USDA Analytical Method	United States Department of Agriculture Animal and Plant Health Inspection Service Wildlife Services National Wildlife Research Center Product Development Program Analytical Chemistry Project	Number: Method 121A Supersedes: none	Date Effective: 08-27-02 Page: 1 of 13

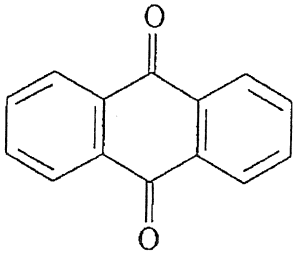
DETERMINATION OF ANTHRAQUINONE RESIDUES (10-1400 ppm) IN SUNFLOWER SEEDS TREATED WITH A SPRAY APPLICATION

I. CHEMICAL DATA (ACTIVE INGREDIENT)

Common Name: Anthraquinone

Alternative Names: 9,10-anthracenedione, 9,10-anthraquinone, 9,10-dioxoanthracene, Morkit

Structure:



Anthraquinone

Dennis J. Kohler Developed By	8-27-02 Date	[illegible] Approved By	8-27-02 Date
Dennis J. Kohler Validated By	8-27-02 Date	[illegible] QA/QC Specialist	8/27/02 Date

I. CHEMICAL DATA (Continued)

Anthraquinone

Formula:	$C_{14}H_8O_2$
MW:	208.22
MP:	286 °C
Physical State:	light yellow crystals
Solubility:	insoluble in water, soluble in alcohol, soluble in chloroform and benzene

II. MATRICES TESTED

This method was developed and validated for the determination of 10.0 to 1400 ppm anthraquinone in sunflowers seeds.

III. GENERAL METHOD

Sunflower seed samples (10-15 gram portions) are ground and 0.25 g replicates are extracted with chloroform. The extracts are filtered and 1.00 mL of each sample extract is then vortex mixed, sonicated, and centrifuged. Each sample extract is passed through a aminopropyl solid phase extraction (SPE) column followed by evaporation of the solvent. The residue is reconstituted with 1.00 mL of methanol, carefully filtered and transferred into a HPLC vial, capped and analyzed by HPLC.

IV. REAGENTS

	Name	CAS Registry Number
1.	Methanol	67-56-1
2.	Water, HPLC Grade	7732-18-54
3.	Anthraquinone	84-65-1
4.	Chloroform	67-66-3

V. EQUIPMENT

1. Fisher Centrific Centrifuge #1 SN- 4650
2. Bransonic 32 Sonicator
3. Mettler AE 100 Analytical Balance SN - D14532
4. Hand-held grinder (Model Krups #203)
5. 1.00 mL Hamilton syringe
6. IST 500 mg Aminopropyl in 3-mL SPE columns
7. Zymark RapidTrace SPE workstation

VI. SOLUTIONS

Diluent: NA

VII. STANDARD PREPARATION

Concentrated Standard Solution: Accurately weigh 50 mg of anthraquinone reference standard and quantitatively transfer to a 10-mL volumetric flask. Dissolve and dilute to volume with chloroform. The anthraquinone concentration of this solution is about 5,000 µg/mL.

Intermediate Standard Solution: Accurately transfer a 0.100 mL aliquot of the 5,000 µg/mL standard into a 10-mL volumetric flask and dilute to volume with chloroform and mix well. The concentration of this solution is about 50.0 µg/mL.

Working Standard Solution: Accurately transfer a 0.200 mL aliquot of the intermediate standard solution (50.0 µg/mL in chloroform) into a 10-mL volumetric flask, dilute to volume with methanol and mix well. The anthraquinone concentration of this solution is about 1.00 µg/mL.

VIII. SAMPLE PREPARATION

1. Grind each sample into a powder with a hand-held electric grinder.

2. Accurately weigh a 0.25 to 0.27 g sample into a 25-mL glass screw top tube.

3. Add 10 mL of chloroform from a repipette. Seal the tube with a screw cap and vortex mix the sample.

4. Place the tubes in an ultrasonic bath for 20 minutes in a glass beaker. (Do not use the Bransonic 5200 ultrasonic bath). Shake the tubes by hand for a couple of seconds. Place back in the ultrasonic bath for 20 additional minutes. Repeat the last step one more time.

5. Centrifuge the sample for 5 minutes.

6. Using a Pasteur pipette and bulb, transfer 2 to 3 mL of extract into a 10-mL syringe with a 0.45 μm Teflon filter disk and collect the filtrate in a 10-mL tube.

