July 18, 2017

#723

Food and Drug Administration
Center for Food Safety & Applied Nutrition
Office of Food Additive Safety (HFS-255)
5100 Paint Branch Parkway
College Park, MD 20740-3835

Attention: Mr. Richard Bonnette
Re: GRAS Notification – Free N Clear™

Dear Richard:

On behalf of Marvel Technologies USA, LLC, we are submitting for FDA review our GRAS notification and the enclosed CD, containing a GRAS notification for Free N Clear™. An Expert Panel of qualified persons was assembled to assess the composite safety information of the subject substance with the intended use as a food processing aid. The attached documentation contains the specific information that addresses the safe human food uses for the subject notified substance as discussed in the GRAS guidance document.

If additional information or clarification is needed as you and your colleagues proceed with the review, please feel free to contact me via telephone or email.

We look forward to your feedback.

Sincerely,

(b) (6)

Jack A. Wheeler
CEO
Marvel Technologies USA, LLC
1224 Columbia Avenue, Suite 104
Franklin, Tennessee 37064
615-261-8084
marvelfnc@gmail.com

DOSSIER IN SUPPORT OF THE GENERALLY RECOGNIZED AS SAFE (GRAS) STATUS OF BENZALKONIUM CHLORIDE AS A COMPONENT OF FREE N CLEAR™ AS A PROCESSING AID FOR LETTUCE AND CARROTS

July 18, 2017

Panel Members

Douglas L. Archer, Ph.D.

Ed Carmines, Ph.D. D.

Reid Patterson, D.V.M., Ph.D.

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GRAS EXEMPTION CLAIM

No Claim of Exemption Requirement since this notification is GRAS

Signed:

(b) (6)

Jack A. Wheeler
CEO
Marvel Technologies USA, LLC
1224 Columbia Avenue, Suite 104
Franklin, Tennessee 37064

1) Name and Address of Notifier

Marvel Technologies USA, LLC
1224 Columbia Avenue, Suite 104
Franklin, Tennessee 37064

2) CERTIFICATION

The undersigned author of this document – a dossier in support of the GRAS determination for the use of benzalkonium chloride (BAC) as a component of Free N Clear™ (FNC), when diluted to a 2% use solution to be used as a processing aid for lettuce and carrots – hereby certify that, to the best of his knowledge and belief, this document is a complete and balanced representation of all available information, favorable as well as unfavorable, known by the author to be relevant to evaluation of the substance described herein.

(b) (6)

Jack A. Wheeler Date: 7/18/2017

COMMON NAME OF NOTIFIED SUBSTANCE, AND IDENTITY

The common name of benzalkonium chloride, for the purposes of this GRAS Notification has been defined as: **Benzalkonium Chloride (70% composed of C12 and C14)**

Detailed Information about the Identity of the Notified Substance

Benzalkonium chloride (BAC – Figure 1) a member of the class of quaternary ammonium compounds. It is a mixture of alkylbenzyl dimethylammonium chlorides of the general formula [$C_6H_5CH2N(C^u3)_{2}$-•R]C1, with the R group including noctyl (n-C_8H17; C8) and extending through higher homologues. The average molecular weight of BAC is 360[2]. BAC has been assigned a number of different CAS numbers, depending on the numbers of carbons in the R groups. In USP-BAC (CAS No. 8001-54-5), the R groups of n-$C_{12}H_{25}$, n-$C_{14}H_{29}$ and n-$C_{16}H_{33}$ (C12, C14 and C16) predominate, with the total amount of the C12 and C14 homologs not less than 70.0% of the total alkylbenzyl dimethylammonium content.

(*BAC 50 was selected to blend with an aqueous solution; BAC 80 typically requires alcohol for blending, ingredients are based on weight measures, not percent.)

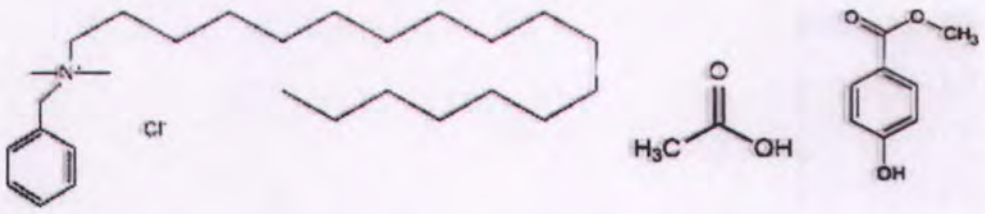

Figure 1: Structures of benzaikonium chloride (BAC), acetic acid, and methyl paraben, respectively.

CONDITIONS OF USE

Benzalkonium Chloride will be used as a component of Free N Clear™ (FNC) which is used as a processing aid for lettuce and carrots when diluted to a 2% use solution (2% FNC solution with a 5 minute exposure). Such use could potentially increase the daily dietary (aggregate) exposure to BAC to 0.0530 mg/kg bw/day, maximum of 200 ppm. EPA safe daily level is 44 ppm; Kansas State University testing shows residuals < 10 ppm of BAC and < 5 ppm of Methyl Paraben. EPA safe daily intake for BAC is 44 ppm. As listed, residual BAC of Free N Clear is <10 ppm.

BASIS OF GRAS DETERMINATION

Pursuant to 21 CFR § 170.3, benzalkonium chloride, as a component of Free N Clear™ (FNC), has been determined GRAS by scientific procedures for its intended conditions of use. The safety of benzalkonium studies (OEDC Guideline No. 471 and 474) on 2% FNC use dilution solution and acute, subcronic, chronic and reproductive developmental toxicity studies on benzalkonium chloride. This determination is based on the views of experts who are qualified by scientific training and experience to evaluate the safety of substances used as ingredients in food.

AVAILABILITY OF INFORMATION

The data and information that serve as a basis for this GRAS determination are available for FDA review and copying at reasonable times at:

MARVEL TECHNOLOGIES USA, LLC, 1224 COLUMBIA AVENUE SUITE 104, FRANKLIN, TN 37064

Alternatively, data and information that serve as a basis for this GRAS determination may be sent to FDA upon request.

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Part 2

Accepted Identity Specifications for BAC and Methyl Paraben

Benzalkonium Chloride CAS No. 8001-54-5
Methyl Paraben CAS No. 99-76-3

A. Manufacturing Processes for BAC and Methyl Paraben

Scientific and Patent Literature
Patent Number 8,268,337 ; issued September of 2012

FNC is manufactured at ambient temperature by combining food grade glacial acetic acid, food grade methyl paraben and USP grade BAC 50% solution, chosen for aqueous blend, in precise amounts and in a defined sequence. FNC may be mixed in either high density polyethylene (HDPE) or stainless steel tubs. For a batch with a total volume of 6.3 gallons, methyl paraben (118 g, FCC grade) is added to glacial acetic acid (236 g) and stirred until dissolved. USP-BAC solution (118 g) is then stirred into the mixture, until dissolved. Water from the municipal water supply of Memphis, TN is added until a final volume of 6.3 gallons is reached. Larger batches (up to 300 gallons) may be prepared, by proportionately increasing the amounts of methyl paraben, glacial acetic acid, USP-BAC solution and water used for a 6.3 gallon batch (see above) to the desired volume. The water supply for Memphis, TN meets or exceeds standards set by the Environmental Protection Agency (EPA) for possible contaminants. The final product, FNC is sold in 55 gallon plastic drums. 275 gallon plastic totes or metal rail cars (with liners) for up to 20,000 gallon quantities. The recommended storage temperature for FNC is room temperature. The concentrated FNC will be diluted 1:50 by the user (for a 2% solution), prior to application to carrots or lettuce in processing facilities. Users will be directed to submerge carrots or lettuce in the 2% FNC solution (as incorporated into the wash solution) for five minutes and change solutions daily or when the lot number changes (whichever is sooner). Users will be directed to divert used 2% FNC to water treatment facilities, as per local requirements. The temperature of the product (carrots and lettuce) does not effect the solution; 40°F to room temperature is the suggested range of temperature for storage.

Product Specifications and Supporting Methods

An analysis was conducted by Microbac Laboratories Inc., Wilson, NC to determine the concentration of BAC that would remain on carrots or lettuce after use of 2% FNC as a processing aid. Two heads of organic, romaine lettuce and a bag of organic baby carrots purchased from Harris Teeter, Wilson, NC were used in the test. FNC (lot 214) was diluted 1:50 with tap water to make approximately 325 ml of a 2% solution. An aliquot of the 2% FNC solution was prepared by filtering an aliquot through a polyvinylidene difluoride (PVDF) filter into an HPLC auto sampler vial. The heads of lettuce were placed into two separate gallon-size Ziploc bags. Carrots were

allocated into two different gallon-size Ziploc bags (12/bag). The 2% FNC solution (75 ml) was then placed into each one of the bags. Each sample was shaken for approximately 30 seconds and was allowed to sit at room temperature for approximately three hours.

Table 4. Summary of BAC analyses on carrots and lettuce

Sample	BAC (ppm)	Average BAC (ppm)
BAC 2% solution	*75.993*	*75.993*
Carrot (Sample 1)	*13.167*	*13.163*
Carrot (Sample 2)	*13.160*	
Lettuce (Sample 1)	*22.109*	*18.866*
Lettuce (Sample 2)	*15.624*	

BAC = benzalkonium chloride ; ppm = parts per million

Common Name of Notified Substance

The common name of benzalkonium chloride, for the purpose of this GRAS Notification has been defined as: **Benzalkonium Chloride (70% composed of C12 and C14)**

Chemistry of BAC and Methyl Paraben

B. Description and Specifications

The physical and chemical properties for FNC are provided in Table 2. FNC is a clear, colorless liquid with a slight vinegar odor. FNC is readily soluble in cold and hot water and very soluble in alcohol. The BAC component of FNC is a quaternary salt that has a low vapor pressure (3.53 x 10"' mm Hg) [6]; therefore, it will not volatize under expected storage conditions.

Table 2. Physical and chemical properties of Free N Clear™ Concentrate*

Characteristic	Value
Physical state	*Liquid*
Color	*Colorless*
Appearance	*Clear*
Odor	*Slight vinegar*
Specific Gravity	*0.Q8 - i .049*
Solubility in water	*Complete*
pH	*3.06 - 3.45*
Freezing point	*30.2°F*
Boiling Point	*212°F*

*information obtained from J. Wheeler, Marvel Technologies, personal communication, December 14. 2012.

Permissible concentrations of the components BAC, acetic acid and methyl paraben in FNC are shown in Table 3. Measured concentrations of BAC. methyl paraben and acetic acid in 1:50 dilutions (2% solutions) of lots of FNC meeting specifications (lots 44 and 214) averaged 74 ppm (74 mg/L or 0.0074%), 80.5 ppm (80.5 mg/L or 0.00805%) and 239 ppm (239 mg/L or 0.0239%), respectively.

B.1. Background

Table 3 Specifications for Free N Clear™ Concentrate

			Batch Analysis Results (N=2*)	
Analysis	Method	Specification	Range	Average
Benzalkonium chloride (BAC)	*Microbac*	*2700 ± 200 ppm*	*2638 (Lot 214) - 2777 (Lot 44)*	*2708*
Acetic acid	*NREL/TP-510-42623*	*8200 ± 1000 ppm*	*73 83 (Lot 44) - 9011 (Lot 214)*	*8197*
Methyl paraben	*Modified Agilient*	*2900 ± 215 ppm*	*2703 (Lot 214) 3029 (Lot 44)*	*2866*

* Lots 44 (prepared 11/2011) and 214 (prepared 11/2012) were analyzed on January 11, 2013 ; ppm = parts *per m*illion

B.1.2 Background Information on Chemistry

The methods used to detect BAC, acetic acid and methyl paraben for verification of the components within FNC are as follows:

BAC: Modified USP 34/29 methodology. The mobile phase for the high performance liquid chromatograph (HPLC) was a 50:50 mixture of methanol: 7.5 mM K_2HPO_4, pH 3 (68:32) and methanol. The flow rate of the mobile phase was 1.0 rnl/min, the injection volume was 5 µl, and the UV detector was set to 260 nm. A four point curve from 30 - 600 ppm was used for calibration. A coefficient of determination of 0.9996 was obtained and percent recovery was 99.9 - 101.7%, demonstrating that the method accurately detected BAC.

Acetic Acid: NREL/TP-510-42623. A four point curve from 5 – 100 ppm was used for calibration. A coefficient of determination of 0.9999 was obtained and percent recovery was 99.2 - 99.8%, demonstrating that the method accurately detected acetic acid.

Methyl Paraben: Modified Agilent method. The method was modified by use of a larger column (4.6 mm x 250 mm). A six point curve from 10 - 500 ppm was used for calibration. A coefficient of determination of 0.9998 was obtained and percent recovery was 100.6 - 101.8%, demonstrating that the method accurately detected methyl paraben.

B. 2. Mixture

Free N Clear™ (FNC) is a proprietary mixture[1] composed of benzalkonium chloride (BAC), acetic acid, and methyl paraben in an aqueous base. The concentrated form of FNC is prepared from mixing specified amounts of United States Pharmacopeia (USP)-grade benzalkonium chloride (BAC) solution, methyl paraben, acetic acid and water. This concentrate is diluted 50:1 (2% FNC solution) for use. A 2% FNC solution contains 96 - 100 ppm (0.01%) USP-BAC. The intended use of 2% FNC is as a processing aid on lettuce and carrots at processing facility. The safety in use of BAC as a component of a 2% FNC solution is the primary focus of this dossier, as acetic acid and methyl paraben are safe and suitable ingredients already approved for use in foods. This dossier is a summary of the scientific evidence that supports the general recognition that the residual amount of BAC on lettuce and carrots after use of a 2% FNC solution as a processing aid is safe for human consumption.

B. 3 Solution

Free N Clear™ (FNC) is a clear, colorless solution prepared from mixing 118 g of USP-grade benzalkonium chloride (BAC) solution, 236 g of acetic acid and 118 g of methyl paraben (synonym: methyl /hydroxybenzoate or 4-hydroxybenzoic acid, methyl ester) and adding water to 6.3 gallons. The structures of BAC (CAS No. 8001-54-5),[i] acetic acid (CAS No. 64-19-7) and methyl paraben (CAS No. 99-76-3) and are shown in Figure 1. The three ingredients of FNC act together - BAC is a quaternary ammonium cationic surfactant which exhibits antimicrobial activity towards a wide variety of bacteria[2], methyl paraben is an adjuvant which may act in

[2]

combination or synergism with other antimicrobial agents[1], and acetic acid is a weak organic acid that helps maintain pH of FNC at the desired level. FNC will be used as a processing aid applied to lettuce and carrots at a concentration of up to 2% in water.

Test references:

1) Remarkable Kill **(APPENDIX 3 – page 14)** of 85.4% reduction using 2% FNC solution for 5 minutes exposure, as tested by Kansas State University (KSU) Food Science Institute
2) Residuals **(APPENDIX 2 – page 41,51)** Test performed by KSU Olathe Division

B.4 Chemistry

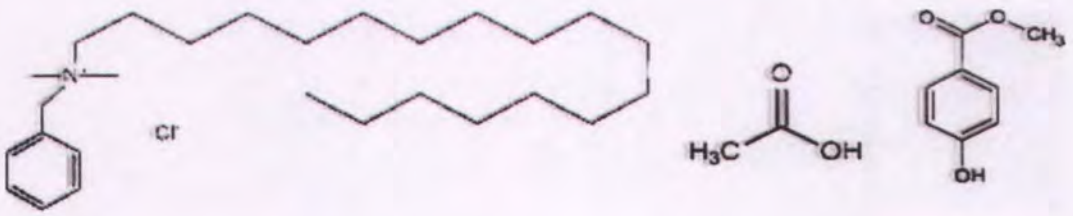

Figure 1: Structures of benzaikonium chloride (BAC), acetic acid, and methyl paraben, respectively.

Marvel selected:
A. Acetic Acid to manage pH
B. BAC to kill the bacteria
C. Methyl Paraben to mix/blend the solution

BAC, a member of the class of quaternary ammonium compounds, and may be referred to as alkyl dimethyl benzyl ammonium chloride (ADBAC) or alkylbenzyl dimethylammonium chloride. It is a mixture of alkylbenzyl dimethylammonium chlorides of the general formula [$C_6H_5CH2N(C^u3)_2$-•R]C1, with the R group including noctyl (n-C_8H17; C8) and extending through higher

homologues. The average molecular weight of BAC is 360[2]. BAC has been assigned a number of different CAS numbers, depending on the numbers of carbons in the R groups. In USP-BAC (CAS No. 8001-54-5), the R groups of n-$C_{12}H_{25}$, n-$C_{14}H_{29}$ and n-$C_{16}H_{33}$ (C12, C14 and C16) predominate, with the total amount of the C12 and C14 homologs not less than 70.0% of the total alkylbenzyl dimethylammonium content. A directly added food ingredient affirmed as GRAS for used in human food, is acetic acid, with its level of use limited only by current good manufacturing practice (cGMP).[3] Maximum levels of use of acetic acid that are consistent with cGMP are 9.0% for condiments and relishes, 3.0% for gravies and sauces, 0.8% for cheeses and dairy product analogs, 0.6% for meat products, 0.5% for fats, oils, and chewing gum, 0.25% for baked goods, and 0.15% for all other food categories.[4] Acetic acid is also approved for use as an indirect food additive in sanitizers used to control the growth of microorganisms, with the level of use limited only by cGMP.[5] As the concentration of acetic acid in a 2% solution of FNC (0.0239%, see below) is encompassed by the FDA GRAS affirmation for acetic acid, which permits use of up to 0.15% acetic acid in foods such as produce, the use of acetic acid as a component of FNC for use as a processing aid for lettuce and carrots is considered GRAS by reference. Therefore, in this dossier, information about acetic acid will be limited to a discussion of the level in FNC. Methyl paraben is widely used as a preservative[6] in food products, cosmetics and over-the-counter (OTC) pharmaceutical formulations. It can be used alone or in combination with other parabens, or with other antimicrobial agents. Methyl paraben is GRAS when used as a chemical preservative in foods at a limit of 0.1% (1000 ppm) under cGMP (21 CFR §184.1490). As the concentration of methyl paraben is considered GRAS by reference, therefore, in this dossier, information about methyl paraben will be limited to a discussion of the level utilized in the formation of FNC, and testing by Kansas State University Food Science Institute showing bacteria kill and residual parts per million.

C. Physical or Technical Effects of the Free N Clear™ Chemistry

C.1. When used as a processing aid at a carrot or lettuce wash station, some insignificant amount of GRAS product remains. This does not effect the tissue, taste or color.

C.2. No foreign chemistry is created

C.3. Residual of BAC <10 PPM. The EPA standard lists 44 PPM as safe daily intake.

* Regulatory History

BAC is commonly used in cosmetics as a foaming, cleansing and bactericidal agent at concentrations up to 5.0%. Lower concentrations (typically 0.002 - 0.02%) are used as a preservative for ophthalmic, optic, nasal and/or dermal OTC drug preparations. BAC may also be utilized as hard surface cleaner, wood preservative, or pesticide for nursery ornamentals, turf, decorative ponds, or swimming pools. Currently, the only food use of BAC is as component of adhesives for food packaging. The related Quaternary ammonium salt cetylpyridinium chloride (CPC) is used as an antimicrobial agent to treat the surface of poultry carcasses.

Table 1. Regulatory status of BAC (FDA)

Agency	Identification	Permitted functionality	Use limits	Reference
FDA	BAC (Alky 1 (CI0-20) Dimethylbenzyl ammonium chloride)	Component of packaging adhesive	cGMP	21CFR §175.105
FDA	BAC (CAS No. 8001-54-5)	Auricular (otic) preparations	< 0.02%	Inactive Ingredient for Approved Drug Products*
		Inhalation solution	< 20%	Do.
		Intralesional and intramuscular injectable	< 0.02%	Do.
		Nasal spray	<0.11%	Do.
		Ophthalmic suspension and ointment	< 0.02%	Do.
		Topical lotion	<0.1%	Do.
		Topical shampoo	< 0.2%	Do.
		Topical suspension and drops solution	< 0.01%	Do.
		Topicsl solution	$< 7.5x10^{-3\%}$	Do.
		Nasal solution	< 1.0%	Do.
		Nasal solution, spray	< 0.02%	Do.
		Ophthalmic solution	< 2.0%	Do.

BAC = Benzaikonium chloride; CAS = Chemical Abstract Service: CFR = Code of Federal Regulations, 2010; Do = Same as above: cGMP = current Good Manufacturing Practice: FDA = US Food and Drug Administration: GRAS = Generally Recognized As Safe: NS = not stated.
*** htto://www.accessdata.fda.gov/scripts/cder/iig/index.cfm: site visited May 28. 2013.**

D. Any Toxicology Effects

1. No known toxic effects from marvel Free N Clear™ solutions when used as directed (2%)
2. No physical changes observed
3. Product listed as nontoxic

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Part 3

DIETARY EXPOSURE

(a) Dietary exposure; all sources of BAC in diet

(b) No reaction products in current testing

(c) No contaminants or by-products discovered

(d) Source of food data by *What We Eat In America* – continuing data study

(e) Estimated consumption of 1 toss salad and 5 baby carrots per day

Part 3 (a) Dietary exposure; all sources of BAC in diet

The intake profile (amount and frequency) by individuals in USDA's What We Eat in America (WWE1A) Continuing Survey of Food Intakes by Individuals 2007 - 2008 was used to calculate the estimated daily intake (EDI) of BAC for individuals consuming the foods that will be processed with Free N Clear™ per this GRAS determination (i.e., carrot or lettuce products). The consumption of BAC from ingestion of the processed lettuce and carrot products was determined using the residual amount of BAC remaining in the lettuce or carrots after treatment with 2% FNC for three hours (18.866 ppm or 13.163 ppm, respectively). The amount of BAC remaining on the produce after treatment is expected to be a worst case scenario, as the recommended exposure time with 2% FNC is five minutes. The means and 90th **percentile** EDIs were calculated for; (1) current intake of BAC from other sources (current); (2) BAC intake following use of 2% FNC on lettuce and carrots and; (3) total estimated EDI from current sources combined with levels from use of 2% FNC on lettuce and carrots. Current daily dietary aggregate exposure to BAC from direct and indirect food contact as well as drinking water exposures for adults is estimated at 0.0066 mg/kg bw/day. The results of the analysis show that the total estimated mean and 90th **percentile** weighted aggregate consumption of BAC from use of a 2% solution of FNC as a processing aid for "eaters only" of carrots and lettuce is 0.025 mg/kg bw/day and 0.053 mg/kg bw[26] /day, respectively (Table 5). *The Environmental Protection Agency (EPA's) level of concern for chronic dietary aggregate exposure to BAC is 0.44 mg/kg bw/day,* which is approximately eight times higher than estimated 90th **percentile** consumption by people who will eat lettuce and carrots processed with a 2% solution of FNC.

Table 5 BAC current intake, predicted intake and total intake (predicted + current) for individuals consuming lettuce and carrots processed with a 2% solution of Free N Clear™

	Per User	(mg/kg bw/day)
BAC intake from:	Mean	90^{lh} Percentile
Current consumption from food and drinking water	0.0066	0.0132
Of Free N Clear™ on lettuce and carrots	0.0186	0.0398
Total from conventional food (current + added)	0.0252	0.0530

Table 8

STUDY OF BENZALKONIUM CHLORIDE

LAB	PRODUCT	EXPOSURE	RESDIUAL PPM ON LETTUCE	EXPOSURE TIME
MICROBAC LAB	MARVEL FNC™	2% SOLUTION	18.866 PPM	3 HOURS
KANSAS STATE UNIVERSITY (KSU)	MARVEL FNC™	2% SOLUTION	< 10 PPM	5 MINUTES

DAILY INTAKE	PRODUCT	EPA SAFE LEVEL IS 44 PPM
WATER, FOOD ETC. 0.0066 PPM Salad and Carrots 0.0530	1 – TOSS SALAD 5 – BABY CARROTS	RESIDUAL BAC ON LETTUCE/CARROTS IS < 10 PPM AS PER KANSAS STATE UNIVERSITY TESTING
BAC DAILY INTAKE 0.0596 PPM	1 – TOSS SALAD 5 – BABY CARROTS	

Estimate – Average person consumes 1- toss salad per day and 5 raw baby carrots per day

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Part 4

PART FOUR NOT APPLICABLE AS PER FDA (RB)

TABLE OF CONTENTS

Part 5

PART FIVE NOT APPLICABLE AS PER FDA (RB)

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Part 6 NARRATIVE

The basis for conclusion of GRAS Status

(a) SAFETY

1. Non toxic as 90 rat study, efficient kill, and safe residuals

2. Specific data and information (safety studies pgs. 87 and 97)

(b) BASIS FOR CONCLUSION

1. There is a great need for a wash solution processing aid for carrots and lettuce
2. Major food companies recalling carrots and lettuce due to problems with E. coli, salmonella and listeria
3. FDA was questioned to find most respected testing to validate non-toxic use of liquid disinfectant (The 90 day rat consumption program was suggested.)
4. Testing Results:
   a. 90 day rat study by Charles River Laboratories (Free N Clear™ was non toxic, external, liver, bone marrow
   b. Microbac Laboratories of BAC bacteria kills and residuals
   c. Kansas State University Food Science Institute kill times to exposed bacteriaof E. coli, salmonella, listeria (using 2% Free N Clear™ solution for 5 minute exposure) 85.4% all bacteria killed in 5 minutes
   d. Kansas State University (Food Science Lab in Olathe Kansas – residuals after 2% exposure of 5 minutes) BAC <10 PPM, methyl paraben <5 PPM ; EPA level of 44PPM daily intake

5. Chemistries of creating blend for Free N Clear™ are available from Lonza Chemical Company, stable and blend is safe for human consumption. Acetic acid and methyl paraben are listed as GRAS

6. Filing for GRAS listing as processing aid for solution used at the processing locations for carrots and lettuce. Respect for scientific testing and professional opinions of industry connected professionals

(c) Specifications for food grade materials
   1. No negatives found against Marvel Free N Clear™
   2. We have reviewed and not found any inconsistent information against Marvel Free N Clear™

(d) Not any information exempt from disclosure of patent protected, #8,268,337

(e) Basis for GRAS conclusion, even if they had no known data or information

(f) CONCLUSION (attached)

Following a critical evaluation of the information available, the Expert Panel has determined that, based on common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food, there is reasonable certainty that BAC is safe under the intended conditions of use, and is therefore Generally Recognized As Safe (GRAS), by scientific procedures, when used as an ingredient of Free N Clear™ (FNC), when a 2% solution of FNC is used as a processing aid on lettuce and carrots.

It is our opinion that other experts qualified by scientific training and experience to evaluate the safety of food and food ingredients would concur with these conclusions.

10. SIGNATURES

(b) (6)

8/9/13
Date

Douglas L. Archer, Ph.D.
Associate Dean for Research, UF/IFAS
University of Florida

(b) (6)

8/12/13
Date

Edward Carmines, Ph.D.
Consultant

(b) (6)

8/15/13
Date

D. Reid Patterson, D.V.M., Ph.D.
Diplomate, A.C.V.P., A.C.L.A.M., A.B.T.
Fellow, A.T.S., I.A.T.P.
Reid Patterson Consulting, Inc.
President

A. Safety Studies on BAC

The means and 90th percentile EDIs were calculated for; (1) current intake of BAC from other sources (current); (2) BAC intake following use of 2% FNC on lettuce and carrots and; (3) total estimated EDI from current sources combined with levels from use of 2% FNC on lettuce and carrots. Current daily dietary aggregate exposure to BAC from direct and indirect food contact as well as drinking water exposures for adults is estimated at 0.0066 mg/kg bw/day.

The results of the analysis show that the total estimated mean and 90th percentile weighted aggregate consumption of BAC from use of a 2% solution of FNC as a processing aid for "eaters only" of carrots and lettuce is 0.025 mg/kg bw/day and 0.053 mg/kg bw[26] /day, respectively (Table 5). The Environmental Protection Agency (EPA's) level of concern for chronic dietary aggregate exposure to BAC is 0.44 mg/kg bw/day, which is approximately eight times higher than estimated 90th percentile consumption by people who will eat lettuce and carrots processed with a 2% solution of FNC.

Table 5 BAC current intake, predicted intake and total intake (predicted + current) for individuals consuming lettuce and carrots processed with a 2% solution of Free N Clear™

	Per User	(mg/kg bw/day)
BAC intake from:	Mean	90lh Percentile
Current consumption from food and drinking water*	0.0066	0.0132**
Possible maximum consumption with use of 2 % solution of Free N Clear™ on lettuce and carrots	0.0186	0.0398
Total from conventional food (current + added)	0.0252	0.0530

EPA estimate [6]: ** Estimated as two times the mean: BAC = benzalkonium chloride.

ABSORPTION, DISTRIBUTION, METABOLISM AND ELIMINATION (ADME)

No information about the absorption, distribution, metabolism and elimination (ADME) of Free N Clear™ was located. However, information about the ADME of BAC is available. The distribution and disposition of BAC after a single oral dose of a BAC-containing product commercially available in Japan (Osvan®) has been studied in rats [15,16]. A dose of 2.5 ml/kg Osvan® (250 mg/kg BAC) was administered to 30 fasted rats by stomach tube, and blood samples were collected by cardiac puncture 1, 2, 4, 8 and 24 hours later (six rats/time point). Rats were terminated and the lung, liver and kidneys were harvested. The authors noted that

concentrations of BAC in blood and tissues were substantially higher in animals that aspirated the BAC-containing product, suggesting that BAC is absorbed by the pulmonary blood vessels if inhaled. In animals that did not aspirate BAC, concentrations of BAC in blood and tissues were relatively low (0.01 - 1 u.g/g), and did not increase over time, suggesting that only a small amount of orally administered BAC is absorbed through the gastrointestinal tract of rats. Because BAC is a large, positively charged molecule it is poorly absorbed and likely eliminated largely in feces, similar to other quaternary ammonium compounds.

Acute toxicity

Reported oral LD_{50} values for BAC in mice and rats are 150 mg/kg bw and 234 -300 mg/kg bw, respectively. In a study designed to assess the pharmacokinetics of orally administered BAC, four out of 34 rats receiving 250 mg/kg BAC by gavage died from respiratory toxicity within a 24 hour period. Approximately 50% of the rats exhibited symptoms of respiratory toxicity. The authors suspected that the fatalities and respiratory toxicity were due to aspiration of gavaged material. If no aspiration occurred, the rats appeared normal. In humans, the lethal oral dose ranges from 100 - 400 mg/kg bw (10 - 15% solution). A 70- year old woman who drank a solution containing approximately 200 mg/kg BAC survived after receiving supportive care at a hospital. The literature search revealed one case of acute respiratory toxicity in humans after ingestion of a solution containing 10% BAC. Respiratory insufficiency was reported in a two year old girl after ingesting two teaspoons of an antiseptic solution containing 10% BAC. She developed chemical pneumonitis and upper GI tract bleeding within a week of exposure, but recovered after treatment in an intensive care unit. Therapies included assisted ventilation, enteral feeding, intravenous infusion of a dextrose- electrolyte solution, and treatment with an H_2 receptor antagonist, steroid and antibiotic.

Subchronic and chronic toxicity

The subchronic or chronic oral toxicity of BAC has been examined in a number of studies that were conducted prior to the adoption of Good Laboratory Practice (GLP) guidelines (Table 6). Early studies with BAC of unknown purity indicate variable NOAELs for BAC, depending on concentration, method of administration and/or vehicle. Whereas one two year dietary study in rats showed no adverse effects at up to approximately 125 mg BAC/kg bw/day (0.25% or 2500 ppm in the diet), another showed decreased growth at approximately 31.5 mg/kg bw/day (0.063% or 630 ppm in the diet). In both studies, increased mortality, decreased weight gain,

diarrhea, gastritis and gross and microscopic changes in the stomach and small intestine were noted in rats administered approximately 250 mg/kg bw/day (0.5% in the diet) BAC. Conversely, no overt adverse effects were found in rats ingesting feed containing 3000 mg/kg bw/day BAC for 4 - 5 weeks. Although results of these early studies provide some information about the toxicity of BAC, they are not of sufficient quality for risk assessment purposes. Results of subchronic or chronic toxicity studies in rats, dogs or guinea pigs involving administration of BAC *via* gavage are also somewhat variable, but in general show lower NOAELs than when administered in the diet. In dogs, the 52-week oral (gavage) NOAELs for BAC when diluted in water or milk as the vehicle are < 12.5 mg/kg bw/day and 25 mg/kg bw/day, respectively. No overt signs of toxicity are observed in guinea pigs exposed to 25 mg BAC/kg bw/day by gavage (water vehicle) for one year. Whereas one study showed histological changes in the stomach and intestine of rats administered 5-25 mg BAC/kg bw/day for two years by gavage (water vehicle), another showed no effects of 50 mg BAC/kg bw/day when administered by gavage (milk or water vehicle).

