10761 King William Drive | Dallas, TX 75220
Phone: 469-484-1927 | Fax: 469-484-1930 | www.USPlabsDirect.com

Contains Trade Secrets and
Confidential Commercial Information
Not For Public Disclosure

September 28, 2012

Quyen Tien
Division of Enforcement
Office of Compliance (HFS-608)
Center for Food Safety and Applied Nutrition
Food and Drug Administration
5100 Paint Branch Parkway
College Park, Maryland 20740-3835

Re: Warning Letter No. 285519 (April 24, 2012)

Dear Mr. Tien:

We received a warning letter from Michael W. Roosevelt (the Roosevelt letter) dated April 24, 2012, regarding the marketing of our dietary supplements that contain the dietary ingredient 1,3-DMAA, and responded to that letter on May 15, 2012. Subsequent to that response, we have also had an opportunity to meet with FDA officials in order to obtain a more complete understanding of the issues raised in the Roosevelt letter. As promised in our May 15 response, we are providing additional information that has become available since sending that response.

I. Four Analytical Studies Have Now Found That 1,3-DMAA is a Natural Constituent of Geranium

In Section I(B) on pages 3-4 of our response of May 15, we described and submitted in Appendices 4-7 three independent scientific analytical studies that have detected and quantified 1,3-DMAA as a constituent of geranium from specific regions in China. At the time, the two most recent of those studies had not yet been subjected to peer review and publication: the Intertek Study (subsequently published under the name of the principal investigator Li) and the Simone Study (now accepted for publication under the name of the principal investigator Fleming). The Li Study is attached in the form in which it has been published following peer review, in Appendix A to this supplemental response. The Fleming Study is attached in the form in which it has been accepted for publication following peer review, in Appendix B. As noted in our May 15 response, both studies detect and quantify the amount of 1,3-DMAA found in Chinese geranium.

10761 King William Drive | Dallas, TX 75220
Phone: 469-484-1927 | Fax: 469-484-1930 | www.USPlabsDirect.com

Subsequent to our May 15 response, we asked a respected independent analytical chemist to evaluate both the three studies that have detected and quantified the amount of 1,3-DMAA in geranium (Ping, Li, and Fleming) and the two studies that FDA officials have said did not detect the presence of 1,3-DMAA in geranium (Zhang and ElSohly). Attached in Appendix C is the report prepared by Thomas D. Gauthier, Ph.D., Senior Science Advisor at Environ International Corporation. As his report relates, a detailed analysis of the Zhang and ElSohly studies, including the chromatograms made publicly available by the publisher but not included in the published version of the Zhang study, demonstrates that 1,3-DMAA was in fact found in geranium in one of those studies (ElSohly), and may have been found in the other study (Zhang), although the amount was not quantified in either study.

Thus, there are now four scientific studies that have detected 1,3-DMAA in natural geranium and a fifth study in which it may have been detected.

II. The Status of the Animal Toxicity Studies of 1,3-DMAA in Rats and Rabbits is Nearing Completion

In Section IV(B) of our May 15 response, we informed you that, in addition to the published animal studies on 1,3-DMAA cited in footnotes 38 and 39 and the recently-completed oral LD-50 studies in rats and rabbits, oral subchronic 90-day studies in rats and rabbits were also underway.

At the time of that response, we informed you about the results of the oral LD-50 studies, but the final reports on those studies had not yet been completed. Two maximum tolerated dose (MTD) studies were conducted by Clintox on 1,3-DMAA, one on rats and the other on rabbits. Using well-accepted scientific extrapolations based on probit analysis, the results of those MTD studies have been used to calculate the LD-50 data shown on Page 17 of our May 15 response. The MTD study on rats is attached in Appendix D and the MTD study on rabbits is attached in Appendix E. The Summary Report for the LD-50 calculation in rats is in Appendix F and in rabbits is in Appendix G. Finally, we are also attaching a summary report on the 90-day study in rats at day 90 in Appendix H and a status report on the 90-day study in rabbits at week 10 in Appendix I. All of these data confirm the safety of 1,3-DMAA when used at the levels contained in our dietary supplement products and in accordance with the directions for use and warnings.

III. Conclusion

The information contained in this supplemental response to the April 24, 2012 Roosevelt letter provides further confirmation of the safety and legality of 1,3-DMAA as used in our products. We will provide final reports on the animal subchronic toxicity testing as soon as they become available.

10761 King William Drive | Dallas, TX 75220
Phone: 469-484-1927 | Fax: 469-484-1930 | www.USPlabsDirect.com

IV. Confidentiality

The information in this submission constitutes trade secrets and confidential information that is exempt from public disclosure under 5 U.S.C. § 1905 and Section 301(j) of the Federal Food, Drug, and Cosmetic Act, 21 U.S.C. § 331(j).

Sincerely yours,

Jonathan V. Doyle
President

Enclosures

Study code: CB-ULD-SCTDMPN-01

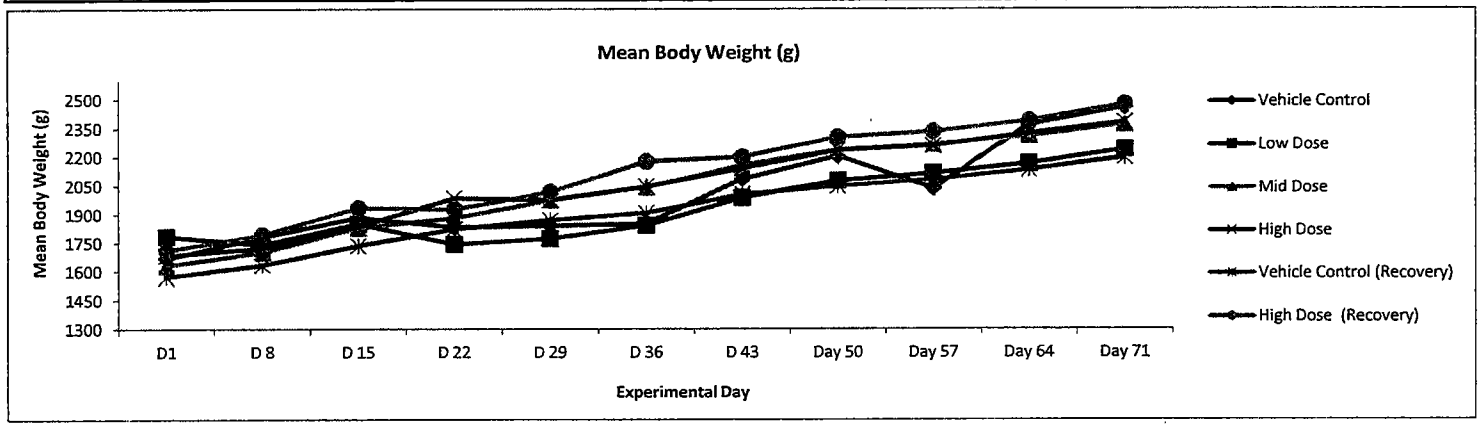
Clintox biosciences

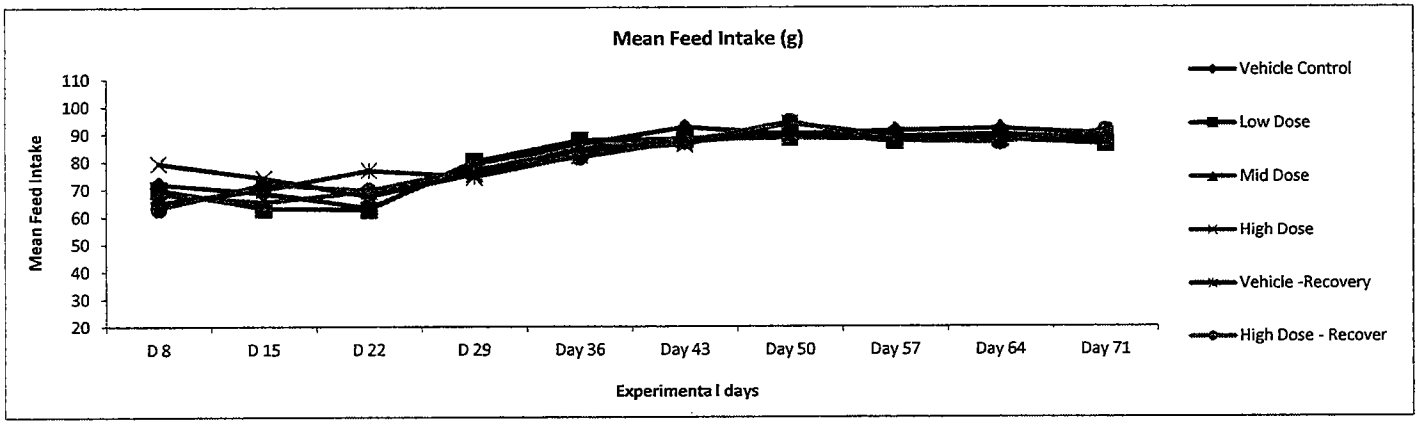
STATUS REPORT

STUDY TITLE	90 - DAY REPEATED DOSE TOXICITY STUDY OF 1, 3 DIMETHYLPENTYLAMINE ADMINISTERED BY ORAL GAVAGE IN NEW ZEALAND WHITE RABBITS WITH RECOVERY PERIOD OF 14 DAYS

Date of reporting	20-Sep-12
Week of Reporting	10

Group Name	Group Description	Dose (mg/kg)	No of animals/			% Mortality (%)			% Survival
			M	F	Total	M	F	Total	
Group I	Vehicle control	0.00	4	4	8	0%	0%	0%	100%
Group II	Low dose	3.13	4	4	8	0%	0%	0%	100%
Group III	Mid dose	6.25	4	4	8	0%	0%	0%	100%
Group IV	High dose	12.50	4	4	8	0%	0%	0%	100%
Group V	Vehicle Control Recovery	0.00	4	4	8	0%	0%	0%	100%
Group VI	High Dose Recovery	12.50	4	4	8	0%	0%	0%	100%





Prepared by	Mr. Madhusudhan
Verified by	Dr. Gunindra
Approved by	Mr. Srinivas Ventrapragada

SUMMARY REPORT FOR 1, 3 DMPA-RAT

Clintox bioservices

CB-ULD-SCTDMPW-01 (1, 3-DMPA-RAT):

1 TITLE

90-day repeated dose toxicity study of 1, 3 Dimethylpentylamine administered by oral gavage in male and female Wistar rats.

2 OBJECTIVE

The objective of this study is to assess the toxicity profile of 1, 3 dimethylpentylamine after repeated administration by oral gavage for 90 days in Wistar rats. The toxicity will be assessed in terms of general behavior, mortality, serum biochemistry, hematology, and histopathology examinations.

3 STUDY DESIGN

The study mainly consisted of one vehicle control group and three treatment groups and two recovery groups. The three treatment groups included low dose (3.13 mg/Kg BW), mid dose (6.25 mg/Kg) and high dose (12.50 mg/Kg). The recovery groups include vehicle control recovery and high dose recovery (12.50 mg/Kg). The animals were dose repeatedly for 90-days with respective concentration by oral gavage.

Group Name	Group Description	Dose (mg/kg)	No of animals/ group			No of Mortality (%)			% Survival
			M	F	Total	M	F	Total	
Group I	Vehicle control	0.00	10	10	20	0%	0%	0%	100%
Group II	Low dose	3.13	10	10	20	0%	0%	0%	100%
Group III	Mid dose	6.25	10	10	20	0%	0%	0%	100%
Group IV	High dose	12.50	10	10	20	0%	0%	0%	100%
Group V	Vehicle control Recovery	0.00	5	5	10	0%	0%	0%	100%
Group VI	High dose Recovery	12.50	5	5	10	0%	0%	0%	100%

4 OBSERVATIONS

The animals were observed daily for clinical signs for a period of 90 days for the main groups and 104 days for recovery groups. The observations were recorded once daily.

5 MORTALITY

Mortality was observed daily twice, once morning and evening and was duly recorded. No mortality was recorded during the study.

6 GENERAL OBSERVATION

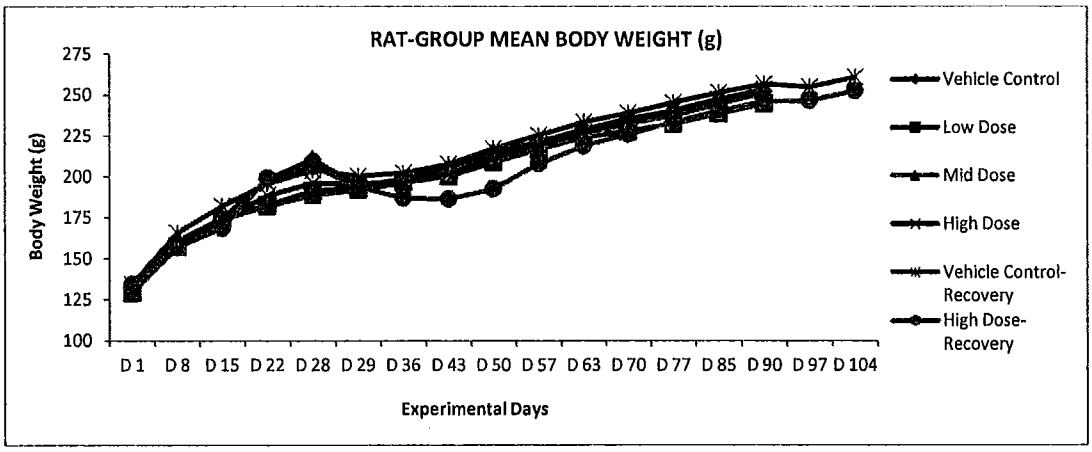
All animals were observed daily by trained study personnel for any general behavioral and physical changes. The regular observation included, but not limited to, natural orifices, skin condition, mortality, gait, movement, neurological disorders, eye condition, faeces and urine conditions. These observations were observed once daily outside the home cage, Observations were recorded, using scoring system using organizational SOP's. Observations like skin, fur, eyes, mucous membranes, presence or absence of secretions and excretions

and autonomic activity, lacrimation, piloerection, pupil size, unusual respiratory pattern, changes in gait, posture were also recorded.

No behavioral abnormalities were observed in all the experimental animals after dosing till the end of the experimental period.

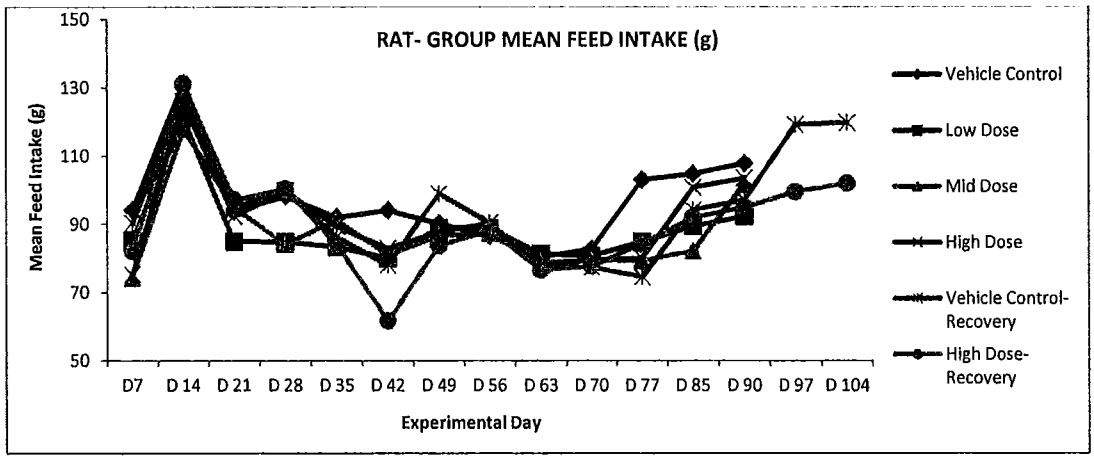
7 BODY WEIGHT

Body weight of each animal was recorded once in a week during the entire study period. A normal body weight gain was observed across all groups during the entire study period.



8 FEED INTAKE

Feed intake of each animal was recorded once in a week during the entire study period. The mean feed intake was found to be normal during the study period.



Hematology, Clinical Chemistry, Urine analysis and histopathology are under process.

SUMMARY REPORT FOR LD_{50}

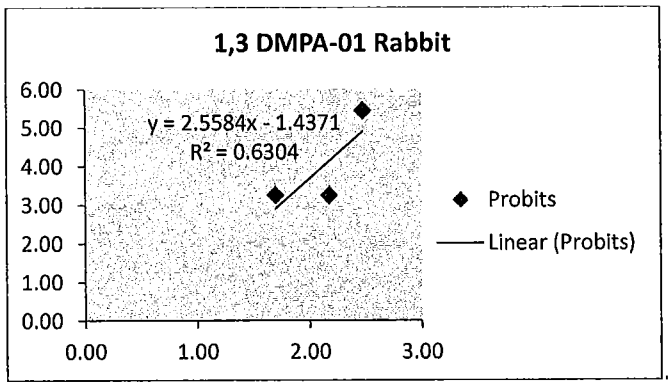
Clintox bioservices

CB-ULD-ATDMPN-01 (1, 3 DMPA-RABBIT):

The study mainly consisted of one control group and three treatment groups. The three treatment groups included low dose (75mg/Kg BW), mid dose (150mg/Kg) and high dose (300mg/Kg). The animals were given a single dose of respective concentration by oral gavage and observed for a period of 14 days to determine the maximum tolerated dose (MTD). Four mortalities were reported in the high dose group. Hence the maximum tolerated dose was found to be between 150 and 300 mg/Kg BW. The LD_{50} calculated from probit analysis is 324.33 mg/kg.

CB-ULD-ATDMPN-01 (1,3 DMPA Rabbit)							
Treatment Groups	No of animals/ Group	Dose mg/Kg	Log Dose	No of Mortalities	% Dead	*Corrected %	Probits
1	6	50	1.70	0	0	4	3.25
2	6	150	2.18	0	0	4	3.25
3	6	300	2.48	4	66.66667	67	5.4

Log Dose	Probits
1.70	3.25
2.18	3.25
2.48	5.44



Formula for correction of 0% mortality = 100 (0.25/n)
Where n = total number of animals.
Where y = 5 (Since we are calculating LD_{50})
Y = 2.5584x-1.4371 ⇒ 2.5584x = 5+1.4371 ⇒ X = 6.4371/2.5584 ⇒ X = 2.5161
Antilog x = Antilog 2.5161 = LD_{50} ⇒ Therefore, LD_{50}=324.34 mg/Kg.

RESULT: The calculated LD_{50} obtained is 324.34 mg/Kg.

SUMMARY REPORT FOR LD_{50}

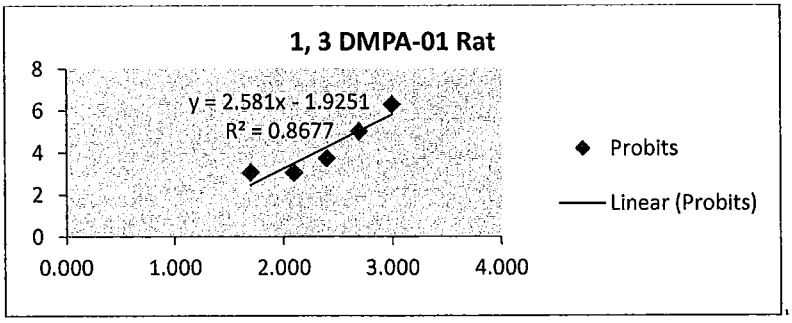
Clintox bioservices

CB-ULD-ATDMPW-01 (1, 3-DMPA-RAT):

The study mainly consisted of one control group and three treatment groups. The three treatment groups included low dose (50mg/Kg BW), mid dose (125mg/Kg) and high dose (250mg/Kg). The animals were given a single dose of respective concentration by oral gavage and observed for a period of 14 days to determine the maximum tolerated dose (MTD). One mortality was reported in the high dose group. Hence the maximum tolerated dose was found to be between 125 and 250 mg/Kg BW. The LD_{50} calculated from probit analysis is 481.94 mg/kg.

CB-ULD-ATDMPW-01 (1,3 DMPA Rat)							
Treatment Groups	No of animals/ Group	Dose mg/Kg	Log Dose	No of Mortalities	% Dead	*Corrected %	Probits
1	10	50	1.699	0	0	2.5	3.035
2	10	125	2.097	0	0	2.5	3.035
3	10	250	2.398	1	10	10	3.72

Log Dose	Probits
1.699	3.035
2.097	3.035
2.398	3.72



Formula for correction of 0% mortality = 100 (0.25/n)
Where n = total number of animals.
Where y = 5 (Since we are calculating LD_{50})
Y=2.581x-1.9251 → 2.581x=5+1.9251 → X=6.9251/2.581 → X=2.6831
Antilog x = Antilog 2.6831 = LD_{50} → Therefore, LD_{50}=481.948 mg/Kg.

RESULT: The calculated LD_{50} obtained is 481.94 mg/Kg.

REPORT

ON

DETERMINATION OF MAXIMUM TOLERATED DOSE (MTD) OF

1, 3- DIMETHYLPENTYLAMINE IN NEW ZEALAND WHITE

RABBITS

SUBMITTED BY

ClintoxBioservices Pvt. Ltd
Plot # 4B, Phase I,
Alexandria Knowledge Park, Turkapally
Genome Valley, Hyderabad, India –500 078

DECLARATION

This study was conducted at Clintox Bioservices Pvt. Ltd, Shameerpet, Hyderabad, India. All procedures were conducted in accordance with Standard Operating Procedures at Clintox Bioservices. No occurances or results have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study. The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

Dr. Prashant Deshmukh
Study Director

Date: 10 May 2012

With the participation of:

Mr. Praveen Thirunahari
Mr. Bhaskar Banoth

This report has been reviewed by:

Dr. Sekhar Bathina, PhD
Quality Assurance

Date: 10 May 2012

Clintox bioservices

QUALITY ASSURANCE STATEMENT

I declare that the report on *"DETERMINATION OF MAXIMUM TOLERATED DOSE (MTD) OF 1,3-DIMETHYLPENTYLAMINE IN NEW ZEALAND WHITE RABBITS"* was audited by Quality Assurance Unit and is in conformity with the protocols, standard operating procedures and this report accurately reflects the methods and findings.

Dr. Sekhar Bathina, PhD
Quality Assurance

Date: 10 May 2012

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS

ABBREVIATION	DETAILS
GLP	Good Laboratory Practice
G	Gram
Kg	Kilogram
Bwt	Body weight
S No	Serial Number
F	Female
mL	Milliliter
°C	Degree Celsius
%	Percentage
Mg	Milligram
NAD	No Abnormalities Detected
No.	Number
H	Hour/hours
SOP	Standard Operating Procedure
IAEC	Institutional Animal Ethics Committee
CPCSEA	Committee for the Purpose of Control and Supervision on Experiments on Animals

1 STUDY SUMMARY

The purpose of this study was to determine Maximum Tolerated Dose (MTD) of 1,3-Dimethyl pentylamine in New Zealand White rabbits.