7. Transfer 1.00 mL of the filtered extract to a second 10-mL glass tube.

8. Place each tube with extract on the Zymark RapidTrace SPE workstation and run the method named AQSFLOW.SPE. See page 13.

9. Evaporate the extract to dryness on the N-Evaporator.

10. Reconstitute the residue to 1.00 mL with methanol and vortex mix. Sonicate the samples horizontally and rotate for 10 seconds individually, then sonicate the tube for 10 minutes vertically in a glass beaker. Centrifuge the samples for 1 minute.

11. Filter a portion of the final solution through a 0.45 μm Teflon disk into an amber HPLC vial, and seal with a crimp cap. Analyze each sample by HPLC.

IX. PROCEDURE

Repeatedly inject 10 µL of the working standard to determine the HPLC system suitability prior to analysis. Inject 10 µL of each sample and record the anthraquinone peak response.

X. HPLC CONDITIONS

Mobile Phase:	80% Methanol 20% Water
Flow Rate:	1.0 mL/min
Oven Temperature:	40 °C
Column:	Keystone ODS/H , 25.0 cm x 4.6 mm i.d., 5 µm or equivalent with 1.5 x 4.6 mm i.d. guard column.
Injection Volume:	10 µL
Detector:	UV @ 254 - primary wavelength UV @ 325 - secondary wavelength
Run Time:	20 minutes (analyte retention time is approximately 8.3 minutes) for standards and samples.

Note : Operating conditions should be adjusted to obtain optimum response and reproducibility.

XI. SYSTEM SUITABILITY

System suitability is demonstrated when the relative standard deviation (rsd) of the anthraquinone chromatographic peak response is $\leq$ 2.0% for five consecutive injections of the working standard.

XII. DATA ANALYSIS AND CALCULATIONS

Anthraquinone Residue Calculation:

$$ppm\ Analyte = \frac{Area_{sample}}{Area_{std.}} \times Conc._{std.}\ (\mu g/mL) \times \frac{10.00\ mL}{Sample\ wt.\ (g)}$$

where:

$Area_{sample}$ and $Area_{std.}$ = the anthraquinone chromatographic responses from the sample and working standard solutions, respectively.

$Conc._{std.}$ = the concentration of the anthraquinone fortification standard solutions (µg/mL)

Sample wt. = the sample weight of the homogenized sunflower seeds 0.25 to 0.27 (g)

EXAMPLE CALCULATION

A sunflower seed sample weighing 0.2515 g was assayed for anthraquinone using the procedures described in this method. The anthraquinone chromatographic response (@ 254 nm) from the final solution was 85.321 area units. A fortification standard with a anthraquinone concentration of 0.248 µg/mL was chromatographed and its area response was 30.52 area units.

The concentration of anthraquinone is calculated as follows:

$$ppm\ Anthraquinone = \frac{85.321}{30.52} \times 0.248\ \mu g/mL \times \frac{10.00\ mL}{0.2515\ g}$$

$$= \underline{27.6\ ppm}$$

XIII. METHOD VALIDATION

The validation data were generated using a Hewlett-Packard 1050 HPLC equipped with a Multiple Wavelength Detector. The HPLC conditions used were given in Section X.

Response Linearity

Response Linearity was established in Method 108A
Two sets of five anthraquinone standard solutions ranging from 0.025 to 20.0 µg/mL were prepared. Data were collected from duplicate injections of each solution, and a plot was constructed of analyte peak response (y-axis) vs. anthraquinone concentration (x-axis). A linear regression was performed on the data set using SAS Version 6.12. The regression statistics were as follows:

254 nm $r^2 = 1.0000$

Log(analyte response) vs. Log (anthraquinone concentration):

254 nm slope = 0.992586 $r^2 = 0.9996$

Result: A linear and proportional relationship exists between chromatographic peak response and anthraquinone concentration from 0.025 µg/mL to 20.0 µg/mL. Single point calibration is valid in this range.

Matrix Interference
Seven control sunflower seed matrix samples were analyzed according to the procedures described.

Results: No chromatographic interferences were observed. Chromatograms of a 0.248 µg/mL anthraquinone fortification standard solution, a control sunflower seed sample and a sunflower seed sample fortified at 9.58 ppm are attached.

XIII. METHOD VALIDATION (Continued)

Instrument Limit of Detection (ILOD)

Instrument Limit of Detection was established in Method 108A.
The instrument limit of detection (ILOD) was estimated from the chromatographic response of the reagent blank and the response of a 0.025 µg/mL anthraquinone standard. The ILOD is defined as the concentration of anthraquinone required to generate a signal equal to 3X the baseline noise (measured peak-to-peak) observed in the reagent blank. Under the conditions stipulated in the method, the ILOD for anthraquinone is equal to 0.0078 µg/mL at 254 nm.