EPA FIFRA guideline subchronic toxicity studies in rats and mice and chronic toxicity studies in dogs, mice and rats have been conducted on materials described as "ADBAC C12- 16" or "ADBAC C12-18", in support of an EPA high production volume (HPV) test plan for ADBAC published online in 2011. The test substance was administered by the dietary route in all of the studies. Summaries of these unpublished studies are provided in an appendix to the test plan. These tests apply to USP-BAC (CAS No. 8001-54-5), as it predominantly contains.

ADBAC C12-16, but also contains 3% ADBAC C18. The results of these studies are similar to those of the published, non-GLP studies conducted from 1948 - 1961 (see paragraph above).

The author of the summaries stated that the NOAELs for subchronic (93 - 96 day) toxicity in rats and mice were 1000 ppm (approximately 70 mg/kg bw/day in rats and 192 mg/kg bw/day in mice). All of the mice and most of the rats did not survive exposure to next highest dose (4000 ppm; approximately 280 mg/kg bw/day in rats and 768 mg/kg bw/day in mice). The summary writer mentioned that clinical signs of toxicity, decreased food consumption and body weights, gross necropsy findings (principally ileus consisting of distended fluid and gas-filled viscera) and histopathologic effects (related to the gastrointestinal changes) were observed in rats exposed to 4000 ppm concentration. In mice exposed to 4000 ppm, clinical signs of toxicity were restricted to the animals that died, and were related to general cachexia and gross necropsy observations of increased amounts of liquid or semisolid material throughout the gastrointestinal tract. The summary writer also stated that the NOAELs for chronic toxicity in dogs, rats and mice were 14, 50 and 82 mg/kg bw/day, respectively. In rats and mice, the

NOAELs for carcinogenicity were the highest doses given (102 and 259 mg/kg bw/day, respectively). Therefore, it was concluded that BAC was not a carcinogen under the conditions of the studies. Responses observed at the lowest observable adverse effect levels (LOAEL) in chronic toxicity/carcinogenicity studies in dogs, rats and mice (35, 102 or 259 mg/kg/day, the highest doses tested) were limited to changes in body weight, food consumption and/or plasma cholesterol. Altogether, results of guideline studies in dogs, rats and mice are consistent and show that the NOAELs for chronic toxicity (effects on body weight, food consumption and/or plasma cholesterol) are 14, 50 and 82 mg/kg bw/day, respectively. The minimum NOAEL for chronic toxicity(14mg/kgbw/day)is264timeshigherthantheestimated90thpercentileintakeof BAC from all sources (0.0530 mg/kg bw/day), including lettuce and lettuce and carrots processed with a 2% solution of FNC. In rats and mice, BAC is not carcinogenic at the highest doses tested (102 and 259 mg/kg bw/day, respectively). The EPA also has concluded that BAC is not carcinogenic.

Table 6. Subchronic and Chronic Oral Toxicity Studies Conducted on BAC

Species/Strain/ Guideline (Number *per* group)*	Dose/Route	Duration	Results/Notes	Reference
White Rat (strain not specified) Non-guideline study	3% (3000 mg/kg bw/day). Dietary administration	28 - 35 days	NOAEL = 3% (300 mg/kg bw/day). No overt adverse effects noted.	Harshbarger [24]
Osborne-Mendel Rat Non guideline study *N* = 12 males/group	0, 0.063% (63 mg/kg bw/day), 0.125% (250 mg/kg bw/day), 0.25% and 0.5%. Dietary administration	Two years	NOAEL< 0.063 % (63 mg/kg bw/day) LOAEL = 0.063%: Growth suppression 0.25 %: Diarrhea, bloating of the abdomen, brown syrupy material in intestine, distension of cecum, foci of necrosis in GI tract. 0.5%: All rats died within 10 weeks	Fitzhugh and Nelson [23]
Yale/Sherman/Wistar Rat Non guideline study *N* = 12-13/sex/group	5, 12.5, 25 mg/kg bw/day *via* gavage	Two years	25 mg/kg bw/day: decreased body weight. Increased cell growth in gastric mucosa at unspecified doses	Shelanski [26]
"Albino" Rat Non guideline study (*N* = 12/sex/group)	0, 0.015%, 0.031%, 0.062%, 0.125%, 0.25% and 0.5%. Dietary administration	Two years	NOAEL = 0.125% (125 mg/kg bw/day) 0.5%: 50% survival. Diarrhea, brown viscid substance in upper GI tract, gastritis, mucosal necrosis of GI tract	Alfredson *et al.* [19]
SD Rat Non guideline study *N* = 10 males/group	50 or 100 mg/kg bw/day *via* gavage (water or milk vehicle)	12 weeks	50 mg/kg bw/day: "well tolerated" 100 mg/kg bw/day: Reduced weight gain, increased mortality[a]	Coulston *et al.* [25]

SD Rat EPA FIFRA 82-1 guideline study	100 ppm (7 mg/kg bw/day), 500 ppm (35 mg/kg bw/day) 1000 ppm (70 mg/kg bw/day),	95 - 96 days	NOAEL = 1000 ppm (70 mg/kg bw/day) LOAEL could not be determined due to 100% mortality at 4000 ppm	Van Miller and Weaver [28]

Species/Strain/ Guideline (Number *per* group)*	**Dose/Route**	**Duration**	**Results/Notes**	**Reference**
(OPPTS 870.3100) *N* = 10 sex/group, minimum**	4000 and 8000 ppm.[b] Dietary administration		Increased mortality, gross and histological changes in the GI tract at 4000 and 8000 ppm.	
SD Rat EPA FIFRA 83-5 guideline study (OPPTS 870.4300) *N* = 50 sex/group, minimum**	300 ppm (15 mg/kg bw/day), 1000 ppm (50 mg/kg bw/day), 2000 ppm (102 mg/kg bw/day). Dietary administration	Two years	NOAEL (repeated dose toxicity) = 1000 ppm (50 mg/kg bw/day) NOAEL (carcinogenicity) = 2000 ppm (102 mg/kg bw/day) LOAEL (repeated dose toxicity) = 2000 ppm (102 mg/kg bw/day). Reduced body weight and food consumption	Gill *et al.* [29]
CD-1 Mouse EPA FIFRA 82-1 guideline study (OPPTS 870.3100) *N* = 10 sex/group, minimum**	100 ppm (20 mg/kg bw/day), 500 ppm (94 mg/kg bw/day), 1000 ppm (192 mg/kg bw/day), 4000, 8000 ppm[b]. Dietary administration	93-94 days	NOAEL = 1000 ppm (192 mg/kg bw/day) LOAEL could not be determined due to high mortality at 4000 ppm. Increased mortality, gross and histological changes in the GI tract at 4000 and 8000 ppm.	Van Miller and Weaver [28]
CD-1 Mouse EPA FIFRA 83-2 guideline study (OPPTS 870.4200) *N* = 50 sex/group, minimum**	100 ppm (16 mg/kg bw/day), 500 ppm (82 mg/kg bw/day) and 1500 ppm (259 mg/kg bw/day). Dietary administration	78 weeks	NOAEL (repeated dose toxicity) = 500 ppm (82 mg/kg bw/day) NOAEL (carcinogenicity) = 1500 ppm (259 mg/kg bw/day) LOAEL (repeated dose toxicity) = 1500 ppm (259 mg/kg bw/day). Reduced body weights and body weight gains	Gill *et al.* [29]
Guinea pig	Non guideline study	*N* = 10/sex/group		5, 12.5, 25 mg/kg

Species/Strain/ Guideline (Number *per* group)*	Dose/Route	Duration	Results/Notes	Reference
Mongrel Dog Non guideline study *N* = 1-2/group	0.031, 0.062, 0.12, 0.25, 0.5, 1%. Dietary administration	15 weeks	NOAEL = 0.12%. LOAEL = 0.25%. Decreased body weight at ≥ 0.25%. Moribundity, mortality and gross and histological changes in the GI tract at ≥ 0.5%.	Alfredson *et al.* [19]
Beagle Dog Non guideline study *N* = 3/group	12.5, 25 and 50 mg/kg bw/day *via* gavage (10% solution in water or milk vehicle)	One year	Water vehicle: NOAEL < 12.5 mg/kg bw/day. Gross and microscopic changes in the intestine at12.5 mg/kg bw/day. Vomiting and increased mortality at higher doses Milk vehicle: NOAEL 25 mg/kg bw/day. Gross changes in the intestine at 50 mg/kg bw/day	Coulston *et al.* [25]
Beagle Dog EPA FIFRA 83-1(b) guideline study (OPPTS 870.4100) *N* = 4/sex/group	120 ppm (4 mg/kg bw/day), 400 ppm (14 mg/kg bw/day) and 1200 ppm (35 mg/kg bw/day). Dietary administration	One year	NOAEL = 400 ppm (14 mg/kg bw/day) LOAEL = 1200 ppm (35 mg/kg bw/day). Decreased body weight, food consumption and cholesterol	Goldenthal [30]

EPA = Environmental Protection Agency; FIFRA = Federal Insecticide, Fungicide and Rodenticide Act; GI = gastrointestinal; LOAEL = lowest observed adverse effect level; NOAEL = no observable adverse effect level; ppm = parts *per* million; SD = Sprague-Dawley

*If available from reference; ** According to guideline. Numbers of animals tested were not mentioned in the reference. [a] Unclear if tissue examinations were performed; [b] Due to mortality in the 4000 and 8000 ppm groups, actual doses could not be calculated.

Genotoxicity

The genotoxicity of BAC has been evaluated in several *in vitro* assays. Results of bacterial mutagenicity studies with BAC are overwhelmingly negative, however, chromosome aberration studies are variable. Results of one study show that concentrations of up to 30 µM BAC (approximately 10.8 mg/L) are not clastogenic in a Syrian Hamster Embryo (SHE) cell study. Another study showed that 1 or 3 mg/L BAC caused an equivocal increase in sister chromatid exchanges in SHE cells. A 1.0 mg/L concentration of BAC increased the frequency of micronuclei in cultured human lymphocytes. DNA damage occurs in cultured human respiratory epithelial cells exposed to 0.02% BAC (200 mg/L). In rats administered 250 mg/kg BAC by the oral route, the concentration of BAC in plasma and tissues is approximately 0.1 – 1 µg/g (mL), which is approximately equal to the lowest concentration of BAC that caused genetic toxicity *in vitro* (1 mg/L). As mentioned in Section 6.1.3 above, a 20 mL/kg dose of 2% FNC (which delivers a BAC dose of 1.92 mg BAC/kg bw) does not increase the frequency of micronuclei in bone marrow of rats. These findings suggest that the plasma and tissue concentrations that could arise from rats exposed to 1.92 mg BAC/kg bw from a 2% solution of FNC (an estimated maximum of 7.7 µg/L) are substantially lower than those that produce genotoxicity *in vitro*. The plasma concentration of BAC in people consuming lettuce and carrots processed with 2% FNC would be even lower

than 7.7 µg/L, as the total amount of BAC consumed by these individuals (0.053 mg/kg bw/day) is 36-times lower than the BAC dose associated with this plasma concentration in rats (1.92 mg BAC/kg bw/day). In conclusion, the maximum tissue and plasma concentrations of BAC in people consuming 90th percentile quantities of lettuce and carrots processed with 2% FNC would be substantially lower than the concentrations of BAC that are clastogenic in some *in vitro* assays. Therefore, the positive results of some *in vitro* clastogenicity studies with high concentrations of BAC are not relevant for the safety assessment of BAC in 2% FNC.

Reproductive or Developmental Toxicity

The potential for Free N Clear ™ to cause reproductive or developmental toxicity has not been tested. Studies conducted prior to adoption of GLP guidelines showed no overt adverse effects of up to 25 mg/kg bw/day BAC on "fertility"[32] of rats or guinea pigs.

Four studies have been conducted to examine the developmental toxicity of BAC (Table 7). An EPA FIFRA guideline two generation reproductive toxicity study in Sprague-Dawley rats has been conducted on a material described as "ADBAC C12-16" in support of an EPA high production volume (HPV) test plan published online in 2011. This test applies to USP-BAC (CAS No. 8001-54-5), as it predominantly contains ADBAC C12-16. A summary of the unpublished study is provided in an appendix to the published EPA HPV test plan for ADBAC. The results of this study are similar to those of the published, non-GLP study conducted by Shelanski (see paragraph above). Doses used in the study were 300, 1000 and 2000 ppm in feed (approximately 22, 73 or 145 mg/kg bw/day, respectively). The author of the summary stated that the no observable effect level (NOEL) for toxicity to parental animals or offspring was 1000 ppm (73 mg/kg bw/day). Effects noted at the lowest observable effect level (LOEL) of 2000 ppm (145 mg/kg bw/day) were reduced body weight or reduced body weight gain of parental animals and pups. Reproductive parameters were not affected by treatment with up to 2000 ppm (145 mg/kg bw/day).

Table 7. Developmental Toxicity of BAC

Species/Strain/ Guideline (Number *per* group)*	Dose/Route	Duration	Results/Notes	Reference
Wistar Rat Study comparable to OECD Guideline 414 *N* = 22 - 37/group	0, 5, 15, 50 g/kg bw/day	Treatment: GD 6-15 Termination: GD 20	NOEL (maternal toxicity): 15 mg/kg bw/day NOEL (developmental toxicity): 50 mg/kg bw/day LOEL (maternal toxicity):50 mg/kg bw/day. Increased mortality.	Knickerbocker and Stevens [37]
SD Rat EPA FIFRA 83-3 guideline study (OPPTS number 870.3700) *N* = 25/group	0, 10, 30, 100 mg/kg bw/day	Treatment: GD 6-15 Termination; GD 21	NOEL (maternal toxicity): 10 mg/kg bw/day NOEL (developmental toxicity): 100 mg/kg bw/day LOEL (maternal toxicity): 30 mg/kg bw/day. Reduced food consumption, audible respiration. Perioral wetness noted at 100 mg/kg bw/day.	Neeper-Bradley [36]
ICR/JCL Mouse Non guideline study *N* = 5 - 20/group	0.001, 0.05, 0.1, 3, 10, 30 mg/kg bw BAC	Treatment: GD 0-6 Termination: GD 13 or Treatment: GD 0-17 Termination: GD 17	NOAEL (developmental toxicity): 30 mg/kg bw/day	Momma *et al.* [38]
NZW Rabbit EPA FIFRA 83-3 guideline study (OPPTS number 870.3700) *N* = 16/group	0, 1, 3 or 9 mg/kg bw/day	Treatment: GD 6-18 Termination; GD 29	NOEL (maternal toxicity): 3 mg/kg bw/day NOEL (developmental toxicity): 9 mg/kg bw/day LOEL (maternal toxicity): 9 mg/kg bw/day. Hypoactivity, labored respiration	Neeper-Bradley [36]

EPA FIFRA guideline developmental toxicity studies have been conducted in Sprague- Dawley rats and New Zealand White Rabbits with a material described as "ADBAC C12-16", and a study comparable to OECD Guideline 414 has been conducted in Wistar rats with "ADBAC C12-18". These studies were performed in support of an EPA high production volume (HPV) test plan for ADBAC published online in 2011. These tests apply to USP-BAC (CAS No. 8001-54-5), as it predominantly contains ADBAC C12-16, but also contains 3% ADBAC C18. Summaries of the studies are provided in an appendix to the test plan. ADBAC was provided by gavage for all of the studies. NOELs in the studies performed with Wistar or SD rats were 15 or 10 mg/kg bw/day for maternal toxicity and 50 or 100 mg/kg bw/day for developmental toxicity (respectively). In SD rats, doses of 30 mg/kg bw/day or 100 mg/kg bw/day from GD 6-15 were associated with reduced food consumption during GD 6-9. However, there was no effect of either of these doses on maternal or fetal body weight or any index of reproductive or developmental toxicity measured in the study. In the study performed in Wistar rats, three dams exposed to 50 mg/kg bw/day died. No other adverse effects of treatment were mentioned in the HPV summary. It is unclear whether the deaths were due to gavage error or treatment with BAC. However, it should be noted that no deaths were reported in SD rats exposed to up to 100 mg/kg bw/day BAC. In New Zealand rabbits NOELs for maternal and developmental toxicity were 3 mg/kg bw/day and 9 mg/kg bw/day (highest dose provided), respectively. Hypoactivity and labored or audible respiration were observed in rabbits exposed to 9 mg/kg bw/day. Based on information provided for these studies, it is reasonable to assign a NOEL for developmental toxicity in rats and rabbits of 100 mg/kg bw/day and 9 mg/kg bw/day BAC, respectively.

Other toxicity studies

BAC has been tested for the ability to cause eye irritation, skin irritation and sensitization. The results of the studies are not critical to the determination of safety of a GRAS ingredient, but help identify whether certain precautions should be taken during handling.

Eye irritation

Microscopic changes in the corneal epithelium are observed in eyes of rabbits after ocular exposure to ≥ 0.01% (100 ppm) BAC. In humans, solutions of 0.03 - 0.04% BAC (300 - 400 ppm) may cause reversible eye irritation. A 0.02% (200 ppm) solution of BAC is generally not irritating to human eyes; however, a few cases of conjunctival redness have been reported at this

concentration. Use of eye protection will be recommended for workers handling FNC concentrate, as the amount of BAC in the concentrate is greater than 200 ppm. Skin irritation BAC is irritating to human skin at concentrations of 1 - 10%. The maximum concentration of BAC that does not produce irritation to intact skin is 0.1% (1000 ppm). Concentrations < 0.1% have caused irritation in people with contact dermatitis or broken skin. In an annual review of cosmetic ingredient safety conducted in 2006 and published in 2008, the Cosmetic Ingredient Review (CIR) Expert Panel stated that up to 0.1% BAC is safe for use as a cosmetic ingredient in certain products that are applied to skin or eyes. Use of gloves will be recommended for workers handling FNC concentrate, as the concentration of BAC in the concentrate is greater than 0.1%.

Sensitization

Various studies involving repeated dermal or intradermal applications of BAC and challenge with 0.01 - 0.3% (100 - 3000 ppm) solution have shown that BAC can induce sensitization in guinea pigs and mice. Skin sensitization has been noted in patients tested with BAC concentrations ranging from 0.01 to 0.7% (100 - 7000 ppm). However, in patch tests carried out in the general population and in healthy volunteers, no sensitivity to 0.1% (1000 ppm) BAC was detected. It has been suggested that the sensitization response to BAC is not mediated by an immune response, but to the irritant properties of BAC. Inhalation of nebulizer solutions containing 250 ppm BAC has been associated with bronchoconstriction in some patients with asthma. A study involving 30 subjects with bronchial asthma and ten normal controls inhaling up to three 600 μg nebulized doses of BAC in a jet nebulizer showed that BAC exposure did not cause bronchoconstriction (defined as a ≥ 15% decrease in forced expiratory volume over one second (FEV1)) in normal subjects, but did in 6/30 (20%) of the asthmatics. The *percentage* decrease in FEV1 was significantly higher in asthmatics (2.69%, 5.36% and 5.30% after each dose) than normal subjects ($p < 0.05$), and significantly higher in asthmatics with higher bronchial hyper-responsiveness (BHR) than those with lower BHR. BAC-induced bronchoconstriction was reversed with a short-acting β-2 agonist. The study suggest that bronchoconstriction to BAC does not occur in nonasthmatics, but may in asthmatics (particularly in those with high airway sensitivity). Cases of bronchoconstriction or asthma have been reported in people occupationally exposed to BAC. A statistically significant association has been found between the prevalence of mild bronchial responsiveness in pig farmers and use of BAC as a disinfectant. A 22-year old woman working in a factory that manufactured

cleaning solutions developed wheezing, shortness of breath and a skin rash, which were precipitated by exposure to a toilet bowl cleaner containing BAC. Three nurses developed symptoms of asthma after handling disinfectant solutions containing BAC. Concentrations of BAC in the disinfectant solutions were reported in two of the cases - 10% and 40%. In all cases mentioned above, the diagnosis of BAC-induced bronchoconstriton was confirmed by bronchial challenge tests. The mechanism by which BAC causes bronchoconstriction is unknown. As intradermal administration of BAC can cause skin sensitization, it has been suggested that the mechanism of BAC-induced bronchoconstriction is mediated by IgE. However, as BAC can elicit non-IgE mediated histamine release from rat mast cells and the BAC-induced bronchoconstriction response can be blocked by antihistamines, the bronchoconstrictor response is likely mediated by a non-immunological response involving release of histamine. A placebo-controlled study in twelve asthmatic subjects showed that use of terfenadine (an H1-receptor antagonist) inhibited the initial, 5-minute bronchoconstrictor response to BAC by 40%, but had a minimal effect on the longer term (45-minute) response. The authors concluded that the major mechanism by which BAC exerts its adverse effects on the airways of asthmatics does not involve histamine release. However, as terfenadine does not block H2 receptors, it is altogether possible that the effects of BAC on the airways were mediated by an H2-dependent pathway. Other possible mechanisms include BAC-induced release of lipid-derived mediators (*e.g.* prostaglandins) from mast cells into airways or direct stimulation of nerve endings, irritant receptors or bronchial smooth muscle. Although skin sensitization and bronchoconstriction in response to BAC exposure are rare and have occurred at concentrations higher than the concentration of BAC in FNC concentrate, use of gloves and respiratory protection will be recommended for workers handling FNC concentrate. These protections will not be required for consumers of lettuce or carrots processed with 2% FNC, as the concentrations of BAC remaining in the foods after processing are substantially lower than the concentrations of BAC shown to cause skin sensitization or bronchoconstriction.

OTHER CONSIDERATIONS

Possible tolerance of *E. coli* to BAC

The concentration of BAC in 2% FNC and the residual levels on carrots and lettuce are highly unlikely to result in increased resistance to either BAC or antibiotics. Reported minimum inhibitory concentrations (MIC) of BAC against *E. coli* are 6.2 ppm to 13 ppm. Exposure of *E. coli* to a sub minimal inhibitory concentration (25% below the MIC) of BAC has been shown to select for a small population (approximately 1-5% of the initial population) to survive and regain similar morphology and growth rate as non-exposed cells. This subpopulation maintains tolerance to BAC after serial transfers in medium without BAC. Experiments *in vitro* have shown that *E. coli* grown in medium initially containing 2 ppm BAC and passaged in medium containing incremental concentrations of BAC develop decreased sensitivity to BAC and antibiotics such as chloramphenicol, florfenicol, ciprofloxacin, nalidixic acid, ampicillin and cefoxatime. The concentration of BAC in a 2% solution of FNC (75 - 96 ppm) is much higher than 2 ppm, suggesting that use of 2% FNC in lettuce and carrot processing plants will not lead to growth of *E. coli* that is tolerant to BAC in the processing plants. Further, residual concentrations of BAC remaining on lettuce and carrots processed with 2% FNC (19 and 13 ppm, respectively) are higher than 1-3 ppm, suggesting that use of 2% FNC as a processing aid will not lead to development of antibiotic-resistant strains of *E. coli* in humans ingesting the treated lettuce or carrots.

Potential Environmental Effects

The proposed use of a 2% solution of FNC will not significantly alter the concentration or distribution of BAC which is naturally present in the environment. This conclusion is based on the following:

- Users will be directed to divert used 2% FNC to water treatment facilities, as *per* local requirements;
- BAC has a strong tendency to bind to soil and sediment and will bind to sludge in water treatment plants, removing BAC from water ;
- Under appropriate conditions and concentrations, BAC degrades into 60% CO_2 within 13 days and is therefore considered readily biodegradable (EPA, 2006)
- Based on physical properties and results of biodegradability studies BAC is expected to be removed from water before it reaches aquatic ecosystems.

EVALUATION

Free N Clear™ (FNC) is a proprietary mixture composed of benzalkonium chloride (BAC), acetic acid, and methyl paraben in an aqueous base. The concentrated form of FNC is prepared by mixing specified amounts of USP benzalkonium chloride (BAC) solution (50% concentration), methyl paraben, acetic acid and water. FNC is stable at room temperature for 12 to 16 months. FNC concentrate is diluted 1:50 (*i.e.*, a 2% FNC solution) for use. Both acetic acid and methyl paraben are safe and suitable ingredients already approved for use in foods; therefore, the safety of BAC as a component of a 2% FNC solution is the primary focus of this dossier. A 2% FNC solution contains 96 - 100 ppm USP-BAC (Review all references to PPM of BAC through out testing. BAC is currently approved for use as a component of adhesives for food packaging, and for several non-food uses. BAC-containing formulations that are added directly to water or used to treat hard nonporous surfaces, wood, plants or turf are regulated as pesticides. FNC is not subject to regulation as a pesticide when used in processing plants as a processing aid for lettuce or carrots, as it is not being used on a pest.

The intended use of 2% FNC is as a processing aid for lettuce and carrots. Users will be directed to submerge carrots or lettuce in the 2% FNC solution for five minutes and change solutions daily or when the lot number changes (whichever is sooner). Users also will be directed to divert used 2% FNC solution to their water treatment facility before discharge into environmental water to minimize exposure to aquatic organisms.

FNC is nonmutagenic in a bacterial reverse mutation assay in the presence or absence of metabolic activation. In rats, up to 20 mL/kg bw/day of a 2% solution of FNC (maximum dose of 1.92 mg USP-BAC/kg bw/day) does not cause clastogenicity. The NOAEL for 2% FNC in a subchronic (91-day) oral toxicity study in rats is 5000 mg/kg bw/day (the highest dose administered), or 0.48 mg/kg bw/day in terms of BAC content. Guideline studies indicate that the NOAELs for subchronic toxicity of BAC in rats and mice are 70 and 192 mg/kg bw/day, respectively, and for chronic toxicity in dogs, rats and mice are 14, 50 and 82 mg/kg bw/day, respectively. The NOAEL for reproductive toxicity in mice is 30 mg BAC/kg bw/day, and for developmental toxicity in rats and rabbits is 100 mg BAC/kg bw/day and 9 mg BAC/kg bw/day, respectively. In rats and mice, the NOAELs for carcinogenicity were the highest doses given (102 and 259 mg/kg bw/day, respectively). The EPA has concluded that BAC is not a carcinogen.

Use of gloves and respiratory protection will be recommended for workers handling Free N Clear™ concentrate, as the amount of BAC in the concentrate could cause eye, skin or respiratory irritation or skin sensitization. The concentrations of BAC in carrots and lettuce exposed to FNC for three hours are 13.163 and 18.166 ppm, respectively. The residual amounts of BAC remaining on the lettuce and carrots are estimated to be a worst case scenario for use of 2% FNC as a processing aid, as the recommended processing time for treatment is five minutes, rather than three hours. Combining the 90th *percentile* consumption of BAC retained on lettuce and carrots processed with 2% FNC (0.0398 mg/kg bw/day) with the current 90th *percentile* current consumption level of BAC from conventional foods (0.0132 mg/kg bw/day), the estimated 90th *percentile* intake of BAC from conventional foods is 0.0530 mg/kg bw/day. This theoretical intake level represents a conservative estimate because it is unlikely that an individual would consume BAC at its current estimated 90th *percentile* exposure level plus the 90th p*ercentile* exposure level from lettuce and carrots processed with 2% FNC. Even this conservative estimate, however, is 8 - 9 times less than the EPA's oral concern level of 0.44 mg BAC/kg bw/day and the NOAEL for the 91-day rat study for 2% FNC in terms of BAC (0.48 mg BAC/kg bw/day), 264 times the lowest NOAEL for chronic toxicity of BAC (14 mg/kg bw/day in dogs), 1925 times the lowest NOAEL for carcinogenicity of BAC (102 mg/kg bw/day in rats) and 167 times the lowest NOAEL for reproductive or developmental toxicity of BAC (9 mg/kg bw/day in rabbits).

B. Safety Studies on Methyl Paraben

Mythel Paraben is listed by FDA as GRAS

Methyl paraben is widely used as a preservative in food products, cosmetics and over-the counter (OTC) pharmaceutical formulations. It can be used alone or in combination with other parabens, or with other antimicrobial agents. Methyl paraben is GRAS when used as a chemical preservative in foods at a limit of 0.1% (1000 ppm)8 under cGMP (21 CFR §184.1490). As the concentration of methyl paraben in a 2% solution of FNC is encompassed by the GRAS affirmation for methyl paraben, methyl paraben is considered GRAS by reference. Therefore, in this dossier, information about methyl paraben will be limited to a discussion of the level utilized in the formation of FNC, and testing by Kansas State University Food Science Institute showing bacteria kill and residual parts per million.

Acute toxicity

The dose of Free N Clear^{TM} (or a 2% FNC solution) that causes 50% mortality in rats or mice (LD50 value) has not been determined. A preliminary dose range finding test for a micronucleus study in rats showed that Sprague Dawley rats to not die or exhibit clinical signs of distress after daily gavage exposure to 20 ml/kg bw of a 2% FNC solution for three days (which delivers a dose of approximately 1.92 mg USP-BAC/kg bw/day.

Subchronic toxicity

The subchronic (91-day) oral toxicity of a 2% FNC solution has been tested in a study performed in accordance with the OECD Guideline No. 408. Groups of ten Sprague-Dawley rats/sex were administered 0.5, 1.0 or 5.0 mL/kg bw/day (500, 1000 or 5000 mg/kg bw/day)[29] of the 2% FNC solution by gavage, once daily for 91 days. The test substance was administered as received (1:50 dilution of FNC concentrate in water). A control group of ten rats/sex was administered 5.0 ml/kg bw/day reverse osmosis deionized water by gavage. All animals survived until study termination and there were no test article-related clinical or ophthalmologic findings. There were no statistically significant differences in food consumption between treated animals or controls at any time. A statistically significant increase in mean body weight of males provided 500 mg/kg bw/day ($p < 0.05$) was observed on Day 36, but at no other time point. Mean body weight of females given 5000 mg/kg bw/day were slightly lower (1 - 5%) than control at most time points; however there were no statistically significant differences between mean body weights of control or treated females (any dose group) at any time point. Mean body weight gains of males given 500 mg/kg bw/day were significantly higher than control ($p < 0.05$) from Days 1 to 8 and mean body weight gains of females exposed to 5000 mg/kg bw/day were significantly lower than control ($p < 0.05$) from Days 8 to 15. Due to their isolated nature, small magnitude, and lack of dose response or similar directional change in both sexes, any differences in body weight or body weight gain between control and treated males or females are not considered to be related to administration of the test material. There were no toxicologically relevant changes in hematology or coagulation parameters, clinical chemistry or urinalysis. There was an increase in the number of high dose (5000 mg/kg bw/day) males or females exhibiting nitrite-positive urine (a marker for urinary

tract infection). Animals that exhibited nitrite-positive urine did not have corresponding increases in numbers of bacteria or white blood cells in urine, suggesting that the nitrite results were falsely positive for bacterial infection. It is possible that the positive nitrite response was due to an end-product of BAC metabolism. The only statistically significant change in any parameter was a decrease in serum aspartate aminotransferase (AST) in females provided 1000 mg/kg bw/day compared to controls. Decreases in liver enzymes are not considered toxicologically relevant and the mean values were well within the range of historical control values for similar-aged rats of the same strain. There was no toxicological effect of the test material on organ weights, gross pathology or histopathology. The only statistically significant differences in organ weights between treated and control animals were decreased heart/body weight and lung/body weight ratios of males given 500 mg/kg bw/day and increased epididymides/brain weight ratio of males given 5000 mg/kg bw/day (58.6 ± 9.1 in control and 67.2 ± 4.4 in treated). All differences in organ weight were considered to be spurious or attributed to normal biologic variation given their lack of microscopic correlates and dose-response relationship. Incidences of gross and microscopic findings were similar between groups. One female in the control group showed evidence of gross changes in the abdominal cavity, spleen and pancreatic and mediastinal lymph nodes, and inflammation in the spleen, lung, liver and lymph nodes. The etiology of the pathological findings in this animal was not apparent. All gross and microscopic findings were considered to be incidental or spontaneous background changes of no toxicologic significance. Results of the study show that the subchronic (91-day) oral no observable adverse effect level (NOAEL) of a 2% FNC solution is 5000 mg/kg bw/day in rats, the highest dose administered. The daily dose of USP-BAC that was delivered to rats exposed to the 5000 mg/kg bw/day dose of 2% FNC solution (the NOAEL), was 0.48 mg BAC/kg bw/day.