The study was conducted in 24 (12M + 12F) New Zealand White rabbits. All animals were quarantined for at least 7 days prior to experimentation, and then randomized and divided into four groups of 6 animals (3M+3F) in each group. The four groups were designated as Group I (Vehicle Control), II (50 mg/Kg), III (150 mg/Kg)and IV (300 mg/Kg). The Vehicle Control animals were treated with Water for Injection while the treatment animals were treated with respective concentrations of 1,3-Dimethylpentylamine.

All animals were observed daily by trained personnel for any overt toxicity. The regular general observations included body weight, feed intake, mortality and natural orifices. Clinical observations included tremor, convulsion, piloerection, salivation, lacrimation, respiration, activity, arched back, distended abdomen, skin condition, fur, mucous membrane, presence or absence of secretions, eye condition, tail elevation, motor activity, posture, gait, urination and defecation, neurological disorders, presence of clonic and tonic movements. Observations were accurately recorded as per the scoring systems defined by organizational SOP. Mortality was observed and duly recorded daily for 14 days. Gross pathological examination was carried out on all animals. Heart, lung, liver, kidneys, spleen, stomach, large intestine, small intestine, brain and skin were examined for any macroscopic lesions and the observations were duly recorded.

Nasal discharge, salivation, tremors, convulsions and tachycardia were observed in animals treated with 300 mg/Kg where as a few animals treated with 150 mg/Kg exhibited tremors until 6 hrs after dosing. Those which were dosed with 50 mg/Kg and vehicle did not show any behavioural aberrations during physical examination.

A consistent increase in body weight and feed intake was observed across all the groups.

Four preterminal mortalities were observed in the group treated with 300 mg/Kg. Two deaths were recorded within three hours after dose administration where as one mortality each were recorded 3 and 4 hours after dosing.

Gross pathological observations indicated no changes in animals treated with vehicle, 50 and 150 mg/kg. Animals which were reported dead after treating with 300 mg/kg showed reddish discolouration in the lungs, multiple watery cyst on right median lobe of liver and focal white patches, spleen showed focal whitish deposit on surface, stomach showed glandular mucosa with focal hyperaemia during gross necropsy.

Based on the experimental results in this study it may be concluded that the maximum tolerated dose (MTD) of 1,3-Dimethylpentylamine in New Zealand White rabbits when treated with a single oral dose is between 150 mg/Kg and 300 mg/Kg body weight.

2 STUDY OVERVIEW

2.1 STUDY OBJECTIVE

The objective of this study was to determine Maximum Tolerated Dose (MTD) of orally administered 1,3-Dimethylpentylamine in New Zealand white rabbits. The MTD was assessed in terms of general observations, clinical signs, body weight, feed intake, mortality and gross pathological examination.

2.2 STUDY SCHEDULE

Study title: *"Determination of Maximum Tolerated Dose (MTD) of 1,3- Dimethylpentylamine in New zealand white rabbits."*

Study Code	:	CB-ULD-ATDMPN-01
Study Schedule	:	
Acclimatization Start Date	:	31 March 2012
Acclimatization End Date	:	10 April 2012
Test Article Dosing Date	:	11 Apri 2012
Observations Period	:	11 April 2012 to 24 April 2012
Date of Sacrifice	:	25 April 2012
Draft Report Submission	:	05 May 2012
Final Report Submission	:	10 May 2012
Study Facility	:	ClintoxBioservices Pvt. Ltd Alexandria Knowledge Park,Turkapally Genome Valley, Hyderabad, India –500 078
Study Sponsor	:	USP Labs, 10761 King William Drive Dallas, TX 75220, USA

2.3 IAEC APPROVAL

The study was initiated after obtaining due approval of the protocol from IAEC.

2.4 TEST SYSTEM

Species	Rabbit
Strain	NewZealand white
Justification for the species selection	As per sponsor's requirement
Source	Sainath Agencies, Hyderabad
Number of animals for study	24 (12 M + 12 F)
Age (Weeks) /Average weight	2-3 kg
Sex	Male and Female

2.5 METHOD OF IDENTIFICATION

Animals were identified by ear markings and cage identification system in standard stainless steel rabbit cages. All animals were identified separately during quarantine and experimental period.

2.6 HOUSING AND ENVIRONMENTAL CONDITION

Each animal were housed separately in standard stainless steel rabbit cages in Animal Room No.4 during acclimatization and in Animal Room No.1 during the experimental period. They were maintained at a 12h light - dark cycle. The temperature was maintained at 22 (±3)°C while the relative humidity of 30 - 70% with 15 air changes per hour was maintained in the animal rooms.

2.7 QUARANTINE AND ACCLIMATIZATION

The animals were quarantined to laboratory conditions for at least seven days in quarantine room and acclimatized for two days in experimental room prior to initiation of the experiment and were observed for general observation, mortality and body weight and feed intake.

2.8 FEED

Nutritionally balanced autoclaved pelleted feed provided to animal *ad libitum,* was procured Rayans Biotechnologies Private Ltd. Hyderabad.

2.9 WATER

Autoclaved normal drinking water was provided to the animals *ad libitum* through out the quarantine and experimental period.

3 EXPERIMENTAL PROCEDURE

3.1 RANDOMIZATION

Total 24 (12M+12F) New Zealand white rabbits were randomized into four groups of 6 animals (3M+3F) each. The randomization procedure was performed as per the SOP for Randomization based on animal body weights at the end of quarantine period. The body weight variation among the groups did not exceed ± 20% of the mean body weight for each group.

3.2 TEST ARTICLE

IUPAC Name	4-methylhexan-2-amine
Common Name	1,3 dimethylpentylamine
Source	Smartchem (Beijing) Ltd.
Physical appearance	Fine hygroscopic white powder
Lot number	20110912
Concentration	99.4%
Manufactured date	28th September 2011
Storage condition	Store in air tight container away from moisture

3.3 TEST VEHICLE

Common Name	Water for Injection
Physical appearance	Colourless solution
Batch Number	1WT-1140
Manufactured date	February 2011
Source	Parenteral Drugs (I) Ltd. Baddi, HP
Storage condition	Room Temperature

3.4 EXPERIMENTAL DESIGN

Group	Species	Strain	Number of animals	Dose (mg/kg)	Route of administration	Duration of Observation (Days)
I (Vehicle Control)	Rabbit	NZW	3M+3F	0	Oral	14
II	Rabbit	NZW	3M+3F	50	Oral	14
III	Rabbit	NZW	3M+3F	150	Oral	14
IV	Rabbit	NZW	3M+3F	300	Oral	14

3.5 PREPARATION OF DOSE AND DOSING

3.5.1 PREPARATION OF TEST ARTICLE FORMULATION FOR DOSING

The test article was provided by the sponsor and formulation was prepared at Clintox Bioservices as per SOP.

3.5.2 VEHICLE FOR DOSING

Commercially available Water for Injection (WFI) was used as a vehicle.

3.5.3 ROUTE OF ADMINISTRATION

The test article and vehicle were administered orally through the stomach feeding tube of size 28 (FG-05) 1.70 mm. The test article dose for individual animal was adjusted based on the body weights recorded on the day of dosing.

3.5.4 RATIONALE FOR ROUTE OF ADMINISTRATION

The route of administration was chosen as the intended route for clinical use of the test article.

4 OBSERVATIONS

The animals were observed for a period of 14 days. The following observations were recorded.

4.1 MORTALITY

Mortality was observed daily for 14 days and was duly recorded.

4.2 GENERAL OBSERVATION

Animals were observed for clinical signs at 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours post dosing on day 1 and once daily for 14 days.

All animals were observed daily by trained personnel for any general behavioral and physical changes. The regular observation included observation of natural orifices for any abnormal secretions, skin condition, mortality, gait, movement, neurological disorders, eye condition, faeces, convulsions, tremors, activity rate, restlessness, grooming, sleep, aggressiveness, salivation, diarrhoea, respiration, appetite, thirst and urine conditions. These observations were made daily. Observations were accurately recorded, using scoring systems as per organizational SOP. Other observations such as fur condition, mucous membranes, presence or absence of secretions and

excretions and autonomic activity, lacrimation, piloerection, pupil size, posture and response to handling as well as the presence of clonic or tonic movements, repetitive circling, bizarre behavior, self-mutilation and walking backwards were also recorded.

4.3 BODY WEIGHT

Body weight of each animal was recorded on the day of dosing and once daily for 14 days during the experimental period.

5 SACRIFICE

After the completion of 14 day observation period, all animals were euthanised following organizational SOP.

6 NECROPSY AND GROSS EXAMINATION

Necropsy and gross pathological examination was carried out on heart, lung, liver, kidneys, spleen, stomach, large intestine, small intestine, brain, skin, thymus, adrenal glands, duodenum, jejunum, caecum, colon and rectum of all the experimental animals that were euthanized at the termination of the study as well as of those which died during the study period and examined for any macroscopic lesions and the observations were duly recorded.

7 DISPOSAL

As per regulatory requirements, the carcasses were sent to an authorized biomedical waste disposal facility - G.J.Multiclave Pvt. Ltd – an agency approved for animal collection and disposal by A. P. Pollution Control Board, Government of Andhra Pradesh, India.

8 GOOD LABORATORY PRACTICE

The study was performed in compliance with The Principles of Good Laboratory Practice (1998) of the Organisation for Economic Cooperation and Development (OECD).

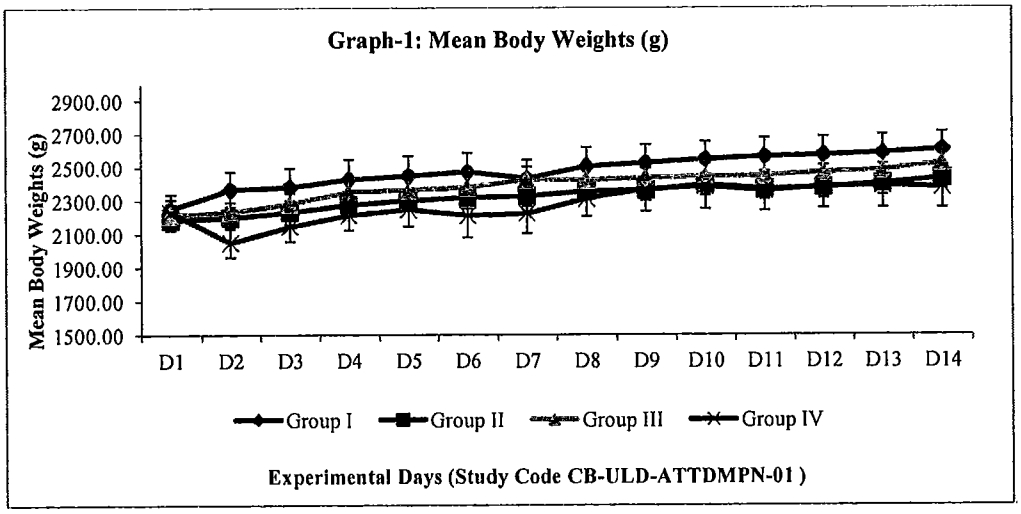
9 QUALITY ASSURANCE

The Quality Assurance Unit (QAU) reviewed, investigated and audited the study during different phases of its execution including raw data, draft and final reports.

RESULTS AND CONCLUSION

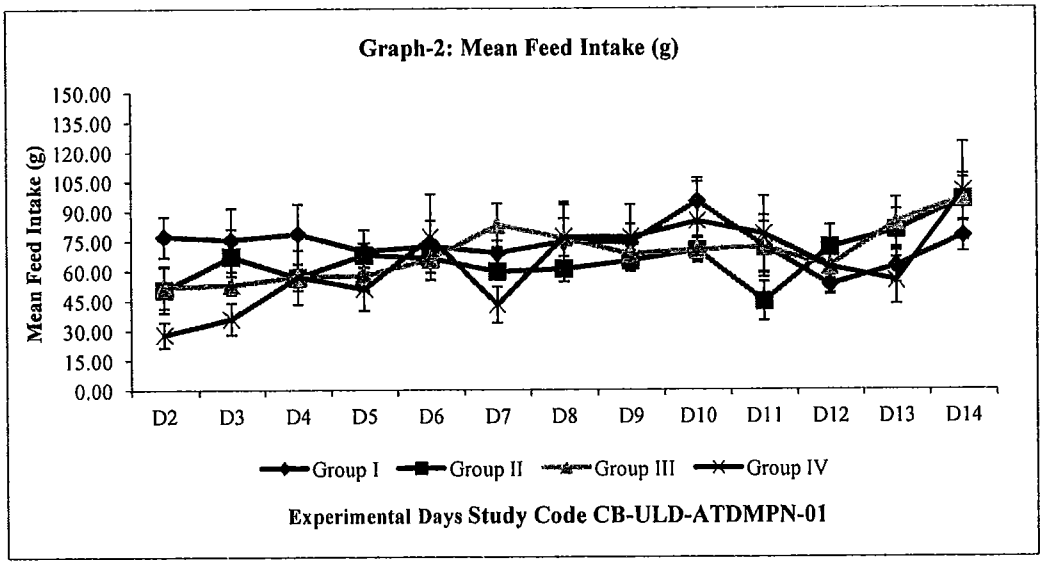
10 RESULTS

10.1 BODY WEIGHT



The mean body weights of animals treated with 50, 150 and 300 mg/kg of 1,3-dimethyl pentylamine showed a consistent increase during experimental period. There was no adverse observation in mean body weights of all above treated groups during the study period.

10.2 FEED INTAKE



The mean feed intake was normal in all animals treated with 50, 150 and 300 mg/kg of 1,3-dimethyl pentyl amine. Though there was a decrease in feed intake in the treatment groups in the initial stages after treatment, all animals recovered during the study period.

10.3 PHYSICAL EXAMINATIONS

Few animals treated with 150 mg/kg showed tremors after 1 hour of test drug adminstration until 6 hours. Animals treated with 300 mg/kg showed nasal discharge, salivation, tremors, convulsions and tachycardia. These animals were found to succumb to the conditions during the study. No behavioral abnormalities were observed in control animals and those treated with 50 mg/kg throughout the study period.

10.4 MORTALITY

Four pre-terminal mortalities were observed in the group treated with 300 mg/Kg. Two deaths were recorded within three hours of dose administration while mortality each were recorded 3 and 4 hours after dosing.

10.5 NECROPSY

At the termination of the study, all the treated animals that survived were euthanized for gross necropsy. No gross pathological changes were observed in animals treated with vehicle, 50 and 150 mg/kg. However, in the group treated with 300 mg/kg gross changes observed in animals that died pre-terminally are congestion of the lungs, multiple watery cyst on right median lobe of liver with small white patches on the surface which were focal, spleen showed white fibrinous covering which were focal, stomach showed glandular mucosa with focal hyperaemia.

11 CONCLUSION

Based on the experimental results in this study, it may be concluded that the Maximum Tolerated Dose (MTD) of 1,3-Dimethylpentylamine in New Zealand White rabbits when treated with a single oral dose is between 150 mg/Kg and 300 mg/Kg body weight.

CONTENTS OF TABLE

Table No 1: Dosing Schedule

Group	Cage NO	Animal Code		Sex	Test Article	Dose (mg/kg)	Drug Conc. (ml/kg)	Body Weights (g)	Dose ml/Animal
Group - I	1	RBMQ007	T001	Male	Water for injection	0.00	2.00	2042.00	4.084
	2	RBMQ012	T002					2016.00	4.032
	3	RBMQ008	T003					2302.00	4.604
	4	RBFQ020	T004	Female				2348.00	4.696
	5	RBFQ015	T005					2200.00	4.400
	6	RBFQ023	T006					2600.00	5.200
Group -II	7	RBMQ003	T007	Male	1,3 dimethyl pentylamine	50.00	2.00	2092.00	4.184
	8	RBMQ004	T008					2062.00	4.124
	9	RBMQ005	T009					2232.00	4.464
	10	RBFQ016	T010	Female				2366.00	4.732
	11	RBFQ021	T011					2024.00	4.048
	12	RBFQ018	T012					2330.00	4.660
Group-III	13	RBMQ001	T013	Male	1,3 dimethyl pentylamine	150.00	2.00	2074.00	4.148
	14	RBMQ010	T014					2114.00	4.228
	15	RBMQ002	T015					2274.00	4.548
	16	RBFQ019	T016	Female				2306.00	4.612
	17	RBFQ025	T017					2066.00	4.132
	18	RBFQ024	T018					2458.00	4.916
Group - IV	19	RBMQ006	T019	Male	1,3 dimethyl pentylamine	300.00	2.00	2194.00	4.388
	20	RBMQ009	T020					2126.00	4.252
	21	RBMQ011	T021					2026.00	4.052
	22	RBFQ014	T022	Female				2270.00	4.540
	23	RBFQ022	T023					2202.00	4.404
	24	RBFQ017	T024					2570.00	5.140

Table No 2: Mean ± SE body weight (g) in all the groups during entire experimental period

Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Group I	2251.3±88.5	2367.6±107	2382.3±113.4	2428.6±119.2	2448.6±120.8	2472.3±114.9	2433.3±111.9
Group II	2184.3±59.3	2199.3±67.4	2230.6±70.3	2276.3±65.3	2303.0±64.2	2319.3±66.4	2328.3±61.2
Group III	2215.3±64	2231.0±60.9	2283.0±65	2353.0±75.1	2364.6±75.9	2380.0±82.8	2424.6±79.7
Group IV	2231.3±75.6	2051.0±88.3	2146.0±88.9	2214.0±88.9	2248.0±100.4	2212.0±130.4	2225.0±119.5

Groups	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Group I	2505.6±113.2	2525.6±110.1	2548.3±107.6	2564.3±113.6	2574.3±111.1	2585.3±112.3	2607.3±106.7
Group II	2356.6±55.8	2362.6±60.2	2396.3±62.1	2370.6±58.7	2380.3±63.5	2404.6±68.3	2434.0±62.9
Group III	2424±82.7	2435.3±82.1	2445.6±82.4	2448.3±92.2	2469.6±85.7	2482.5±82.7	2525.0±73.3
Group IV	2313.0±105.6	2368.0±129.3	2387.0±134.5	2359.0±118.3	2386.0±128.1	2391.0±131	2381.0±122.9

Table No 3: Mean ± SE feed intake (g) in all the groups during entire experimental period

Groups	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Group I	77.3±10.2	75.6±15.9	78.6±15	70±10.6	72.3±13	69±6.3	74.6±11.8
Group II	50.6±11.4	67.3±13.8	56.6±4.3	68.0±6	66.3±7.1	59.6±2.9	61.0±6.4
Group III	52.0±10.6	53.0±4.6	57.0±6.7	57.6±7.5	65.6±10	83.0±11	76.3±17.1
Group IV	28.0±6.4	36.0±8	57.0±13.6	51.0±10.9	77.0±21.5	43.0±9	77.0±17.6

Groups	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Group I	74.3±9.2	95±11.7	72.3±15.4	53±4.8	62.3±8.5	77.5±7.8
Group II	65.0±5.2	70.3±6.1	44.6±9.7	71.6±11.3	80.5±10.4	95.8±10.8
Group III	68.3±7.4	70.6±6.6	71.6±12.8	62.3±6.4	84.3±12.4	96.6±11.9
Group IV	77.0±16.3	85.0±19.8	78.5±19	62.0±13.3	55.0±11.6	100.0±24.7

Appendix1: Details of gross necropsy of all Group-I and Group-II animals.

Group	Animal code		Neropsy Date	General	Skin	Body Cavities	Kidneys	Heart	Lungs	Liver	Spleen	Stomach	Small Intestine	Large Intestine	Brain	Other Observation
Group I	RBMQ007	T001	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBMQ012	T002	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBMQ008	T003	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ020	T004	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ015	T005	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ023	T006	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Group-II	RBMQ003	T007	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBMQ004	T008	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBMQ005	T009	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ016	T010	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ021	T011	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ018	T012	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

Appendix 2 : Details of gross necropsy of all Group-III and Group-IV animals.

