United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science

SOP No: **CLG-BSP.01**		Page 1 of 15
Title: **Determination of Benzoic Acid, Sorbic Acid, and Methyl, Ethyl, Propyl, and Butyl Parabens by HPLC**		
Revision: .01	Replaces: Guidebook Method, May 1993	Effective: 11-1-04

Contents

A. INTRODUCTION

1. Theory

The antimicrobial preservatives benzoic acid, sorbic acid, and parabens are not permitted in fresh meat products or in seasoning mixtures used in the formulation of these products.

Ten grams of thoroughly comminuted meat samples are extracted with 70 mL ethanol. After filtration, extracts are analyzed by reverse phase liquid chromatography, using a 254 or 280 nm UV detector.

2. Applicability

This method is applicable for meat products at levels listed in I. 1.

B. EQUIPMENT

Note: Equivalent apparatus and instrumentation may be substituted for any of the following.

1. Apparatus

a. Magnetic stirrer - Variable-speed, Nuova 7 Thermolyne, Sybron Corp., Dubuque, IA.

b. Stirring bar - Plastic-coated, magnetic, 1" long.

c. Filter paper - Reeve Angel grade 802, Sargent-Welch Scientific Co., Skokie, IL, fast-filtering and medium porosity (18.5 cm).

d. Solvent clarification kit - With Durapore filters (0.4 µm, 47 mm), but without pump and filtering flask, Waters Associates, Milford, MA.

e. Evaporator - N-Evap, Model III, Organomation Associates, Inc., South Berlin, MA.

f. Sample filtration apparatus - Stainless steel Swinney filter holder, with 0.45 - 0.50 µm, Fluoropore, Nylon 66 or PTFE filters, Millipore Corp., Bedford, MA.

g. pH meter - Fisher Accumet, Model 210, Fisher Scientific Co., Pittsburgh, PA.

2. Instrumentation

a. Liquid chromatograph

i. Waters Alliance LC system consisting of a 2690 Separations Module.

ii. Model 996 PhotoDiode Array Detector.

iii. Integrator or integrating software.

iv. Column - 15cm x 4.6mm ID, Luna C-18 (2), 5 µm spherical silica (Phenomenex).

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for the following.

1. Reagents

a. Anhydrous ethanol (formula 3A) - J.T. Baker.

b. Methanol - Distilled in glass, UV grade, Burdick and Jackson laboratories Inc., Muskegon, MI.

c. Glacial acetic acid - HPLC grade, J.T. Baker.

d. Deionized water (DI) - Low organic content and specific resistance ≥ 10 megaohms/cm to avoid problems with extraneous interferences. Milli-Q Water Purification System, Millipore Corp.

e. Ammonium acetate - HPLC grade, Fisher Scientific Co.

f. Phosphoric acid - reagent grade, Mallinkrodt, #MK2796.

g. Potassium Phosphate monobasic (K_2PO_4) - analytical reagent grade, Mallinkrodt, #MK7100.

h. Acetonitrile - distilled in glass, Burdick & Jackson, Cat # AH015-4.

2. Solutions

a. LC mobile phase

i. Mobile Phase A (Buffer solution):

Add approximately 200 - 300 mL of deionized water to a 1 L volumetric flask. Add 15 mL acetic acid to the flask and mix. Weigh 15 g of ammonium acetate into a beaker, and dissolve in a 100 mL of deionized water. Transfer aqueous ammonium acetate solution to the 1L flask containing the aqueous acetic acid. Mix and bring to volume with deionized water. Mix well and filter.

ii. Mobile Phase B (Methanol):

Sparge or degas as necessary.

iii. 70% Ethanol:

Add 700 mL of anhydrous ethanol to a graduated cylinder, and add DI water to adjust volume to 950 mL. Mix well, and transfer to storage container.

iv. 80% ethanol:

Add 800 mL of anhydrous ethanol to a graduated cylinder, and add DI water to adjust volume to 950 mL. Mix well and transfer to storage container.

