Method 27.0

Total Hexane Content in Ground Paprika (Headspace Gas Chromatography)

Purpose: To determine the total amount of hexane present in ground paprika. The method has a quantitation limit of 2 ppm total hexanes when at least three of the individual hexane isomers are detected.

A. Apparatus:

1. Gas chromatograph equipped with:
   a. split/splitless capillary injector
   b. Headspace autosampler, capable of thermostating samples for automated sample injection
   c. Flame ionization detector
   d. Electronic integrator or chromatography data station

2. Chromatography column, 30 m x 0.25 mm i.d. Stationary phase: methyl polysiloxane (5% phenyl, "DB-5"), film thickness 1 µm

3. Balance, readable to 0.001 g

4. Headspace vials, nominally 20 mL size with aluminum- or PTFE-coated silicone rubber septa (rated for over 100ºC), seals and crimping pliers

5. Volumetric pipets to deliver between 25-500 µL and 1-10 mL

6. Volumetric flasks, 100 mL size, Class A

7. Electric ovens, maintained at 60° and 110°C (see Note 1)

8. Gas tight syringe, 100 and 1000 µL

9. Gloves, suitable for handling hot syringes (see Note 1)

B. Reagents:

1. High purity mixture of hexanes used in industrial extraction. See Note 2.

2. Toluene and ethyl acetate, ACS grade, must not contain impurities that would co-elute with components of technical hexanes.

3. Compressed gases:
   a. Helium, minimum purity 99.99 mol%, dried and containing less than 10 mg O_2/kg
   b. Hydrogen, minimum purity 99.95 mol%

c. Air, dry and hydrocarbon free

C. Preparation of Sample:

1. To prevent loss of solvent from samples, store them in glass containers at –20°C until they can be analyzed. Prepare samples in duplicate. Accurately weigh 5.0 ± 0.1 g of sample into a headspace vial. Add 2.5 mL of the ethyl acetate solution (see step 1 below) into the sample and seal with a crimped aluminum cap and septum. The sample is ready for GC analysis.

D. Procedure (See Appendix for note on standard additions method of calibration)

1. Prepare an ethyl acetate internal standard solution. Weigh about 200 mg (record to ± 1 mg) of ethyl acetate into a 100.0 ml volumetric flask and dilute to volume with water. Mix well to dissolve completely. Dilute 0.500 mL of this solution to 100.0 mL in water. Calculate the concentration of ethyl acetate in µg/mL by dividing the milligrams of ethyl acetate by 20.0. The calculated concentration of ethyl acetate will be approximately 10 µg/mL. Record this value.

2. Prepare a technical hexane stock standard solution. See Note 2. Weigh about 500 mg (record to ± 1 mg) of technical hexanes into a 100 mL volumetric flask and dilute to volume with toluene. Mix well. Dilute 2.00 mL of this solution to 100.0 mL in toluene (or alternatively, 1.00 mL to 50.0 mL). Calculate the concentration of total hexanes in µg/mL by dividing the milligrams of hexanes by 5.00. The calculated concentration of total hexanes will be approximately 100 µg/mL. Record this value.

3. Prepare the following series of standards containing 0, 1, 2, 5 and 10 µg /g paprika and an internal standard concentration of 5 µg /g of paprika. Make sure additions are done in the following order. Add 5.0 ± 0.1 g of <u>hexane- free</u> ground paprika to five separate headspace vials. Next pipet 0, 50, 100, 250 and 500 µL of the stock total hexane solution onto the paprika in the separate headspace vials in a manner that is as consistent as possible. Next pipet 2.5 mL of stock ethyl acetate internal standard solution into each headspace vial. Seal each vial with a crimped aluminum cap and septum. These standards are ready for GC analysis.

4. To calculate the concentration of hexanes in each standard (µg/g paprika), multiply the concentration of the hexane stock standard (step 2) by 0, 0.01, 0.02, 0.05, 0.1, respectively. To calculate the concentration (µg/g paprika) of ethyl acetate internal standard in the standards, multiply the concentration of the ethyl acetate stock standard (step 1) by 0.5. Record these values.

5. Automated headspace conditions:

a.	Sampling thermostatting temperature:	110°C
b.	Sample thermostatting time:	90 min (180 min better)
c.	Needle temperature:	105°C
d.	Transfer line temperature:	115°C
e.	GC cycle time:	30 min
f.	Pressurization time:	1 min
g.	Injection time:	0.06 min
h.	Withdraw time:	0.2 min

6. Gas chromatographic conditions:

a.	Injector temperature:	180 °C
b.	Detector temperature:	250 °C
c.	Column temperature:	40 °C
d.	Carrier gas:	Helium
e.	Column flow rate:	0.8 mL/min
f.	Make-up gas:	Helium
g.	Make-up gas flow rate:	30 mL/min
h.	Split flow:	15 mL/min
i.	Split ratio:	15/0.8 = 18.75
j.	Detector gases	
	1. Hydrogen:	30 mL/min
	2. Air:	300 mL/min