Group	Animal code		Neropsy Date	General	Skin	Body Cavities	Kidneys	Heart	Lungs	Liver	Spleen	Stomach	Small Intestine	Large Intestine	Brain	Other Observation
Group - III	RBMQ001	T013	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBMQ010	T014	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBMQ002	T015	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ019	T016	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ025	T017	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD		NAD	NAD	NAD	NAD
	RBFQ024	T018	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Group - IV	RBMQ006	T019	11-Apr-12	Severe salivation	NAD	NAD	NAD	NAD	NAD	Multiple watery cyst on right median lobe	Whitish deposit on surface,focal	Gladular mucosa -Focal hyperaemia.	NAD	NAD	NAD	NAD
	RBMQ009	T020	11-Apr-12	Severe salivation	NAD	NAD	NAD	NAD	Reddish discolouration	NAD	NAD	Gladular mucosa :hyperaemia.	NAD	NAD	NAD	NAD
	RBMQ011	T021	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ014	T022	25-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RBFQ022	T023	11-Apr-12	Severe nasal discharge & salivation	NAD	NAD	NAD	NAD	NAD	NAD	NAD	Gladular mucosa :thinning	NAD	NAD	NAD	NAD
	RBFQ017	T024	11-Apr-12	NAD	NAD	NAD	NAD	NAD	NAD	Right median lobe white patches focal	NAD	Gladular mucosa :hyperaemia	NAD	NAD	NAD	NAD

Appendix 3 : Details of body weight (g) and net body weight gain (loss) in g of all the groups during entire experimental period

	Individual body weights		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Net BW'T Gain(loss)	Mean Net BW'T Gain(loss)
GROUP	ANIMAL CODE																	
Group -I	RBMQ007	T001	2042.00	2110.00	2116.00	2198.00	2262.00	2268.00	2232.00	2276.00	2322.00	2346.00	2346.00	2352.00	2354.00	2402.00	360.00	356.00
	RBMQ012	T002	2016.00	2064.00	2032.00	2014.00	1996.00	2070.00	2098.00	2116.00	2156.00	2196.00	2204.00	2232.00	2252.00	2274.00	258.00	
	RBMQ008	T003	2302.00	2560.00	2598.00	2650.00	2640.00	2666.00	2358.00	2692.00	2704.00	2734.00	2766.00	2780.00	2792.00	2806.00	504.00	
	RBFQ020	T004	2348.00	2490.00	2522.00	2568.00	2620.00	2658.00	2650.00	2694.00	2704.00	2748.00	2752.00	2760.00	2766.00	2800.00	452.00	
	RBFQ015	T005	2200.00	2264.00	2298.00	2356.00	2370.00	2368.00	2416.00	2428.00	2412.00	2416.00	2418.00	2424.00	2426.00	2456.00	256.00	
	RBFQ023	T006	2600.00	2718.00	2728.00	2786.00	2804.00	2804.00	2846.00	2828.00	2856.00	2850.00	2900.00	2898.00	2922.00	2906.00	306.00	
Group-II	RBMQ003	T007	2092.00	2120.00	2138.00	2190.00	2210.00	2172.00	2206.00	2252.00	2248.00	2264.00	2296.00	2320.00	2338.00	2346.00	254.00	249.67
	RBMQ004	T008	2062.00	2142.00	2196.00	2218.00	2226.00	2196.00	2196.00	2220.00	2222.00	2278.00	2250.00	2242.00	2250.00	2256.00	194.00	
	RBMQ005	T009	2232.00	2306.00	2326.00	2408.00	2444.00	2438.00	2466.00	2448.00	2458.00	2492.00	2480.00	2494.00	2486.00	2536.00	304.00	
	RBFQ016	T010	2366.00	2400.00	2344.00	2332.00	2452.00	2504.00	2506.00	2490.00	2494.00	2508.00	2536.00	2554.00	2558.00	2556.00	190.00	
	RBFQ021	T011	2024.00	1940.00	1952.00	2036.00	2070.00	2150.00	2180.00	2228.00	2220.00	2242.00	2188.00	2176.00	2196.00	2292.00	268.00	
	RBFQ018	T012	2330.00	2288.00	2428.00	2474.00	2416.00	2456.00	2416.00	2502.00	2534.00	2594.00	2474.00	2496.00	2600.00	2618.00	288.00	
Group -III	RBMQ001	T013	2074.00	2108.00	2128.00	2166.00	2170.00	2178.00	2190.00	2168.00	2218.00	2232.00	2206.00	2244.00	2250.00	2322.00	248.00	308.33
	RBMQ010	T014	2114.00	2144.00	2190.00	2238.00	2268.00	2340.00	2358.00	2334.00	2376.00	2370.00	2324.00	2362.00	2403.00	2454.00	340.00	
	RBMQ002	T015	2274.00	2268.00	2308.00	2394.00	2388.00	2418.00	2446.00	2434.00	2424.00	2448.00	2468.00	2496.00	2504.00	2512.00	238.00	
	RBFQ019	T016	2306.00	2306.00	2364.00	2448.00	2456.00	2478.00	2520.00	2502.00	2510.00	2556.00	2558.00	2562.00	2572.00	2616.00	310.00	
	RBFQ025	T017	2066.00	2084.00	2158.00	2216.00	2228.00	2162.00	2288.00	2338.00	2294.00	2284.00	2302.00	2326.00	2342.00	2414.00	348.00	
	RBFQ024	T018	2458.00	2476.00	2550.00	2656.00	2678.00	2704.00	2746.00	2768.00	2790.00	2784.00	2832.00	2828.00	2824.00	2832.00	366.00	
Group -IV	RBMQ006	T019	2194.00	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	0.00	77.67
	RBMQ009	T020	2126.00	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	0.00	
	RBMQ011	T021	2026.00	1898.00	1992.00	2060.00	2074.00	1986.00	2018.00	2130.00	2144.00	2154.00	2154.00	2164.00	2164.00	2168.00	142.00	
	RBFQ014	T022	2270.00	2204.00	2300.00	2368.00	2422.00	2438.00	2432.00	2496.00	2592.00	2620.00	2564.00	2608.00	2618.00	2594.00	324.00	
	RBFQ022	T023	2202.00	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	0.00	
	RBFQ017	T024	2570.00	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	Died	0.00	

Table No 4:Study Coding system

Study Code	CB-ULD-ATDMPN-01	
	C	Clintox
	B	Bioservices
	ULD	USP Labs Direct
	AT	Acute Toxicity
	DMP	Dimethylpentylamine
	N	New Zealand white rabbit.
	01	First Toxicology study

Table No 5 :Animal Coding system

Animal Code	RBMQ006T019 & RBFQ014T022	
	RB	Rabbit
	M	Male
	F	Female
	Q	Quarantine
	T	Treatment
	001	Animal Numbers

REPORT

ON

DETERMINATION OF MAXIMUM TOLERATED DOSE (MTD) OF

1, 3-DIMETHYLPENTYLAMINE IN WISTAR RATS

SUBMITTED BY

ClintoxBioservices Pvt. Ltd
Plot # 4B, Phase I,
Alexandria Knowledge Park, Turkapally
Genome Valley, Hyderabad, India –500 078

DECLARATION

This study was conducted at Clintox Bioservices Pvt. Ltd, Shameerpet, Hyderabad, India. All procedures were conducted in accordance with Standard Operating Procedures at Clintox Bioservices. No circumstances have been left unreported which may have affected the quality or integrity of the data or which might have a potential bearing on the validity and reproducibility of this study. The Study Director accepts overall responsibility for the technical conduct of the study as well as the interpretation, analysis, documentation and reporting of the results.

Dr. Prashant Deshmukh
Study Director

Date: 10 May 2012

With the participation of:

Mr. Praveen Thirunahari
Mr. Bhaskar Banoth

This report has been reviewed by:

Dr. Sekhar Bathina, Ph D
Quality Assurance

Date: 10 May 2012

QUALITY ASSURANCE STATEMENT

I declare that the report on *"DETERMINATION OF MAXIMUM TOLERATED DOSE (MTD) OF 1,3- DIMETHYLPENTYLAMINE IN WISTAR RATS"* was audited by Quality Assurance Unit and is in conformity with the protocols, standard operating procedures and this report accurately reflects the methods and findings.

Dr. Sekhar Bathina, Ph D
Quality Assurance

Date: 10 May 2012

TABLE OF CONTENTS

ACRONYMS AND ABBREVIATIONS

ABBREVIATION	DETAILS
GLP	Good Laboratory Practice
g	Gram
kg	Kilogram
Bwt	Body weight
S No	Serial Number
F	Female
mL	Milliliter
°C	Degree Celsius
%	Percentage
mg	Milligram
NAD	No Abnormalities Detected
No.	Number
hr.	Hour/hours
SOP	Standard Operating Procedure
IAEC	Institutional Animal Ethics Committee
CPCSEA	Committee for the Purpose of Control and Supervision on Experiments on Animals

1 STUDY SUMMARY

The purpose of this study was to determine Maximum Tolerated Dose (MTD) of 1,3-Dimethylpentylamine in Wistar rats.

The study was conducted in 40 (20M+ 20F) Wistar rats. All animals were quarantined for least 7 days prior to experimentation, and then randomized and divided into four groups of 10 animals (5M+5F) in each group. The four groups were designated as Group I (Vehicle Control), Group II (50 mg/Kg), Group III (125 mg/Kg) and Group IV (250 mg/Kg). The vehicle control animals were treated with Water for Injection while the treatment animals were treated with respective concentrations of 1,3-Dimethylpentylamine.

All animals were observed daily by trained personnel for any overt toxicity. The regular general observations included body weight, feed intake, mortality and natural orifices. Clinical observations included tremor, convulsion, piloerection, salivation, lacrimation, respiration, activity, arched back, distended abdomen, skin condition, fur, mucous membrane, presence or absence of secretions, eye condition, tail elevation, motor activity, posture, gait, urination and defecation, neurological disorders, presence of clonic and tonic movements. Observations were accurately recorded as per the scoring systems defined by organizational SOP. Mortality was observed and duly recorded daily for 14 days. Gross pathological examination was carried out on all animals. Heart, lung, liver, kidneys, spleen, stomach, large intestine, small intestine, brain and skin were examined for any macroscopic lesions and the observations were duly recorded.

All animals treated with different concentrations of 1,3-Dimethylpentylamine showed a consistent increase in the mean body weight. Piloerection, tail elevation, nasal discharge and hyperactivity were observed in all the treated groups whereas tremors were observed in the group dosed with 250 mg/Kg.

One preterminal mortality was reported from group treated with 250 mg/Kg within 26 hrs of dosing, where as the lower dose groups did not show any mortalities.

Necropsy findings indicate that hyperemia was present in the stomach glandular mucosa, congestion of lungs, liver and adrenals, presence of blood clots in the cranial cavity and ballooning of the small intestine with reddish yellow fluid was observed.

Based on the results of this study, it may be concluded that the Maximum Tolerated Dose (MTD) of 1,3-Dimethylpentylamine in Wistar rats when treated with single oral dose is between 125 and 250 mg/Kg body weight.

2 STUDY OVERVIEW

2.1 STUDY OBJECTIVE

The objective of this study was to determine Maximum Tolerated Dose (MTD) of orally administered 1,3-dimethylpentylamine in Wistar rats. The MTD was assessed in terms of general observations, clinical signs, body weight, feed intake, mortality and gross pathological examination.

2.2 STUDY SCHEDULE

Study title: *"Determination of Maximum Tolerated Dose (MTD) of 1, 3-Dimethylpentylamine in Wistar rats."*

Study Code	:	CB-ULD-ATDMPW-01
Study Schedule	:	
Acclimatization Start Date	:	5 March 2012
Acclimatization End Date	:	14 March 2012
Test Article Dosing Date	:	15 March 2012
Observations Period	:	15 March 2012 to 28 March 2012
Date of Sacrifice	:	29 March 2012
Draft Report Submission	:	04 May, 2012
Final Report Submission	:	10 May, 2012
Study Facility	:	ClintoxBioservices Pvt. Ltd Alexandria Knowledge Park,Turkapally Genome Valley, Hyderabad, India –500 078
Study Sponsor	:	USP Labs, 10761 King William Drive Dallas, TX 75220, USA

2.3 IAEC APPROVAL

The study was initiated after obtaining due approval of the protocol from IAEC.

2.4 TEST SYSTEM

Species	Rat
Strain	Wistar
Justification for the species selection	As per sponsor's requirement
Source	Mahaveera Enterprises, Hyderabad
Number of animals for study	40 (20 M + 20 F)
Age (Weeks)	7-8 Weeks
Sex	Male and Female

2.5 METHOD OF IDENTIFICATION

Animals were identified by tail marking and cage identification system in individually ventilated cages. All animals were identified separately during quarantine and experimental period.

2.6 HOUSING AND ENVIRONMENTAL CONDITION

Three male rats and three female rats were housed separately whereas the remaining two male and two female rats were also housed seperately in Individually Ventilated Cages (Tecniplast, Italy) in Animal Room No.3 during acclimatization and experimental period. They were maintained at a 12h light - dark cycle. The temperature was maintained at 22(±3)°C while the relative humidity of 30 - 70% with 15 air changes per hour was maintained in the animal rooms.

2.7 QUARANTINE AND ACCLIMATIZATION

The animals were quarantined to laboratory conditions for at least seven days in quarantine room and acclimatized for two days in experimental room prior to initiation of the experiment and were observed for general observation, mortality and body weight and feed intake.

2.8 FEED

Nutritionally balanced autoclaved pelleted feed provided to animal *ad libitum,* was procured from Nutrivet Life Sciences, Sinhagad Road, Pune.

2.9 WATER

Autoclaved normal drinking water was provided to the animals *ad libitum* through out the quarantine and experimental period.

3 EXPERIMENTAL PROCEDURE

3.1 RANDOMIZATION

Total 40 (20M + 20F) wistar rats were randomized into four groups of 10 animals each (5M + 5F). The randomization procedure was performed as per the SOP for Randomization based on animal body weights at the end of quarantine period. The body weight variation among the groups did not exceed ± 20% of the mean body weight for each group.

3.2 TEST ARTICLE

IUPAC Name	4-methylhexan-2-amine
Common Name	1,3 dimethylpentylamine
Source	Smartchem (Beijing) Ltd.
Physical appearance	Fine hygroscopic white powder
Lot number	20110912
Concentration	99.4%
Manufactured date	28th September 2011
Storage condition	Store in air tight container away from moisture

3.3 TEST VEHICLE

Common Name	Water for Injection
Physical appearance	Colourless solution
Batch Number	1WT-1140
Manufactured date	February 2011
Source	Parenteral Drugs (I) Ltd. Baddi, HP
Storage condition	Room Temperature

3.4 EXPERIMENTAL DESIGN

Group	Species	Strain	Number of animals	Dose (mg/kg)	Route of administration	Duration of Observation (Days)
I (Vehicle Control)	Rat	Wistar	5M+5F	0	Oral	14
II	Rat	Wistar	5M+5F	50	Oral	14
III	Rat	Wistar	5M+5F	125	Oral	14
IV	Rat	Wistar	5M+5F	250	Oral	14

3.5 PREPARATION OF DOSE AND DOSING

3.5.1 PREPARATION OF TEST ARTICLE FORMULATION FOR DOSING

The test article was provided by the sponsor and formulation was prepared at Clintox Bioservices as per SOP.

3.5.2 VEHICLE FOR DOSING

Commercially available Water for Injection (WFI) was used as a vehicle.

3.5.3 ROUTE OF ADMINISTRATION

The test article and vehicle were administered through the oral gavage. The test article dose for individual animal was adjusted based on the body weights recorded on the day of dosing.

3.5.4 RATIONALE FOR ROUTE OF ADMINISTRATION

The route of administration was chosen as the intended route for clinical use of the test article.

4 OBSERVATIONS

The animals were observed for a period of 14 days. The following observations were recorded.

4.1 MORTALITY

Mortality was observed daily for 14 days and was duly recorded.

4.2 GENERAL OBSERVATION

Animals were observed for clinical signs at 15 minutes, 30 minutes, 1 hour, 2 hours, 4 hours, 6 hours post dosing on day 1 and once daily for 14 days.

All animals were observed daily by trained personnel for any overt toxicity. The regular observation included observation of natural orifices for any abnormal secretions, skin condition, mortality, gait, movement, neurological disorders, eye condition, faeces, convulsions, tremors, activity rate, restlessness, grooming, sleep, aggressiveness, salivation, diarrhoea, respiration, appetite, thirst and urine conditions. These observations were made daily outside the cage. Observations were accurately recorded, using scoring systems as per organizational SOP. Other observations such as fur condition, mucous membranes, presence or absence of secretions and excretions and autonomic activity, lacrimation, piloerection, pupil

size, posture and response to handling as well as the presence of clonic or tonic movements, repetitive circling, bizarre behavior, self-mutilation and walking backwards were also recorded.

4.3 BODY WEIGHT

Body weight of each animal was recorded on the day of dosing and once daily for 14 days during the experimental period.

4.4 SACRIFICE

After the completion of 14 days observation period, all animals were euthanised following organizational SOP.

5 NECROPSY AND GROSS EXAMINATION

Necropsy and gross pathological examination was carried out on heart, lung, liver, kidneys, spleen, stomach, large intestine, small intestine, brain, skin, thymus, adrenal glands, duodenum, jejunum, caecum, colon and rectum of all the experimental animals that were euthanized at the termination of the study as well as of those which died during the study period and examined for any macroscopic lesions and the observations were duly recorded.

6 DISPOSAL

As per regulatory requirements, the carcasses were sent to an authorized biomedical waste disposal facility - G.J.Multiclave Pvt. Ltd – an agency approved for animal collection and disposal by A. P. Pollution Control Board, Government of Andhra Pradesh, India.

7 GOOD LABORATORY PRACTICE

The study was performed in compliance with The Principles of Good Laboratory Practice (1998) of the Organisation for Economic Cooperation and Development (OECD).

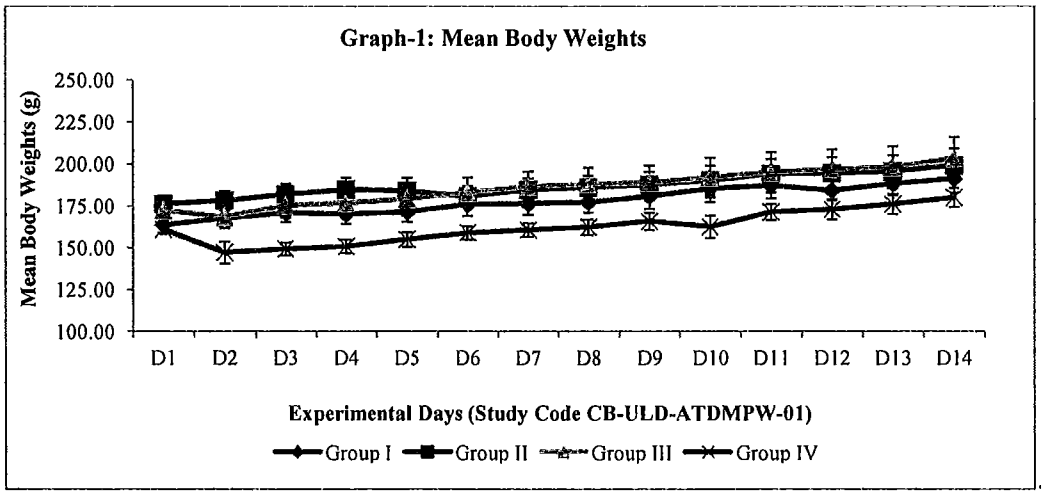
8 QUALITY ASSURANCE

The Quality Assurance Unit (QAU) reviewed, investigated and audited the study during different phases of its execution including raw data, draft and final reports.

RESULTS AND CONCLUSION

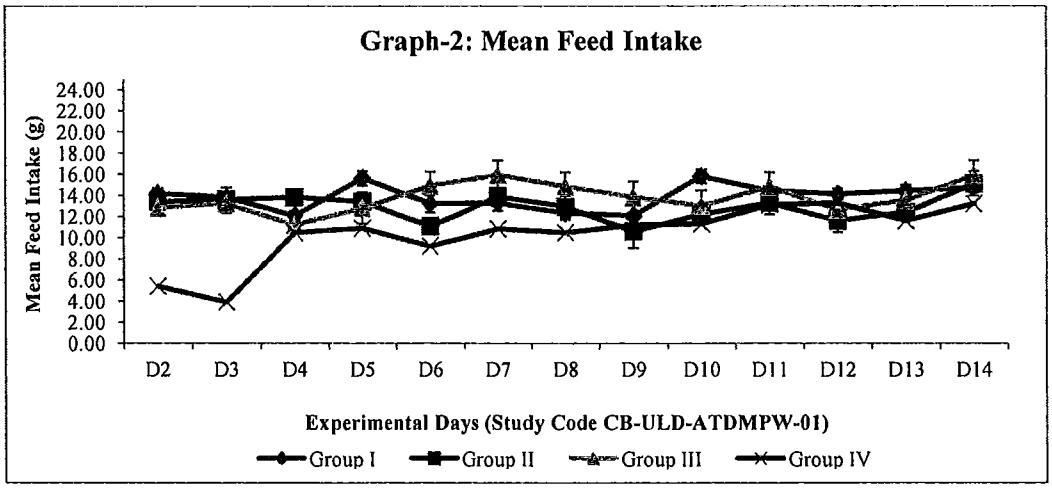
9 RESULTS

9.1 BODY WEIGHT



The mean body weights of animals treated with 50, 125 and 250 mg/kg of 1,3-dimethylpentylamine showed a consistent increase which was less than 10% during experimental period as compared to control. There was no adverse observation recorded in mean body weights in all the above treated groups during the study period.

9.2 FEED INTAKE



The mean feed intake was normal in all animals treated with 50 and 125 mg/kg of 1,3-dimethyl pentylamine except for the animals treated with 250 mg/Kg were the feed intake appeared to be reduced until Day 3 that gradually increased which may have been a dose related response.

9.3 PHYSICAL EXAMINATIONS

All the treated animals showed piloerection, tail elevation, nasal discharge and hyperactivity. Those treated with 250 mg/Kg showed the presence of tremors. No behavioral abnormalities were observed in the animals of Vehicle control group.

9.4 MORTALITY

One mortality was recorded at 25 hrs after dosing in group treated with 250 mg/Kg where as no mortalities were recorded from groups treated with 125 mg/Kg and 50 mg/Kg.

9.5 NECROPSY

At the termination of the study, all the treated animals were euthanized for gross necropsy. Gross pathological changes were not seen in animals treated with vehicle, 50 and 125 mg/kg. Where as Animals treated with 250 mg/kg showed lungs and liver discolouration with point foci in lungs, stomach glandular mucosa showed hyperaemia and small intestine showed ballooning containing reddish yellow fluid during gross necropsy.

9.6 DISCUSSION

1,3-Dimethylpentylamine is used as a dietary supplement for weight loss, body building etc. Literature suggests 1,3-DMPA has a LD_{50} of 72500µg/Kg in rats. They are potential convulsants and can cause an increase in blood pressure. The results obtained during the study indicates that this molecule showed tremors which at higher concentrations may have induced convulsions. Body weight recorded in the treatment groups suggests that an increase in concentration of the test article may result in reducing body weights with proportionately reduced consumption of food. Though an increase in concentration can results in reduced body weights, a proportionately increased toxicity in the lungs and gastrointestinal tract may be observed at high concentrations resulting in mortalities. Hence results suggests that the Maximum Tolerated Dose of orally administered 1,3-Dimethypentylamine in Wistar rats after single administration may be between 125 and 250 mg/Kg body weight.

10 CONCLUSION

From the above results, it may be concluded that the Maximum Tolerated Dose (MTD) of 1,3-Dimethylpentylamine is between 125 and 250 mg/Kg in Wistar rats.