D. STANDARDS

1. Source
   a. Propyl Paraben - USP, #57700.
   b. Potassium Sorbate - Supelco, #4-7848.
   c. Benzoic Acid - Supelco, #4-7849.
   d. Methyl Paraben - Supelco, #4-7889.
   e. Butyl Paraben - Supelco, #4-7891.
   f. Ethyl Paraben - Supelco, #20245838.

2. Preparation of Standards
   a. Stock standards (4.0 mg/mL benzoic acid, 0.16 mg/mL sorbic acid, and 0.40 mg/mL each methyl, ethyl, propyl, and butyl parabens):
      i. Weigh 400.0 mg benzoic acid and 16.0 mg of sorbic acid into a 100 mL volumetric flask. Add approximately 50 mL 70% ethanol to dissolve, and dilute to volume with 70% ethanol.
      ii. Weigh 40.0 mg each of methyl, ethyl, propyl, and butyl parabens into a 100 mL volumetric flask. Add approximately 50 mL 70% ethanol to dissolve, and dilute to volume with 70% ethanol.

b. HPLC standards

Combine 0.0, 0.25, 0.50 and 1.0 mL of each stock standard into 50 mL volumetric flasks and dilute to volume with 70% ethanol. These dilutions prepare standard solutions at the following concentrations:

	STD 0 (µg/mL)	STD 1 (µg/mL)	STD 2 (µg/mL)	STD 3 (µg/mL)
Benzoic acid	0.0	20.0	40.0	80.0
Sorbic acid	0.0	0.80	1.60	3.20
Methyl Paraben	0.0	2.00	4.00	8.00
Ethyl Paraben	0.0	2.00	4.00	8.00
Propyl Paraben	0.0	2.00	4.00	8.00
Butyl Paraben	0.0	2.00	4.00	8.00

3. Storage Conditions

All standards are to be kept in screw cap glass bottles and refrigerated.

4. Stability

Working standards: 12 months.

E. SAMPLE PREPARATION

Process the sample using a commercial type food processor until a homogeneous mixture is obtained.

F. ANALYTICAL PROCEDURE

1. Determination

a. Weigh 10 grams of sample into a 50 mL polypropylene centrifuge tube. Select a "blank" meat sample for a negative control and fortification.

Fortify the recovery with 1.0 mL of each stock standard (D.2.a.i-ii.): 400 ppm benzoic acid, 16 ppm sorbic acid, and 40 ppm of each paraben.

b. Add 35 mL of 80% EtOH, cap tube, and shake or tissuemize to break up sample. Place tube(s) on a shaker for 10 min at low speed and the centrifuge samples at 2000 - 2500 rpm for 5 min.

c. Filter the solution through filter paper into a 100 mL volumetric flask. Repeat steps b - e combining the extraction solutions in the volumetric flask. Rinse the tube(s) with 20 mL of 80% EtOH and add to the filter paper. Adjust the volume to 100 mL with 70% EtOH, stopper, and mix well.

d. Filter 4 - 5 mL through a 0.45 - 0.5 µm syringe filter or LC centrifuge filtering systems.

e. Transfer an appropriate amount of filtrate to an autosampler vial and/or insert for analysis.

f. Save remaining filtrate for GC/MS confirmatory analysis.

2. Instrumental Settings

Note: Instrument conditions and retention times will be dependent on the equipment and the column used for analysis.

a. Mobile phase gradient profile :

A: 1.5% acetic acid + 1.5% ammonium acetate in DI water.

B: 100% methanol.

Time	**Flow (mL/min)**	**A (%)**	**B (%)**
Initial	1.0	90	10
25.0	1.0	30	70
28.0	1.0	30	70
31.0	1.0	90	10
40.0	1.0	90	10

b. Inject 10 - 20 µL portions each of sample extracts and mixed standards and program solvent as described.

c. Typical injection sequence:

i. Standards

ii. Recovery

iii. Blank

iv. Samples

v. Standard(s)

d. Under conditions used, retention times (min) of all 6 preservatives are as follows:

Benzoic acid	11.9
Sorbic acid	14.3
Methyl paraben	16.7
Ethyl paraben	20.6
Propyl paraben	24.1
Butyl paraben	27.0

3. Chromatograms

See Section K for chromatograms.