Method Limit of Detection (MLOD)

The method limit of detection (MLOD) was estimated from the mean chromatographic response of seven control sunflower seed matrix samples and the response of seven control sunflower seed matrix samples fortified at a mean of 9.75 µg/g anthraquinone. The MLOD is defined as the concentration of anthraquinone required to generate a signal equal to 3X the baseline noise (measured peak-to- peak) observed in the control sample. Under the conditions stipulated in the method, the mean MLOD for anthraquinone in sunflower seed is equal to 2.50 µg/g at 254 nm.

Fortification of Control Matrices

High Level Fortification Standard: Accurately transfer a 0.070 mL aliquot of the 5000 µg/mL concentrated stock solution into a 25-mL glass tube and dilute to volume with 10.0 mL of chloroform and mix well. The anthraquinone concentration of this solution is about 35.0 µg/mL.

Low Level Fortification Standard: Accurately transfer a 0.0500 mL aliquot of the 50.0 µg/mL concentrated stock solution into a 25-mL glass tube and dilute to volume with 10.0 mL of chloroform and mix well. The anthraquinone concentration of this solution is about 0.250 µg/mL.

XIII. METHOD VALIDATION (Continued)

1400 μg/g Anthraquinone Fortification Procedure: To each 0.25 g portion of control sunflower seed, add a 70.0 μL aliquot of the 5000 μg/mL standard solution in chloroform. Allow the solvent to evaporate. Proceed with the extraction procedure as described above.

10.0 μg/g Anthraquinone Fortification Procedure: To each 0.25 g portion of control sunflower seed, add a 50 μL aliquot of the 50.0 μg/mL standard solution in chloroform. Allow the solvent to evaporate. Proceed with the extraction procedure as described above.

Bias and Repeatability

Replicate control sunflower seed samples were fortified with anthraquinone and assayed according to the procedures in this method. The recovery and precision data are given on the next page:

Sunflower Seeds

Sample #	10.0 ppm	1400 ppm
1	89.4	87.1
2	92.9	90.4
3	107	85.3
4	87.4	87.6
5	97.4	88.1
6	91.2	87.4
7	94.8	89.0
Mean	94.(3)%	87.(8)%
s =	6.5%	1.6%
cv =	6.9%	1.8%

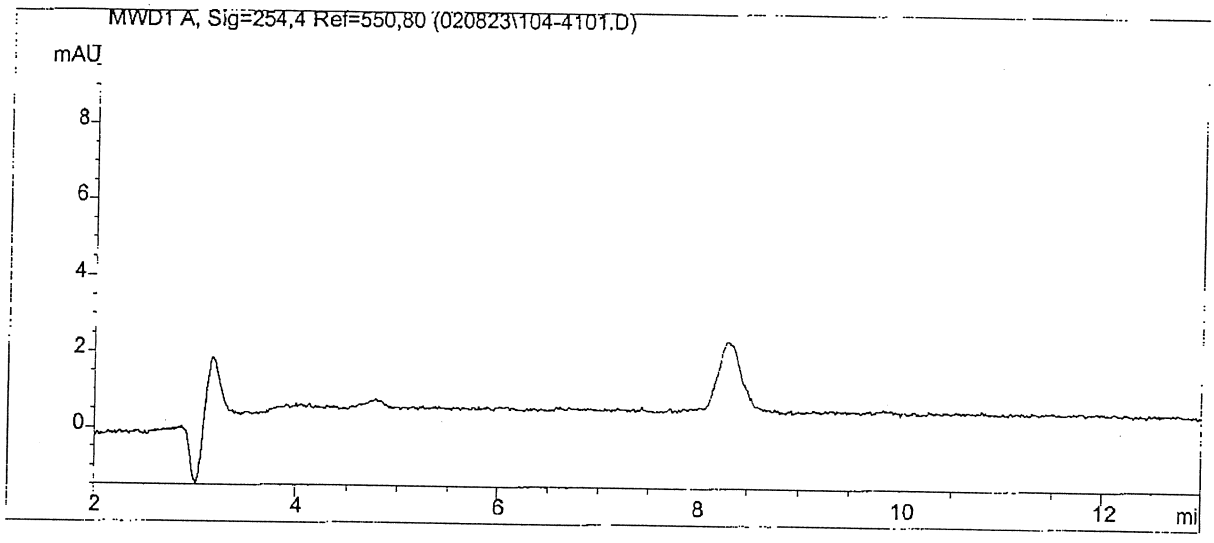
XIII. REFERENCES

Analytical Services Project Number: 02-061
Analytical Chemistry Project Notebooks: AC94: pp. 137 - 142
QC21: pp. 28 - 30