LIST OF TABLES

Table No 1: Dosing Schedule of Group-I and Group-II

Group	Cage NO	Animal no		Sex	Test Article	1,3 dimethylpentylamine Dose (mg/Kg)	Drug Conc. (ml/kg)	Body Weights (g) (DAY 1)	Dose ml/Animal (Day 1)	Body Weights (g) (DAY 2)	Dose ml/Animal (Day 2)
Group -I	1	RMQ018	T001	Male	WFI	0.00	0.00	168	0.42	174	0.44
		RMQ009	T002					198	0.50	204	0.51
		RMQ005	T003					172	0.43	174	0.44
	2	RMQ013	T004					158	0.40	162	0.41
		RMQ019	T005					170	0.43	176	0.44
	3	RFQ042	T006	Female				154	0.39	156	0.39
		RFQ023	T007					154	0.39	158	0.40
		RFQ031	T008					150	0.38	156	0.39
	4	RFQ036	T009					148	0.37	148	0.37
		RFQ041	T010					164	0.41	168	0.42
Group -II	17	RMQ054	T011	Male	1,3 dimethylpentylamine	50.00	2.50	184	0.46	188	0.47
		RMQ053	T012					188	0.47	198	0.50
		RMQ056	T013					184	0.46	192	0.00
	18	RMQ045	T014					194	0.49	196	0.49
		RMQ047	T015					194	0.49	200	0.50
	19	RFQ063	T016	Female				166	0.42	168	0.42
		RFQ066	T017					156	0.39	156	0.00
		RFQ060	T018					162	0.41	162	0.41
	20	RFQ065	T019					172	0.43	168	0.00
		RFQ059	T020					162	0.41	154	0.00

Table No 2: Dosing Schedule of Group-III and Group-IV

Group	Cage NO	Animal no		Sex	Test Article	1,3 dimethyl pentylamine Dose (mg/Kg)	Drug Conc. (mg/ml)	Body Weights (g) (DAY 1)	Dose ml/Animal	Body Weights (g) (DAY 8)	Dose ml/Animal (Day 8-14)
Group -III	21	RMQ050	T021	Male	1,3 dimethyl pentylamine	125.00	2.50	188	0.47	190	0.00
		RMQ049	T022					172	0.43	168	0.00
		RMQ052	T023					176	0.44	174	0.00
	22	RMQ055	T024					192	0.48	198	0.00
		RMQ046	T025					192	0.48	194	0.00
	23	RFQ058	T026	Female				158	0.40	150	0.00
		RFQ067	T027					178	0.45	168	0.42
		RFQ064	T028					160	0.40	150	0.38
	24	RFQ061	T029					160	0.40	154	0.39
		RFQ057	T030					146	0.37	138	0.00
Group -IV	5	RMQ022	T031	Male	1,3 dimethyl pentylamine	250.00	2.50	162	0.41	150	0.38
		RMQ011	T032					172	0.43	170	0.43
		RMQ015	T033					178	0.45	168	0.42
	6	RMQ004	T034					158	0.40	160	0.40
		RMQ006	T035					158	0.40	104	0.26
	7	RFQ044	T036	Female				148	0.37	120	0.30
		RFQ030	T037					156	0.39	148	0.37
		RFQ028	T038					156	0.39	146	0.37
	8	RFQ029	T039					158	0.40	146	0.37
		RFQ025	T040					164	0.41	160	0.40

Table No 2: Mean ± SE body weight (g) in all the groups during entire experimental period

Groups	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Group I	163.6±4.66	167.6±5	170.8±5.39	170±5.68	171.4±5.9	175.8±6.64	176.4±6.61	177.2±6.1	180.8±7.5	185.2±8.04	187±7.54	184.2±5.83	188.2±6.82	191.2±8.3
Group II	176.2±4.5	178.2±5.79	182±6.29	184.6±7.16	184±7.75	180.6±6.13	184.6±6.81	186±7.19	187.6±7.81	190.2±8.67	194±8.79	194.4±9.52	195.4±9.71	199±10.22
Group III	172.2±5.01	168.4±6.53	175.2±7.19	176.6±7.57	179±8.5	183±8.93	186.4±9.16	187.8±10.06	188.8±10.42	191.8±11.77	195±11.95	196.6±12.02	198±12.63	203.2±12.73
Group IV	161±2.72	147.2±6.58	149.33±3.86	150.88±4.03	155±4.41	158.88±3.92	160.66±4.17	162.22±4.62	165.77±5.12	162.44±6.66	171.33±4.82	172.88±6.08	176±6	180±5.61

Table No 3: Mean ± SE feed intake (g) in all the groups during entire experimental period

Groups	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14
Group I	14.2±0.46	13.8±0.94	12.1±0.43	15.6±0.68	13.2±0.78	13.25±0.69	12.3±0.57	12.1±1.26	15.8±0.64	14.4±0.37	14.1±0.5	14.4±0.58	14.7±0.44
Group II	13.4±0.37	13.6±0.79	13.8±0.61	13.4±0.37	11.1±0.73	13.9±0.52	12.9±1.05	10.6±1.55	12.2±0.84	13.3±1.1	11.6±1.06	12.4±0.81	15.1±1.14
Group III	12.8±0.53	13.2±0.84	11.2±0.79	12.8±1.48	14.9±1.32	15.9±1.38	14.8±1.37	13.8±1.51	13±1.45	14.8±1.37	12.6±1.22	13.5±0.99	15.9±1.4
Group IV	5.4±0.37	3.9±0.76	10.5±1.75	10.9±1.33	9.2±1.12	10.85±1.28	10.5±1.25	11.2±1.36	11.3±1.46	13.1±1.58	13.3±1.64	11.6±1.31	13.2±1.51

Appendix1: Details of gross necropsy of all Group-I and Group-II animals.

Group	Animal no		Neropsy Date	Number of Days Alive	General	Skin	Body Cavities	Kidneys	Adrenals	Heart	Lungs	Liver	Spleen	Stomach	Small Intestine	Large Intestine	Brain	Other Observation
Group I (Vehicle control)	RMQ018	T001	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ009	T002	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ005	T003	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ013	T004	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ019	T005	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ042	T006	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ023	T007	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ031	T008	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ036	T009	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ041	T010	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Group II (50 mg/kg)	RMQ050	T021	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ049	T022	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ052	T023	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ055	T024	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ046	T025	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ058	T026	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ067	T027	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ064	T028	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ061	T029	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ057	T030	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

Appendix 2 : Details of gross necropsy of all Group-II and Group-III

Group	Animal no		Neropsy Date	Number of Days Alive	General	Skin	Body Cavities	Kidneys	Adrenals	Heart	Lungs	Liver	Spleen	Stomach	Small Intestine	Large Intestine	Brain	Other Observation
Group III (125 mg/kg)	RMQ054	T031	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ053	T032	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ056	T033	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ045	T034	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ047	T035	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ063	T036	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ066	T037	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ060	T038	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ065	T039	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ059	T040	29-Mar-12	14	NAD	NAD	NAD	NAD		NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
Group IV (250 mg/kg)	RMQ022	T031	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ011	T032	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ015	T033	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ004	T034	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	Reddish discolouration	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RMQ006	T035	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	Pin point white foci (raised), multiple	NAD	NAD	NAD	NAD	NAD	NAD	NAD

	RFQ044	T036	16-Mar-12	1	NAD	NAD	NAD	NAD	NAD	NAD	NAD	Reddish discolouration	NAD	Glandular mucosa-hyperaemia	Ballooning & containing reddish yellow fluid.	NAD	NAD	NAD
	RFQ030	T037	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ028	T038	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ029	T039	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD
	RFQ025	T040	29-Mar-12	14	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD	NAD

Appendix 4 : Details of body weight (g) and net body weight gain (loss) in g of all the groups during entire experimental period

Details of body weight (g) and net body weights gain (loss) in g of all the groups during entire 14 days experimental period

	Individual body weights		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Day 11	Day 12	Day 13	Day 14	Net BW'T Gain(loss)	Mean Net BW'T Gain(loss)
GROUP	**ANIMAL CODE**																	
Group I Vehicle Control	RMQ018	T001	168.00	174.00	179.00	178.00	180.00	182.00	186.00	192.00	192.00	200.00	234.00	198.00	204.00	208.00	40.00	**27.60**
	RMQ009	T002	198.00	204.00	209.00	208.00	210.00	222.00	218.00	192.00	226.00	236.00	198.00	208.00	220.00	238.00	40.00	
	RMQ005	T003	172.00	174.00	180.00	180.00	188.00	192.00	194.00	202.00	198.00	202.00	200.00	196.00	206.00	214.00	42.00	
	RMQ013	T004	158.00	162.00	167.00	168.00	170.00	174.00	176.00	184.00	186.00	186.00	192.00	196.00	192.00	196.00	38.00	
	RMQ019	T005	170.00	176.00	180.00	184.00	182.00	188.00	192.00	200.00	200.00	204.00	208.00	204.00	212.00	212.00	42.00	
	RFQ042	T006	154.00	156.00	154.00	158.00	150.00	158.00	156.00	156.00	156.00	158.00	164.00	166.00	170.00	170.00	16.00	
	RFQ023	T007	154.00	158.00	162.00	162.00	160.00	160.00	160.00	160.00	158.00	162.00	166.00	164.00	162.00	164.00	10.00	
	RFQ031	T008	150.00	156.00	159.00	154.00	156.00	156.00	158.00	156.00	160.00	164.00	166.00	164.00	164.00	162.00	12.00	
	RFQ036	T009	148.00	148.00	151.00	146.00	154.00	156.00	154.00	154.00	156.00	160.00	160.00	162.00	168.00	166.00	18.00	
	RFQ041	T010	164.00	168.00	167.00	162.00	164.00	170.00	170.00	176.00	176.00	180.00	182.00	184.00	184.00	182.00	18.00	
Group II 50 mg/kg	RMQ054	T011	184.00	188.00	196.00	204.00	208.00	192.00	200.00	204.00	212.00	226.00	220.00	224.00	226.00	236.00	52.00	**22.80**
	RMQ053	T012	188.00	198.00	204.00	206.00	206.00	194.00	198.00	204.00	208.00	214.00	218.00	220.00	228.00	226.00	38.00	
	RMQ056	T013	184.00	192.00	202.00	210.00	212.00	194.00	202.00	208.00	212.00	208.00	222.00	228.00	230.00	236.00	52.00	
	RMQ045	T014	194.00	196.00	200.00	206.00	206.00	206.00	212.00	210.00	210.00	214.00	218.00	218.00	212.00	218.00	24.00	
	RMQ047	T015	194.00	200.00	200.00	202.00	202.00	204.00	208.00	208.00	210.00	214.00	220.00	220.00	220.00	224.00	30.00	
	RFQ063	T016	166.00	168.00	166.00	166.00	160.00	166.00	164.00	166.00	162.00	162.00	166.00	166.00	166.00	168.00	2.00	
	RFQ066	T017	156.00	156.00	156.00	154.00	158.00	156.00	160.00	160.00	160.00	160.00	164.00	160.00	162.00	164.00	8.00	
	RFQ060	T018	162.00	162.00	164.00	164.00	156.00	160.00	162.00	158.00	162.00	160.00	162.00	160.00	158.00	160.00	-2.00	
	RFQ065	T019	172.00	168.00	172.00	172.00	172.00	174.00	180.00	180.00	180.00	182.00	186.00	186.00	188.00	194.00	22.00	
	RFQ059	T020	162.00	154.00	160.00	162.00	160.00	160.00	160.00	162.00	160.00	162.00	164.00	162.00	164.00	164.00	2.00	

Group III 125 mg/kg	RMQ050	T021	188.00	190.00	200.00	196.00	206.00	214.00	216.00	222.00	226.00	234.00	242.00	244.00	244.00	250.00	62.00	**31.00**
	RMQ049	T022	172.00	168.00	184.00	180.00	188.00	194.00	198.00	206.00	206.00	214.00	214.00	218.00	224.00	230.00	58.00	
	RMQ052	T023	176.00	174.00	182.00	180.00	188.00	196.00	200.00	202.00	204.00	208.00	212.00	214.00	216.00	224.00	48.00	
	RMQ055	T024	192.00	198.00	204.00	210.00	216.00	220.00	222.00	226.00	226.00	238.00	236.00	236.00	238.00	244.00	52.00	
	RMQ046	T025	192.00	194.00	202.00	212.00	214.00	216.00	224.00	226.00	230.00	234.00	242.00	244.00	250.00	252.00	60.00	
	RFQ058	T026	158.00	150.00	154.00	152.00	156.00	158.00	160.00	156.00	160.00	160.00	162.00	162.00	166.00	172.00	14.00	
	RFQ067	T027	178.00	168.00	172.00	174.00	168.00	170.00	174.00	174.00	170.00	168.00	170.00	172.00	172.00	176.00	-2.00	
	RFQ064	T028	160.00	150.00	154.00	156.00	154.00	158.00	160.00	158.00	156.00	156.00	160.00	156.00	158.00	162.00	2.00	
	RFQ061	T029	160.00	154.00	160.00	164.00	156.00	160.00	164.00	162.00	166.00	162.00	164.00	166.00	164.00	172.00	12.00	
	RFQ057	T030	146.00	138.00	140.00	142.00	144.00	144.00	146.00	146.00	144.00	144.00	148.00	154.00	148.00	150.00	4.00	
Group IV 250 mg/kg	RMQ022	T031	162.00	150.00	149.00	154.00	154.00	156.00	160.00	164.00	168.00	164.00	172.00	172.00	174.00	178.00	16.00	**18.60**
	RMQ011	T032	172.00	170.00	167.00	158.00	162.00	170.00	174.00	174.00	182.00	186.00	190.00	200.00	198.00	200.00	28.00	
	RMQ015	T033	178.00	168.00	164.00	170.00	178.00	180.00	184.00	186.00	190.00	190.00	200.00	204.00	206.00	210.00	32.00	
	RMQ004	T034	158.00	160.00	160.00	168.00	174.00	172.00	172.00	178.00	184.00	182.00	176.00	184.00	194.00	194.00	36.00	
	RMQ006	T035	158.00	104.00	137.00	142.00	145.00	150.00	154.00	156.00	160.00	162.00	168.00	170.00	176.00	180.00	22.00	
	RFQ044	T036	148.00	120.00	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	DEAD	28.00	
	RFQ030	T037	156.00	148.00	140.00	140.00	144.00	148.00	148.00	150.00	154.00	132.00	158.00	160.00	160.00	162.00	6.00	
	RFQ028	T038	156.00	146.00	141.00	138.00	142.00	148.00	150.00	142.00	146.00	134.00	160.00	154.00	156.00	160.00	4.00	
	RFQ029	T039	158.00	146.00	134.00	136.00	140.00	146.00	146.00	150.00	148.00	152.00	154.00	152.00	158.00	166.00	8.00	
	RFQ025	T040	164.00	160.00	152.00	152.00	156.00	160.00	158.00	160.00	160.00	160.00	164.00	160.00	162.00	170.00	6.00	

Table No 7:Study Coding system

Study Code	CB-ULD-ATDMPW-01	
	C	Clintox
	B	Bioservices
	ULD	USP Labs Direct
	AT	Acute Toxicity
	DMP	Dimethyl Pentyl amine
	W	Wistar rat
	01	First Toxicology study

Table No 8 :Animal Coding system

Animal Code	RFQ057T060 & RMQ055T054	
	R	Rat
	F	Female
	M	Male
	Q	Quarantine
	T	Treatment
	001	Animal Numbers

Evidence for the Presence of 1,3-Dimethylamylamine (1,3-DMAA) in Natural Geranium Products

Prepared for:
USPlabs LLC
Dallas, Texas

Prepared by:
Thomas D. Gauthier, Ph.D.
Senior Science Advisor

ENVIRON International Corporation
Tampa, Florida

Date:
September, 2012

Contents

1 Introduction

1,3-Dimethylamylamine (1,3-DMAA), also known as 1,3-dimethylpentylamine and methyl hexaneamine (MHA) is an aliphatic amine with stimulant properties that is reportedly found naturally in geranium plants (*Pelargonium graveolens*). The presence of 1,3-DMAA in geranium plants was first reported in a paper published in the Journal of Guizhou Institute of Technology by Ping et al. (1996). The authors detected the presence of 1,3-DMAA at a concentration of 0.66% in geranium oil isolated from fresh stem and leaves from the *P. graveolens* plant collected from the Rongjang region of Guizhou Province in China. The purpose of that study was to identify the main components of the essential oil in order to provide a basis for breed selection and evaluating the quality of the extracted oil. However, it has since become increasingly cited as evidence for the existence of 1,3-DMAA as a natural product in certain species of geranium. In the Ping et al. study, the authors used gas chromatography-mass spectrometry (GC-MS) to identify the major compounds in the oil; but some have questioned the identification of DMAA in the oil based on apparent mislabeling of the reported chromatogram, inconsistent chromatographic elution order, and the lack of confirmation using a known standard (Lisi et al., 2011; Zhang et al., 2012).

More recently, a number of additional studies have been undertaken with the expressed purpose of establishing whether or not 1,3- DMAA is present as a natural product in geranium plants and geranium-derived oils (Lisi et al., 2011; Zhang et al., 2012; ElSohly et al., 2012; Li et al., 2012; Fleming et al., 2012). The relevancy of this issue has increased recently since the U.S. Food and Drug Administration (FDA) warned in April, 2012 that synthetically-produced 1,3-DMAA is not eligible to be used as an active ingredient in dietary supplement because it is not classified as a "dietary ingredient", a category which includes botanicals (FDA, 2012).

This report contains a review of recent studies and an overall evaluation of the existing evidence for the presence of 1,3-DMAA in natural geranium products.

2 Evaluation of Recent Studies

2.1 Zhang et al. Study (2012)

In July, 2012, Wiley Online published a paper by Zhang et al. (2012) entitled "1,3-Dimethylamine (1,3-DMAA) in supplements and geranium products: natural or synthetic?" There were actually two versions of this paper submitted for publication. After the first version was submitted and a pre-publication press release was published online, the paper was retracted by the authors and re-submitted at a later date.

The authors analyzed eight different geranium oils for the presence of 1,3-DMAA using two different mass spectrometric methods. The geranium oils were purchased from GNC and Amazon and extracted using a multi-step procedure. The oils were treated by first adding water and concentrated HCL solution, and then extracting twice with a 50/50 mixture of hexane/ethyl acetate. After each extraction step, the organic phase was discarded. The aqueous phase was then treated with a 50% NaOH solution and re-extracted three more times with a 50/50 hexane/ethyl acetate solution. The organic portion was retained, evaporated to dryness, and reconstituted in methanol containing 0.1 % formic acid for injection into the HPLC. Because 1,3-DMAA has an appreciable vapor pressure at room temperature (0.65 mm Hg at 25 °C), evaporation to dryness is likely to have an adverse effect on extraction efficiency, which was not reported by the authors.

The extracts were analyzed using two different mass spectrometric methods: an HPLC-electrospray ionization-linear ion trap (HPLC-ESI-LIT) method, and an HPLC-electrospray ionization-triple quadrupole (HPLC-ESI-QQQ) method. In the HPLC-ESI-LIT method, separation of the 1,3-DMAA from other geranium oil components was accomplished using a LARIHC CF6-P chromatography column and a 90/10 acetonitrile/methanol mobile phase containing 0.1% formic acid. The LARIHC CF6-P is an alkyl derivatized cyclofructan 6 chiral stationary phase, invented by Dr. Armstrong (one of the authors of the paper), with reported capability of separating simple aliphatic racemic amines. However, no such separation of 1,3-DMAA was observed in the chromatogram presented in the supplementary material provided by the authors (Zhang et al., 2012, Figure 4).

1,3-DMAA has two chiral centers resulting in two pairs of enantiomers with each enantiomeric pair composing a diastereomer. In the R-S system of nomenclature, one diastereomer comprises the S,S- and R,R-configurations and the other diastereomer comprises the R,S- and S,R-configurations. Note that enantiomers are mirror images of each other (like left and right hands) and have similar physical and chemical properties except for how they react with other chiral compounds and how they rotate plane polarized light. An equal (50:50) mixture of enantiomers is referred to as a racemic mixture or racemate. Racemates are difficult to separate and often require reaction with a chiral reagent to achieve separation. In contrast, diastereomers have different physical and chemical properties and are often readily separated using conventional techniques.

For example, Zhang et al. (2012) were able to separate all four isomers of 1,3-DMAA in 13 commercially available nutritional supplements and two synthetic standards using a GC fitted with an Astec ChiralDex G-DM column (30m x 0.25mm ID x 0.2µm) and flame ionization detector (FID). The authors reported that diastereomeric ratios of 1,3-DMAA measured in the 13 nutritional supplements ranged from 1.23 to 1.43 and were similar to the diastereomeric ratios of 1.22 ± 0.06 and 1.42 ± 0.09 reported for the two synthetic standards. In addition the authors reported that all enantiomers in the nutritional supplements and synthetic standards were racemic (Zhang et al., 2012).

However, only a single peak was detected in spiked geranium oil samples using the HPLC-ESI-LIT method as indicated in Figure 4 of the supporting materials accompanying the paper. Detection of 1,3-DMAA was accomplished with a linear ion trap mass spectrometer using single ion monitoring at a m/z of 116.2 with electrospray ionization (ESI) in the positive mode. ESI is a soft ionization technique and the m/z of 116.2 corresponds to the 1,3-DMAA ammonium ion. One disadvantage of ESI is the potential for ion suppression, which was noted by the authors. Ion suppression is a matrix effect which can adversely affect system performance and detection limits. The limit of detection (LOD) reported for the spiked geranium oil using the HPLC-ESI-LIT method was 50 ppb (assumed to be by mass). The authors reported no 1,3-DMAA was detected in any of the 8 geranium oil samples using this method.

A second method used for analysis of 1,3-DMAA in geranium oil involved a derivatization step prior to analysis. The dried extract was dissolved in a 2:1 acetone/sodium carbonate (3M) solution to which was added 25 mg of 1-dimethylamino-naphthalene-5-sulfonyl chloride (dansyl chloride). Derivatization using dansyl chloride typically offers increased sensitivity with ESI mass spectrometry methods. Dichloromethane (DCM) was added to the derivatization solution and the organic layer as washed twice with deionized water. The solvent was then removed under vacuum and the residue including the derivatized product was dissolved in acetonitrile prior to analysis.

Separation of the derivatized product was performed using a XB-C18 column with a 70/30 acetonitrile/water mobile phase with the water phase containing 0.1% trifluoroacetic acid. This column should have been capable of resolving the pair of derivatized 1,3-DMAA diastereomers; however, again only a single peak was identified in the chromatogram of the spiked geranium oil sample (presented in Figure 5 of the supporting materials accompanying the paper). Note that this figure indicates that the oil sample was spiked with 30 ppb DMAA whereas the experimental write-up indicates samples were spiked with 100 ppb DMAA. Detection was accomplished using multiple reaction monitoring of the m/z 349 to 171 transition corresponding to the loss of the primary dansyl fragment. The LOD reported for the spiked geranium oil using the HPLC-ESI-QQQ method was reported to be 10 ppb (assumed to be by mass). The authors note that the LOD refers to the concentration of the neat 1,3-DMAA and not the derivatized product and report that no 1,3-DMAA was detected in any of the 8 geranium oil samples using this method. It is unclear how the authors established a LOD for either method.

Curiously, a pre-publication press release of this paper with the same title from the same group noted that "trace levels of the stimulant were detected in only 2 geranium products, with the

concentrations lower than 10 part per million. The two geranium oils contained a very small amount of 1,3-DMAA, with 7 mg/kg in one and 3 mg/kg in the other." (Physical Science News, 2012). Indeed, LC-MS chromatograms of a dansyl-DMAA standard from ChromaDex and a dansylated Now Foods geranium oil originally provided as supplementary material to the published paper (reproduced here in Figure 1) showed partial chromatographic separation of the derivatized 1,3-DMAA diastereomers with a diastereomeric ratio of 1.42 for the ChromaDex standard (consistent with information presented in the paper) and a diastereomeric ratio of ~0.8 for the Now Foods geranium oil.

Apparently, the supplementary material originally provided with the final version of the paper was published in error (Watson, 2012) and a revised version was later posted to the website. Interestingly, the original version of the supplementary material also included a calibration curve and validation procedure for an HPLC-Fluorescence detection method that is not presented in the published paper. Reaction with dansyl chloride generates a blue or blue-green fluorescent sulfonamide product that is often amenable to fluorescence detection.

When questioned about the detection of 1,3-DMAA in two of the oil samples, one of the authors reportedly explained that the concentrations were in error (actually present at tens of parts per billion rather than parts per million) and due to contamination of the instruments (Watson, 2012). However it is unclear how contamination of the instrument would lead to a different diastereomeric ratio of 1,3-DMAA isomers as shown in the chromatogram for the dansylated Now Foods geranium oil. It also appears that data may have been generated using a fluorescence detection technique – but these data were not reported in the final version of the paper.

2.2 ElSohly et al. Study (2012)

In another study, ElSohly et al. (2012) analyzed samples of authenticated plant materials and extracted oils from *P. graveolens* as well as a number of commercial oils using gas chromatography-mass spectrometry (GC-MS) and two different LC-MS-MS methods: HPLC-ESI-QQQ and an ultra performance liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-QTOF-MS) method.

The oil samples were mixed with hexane and extracted with 1 N HCl. The hexane layer was discarded and the aqueous layer was re-extracted with hexane, after which the hexane layer was again discarded. The aqueous layer was adjusted to a pH of 9-10 with 10 N KOH and extracted with DCM. The DCM was then transferred to a separate vial, evaporated to a volume of 1 mL and derivatized with heptafluorobutyric anhydride (HFBA). After 1 hour, the solution was mixed with 2 N NaOH and 1.5 M Na_2CO_3, and after an additional 5 minutes, chloroform was added and mixed. The organic layer was then separated for analysis by GC-MS.