Genotoxicity- Free N Clear™ Solution

Free N Clear™ was non mutagenic in a bacterial reverse mutation (OECD Guideline 471) assay using a plate incorporation (experiment 1) and a preincubation (experiment 2) method. Strains used in the study included *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 and *Escherichia coli* (*E. coli*) WP2uvrA. Each assay was conducted in the presence and absence of metabolic activation with S9 mix prepared from the S9 microsomal fraction of the livers of

Aroclor 1254-treated adult, male Fischer rats. The test substance (a 2% solution of FNC) was diluted in saline (0.9%) solution and added to plates within one hour of dilution. Preliminary toxicity tests using *S. typhimurium* TA100 indicated that the maximum concentrations of test material that would not produce excessive cytotoxicity were 100% (undiluted) in the presence of S9 mix and 50% in the absence of S9 mix. Therefore, these concentrations were selected as the highest test concentrations with or without S9 (respectively). Additional concentrations tested in the presence or absence of S9 were 50%, 25%, 12.5%, 6.25%, 3.13% and 1.56%. Saline (0.9%) was used as the vehicle control. The test substance did not induce any significant or dose- dependent increases in the numbers of revertant colonies in any strain tested in the absence or presence of S9 mix. No precipitation was noted in any strain, under any condition. In the plate incorporation experiment (experiment 1), toxicity occurred in all strains exposed to the 50% concentration in the absence of S9 mix and in strain TA1537 exposed to the 25% concentration in the absence of S9 mix. In the presence of S9 mix, toxicity was observed in stains TA1535, TA1537 and TA100 exposed to 100% test substance. The concentrations that were toxic to cells in experiment 1 were also toxic to the same strains in experiment 2, with additional strains affected by the 25% concentration in the absence of S9 mix. Therefore, concentrations that were tested extended into the toxic range. The test was considered valid because at least two of the vehicle control plates were within historical control values for mean numbers of revertant colonies, at least two of the positive control plates for each strain and activation state exhibited at least 2-fold increases in revertant colonies, and no toxicity or contamination occurred at four or more concentration levels. The ability of Free N Clear™ Solution to cause clastogenicity was tested in a rat micronucleus test that complied with OECD Guideline No. 474. The test substance was a 2% solution of FNC. Groups of male and female Sprague-Dawley rats were dosed by gavage with 0 mL/kg bw (n = 5/sex), 5 mL/kg bw (n = 5 males), 10 mL/kg bw (n = 5 males) or 20 mL/kg bw (n = 7/sex) test substance or 50 mg/kg cyclophosphamide (n = 3 males) at 0, 24, and 48 hours. All doses of 2% solution of FNC used in the study (5 g/kg, 10 g/kg or 20 g/kg[29]) exceeded the limit dose of 2g/kg recommended by OECD Guideline No. 474. Further, the gavage volume of 2% FNC solution used for the 20 mL/kg bw dose (2 mL/100 g bw) was the maximum volume recommended by the Guideline. The 20 mL/kg bw dose of the 2% solution of FNC delivered a BAC dose of 1.92 mg BAC/kg bw. The

vehicle control was water for irrigation (20 mL/kg). No animal deaths or adverse clinical signs occurred in the any of the dose groups. Animals were euthanized 24 hours after the final dose and bone marrow samples were taken from one femur of each animal. Two-thousand polychromatic erythrocytes (PCE) *per* animal were scored for micronuclei and the frequency of micronucleated PCE (MN-PCE) determined. The numbers of micronucleated normochromatic erythrocytes (MN-NCE) in mature red blood cells were also recorded. The PCE/NCE ratio (a measure of systemic toxicity) was determined by counting a minimum total of 1000 erythrocytes (PCE + NCE) *per* marrow preparation. The test was judged positive if any test group exhibited a greater than 10% increase in MN-PCE over the expected historical control range (0.04 ± 0.05%). There was no indication that any of the doses of test material (5, 10 or 20 mL/kg/day) increased the frequency of MN-PCE. The highest MN-PCE frequency recorded for the test item was in the high dose group, where an incidence of 0.03% was observed for male or female animals (or both sexes combined). This range is within the expected negative control range for CD rats. There was no indication of bone marrow toxicity in any of the test item dose groups. The assay was considered acceptable as the MN-PCE frequencies for the negative control rats were within the expected historical range and an adequate positive control response was obtained for at least two animals, as well as the positive control dose group as a whole.

RINSING AFTER EXPOSURE TO FNC

Since industry guide is "no rinse required" on items exposed to quats 200 ppm or less
And FNC never used BAC with a level of 200 ppm, then FNC is non-toxic/no rinse required.

Primary uses (lettuce and carrots) - Uses will be large volume processing locations, 5 – 500 gallons of use solution and residuals are less than 5 ppm methyl paraben and less than 10 ppm of BAC. DISPOSAL of FNC as a processing aid is safe, non-toxic to water regulations of any location.

Reproductive or Developmental Toxicity

The potential for Free N Clear™ to cause reproductive or developmental toxicity has not been tested. Studies conducted prior to adoption of GLP guidelines showed no overt adverse effects

of up to 25 mg/kg bw/day BAC on "fertility"[32] of rats or guinea pigs.Four studies have been conducted to examine the developmental toxicity of BAC (Table 7). An EPA FIFRA guideline two generation reproductive toxicity study in Sprague-Dawley rats has been conducted on a material described as "ADBAC C12-16" in support of an EPA high production volume (HPV) test plan published online in 2011. This test applies to USP-BAC (CAS No. 8001-54-5), as it predominantly contains ADBAC C12-16. A summary of the unpublished study is provided in an appendix to the published EPA HPV test plan for ADBAC. The results of this study are similar to those of the published, non-GLP study conducted by Shelanski (see paragraph above). Doses used in the study were 300, 1000 and 2000 ppm in feed (approximately 22, 73 or 145 mg/kg bw/day, respectively). The author of the summary stated that the no observable effect level (NOEL) for toxicity to parental animals or offspring was 1000 ppm (73 mg/kg bw/day). Effects noted at the lowest observable effect level (LOEL) of 2000 ppm (145 mg/kg bw/day) were reduced body weight or reduced body weight gain of parental animals and pups. Reproductive parameters were not affected by treatment with up to 2000 ppm (145 mg/kg bw/day). Doses of 0.001, 0.05, 0.1, 3, 10 and 30 mg/kg bw BAC (Japanese Pharmaceutical Grade) were tested for the ability to cause developmental toxicity in mice in three separate experiments. In the first experiment, 3, 10 or 30 mg/kg bw/day BAC or vehicle (purified water) was administered by gavage to groups of 9 - 12 pregnant mice from Gestation Days 0 - 6. There was no effect of any dose of BAC on body weight, food consumption or the general appearance of the dams. No significant differences were observed between the control group and the respective treatment groups for pregnancy rate, implantation number, numbers of dead or resorbed fetuses, numbers of viable fetuses, sex ratio or viable fetus body weight. External abnormalities were not observed in any group. The authors stated that there was a trend for decreased pregnancy rate in the 10 and 30 mg/kg bw/day treated groups and a trend for increased numbers of dead and resorbed fetuses in the 30 mg/kg bw/day treated groups; however, values were not statistically significant from controls. In additional experiments, lower doses of 1, 50 or 100 μg/kg bw/day BAC were administered to mice from Gestation Days 0 - 6 and 1 or 50 μg/kg bw/day BAC was administered Gestation Days 0 - 18 (entire pregnancy period). Significant differences were not observed for any variable measured between treated groups and the control group. The authors concluded that the results of their experiments suggested that in mice, exposure to the high dose of BAC (30 mg/kg bw/day) caused inhibition of implantation or abortion, but that doses < 100 μg/kg bw/day (the highest dose

administered in the second experiment) had no effect on reproductive function. However, in the first experiment, the number of implantations, abortions or any other reproductive toxicity parameter measured in the mice exposed to 30 mg/kg bw/day was not significantly different from control. Based on the data reported in the study, the NOAEL for reproductive toxicity in mice is 30 mg/kg bw/day, the highest dose administered in the first experiment.

F. CONCLUSION

Following a critical evaluation of the information available, the Expert Panel has determined that, based on common knowledge throughout the scientific community knowledgeable about the safety of substances directly or indirectly added to food, there is reasonable certainty that BAC is safe under the intended conditions of use, and is therefore Generally Recognized As Safe (GRAS), by scientific procedures, when used as an ingredient of Free N Clear™ (FNC), when a 2% solution of FNC is used as a processing aid on lettuce and carrots.

It is our opinion that other experts qualified by scientific training and experience to evaluate the safety of food and food ingredients would concur with these conclusions.

10. SIGNATURES

(b) (6)

8/9/13
Date

Douglas L. Archer, Ph.D.
Associate Dean for Research, UF/IFAS
University of Florida

(b) (6)

8/12/13
Date

Edward Carmines, Ph.D.
Consultant

(b) (6)

8/15/13
Date

D. Reid Patterson, D.V.M., Ph.D.
Diplomate, A.C.V.P., A.C.L.A.M., A.B.T.
Fellow, A.T.S., I.A.T.P.
Reid Patterson Consulting, Inc.
President

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Part 7 SUPPORTING DATA AND INFORMATION

(a) Safe under conditions of intended use –

1. Remarkable Kill (85.4% in 2% solution with 5 minute exposure)
   * see Kansas State University kill testing; Appendix 3

2. Residual of BAC (<10 PPM) , after kill
   * see Kansas State University (Olathe), testing for residuals; Appendix 2

(b) ALL DATA IS AVAILABLE Safe under conditions of its intended use, as described in accordance with 170.250 (a)(1)

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APPENDIX 1

SAFETY STUDIES

(Published in the Journal of Food Science)

Pages 000052-000060 have been removed in accordance with copyright laws. The removed reference citation is:

Dolan, L. C., Wheeler, J. A. and Burdock, G. A. (2013), **Safety Studies Conducted on a Sanitizing Agent Containing Benzalkonium Chloride,** J Food Sci 2013 Jan 21;78(1):T119-27

APPENDIX 2

RESIDUALS

(Kansas State University, Olathe)

Methyl Paraben Residue and Wash Water Results

Reagents and Standards
An aqueous benzalkonium chloride solution (50% benzalkonium chloride, 10% ethyl alcohol, and 40% water), powdered 100% methyl paraben, and 100% acetic acid were provided by Marvel Technologies for use with romaine and iceberg lettuce residue analysis and method validation. All solvents were purchased from Fisher Scientific (Waltham, MA) and classified as UPLC UV grade and suitable for mass spectrometry. Two milliliter glass vials and DisQuE™ QuEChERS extraction materials (AOAC certified) were purchased from Waters (Milford, USA). One milliliter plastic syringes and 0.2 micron filters were purchased from VWR International (Radnor, USA).

Wash Water Analyses

Benzalkonium Chloride

Chromatographic System

The operating procedure for determining free benzalkonium chloride in wash solutions was based on a previous vegetable extraction method by Diez et al. (2016) and adapted for aqueous solutions. A Waters Acquity UPLC with a HSS T3 1.8-µm 2.1 x 100mm analytical column, set at a temperature of 35°C, was used to facilitate separation of benzalkonium chloride. The mobile phases contained (A) water and (B) methanol, each containing 0.1% of formic acid to improve ionization, and ran isocratically with a flow rate of 0.613mL/minute. Each injected sample ran for a total of 4 minutes. A 1 µL loop was used for injection. The chromatographic system was equipped with a binary solvent manager, and a photodiode array detector (PDA), set at 258nm, which was used to detect benzalkonium chloride. Empower 3 Software from Waters was used to identify and quantitate samples.

Creating Wash Solutions

Wash solutions were made according to the directions of Marvel Technologies USA, LLC (Franklin, TN). Benzalkonium chloride (0.04 pounds), methyl paraben (0.04 pounds), and acetic acid (0.08 pounds) were added to 0.5 gallons of water, where the volume was filled to 1 gallon, to create a 100% solution of Free N Clear® (FNC). 1%, 2%, and 3% wash solutions were made accordingly to the volume of water used during washing.

Results of Wash Solutions from Pathogen Reduction Study (all results are in parts per million).

Inoculation Study - Benzalkonium Chloride					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	53.05	52.49	58.03	54.52	3.05
1% Wash Post Wash	50.20	49.60	53.94	51.25	2.35
2% Wash Pre Wash	83.69	87.43	92.83	87.98	4.60
2% Wash Post Wash	77.75	81.70	88.37	82.61	5.37
3% Wash Pre Wash	123.67	121.40	138.02	127.70	9.01
3% Wash Post Wash	115.01	115.70	138.94	123.22	13.62

Results of Wash Solutions from Residue Study (all results are in parts per million).

Residue Study - Benzalkonium Chloride					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	67.86	68.25	63.36	66.49	2.72
1% Wash Post Wash	65.19	50.24	59.56	58.33	7.55
2% Wash Pre Wash	112.05	102.35	116.37	110.26	7.18
2% Wash Post Wash	106.15	98.72	111.22	105.36	6.29
3% Wash Pre Wash	170.11	166.91	178.57	171.86	6.02
3% Wash Post Wash	161.07	153.72	151.23	155.34	5.12

Methyl Paraben

Chromatographic System

Analyzing and determining the concentration of free methyl paraben in wash solutions was based on a HPLC method from Marvel Technologies and adapted for a Waters Acquity UPLC. A Waters BEH C18 1.7µm 2.1 x 100mm was employed to facilitate separation of methyl paraben. The mobile phases contained (A) water and (B) methanol, each containing 0.1% formic acid to improve ionization, and ran isocratically with a 50% mixture of each mobile solvent. The flow rate was 0.400 mL/minute. A 1 µL loop was used for injection. The chromatographic system was equipped with a binary solvent manager, and a photodiode array detector (PDA), set at 254nm, which was used to detect methyl paraben.

Results of Wash Solutions from Pathogen Reduction Study (all results in parts per million).

Inoculation Study - Methyl Paraben					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	45.95	66.93	77.11	63.33	15.89
1% Wash Post Wash	67.81	61.80	71.83	67.15	5.05
2% Wash Pre Wash	127.99	135.35	136.37	133.24	4.57
2% Wash Post Wash	114.84	114.62	130.21	119.89	8.94
3% Wash Pre Wash	189.55	182.04	229.00	200.20	25.23
3% Wash Post Wash	186.77	170.66	201.77	186.40	15.56

Results of Wash Solutions from Residue Study (all results are in parts per million).

Residue Study - Methyl Paraben					
	Rep 1	Rep 2	Rep 3	Mean	Standard Deviation
1% Wash Pre Wash	95.45	101.18	90.41	95.68	5.39
1% Wash Post Wash	94.27	100.07	88.45	94.26	5.81
2% Wash Pre Wash	183.19	165.22	160.79	169.73	11.86
2% Wash Post Wash	158.91	164.35	154.31	159.19	5.03
3% Wash Pre Wash	276.51	286.34	289.57	284.14	6.80
3% Wash Post Wash	233.22	275.02	232.93	247.06	24.22

Residue Analyses

Benzalkonium Chloride

Chromatographic System

The procedure for determining benzalkonium chloride on the surface of romaine and iceberg lettuce leaves was based on a published paper by Diez et al. A Waters Acquity UPLC with an Atlantis® T3 3.0-µm 2.1 x 100mm analytical column was used to separate benzalkonium chloride. The flow rate was set at 0.400mL/ minute and the column temperature was set at 40°C. The mobile phases contained (A) water and (B) methanol, each containing 0.1% of formic acid to improve ionization. A gradient program was used and started at 35% A and gradually went to 0% A (100% B) over the first three minutes. This was held for four minutes. Over the next one minute, Mobile phase A was slowly brought back to 35% and held there for four minutes for a total run time of 12 minutes. The 12 minute run time was set with the aim of removing potential matrix interferences from the column. A 1 µL loop was used for injection. All samples were analyzed using an Acquity QDa detector using mass spectrometer.

Mass Spectrometer Settings

The mass 304 daltons was analyzed and corresponded with the benzalkonium chloride molecule that has a twelve carbon alkyl chain. Ionization was performed in positive mode with a cone voltage of 15 V. The capillary voltage was set at 1.5 kV.

Extraction

The extraction protocol was in accordance with QuChERS. A 5 g lettuce sample was placed into a Nalgene centrifuge tube followed by the addition of 15 mL of acetonitrile with 1% acetic acid. The lettuce/acetonitrile with acetic acid solution was hand-mixed for one minute. The DisQue™ salt mixture (6 g anhydrous magnesium sulfate and 1.5 g sodium acetate) was added to the solution and then vigorously hand-mixed for thirty seconds. This mixture was the centrifuged at 2,700 RPM at 4 °C for 5 minutes to obtain a well-defined solid-liquid phase separation. A 1 mL aliquot was then transferred to a clean-up tube consisting of 150 mg of magnesium sulfate and 50 mg of a primary secondary amine (PSA) bonded silica. Further centrifuging at 5,000 RPM for 1 minute to get another solid-liquid phase separation. The supernatant was

removed with a plastic syringe and filtered through a 0.20 micron PTFE filter before being placed in a 2-mL glass vial where UPLC analysis could occur.

Analysis of Unknown Samples
A standard curve was made using extraction liquid that included the lettuce matrix. The extraction protocol was followed using unwashed, fresh lettuce and the supernatant from the finished method was used. The supernatant was then spiked with either 5ppm, 10ppm, or 15ppm of benzalkonium chloride and used to generate a standard curve.

Methyl Paraben

Chromatographic System
The same chromatographic settings were utilized as the wash water analysis.

Extraction
The same extraction protocol used for benzalkonium chloride.

Analysis of Unknown Samples
The analysis of unknown samples was the same as benzalkonium chloride.

Methyl paraben residue on romaine lettuce after washing (all results are in parts per million).

Methyl Paraben on Romaine Lettuce Post Washing				
	Rep 1	Rep 2	Rep 3	Mean
Day 0, 0% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 0, 1% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 0, 2% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 0, 3% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 3, 0% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 3, 2% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 7, 0% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Day 7, 2% FNC Wash	<5 ppm	<5 ppm	<5 ppm	<5 ppm
Prewashed Iceberg	<5 ppm			

Methyl paraben residue on iceberg lettuce after washing (all results are in parts per million).

Methyl Paraben on Iceberg Lettuce Post Washing

	Rep 1	Rep 2	Rep 3	Mean
Day 0, 0% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 0, 1% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 0, 2% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 0, 3% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 3, 0% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 3, 2% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 7, 0% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Day 7, 2% FNC Wash	**<5 ppm**	**<5 ppm**	**<5 ppm**	**<5 ppm**
Prewashed Iceberg	**<5 ppm**			

Results

Determining the residual amount of both benzalkonium chloride and methyl paraben, after washing with various concentrations of FNC, was done by creating standard curves to evaluate the peak area of known concentrations. As lettuce contains many compounds that may interfere with detection by the UPLC machine, the standards need to be as representative of the unknown samples as possible. Therefore, romaine lettuce (and iceberg lettuce, respectively) was purchased from the store and extracted in the same manner as the 5-g samples in the shelf life study. The supernatant (i.e. lettuce matrix) of the finished extraction was used as the diluent for the 5-15ppm standards for residue analysis. A 5ppm standard made using extraction supernatant yielded a distinct peak for methyl paraben as shown in figure 3-1. Lower concentration of methyl paraben (e.g. 2.5ppm) were evaluated but no clear, intense peak was generated making 5ppm the limit of detection for residue analysis. As figure 3-2 shows, injecting the lettuce matrix (sans benzalkonium chloride or methyl paraben) yields no peak clarity or intensity. Based on the y-axis, figure 3-2 is zoomed in on background, presumable of lettuce debris. Importantly, even a low concentration of methyl paraben (5ppm) in the lettuce matrix generates a clear peak for residue analysis meaning unknown samples with clear peaks would have some value of methyl paraben greater than 5ppm. As shown in figure 3-4, lettuce subjected to a 3% FNC wash (the highest concentration) yielded a chromatogram with no distinct peaks. In fact, the chromatogram resembles that of figure 3-2 of just the lettuce matrix (sans benzalkonium chloride and methyl paraben) leading to a conclusion that the amount of methyl paraben on lettuce subjected to a 3% FNC wash is near zero. As the lettuce matrix did not have a distinct peak, it could not be included in the standard curve (figure 3-3) so actual concentration of methyl paraben could not be detected lower than 5ppm.

Conclusion

This study found there to be little (<5 ppm) methyl paraben remaining on romaine or iceberg lettuce washed with up to 3% FNC. This is based on the high resemblance of UPLC chromatograms of just lettuce and lettuce subjected to 3% FNC. The residue on washed romaine and iceberg lettuce is therefore acceptable according to CFR Chapter 21 §184.1490.

Figures

Figures 1-1 – 1-4 were used in the determination of the benzalkonium chloride and methyl paraben concentrations in the wash waters used in the pathogen reduction and shelf life study. Figures 3-1 – 3-4 were used in the determination of methyl paraben residue.

SAMPLE INFORMATION

Sample Name:	R1 2%FNC pre wash	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Patho reduc study wash water
Vial:	1:A,8	Acq. Method Set:	BAC Marvel tech method
Injection #:	1	Processing Method	07Sept16 Patho reduc wash
Injection Volume:	1.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	4.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	9/7/2016 12:53:13 PM CDT		
Date Processed:	9/8/2016 12:29:49 PM CDT		

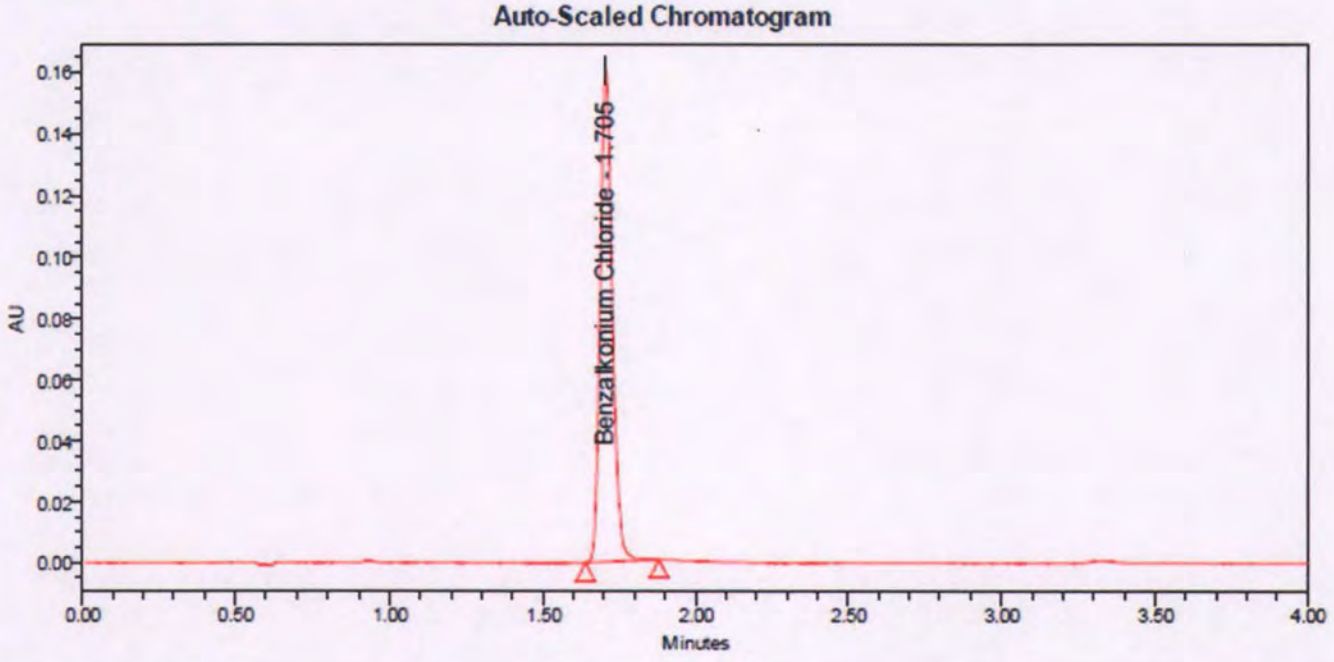

Figure 1-1. The above figure shows an example chromatogram of benzalkonium chloride in FNC wash solutions.

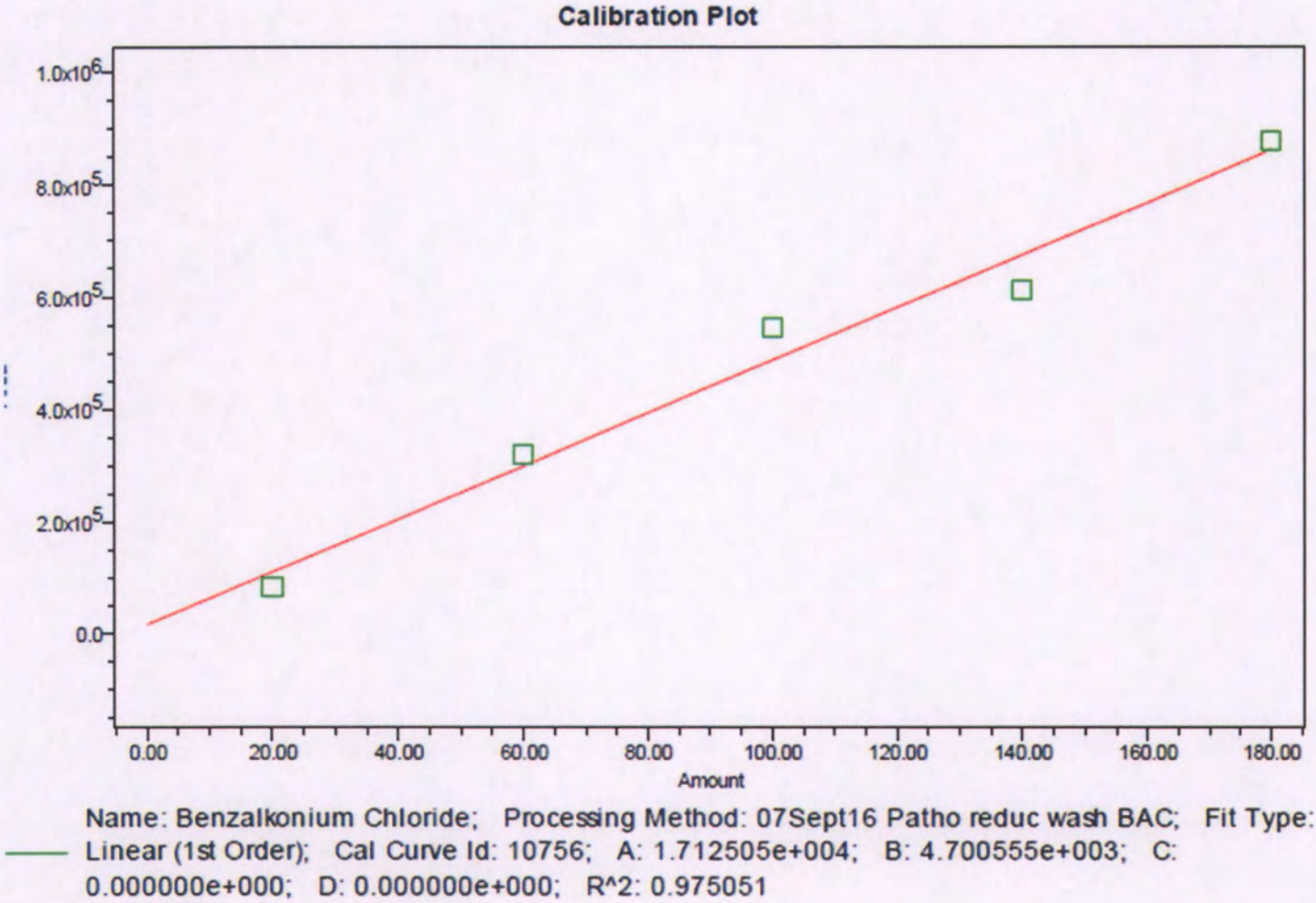

Figure 1-2. The above figure shows the standard curve used to analyze the amount of benzalkonium chloride in FNC wash solutions.

SAMPLE INFORMATION

Sample Name:	R3 1%FNC pre wash	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Patho reduc MP wash water II
Vial:	1:C,2	Acq. Method Set:	BAC Marvel tech method
Injection #:	1	Processing Method	09Sept16 MP wash water inoc
Injection Volume:	1.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	4.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	9/9/2016 2:40:40 PM CDT		
Date Processed:	9/9/2016 4:18:44 PM CDT		

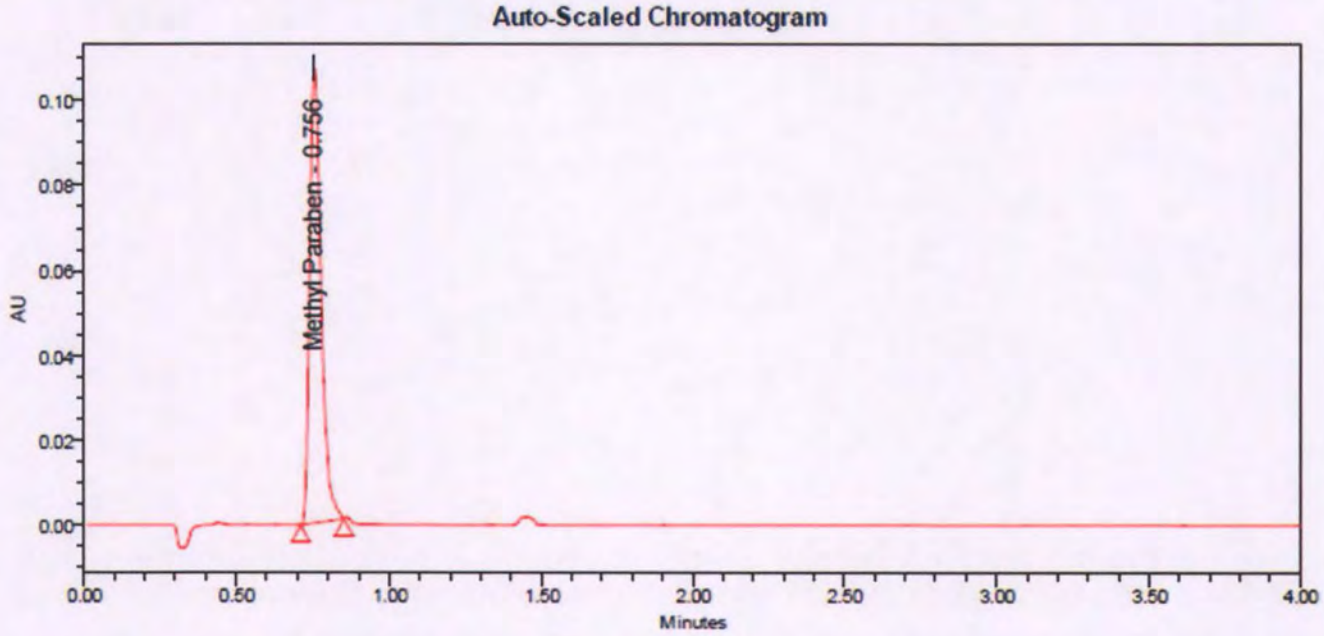

Figure 1-3. This above figure shows an example methyl paraben peak in FNC wash solutions.