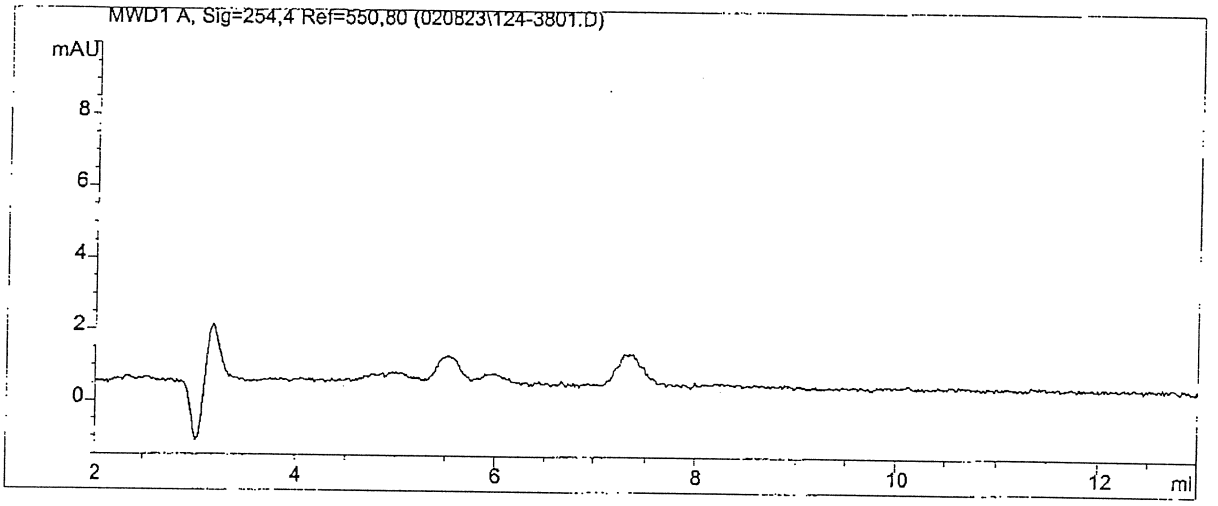
XIV. STANDARD OPERATING PROCEDURES

The following SOP's were applicable at the time of method validation:

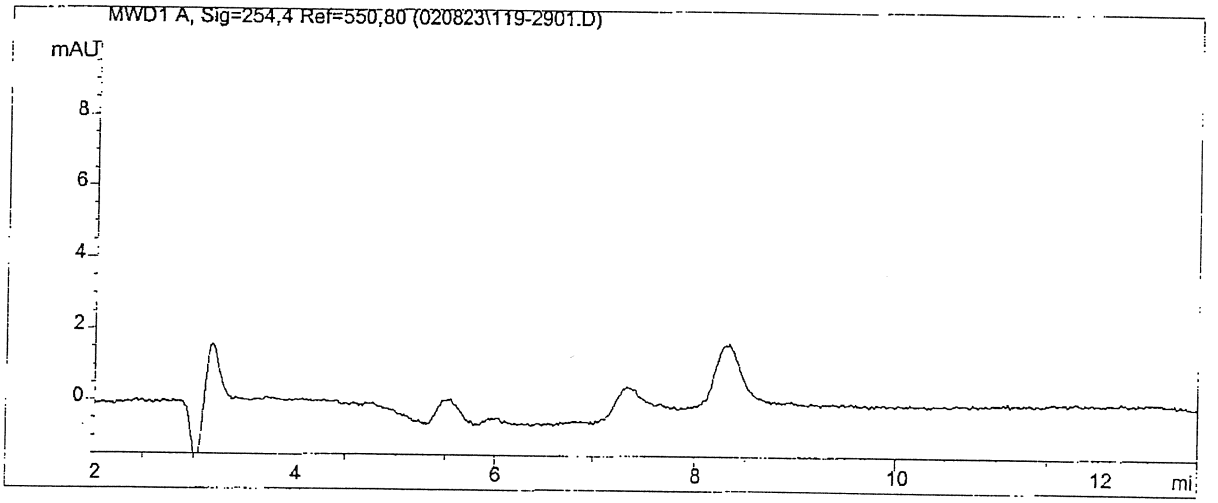
CH 001	CH 002.01	CH 003.02	CH 004	CH 005
CH 006	CH 007	CH 008	CH 009	CH 010
CH 012	CH/CO 001.01	CH/CO 002	WRC-380.R1	WRC-402.R1
HS 001.01	HS 004	HS 006.01	HS 008	HS 009
IE 002	IE 003	IE 005	IE 006	IE 007
IE 008	IE 009	IE 010	IE 011	IE 013
IE 014	IE 018	WRC-228.R6		