Plant materials were extracted with a 0.1 N HCl-methanol solution. After evaporating the initial solvent, the residue was taken up in 1N HCl and extracted with DCM. The acidic layer was retained, adjusted to pH 9-10 using 10 N KOH, and extracted with DCM. The DCM layer was then treated as described above for the oil samples. The extraction efficiency was determined

to be approximately 35% based on recovery of the internal standard (2-amino-6-methylheptane).

Separation of the 1,3-DMAA-HFB derivative was carried out on the GC using an Agilent J&W DB-5MS (25 m x 0.2 mm x 0.33 µm) column. The mass spectrometer was operated in the selected ion monitoring mode at m/z 240, 282 and 296. Based on standard chromatograms reported in Figure 3 of the paper, the response factor for the m/z 240 ion is about 100 times greater than the response factors for the m/z 282 and 296 ions. Despite their poor sensitivity, the authors relied on all three ions for positive identification, which limited the LOD to 100 ppb.

As shown in Figure 2, the method achieved excellent separation of the pair of 1,3-DMAA diastereomers with an estimated diasteriomeric ratio of 1.13 based on measured peak heights. Although the authors reported that "none of the authenticated *P. graveolens* essential oils or plant material, nor any commercial volatile oil of Pelargonium (geranium oil) contain MHA at detectable levels," inspection of the selected ion chromatograms for the control and spiked geranium oil samples suggests the qualitative presence of 1,3-DMAA at concentrations below 100 ppb. Presented in Figure 3 is an overlay of selected ion chromatograms recorded at m/z 240 for a "negative geranium oil control" (presented in ElSohly Figure 4) and a "negative geranium oil" spiked at 0.1 ppm (100 ng/mL) 1,3-DMAA (presented in ElSohly Figure 5).

The chromatograms are displaced slightly in the overlay due to the retention time shift observed between the two runs and have different scales on the y axis. This shift in retention times is consistent with the shift in the retention times observed for the internal standard peak. As indicated in Figure 3, spiking with 1,3-DMAA causes a noticeable increase in two of the peaks observed in the geranium oil control sample suggesting the presence of 1,3-DMAA (showing both diastereomers) in this sample. The estimated diasteriomeric ratios are similar at 1.14 and 1.22 in the spiked and un-spiked samples. The slight difference in estimated diasteriomeric ratios likely reflects the natural variability in this parameter and/or differences between the natural product and synthetic standard.

The picture is less clear at m/z 296 (see Figure 4) due to the poorer sensitivity for this ion (i.e. the spike level is near the limit of detection for this ion). There is a noticeable increase in one peak at a retention time near 3.95 minutes in the spiked sample, but we do not see a recognizable pair of diastereomers at a ratio of ~ 1.15 in the spiked sample. At m/z 282 (not shown in this report), there is a recognizable pair of diastereomers in the spiked sample but the peak intensity appears to be within about a factor of 5 to 10 times the baseline noise consistent with the reported limit of detection of 100 ppb (i.e. at the spike level). Thus, while the more sensitive m/z 240 ion clearly suggests the presence of 1,3-DMAA in the un-spiked geranium oil sample, the low concentration (less than 100 ppb) is not observed in the less sensitive m/z 296 and 282 ion chromatograms.

The chromatograms presented for the extracted plant material (ElSohly Figure 6) appear similar to the chromatograms presented for the geranium oil (ElSohly Figure 4). Overlays of the

chromatograms for the extracted plant material with chromatograms for the spiked geranium oil[1] also suggest the qualitative presence of 1,3-DMAA in the extracted plant material at m/z 240 (see Figure 5). The estimated diasteriomeric ratio in the un-spiked plant material at m/z 240 is 1.21, similar to the un-spiked geranium oil sample. Once again, monitoring at m/z 296 and 282 appears to be unsuitable for low level (less than 100 ppb) detection of DMAA in this study (e.g. Figure 6).

ElSohly et al. (2012) also used two different LC-MS-MS techniques for analysis of the geranium oils and authenticated plant material extracts. The first method employed an HPLC with a triple quadrupole mass spectrometer equipped with an ESI source (HPLC-ESI-QQQ) – similar to the method used by Zhang et al. The same extraction procedure used for GC-MS analysis was used for HPLC-ESI-QQQ analysis except that the no derivatization step took place and the final DCM solution was evaporated to dryness and taken up in methanol for injection onto the HPLC column. Again, evaporation to dryness may have adversely affected extraction efficiency.

Separation of the 1,3-DMAA was performed using a Synergi Hydro-RP column C18 column and a binary solvent gradient using a water/acetonitrile mobile phase with each solvent containing 0.1% formic acid. Detection was accomplished using multiple reaction monitoring of the m/z 116 to 57 and m/z 116 to 41 transitions corresponding to the loss of hydrocarbon fragments. The authors report a LOD and LOQ of 2.5 ppb for this method; although no description of how this LOD was determined. The MRM chromatograms for un-extracted 1,3-DMAA showed a single large peak at a retention time of 4.2 minutes indicating no chromatographic separation of the 1,3-DMAA diastereomers. The authors present representative chromatograms for oil samples, plant materials and three different commercial products - but the reproduction quality of the chromatograms in the paper is poor. Nevertheless, similar peaks at a retention time of 4.2 minutes are observed in the chromatograms of *P. graveolens* and authenticated oil of *P. graveolens* (ElSohly Figures 11A, 11B and 12A) suggesting qualitative presence of 1,3-DMAA in the plant materials.

A second LC-MS-MS method used for confirmatory analysis involved ultra performance liquid chromatography (UPLC) with an ESI source and quadrupole time-of-flight (QToF) mass spectrometer for detection (UPLC-ESI-QToF). Separation was performed using an UPLC BEH C18 column (2.1 x 50 mm x 1.7 µm). Detection was accomplished using selected ion monitoring at m/z = 116.1439. The LOD for this method was estimated at 10 ppb; although no description is given as to how this LOD was established. The authors also present no chromatograms or mass spectra for any of the analyses but report that "all authenticated *P. graveolens* plant material, authenticated *P. graveolens* volatile oils and commercial geranium oil purchased on the open market were negative (less than 10 ppb) for MHA."

In Table II of their paper, ElSoley et al. summarize the results of their investigation and curiously distinguish between two terminologies in reporting results for the LC-MS-MS analyses: "ND" defined as not detected (below LOD of 10 ng/mL or 10 ng/g), and "< 10 ng/mL" which remained

[1] The authors do not present chromatograms for spiked plant materials.

undefined. The distinction suggests that assigning an "ND" indicates that 1,3-DMAA was not detected at a concentration above the limit of detection of 10 ng/mL, and assigning a "< 10 ng/mL" indicates that 1,3-DMAA was detected at a concentration less than 10 ng/mL. This interpretation would be consistent with the authors' summary of analyses of dietary supplements (Products A, B and C) which were reported to contain 1,3-DMAA at concentrations of >10 mg/g, > 2 mg/g and <1 mg/g, respectively.

It is unclear why the authors choose 10 ng/g or 10 ng/mL as the LOD in the summary table when no chromatographic data are presented for this method (UPLC-ESI-QToF), and a lower LOD (2.5 ng/mL) was achieved using the HPLC-ESI-QQQ methodology.

2.3 Li et al. Study (2012)

More recently, Li et al (2012) reported positive detection of 1,3- and 1,4-DMAA in geranium plant tissues at 13 to 365 ng/g and 3 to 35.3 ng/g, respectively; and in geranium oil samples at 167 to 13,271 ng/g and 220 ng/g (in one sample), respectively.

Similar to Zhang et al. study, Li et al. (2012) used a HPLC-ESI-QQQ method for determination of 1,3-DMAA in plant extracts and geranium oils. One major difference between the Zhang and Li studies is in the extraction step. Li et al. used a more simplified approach involving a single hexane extraction step that minimizes potential loss pathways. For geranium oils, the oil is mixed with hexane and 0.5 M HCl and shaken at high speed for 5 minutes. The aqueous layer is then separated, further diluted with HCl as necessary, and filtered with a 0.45 µm nylon filter prior to injection in the HPLC. For geranium plant materials, the leaves and stems are first ground into fine pieces and extracted by sonication with 0.5 M HCl. After centrifugation, the aqueous layer is extracted once with hexane, then separated, further diluted with HCl as necessary, and filtered with a 0.45 µm nylon filter prior to injection in the HPLC. Spiked samples of geranium plants at 5, 10, 20 and 40 ng/g were extracted with an efficiency of 85 to 105% in the Li et al. study. The single liquid-liquid extraction step with hexane was found to improve method performance by reducing sample matrix and ion suppression effects.

In contrast, as noted above, Zhang et al. performed five separate organic solvent extractions and evaporated the sample to dryness prior to reconstituting the sample in methanol. Because each extraction step is not 100% efficient, there is a potential loss of analyte each time an extraction takes place and a layer is discarded. Zhang et al. did not report extraction efficiency in their study. ElSohly performed four separate extraction steps and reported an extraction efficiency of "approximately 35%."

Separation of the DMAA was performed using a C18 column and an 85/15 water/acetonitrile mobile phase with the water phase containing 0.1% formic acid. Detection was accomplished using multiple reaction monitoring of the m/z 116 to 57 and m/z 116 to 99 transitions corresponding to the loss of hydrocarbon fragments. Using this method, the authors were able to achieve a mass detection limit of 1 to 2 picograms (pg) corresponding to a method quantitation limit of 1 to 2 ng/g wet weight based on a 3:1 signal-to-noise ratio.

The authors achieved good separation of 1,3-DMAA diastereomers (two peaks) along with 1,4-DMAA (one peak) using this method and reported similar collision-induced dissociation (CID) spectra for the same precursor ion (m/z = 116) for all three peaks. The authors note that the 1,3-DMAA diastereomers were "present in equal amounts and which are identical in all tested samples, including the standard reference"; although, inspection of the chromatograms reported in Li et al Figures 2, 4 and 5 show diastereomeric peak height ratios of 1.2 to 1.4 (for example see Li et al. Figure 2A reproduced here in Figure 7), which would be consistent with diastereomeric ratios of 1,3-DMAA reported in 13 nutritional supplements by Zhang et al. (2012) and in samples analyzed by ElSohly et al. (2012). Only the chromatogram presented in Li et al. Figure 6 suggests equal amounts of diastereomers based on peak height ratios.

There appears to be some confusion in the paper regarding interpretation of the diastereomeric ratios and whether or not a racemic mixture of DMAA enantiomers is present in the extracted plant materials. As described earlier, an equal mixture of enantiomers is referred to as a racemic mixture or racemate. Racemates only refer to pairs of enantiomers – not pairs of diastereomers. Because the authors did not use a chiral chromatography column to achieve complete separation of all the DMAA isomers, they were not able to separate the two DMAA peaks into their respective pairs of enantiomers. As Zhang et al. (2012) have shown, DMAA can be separated into two pairs of enantiomers using a chiral chromatography column. In this study, each pair of DMAA enantiomers appears as a single peak; thus there is no information regarding enantiomeric ratios and no information as to whether or not 1,3-DMAA is present as a racemic mixture. Yet the authors suggest this study demonstrates the presence of a racemate in a plant tissue. As previously noted, concentrations of 1,3-DMAA in geranium oil samples ranged from 167 to 13,271 ng/g. The geranium oil samples were obtained from the Jiangxi Ji'an Hengcheng Flavor Oil Factory (Ji'an Jiangxi Province, China). It is unclear if the 1000-fold difference in concentrations observed for the geranium oil samples reflects a difference in type of oil, source of geranium plant material, method of processing, or other potential factors.

2.4 Fleming et al., 2012

In a similar study, Fleming et al. (2012) analyzed geranium plants harvested during three different seasons and from three different areas of China (Changzhou, Guiyang and Kunming) for the presence of 1,3- and 1,4-DMAA using an HPLC-ESI-QQQ method.

The extraction procedure was adapted from the procedure reported by Li et al. (2012). Geranium plant materials are first extracted with 0.5 N HCl by sonication. After centrifugation, the aqueous layer was extracted once with hexane, then separated, further diluted with HCl as necessary, and filtered with a 0.45 µm nylon filter prior to injection in the HPLC.[2] In this study, the extraction efficiency of spiked geranium samples ranged from 54 to 107 % for 1,3-DMAA and 63 to 86% for 1,4-DMAA.

[2] Note that samples were first analyzed without the hexane extraction step. This step was later added to reduce matrix effects. Thus, results are reported for some samples with and without the hexane extraction step.

Separation of the DMAA was performed using a Kinetex C18 column and an 82/18 water/acetonitrile mobile phase with the water phase containing 1% formic acid. Detection was accomplished using multiple reaction monitoring of the m/z 116 to 57 and m/z 116 to 99.7 transitions corresponding to the loss of hydrocarbon fragments. Using this method, the authors were able to achieve method detection limits for 1,3- and 1,4-DMAA in the plant extract on the order of 0.6 to 3.2 µg/L (ppb) – corresponding to a detection limit of about 20 ng/g in the plant material.

The authors detected both 1,3-DMAA diastereomers in the chromatograms and recorded diastereomeric ratios of 1.14 ± 0.08 in the standards and ratios of 1.10 ± 0.01 in Changzhou S11-1 sample, 1.25 ± 0.03 in Changzhou S11-2 sample, 1.02 in Changzhou 1 sample, and 1.16 ± 0.1 in Changzhou 3 sample – suggesting a natural variability in diastereomeric ratios.

The Chiangzhou S-11 plant material was obtained from Intertek Laboratories and derived from the same "Jiangsu" sample reported by Li et al. (2012). For this sample, Fleming et al. reported a 1,3-DMAA concentration of 94.7 ± 15.1 ng/g without the hexane extraction step and 254 ± 17 ng/g with the hexane extraction step. In comparison, Li et al. reported 165 ng/g for a sample collected from the same plant material. Similarly, Fleming et al. reported a 1,4-DMAA concentration of 13.5 ± 1.8 ng/g without the hexane extraction step and 39.8 ng/g with the hexane extraction step; and Li et al. reported a concentration of 35.3 ng/g for a sample collected from the same plant material. The similarity in results achieved by two different laboratories analyzing subsamples from the same plant material using similar methods provides confirmatory evidence for the presence of 1,3- and 1,4-DMAA in certain Chinese species of geranium.

Other samples of plant material obtained from Chiangzhou, China also contained 1,3- and 1,4-DMAA. Fleming et al. detected 213 ng/g 1,3-DMAA and 52 ng/g 1,4-DMAA in one sample harvested in March, 2012 (Chiangzhou 1) and 68.8 ± 36.5 ng/g 1,3-DMAA and 118 ± 45 ng/g 1,4-DMAA in a second sample harvested in May, 2012 (Chiangzhou 3) reflecting plant-to-plant variability or possibly seasonal effects.

Some of the samples were re-analyzed using the method of standard additions, which is often used to minimize matrix effects in complex samples such as biological materials. Using the method of standard additions, Fleming et al. detected slightly higher levels of 1,3- and 1,4-DMAA in the Chiangzhou 3 sample at concentrations of 97 ± 20 ng/g and 162 ± 48 ng/g, respectively. Slightly higher levels of 1,3- and 1,4-DMAA were also detected in the Changzhou S11-2 sample at 496 ± 46 ng/g and 68 ± 7 ng/g, respectively.

In other samples of geranium plant materials obtained from China, 1,3- and 1,4-DMAA were detected at or below the detection limit near 20 ng/g. 1,3-DMAA was detected in one geranium plant sample from Kunming, China near the detection limit of 20 ng/g but the duplicate analysis was below the method detection limit. Concentrations of 1,3-DMAA and 1,4-DMAA in all other samples from Kunming and Guiyang China were below the detection limit of approximately 20 ng/g.

2.5 Lisi et al. Study (2011)

Finally, in a short paper prepared by Lisi et al. (2011), five different commercially available geranium oils were analyzed for the presence of DMAA using gas chromatography (GC) with mass spectrometry (MS) detection (GC-MS). The sources of geranium oil in the five samples were reported to be Egypt, France and New Zealand.

The oil samples were extracted with 1M HCl and t-butylmethyl ether (TBME). The TBME layer was discarded and the aqueous layer was re-extracted twice with TBME. The aqueous layer was made basic using 6 M KOH and amended with hexane and pentafluorobenzylchloride (PFBCL) as a derivatizing agent. The solution was then mixed and the hexane layer was removed and evaporated to dryness. The residue was then reconstituted in ethyl acetate for injection onto the GC column. Extraction efficiencies were not reported.

Separation of the DMAA derivative was performed using an Agilent HP Ultra 2 (17m x 0.2mm ID x 0.25µm) column. Semi-quantitative analysis was based selected ion monitoring at m/z 238. Separation of both DMAA diastereomers was achieved using this method; although the authors incorrectly refer to the two peaks as representing enantiomers. The authors report that DMAA was not found in any of the five geranium oils, but no detection limits were reported and no standard concentrations or calibration data are presented.

3 Summary and Conclusions

The recent studies summarized in this report provide conflicting evidence that 1,3-DMAA is found naturally in certain species of geranium plants. However, differences in the source of geranium oils and plant materials, extraction procedures, and methods of analysis may account for the conflicting results. The genus *Pelargonium* includes more than 270 distinct species, most of which are indigenous to South Africa, but many of which are now widely cultivated in Russia, Egypt, India and China (Republic of South Africa, 2009). The plant is cultivated for the production of essential oil and a variety of cultivars exist including the rose scented Chinese and Algerian hybrids of *P. graveolens* in addition to the well-known Bourbon type produced on Reunion Island (Republic of South Africa, 2009). Thus, it is not surprising that some plant and extracted oil samples contain 1,3-DMAA whereas others do not. Based on review of these studies, processing may also play a role as 1,3-DMAA is more likely to be found in oil samples extracted in the lab from plant stems and leaves than in commercially available products sold for medicinal use and aromatherapy (Saraswathi et al., 2011). The reason for this may be due to commercial extraction procedures designed to enhance recovery of essential oils rather than the more volatile 1,3-DMAA.

Three of the studies evaluated in this report included analysis of leaves and stems from geranium plants harvested from China and India. Li et al (2012) detected 1,3-DMAA in authenticated plant materials collected from three areas of China (Yunnan, Jiangsu and Guizhou) and Fleming et al. (2012) detected 1,3-DMAA in authenticated plant materials from Chiangzhou, China and, at low levels, in plant materials from Guiyang, China. However, no 1,3-DMAA was detected in plant materials harvested from Kunming China. ElSohly et al. (2012) analyzed authenticated plant materials from India and the United States (Mississippi) and reported no detectable amounts of 1,3-DMAA (much less than the LOD) at a detection limit of 100 ppb. However, inspection of the chromatograms suggests the qualitative presence of 1,3-DMAA at levels below 100 ppb in samples of the authenticated plant material. The authors also reported no detection of 1,3-DMAA using an HPLC technique with much lower detection limits (2.5 ppb), however, this technique was incapable of resolving the 1,3-DMAA diastereomers. A third technique (UPLC-ESI-QTOF) with an estimated detection limit of 10 ppb was also used to analyze samples, but no data were presented for this method. However, the summary table provided by the authors suggests that 1,3-DMAA was detected in dried plant stems at a level below 10 ppb. Thus, all three studies involving extraction of plant materials provide quantitative or qualitative support for the natural presence of 1,3-DMAA in geranium plants.

Four of the reviewed studies involved analysis of commercially available geranium oils. Zhang et al. (2012) analyzed eight different geranum oils sourced from China and Egypt. Lisi et al. (2011) analyzed five geranium oils sourced from Egypt, France, and New Zealand. ElSohly et al (2012) analyzed 20 different samples of commercial geranium oils but did not report sources; and Li et al. (2012) analyzed geranium oil samples sourced from a flavor oil factory in Jiangxi Province, China. Only Li et al. detected 1,3-DMAA in a commercial oil product.

All eight geranium oil samples analyzed by Zhang et al. were reportedly extracted from geranium plants using steam distillation. Lisi et al. (2011) suggest that due to the volatility of 1,3-DMAA, steam distillation may not be a suitable technique for extracting 1,3-DMAA along with the oil product and that the alternative cold-pressed process provides a better chance of

retaining 1,3-DMAA in the oil. Zhang et al. reported no detection of 1,3-DMAA in any of the geranium oil samples using an HPLC-ESI-LIT technique with a detection limit of 50 ppb; and no detection of 1,3-DMAA using an alternative HPLC-ESI-QQQ technique at a detection limit of 10 ppb. The authors used a somewhat elaborate multi-step extraction technique for the oil samples and did not report extraction efficiencies for the method. The HPLC-ESI-LIT method employed a chiral stationary phase chromatography column that is reportedly capable of adequate retention of primary amines; however, the authors were unable to achieve separation of 1,3-DMAA enantiomers nor diastereomers in standards. Similarly, only a single peak was recorded using the HPLC-ESI-QQQ method.

Curiously, a pre-publication press release of the original manuscript submitted by the authors reported the detection of 1,3-DMAA diastereomers in two geranium oil products at concentrations of 3 and 7 mg/kg. This result was later attributed to contamination. In addition, LC-MS chromatograms of a dansyl-DMAA standard from ChromaDex and a dansylated Now Foods geranium oil originally provided as supplementary material to the published paper showed partial chromatographic separation of the derivatized 1,3-DMAA diastereomers with a diastereomeric ratio of ~0.8 for the Now Foods geranium oil. It is unclear how contamination could lead to a diastereomeric ratio distinct from the reported standards.

Lisi et al. (2011) analyzed five geranium oil samples from Egypt, France and New Zealand; three of the samples were extracted using steam distillation. 1,3-DMAA was not detected in any of the oil samples but no detection limits were reported. ElSohly et al. (2012) also reported no detectable amounts of 1,3-DMAA at a detection limit of 100 ppb using one method. However, published chromatograms for one of the oil samples before and after spiking with 1,3-DMAA at the detection limit suggests the qualitative presence of 1,3-DMAA in the original sample. In addition, the summary table included in the paper suggests that 1,3-DMAA was detected in 25% (5 out of 20) commercial oil samples at levels below 10 ppb.

Finally, Li et al. (2012) detected 1,3- and 1,4-DMAA in geranium oil samples obtained from a Flavor Oil Factory in Ji'an, Jiangxi Province, China. Concentrations of 1,3-DMAA ranged from 167 to 13,271 ng/g in three samples. 1,4-DMAA was only detected in one of the samples at a concentration of 220 ng/g. Except for the one geranium oil sample analyzed by Li et al. (2012), concentrations of 1,3-DMAA detected in geranium plant materials and extracted oils are generally less than 500 ppb.