4. Optional second column identification

Note: If a presumptive positive is found during initial analysis a second column identification may be used before proceeding to the confirmatory method.

a. Column: Zorbax SB-Phenyl, 4.6 x 150mm, 3.5u, Agilent # 863953-912

b. Mobile Phase: 0.05M KH_2PO_4 + 0.1% H_3PO_4 in 25:75 acetonitrile:water

Weigh 6.8 grams of KH_2PO_4 and transfer to a 1 liter volumetric flask. Add 500 - 600 mL of deionized water. Pipet 1 mL of conc. phosphoric acid into the flask and mix well to dissolve. Add 250 mL of acetonitrile, mix well, and let solution come to room temperature. Dilute to volume with deionized water, mix well, and transfer to storage bottle.

c. Instrument settings

(a) Linear gradient - 40 ^{o}C.

Time (min)	Flow	%A
Initial	1.0 ml/min	100
8.00	1.0	100
9.00	1.5	100
23.00	1.5	100
24.00	1.0	100

(b) Inject 10 - 20 µL portions each of sample extracts and mixed standards and program solvent as described.

(c) Under conditions used, retention times (min) of all 6 preservatives are as

follows:

Compound	Retention Time (min)
Sorbic acid	4.8
Benzoic acid	5.0
Methyl paraben	6.0
Ethyl paraben	9.0
Propyl paraben	13.4
Butyl paraben	22.0

G. CALCULATIONS

Calculate concentration of each preservative in sample as follows:

Using peak areas or peak heights and concentrations of standards, construct linear standard curve for each compound based on formula

$y = mx + b$,

where x is peak area or height,

y is concentration (ppm),

m is slope, and

b is the y intercept.

Calculate recovery of fortified sample and sample results.

H. SAFETY PRECAUTIONS AND INFORMATION

1. Required Protective Equipment - Safety glasses, appropriate gloves, and lab coat.

2. Hazards

Reagents	*Hazard*	*Recommended Safe Procedures*
Ethanol, Methanol, Acetonitrile	These solvents may be flammable and may produce toxic effects to skin, eyes, and the respiratory system.	Keep in well closed containers in a cool place and away from fire. Use it in well ventilated hood.
Acetic acid, Phosphoric acid	Caustic: may cause irreversible skin and eye damage.	Wear protective equipment. Avoid contact with skin.

3. Disposal Procedures

Reagents	*Hazard*	*Recommended Safe Procedures*
Ethanol, Methanol, Acetonitrile	See above	Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Acetic acid, Phosphoric acid	See above	Collect waste in a tightly sealed container and store away from non-compatibles in a cool, well ventilated, acid liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

Analyte	*Analytical Range (ppm)*	*Acceptable Recovery*
Benzoic Acid	200 - 800	70 - 105
Sorbic Acid	8 - 32	70 - 105
Methyl Paraben	20 - 80	70 - 105
Ethyl Paraben	20 - 80	70 - 105
Propyl Paraben	20 - 80	70 - 105
Butyl Paraben	20 - 80	70 - 105

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	Sample weight	10 g $\pm$ 0.5 g
b.	Final volume	100 mL $\pm$ 0.08 mL

3. Readiness To Perform (FSIS Training Plan)

   a. Familiarization

      i. Phase I: Standards- Duplicate standard curves on each of 3 consecutive days, which will include the following:

	STD 0 (µg/ml)	STD 1 (µg/ml)	STD 2 (µg/ml)	STD 3 (µg/ml)
Benzoic acid	0.0	20.0	40.0	80.0
Sorbic acid	0.0	0.80	1.60	3.20
Methyl Paraben	0.0	2.00	4.00	8.00
Ethyl Paraben	0.0	2.00	4.00	8.00
Propyl Paraben	0.0	2.00	4.00	8.00
Butyl Paraben	0.0	2.00	4.00	8.00

ii. Phase II: Fortified samples - At least 3 replicates of all analytes fortified between the levels listed in I.1. Analytical Range.

Note: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

(a) 6 samples fortified at a level specified in I.1.

(b) Report analytical findings to the Quality Assurance Manager (QAM).