E. Calculations:

1. Most paprika samples will have detectable peaks in the hexane region of the chromatogram. Hence, the summed relative peak areas (A_{hex}/A_{EtOAc}) in the spiked standards must be corrected by subtraction of the background relative area found in the 0 µg/g standard. After correcting the relative areas, plot the relative area times internal standard concentration vs. the concentration of hexanes to obtain the best fit line for the following equation (force intercept through zero). See Notes 3 & 4 for comments on calibration range and typical chromatography results.

$$\left(\frac{A_{hexanes}}{A_{EtOAc}} - \frac{A'_{hex}}{A'_{EtOAc}} \right) C_{EtOAc} = m\, C_{hexanes}$$

where A = areas of hexane peaks and ethyl acetate in standard samples
A' = areas of hexane interference peaks and ethyl acetate in matrix sample
C = concentrations of total hexanes and ethyl acetate, respectively, in the spiked standards (in µg/g paprika)

m = slope of the regression line

2. Use the calibration slope (m) determined above to calculate the sample results with the following equation.

$$C_{hexanes} = \left(\frac{A_{hexanes}}{A_{EtOAc}}\right)\left(\frac{C_{EtOAc}}{m}\right)$$

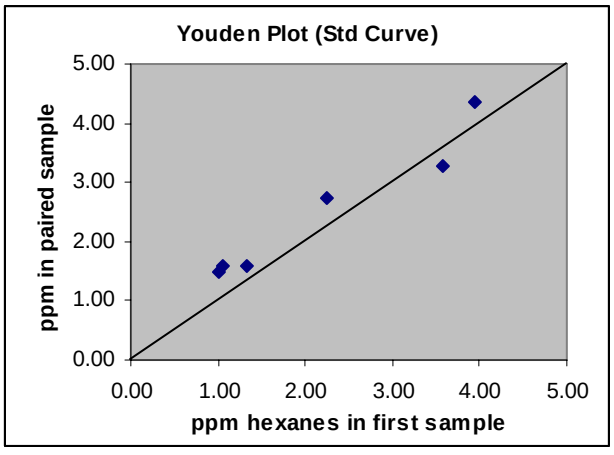
where C =concentration of hexanes or ethyl acetate, respectively, in µg/g paprika
A = chromatographic areas of hexane peaks and ethyl acetate, respectively
m = slope of the regression line

F. Statistics:

1. Previous collaborative studies have demonstrated relative standard deviation (RSD) in this method of between 5-10% for extracted rapeseed and soybean with 300-500 ppm hexanes.(see ref. 1) Method reproducibility at low levels was determined in an ASTA collaborative study of paprika samples spiked at nominal levels of 1.5 and 3.5 ppm (three participating labs, Jan 2008).(see ref. 2)

Mean (ug/g)	1.3 ppm	3.4 ppm
Std dev (all results)	0.3 ppm	0.8 ppm
RSD	24%	26%

2. High and low level results were consistent and well distributed for all labs in double blind pair assessment.



3. Minor paprika peaks that coeluted with hexanes under these chromatography conditions typically amounted to an equivalent quantity of about 1 ppm hexanes. These were confirmed to be different than hexanes by mass spectrometric

detection. Based upon these findings, a lower quantitation limit of 2 ppm hexanes was recommended.

4. Positive results between 2-5 ppm hexanes must be confirmed by the presence of at least three of the individual hexane isomers. If three or more isomers cannot be found, then reporting a positive result at these low levels would require additional analysis that is beyond the scope of this method (e.g., mass spectrometric detection).