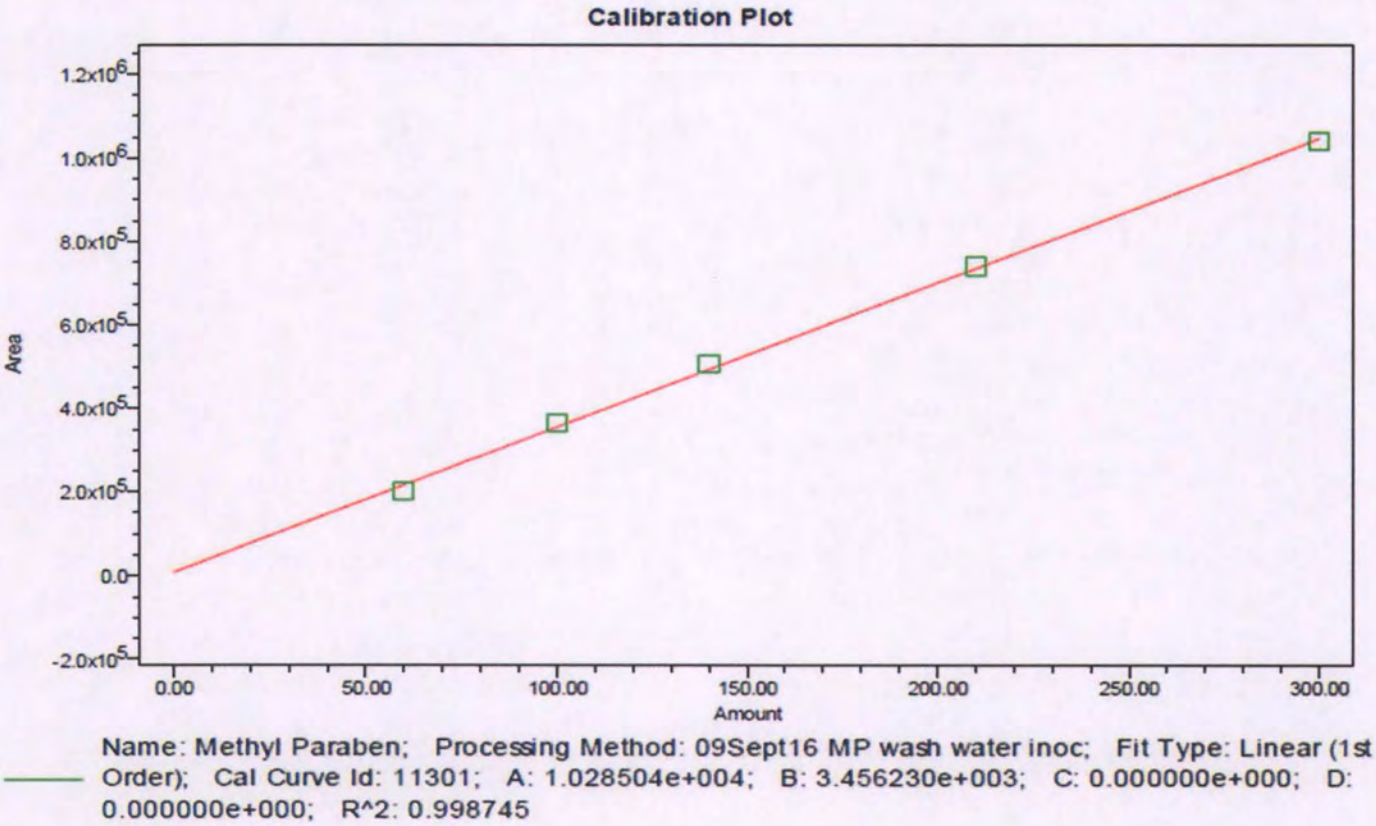

Figure 1-4. The above figure shows the standard curve used to analyze the amount of methyl paraben in FNC wash solutions.

SAMPLE INFORMATION

Sample Name:	5ppm standard	Acquired By:	System
Sample Type:	Standard	Sample Set Name	22Sept Methyl Paraben Residue
Vial:	1:A,3	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	22Sept16 mp resid proc met
Injection Volume:	1.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	9/22/2016 12:56:56 PM CDT		
Date Processed:	9/22/2016 3:26:45 PM CDT		

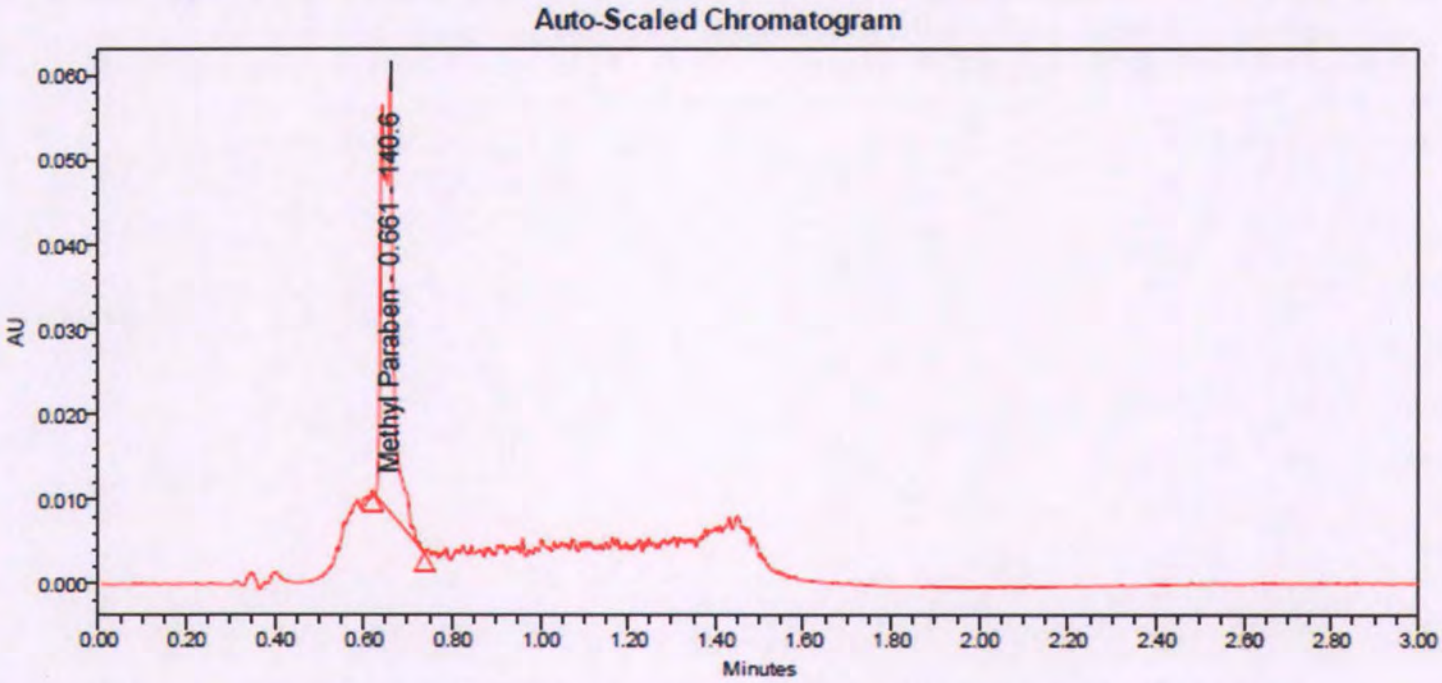

Figure 3-1. Five parts per million methyl paraben standard in lettuce matrix. A clear peak with moderate intensity. Lower concentrations than 5ppm resulted in a lack of peak clarity. As a result, the limit of detection for methyl paraben is 5ppm.

SAMPLE INFORMATION

Sample Name:	0ppm standard	Acquired By:	System
Sample Type:	Standard	Sample Set Name	22Sept Methyl Paraben Residue
Vial:	1:A,1	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	22Sept16 mp resid proc met
Injection Volume:	1.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	9/22/2016 12:52:49 PM CDT		
Date Processed:	9/22/2016 3:25:27 PM CDT		

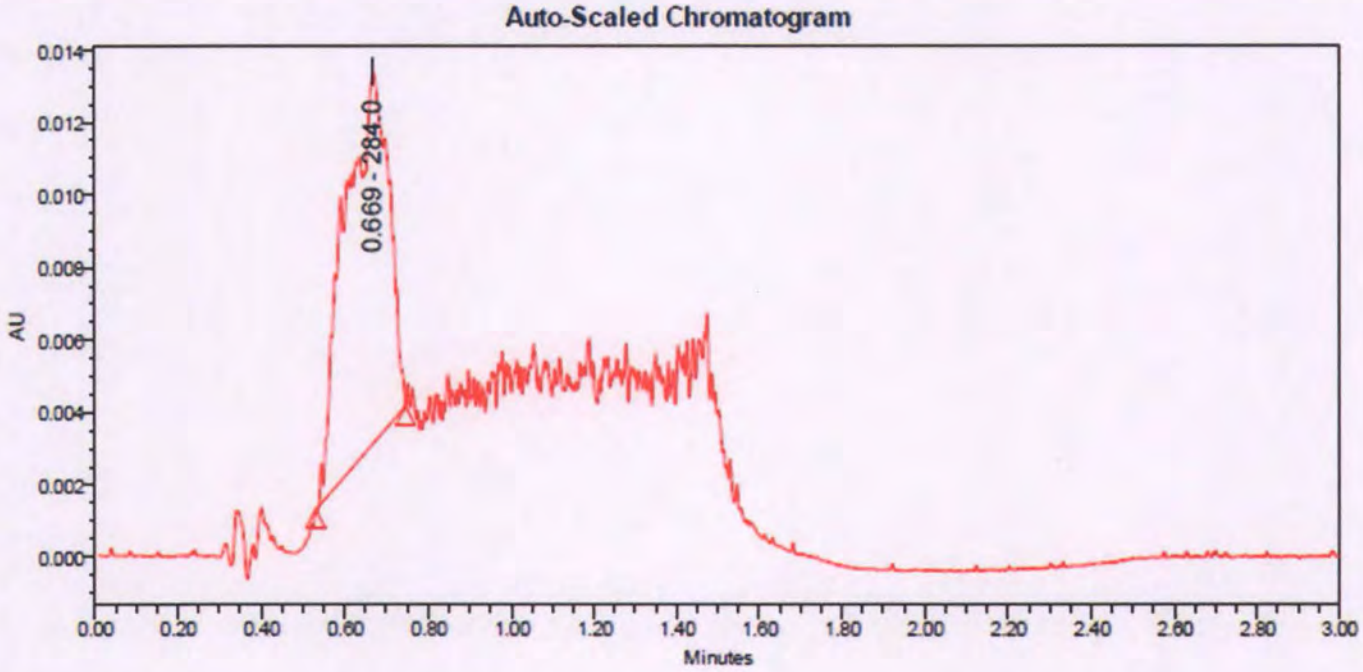

Figure 3-2. Injected lettuce matrix for methyl paraben and benzalkonium chloride residue determination. This injection shows just the contents of lettuce (sans methyl paraben and benzalkonium chloride). There is very low peak intensity and no distinguishable peak.

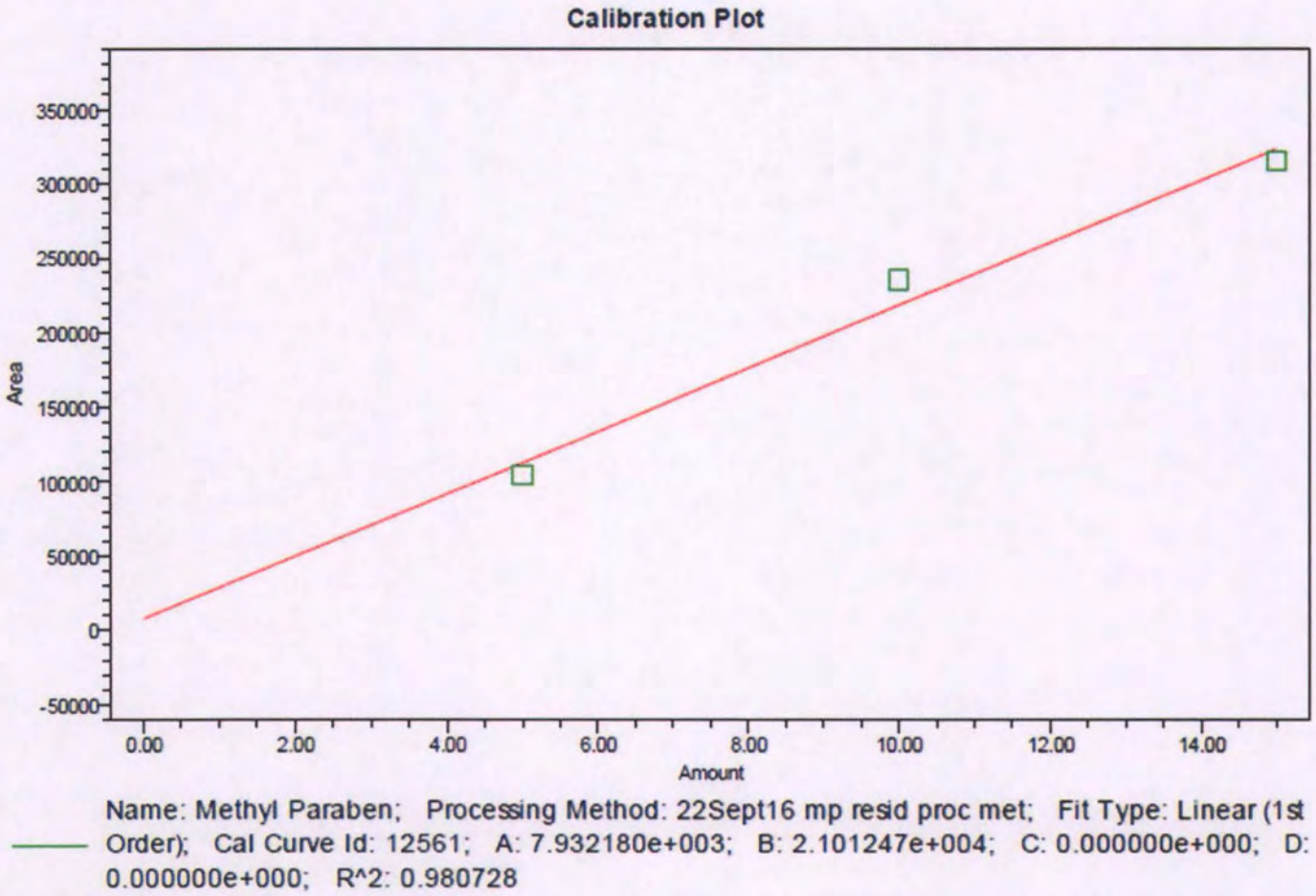

Figure 3-3. Standard curve for methyl paraben residue calculation. As 0 ppm methyl paraben lacked a peak, it could not be included in the standard curve.

SAMPLE INFORMATION

Sample Name:	D0 R1 3% wash Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	22Sept Methyl Paraben Residue
Vial:	1:A,8	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	22Sept16 mp resid proc met
Injection Volume:	1.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	9/22/2016 1:21:45 PM CDT		
Date Processed:	9/22/2016 3:28:44 PM CDT		

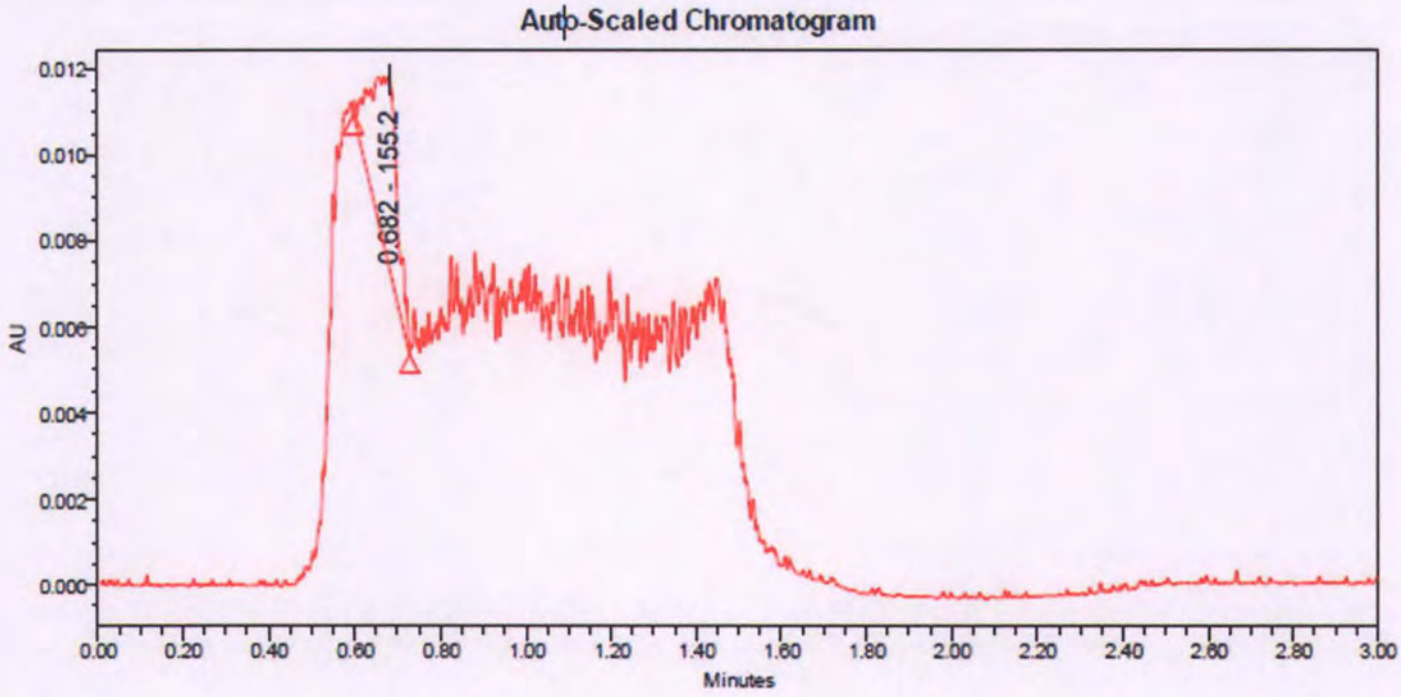

Figure 3-4. This injection shows an example chromatogram from a methyl paraben extraction. This lettuce extracted had been subjected to a 3% FNC wash but shows no discernable peak with intensity. This chromatogram looks very similar as just the lettuce matrix injection, figure 3-2 leading to the conclusion the actual parts per million on the lettuce (per gram) was very close to zero (i.e. <5ppm)

Benzalkonium chloride and methyl paraben residues on fresh-cut romaine and iceberg lettuce subjected to a 2% Free N' Clear™M (FNC) wash for 5 minutes followed by a 1 minute potable water wash

Determining the Amount of Benzalkonium Chloride in Wash Water and on Lettuce

Chromatographic System

The procedure for determining benzalkonium chloride was based on a published paper. A Waters™(Milford, MA) Acquity UPLC with an Atlantis® T3 3.0-µm 2.1 x 100mm analytical column was used to separate benzalkonium chloride. The flow rate was set at 0.400mL/ minute and the column temperature was set at 40°C. The mobile phases contained (A) water and (B) methanol, each containing 0.1% of formic acid to improve ionization. A gradient program was used and started at 35% A and gradually went to 0% A (100% B) over the 5 minute run time. A 1 µL loop was used for injection. All samples were detected using an Acquity QDa detector using mass spectrometry.

Mass Spectrometer Settings

The mass 305.71 daltons was analyzed and corresponded with the benzalkonium chloride molecule that has a twelve carbon alkyl chain. Ionization was performed in positive mode with a cone voltage of 15 V. The capillary voltage was set at 1.5 kV.

Extraction

To accurately quantitate benzalkonium chloride and methyl paraben in fresh-cut romaine or iceberg tissue, an extraction of the compound needed to take place. The extraction protocol was in accordance with QuChERS (Waters, Milford, MA), per the previously published method. A 5g lettuce sample was placed into a 20mL Nalgene (Rochester, NY) centrifuge tube followed by the addition of 15 mL of acetonitrile with 1% acetic acid. The lettuce and acetonitrile with acetic acid solution was hand-mixed vigorously for one minute. A DisQueTM salt mixture(6 g

anhydrous magnesium sulfate and 1.5 g sodium acetate) was added to the solution and then vigorously hand-mixed again for another thirty seconds. This mixture was the centrifuged at 2,700 RPM at 4 °C for 5 minutes to obtain a well-defined solid-liquid phase separation. A 1 mL aliquot was then transferred to a clean-up tube consisting of 150 mg of magnesium sulfate and 50 mg of a primary secondary amine (PSA) bonded silica. Further centrifuging at 5,000 RPM for 1 minute to get another solid-liquid phase separation. The supernatant was removed with a plastic syringe and filtered through a 0.20 micron PTFE filter before being placed in a 2-mL glass vial and injected into the UPLC.

Analysis of Lettuce Samples for Residue

The extraction protocol was followed using unwashed, fresh lettuce and the supernatant from the finished method was used as the diluent for standard making of either methyl paraben and benzalkonium chloride. The supernatant was then spiked with either 10ppm or 15ppm of benzalkonium chloride and used to generate a standard curve and an equation. Standards of 5ppm, 10ppm, and 15ppm were used for methyl paraben analysis. The peak area generated from the unknown sample could then be fit into the standard curve equation to obtain the concentration of benzalkonium chloride or methyl paraben on the lettuce tissue.

Determining the Amount of Methyl Paraben in Wash Water and Lettuce

Chromatographic System

Analyzing and determining the concentration of free methyl paraben in lettuce was based on a HPLC method from Marvel Technologies and adapted for a WatersTM Acquity UPLC. A WatersTM BEH C18 1.7µm 2.1 x 100mm was employed to facilitate separation of methyl paraben. The mobile phases contained (A) water and (B) methanol, each containing 0.1% formic acid to improve ionization, and ran isocratically with a 50% mixture of each solvent. The flow rate was 0.400 mL/minute. A 1 µL loop was used for injection. The chromatographic system was

equipped with a binary solvent manager, and a photodiode array detector (PDA), set at 254nm, which was used to detect methyl paraben.

Benzalkonium Chloride Chromatograms for Determining Residue on Romaine and Iceberg Lettuce

Benzalkonium Chloride Residues on Iceberg Lettuce

Replication 1: <10.0 ppm

Replication 2: <10.0 ppm

Replication 3: <10.0 ppm

Benzalkonium Chloride Residues on Romaine Lettuce

Replication 1: <10.0 ppm

Replication 2: <10.0 ppm

Replication 3: <10.0 ppm

SAMPLE INFORMATION

Sample Name:	0ppm BAC	Acquired By:	System
Sample Type:	Standard	Sample Set Name:	BAC 5min wash residue analysis
Vial:	2:D,1	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 2:47:13 PM CST		
Date Processed:	12/2/2016 4:00:34 PM CST		

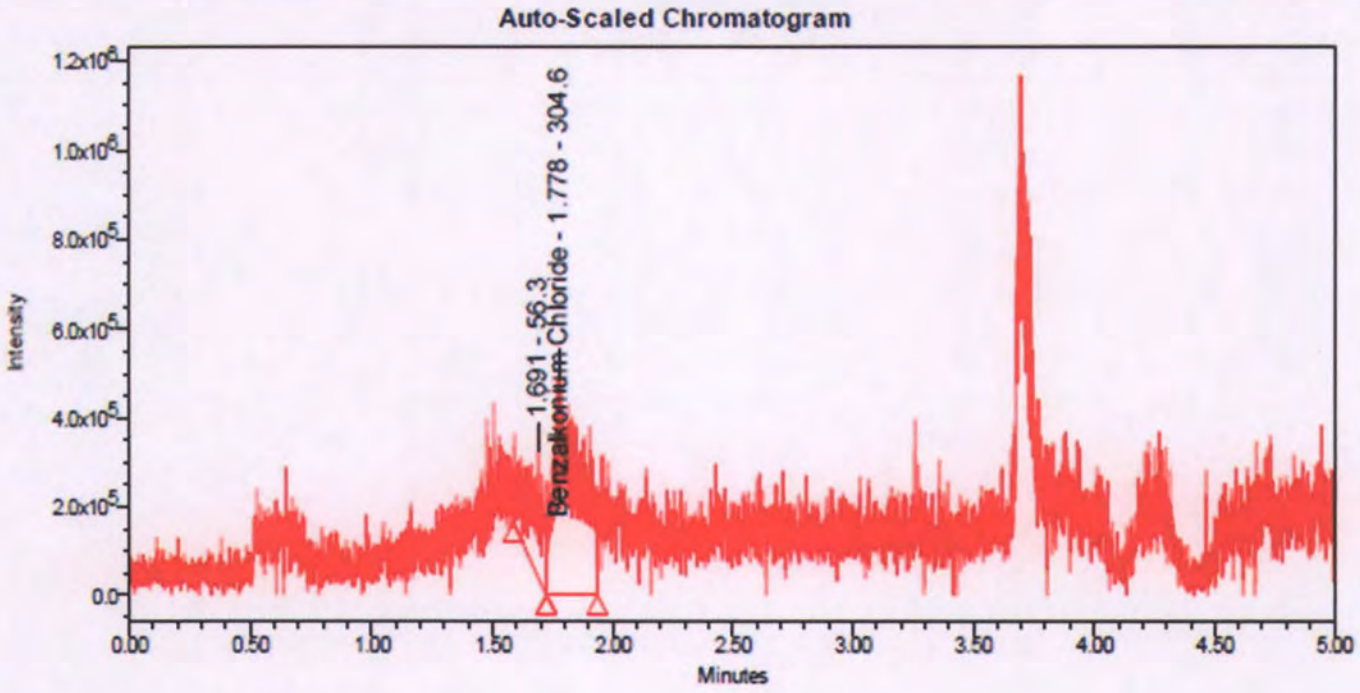

Figure 1-1. Zero parts per million benzalkonium chloride with lettuce matrix.

SAMPLE INFORMATION

Sample Name:	10ppm BAC	Acquired By:	System
Sample Type:	Standard	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:D,3	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 2:59:25 PM CST		
Date Processed:	12/2/2016 4:00:48 PM CST		

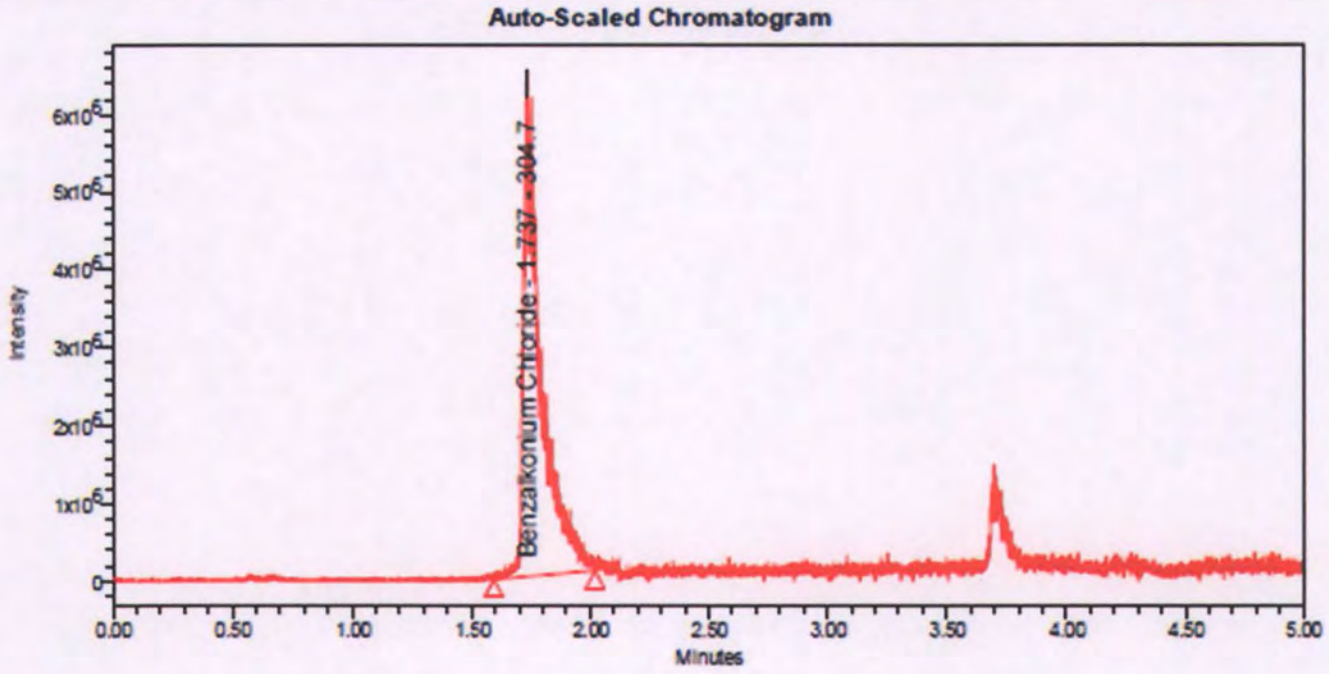

Figure 1-2. Ten parts per million benzalkonium chloride with lettuce matrix.

SAMPLE INFORMATION

Sample Name:	15ppm BAC	Acquired By:	System
Sample Type:	Standard	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:D,4	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 3:05:31 PM CST		
Date Processed:	12/2/2016 3:58:59 PM CST		

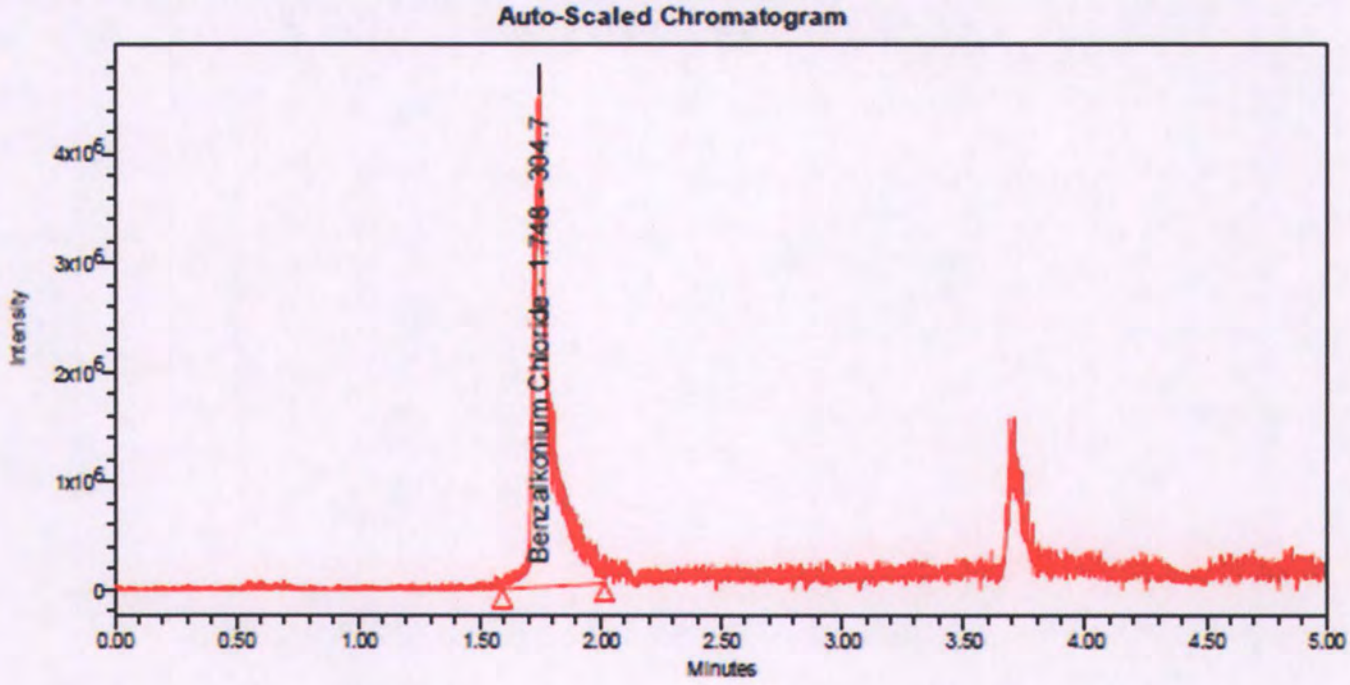

Figure 1-3. Fifteen parts per million benzalkonium chloride with lettuce matrix.

SAMPLE INFORMATION

Sample Name:	R1 Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:D,5	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	305.7Da@3
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 3:11:37 PM CST		
Date Processed:	12/2/2016 5:29:55 PM CST		

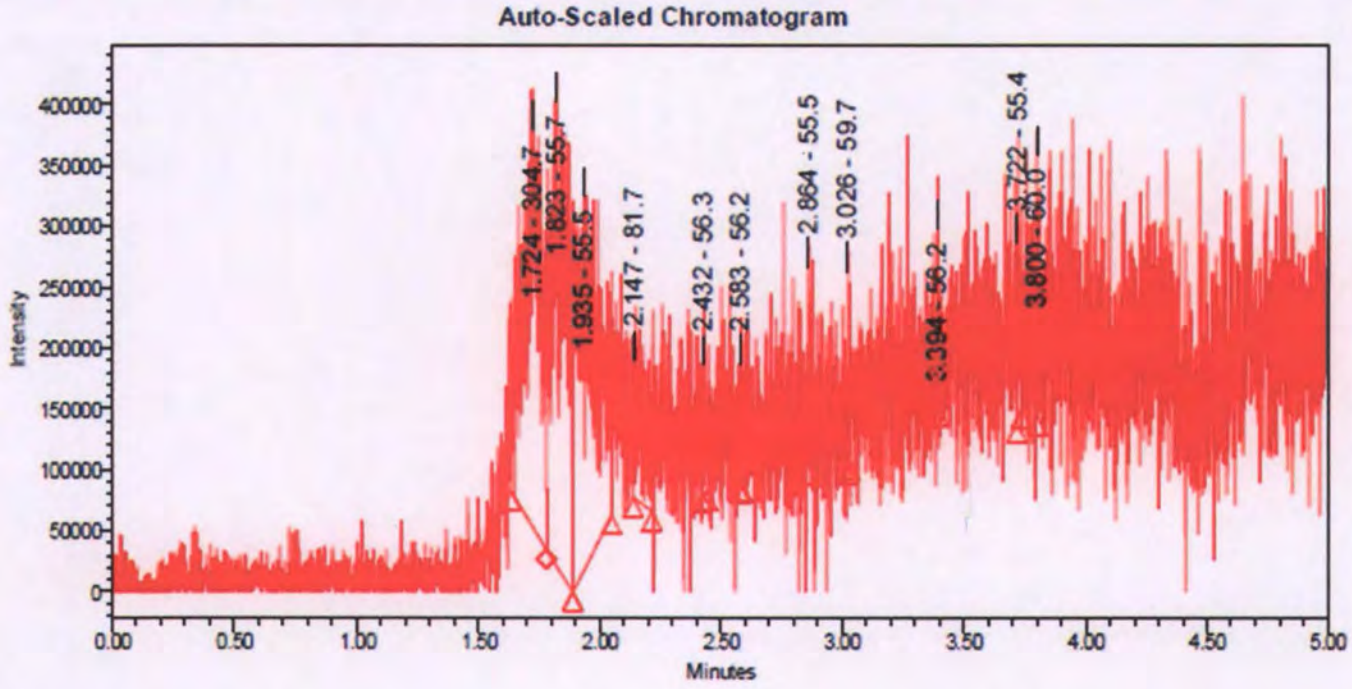

Figure 1-4. Romaine lettuce subjected to 2% FNC for five minutes (Rep 1).