(c) Letter from QAM is required to commence official analysis.

b. Acceptability criteria.

Refer to Section I.1 above.

4. Intralaboratory Check Samples

Refer to Section I.1 above.

a. System, minimum contents.

i. Frequency:

(a) 1 sample weekly per analyst as samples analyzed.

(b) Random replicates may be chosen by the supervisor or his/her designee.

(c) Records are to be maintained.

b. Acceptability criteria.

Refer to Section I.1 above.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrix: Meat and meat products.

b. Sample storage: 24 months frozen or 1 - 3 weeks refrigerated.

e. Condition of sample upon receipt: Unspoiled and sealed from air.

6. Sample Set

With each set of official samples to be analyzed, process

a. Negative control,

b. Fortified control, and

c. Samples

7. Sensitivity

a. Minimum proficiency level (MPL): See below.

Compound	MPL (ppm)
Benzoic acid	200
Sorbic acid	8
Methyl Paraben	20
Ethyl Paraben	20
Propyl Paraben	20
Butyl Paraben	20

J. WORKSHEET

An example of a worksheet for Benzoates is on the following page.

Benzoates

Analyst: Balance # Ethyl Acetate #
Started: LC System # 70% ethanol #
Completed:

Standard Curve Linear Regression
BSPstd0- ppm ht. or area m=
BSPstd1- b=
BSPstd2- r=
BSPstd3- s=

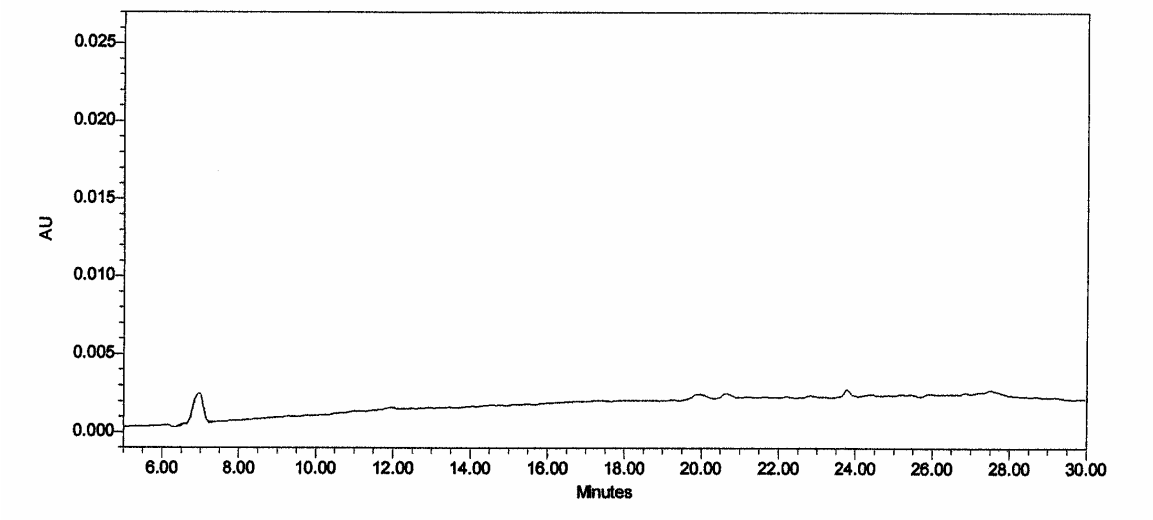
Sample	Sample Weight	Final Volume	Height or Area	ppm Benzoic Acid	ppm Sodium Benzoate	% Recovery	GC/MS Confirmation?
Blank							
Recovery							

Comments:

