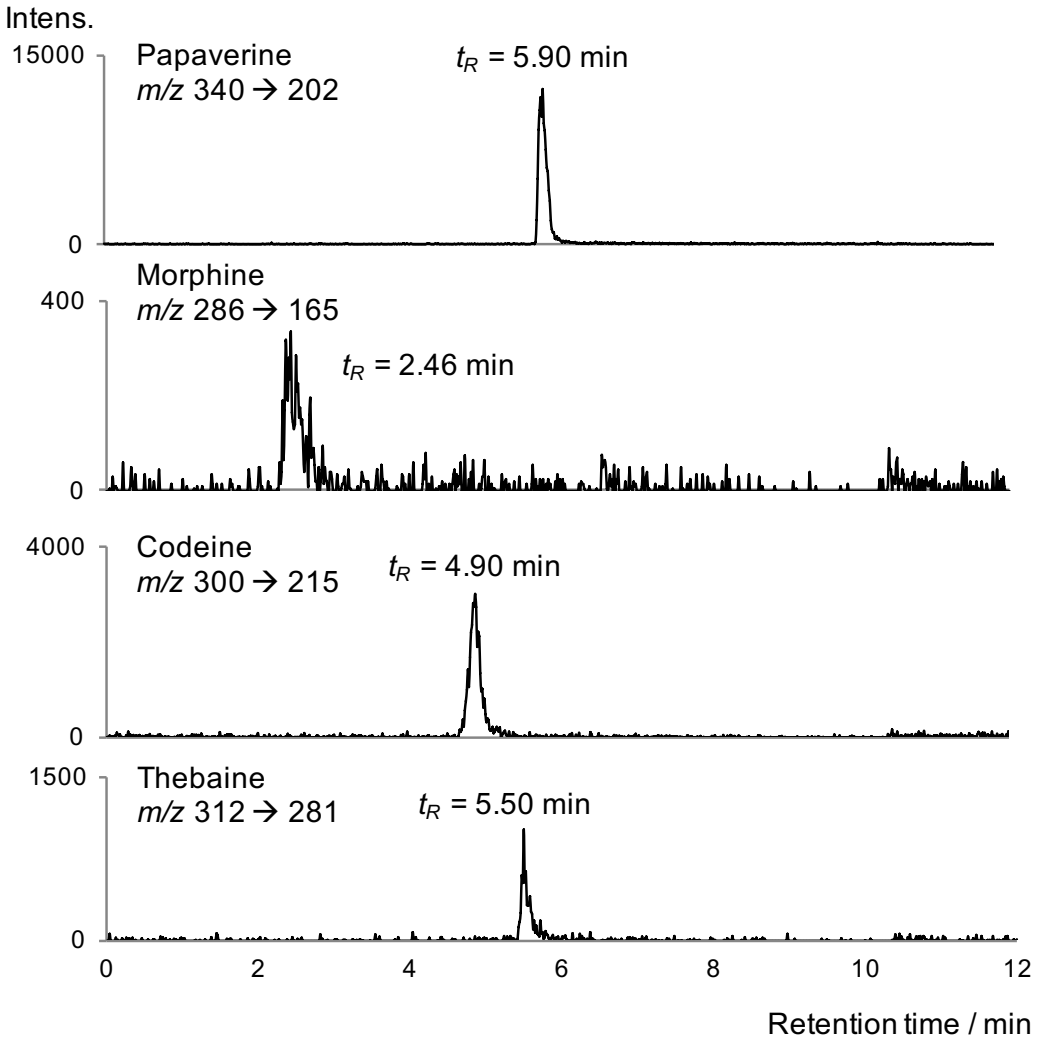

Figure S-5. SRM chromatograms of 25 pg of each of the four opiate standards.

References

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