SAMPLE INFORMATION

Sample Name:	R2 Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:D,6	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 3:17:45 PM CST		
Date Processed:	12/2/2016 5:22:41 PM CST		

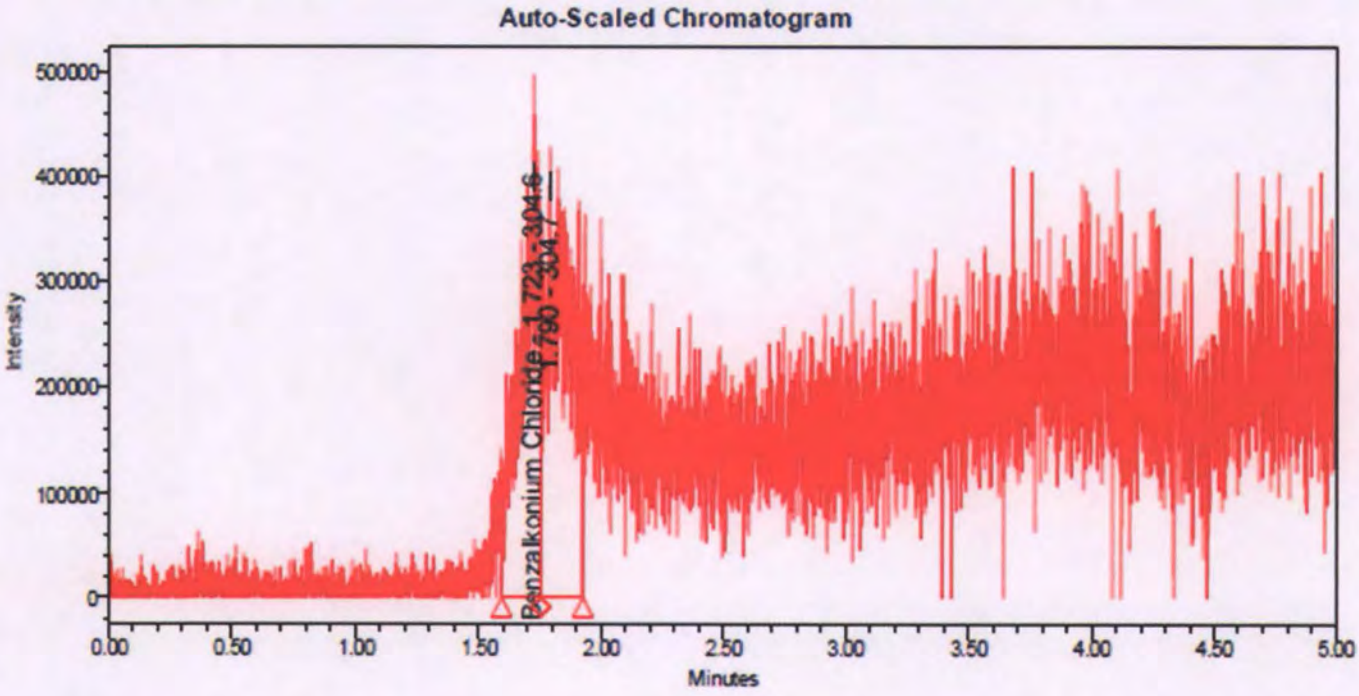

Figure 1-5. Romaine lettuce subjected to 2% FNC for five minutes (Rep 2).

SAMPLE INFORMATION

Sample Name:	R3 Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:D,7	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
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Date Processed:	12/2/2016 4:01:41 PM CST		

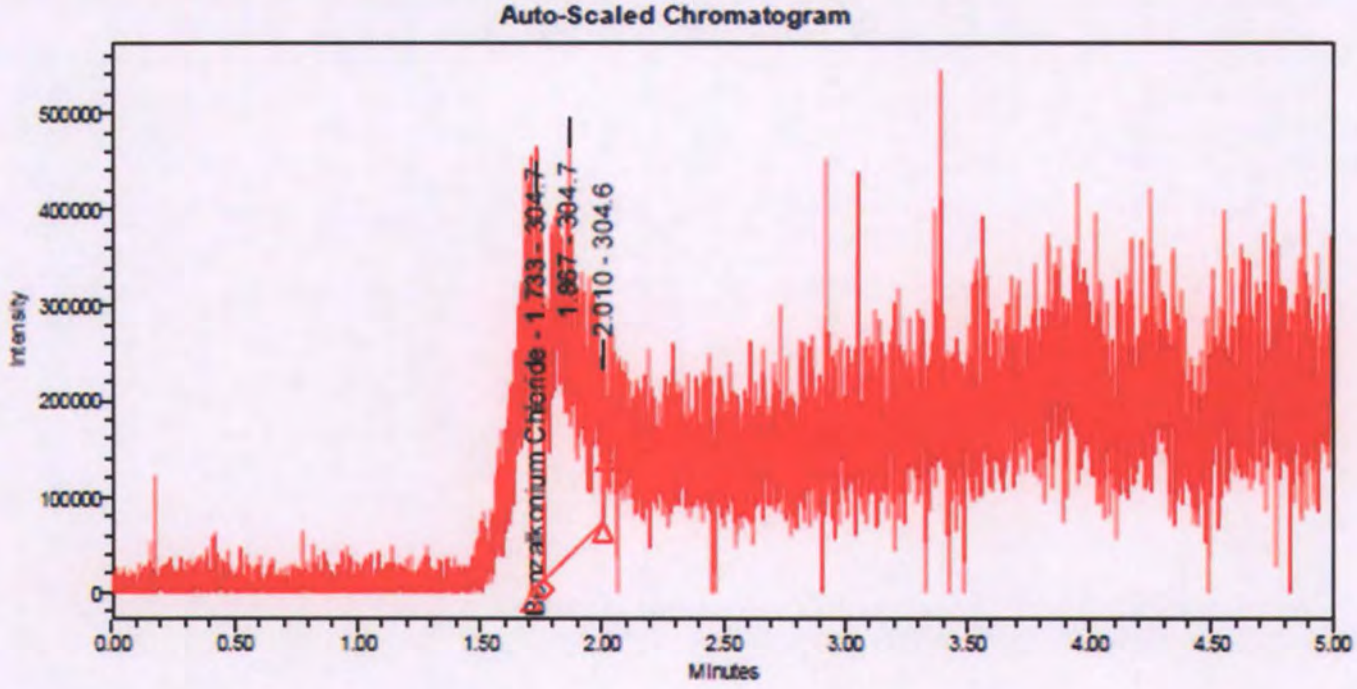

Figure 1-6. Romaine lettuce subjected to 2% FNC for five minutes (Rep 3).

SAMPLE INFORMATION

Sample Name:	R1 Iceberg	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	R1 iceberg Redo II
Vial:	2:D,8	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue R1 Iceberg Rerun
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 4:46:21 PM CST		
Date Processed:	12/2/2016 5:24:55 PM CST		

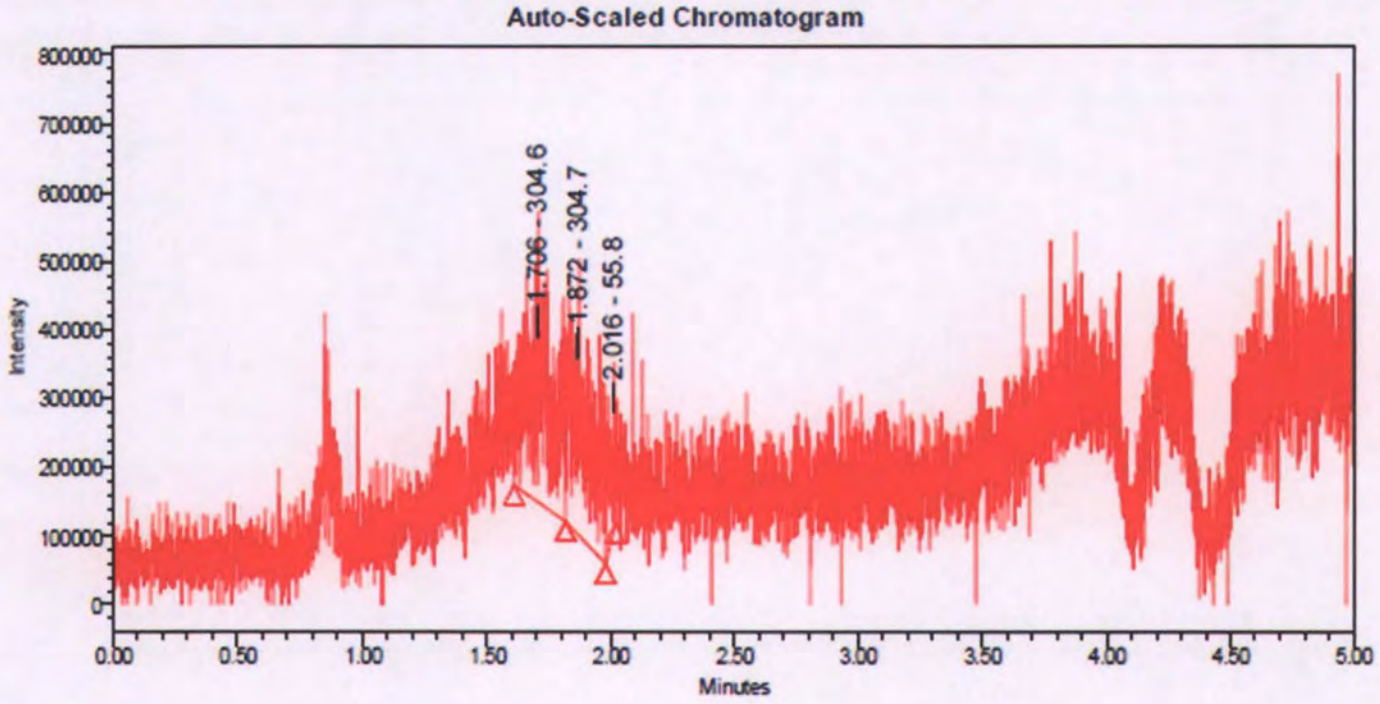

Figure 1-7. Iceberg lettuce subjected to 2% FNC for five minutes (Rep 1).

SAMPLE INFORMATION

Sample Name:	R2 Iceberg	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:E,1	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 3:36:06 PM CST		
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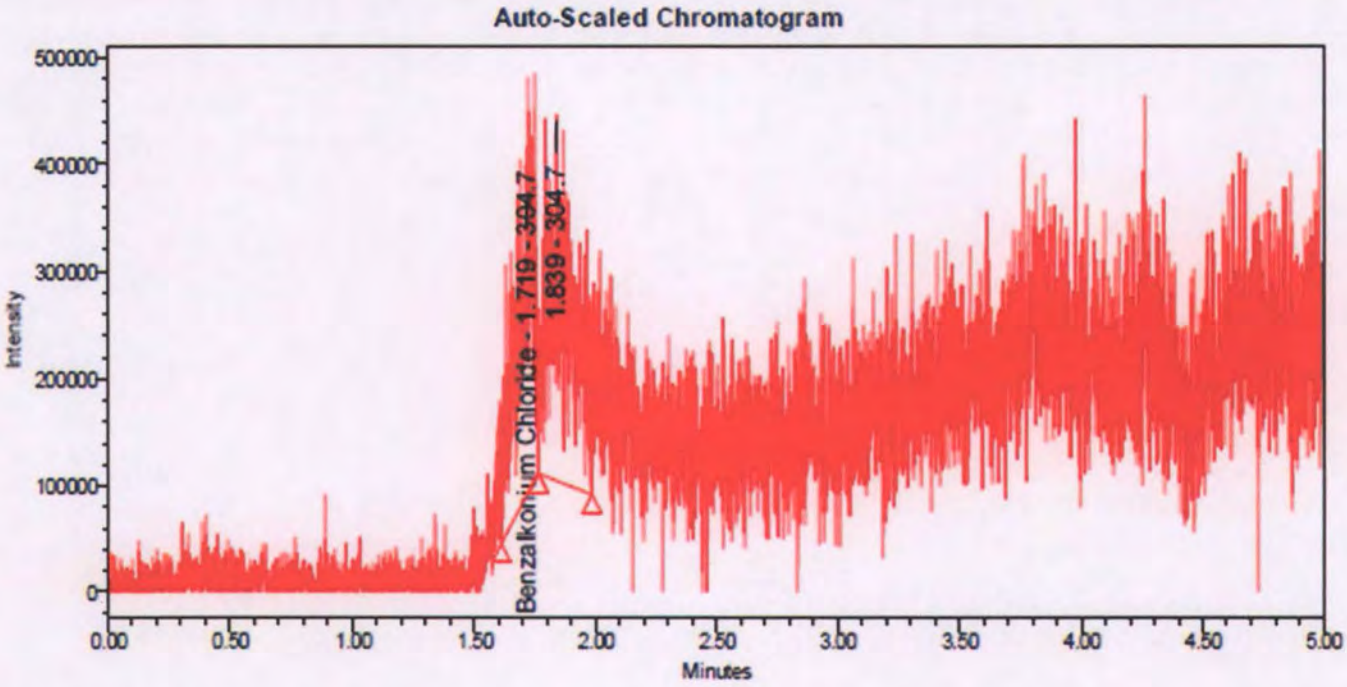

Figure 1-8. Iceberg lettuce subjected to 2% FNC for five minutes (Rep 2)

SAMPLE INFORMATION

Sample Name:	R3 Iceberg	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	BAC 5min wash residue analysis
Vial:	2:E,2	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC residue
Injection Volume:	10.00 ul	Channel Name:	305.7Da
Run Time:	5.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 3:42:11 PM CST		
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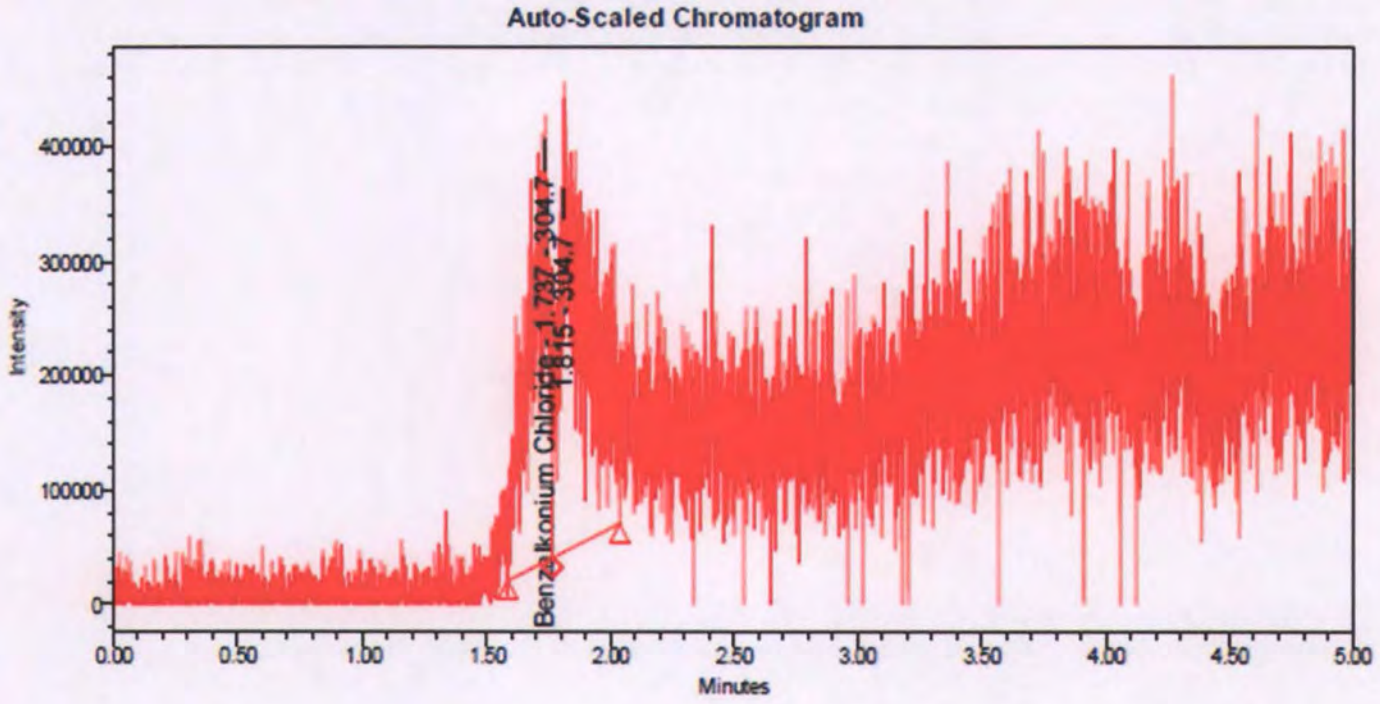

Figure 1-9. Iceberg lettuce subjected to 2% FNC for five minutes (Rep 3).

CONCLUSIONS

Figure 1-1 illustrates a chromatogram of just extracted lettuce (no benzalkonium chloride) that highly resembles figures 1-4 – 1-9. As figure 1-2 illustrates, a benzalkonium chloride concentration of 10 parts per million generates a clear, intense peak. **A conclusion can be drawn that the concentration of benzalkonium chloride remaining on romaine and iceberg lettuce subjected to 2% FNC for 5 minutes is less than 10 parts per million.**

Methyl Paraben Chromatograms for Determining Residue on Romaine and Iceberg Lettuce

Methyl Paraben Residues on Iceberg Lettuce

Replication 1: <5.0 ppm

Replication 2: <5.0 ppm

Replication 3: <5.0 ppm

Methyl Paraben Residues on Romaine Lettuce

Replication 1: <5.0 ppm

Replication 2: <5.0 ppm

Replication 3: <5.0 ppm

Figure 1-10. Zero parts per million methyl paraben with lettuce matrix

SAMPLE INFORMATION

Sample Name:	0ppm	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,1	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	12/2/2016 5:17:18 PM CST		
Date Processed:	12/2/2016 7:46:12 PM CST		

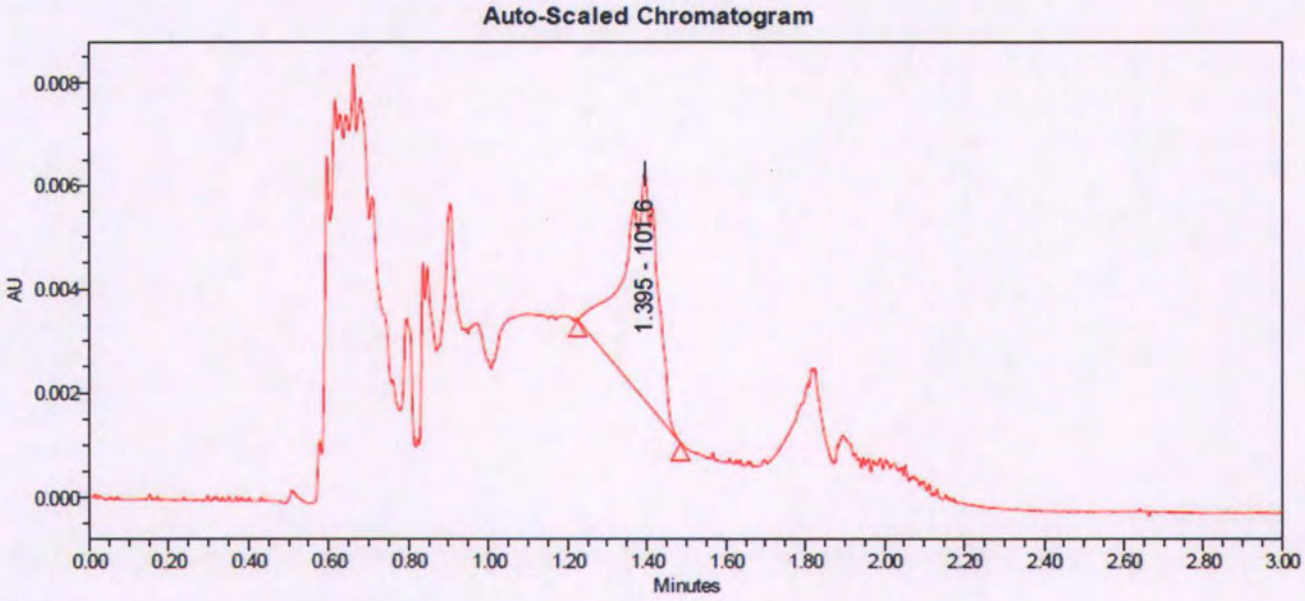

Figure 1-10. Zero parts per million methyl paraben with Lettuce matrix

Empower 3 SOFTWARE

Untitled

SAMPLE INFORMATION

Sample Name:	5ppm MP	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,2	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
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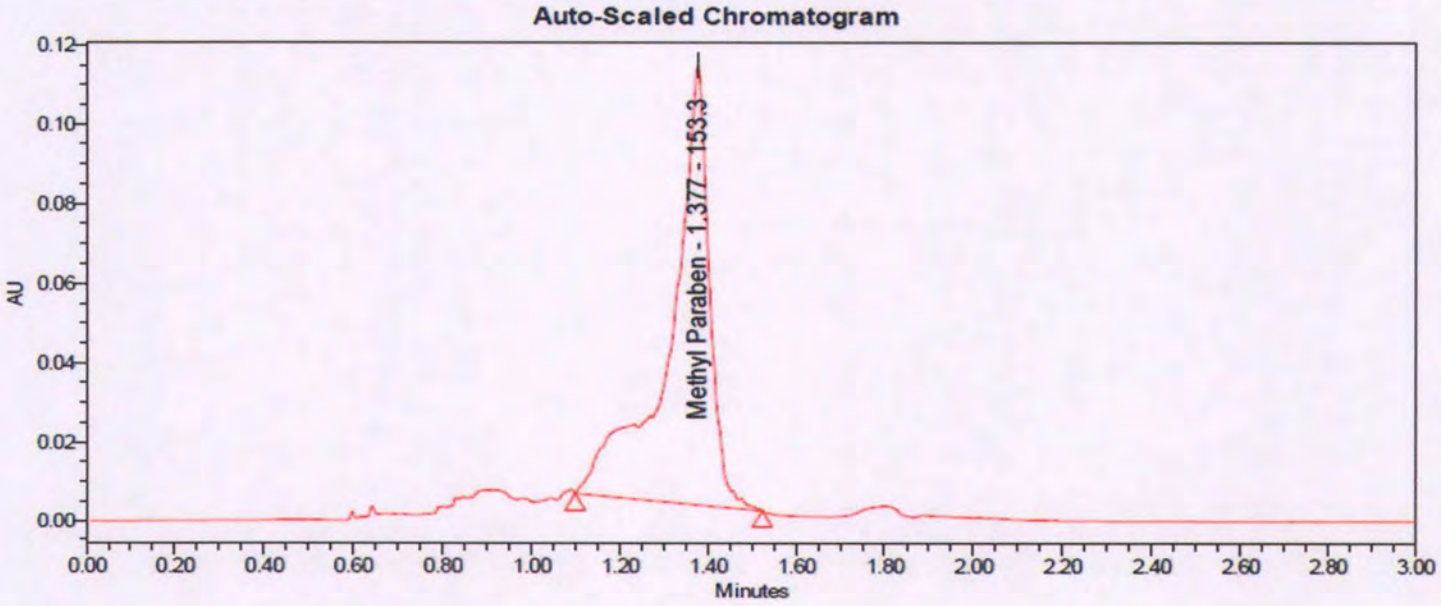

Figure 1-11. Five parts per million methyl paraben with lettuce matrix.

SAMPLE INFORMATION

Sample Name:	10ppm MP	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,3	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
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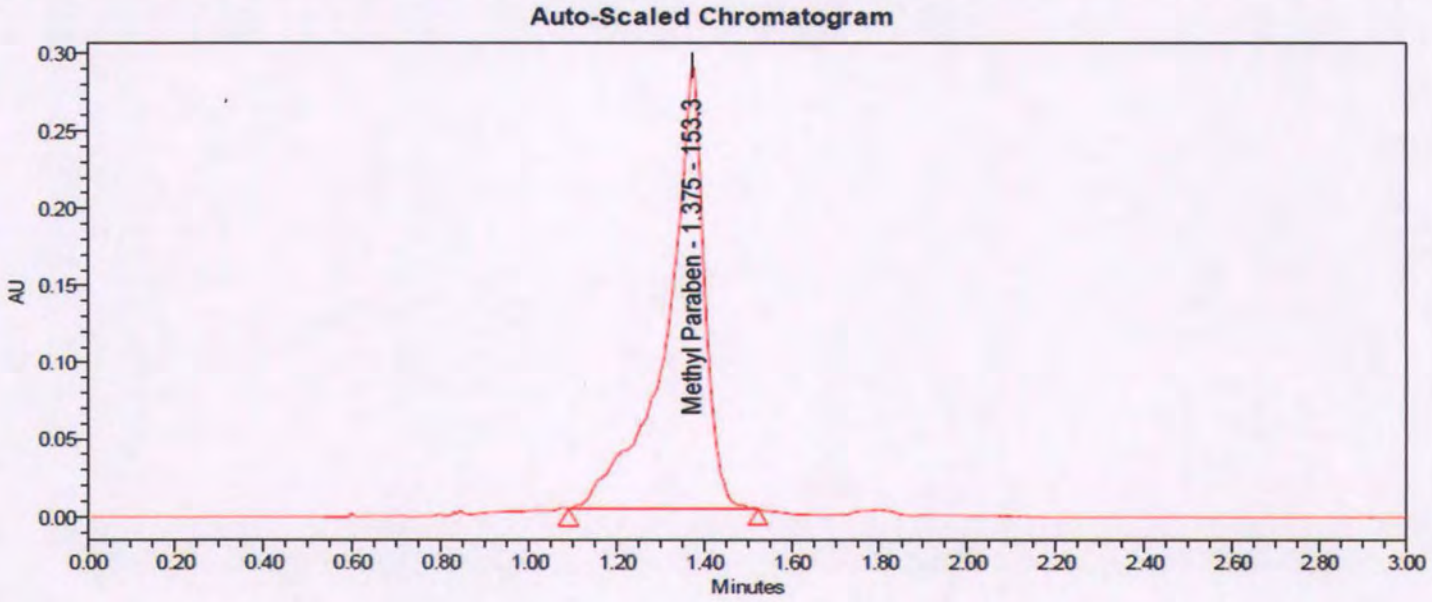

Figure 1-12. Ten parts per million methyl paraben with lettuce matrix.

SAMPLE INFORMATION

Sample Name:	15ppm MP	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,4	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	12/2/2016 5:30:10 PM CST		
Date Processed:	12/2/2016 7:45:10 PM CST		

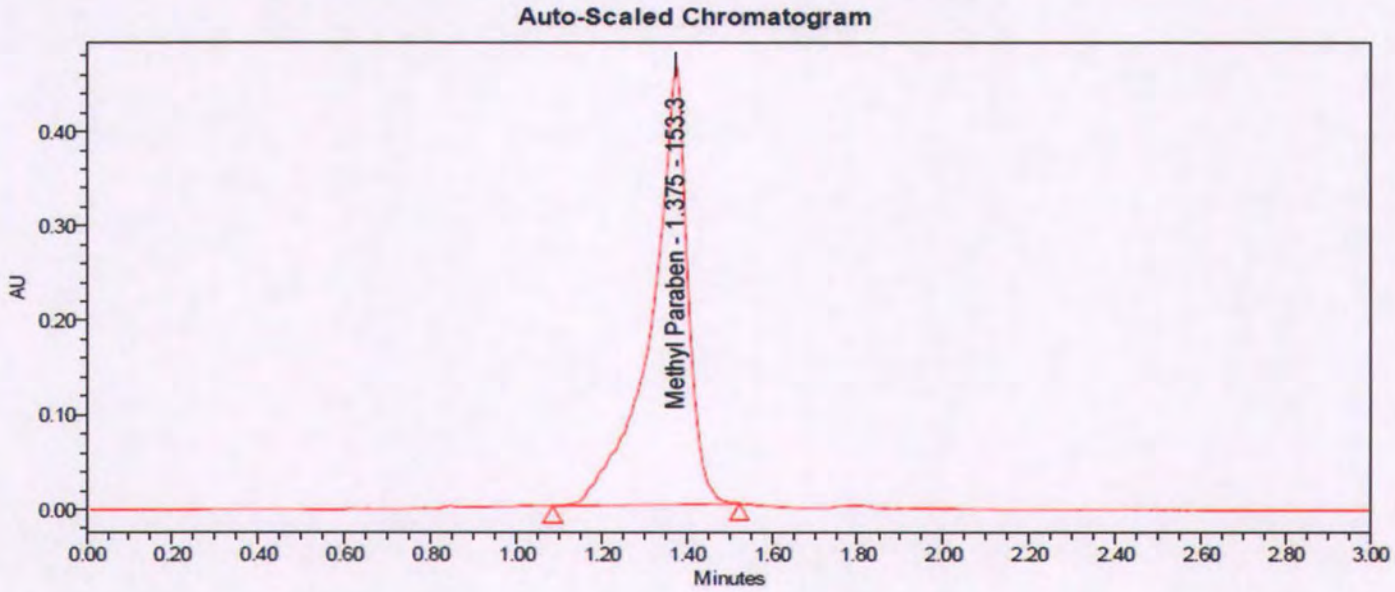

Figure 1-13. Fifteen parts per million methyl paraben with lettuce matrix.

SAMPLE INFORMATION

Sample Name:	R1 Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,5	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	12/2/2016 5:34:29 PM CST		
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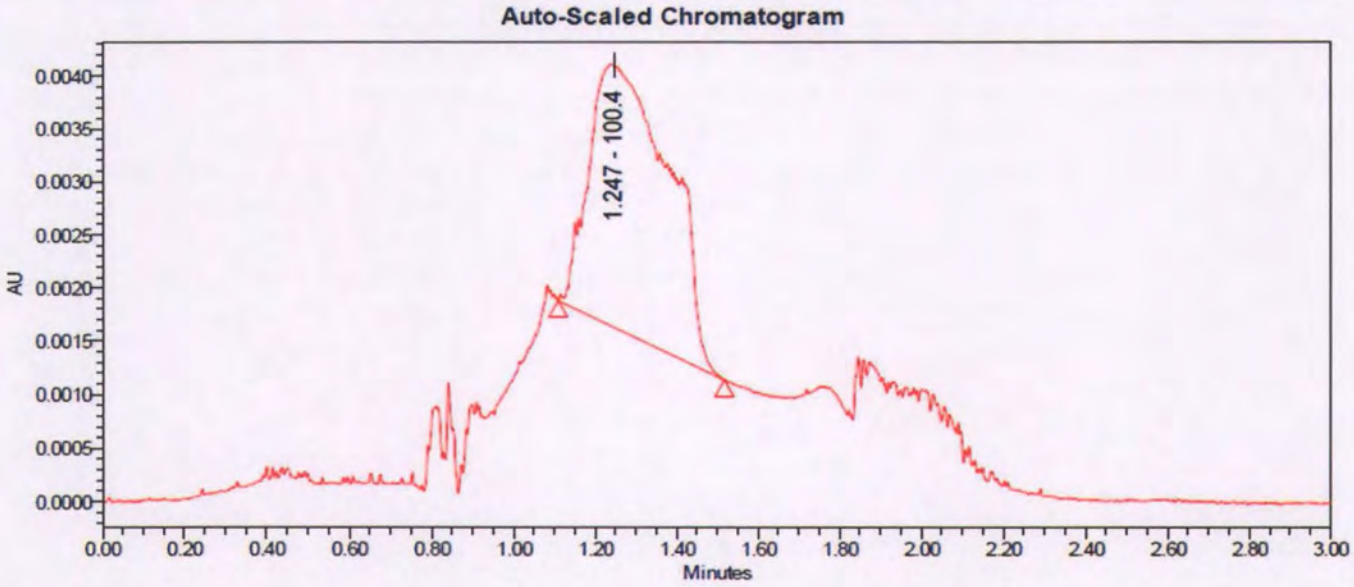

Figure 1-14. Romaine lettuce subjected to 2% FNC for five minutes (Rep 1).