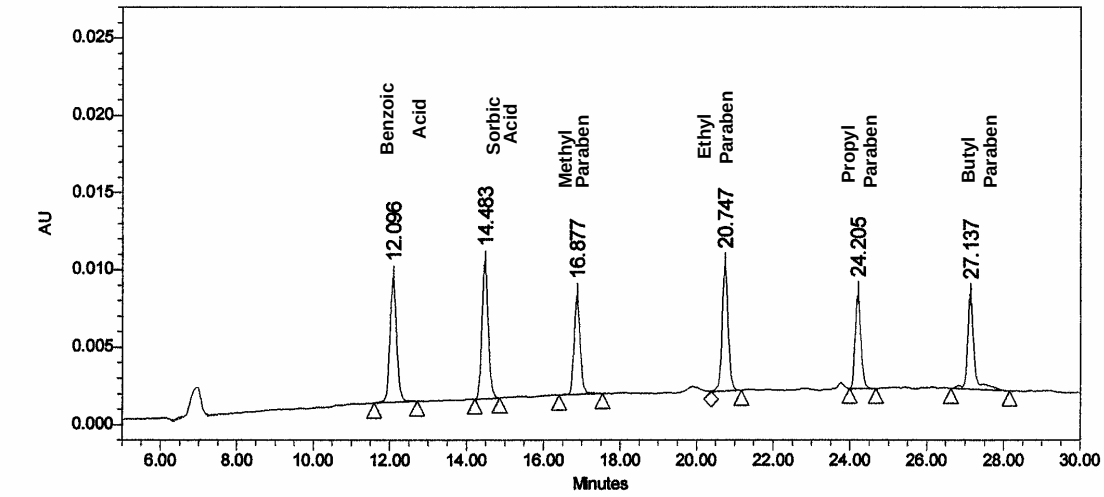
K. APPENDIX

1. Chromatograms

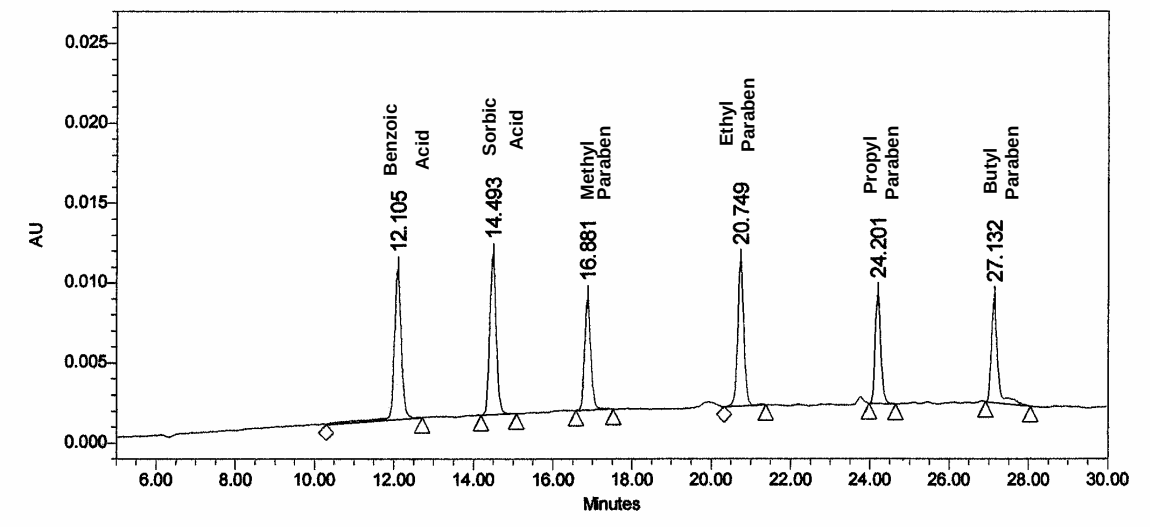
   a. Blank Ground Beef



   b. Ground Beef Recovery fortified at Std 2 Level: Benzoic Acid (40 ppm), Sorbic Acid (1.60 ppm), Methyl Paraben (4.00 ppm), Ethyl Paraben (4.00 ppm), Propyl Paraben (4.00 ppm), and Butyl Paraben (4.00 ppm).



c. Standard at STD 2 Level. (See above chromatogram for concentrations).



2. Reference:

Ali, M. Sher. J. Assoc. Off. Anal. Chem., 1985, 68. 488-492.

Approved by:	**Date Approved:**
Bill Koscinski	10-6-04
Gina McLeroy	10-6-04
Jess Rajan	10-7-04
Charles Pixley	10-7-04
Phyllis Sparling	10-7-04

Approval signatures on file.