Only Zhang et al. (2012) report separation of 1,3-DMAA into its four stereoisomers accomplished using a GC-FID fitted with a chiral stationary phase. However, this technique was used to analyze nutritional supplements and not geranium plant materials. Thus, no data has been presented in any of the five studies regarding the enantiomeric purity of 1,3-DMAA extracted from geranium plants and oils. However, all five studies reported separation of 1,3-DMAA into its diastereomers, and diastereomeric ratios were similar for 1,3-DMAA found in commercially obtained standards, nutritional supplements, and extracted geranium plant materials and geranium oils.

Overall, based on my review of these five recent studies, there is clear and convincing evidence that 1,3-DMAA is found naturally in some, but not all, geranium plants and extracted geranium oils. Quantitative (Li et al., 2012; Fleming et al., 2012) and/or qualitative (ElSohly et al., 2012)

evidence for the presence of 1,3-DMAA in plant materials is more likely to be found in studies involving extraction of the plant materials in the lab rather than analysis of commercially available products sold for medicinal use and aromatherapy. This is likely due to differences in processing. Steam distillation, which appears to be a preferred extraction procedure for commercial oils, may not be suitable for retention of the more volatile DMAA. In the lab, detection of 1,3-DMAA appears to be favored by extraction procedures involving fewer extraction steps which minimizes potential for losses. In addition, studies reporting negative findings for the presence of 1,3-DMAA all include extraction procedures involving an evaporation to dryness step or solvent removal under vacuum, which can adversely affect extraction efficiency due to the volatility of 1,3-DMAA.

Finally, these studies show that there is considerable plant-to-plant variability in DMAA content. In plant materials where 1,3-DMAA has been detected, which are primarily sourced from China, concentrations are generally less than 500 ppb. However, some plants from China contain no detectable levels of DMAA while one geranium oil sourced from China contained over 13 ppm 1,3-DMAA.

4 References

Zhang, Y., R.M. Woods, Z.S. Breitbach and D.W. Armstrong. 2012. 1,3-Dimethylamine (DMAA) in supplements and geranium products: natural or synthetic? *Drug Testing and Analysis*, published online 12 JUL 2012; http://onlinelibrary.wiley.com/doi/10.1002/dta.1368/pdf

ElSohly, M.A., W. Gul, K.M. ElSohly, T.P. Murphy, A. Weerasooriya, A.G. Chittiboyina, B. Avula, I. Khan, A. Eichner and L.D. Bowers. 2012. Pelargonium oil and methyl hexaneamine (MHA): Analytical approaches supporting the absence of MHA in authenticated *Pelargonium graveolens* plant material and oil. *Journal of Analytical Toxicology; first published June 25;* 00:1-15.

Fleming, H.L., P.L. Ranaivo and P.S. Simone. 2012. Analysis and confirmation of 1,3-DMAA and 1,4-DMAA in geranium plants using high performance liquid chromatography with tandem mass spectrometry at ng/g concentrations. *Analytical Chemistry Insights* (submitted for publication).

Li, J.S., M. Chena and Z.C. Li. 2012. Identification and quantification of dimethylamylamine in geranium by liquid chromatography tandem mass spectrometry. *Analytical Chemistry Insights* 7: 47-58.

Lisi, A., N. Hasick, R. Kazlauskas and C. Goebel. 2011. Studies of methlhexaneamine in supplements and geranium oil. *Drug Testing and Analysis*. Short Communication, published online.

Physical Science News. 2012. IMPORTANT NOTICE - PUBLICATION POSTPONED: Stimulant Marketed as "Natural" in Sports Supplement Actually of Synthetic Origin. May 28, 2012 at 6:44 AM.

Ping, Z., Q. Jun and L. Qing. 1996. A study of the chemical constituents of geranium oil. *Journal of Guizhou Institute of Technology* 25:82-85.

Republic of South Africa: Department of Agriculture Forestry and Fisheries. 2009. Rose Geranium Production, Department of Agriculture, Plant Production. June.

Saraswathi, J., K. Venkatesh, N. Baburao, M. Hameed Hilal and A. Roja Rani. 2011. Phytopharmacological importance of *Pelargonium* species. *Journal of Medicinal Plants Research* 5(13): 2587-2598.

U.S. Food and Drug Administration (FDA). 2012. FDA News Release. April 27. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm302133.htm

Watson, E. 2012. USP Labs promises new data that 'definitively' proves presence of DMAA in geranium. July 16. http://www.nutraingredients-usa.com/content/view/print/654887

Figures

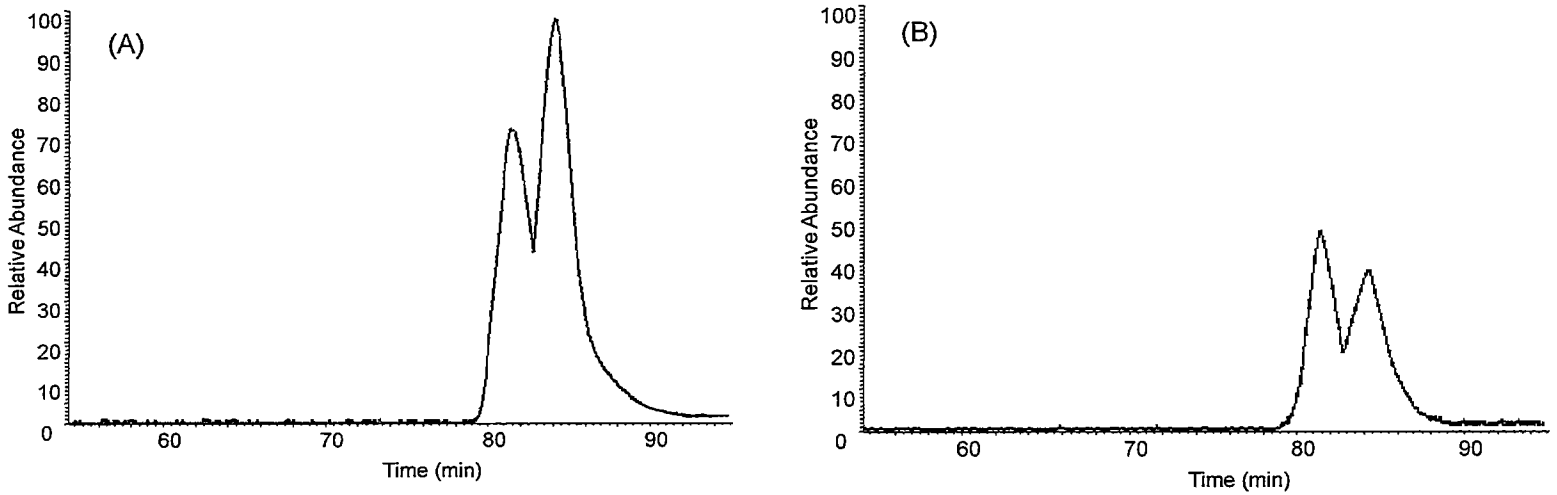


Figure 1: LC-MS chromatograms of (A) dansyl-DMAA standard from ChromaDex and (B) dansylated Now Foods geranium oil originally provided in supplementary material accompanying the Zhang et al. paper.

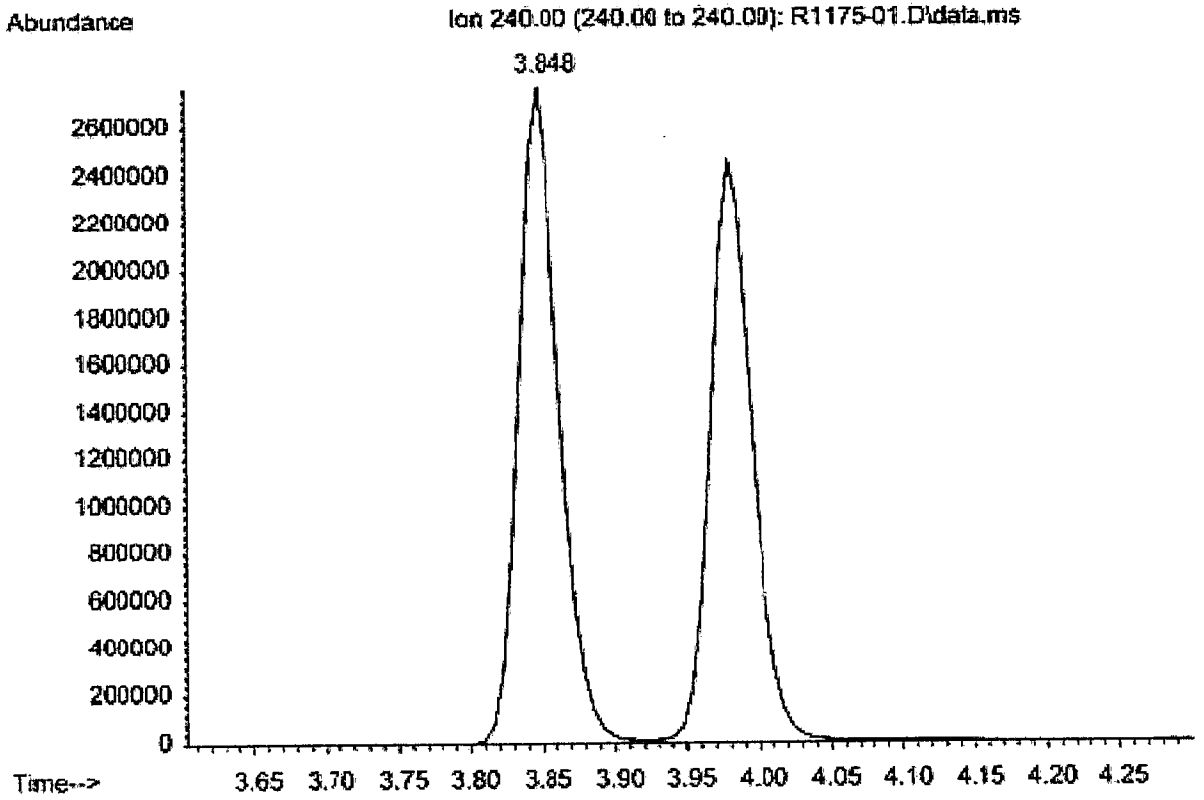


Figure 2. GC-MS selected ion chromatogram of the DMAA-HFB derivative for a 1 µg standard (ElSohly – Figure 3). Estimated diastereomeric ratio based on peak heights is 1.13.

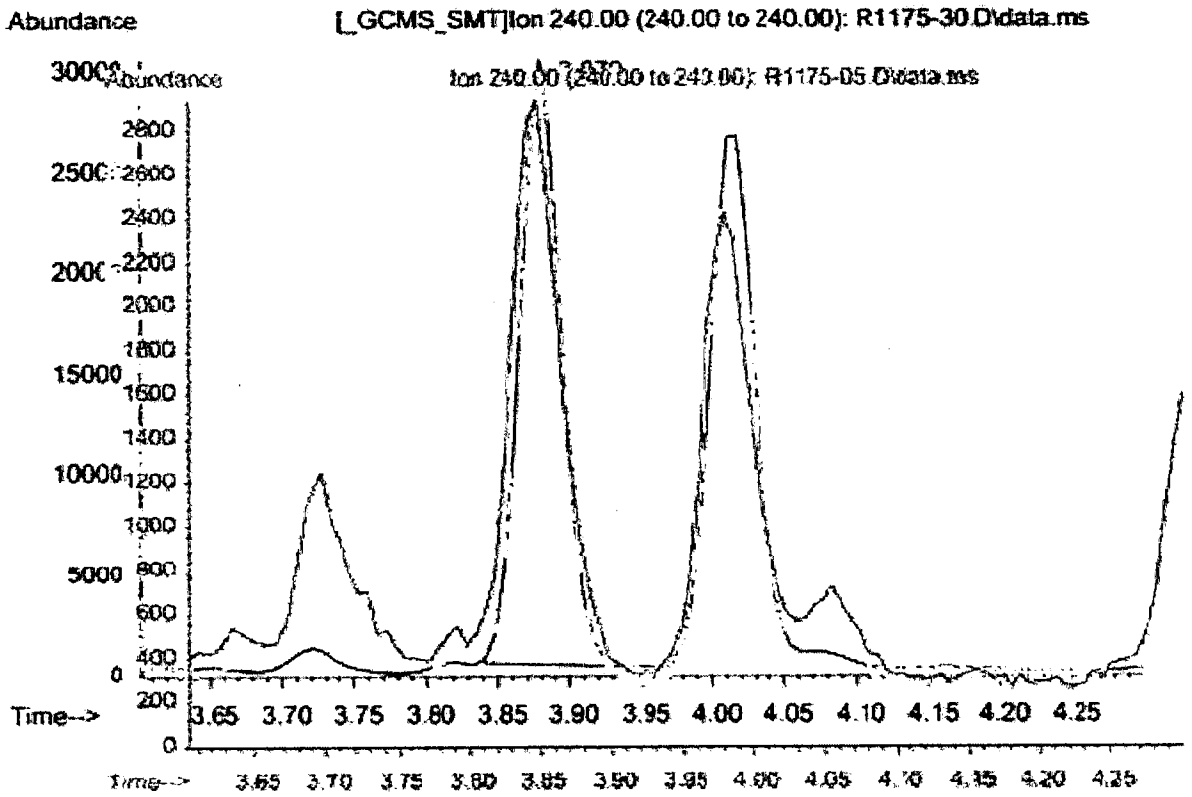


Figure 3. Overlay of GC-MS selected ion chromatograms of the DMAA-HFB derivative recorded at m/z 240 for geranium oil sample (recorded in red – ElSohly Figure 4) and geranium oil sample spiked at 0.1 ppm DMAA (recorded in black – ElSohly Figure 5). The observed retention time shift in the two spectra is consistent with the retention time shift for the internal standard.

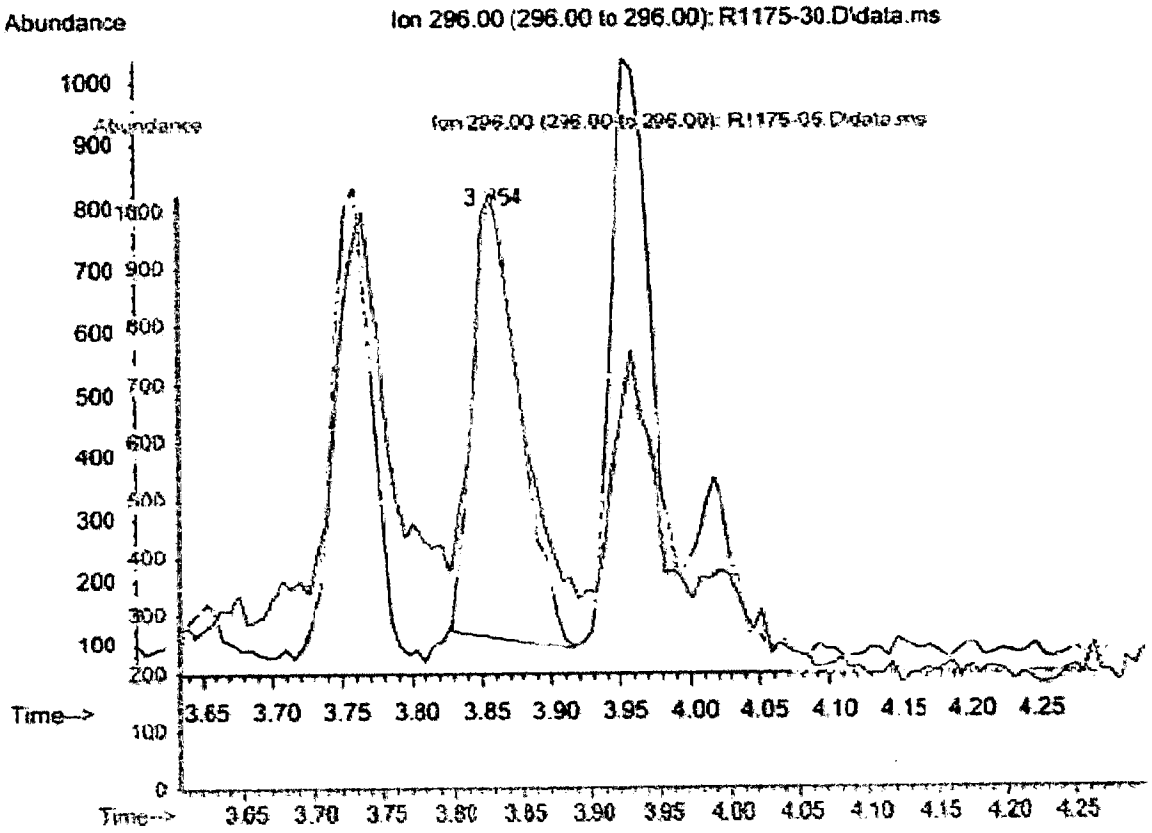


Figure 4. Overlay of GC-MS selected ion chromatograms of the DMAA-HFB derivative recorded at m/z 296 for geranium oil sample (recorded in red – ElSohly Figure 4) and geranium oil sample spiked at 0.1 ppm DMAA (recorded in black – ElSohly Figure 5). The observed retention time shift in the two spectra is consistent with the retention time shift for the internal standard.

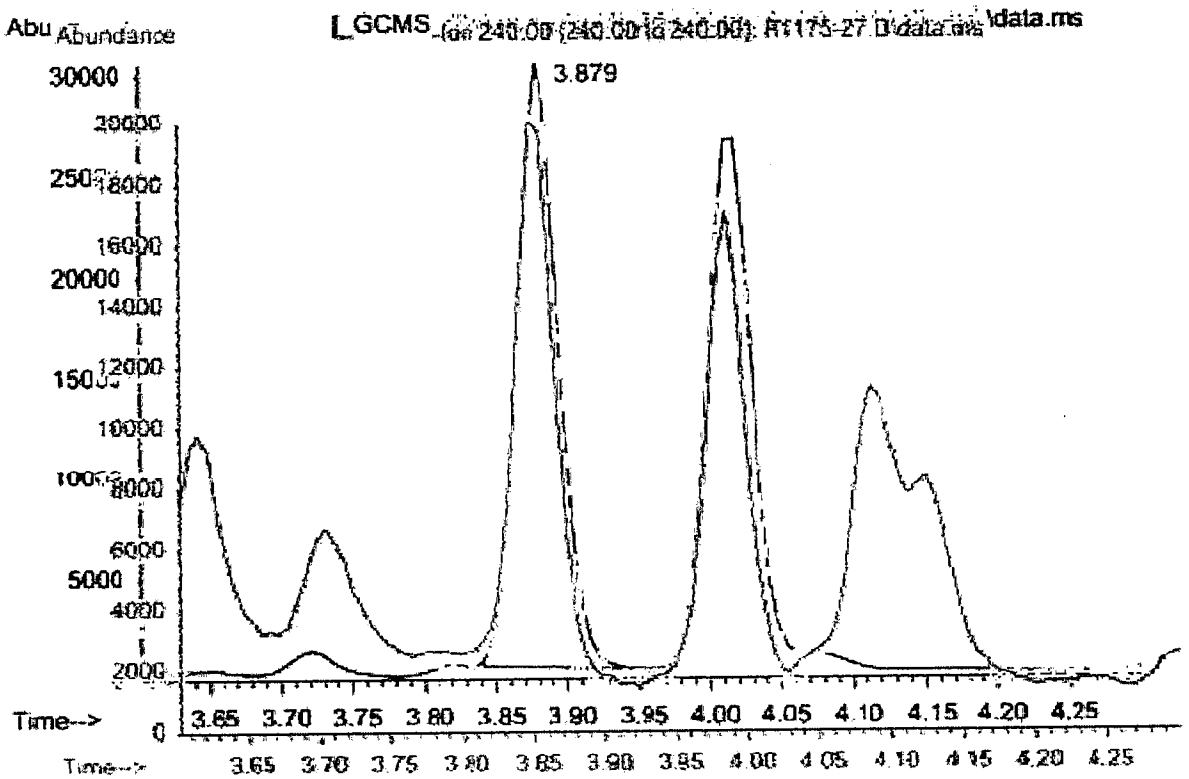


Figure 5. Overlay of GC-MS selected ion chromatograms of the DMAA-HFB derivative recorded at m/z 240 for the extract of authenticated *P. graveolens* plant material (recorded in red – ElSohly Figure 6) and geranium oil sample spiked at 0.1 ppm DMAA (recorded in black – ElSohly Figure 5). The observed retention time shift in the two spectra is consistent with the retention time shift for the internal standard.

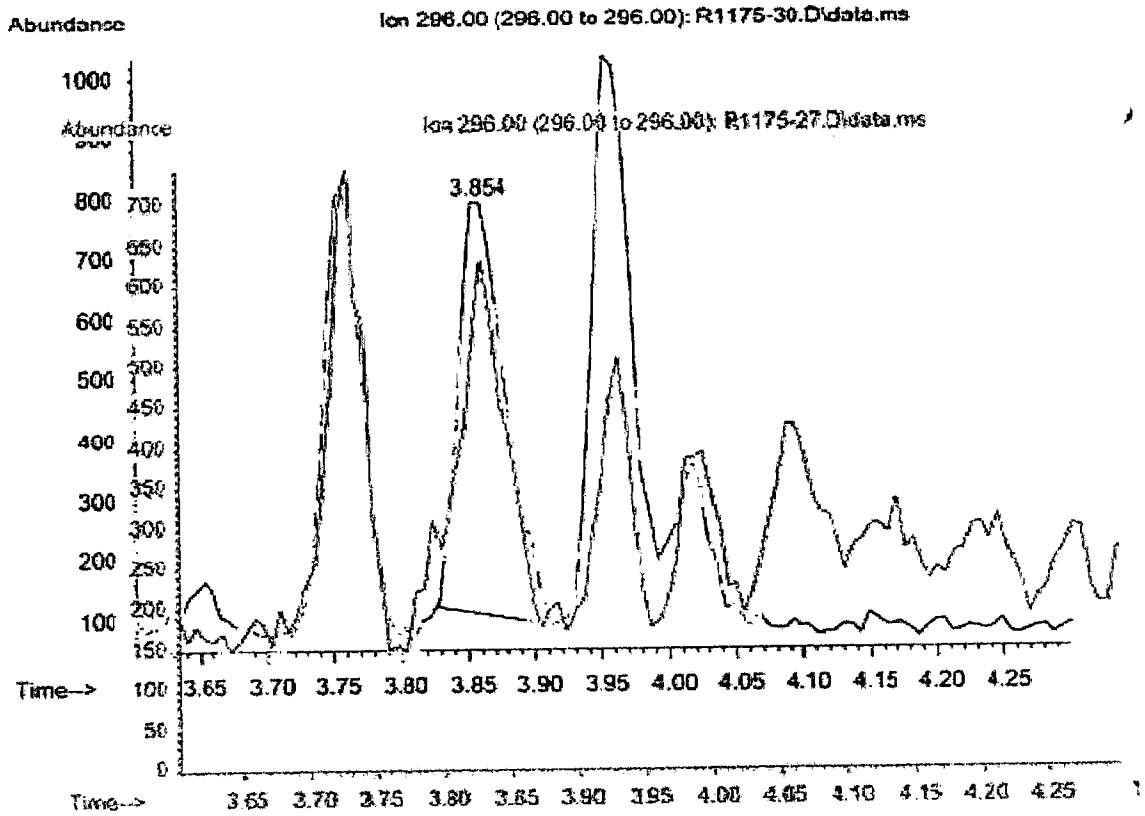


Figure 6. Overlay of GC-MS selected ion chromatograms of the DMAA-HFB derivative recorded at m/z 296 for the extract of authenticated *P. graveolens* plant material (recorded in red – ElSohly Figure 6) and geranium oil sample spiked at 0.1 ppm DMAA (recorded in black – ElSohly Figure 5). The observed retention time shift in the two spectra is consistent with the retention time shift for the internal standard.