SAMPLE INFORMATION

Sample Name:	R2 Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,6	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
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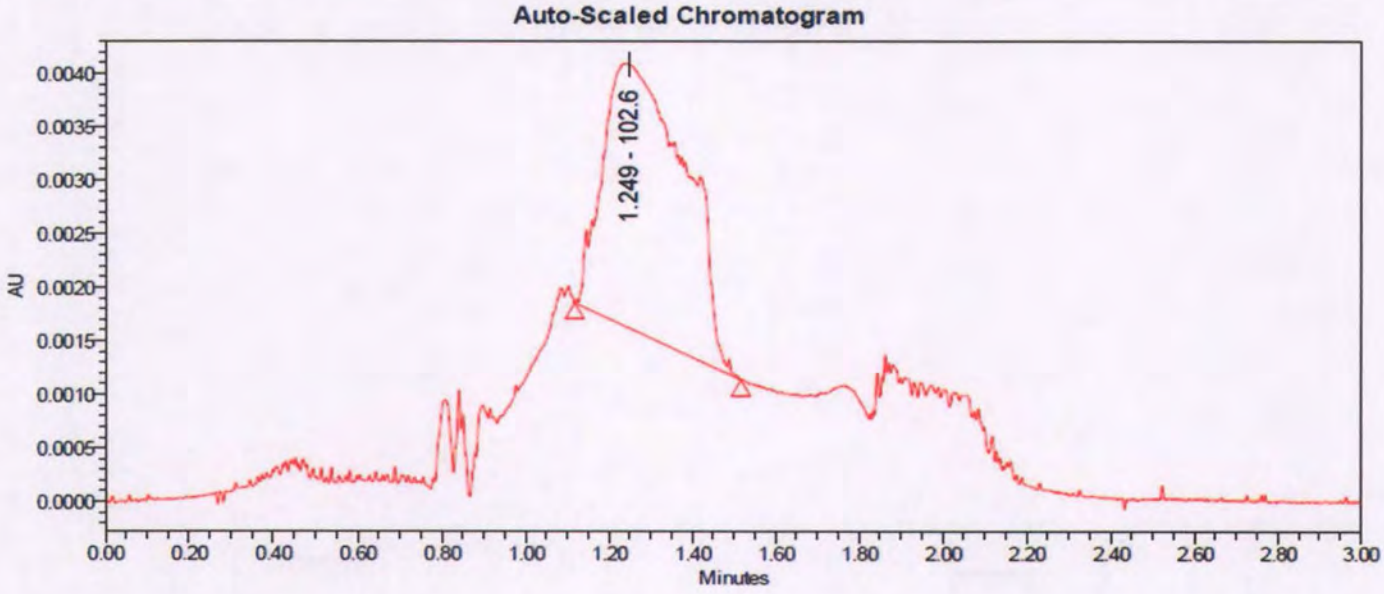

Figure 1-15. Romaine lettuce subjected to 2% FNC for five minutes (Rep 2).

SAMPLE INFORMATION

Sample Name:	R3 Romaine	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,7	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
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Date Processed:	12/2/2016 7:45:34 PM CST		

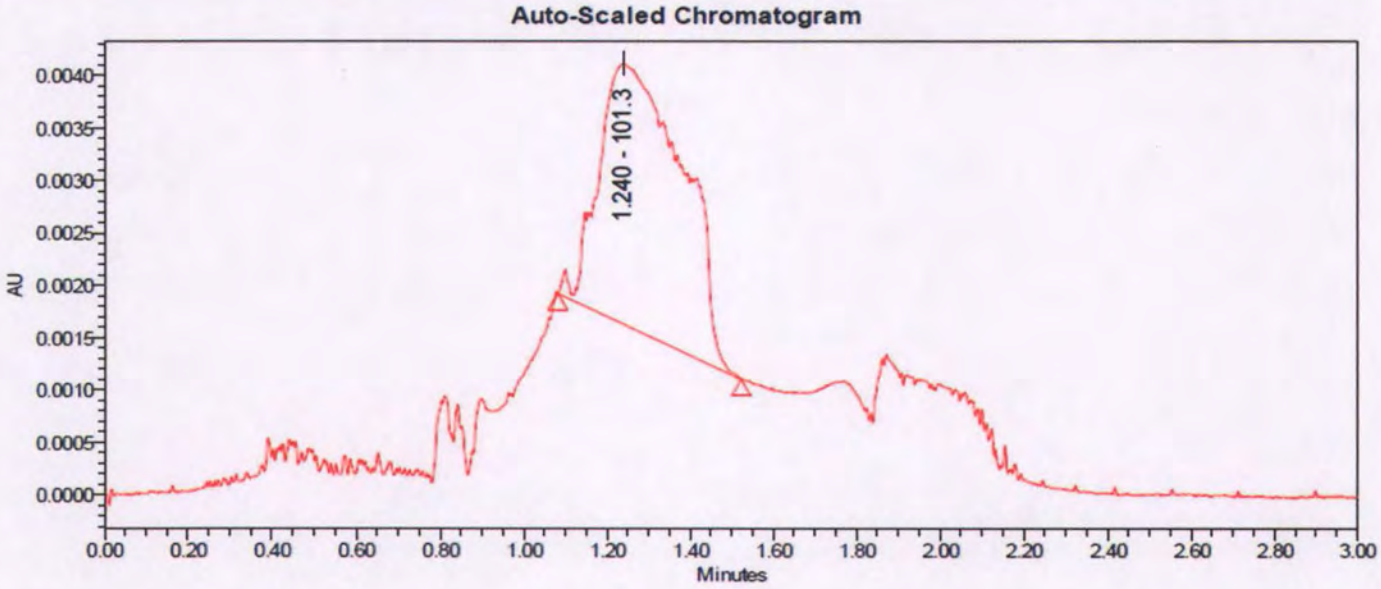

Figure 1-16. Romaine lettuce subjected to 2% FNC for five minutes (Rep 3).

SAMPLE INFORMATION

Sample Name:	R1 Iceberg	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:D,8	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
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Date Processed:	12/2/2016 7:45:35 PM CST		

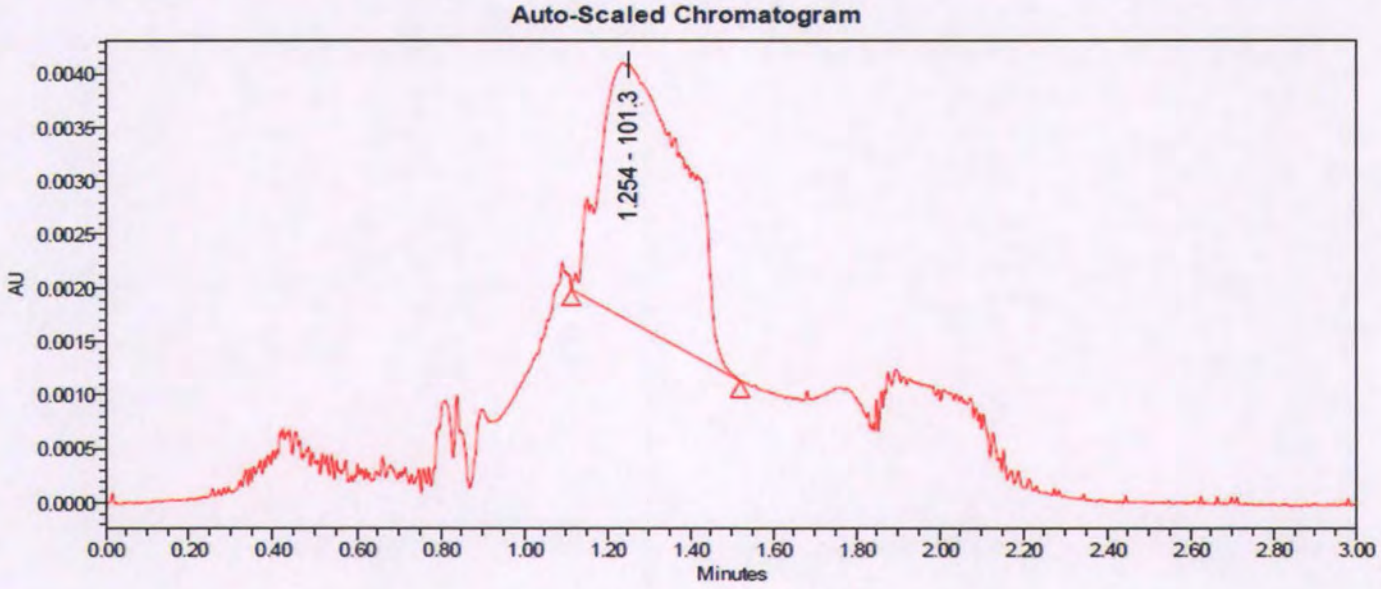

Figure 1-17. Iceberg lettuce subjected to 2% FNC for five minutes (Rep 1).

SAMPLE INFORMATION

Sample Name:	R2 Iceberg	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:E,1	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
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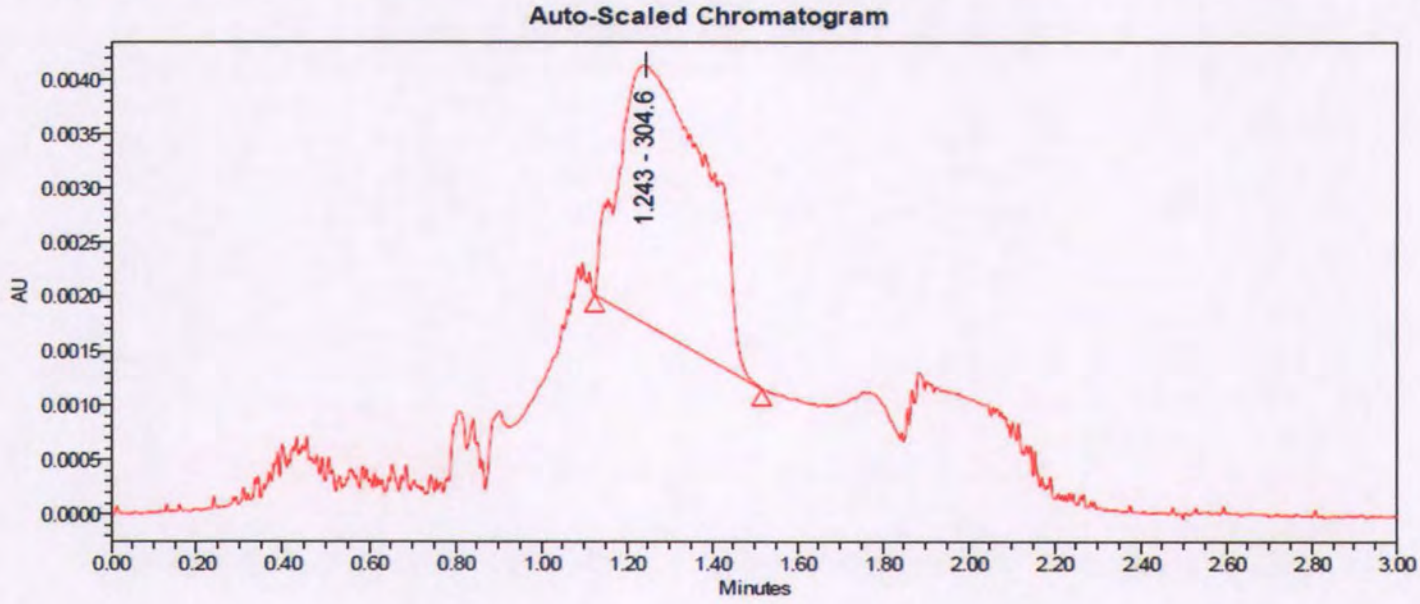


Figure 1-18. Iceberg lettuce subjected to 2% FNC for five minutes (Rep 2).

SAMPLE INFORMATION

Sample Name:	R3 Iceberg	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	Methyl paraben residue
Vial:	2:E,2	Acq. Method Set:	Methyl Paraben
Injection #:	1	Processing Method	test
Injection Volume:	10.00 ul	Channel Name:	PDA Ch1 258nm@4.8nm
Run Time:	3.0 Minutes	Proc. Chnl. Descr.:	PDA Ch1 258nm@4.8nm
Date Acquired:	12/2/2016 5:56:01 PM CST		
Date Processed:	12/2/2016 7:45:37 PM CST		

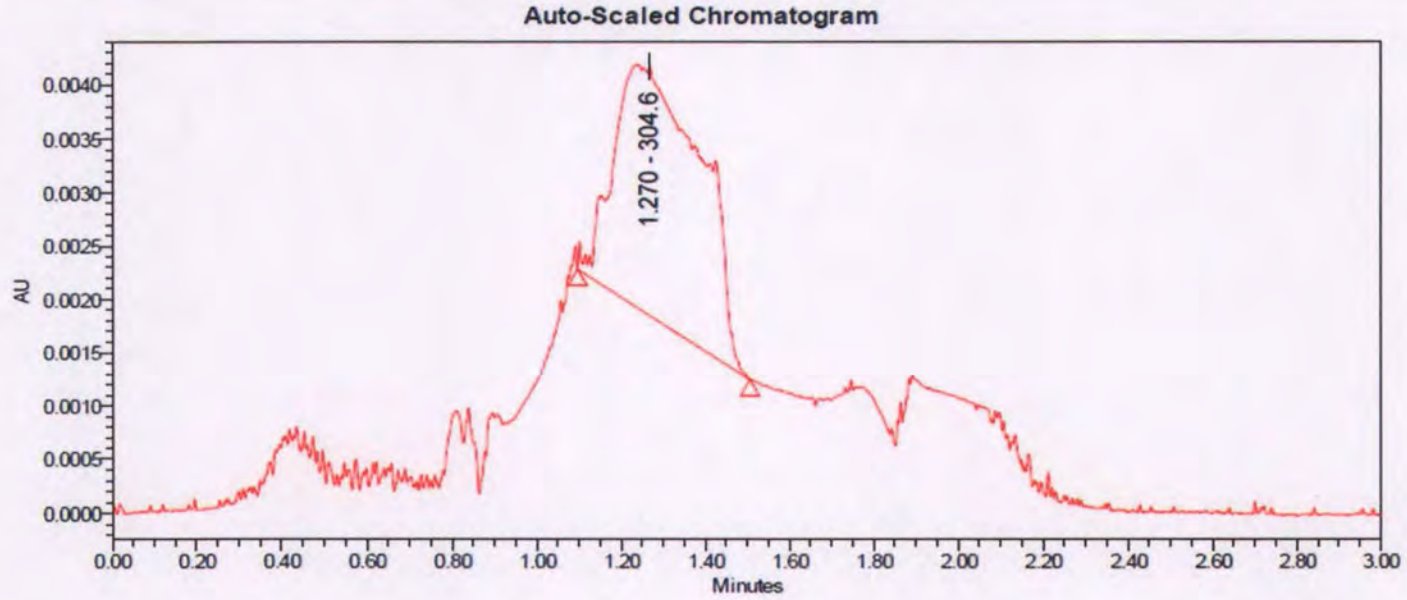


Figure 1-19. Iceberg lettuce subjected to 2% FNC for five minutes (Rep 3).

CONCLUSIONS

Figure 1-10 illustrates a chromatogram of just extracted lettuce (no methyl paraben) that resembles figures 1-14 – 1-19. Figures 1-14 – 1-19 also have very little peak intensity. As figure 1-11 illustrates, a methyl paraben concentration of 5 parts per million generates a clear, intense peak. **A conclusion can be drawn that the concentration of methyl paraben remaining on both romaine and iceberg lettuce subjected to 2% FNC for 5 minutes is less than 5 parts per million.**

Methyl Paraben Concentrations in Wash Water

Replication 1, Pre Wash: 170.51 ppm

Replication 1, Post Wash: 151.26 ppm

Replication 2, Pre Wash: 161.50 ppm

Replication 2, Post Wash: 157.58 ppm

Replication 3, Pre Wash: 174.29 ppm

Replication 3, Post Wash: 162.35 ppm

Below are chromatograms supporting these data.

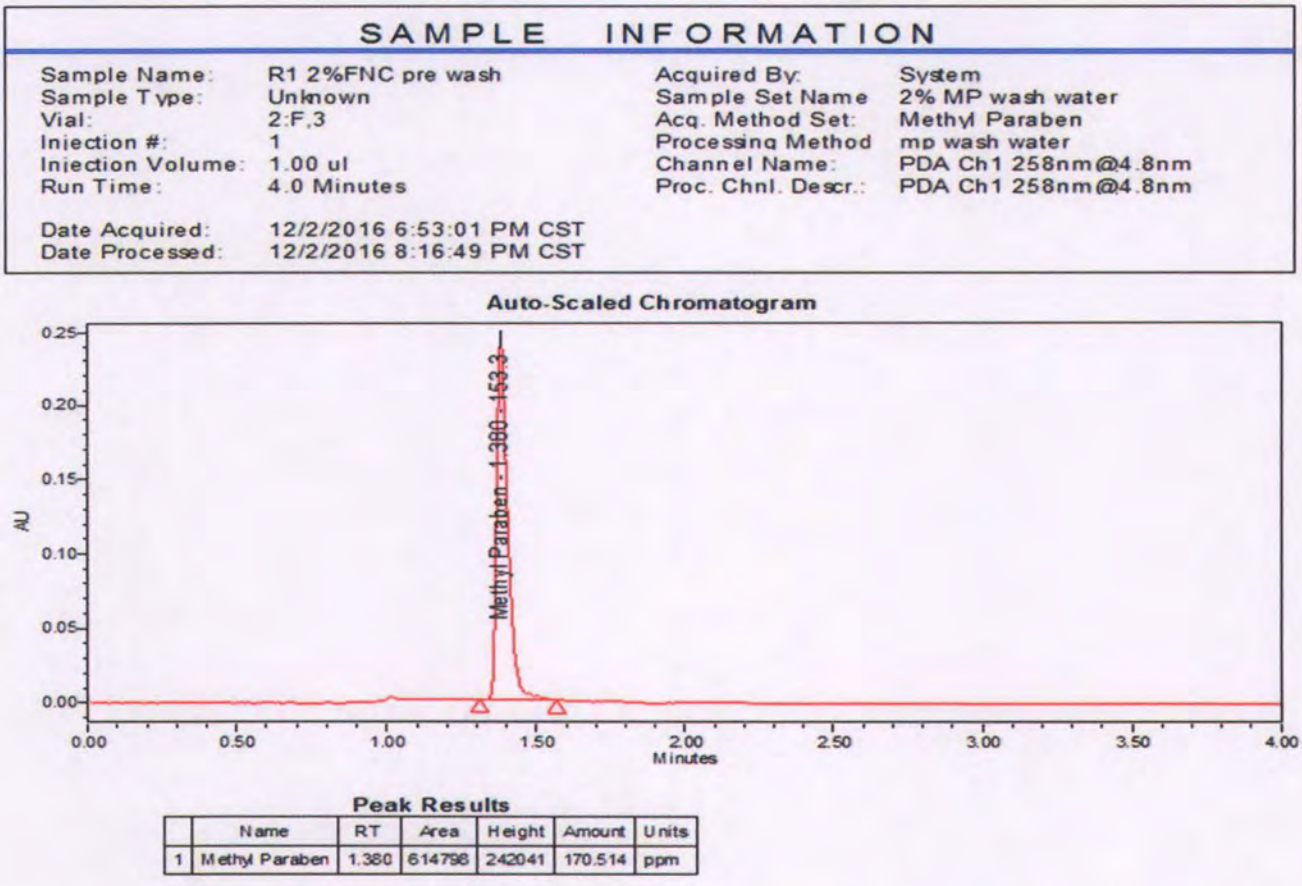


Figure 1-20. Example methyl paraben chromatogram.

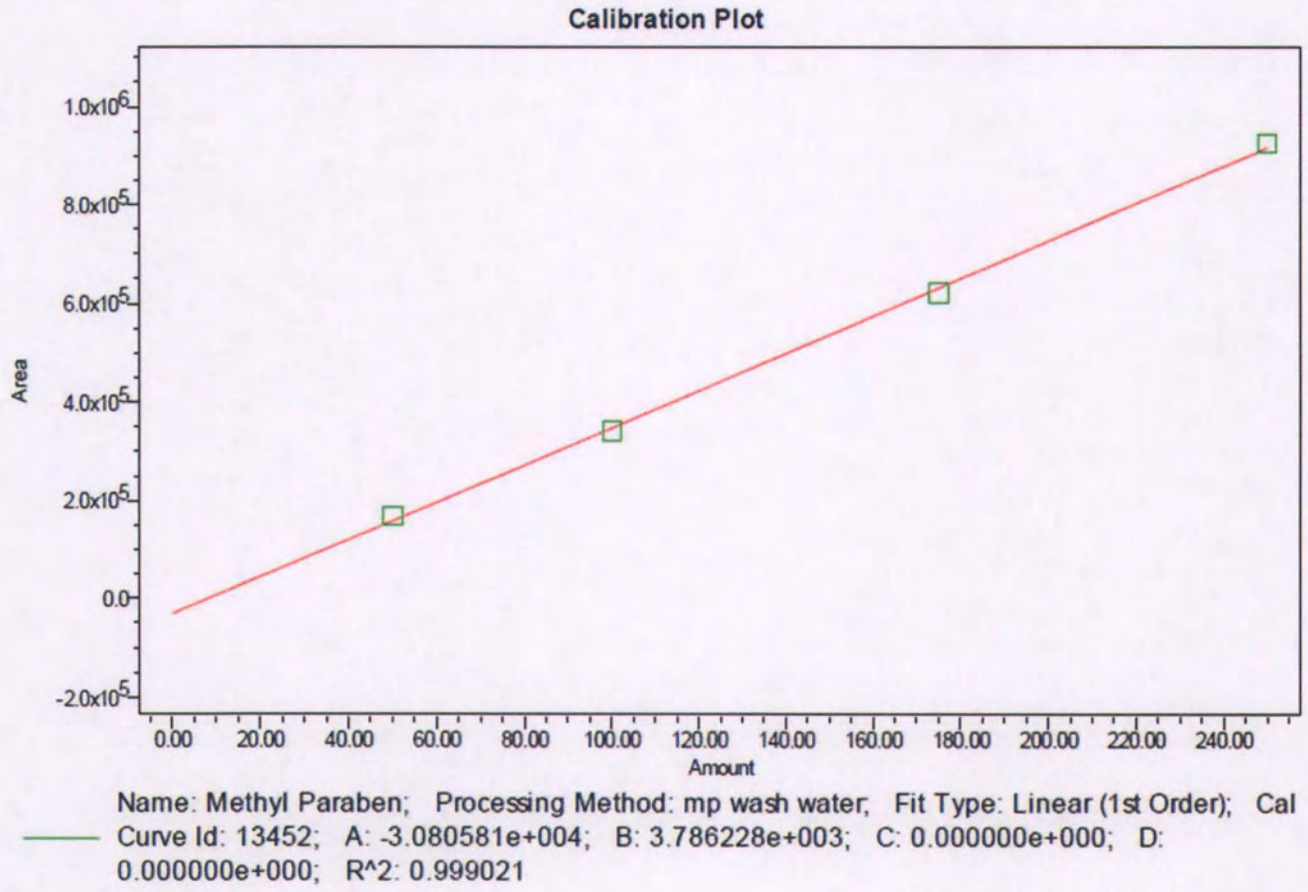


Figure 1-21. Standard curve used for determining methyl paraben concentrations in wash water.

Benzalkonium Chloride Concentrations in Wash Water

Replication 1, Pre Wash: 78.35 ppm

Replication 1, Post Wash: 82.45 ppm

Replication 2, Pre Wash: 86.77 ppm

Replication 2, Post Wash: 79.69 ppm

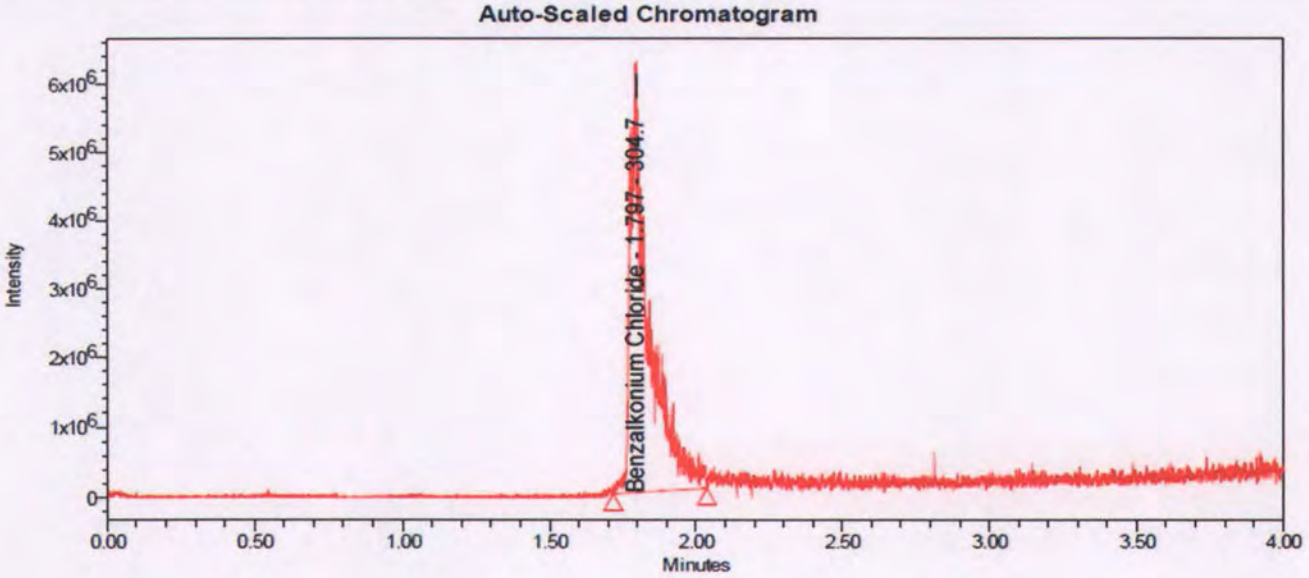
Replication 3, Pre Wash: 81.75 ppm

Replication 3, Post Wash: 78.89 ppm

Below are chromatograms supporting these data.

SAMPLE INFORMATION

Sample Name:	R3 2%FNC pre wash	Acquired By:	System
Sample Type:	Unknown	Sample Set Name	2% BAC washwater
Vial:	2:F,7	Acq. Method Set:	BAC_Method Set
Injection #:	1	Processing Method	BAC wash water
Injection Volume:	1.00 ul	Channel Name:	305.7Da
Run Time:	4.0 Minutes	Proc. Chnl. Descr.:	QDa Positive Scan MS 305.71
Date Acquired:	12/2/2016 8:11:54 PM CST		
Date Processed:	12/2/2016 8:32:29 PM CST		



Peak Results

	Name	RT	Area	Height	Amount	Units
1	Benzalkonium Chloride	1.797	26195364	5776758	81.745	ppm

Figure 1-22 Example benzalkonium chloride chromatogram.

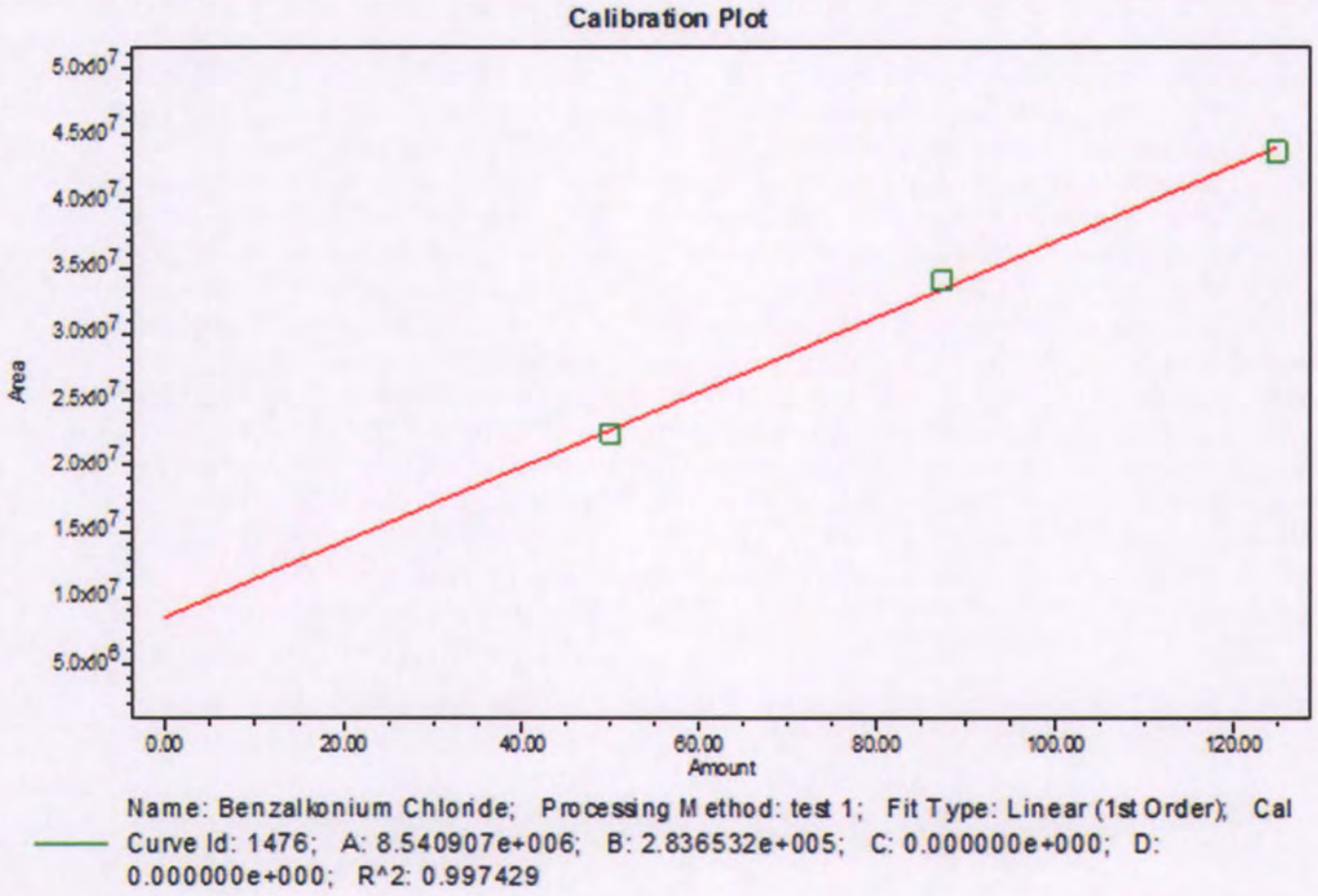


Figure 1-23. Standard curve used for determining benzalkonium chloride concentrations in wash water.