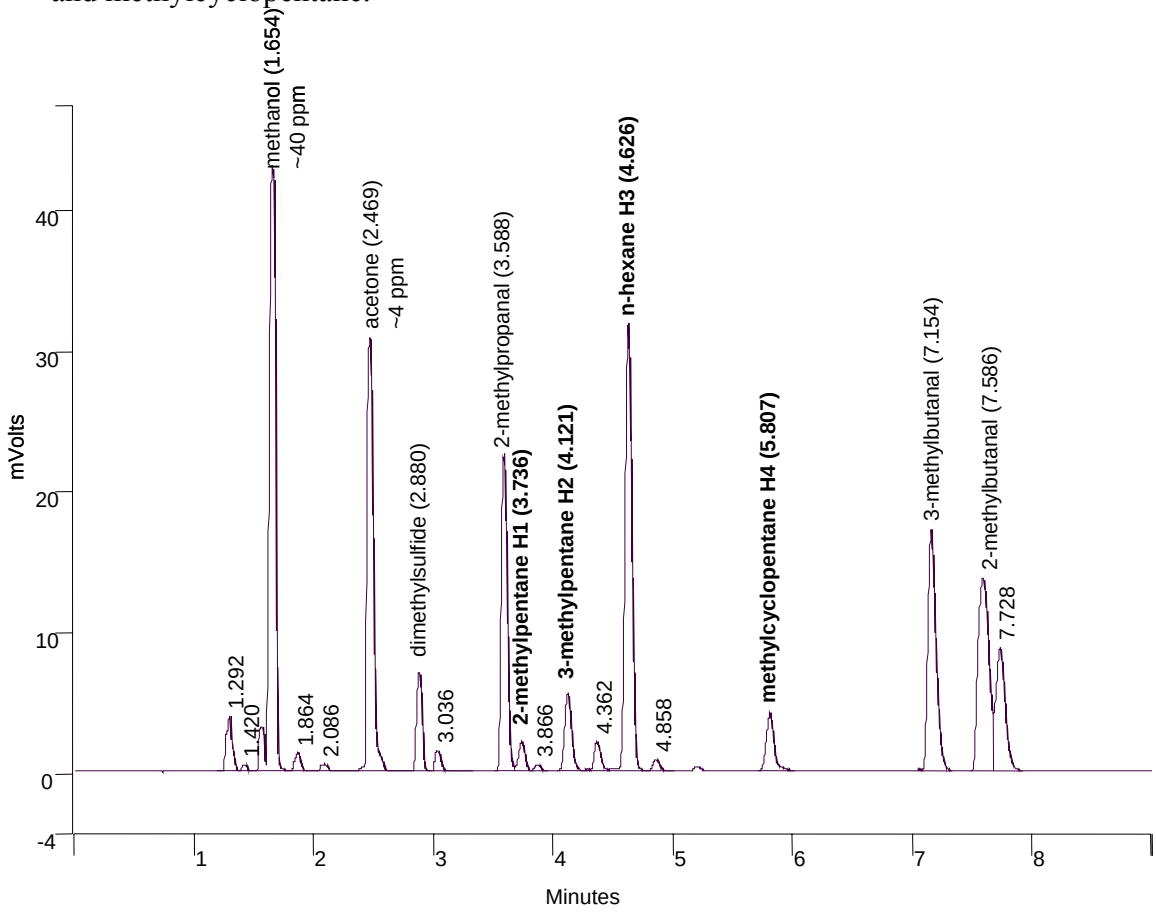
G. Notes:

1. If the sample is injected manually, follow these steps. Place the sample vial in the oven at 110 °C for 90 minutes (when running standard solutions keep them in the oven for 10 minutes). Make sure that all samples and standards remain in the oven for the same length of time. Warm the gas tight syringe to 60 °C. Remove the vial from the oven and allow cooling for 2 minutes. Agitate the sample by inverting. With the warmed gas tight syringe, take exactly 0.5 mL of headspace and inject immediately into the gas chromatograph. Gloves should be used when handling the hot syringe.

2. The hydrocarbons that are typically found in technical grade hexane are 2-methylpentane, 3-methylpentane, methylcyclopentane, cyclohexane, n-hexane, etc. For example, hexanes mixture (98.5% min purity, ACS reagent grade) is available from Sigma-Aldrich. For purpose of measurement by volume, the density of technical hexane is assumed to be 0.67 g/mL. The method can be calibrated optionally with individual hexane isomers such as 2-methylpentane 3-methylpentane, n-hexane, and methylcyclopentane. Each component should be over 95% pure. Accurately weigh and combine each isomer into a single stock solution that contains approximately 100 µg/mL of total hexanes. Calculate the final concentration of each component in the final standards. Calibrations should be appropriately based upon independent peak areas and component concentrations for each component.

3. Detector response may be non-linear for concentrations that exceed 10 ppm. Measured values that exceed the calibrated range should be reported as "> 10 ppm."

4. Chromatogram of paprika containing 14 ppm hexanes. Hexane components are annotated in boldface type. Ethyl acetate (not shown) elutes between n-hexane and methylcyclopentane.



H. References:

1. This method was initially adapted from AOCS Official Method Ba14-87 (1997). Collaborative study results published in *Pure Appl. Chem.* **1987,** *59(10)*, 1407.
2. Williams, D.; Burroughs, L.; and Oliver, N. "Summary of Collaborative Round #2 for Revision of ASTA Method 27.0," report to the ASTA Technical Committee, Jan 7, 2008.

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Appendix. Alternative Method of Standard Addition

Note: During preparations for collaborative trial, an alternative method of quantitation by standard addition was proposed. In the final collaborative trial of this option, the pooled standard deviation for results between 1-3 ppm was ±1.0 ppm. In the absence of additional intralaboratory validation, results between 1-3 ppm by standard additions should be confirmed by standard curve.

Quantitation by standard additions is performed as follows.

1. No preparation of separate calibration standards (Section D) is necessary.
2. For each unknown sample (Section C), prepare an equivalent sample of the same material but add 250 µL of the stock hexane standard solution (Step D.2) just before adding 2.5 ml of ethyl acetate internal standard solution (Step D.1).
3. Run unknowns and spiked unknowns in duplicate.
4. Final results are calculated as follows. Linear response is assumed.

$$C_x = \left(\frac{A_x}{A_{spike} - A_x} \right) C_{spike}$$

Where:

C_x = concentration of hexanes in the unknown sample (ppm or µg/g)

A_x = area of all hexane peaks in the unknown sample divided by area of the ethyl acetate peak

A_{spike} = area of all hexane peaks in the spiked sample divided by area of the ethyl acetate peak

C_{spike} = concentration of the total hexanes added to the spiked sample (ppm or µg/g). This is equal to the concentration of the stock hexane standard solution (Step D.2) times 0.05.