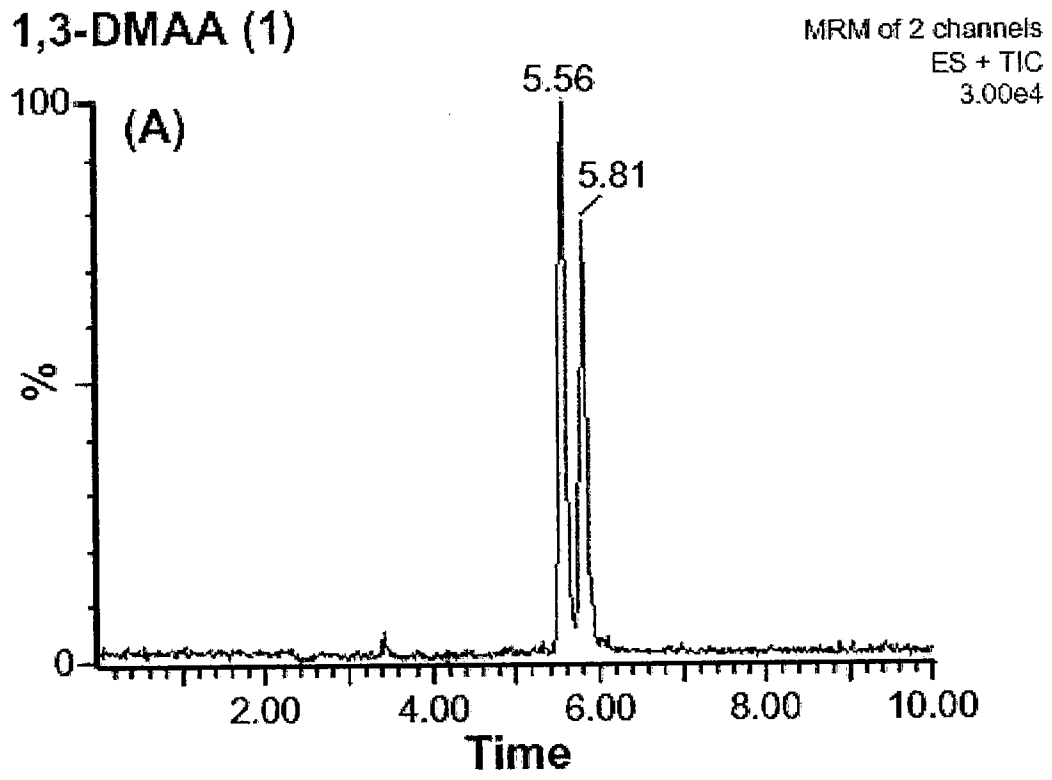


Figure 7. MRM Chromatogram of 1,3-DMAA diastereomers reported by Li et al. 2012 (Figure 2A) in a 2 ng/mL solution. Estimated diastereomeric ratio based on peak heights is 1.27.

ENVIRON

Thomas D. Gauthier, PhD | Principal

Tampa, Florida
+1 813 628 4325 | tgauthier@environcorp.com

Dr. Thomas Gauthier has over 20 years of experience in analytical and environmental chemistry, focusing on the transport and fate of chemicals in the environment, historical dose reconstruction, source identification and the statistical analysis of environmental chemistry data. He has considerable experience addressing issues related to PAH contamination at former MGP sites, metals in soils, chlorinated solvents in groundwater and volatile organic chemicals in indoor air; and he has worked on numerous environmental forensic investigations concerning a wide variety of contaminants, including dioxins and furans, lead, petroleum, polychlorinated biphenyl s (PCBs), metals, pesticides and PCE. He is the author of more than 25 publications and presentations, including the chapter on "Statistical Methods" found in *Introduction to Environmental Forensics*, published by Academic Press.

EDUCATION

1986 PhD, Analytical Chemistry, University of New Hampshire

1982 BS, Chemistry, Merrimack College

EXPERIENCE

Environmental Forensics

- Investigated the source of corrosion and odors in homes containing Chinese drywall. Identified elemental sulfur in mined gypsum as the cause of the corrosion; developed a laboratory method for analysis of elemental sulfur in drywall; and developed a non-destructive technique for identifying corrosive drywall in the home (patent pending).
- Examined PAH fingerprinting analysis of tar samples unearthed during building construction and offered deposition testimony related to the source of the tar material. (Merco Group vs. Tampa Electric Company; Case No. 04-22909 CA 09).
- Analyzed ratios of concentrations of PCE and its biodegradation products (TCE and DCE) in groundwater to determine the relative contribution to a large PCE plume from a small release at a dry cleaning facility located down gradient from the primary source.
- Used cluster analysis and principal components analysis to distinguish sources of PAHs in sediments located adjacent to a former manufactured gas plant and former coal tar distillation facility.
- Evaluated the hypothesis that anaerobic dechlorination of PCBs was naturally occurring in Hudson River sediments by examining PCB congener profiles as a function of depth in sediment cores and comparing these data to PCB congener profiles for commercial Aroclor mixtures using multiple linear regression techniques.
- Investigated coal-tar based parking lot sealants as a potential source of PAHs in Austin, Texas stream sediments. Prepared double ratio plots and used principal components analysis to compare PAH profiles in stream sediments with coal-tar sealed parking lot runoff samples.
- Examined congener profiles using principal component analysis to distinguish sources of dioxins and furans at a wood treating facility.
- Performed a statistical analysis of lead in surface soils at residential properties surrounding a former dump in order to assess the potential contributions from urban background, house paint and contaminated soils originating from the dump.

- Examined the results of advanced hydrocarbon fingerprinting analyses to determine the source of an oil sheen appearing on the Allegheny River.
- Reviewed historical operating practices in relation to the nature and extent of contamination at MGP sites in Massachusetts and New York to establish whether the contamination was the result of accidental releases or intentional disposal.
- Compared x-ray fluorescence and atomic absorption methods for analysis of lead, cadmium and zinc in soils and dust at the Palmerton Zinc Pile Superfund Site. Assessed quality of the data and performed statistical analysis to determine sources of elevated metal concentrations.
- Performed a statistical analysis of indoor air samples collected in homes built over a former refinery site. Compared relative concentrations of contaminants in indoor air with relative concentrations detected in soil gas samples collected beneath the site and determined that indoor air contaminants were unrelated to former refinery operations.
- Examined the spatial distribution of pesticides in soil and groundwater at a former pesticide formulation plant in California in order to determine the impact of historical waste disposal practices.
- Investigated the source and timing of gasoline storage tank releases using a variety of environmental forensic techniques at convenience stores located in Berkeley, California, Houston, Texas and Windham, Maine.
- Examined dioxin fingerprint in boiler ash generated from combustion of solvent waste and compared it to the fingerprints associated with background levels in sediments and soils to determine contributions from the boiler.

Risk Assessment/Exposure Assessment

- Performed a multi-pathway risk assessment and calculated risk-based cleanup levels for residential exposures to chlorinated solvents in groundwater used for irrigation purposes.
- Estimated risks from exposure to volatile organic compounds and polycyclic aromatic hydrocarbons at a former manufactured gas plant site in central Florida.
- Developed a probabilistic risk model in Excel using Crystal Ball software for the local population surrounding a former beryllium metal manufacturing facility.
- Estimated risks from exposure to chlorinated solvents in groundwater via the soil vapor intrusion pathway using the Johnson & Ettinger Model.
- At a western US Superfund site, estimated the range of vinyl chloride concentrations in air that would result in an unacceptable cancer risk for an off-site receptor using Monte Carlo simulation techniques.
- Calculated risk-based clean up levels for explosive compounds in groundwater at a site in Utah. Prepared a comprehensive literature review on the uptake and bioconcentration of RDX by plants.
- Assessed the risks to nearby workers from potential exposures to chlorinated solvents in groundwater at a former circuit board manufacturing site located in central Florida.
- Developed alternative soil cleanup target levels (SCTLs) for arsenic and PAHs in soil under a recreational exposure scenario in support of a remedial action plan for a redevelopment project in Florida.
- Estimated the health risks from exposure to 1,1-DCE in indoor air at residences located above a groundwater contamination plume.
- Performed a statistical analysis of lead concentrations measured in soils collected from a former battery breaking site. These data were used to perform a health risk assessment for the site.

- Developed a Monte Carlo simulation model to predict blood lead concentrations in children exposed to lead in soil, dust, water and food. Model is based upon the EPA LEAD model.
- Performed a sensitivity analysis on an empirical model designed to predict soil lead clean-up levels based on blood lead levels. Interpreted the results of an electron microprobe and selective extraction study designed to assess the bioavailability of lead in soils. Prepared a review on the bioavailability of lead in mining waste for submission to the CDC.
- Determined the health risk of cancer from inhalation of arsenic containing particulates at a primary copper smelter located in southwest United States. Estimated fugitive particulate emissions from the facility, directed air dispersion modeling, and conducted a probabilistic risk assessment using Monte Carlo simulation techniques.
- Estimated household exposures to residents with drinking water wells installed in an aquifer containing low levels of 1,1,1-TCA, 1,1-DCE, and chloroform. Exposures to groundwater contaminants were compared with exposures to contaminants in chlorinated drinking water and naturally occurring radon levels.
- Modeled chemical emissions from three automotive part manufacturing plants in Matamoros, Mexico. Estimated potential exposures to residents in nearby Brownsville, Texas, as part of a toxic tort anencephaly case.

Fate and Transport Analyses/Model Development

- Estimated PAH contributions from coal tar-based pavement sealers on a city-wide scale. Used US Census data coupled with literature-sourced PAH emission rates in a Monte Carlo analysis to generate a probability distribution of PAH mass loadings from various sources.
- Modeled potential vapor emissions released during proposed soil and groundwater remediation scenarios at a chlorinated solvent release site located in central Florida.
- Prepared a report summarizing the environmental impacts of lead released from paper-insulated lead-covered power cables. Examined corrosion processes, environmental chemistry, bioavailability and transport and fate of lead in soil.
- Prepared a comprehensive review of the transport and fate of iron cyanide complexes in manufactured gas plant wastes. Reviewed the chemistry of formation, chemical and biological transformation processes, transport properties and methods of analysis of ferric ferrocyanide and related cyanide species.
- Used the Johnson & Ettinger Model to estimate concentrations of chlorinated solvents in indoor air at residential locations situated above a groundwater plume.
- Modeled infiltration of volatile organic chemicals into basements of homes surrounding a drycleaner facility, and estimated resulting indoor air concentrations for incorporation into a baseline risk assessment.
- Modeled chemical vapor emissions at the Tar Lake (Michigan) wood-tar contamination site for Paramount Inc.
- Served as project manager for a subcontract to design, oversee, and evaluate soil flushing as a remedial alternative for a Superfund site in the midwest. Involved in experimental design, collection of field samples, experiment oversight and report preparation.
- Evaluated the potential for bioremediation of organic constituents in tars produced during production of manufactured gas. Reviewed recent literature on this topic and the results of a treatability study conducted at a former manufactured gas plant site owned by the utility.
- Developed an equilibrium fugacity based model to predict the transport and fate of organic contaminants emitted from municipal waste combustors as part of an evaluation of alternative monitoring and assessment strategies.

- Modeled chemical vapor emissions from the 102nd Street Landfill Site in Niagara Falls, New York, as part of a risk assessment evaluating potential remedial alternatives for the site.
- Investigated the effects of organic cosolvents and surfactants on the transport of volatile organic chemicals in groundwater. Considered effects on the dissolution of non aqueous phase liquids (NAPL) and effects on contaminant retardation factors.

Air Quality Studies

- Conducted indoor air sampling at an elementary school to evaluate the potential for soil vapor intrusion associated with a nearby chlorinated solvent plume.
- Served as project manager for a large multi-year community air monitoring project located in Tallevast, Florida. Analyzed results from SUMMA canister samples and real-time monitoring data to evaluate potential offsite impacts from remediation activities. Prepared a statistical analysis of over 20 months of monitoring data using cluster analysis techniques to identify trends in the data.
- Investigated air quality related issues associated with the expansion of an existing landfill. Examined leachate collection system, landfill gas extraction system and gas flare design in relation to potential air emissions.
- Designed an air sampling program to determine the emission of chemicals from the present landfill cover. Estimated emissions from the proposed landfill expansion based on existing data and air monitoring results.
- Estimated vapor emissions from PCB-contaminated soils at an automotive facility in Region II. Estimated the potential for dioxin formation from the incineration of PCB contaminated soils.
- Estimated volatile organic emissions from three automobile parts manufacturing facilities located in Mexico. Emission estimates were based on mass balance calculations involving purchasing records, waste disposal quantities, material safety data sheet information and production quantities.
- Modeled historical vapor emissions from a wood treating facility based on pentachlorophenol and creosote usage rates.

Testimony Experience

- Merco Group vs. Tampa Electric Company; Case No. 04-22909 CA 09. Offered deposition testimony related to the source of the tar material unearthed during building construction. October 4, 2005.
- BASF Catalysts LLC (f/k/a Engelhard Corp) v. Allstate Insurance Co., et al. No. MID-L-2061-05. Offered three days of deposition testimony as a fact witness in an insurance litigation case. November 9-10, 2009; January 13, 2010.

Other Experience

- Critiqued the State Department of Health's rationale for maintaining state ambient air quality standards more stringent than federal standards. Analyzed ambient air monitoring data, air dispersion modeling results and insurance claims data for the treatment of asthma to support our opinions.
- Served as project manager for a RCRA facility investigation at a former specialty metals manufacturing facility contaminated with radionuclides, metals and chlorinated solvents. Responsible for overall project oversight and report preparation, including human health and ecological risk assessments.
- Served as project manager for a patent litigation case involving the analysis of filtered wood smoke in fish.

Thomas D. Gauthier, PhD

CREDENTIALS

Registrations and Certifications

Member, American Chemical Society

Professional Affiliations and Activities

Reviewer for Environmental Science and Technology and Environmental Forensics

Completed ASTM training course on Risk Based Corrective Action, 1996

Completed "Environmental Project Management" course at Tufts University, 1993

Patents

DBR. No. P2399US. Process and Apparatus for Detecting Sulfur Gases Emitted from Defective Chinese Drywall (patent pending).

PUBLICATIONS & PRESENTATIONS

DeMott, R.P., S.J. Roberts and T.D. Gauthier. 2011. Use of Mass Balance Bounding Estimates and Sensitivity Analysis to Prioritize PAH Inputs in Urban Systems. Paper presented at the Society of Environmental Toxicology and Chemistry 32nd Annual Meeting, Boston, Massachusetts, November 13-17.

Freeman, G., R. DeMott, T. Gauthier, M. Stevenson and J. Hubbard. 2011. Continued Corrosion After Removal of Corrosive Drywall. Journal of Failure Analysis and Prevention 11:265-273.

DeMott, R.P., T.D. Gauthier, J.M. Wiersma and G. Crenson. 2010. Polycyclic Aromatic Hydrocarbons (PAHs) in Austin Sediments after a Ban on Pavement Sealers. Environmental Forensics 11(4): 372 – 382.

Gauthier, T.D., M.C. Masonjones, M.A. Alessandroni, and R. DeMott. 2009. Proposed Mechanism for the Release of reduced Sulfur Compounds from Corrosive Imported Drywall. Paper presented at the Technical Symposium on Corrosive Imported Drywall, Tampa, Florida, November 5-6.

Gauthier, T.D. and R.P. DeMott. 2008. Analysis of PAH Concentrations Detected in Austin Texas Stream Sediments Following a Ban on the Use of Coal Tar Sealers. Paper presented at the Society of Environmental Toxicology and Chemistry 29th Annual Meeting, Tampa, Florida, November 16-20.

Gauthier, T.D. and M. Hawley. 2007. Statistical Methods. In: Introduction to Environmental Forensics, 2nd Edition. B.L. Murphy and R.M. Morrison, Eds. Elsevier/Academic Press, London.

Gauthier, T.D., R.P. DeMott and G. Crenson. 2006. Evaluation of Polycyclic Aromatic Hydrocarbon Sources in Austin Texas Stream Sediments. Poster presented at the Society of Environmental Toxicology and Chemistry 27th Annual Meeting, Montreal Canada, November 5-9.

Gauthier, T.D. 2005. Deriving Florida-Specific Attenuation Factors Using Radon Data. Paper presented at the Florida Air & Waste Management Association 2005 Annual Conference, St. Pete Beach, FL. November 22.

Gauthier, T.D. 2005. Deriving Florida-Specific Attenuation Factors Using Radon Data. Paper presented at the 2005 Florida Brownfields Conference, Jacksonville, FL. October 11.

Gauthier, T.D. 2004. Environmental Impacts of Lead from Paper-Insulated Lead-Covered Cable. EPRI Technical Update Report No. 1009513. April.

Gauthier, T.D., and B.L. Murphy. 2003. Age dating groundwater plumes based on the ratio of 1,1-dichloroethylene to 1,1,1-trichloroethane: an uncertainty analysis. Environmental Forensics 4: 205-213.

Gauthier, T.D., and B.L. Murphy. 2003. Uncertainties in age dating groundwater plumes using 1,1-DCE/1,1,1-TCA ratios. Poster presented at the Battelle In Situ and On-Site Bioremediation Symposium, Orlando, FL. June 2- 5.

Bigham, G., C. Mackay, B. Henry, and T.D. Gauthier. 2002. Fate of mercury deposited in the Everglades. Paper presented at the Air and Waste Management Association – Florida Section 2002 Annual Conference, Jupiter, FL. September 15 – 17.

Gauthier, T.D. 2001. Detecting trends using Spearman's rank correlation coefficient. Environmental Forensics. 2(4):359-362.

Gauthier, T.D. 2001. Statistical Methods. In: Introduction to Environmental Forensics, B.L. Murphy and R.M. Morrison, Eds. Academic Press, London.

Gauthier, T.D., and B.L. Murphy. 2001. Recent Developments in Environmental Forensics: Statistical analysis techniques. Environmental Claims Journal. 13(2): 83-102.

Gauthier, T.D., and B.L. Murphy. 2000. Edible plant bioconcentration factors for RDX. Paper presented at the 2000 Annual Meeting, Society for Risk Analysis, Arlington, VA. December 3-6.

Murphy, B.L., and T.D. Gauthier. 2000. Current Developments in Environmental Forensics: A Survey of Environmental Forensics Topics Presented at Seven Recent Conferences. Environmental Claims Journal. 12(4):113-125.

Murphy, B.L., and T.D. Gauthier. 2000. Dose reconstruction for toxic torts. Environmental Claims Journal. 12(3):161-171.

Murphy, B.L., and T.D. Gauthier. 1999. Forensic analysis of chlorinated solvent contamination data. Environmental Claims Journal. 11(4):81-96.

Murphy, B.L., and T.D. Gauthier. 1999. Determining air emission source contributions to soil concentrations. Environmental Claims Journal. 11(2):143-155.

Gauthier, T. D. and B. L. Murphy. 1998. "Avoiding Sick Buildings at Brownfield Sites," Brownfield News, August.

Butcher, J.B., T.D. Gauthier, and E.A. Garvey. 1997. Use of historical PCB Aroclor measurements: Hudson River fish data. Environmental Toxicology and Chemistry 16(8):1618-1623.

Slayton, T.M., B.D. Beck, K.A. Reynolds, S.D. Chapnick, P.A. Valberg, L.J. Yost, R.A. Schoof, T.D. Gauthier, and L. Jones. 1997. Issues in arsenic cancer risk assessment. Environmental Health Perspectives 104(10): 1012-1014.

Gauthier, T.D. 1996. Application of risk-based corrective action to sites contaminated with chlorinated solvents. Paper presented at the Florida Environmental Expo, Tampa, FL. October.

Shifrin, N.S., B.D. Beck, T.D. Gauthier, S.D. Chapnick, and G. Goodman. 1996. Chemistry, toxicology and human health risk of cyanide compounds in soils at former manufactured gas plant sites. Reg. Tox. Pharm. 23: 106-116.

Bowers, T.S. and T.D. Gauthier. 1995. Use of the output of a lead risk assessment model to establish soil lead cleanup levels. Environmental Geochemistry and Health 16(3): 191-196.

Butcher, J.B. and T.D. Gauthier. 1994. Estimation of residual dense NAPL mass by inverse modeling. Ground Water 32(1): 71-78.

Beck, B.D., G. Goodman, and T.D. Gauthier. 1994. Risk assessment for cyanides in soil at manufactured gas plant (MGP) sites. Paper presented at 1994 Annual Meeting, Society of Toxicology.

Drivas, P.J., P.A. Valberg, and T.D. Gauthier. 1991. Health assessment of air toxics emissions from alternative fuels. Presented at 84th Annual Meeting, Air and Waste Management Association, Vancouver, BC, June.

Thomas D. Gauthier, PhD

Clarke, R.H., J.M. Isner, T.D. Gauthier, K. Nakagawa, F. Cerio, E. Hanlon, H. Brody, E. Gaffney, E. Rouse, and S. DeJesus. 1988. Spectroscopic characterization of cardiovascular tissue. Lasers in Surgery and Medicine 8:45-59.

Gauthier, T.D., R.H. Clarke, and J.M. Isner. 1988. Time resolved plasma photoemission of myocardium with excimer laser excitation. Journal of Applied Physics 64:2736-2741.

Gauthier, T.D., W.R. Seitz, and C.L. Grant. 1987. Effects of structural and compositional variations of dissolved humic materials on pyrene Koc values. Environmental Science & Technology 21 :243-248.

Gauthier, T.D. 1986. "Partitioning of Polyaromatic Hydrocarbons in Natural Waters: Dissolved Organic Matter Effects." Paper #496. Presented at Pittsburgh Conference, Atlantic City, NJ, March.

Gauthier, T.D., E.C. Shane, W.F. Guerin, W.R. Seitz, and C.L. Grant. 1986. Fluorescence quenching method for determining equilibrium constants for polycyclic aromatic hydrocarbons binding to dissolved humic materials. Environmental Science & Technology 20:1162-1166.

Gauthier, T.D. 1985. "A Novel Method for Determining Polyaromatic Hydrocarbon Binding to Dissolved Humic Material." NERM 15. Presented at American Chemical Society, June.

Gauthier, T.D. 1984. "A Fluorescence Quenching Study of the Interaction of Polyaromatic Hydrocarbons with Natural Organics." Presented at Pittsburgh Conference, Atlantic City, NJ, March.