APPENDIX 3

EXECUTIVE SUMMARY
(Kansas State University, Food Science Institute)

Free N Clear® Preliminary Project Summary:

Food Safety & Defense Laboratory (FSDL; Manhattan, KS)

June 2015- January 2016
Prepared for: Marvel Technologies (Jack Wheeler)

Executive Summary (Remarkable Kill)

Marvel Technologies USA, LLC (Franklin, TN) is seeking FDA approval to market Free N Clear® (FNC), a food-grade sanitizer containing benzalkonium chloride (BAC), as an effective antimicrobial wash to control commonly associated pathogens in commercially processed fresh produce. Inoculated laboratory studies were conducted by the FSDL under the direction of Dr. Randall Phebus to scientifically validate the antimicrobial efficacy of different concentrations (0 control, 1%, 2%, and 3% from concentrate) of FNC wash solutions, along with different washing exposure times (1 and 5 min), used as a fresh produce wash to reduce populations of Listeria monocytogenes, Salmonella spp., and Escherichia coli O157:H7 in cut Romaine lettuce, cut Iceberg lettuce, and the bulk FNC wash tank water during operations. Three replications of the inoculated study have now been completed using a single coded lot of make-up FNC components (benzalkonium chloride, methyl paraben, and acetic acid received pre-weighed and mixed to form the FNC concentrate) supplied by Marvel Technologies. A fourth pathogen inoculated cut lettuce replication, utilizing a FNC concentrate prepared with new ingredients, will be conducted in January/February, 2016 to confirm or deny the results found in the previous three replications. FNC-washed lettuce samples and wash tank water samples from these inoculated studies have been collected and are in frozen storage awaiting BAC and methyl paraben residue analysis by Dr. Pliakoni's laboratory in February 2016. Very importantly, there was a realization that the targeted concentration of active ingredient (benzalkonium chloride) in a 2% FNC application solution as prepared following Marvel Technologies' instructions is not 100 ppm as previously declared, but only 50 ppm. This calculation of the active ingredient concentration in a 2% FNC solution is presented in Figure 1. Experimental design, materials and methods, and results from the three replications can be seen below.

Residual BAC and methyl paraben determinations, along with non-inoculated microbial and organoleptic shelf life studies, will be conducted at the K-State Olathe campus throughout February 2016 under the supervision of Dr. Sara Gragg (microbial shelf life focus) and Dr. Eleni Pliakoni (residue determinations and product organoleptic determinations during simulated retail storage). In December 2015 and January 2016, Dr. Pliakoni's laboratory worked to establish appropriate UPLC equipment operational parameters, train technicians on the equipment operation, generate standard concentration to UPLC signal curves for BAC and methyl paraben, and develop and verify an appropriate extraction protocol for BAC and methyl paraben from treated lettuce. Drs. Pliakoni and Gragg will now apply FNC washes to non-inoculated, cut Romaine and Iceberg lettuce to determine treatment effects on shelf life and quality, and these produce types will be analyzed after simulated commercial washing procedures to establish residual BAC and methyl paraben levels post-washing by the use of ultra high performance liquid chromatography. Three replications of these studies are projected to be finished by mid-February, 2016.

Preliminary observations to date indicate that FNC produce wash concentrations of 2 or 3% when used to wash fresh-cut Romaine or Iceberg lettuce result in approximately 1 log CFU/g or less (65 to 97 percent) reductions in *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on the produce directly. However, and very important to the produce industry in their food safety programs, the FNC solutions were extremely effective in controlling any pathogen contamination that would be encountered in the water of large bulk commercial washing operations as the result of contaminated produce entering the system during extended operations. In speaking with two commercial cut lettuce processors, along with two internationally recognized produce safety professors, these findings are very much in line with known/expected results generated from studies using a host of different antimicrobial washes with fresh produce. The industry greatly values the ability to control pathogens in recycled fresh-cut produce wash water, as this is their primary means of preventing large-scale contamination and recall events if a lot(s) of contaminated produce enters into the washing system. Currently, chlorinated water or peroxyacetic acetic acid seem to be the chemical treatments of choice for maintaining pathogen-free wash water; however there are negative issues with each of these treatment options. FNC may provide a cost-effective alternative, especially if our continuing studies in Olathe indicate good shelf life characteristics for the treated and packaged cut lettuce.

Figure 1.

Calculation of parts per million (ppm) of active ingredient (benzylkonium chloride) in a 2% working solution of FNC made from concentrate per Marvel Technologies' guidelines.

FNC Ingredients (g):
18 g methyl paraben
18 g USP-BAC (which is only 50% BAC or 9 g active ingredient as purchased)
36 g acetic acid
3785 g water (1 gal of water mixed with ingredients)

3857 g TOTAL

Active ingredient benzylkonium chloride concentration in a 2% FNC solution:
(g active ingredient / total g of FNC solution)(1000000) = ppm active ingredient
(9g / 3857g)(1000000) = 2333.42 ppm BAC

A 2% mixture is a 50:1 dilution, so to figure out the ppm of active ingredient in a 2% solution we divide the ppm in the concentrated solution (2333.42) by 50.
(2333.42 / 50) = 46.66 ppm benzylkonium chloride in a 2% solution.

One would be correct in saying a 2% solution contained 96-100 ppm of USP-BAC, but the USP-BAC is only 50% benzylkonium chloride. This means that a 2% solution contains only 46- 50 ppm of active ingredient BAC.

Experimental Design (Pathogen-Inoculated Study)

Products:	**Ro**maine (cut)	Iceberg (cut)
FNC Concentrations:	0% 1% 2% 3%	0% 1% 2% 3%
Contact/Immersion Time:	1 min or 5 min (200 g of cut lettuce submerged in 5 gal FNC wash)	
Microorganisms:	*Listeria monocytogenes* *Salmonella* spp. *E. coli* O157:H7	
	* **2 strains each combined into one master inoculum**	
Sanitizing Agent:	0.08 lb acetic acid, 0.04 lb methyl paraben, and 0.04 lb of USP-BAC (50% benzalkonium chloride) were mixed with 1 gallon of water to create the concentrated FNC solution, per Marvel Technologies guidance.	
Replications:	3	

Materials & Methods (Pathogen-Inoculated Study)

Produce and Inoculation: Whole Romaine and Iceberg lettuce was purchased in bulk from Liberty Fruit Co. in Kansas City, KS. The produce was not washed or processed in any way before being transported to the K-State Food Safety and Defense Laboratory in Manhattan, KS and stored at 4°C. The following day, lettuce was cored and cut into ~1 in x 1 in squares. 1000-g batches of each lettuce type were inoculated with a mixed cocktail containing two strains of

Listeria monocytogenes, *Salmonella* Typhimurium, *Salmonella* Newport, and two strains of *E. coli* O157:H7. Lettuce batches were inoculated using a light spray mist procedure to target approximately 7 log colony forming units (CFU)/g of product, which is approximately 10

million cells of each individual pathogen per gram of product. After inoculation, the lettuce was held at room temperature for 30 min to allow bacterial attachment before FNC wash treatments were applied.

Inoculum Preparation: Two strains of *Listeria monocytogenes*, *Salmonella* Typhimurium, *Salmonella* Newport, and two strains of rifampicin resistant *E. coli* O157:H7 were removed from frozen storage and activated by transferring to 10 mL Tryptic Soy Broth (TSB) and incubated at 37°C for 24 h. Aliquots (0.1 ml) of each *Listeria* culture were combined with 200 ml of TSB and 2 strains of each combined into one master inoculum

incubated at 37°C for 24 h. The same transfer steps were done for both *Salmonella* and both *E. coli* cultures separately. Following incubation, cultures were centrifuged (6,000 rpm; -4°C; 15 min) and the resulting cell pellets were rehydrated with 100 ml sterile 0.1% peptone water. One ml from each of the rehydrated pellets (total of 3 ml) was combined with 150 ml 0.1% peptone water to create a combined master inoculum. Five ml of the combined master inoculum was then sprayed onto 1000 g of lettuce to achieve the 7 log CFU/g target for each organism.

Treatment and Application: Concentrated FNC solution was made by combining 0.08 lb acetic acid, 0.04 lb methyl paraben, and 0.04 lb of USP-BAC (which is only 50% benzalkonium chloride active ingredient as explained above) (each chemical was received pre-weighed from Marvel Technologies) with 1 gallon of tap water. This concentrated FNC solution was further mixed with tap water to make four 5-gal treatment tubs (providing working FNC wash solutions of 0%, 1%, 2%, and 3%). New 5-gal treatment tubs were prepared for each of the three experimental replications using the original FNC concentrate. Inoculated Romaine or Iceberg lettuce (200 g portions) were placed in slotted containers and completely submerged for either 1 min or 5 min in the different treatment tubs using ambient wash solution temperature. The wash solution from each of the treatment tubs was sampled after 1 min and 5 min while the inoculated produce was submerged to establish microbial levels in the treatment water itself. To analyze each of these wash solution samples, 20 ml of treatment solution were collected and combined with 20 ml of double strength DE Neutralizing Broth (DNB) to stop further residual antimicrobial effects of the FNC wash. Following FNC treatment, each 200-g batch of lettuce was completely submerged in tap water for 1 min to act as a secondary wash prior to being dewatered using a consumer sized salad spinner.

Approximately 25-30 g of each 200-g batch of FNC treated lettuce was placed in 75 ml of single strength DNB for microbial analysis.

Microbial Analysis: Samples were mixed/homogenized for 1 min in a laboratory blender (PulsifierTM) and serial dilutions prepared in 0.1% peptone water. Dilutions were plated onto 1/2 strength modified Oxford (MOX), xylose lysine desoxycholate (XLD), and sorbitiol MacConkey (SMAC$_{rif}$; 100 ppm rifampicin added) agars to enumerate *L. monocytogenes*, *Salmonella* and *E. coli* O157 populations, respectively. Plates were incubated at 37°C for 24 h. Each sample was also plated in triplicate on tryptic soy agar (TSA) and incubated at 37°C for 6 h before being overlaid with each of the selective agars (MOX, XLD, or SMAC$_{rif}$) to facilitate recovery and enumeration of sublethally injured cells resulting from the antimicrobial treatments. Following overlay, the plates were placed back in the incubator at 37°C for an additional 18 h before counting.

Residue Analysis of FNC-Treated Inoculated Cut Lettuce: From the pathogen-inoculated studies above, samples of FNC treated lettuce that were subsequently water rinsed to simulate expected commercial processing steps were collected and frozen to send to Olathe for BAC and methyl paraben analysis (will be completed in February 2016 as previously detailed). Additionally, samples of each FNC wash solution were collected before and after lettuce treatment to verify concentrations of active ingredients.

Results & Discussion (Pathogen-Inoculated Study)

It is important to note that this report consists of data that has not been statistically analyzed, and additional microbiological data will be collected utilizing a new FNC concentrate next week. This needs to be considered when drawing conclusions from the raw data. Further, wash water samples and treated product samples were collected and frozen and will be chemically analyzed to quantify benzylkonium and methyl paraben concentrations/residues by Dr. Pliakoni's laboratory over the next couple of weeks.

Romaine and Iceberg Results: Pathogen recovery results from the inoculated study are presented in Table 1. Reduction in *L. monocytogenes* contamination levels on cut lettuce ranged from 75 to 97 percent across FNC treatment concentrations of 2 and 3% and 1 and 5 minute wash times; whereas, *Salmonella* and *E. coli* O157:H7 reductions ranged from 77 to 89 percent and 62 to 87 percent, respectively. There did not seem to be a difference between the 1 min and 5 min contact times for any of the FNC wash treatments. A slight increase (but likely not

statistically relevant) in microbial reductions for each of the three pathogen types seemed to occur as FNC treatment concentrations increased. However, when 1%, 2% and 3% FNC treatment recovery counts are compared to the 0% (water only) control, virtually no differences were observed indicating that the very modest pathogen reductions observed on the produce itself is likely attributable mostly to physical detachment of the microbial cells from the lettuce into the bulk wash solution, particularly on the portion of the inoculated pathogen population that was only loosely attached.

Treatment Water Results: Results from the treatment water after washing the highly contaminated cut lettuce can be seen below in Table 1. There seemed to be a slight difference between the two sampling points (1 min and 5 min) for all three FNC solution concentrations, with the 1 min recovery counts being consistently higher than the 5 min recovery counts. This could be due to the added exposure of chemical to the free floating microbes in the treatment water. There was also a huge difference between recovery counts of the 0% (water only) control and the 1%, 2%, and 3% FNC treatment solutions. The 0% control samples had between 3.8 and 4.3 log CFU/ml of viable pathogens remaining at the 5-minute sampling times. On the other hand, the 1%, 2%, and 3% solutions reduced counts of all three pathogen populations to below the detection limit in most cases.

Table 1. Microbial counts (log CFU/g or ml) recovered from inoculated Romaine and Iceberg lettuce, and from FNC wash water, after being treated with FNC used at different concentrations and for different contact times (percent reduction of specific pathogen's initial population level by each treatment is provided in parentheses).

Product	Sample	*Listeria monocytogenes*	*Salmonella* spp.	*E. coli*O157:H7
Romaine	Initial Contamination level	6.6± 0.14	6.9 ± 0.17	6.6± 0.07
	0% 1min	6.0± 0.12 (73.8%)	6.4± 0.19 (64.1%)	6.4± 0.20 (45.5%)
	0% 5min	5.8±0.23 (83.6%)	6.3± 0.12 (75.1%)	6.3± 0.04 (55.9%)
	1% 1min	5.9± 0.34 (81.2%)	6.2± 0.16 (78.3%)	6.2± 0.22 (64.0%)
	1% 5min	5.9± 0.14 (81.0%)	6.2± 0.21 (76.9%).	6.1± 0.08 (69.2%)
	2% 1min	6.0± 0.13 (74.9%)	6.1± 0.02 (84.0%)	6.2± 0.11 (65.2%)
	2% 5min	5.8± 0.17 (85.6%)	5.9± 0.20 (89.2%)	5.9± 0.25 (81.5%)
	3% 1min	5.8± 0.36 (84.1%)	6.2± 0.09 (76.7%)	6.2± 0.13 (61.8%)
	3% 5min	6.0± 0.19 (75.8%)	6.0± 0.14 (86.8%)	6.1± 0.08 (71.4%)
Iceberg	Initial Contamination level	6.8± 0.09	6.9±0.18	6.9± 0.03
	0% 1min	6.2± 0.12 (72.7%)	6.2± 0.21 (79.7%)	6.4± 0.10 (70.2%)
AVG-	0% 5min	6.1± 0.05 (78.1%)	6.1± 0.07 (86.6%)	6.3± 0.07 (75.3%)
	1% 1min	6.3± 0.15 (68.2%)	6.3± 0.14 (78.1%)	6.5± 0.14 (65.5%)
85.4%	1% 5min	5.9± 0.47 (87.1%)	6.2±0.46 (81.2%)	6.4± 0.25 (68.4%)
	2% 1min	5.7± 0.23 (92.1%)	6.0± 0.07 (87.5%)	6.4± 0.21 (72.9%)
Reduction	2% 5min	5.8± 0.18 (90.6%)	6.1± 0.28 (85.1%)	6.2± 0.08 (80.7%)
	3% 1min	5.6± 0.52 (93.0%)	6.1± 0.17 (84.7%)	6.2± 0.19 (82.5%)
	3% 5min	5.3± 0.58 (96.9%)	6.0± 0.34 (88.4%)	6.1± 0.14 (86.6%)
Treatment Water	0% 1min	3.3± 0.25	3.7± 0.28	3.7± 0.28
	0% 5min	3.8± 0.37	4.1± 0.27	4.3± 0.15
	1% 1min	1.0± 0.93	1.3± 1.37	1.1± 1.17
	1% 5min	0.2± 0.14	0.2± 0.14	0.1± 0.14
	2% 1min	0.2±0.14	0.2± 0.14	0.1± 0.14
	2% 5min	0.2± 0.14	0.3± 0.00	0.0± 0.00
	3% 1min	0.4± 0.54	0.8± 0.91	0.6± 0.79
	3% 5min	0.1± 0.14	0.2± 0.14	0.0± 0.00

Note: A 1-log CFU/g microbial population reduction from the initial contamination level equals a 90% overall reduction; a 2-log CFU/g microbial reduction equals a 99% overall reduction, etc.

Average 85.4% REMARKABLE KILL

APPENDIX 4

CHARLES RIVER REPORT

(91 – DAY RAT STUDY)

CHARLES RIVER REPORT SUMMARY

FINAL REPORT Guideline: OECD (408) Testing Facility Study No. 20011912 Sponsor Reference No. 11

A 91-day Study of Free N Clear by Oral Gavage in Rats

SPONSOR:

Marvel Technologies USA, LLC 1224 Columbia Avenue, Suite 104 Franklin, TN 37064 United States

TESTING FACILITY:

Charles River Laboratories

Preclinical Services, Ohio (PCS-OH) 640 North Elizabeth Street Spencerville, OH 45887 United States

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SUMMARY

The objective of this study was to determine the potential toxicity, if any, of Free N Clear when given orally once daily for 91 days to rats. Free N Clear contains benzalkonium chloride (BAC) acetic acid, methyl paraben, and water; BAC is the characterizing ingredient.

The study design was as follows:

Text Table 1
Experimental Design

Group No.	No. of Main Study Animals		Test Material	Free N Clear[a] (mg/kg/day)	BAC Dose Level (mg/kg/day)	BAC Concentration (mg/mL)	As Gavage Dose Volume (mL/kg bw)
	Males	Females					
1	10	10	RODI water	0	0	0	5
2	10	10	Free N Clear	500	0.05	0.1	0.5
3	10	10	Free N Clear	1000	0.1	0.1	1
4	10	10	Free N Clear	5000	0.5	0.1	5

BAC = benzalkonium chloride concentration within the dosing solution; RODI water = reverse osmosis deionized water; bw = body weight.

[a] The product was dosed as received; density was assumed to be 1 g/mL.

The following parameters and end points were evaluated in this study: clinical signs, body weights, body weight changes, food consumption, ophthalmology, clinical pathology parameters (hematology, coagulation, clinical chemistry, and urinalysis), gross necropsy findings, organ weights, and histopathologic examinations. All animals survived until Day 92 (study termination). There were no test article-related clinical findings. There were no toxicologically relevant differences in mean body weights, mean body weight gains, or food consumption during the study. There were no test article-related changes detected during the ophthalmological examinations. There were no toxicologically relevant differences in hematology, coagulation, or clinical chemistry parameters. There were no toxicologically relevant differences in urinalysis parameters noted during the study. There were no organ weight changes or gross or microscopic findings associated with test article administration. In conclusion, once daily oral gavage administration of Free N Clear to Crl:CD(SD) Sprague Dawley rats for 91 days was well tolerated in rats at levels up to and including 5000 mg/kg/day as there were no test article-related findings during the study. Therefore, based on these results, the no-observed-adverse-effect level (NOAEL) was 5000 mg/kg/day, the highest dose tested.

6. INTRODUCTION

The objective of this study was to determine the potential toxicity, if any, of Free N Clear when given orally once daily for 91 days to rats. Free N Clear contains benzalkonium chloride (BAC) acetic acid, methyl paraben, and water; BAC is the characterizing ingredient. The Study Director signed the protocol on 30 Mar 2011, and dosing was initiated on 07 Apr 2011. The in-life phase of the study was completed on 08 Jul 2011. The experimental start date was 31 Mar 2011, and the experimental completion date was 04 Apr 2012. The study protocol, protocol amendment, and deviations are presented in.

MATERIALS AND METHODS

6.1. Test and Control Articles

6.1.1 Test Article

Identification:	Free N Clear
Batch (Lot) No.:	1
Receipt Date:	10 March 2011
Retest Date:	12 Oct 2011 ; stable throughout the course of the study

Purity:	Purity assumed to be 100% for dose calculation purposes
Concentration Mixture:	Product was received from the Sponsor as a 1:50 dilution (2% solution) from concentrate. Concentrate was prepared from mixing 236 g of acetic acid, 118 g of methyl paraben, and 188 g of USP benzalkonium Chloride (BAC) solution (50%) and adding water to 6.3 gallons.
Storage Conditions:	Kept in a controlled temperature area (64°F to 82°F)
Supplier:	Marvel Technologies USA, LLC

6.1.2 Control Article

Reverse osmosis deionized (RODI) water was obtained from the tap in the Testing Facility's Formulations Department.

6.2. Test Article Characterization and Stability

The Sponsor provided to the Testing Facility documentation of the identity, strength, purity, composition, and stability for the test article. Characterization of the test article was provided to the Testing Facility and is presented in BAC characterization conducted by Microbac Laboratories, Inc. prior to study start (Mar 2011) indicated that the samples were 4% lower (average of 96.2 ppm BAC) than the target concentration (100 ppm BAC). In addition, 2 Lot No. 1 samples were shipped to Microbac Laboratories, Inc. for stability testing of BAC after study completion. BAC testing results for stability (Sep 2011) indicated that the samples were 2.49% lower than that of the characterization results prior to study start or 6.5% lower (average of 93.8 ppm) than the target concentration (100 ppm BAC).

8.3 Reserve Samples

For each batch (lot) of test article, a reserve sample (1 mL) was collected and maintained under the appropriate storage conditions by the Testing Facility.

8.4 Test Article Inventory and Disposition

Records of the receipt, distribution, storage, and disposition of test article (including empty containers) were maintained. With the exception of the reserve sample, all unused Sponsor-supplied bulk test article will be returned to the Sponsor (after issuance of the final reports of all studies using these materials). All empty containers were maintained for the duration of the study.

8.5 Analysis of Test Article

Two (2) containers of the test article were shipped to the analytical laboratory during the week of 26 September 2011 for concentration analysis in order to demonstrate stability. The results indicated that the bulk material was stable during the period of use. Any residual/retained analytical samples (and test article used in analysis) were discarded.

8.6. Dose Formulation and Analysis

8.6.1 Preparation of Control Article

The control article, RODI water, was dispensed daily for administration to Group 1 control animals. An adequate amount of the control article was dispensed into daily aliquots, which were stored in a controlled room temperature area until use. Actual room temperature was recorded continuously at 15-minute intervals and the daily averages ranged from 67°F to 82°F. Details of the preparation and dispensing of the control article have been retained in the Study Records.

8.6.2 Preparation of Test Article

The test article, Free N Clear, was administered as received. An adequate amount of the test article was dispensed into daily aliquots, which were stored at controlled room temperature until use. Actual room temperature was recorded continuously at 15-minute intervals and the daily averages ranged from 67°F to 82°F. Details of the preparation and dispensing of the test article have been retained in the Study Records.

8.6.3 Sample Collection and Analysis

The test article was used as received from the Sponsor and RODI water was used as obtained from the Testing Facility's Formulations Department; therefore, samples for dose formulation analysis were not collected by the Testing Facility.

8.7. Test System

8.7.1 Receipt

Forty-four (44) male and 44 female Sprague Dawley Crl:CD(SD) rats were received on 31 Mar 2011 from Charles River Laboratories, Portage, MI. The animals were examined and weighed on the day following receipt.

8.7.2 Justification for Test System and Number of Animals

The Sprague Dawley rat was chosen as the animal model for this study because it is a preferred rodent species for preclinical toxicity testing by regulatory agencies. The total number of animals used in this study is the minimum required to properly characterize the effects of the test article. The number of groups is based on guidelines of the FDA Redbook.[ii] Based on statistical sample size calculations,[iii] the number of animals per group is the minimum necessary to detect a 12.5% difference in body weight as statistically significant when compared to a mean body weight of 250 g with a standard deviation of 20 g at an α level of 0.05 and a β level of 0.1. From a toxicological perspective, this degree of statistical resolution is sufficient for this study design.

8.7.3. Animal Identification

Each animal was identified by a cage card and tail tattoo after randomization.

8.7.4. Environmental Acclimation

The animals were acclimated to their designated housing for at least 7 days before the first day of dosing.

8.7.5. Selection, Assignment, and Disposition

Prior to randomization procedures, the animals were weighed and examined in detail. Animals were then randomly assigned to groups by a stratified randomization scheme designed to achieve similar group mean body weights. For scheduling convenience, males and females were stagger-started by 1 day. The animals were approximately 8 weeks of age at the initiation of dosing with body weights ranging from 232 to 267 grams for the males and 180 to 206 grams for the females.

The disposition of all animals was documented in the study records.

8.7.6. Husbandry

8.7.6.2.1 Housing

The animals were housed individually throughout the study in suspended stainless steel cages. Housing and care were as specified in the USDA Animal Welfare Act (9 CFR, Parts 1, 2, and 3) and as described in the *Guide for the Care and Use of Laboratory Animals* from the National Research Council.[iv]

8.7.6.2 Environmental Conditions

Targeted environmental conditions were as follows: Temperature 64oF to 79oF (18oC to 26oC)

Humidity 50% ± 20% Light Cycle 12-hour light/12-hour dark cycle Ventilation 10 or more air changes per hour with 100% fresh air

Actual room temperature and relative humidity were recorded continuously at 15-minute intervals and the daily averages ranged from 71°F to 76°F (22°C to 24°C) and 45% to 60%, respectively.

8.7.6.3 Food

PMI Nutrition International Certified Rodent Chow No. 5CR4 (14% protein) was provided ad libitum throughout the study, except during designated procedures. The lot number and expiration date of each batch of diet used during the study were recorded. The feed was analyzed by the supplier for nutritional components and environmental contaminants. Results of the dietary analyses were provided by the manufacturer for each lot of diet and are on file at

the Testing Facility. Based on the results of these analyses, there were no contaminants that

would interfere with the conduct or interpretation of the study.

8.7.6.4 Water

Municipal tap water following treatment by reverse osmosis and ultraviolet irradiation was available ad libitum throughout the study. The water is analyzed semi-annually for microbial contamination and for total dissolved solids, hardness, and various environmental contaminants. Results of these analyses are maintained on file at the Testing Facility. Based on the results of the most recent analyses, there were no contaminants that would interfere with the conduct or interpretation of the study.

8.7.6.5 Veterinary Care

Veterinary care was available throughout the study and animals were examined by the veterinary staff as warranted by clinical signs or other changes. All veterinary examinations and recommended therapeutic treatments, if any, were documented in the study records. No veterinary medicinal treatments were administered during the study.

8.8. Experimental Design

Text Table 2 Experimental Design

Text Table 2
Experimental Design

Group No.	No. of Main Study Animals		Test Material	Free N Clear[a] (mg/kg/day)	BAC Dose Level (mg/kg/day)	BAC Concentration (mg/mL)	As Gavage Dose Volume (mL/kg bw)
	Males	Females					
1	10	10	RODI water	0	0	0	5
2	10	10	Free N Clear	500	0.05	0.1	0.5
3	10	10	Free N Clear	1000	0.1	0.1	1
4	10	10	Free N Clear	5000	0.5	0.1	5

BAC = benzalkonium chloride concentration within the dosing solution; RODI water = reverse osmosis deionized water; bw = body weight.
[a] The product was dosed as received; density was assumed to be 1 g/mL.

8.8.1 Administration of Test Materials

The test and control articles were administered to the appropriate animals by once daily oral gavage from Days 1 to 91. The dose volume for each animal was based on the most recent body

weight measurement. The doses were given using a syringe with attached gavage cannula. The first day of dosing was designated as Day 1.

8.8.2 Justification of Route and Dose Concentration Levels

The oral route of exposure was selected because humans will be exposed via the oral route. The dose levels were selected based on theoretical residue levels to which humans could be exposed, as well as doses of benzalkonium chloride that are known to be toxic to rats.

8.9 In-life Procedures, Observations, and Measurements

8.9.1 Mortality/Moribundity Checks

The animals were observed for general health/mortality and moribundity twice daily, once in the morning and afternoon, throughout the study.

8.9.2. Clinical Observations

8.9.2.1 Cage Side Observations

Cage side observations were performed daily within 3 hours following dosing (postdose observations) on Days 1 to 91.

8.9.2.2 Detailed Clinical Observations

Each animal was removed from the cage and observed in detail weekly, beginning Week -1.

8.9.4. Body Weights

Each animal was weighed on the day of randomization (Day -2 for males/Day -3 for females) and on Days 1, 8, 15, 22, 29, 36, 43, 50, 57, 64, 71, 78, 85, and 91. A final fasted body weight was recorded for each animal on the day of scheduled euthanasia (Day 92) for calculation of organ to body weight ratios.

8.9.5. Food Consumption

Food consumption was quantitatively measured for each animal during Days 1 to 8, 8 to 15, 15 to 22, 22 to 29, 29 to 36, 36 to 43, 43 to 50, 50 to 57, 57 to 64, 64 to 71, 71 to 78, 78 to 85, and 85 to 91. The product was dosed as received; density was assumed to be 1 g/mL.

8.8.1. Administration of Test Materials

8.10. Ophthalmic Examinations

Ophthalmological examinations were performed by a board-certified veterinary ophthalmologist prior to in-life initiation (Day -6 for males/Day -7 for females) and during the last week of the treatment period (Day 92 for males/Day 91 for females). The ocular examinations were conducted using a hand-held slit lamp and indirect ophthalmoscope. A short-acting mydriatic solution was used to dilate the eyes and facilitate the indirect ocular examinations.

8.10 Laboratory Evaluations

8.10.1. Clinical Pathology

8.10.1.1. Sample Collection

Blood was collected from the vena cava (under isoflurane anesthesia at gross necropsy). After collection, samples were transferred to the clinical pathology laboratory for processing. Urine was collected overnight by urine collection cages containing water bottles with ball-bearing sipper tubes.

The animals were fasted overnight before scheduled clinical pathology sample collections, but had access to water ad libitum. Samples were collected according to Text Table.

Text Table 3
Samples for Clinical Pathology Evaluation

Group Nos.	Time Point	Hematology	Coagulation	Clinical Chemistry	Urinalysis
1 to 4	Day 92	X	X	X	X

X = sample collected.

8.10.1.2. Hematology

Blood samples were analyzed for the parameters specified in Text Table 3.

Text Table 3
Hematology Parameters

Red blood cell count (erythrocytes)	White blood cell count (leukocytes)
Hemoglobin concentration (Hgb)	Neutrophil count (Segd Neutrophils)
Hematocrit	Lymphocyte count
Mean corpuscular volume (MCV)	Monocyte count
Mean corpuscular hemoglobin concentration (MCHC)	Eosinophil count
Mean corpuscular hemoglobin (MCH)	Basophil count
Reticulocyte count (absolute)	Large unstained cells (Lg Unstain Cell)
Platelet count	Other cells (as appropriate)
Red cell distribution width (Red Dist Width)	

Blood smear slides were prepared for all animals for possible red blood cell morphology evaluation. One slide per animal was prepared, stained, and archived. Slide review was only performed on samples that met flagging criteria to confirm accurate hematology data.

8.10.1.3 Coagulation

Blood samples were processed for plasma, and plasma was analyzed for the parameters listed in Text Table 4.

Text Table 4
Coagulation Parameters

Activated partial thromboplastin time (Activated PTT)	Prothrombin time

8.10.1.4 Clinical Chemistry

Blood samples were processed for serum, and the serum was analyzed for the parameters specified in Text Table 5.

Text Table 5
Clinical Chemistry Parameters

Alanine aminotransferase (AST)	Total protein
Aspartate aminotransferase (ALT)	Albumin
Alkaline phosphatase (Alk Phos'tase)	Globulin
Gamma-glutamyltransferase (GGT, serum)	Albumin/globulin ratio (A/G Ratio)
Total bilirubin	Glucose
Urea nitrogen	Cholesterol
Creatinine	Triglycerides (Triglyceride)
Calcium	Sodium
Phosphorus	Potassium
	Chloride

8.10.1.5 Urinalysis

Urine samples were processed and analyzed for the parameters listed in Text Table 6.

Text Table 6
Urinalysis Parameters

Color	Glucose (GLU)
Clarity	Bilirubin (BIL)
Specific gravity (SG)	Ketones (KET)
Microscopic evaluation of urine sediment	Nitrites (NIT)
Total Volume (Tot Vol)	Leukocytes (LEU)
pH	Blood
Protein (Pro)	Urobilinogen (URO)

8.11. Terminal Procedures

Terminal procedures are summarized in Text Table 7.

Text Table 7
Terminal Procedures

Group No.	No. of Animals		Scheduled Euthanasia Day	Necropsy Procedures			Histology	Histopathology
	M	F		Necropsy	Tissue Collection	Organ Weights		
1	10	10	92	X	X	X	Full Tissue[a]	Full Tissue[a]
2	10	10					Gross Lesions	Gross Lesions
3	10	10					Gross Lesions	Gross Lesions
4	10	10					Full Tissue[a]	Full Tissue[a]

X = procedure conducted; M = males; F = females.
[a] See Tissue Collection and Preservation table for listing of tissues.

8.11.1. Unscheduled Deaths

No animals died during the course of the study.

8.11.2 Scheduled Euthanasia

Study animals were weighed, samples for evaluation of clinical pathology parameters were collected as specified in Section 0, and the animals were euthanized by isoflurane inhalation, followed by exsanguination. When possible, the animals were euthanized rotating across dose groups such that similar numbers of animals from each group, including controls, were necropsied at similar times throughout the day. Animals were fasted (overnight) before their scheduled necropsy.

8.11.3 Necropsy

Study animals were subjected to a complete necropsy examination, which included evaluation of the carcass and musculoskeletal system; all external surfaces and orifices; cranial cavity and external surfaces of the brain; and thoracic, abdominal, and pelvic cavities with their associated organs and tissues. Necropsy examinations were conducted under the supervision of a board-certified veterinary pathologist.