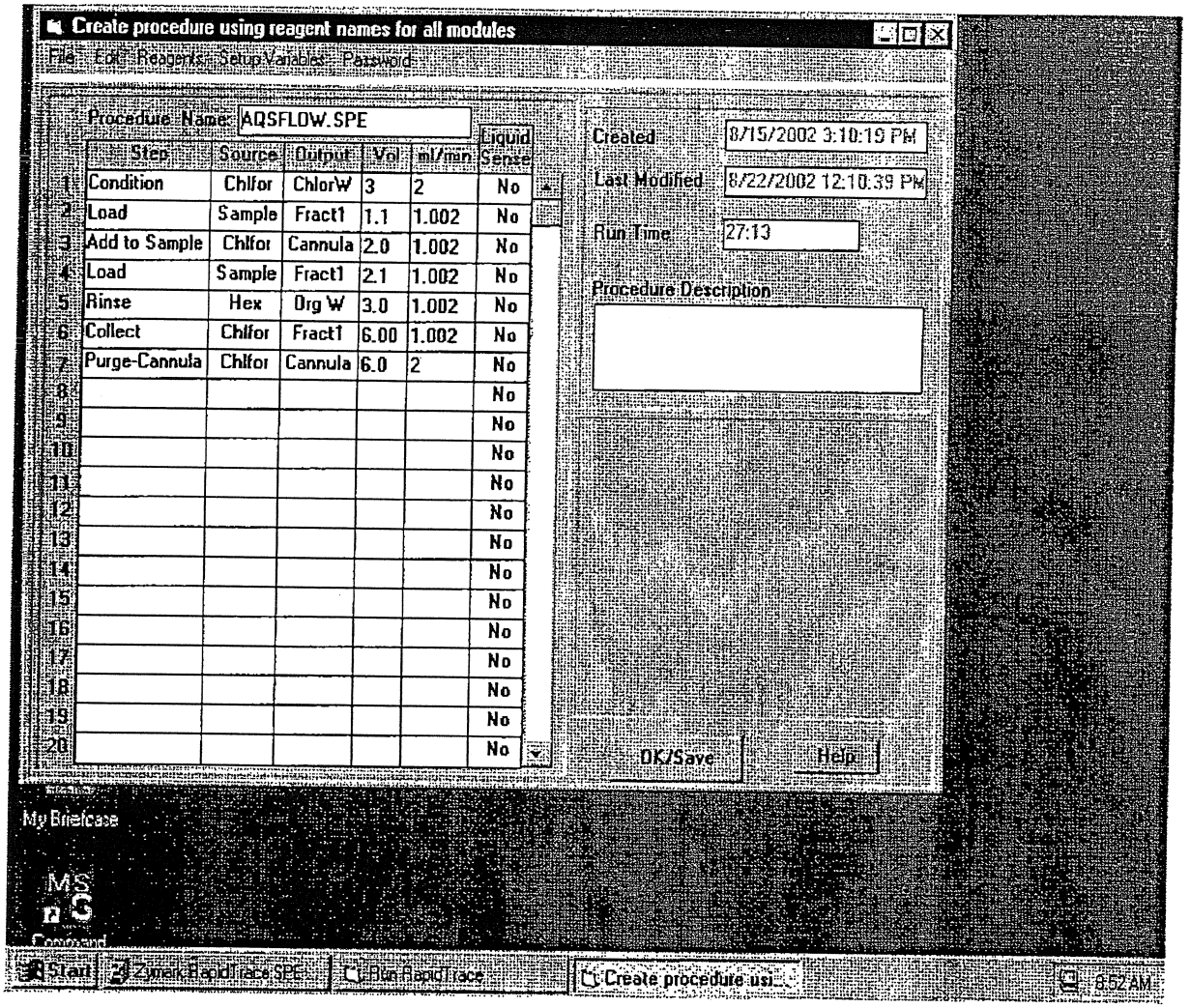
Anthraquinone fortification standard (0.248 µg/mL) chromatographed at 254 nm.



Control sunflower seed sample, extracted and chromatographed at 254 nm.



Fortified (9.58 µg/g) of sunflower seed sample, extracted and chromatographed at 254 nm.



Chlfor = Chloroform

Zymark Rapid Trace Program.