8.11.4. Organ Weights

The organs identified in Text Table 8 were weighed at necropsy for all scheduled euthanasia animals. Paired organs were weighed together. Organ to body weight ratio (using the terminal body weight) and organ to brain weight ratios were calculated.

Brain Epididymis[a] Gland, adrenal [a] Gland, pituitary Gland, prostate Gland, thyroid (including parathyroid)[a] Heart Kidney[a]		Liver Lung Ovary[a] Spleen Testis[a] Thymus Uterus

[a]Paired organ weight

8.11.5 Tissue Collection and Preservation

Representative samples of the tissues identified in Text Table 9 were collected from all animals and preserved in 10% neutral buffered formalin, unless otherwise indicated.

Text Table 9 Tissue Collection and Preservation

Animal identification	Large intestine, colon
Artery, aorta	Large intestine, rectum
Bone marrow smear	Liver
Bone marrow, femur	Lung
Bone marrow, sternum	Lymph node, mandibular
Bone, femur	Lymph node, mesenteric
Bone, sternum	Muscle, skeletal
Brain	Nerve, optic[a]
Cervix	Nerve, sciatic
Epididymis	Ovary
Esophagus	Oviduct
Eye[a]	Pancreas
Gland, adrenal	Skin
Gland, harderian	Small intestine, duodenum
Gland, lacrimal	Small intestine, ileum
Gland, mammary	Small intestine, jejunum
Gland, parathyroid	Spinal cord
Gland, pituitary	Spleen
Gland, prostate	Stomach
Gland, salivary	Testis[b]
Gland, seminal vesicle	Thymus
Gland, thyroid	Tongue
Gross lesions/masses	Trachea
Gut-associated lymphoid tissue	Ureter
Heart	Urinary bladder
Kidney	Uterus
Large intestine, cecum	Vagina

[a] Preserved in Davidson's fixative.
[b] Preserved in Modified Davidson's fixative.

8.11.6 Histology

Tissues were processed at the Testing Facility. Tissues identified in Text Table 9 (except animal identification and bone marrow smears) from animals identified in Text Table 7 were trimmed, embedded in paraffin, sectioned, mounted on glass slides, and stained with hematoxylin and eosin.

8.11.7. Histopathology

Histopathological evaluation was performed by a board-certified veterinary pathologist. Tissues were examined from animals identified in Text Table 7.

7. COMPUTERIZED SYSTEMS

Critical computerized systems used in the study are listed below or presented in the appropriate Phase Report. All computerized systems used in the conduct of this study have been validated; when a particular system has not satisfied all requirements, appropriate administrative and procedural controls were implemented to assure the quality and integrity of data.

Text Table 10
Critical Computerized Systems

System Name	Version No.	Description of Data Collected and/or Analyzed
Compaq Alpha DS10 Computer using the Toxicology Analysis System Customized, General Toxicology Module	1.0.0 or higher	Applicable in-life, clinical pathology, and necropsy data
Systems 600 Apogee Insight System	3.9.1	Temperature and/or humidity (animal rooms, refrigerators, freezers, and compound storage, as applicable)
Instem Life Science Systems, DISPENSE	7.0.3	Test material receipt and accountability
Bayer Advia 120™ Automated Hematology Analyzer	3.1.8.0-MS	Hematology data
Olympus AU640e	7.3	Clinical chemistry data
Stago STA Compact Analyzer	106.10	Coagulation data
Clinitech Advantus Urine Dipstick Analyzer	02.00:007 V1.20	Urinalysis data

The following computer study numbers were used to collect data for the various study phases: 2011912M, male toxicity phase data; 2011912F, female toxicity phase data; and RS1123, acclimation data. The tables and appendices within this report display the applicable computer study number.

8. STATISTICAL ANALYSIS

Statistical analyses were performed for the toxicity phase animals for body weights; body weight changes; food consumption; hematology, coagulation, clinical chemistry, and urinalysis (specific gravity, pH, and total volume); and organ weights. Each data set was subjected to a statistical decision tree. Data sets for each interval were initially analyzed for homogeneity of variance using Levene's test[v] followed by the Shapiro-Wilk test[vi] for normality. A $p < 0.001$ level of significance was required for each test to reject the null hypothesis. If both Levene's test and the Shapiro-Wilk test were not significant, a single-factor parametric ANOVA[vii] was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the parametric ANOVA was significant at $p < 0.05$, Dunnett's test was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

If either Levene's test and/or the Shapiro-Wilk test were significant, then the Kruskal-Wallis non- parametric ANOVA[viii] was applied, with animal grouping as the factor, using a $p < 0.05$ level of significance. If the non-parametric Kruskal-Wallis ANOVA was significant at $p < 0.05$, Dunn's test[ix] was used to identify statistically significant differences between the control group and each test article-treated group using a minimum significance level of $p < 0.05$.

9. RETENTION OF RECORDS, SAMPLES, AND SPECIMENS

All study-specific raw data, documentation, protocol, samples, specimens, and final reports from this study are the property of the Sponsor. These materials were available at the Testing Facility during the progress of the study. When the Final Report is issued, all study-specific raw data, documentation, protocol, samples, specimens, and final reports will be archived at Charles River Laboratories, Preclinical Services, Pennsylvania, Horsham, PA, for a period of 1 year. After this period, the Sponsor will be contacted to determine the disposition of these materials.

Electronic data generated by the Testing Facility will be archived, and the software and hardware required to produce it in a readable form will be maintained and available. All records, samples, specimens, and reports generated from phases or segments performed by Testing Facility-designated subcontractors will be returned to the Testing Facility for archiving.

10. RESULTS AND DISCUSSION

10.1. Mortality

All animals survived until Day 92 (study termination).

10.2. Clinical Observations

There were no test article-related clinical findings. Sporadic/transient findings were noted during the study and included hair loss, reddened areas, urine staining, swelling, scabbing, and dark material around the facial area. These findings were minor in nature and are commonly observed in the laboratory rat in a study of this duration.

10.3 Body Weights and Body Weight Changes

Body weights for Group 2 males were statistically increased as compared to controls on Day 36 during the study. In addition, body weight gains for Group 2 males were increased during Days 1 to 8 and reduced for Group 4 females during Day 8 to 15 as compared to controls. These findings were isolated in nature.

10.4 Food Consumption

There were no statistically significant differences in mean food consumption during the study.

10.5 Ophthalmic Examinations

There were no differences among the dose groups which would indicate a test article-related effect.

10.6 Hematology and Coagulation

There were no statistically significant differences in hematology or coagulation parameters.

10.7 Clinical Chemistry

Statistical differences were limited to a single statistical reduction in aspartate aminotransferase (AST) for Group 3 males as compared to controls. However, all of the mean test values were well within the range of historical control values for rats of this age.

10.8 Urinalysis

For each sex, there were no differences in values of quantitative or qualitative parameters between treated or control animals.

10.9 Gross Pathology

All gross findings including, but not limited to, dilation of the kidney pelvis or dark foci on the lymph nodes did not appear to deviate in intensity or incidence from spontaneous background changes for this strain of rat.

10.10 Organ Weights

There were no organ weight changes related to the test article. In the females, mild increases in the absolute and relative weights of the thymus, as well as mild decreases in the absolute and relative weights of the spleen, were noted at ≥ 500 mg/kg/day. However, these changes were considered to be incidental given their lack of statistical significance, microscopic correlates, and clear dose response. All differences in organ weight, including those that reached the level of statistical significance (decreased heart to body and lung to body weight in males at 500 mg/kg/day, and increased epididymis to brain weight in males at 5000 mg/kg/day) were considered to be spurious or attributed to normal biologic variation.

10.11 Histopathology

There were no test article-related microscopic findings. All microscopic findings were incidental or spontaneous background changes of no toxicologic significance. Note that 1 female in the 0 mg/kg/day group (Animal No. 3666) had evidence of a multisystemic inflammatory process with inflammation observed in the spleen, lung, liver, and lymph nodes.

Conclusion

In conclusion, once daily oral gavage administration of Free N Clear to Crl:CD(SD) Sprague Dawley rats for 91 days was well tolerated in rats at levels up to and including 5000 mg/kg/day as there were no test article-related findings during the study. Therefore, based on these results, the no-observed-adverse-effect level (NOAEL) was 5000 mg/kg/day, the highest dose tested.

Acute toxicity

Reported oral LD_{50} values for BAC in mice and rats are 150 mg/kg bw and 234 -300 mg/kg bw, respectively. In a study designed to assess the pharmacokinetics of orally administered BAC, four out of 34 rats receiving 250 mg/kg BAC by gavage died from respiratory toxicity within a 24 hour period. Approximately 50% of the rats exhibited symptoms of respiratory toxicity. The authors suspected that the fatalities and respiratory toxicity were due to aspiration of gavaged material. If no aspiration occurred, the rats appeared normal.

In humans, the lethal oral dose ranges from 100 - 400 mg/kg bw (10 - 15% solution). A 70- year old woman who drank a solution containing approximately 200 mg/kg BAC survived after receiving supportive care at a hospital. The literature search revealed one case of acute respiratory toxicity in humans after ingestion of a solution containing 10% BAC. Respiratory insufficiency was reported in a two year old girl after ingesting two teaspoons of an antiseptic solution containing 10% BAC. She developed chemical pneumonitis and upper GI tract bleeding within a week of exposure, but recovered after treatment in an intensive care unit. Therapies included assisted ventilation, enteral feeding, intravenous infusion of a dextrose- electrolyte solution, and treatment with an H_2 receptor antagonist, steroid and antibiotic.

Subchronic and chronic toxicity

The subchronic or chronic oral toxicity of BAC has been examined in a number of studies that were conducted prior to the adoption of Good Laboratory Practice (GLP) guidelines (Table 6). Early studies with BAC of unknown purity indicate variable NOAELs for BAC, depending on concentration, method of administration and/or vehicle. Whereas one two year

dietary study in rats showed no adverse effects at up to approximately 125 mg BAC/kg bw/day (0.25% or 2500 ppm in the diet), another showed decreased growth at approximately 31.5 mg/kg bw/day (0.063% or 630 ppm in the diet). In both studies, increased mortality, decreased weight gain, diarrhea, gastritis and gross and microscopic changes in the stomach and small intestine were noted in rats administered approximately 250 mg/kg bw/day (0.5% in the diet) BAC. Conversely, no overt adverse effects were found in rats ingesting feed containing 3000 mg/kg bw/day BAC for 4 - 5 weeks. Although results of these early studies provide some information about the toxicity of BAC, they are not of sufficient quality for risk assessment purposes.

Results of subchronic or chronic toxicity studies in rats, dogs or guinea pigs involving administration of BAC *via* gavage are also somewhat variable, but in general show lower NOAELs than when administered in the diet. In dogs, the 52-week oral (gavage) NOAELs for BAC when diluted in water or milk as the vehicle are < 12.5 mg/kg bw/day and 25 mg/kg bw/day, respectively. No overt signs of toxicity are observed in guinea pigs exposed to 25 mg BAC/kg bw/day by gavage (water vehicle) for one year. Whereas one study showed histological changes in the stomach and intestine of rats administered 5-25 mg BAC/kg bw/day for two years by gavage (water vehicle), another showed no effects of 50 mg BAC/kg bw/day when administered by gavage (milk or water vehicle).

EPA FIFRA guideline subchronic toxicity studies in rats and mice and chronic toxicity studies in dogs, mice and rats have been conducted on materials described as "ADBAC C12- 16" or "ADBAC C12-18", in support of an EPA high production volume (HPV) test plan for ADBAC published online in 2011. The test substance was administered by the dietary route in all of the studies. Summaries of these unpublished studies are provided in an appendix to the test plan. These tests apply to USP-BAC (CAS No. 8001-54-5), as it predominantly contains.

ADBAC C12-16, but also contains 3% ADBAC C18. The results of these studies are similar to those of the published, non-GLP studies conducted from 1948 - 1961 (see paragraph above).

The author of the summaries stated that the NOAELs for subchronic (93 - 96 day) toxicity in rats and mice were 1000 ppm (approximately 70 mg/kg bw/day in rats and 192 mg/kg bw/day in mice). All of the mice and most of the rats did not survive exposure to next highest dose (4000 ppm; approximately 280 mg/kg bw/day in rats and 768 mg/kg bw/day in mice). The summary writer mentioned that clinical signs of toxicity, decreased food consumption and body weights, gross necropsy findings (principally ileus consisting of distended fluid and gas-filled viscera) and histopathologic effects (related to the gastrointestinal changes) were observed in rats exposed to 4000 ppm concentration. In mice exposed to 4000 ppm, clinical signs of toxicity were restricted to the animals that died, and were related to general cachexia and gross necropsy observations of increased amounts of liquid or semisolid material throughout the gastrointestinal tract.

The summary writer also stated that the NOAELs for chronic toxicity in dogs, rats and mice were 14, 50 and 82 mg/kg bw/day, respectively. In rats and mice, the NOAELs for carcinogenicity were the highest doses given (102 and 259 mg/kg bw/day, respectively). Therefore, it was concluded that BAC was not a carcinogen under the conditions of the studies. Responses

observed at the lowest observable adverse effect levels (LOAEL) in chronic toxicity/carcinogenicity studies in dogs, rats and mice (35, 102 or 259 mg/kg/day, the highest doses tested) were limited to changes in body weight, food consumption and/or plasma cholesterol.

Altogether, results of guideline studies in dogs, rats and mice are consistent and show that the NOAELs for chronic toxicity (effects on body weight, food consumption and/or plasma cholesterol) are 14, 50 and 82 mg/kg bw/day, respectively. The minimum NOAEL for chronic toxicity(14mg/kgbw/day)is264timeshigherthantheestimated90thpercentileintakeof BAC from all sources (0.0530 mg/kg bw/day), including lettuce and lettuce and carrots processed with a 2% solution of FNC. In rats and mice, BAC is not carcinogenic at the highest doses tested (102 and 259 mg/kg bw/day, respectively). The EPA also has concluded that BAC is not carcinogenic.

TABLE 6

Table 6. Subchronic and Chronic Oral Toxicity Studies Conducted on BAC

Species/Strain/ Guideline (Number *per* group)*	Dose/Route	Duration	Results/Notes	Reference
White Rat (strain not specified) Non-guideline study	3% (3000 mg/kg bw/day). Dietary administration	28 - 35 days	NOAEL = 3% (300 mg/kg bw/day). No overt adverse effects noted.	Harshbarger [24]
Osborne-Mendel Rat Non guideline study *N* = 12 males/group	0, 0.063% (63 mg/kg bw/day), 0.125% (250 mg/kg bw/day), 0.25% and 0.5%. Dietary administration	Two years	NOAEL< 0.063 % (63 mg/kg bw/day) LOAEL = 0.063%: Growth suppression 0.25 %: Diarrhea, bloating of the abdomen, brown syrupy material in intestine, distension of cecum, foci of necrosis in GI tract. 0.5%: All rats died within 10 weeks	Fitzhugh and Nelson [23]
Yale/Sherman/Wistar Rat Non guideline study *N* = 12-13/sex/group	5, 12.5, 25 mg/kg bw/day *via* gavage	Two years	25 mg/kg bw/day: decreased body weight. Increased cell growth in gastric mucosa at unspecified doses	Shelanski [26]
"Albino" Rat Non guideline study (*N* = 12/sex/group)	0, 0.015%, 0.031%, 0.062%, 0.125%, 0.25% and 0.5%. Dietary administration	Two years	NOAEL = 0.125% (125 mg/kg bw/day) 0.5%: 50% survival. Diarrhea, brown viscid substance in upper GI tract, gastritis, mucosal necrosis of GI tract	Alfredson *et al.* [19]
SD Rat Non guideline study *N* = 10 males/group	50 or 100 mg/kg bw/day *via* gavage (water or milk vehicle)	12 weeks	50 mg/kg bw/day: "well tolerated" 100 mg/kg bw/day: Reduced weight gain, increased mortality[a]	Coulston *et al.* [25]

SD Rat EPA FIFRA 82-1 guideline study	100 ppm (7 mg/kg bw/day), 500 ppm (35 mg/kg bw/day) 1000 ppm (70 mg/kg bw/day),	95 - 96 days	NOAEL = 1000 ppm (70 mg/kg bw/day) LOAEL could not be determined due to 100% mortality at 4000 ppm	Van Miller and Weaver [28]

Species/Strain/ Guideline (Number *per* group)*	**Dose/Route**	**Duration**	**Results/Notes**	**Reference**
(OPPTS 870.3100) *N* = 10 sex/group, minimum**	4000 and 8000 ppm.[b] Dietary administration		Increased mortality, gross and histological changes in the GI tract at 4000 and 8000 ppm.	
SD Rat EPA FIFRA 83-5 guideline study (OPPTS 870.4300) *N* = 50 sex/group, minimum**	300 ppm (15 mg/kg bw/day), 1000 ppm (50 mg/kg bw/day), 2000 ppm (102 mg/kg bw/day). Dietary administration	Two years	NOAEL (repeated dose toxicity) = 1000 ppm (50 mg/kg bw/day) NOAEL (carcinogenicity) = 2000 ppm (102 mg/kg bw/day) LOAEL (repeated dose toxicity) = 2000 ppm (102 mg/kg bw/day). Reduced body weight and food consumption	Gill *et al.* [29]
CD-1 Mouse EPA FIFRA 82-1 guideline study (OPPTS 870.3100) *N* = 10 sex/group, minimum**	100 ppm (20 mg/kg bw/day), 500 ppm (94 mg/kg bw/day), 1000 ppm (192 mg/kg bw/day), 4000, 8000 ppm[b]. Dietary administration	93-94 days	NOAEL = 1000 ppm (192 mg/kg bw/day) LOAEL could not be determined due to high mortality at 4000 ppm. Increased mortality, gross and histological changes in the GI tract at 4000 and 8000 ppm.	Van Miller and Weaver [28]
CD-1 Mouse EPA FIFRA 83-2 guideline study (OPPTS 870.4200) *N* = 50 sex/group, minimum**	100 ppm (16 mg/kg bw/day), 500 ppm (82 mg/kg bw/day) and 1500 ppm (259 mg/kg bw/day). Dietary administration	78 weeks	NOAEL (repeated dose toxicity) = 500 ppm (82 mg/kg bw/day) NOAEL (carcinogenicity) = 1500 ppm (259 mg/kg bw/day) LOAEL (repeated dose toxicity) = 1500 ppm (259 mg/kg bw/day). Reduced body weights and body weight gains	Gill *et al.* [29]
Guinea pig	Non guideline study	*N* = 10/sex/group		5, 12.5, 25 mg/kg

Species/Strain/ Guideline (Number *per* group)*	Dose/Route	Duration	Results/Notes	Reference
Mongrel Dog Non guideline study *N* = 1-2/group	0.031, 0.062, 0.12, 0.25, 0.5, 1%. Dietary administration	15 weeks	NOAEL = 0.12%. LOAEL = 0.25%. Decreased body weight at ≥ 0.25%. Moribundity, mortality and gross and histological changes in the GI tract at ≥ 0.5%.	Alfredson *et al.* [19]
Beagle Dog Non guideline study *N* = 3/group	12.5, 25 and 50 mg/kg bw/day *via* gavage (10% solution in water or milk vehicle)	One year	Water vehicle: NOAEL < 12.5 mg/kg bw/day. Gross and microscopic changes in the intestine at12.5 mg/kg bw/day. Vomiting and increased mortality at higher doses Milk vehicle: NOAEL 25 mg/kg bw/day. Gross changes in the intestine at 50 mg/kg bw/day	Coulston *et al.* [25]
Beagle Dog EPA FIFRA 83-1(b) guideline study (OPPTS 870.4100) *N* = 4/sex/group	120 ppm (4 mg/kg bw/day), 400 ppm (14 mg/kg bw/day) and 1200 ppm (35 mg/kg bw/day). Dietary administration	One year	NOAEL = 400 ppm (14 mg/kg bw/day) LOAEL = 1200 ppm (35 mg/kg bw/day). Decreased body weight, food consumption and cholesterol	Goldenthal [30]

EPA = Environmental Protection Agency; FIFRA = Federal Insecticide, Fungicide and Rodenticide Act; GI = gastrointestinal; LOAEL = lowest observed adverse effect level; NOAEL = no observable adverse effect level; ppm = parts *per* million; SD = Sprague-Dawley

*If available from reference; ** According to guideline. Numbers of animals tested were not mentioned in the reference. [a] Unclear if tissue examinations were performed; [b] Due to mortality in the 4000 and 8000 ppm groups, actual doses could not be calculated.

Genotoxicity

The genotoxicity of BAC has been evaluated in several *in vitro* assays. Results of bacterial mutagenicity studies with BAC are overwhelmingly negative, however, chromosome aberration studies are variable. Results of one study show that concentrations of up to 30 µM BAC (approximately 10.8 mg/L) are not clastogenic in a Syrian Hamster Embryo (SHE) cell study. Another study showed that 1 or 3 mg/L BAC caused an equivocal increase in sister chromatid exchanges in SHE cells. A 1.0 mg/L concentration of BAC increased the frequency of micronuclei in cultured human lymphocytes. DNA damage occurs in cultured human respiratory epithelial cells exposed to 0.02% BAC (200 mg/L). In rats administered 250 mg/kg BAC by the oral route, the concentration of BAC in plasma and tissues is approximately 0.1 – 1 µg/g (mL), which is approximately equal to the lowest concentration of BAC that caused genetic toxicity *in vitro* (1 mg/L). As mentioned in Section 6.1.3 above, a 20 mL/kg dose of 2% FNC (which delivers a BAC dose of 1.92 mg BAC/kg bw) does not increase the frequency of micronuclei in bone marrow of rats. These findings suggest that the plasma and tissue concentrations that could arise from rats exposed to 1.92 mg BAC/kg bw from a 2% solution of FNC (an estimated maximum of 7.7 µg/L) are substantially lower than those that produce genotoxicity *in vitro*. The plasma concentration of BAC in people consuming lettuce and carrots processed with 2% FNC would be even lower

than 7.7 µg/L, as the total amount of BAC consumed by these individuals (0.053 mg/kg bw/day) is 36-times lower than the BAC dose associated with this plasma concentration in rats (1.92 mg BAC/kg bw/day). In conclusion, the maximum tissue and plasma concentrations of BAC in people consuming 90th percentile quantities of lettuce and carrots processed with 2% FNC would be substantially lower than the concentrations of BAC that are clastogenic in some *in vitro* assays. Therefore, the positive results of some *in vitro* clastogenicity studies with high concentrations of BAC are not relevant for the safety assessment of BAC in 2% FNC.

Reproductive or Developmental Toxicity

The potential for Free N Clear^{TM} to cause reproductive or developmental toxicity has not been tested. Studies conducted prior to adoption of GLP guidelines showed no overt adverse effects of up to 25 mg/kg bw/day BAC on "fertility"[32] of rats or guinea pigs.

Four studies have been conducted to examine the developmental toxicity of BAC (Table 7). An EPA FIFRA guideline two generation reproductive toxicity study in Sprague-Dawley rats has been conducted on a material described as "ADBAC C12-16" in support of an EPA high production volume (HPV) test plan published online in 2011. This test applies to USP-BAC (CAS No. 8001-54-5), as it predominantly contains ADBAC C12-16. A summary of the unpublished study is provided in an appendix to the published EPA HPV test plan for ADBAC. The results of this study are similar to those of the published, non-GLP study conducted by Shelanski (see paragraph above). Doses used in the study were 300, 1000 and 2000 ppm in feed (approximately 22, 73 or 145 mg/kg bw/day, respectively). The author of the summary stated that the no observable effect level (NOEL) for toxicity to parental animals or offspring was 1000 ppm (73 mg/kg bw/day). Effects noted at the lowest observable effect level (LOEL) of 2000 ppm (145 mg/kg bw/day) were reduced body weight or reduced body weight gain of parental animals and pups. Reproductive parameters were not affected by treatment with up to 2000 ppm (145 mg/kg bw/day).

Table 7. Developmental Toxicity of BAC

Species/Strain/ Guideline (Number *per* group)*	Dose/Route	Duration	Results/Notes	Reference
Wistar Rat Study comparable to OECD Guideline 414 *N* = 22 - 37/group	0, 5, 15, 50 g/kg bw/day	Treatment: GD 6-15 Termination: GD 20	NOEL (maternal toxicity): 15 mg/kg bw/day NOEL (developmental toxicity): 50 mg/kg bw/day LOEL (maternal toxicity):50 mg/kg bw/day. Increased mortality.	Knickerbocker and Stevens [37]
SD Rat EPA FIFRA 83-3 guideline study (OPPTS number 870.3700) *N* = 25/group	0, 10, 30, 100 mg/kg bw/day	Treatment: GD 6-15 Termination; GD 21	NOEL (maternal toxicity): 10 mg/kg bw/day NOEL (developmental toxicity): 100 mg/kg bw/day LOEL (maternal toxicity): 30 mg/kg bw/day. Reduced food consumption, audible respiration. Perioral wetness noted at 100 mg/kg bw/day.	Neeper-Bradley [36]
ICR/JCL Mouse Non guideline study *N* = 5 - 20/group	0.001, 0.05, 0.1, 3, 10, 30 mg/kg bw BAC	Treatment: GD 0-6 Termination: GD 13 or Treatment: GD 0-17 Termination: GD 17	NOAEL (developmental toxicity): 30 mg/kg bw/day	Momma *et al.* [38]
NZW Rabbit EPA FIFRA 83-3 guideline study (OPPTS number 870.3700) *N* = 16/group	0, 1, 3 or 9 mg/kg bw/day	Treatment: GD 6-18 Termination; GD 29	NOEL (maternal toxicity): 3 mg/kg bw/day NOEL (developmental toxicity): 9 mg/kg bw/day LOEL (maternal toxicity): 9 mg/kg bw/day. Hypoactivity, labored respiration	Neeper-Bradley [36]

FIGURES

TABLES

Rec'd 1-29-17

TABLE OF CONTENTS

Part 6 NARRATIVE

The basis for conclusion of GRAS Status

(a) SAFETY

1. Non toxic as 90 rat study, efficient kill, and safe residuals

2. Specific data and information (safety studies pgs. 87 and 97)

(b) BASIS FOR CONCLUSION

1. There is a great need for a wash solution processing aid for carrots and lettuce
2. Major food companies recalling carrots and lettuce due to problems with E. coli, salmonella and listeria
3. FDA was questioned to find most respected testing to validate non-toxic use of liquid disinfectant (The 90 day rat consumption program was suggested.)
4. Testing Results:
   a. 90 day rat study by Charles River Laboratories (Free N Clear™ was non toxic, external, liver, bone marrow
   b. Microbac Laboratories of BAC bacteria kills and residuals
   c. Kansas State University Food Science Institute kill times to exposed bacteriaof E. coli, salmonella, listeria (using 2% Free N Clear™ solution for 5 minute exposure) 85.4% all bacteria killed in 5 minutes
   d. Kansas State University (Food Science Lab in Olathe Kansas – residuals after 2% exposure of 5 minutes) BAC <10 PPM, methyl paraben <5 PPM ; EPA level of 44PPM daily intake

5. Chemistries of creating blend for Free N Clear™ are available from Lonza Chemical Company, stable and blend is safe for human consumption. Acetic acid and methyl paraben are listed as GRAS

6. Filing for GRAS listing as processing aid for solution used at the processing locations for carrots and lettuce. Respect for scientific testing and professional opinions of industry connected professionals

(c) Specifications for food grade materials
   1. No negatives found against Marvel Free N Clear™
   2. We have reviewed and not found any inconsistent information against Marvel Free N Clear™

(d) Not any information exempt from disclosure of patent protected, #8,268,337

(e) Basis for GRAS conclusion, even if they had no known data or information
(See pages 21 + 22 —

(f) CONCLUSION (attached)

Bonnette, Richard

From: Thomas Ellsworth <tom@marveltechnologiesusa.com>
Sent: Friday, August 18, 2017 10:32 AM
To: Bonnette, Richard
Subject: Re: submission to the GRAS notification program

Richard E. Bonnette
Consumer Safety Office
College Park, MD 20740-3835

Dear Mr. Bonnette,
I am Tom Ellsworth, the president and CEO of Marvel Technologies USA, LLC. I serve solely at the pleasure of the Board of Directors of Marvel Technologies USA, LLC.
I am the contact for the submission of Marvel Free N Clear for GRAS approval by FDA.

We have relocated our offices to:

Marvel Technologies USA, LLC
8161 TN-100
Bellevue, TN 37221

We also have new email addresses:
Tom@marveltechnologies usa.com
Our phone number remains the same: 615-261-8084

Thank you for updating your records on our company.

Best regards,

Tom Ellsworth
President/CEO
Marvel Technologies USA, LLC
8161 TN-100
Bellevue, TN 37221

On Fri, Aug 18, 2017 at 8:44 AM, Bonnette, Richard <Richard.Bonnette@fda.hhs.gov> wrote:

Tom,

I do actually need something from you for our records indicating that you're the contact for this submission going forward - if that's what you intend. It can be a simple email just saying that you're the contact going forward.

Regards,

Richard

Cournoyer, Patrick

From: Thomas Ellsworth <tom@marveltechnologiesusa.com>
Sent: Tuesday, December 12, 2017 1:25 PM
To: Cournoyer, Patrick
Subject: Re: Feedback GRN723

Thank you Patrick. I expected as much.
We are in process of transitioning the management of the company and the submissions from Jack Wheeler to a more qualified individual or team.
I am only in this position for a temporary period during the transition. For these reasons, I would ask that you would cease to evaluate our notice.
If you would let me know the formal way to send you the notice to cease, I will be happy to do that.

Thank you for all the time and effort you and your team put into evaluating the submission and detailing the deficiencies of the submission.
I hope you and your staff have a wonderful holiday season with family and friends, and have a very blessed New Year.

Best regards,

Tom Ellsworth, CEO
Marvel Technologies USA, LLC

On Tue, Dec 12, 2017 at 9:47 AM, Cournoyer, Patrick <Patrick.Cournoyer@fda.hhs.gov> wrote:

> Dear Mr. Ellsworth,
>
> We have identified major deficiencies with your notice that cannot be easily remedied, particularly within our timeframe for submitting amendments to notices (~10 days). We recommend that you send us an email to request that we cease to evaluate your notice. Otherwise, we will issue a letter stating the notice lacks a sufficient basis for a GRAS conclusion.
>
> **General Feedback:**
>
> The notice is poorly written and fails to provide a cogent narrative. Much of the content is not in complete sentences, and typographic errors are ubiquitous. The notice uses terms and phrasing that are not adequately explained, and it lacks appropriate organization. As a consequence, the notice fails to make a logical case for the safe use of BAC and for general recognition of safety.
>
> The notice does not comply with the organization described 21 CFR 170.220. This regulation describes seven parts, and the notice should be organized with each type of information in the appropriate part. Your notice titled several sections "table of contents," however none of these sections is, in fact, a table of contents. Titles and headings throughout the notice are confusing.
>
> Critically, the notice fails to link information to its sources and to cite literature where appropriate. In some instances, the notice appears to cite literature using superscript numerals, but the numerals do not appear to correlate with a list of sources. The notice repurposed text from GRN488, but the text in GRN723 is missing the citations to references that were used in GRN488.

Cournoyer, Patrick

From: Thomas Ellsworth <tom@marveltechnologiesusa.com>
Sent: Friday, December 22, 2017 9:12 AM
To: Cournoyer, Patrick
Subject: Re: GRN723 FDA response letter -cease to evaluate
Attachments: image009.jpg

Patrick,
Thank you for letting me know. He is not authorized to contact you on behalf of Marvel Technologies. I can't really control his actions, I'm sorry for the inconvenience to you.

I hope you get off soon to enjoy the holidays!

Best regards,
Tom

On Wed, Dec 20, 2017 at 4:12 PM Cournoyer, Patrick <Patrick.Cournoyer@fda.hhs.gov> wrote:

> Dear Tom,
>
> I received a voicemail from Jack Wheeler saying he needs help putting together a notice, presumably for BAC. Since you're the listed point of contact for the notice, I won't respond to his voicemail. If Marvel has questions or concerns, you are welcome to contact me.
>
> Best regards,
> Patrick
>
> **From:** Thomas Ellsworth [mailto:tom@marveltechnologiesusa.com]
> **Sent:** Tuesday, December 19, 2017 11:30 AM
> **To:** Cournoyer, Patrick <Patrick.Cournoyer@fda.hhs.gov>
> **Subject:** Re: GRN723 FDA response letter -cease to evaluate
>
> Thank you Patrick.
>
> I hope you and your family have a wonderful Christmas and a great 2018!
>
> Tom