Analytical Chemistry Insights

ORIGINAL RESEARCH

Analysis and Confirmation of 1,3-DMAA and 1,4-DMAA in Geranium Plants Using High Performance Liquid Chromatography with Tandem Mass Spectrometry at ng/g Concentrations

Heather L. Fleming, Patricia L. Ranaivo and Paul S. Simone

Department of Chemistry, The University of Memphis, Memphis, TN, USA.
Corresponding author email: psimone@memphis.edu

Abstract: 1,3-Dimethylamylamine (1,3-DMAA) is a stimulant commercially sold in a variety of dietary supplements as a chemical species derived from geranium plants (Pelargonium graveolens). Whether 1,3-DMAA naturally occurs in geranium plants or other dietary ingredients, it has important regulatory and commercial ramifications. However, the analysis of 1,3-DMAA in geranium plants is not trivial due to low concentrations and a complex environmental matrix, requiring high selectivity and sensitivity. An extraction method combined with high performance liquid chromatography and tandem mass spectrometry is used to determine 1,3-DMAA and 1,4-dimethylamylamine (1,4-DMAA) concentrations in geranium plants with both external calibration and standard addition method. Samples from the Changzhou, Kunming, and Guiyang regions of China during both winter and summer were analyzed for 1,3-DMAA and 1,4-DMAA. The diastereomer ratios of the 1,3-DMAA stereoisomers of a racemic standard and the extracted plant were also quantified.

Keywords: DMAA, geranium, natural product analysis, HPLC, mass spectrometry

Video Abstract Available from http://la-press.com/t.php?i=10445

Analytical Chemistry Insights 2012:7 59–78

doi: 10.4137/ACI.S10445

This article is available from http://www.la-press.com.

Introduction

There has been significant discussion of 1,3-dimethylamylamine (1,3-DMAA) in the literature concerning the presence of 1,3-DMAA in geranium plants (*Pelargonium graveolens*).[1–6] 1,3-DMAA, also known as 4-methyl-2-hexaneamine (MHA), 1,3-dimethylpentylamine, or 2-amino-4-methylhexane can be labeled as geranium extract in dietary supplements. Confirming the presence or absence of 1,3-DMAA as a natural product in geranium plants has important regulatory and commercial consequences for many dietary supplement companies.[7]

The chemical properties and concentrations of 1,3-DMAA and the associated matrix do not allow for simpler LC detection methods (UV-visible absorption or refractive index). Typically, GC-MS analysis requires derivatization to a higher molecular weight to increase boiling point and retention time. The geranium oil and plant matrix are sufficiently complex that most universal detectors, such as refractive index and flame ionization detectors, are likely to encounter significant matrix interferences. Thus, research and analytical effort for 1,3-DMAA analysis has focused on GC-MS[1,3–5] and LC-MS/MS [1,2,4–6] analysis protocols for matrices, such as urine, geranium oil extracts and geranium plants.

The World Anti-Doping Agency requires that compounds with chemical structure and biological activity similar to banned substances must be analyzed by anti-doping laboratories. 1,3-DMAA and 2-aminoheptane (a banned stimulant) have similar chemical structures and physiological stimulant effects (Fig. 1). The laboratory of Saudan[1] developed a high performance liquid chromatography-tandem mass spectrometry (LC-MS/MS) method for detection of 1,3-DMAA in urine samples. The method was calibrated over the range of 50 to 700 ng/mL with excellent intraday precision and accuracy of less than 6%. The results from the Saudan laboratory found that 1,3-DMAA could be detected in urine samples up to 105 hours after administration of a 40 mg dose.

Subsequent research by Vorce et al[2] used LC-MS/MS to confirm 1,3-DMAA as the cause of false positives in amphetamine screening kits used by the United States Department of Defense drug screening laboratories. 1,3-DMAA was suspected due to its inclusion in bodybuilding energy supplements available over the counter. Vorce et al reported that 1,3-DMAA would cause false positives at urine concentrations above 6.0 mg/L and confirmed the presence of 1,3-DMAA concentrations over the 6.0 mg/L limit in 92.3% of the false positive results for amphetamines.

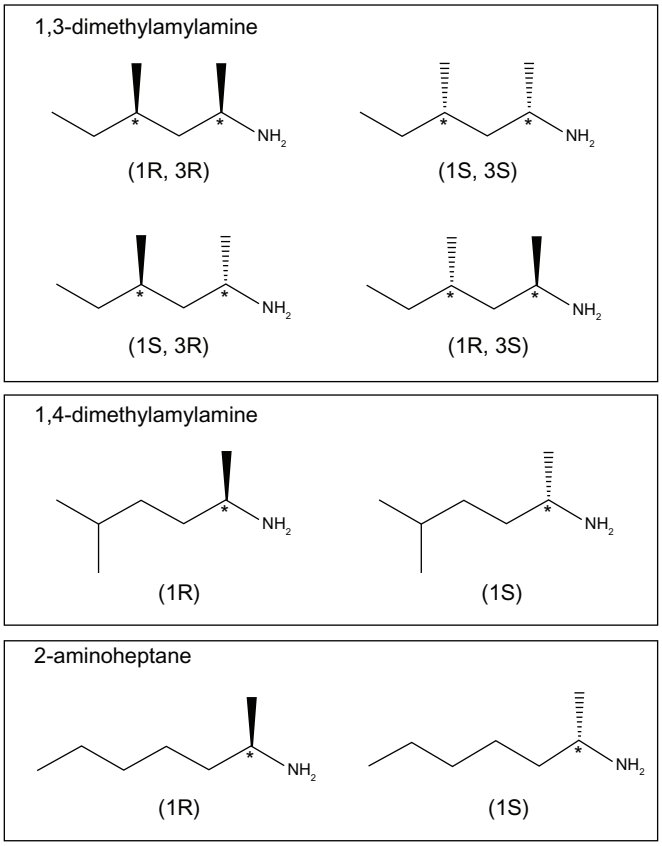


Figure 1. Chemical structures of the stereoisomers of 1,3-DMAA, 1,4-DMAA, and 2-aminoheptane with stereogenic carbons labeled (*) and their respective *(R,S)* configurations.

The laboratory of Lisi[3] conducted an analysis of five geranium oils which had origins in France, Egypt, and New Zealand. The geranium oils were analyzed using a derivatization and extraction procedure for 1,3-DMAA. None of these samples were reported to have 1,3-DMAA, but no limit of detection (LOD) was reported for the method. Supplements containing 1,3-DMAA were then administered and tested in a urine excretion study using a GC with a nitrogen-phosphorous detector. The results showed that 1,3-DMAA is excreted for at least 29 hours in agreement with a previous report.[1]

The research team of ElSohly et al[4] used GC-MS, LC-MS/MS, and high resolution ultra-performance LC with quadrupole-time of flight-MS (UPLC-QTOF-MS) to analyze geranium oils and leaves from India as well as geranium leaves, stems, and freshly extracted

oil from plants grown in Oxford, Mississippi. The GC-MS and LC-MS/MS-based methods used similar extraction procedures with a reported extraction efficiency of 35% (which is relatively low). However, the extraction was shown to have excellent accuracy (75%) and precision (less than 5%) on the control sample using GC-MS analysis. The limits of detection for the GC-MS, LC-MS/MS, and UPLC-QTOF-MS were 0.1 ppm, 2.5 ppb, and 10 ppb, respectively. The GC-MS analysis of the 0.1 ppm spikes of 1,3-DMAA in the geranium oil clearly showed the characteristic double peaks of the 1,3-DMAA diastereomer pairs. The authenticated geranium plant material showed a similar pattern to the spiked geranium oil, whereas the negative geranium oil and authenticated geranium oil did not. The GC-MS chromatograms of the authenticated geranium plant material suggested the presence of the 1,3-DMAA. However, the two more sensitive LC-MS/MS methods did not detect 1,3-DMAA in any of the samples analyzed. The LC-based methods do not exhibit the characteristic diastereomer double peak—possibly due to the chromatographic separation conditions.[2,5,6]

Zhang et al[5] recently reported the analysis of eight different geranium oils, four from China and four from Egypt, and analysis of thirteen dietary supplements containing 1,3-DMAA. The goal of their paper was to determine whether the 1,3-DMAA in dietary supplements had synthetic or natural origins. The supplements were analyzed using GC-FID analysis with a chiral column. The 1,3-DMAA in the standards and supplements were derivatized by pentafluoropropionic anhydride (PFPA). The derivatized stereoisomer separation of 1,3-DMAA by GC-FID was excellent, showing all four stereoisomers present. The GC-FID analysis protocol did not have an LOD reported; however, the calibration curve range was 0.2 to 0.8 mg/mL of 1,3-DMAA. The dietary supplements were reported to contain the same stereoisomer ratios as the synthetic standards.

Zhang et al then used two LC-MS-based methods to analyze the geranium oils for 1,3-DMAA.[5] The LOD of the linear ion trap method (HPLC-ESI-LIT) was 50 ppb and the LOD of the triple quadrupole instrument (HPLC-ESI-QQQ) was 10 ppb for derivatized 1,3-DMAA. The HPLC-ESI-LIT used a chiral-phase HPLC separation column. The HPLC-ESI-QQQ used a standard C18 separation phase. In both methods, 1,3-DMAA was not detected above the LOD, and both lacked the characteristic diastereomer double peak as expected (both possibly due to chromatographic separation choices).

Finally, the research team of Li et al[6] developed an extraction and LC-MS/MS-based method for the analysis of 1,3-DMAA and 1,4-DMAA in geranium plants and oils (three distinct samples of each). The method validation was detailed and conducted according to United States Pharmacopeia guidelines. The traditional instrument LOD[8] reported was 1 to 2 pg/g with a reported method quantification limit (LOQ) of 1 to 2 ng/g in the geranium sample. Li reported concentrations of 1,3-DMAA and 1,4-DMAA as present in three samples of geranium plants ranging from 13 to 365 ng/g and 3 to 35.3 ng/g, respectively. In the geranium oil, Li et al reported all three samples contained 1,3-DMAA ranging from 167 to 13,271 ng/g. In the sample containing 13,271 ng/g of 1,3-DMAA, 1,4-DMAA was detected at 220 ng/g. The other two geranium oil samples did not contain 1,4-DMAA above the LOD.

The research and sample analysis presented here used an adapted extraction and LC-MS-MS analysis[6,9] to analyze both 1,3-DMAA and 1,4-DMAA in geranium plants. Linearity, method detection limit (MDL), accuracy, and precision studies were carried out followed by analysis of geranium plants from 3 distinct regions in China (Changzhou, Guiyang, and Kunming) during winter and summer months. An improved analysis protocol was developed that used standard addition analysis to re-analyze samples and confirm the reported concentrations of 1,3-DMAA and 1,4-DMAA. One of the Changzhou, China, samples was analyzed by another laboratory,[6] and to the best of the authors' knowledge, this represents the first inter-laboratory analysis and confirmation of 1,3-DMAA in an identical geranium sample. Additionally, the diastereomer ratio of 1,3-DMAA in geranium plants was measured and compared with synthetic standards and previously reported research.[5]

Experimental

Chemicals and reagents

All chemicals and reagents have a purity of 97% or greater. All standards and eluent were prepared in reagent-grade water with a resistivity of 18.2 MΩ · cm produced by a Barnstead e-pure four cartridge system.

Glassware was cleaned with concentrated detergent and rinsed with reagent-grade water three times. 1,3-DMAA was purchased from 2A PharmaChem USA (purity confirmed by NMR) and 1,4-DMAA was purchased from Sigma-Aldrich. LC-MS grade acetonitrile and formic acid, HPLC grade ethanol and hexane, and ACS Certified Plus concentrated hydrochloric acid were purchased from Fisher Scientific.

Standard preparation

A combined stock solution was first prepared containing both standards (1,3-DMAA and 1,4-DMAA) with a concentration of 1000 mg/L each in ethanol. An intermediate standard solution is then diluted from the stock to prepare a standard with a concentration of 1000 µg/L in 0.5 N HCl for both 1,3-DMAA and 1,4-DMAA. Two external calibration curves were prepared for each analysis due to the unknown concentrations of 1,3-DMAA. The low range calibration was 1 to 20 µg/L, and the high range calibration was 3 to 100 µg/L. The standard addition spikes curves were prepared by analyzing sample spikes of 1,3-DMAA and 1,4-DMAA at 15.0 µg/L and 25.0 µg/L for each sample.

Sample preparation

Preliminary homogenization and extraction protocol

The preliminary extraction method was adapted from a standard analysis method.[9] The method was scaled from 200 g to 50 g of geranium plant for analysis, and each subsequent step was appropriately scaled by a factor of four. The geranium plants were first cut into pieces having a mass ranging from 40 to 50 g and subsequently placed into a blender. A solution of 15 mL of 0.5 N HCl was added to extract the 1,3-DMAA and 1,4-DMAA analytes present in the plants. The mixture was homogenized at high speed for two minutes, filtered, and re-extracted with 7.5 mL of 0.5 N HCl. Both extracts were combined and diluted to a final volume of 25.00 mL. The solution was then sonicated, filtered, and analyzed by LC-MS/MS. A blank (no geranium plant) and spiked samples containing an additional 10.0 µg/L of the standard solution were also prepared by following the same procedure as those of the plant preparation. The spiked sample provides a percent recovery estimate for each sample matrix.

Optimized homogenization and extraction protocol

The preliminary analysis method was further modified[6] to reduce matrix effects by adding a hexane partitioning step (hexane clean-up step). The geranium samples remained frozen at −20 °C prior to analysis and thawed for sample preparation. The wet geranium leaves and stems were cut into 1 to 2-cm pieces and subsequently ground with a high-speed grinder into finely chopped pieces. Then, 10 g of the chopped sample were weighed and placed into a standard food blender with 80 mL of 0.5 N HCl and homogenized at the highest blend setting for two minutes. The blended mixture was transferred into a 100-mL volumetric flask, and the blade and blender cup were rinsed with 15 mL of 0.5 N HCl and poured into the 100-mL volumetric flask. The blended geranium mixture was extracted by sonication for one hour at 50 °C. This solution was centrifuged at 3700 × g for ten minutes after cooling and filling to volume with 0.5 N HCl. Four mL of the supernatant and 2 mL of hexane were added to a 15-mL glass centrifuge tube with screw cap. This mixture was shaken by a vortex mixer for thirty seconds. The mixture was then centrifuged at 2000 × g for five minutes. The aqueous layer was filtered and analyzed by LC-MS/MS. For all sample analyses, a blank was analyzed with each sample to verify no carryover occurred from the previous analysis. For standard addition analysis, spiked samples were prepared by spiking standard prior to the blending process, such that the final added concentration was 15.0 and 25.0 µg/L in the volumetric flask.

This optimized method added and modified existing steps (grinding, sonication, and centrifuging) to the original extraction protocol to maximize the extraction efficiency of 1,3-DMAA and 1,4-DMAA from the plant matrix. The reduction of plant material extracted and increased volume of extractant resulted in a more practical extraction procedure and minimized sample handling errors. The sonication temperature was increased to 50 °C to increase the breakup and dissolution of the plant material in the acid extract and increase solvation of the analytes. The additional hexane extraction step minimized concentrations of the non-polar plant material in the 0.5 N HCl extraction solution. The non-polar plant material likely caused matrix effects during analysis

by causing ion suppression in the ESI source. The combination of these steps provides an extract that contains a more representative concentration of 1,3-DMAA and 1,4-DMAA and a reduction of matrix effects. This means that the performance of the extraction method improves and this is demonstrated by the large improvement in percent recovery.

HPLC-MS/MS instrumentation

The LC-MS/MS system consists of an Agilent 1100 HPLC system equipped with an autosampler, coupled to a triple quadrupole mass spectrophotometer (Waters Quattro Ultima) operated in ESI+ mode. The injection volume was 100 µL with separation performed on a Phenomenex Kinetex C18 phase column (4.6 × 150 mm, 2.6 µm) with a column temperature set at 25 °C and flow rate at 0.4 mL/min. The HPLC eluent ratio was 82:18 of mobile phase A (1% of formic acid in reagent water) to mobile phase B (acetonitrile). The column effluent was split at a ratio of 1:1 prior to introduction to the mass spectrometer.

The mass spectrometer operating conditions were as follows: the capillary voltage was 3.0 kV, the cone voltage was 20 V, the source temperature was set at 120 °C with a flow of 108 L/hr, and the desolvation temperature was 350 °C with a flow of 635 L/hr. The dwell time was 0.5 second and the interscan delay was 0.1 second. The collision voltage was set to 8 eV with a collision gas (argon) pressure at 7 psi. The detection of the analytes was done using the MRM function with a pair of mass transitions of 116/99.7 m/z and 116/57 m/z to produce a single chromatogram for both 1,3-DMAA and 1,4-DMAA.

All chromatogram integrations were performed with Waters MassLynx MS software. Each chromatogram was prefiltered with a peak-to-peak noise amplitude of 2000. Chromatograms were submitted to a Savitzky Golay[10] smoothing method within the MassLynx software. The Savitzky Golay method takes an average of the intensities of the data points weighted by a quadratic curve.

The LC-MS/MS total analysis time was 10 minutes. Figure 2 presents a typical standard chromatogram of a 20 µg/L standard of 1,3-DMAA and 1,4-DMAA. Additional standards are presented in the supplementary materials (Figs. S1–S3). It is important to mention that the compound 1,3-DMAA has two chiral centers that result in four stereoisomers (Fig. 1). These stereoisomers include two diastereomers that have different physical properties and can be separated. Therefore, 1,3-DMAA is detected as two peaks in the chromatogram. All values referenced to 1,3-DMAA_total or 1,3-DMAA are calculations based on the summation of both peak areas.[2,6,9] The compound 1,4-DMAA exists as two enantiomers which cannot be separated. Therefore, only one peak was detected for 1,4-DMAA.

Results and Discussion

Detection limits, accuracy, precision, and linearity studies

Before sample analysis was conducted, detection limit,[11–13] accuracy,[14] precision,[14] and linearity[8] studies were conducted to evaluate and ensure acceptable instrument performance. The typical practice for United States Environmental Protection Agency (USEPA) MDL studies in the laboratory is to construct a 5-point calibration curve and analyze a check standard halfway between the two lowest calibration points. The USEPA MDL reported here represents the

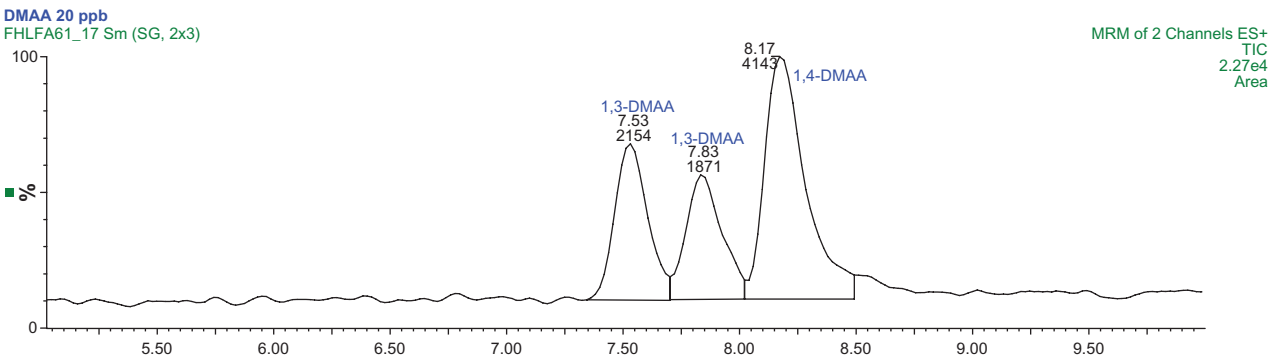


Figure 2. Typical MRM Chromatogram at 20 µg/L each for 1,3 and 1,4-DMAA analytes.
Note: The retention times for the 1,3-DMAA diastereomers are 7.53 and 7.83 minutes, and 1,4-DMAA retention time is 8.17 minutes.

lowest concentration distinguishable from noise and determined on the variation of the analytical signal of a check standard expected to be within a factor of 2 to 5 of the detection limit. At these analytical conditions, the MDL study provides a worst-case estimate of the analyzer performance. The accuracy of the analysis is estimated using the mean percent recovery of the check standard analysis.[14] The precision is estimated as the percent relative standard deviation (% RSD).[14]

Another estimate for the detection limit is the propagation of uncertainty MDL (Unc. MDL).[13] The Unc. MDL is determined using the standard deviations of the slope (m), y-intercept (b), and signal (y) as determined by the LINEST function in Microsoft Excel. These standard deviations are then used to propagate and determine the error on "x" in the linear regression line.[13] The propagated error represents the lowest concentration of analytical significance.

Detailed MDL, accuracy, and precision studies of 1,3-DMAA and 1,4-DMAA are presented in Tables 1 and 2, respectively, for all sample analysis conducted (Analysis Sets 1 to 3). The reported values for Analysis set 1 were based on the preliminary extraction protocol. Analysis Sets 2 and 3 were conducted using a hexane clean-up step as well as standard addition analysis. Typically, an MDL, accuracy and precision study was conducted with two different check standard concentrations prior to each set of sample analysis. For Analysis Sets 1 and 2, the MDLs at 3.0 µg/L were based on the calibration curves from 1 to 20 µg/L (low range calibration). The MDLs at 8.0 µg/L were based on the 3 to 100 µg/L calibration curves (high range calibration). In Analysis Set 3, the calibration curve for the 2.0 µg/L check standard was 1 to 100 µg/L, and the calibration curve for the 3.0 µg/L check standard was 2 to 100 µg/L. The R^2 values for all studies with both DMAA species were greater than 0.99.

The MDL values[11,12] for 1,3-DMAA range from 0.6 to 3.2 µg/L and for 1,4-DMAA, range from 0.8 to 2.7 µg/L. Accuracy[14] for 1,3-DMAA ranges between 60% and 126% and for 1,4-DMAA, ranges between 48% and 127%. The precision (estimated as % RSD)[14] for 1,3-DMAA is in the range of 9% to 35%, and for 1,4-DMAA, precision ranges between 10% and 30%. With the exception of one mean percent recovery analysis in Analysis Set 2, the reported mean percent recoveries and % RSD are within the guidelines set by the USEPA[14] for check standard analysis. The USEPA reports that mean percent recovery can range from 50% to 150%, and the % RSD can be up to 30% when samples are analyzed within a factor of 2 to 5 of the MDL.[14] As the MDL factor decreases, the % RSD of the check standard analysis increases, and below an MDL factor of 2, the % RSD can dramatically increase beyond 30%.[15]

Ideally, MDL, accuracy and precision studies should provide estimates that are similar to each other.[15–17] Further confidence of these MDL values is gained when the USEPA MDLs are compared to the Unc. MDL. Both sets of detection limit values are within 2 µg/L of each other in absolute terms and within a factor of 5 in all cases. This similarity indicates the MDL values for the calibration and analysis protocols are realistic estimates for both 1,3-DMAA and 1,4-DMAA.

A linearity study was conducted to estimate the upper limit of linearity for the LC-MS/MS analysis.[8] A calibration curve was prepared and analyzed over the range of 1 to 250 µg/L for 1,3-DMAA and 1,4-DMAA, with both species being linear over the entire range as evidenced by the excellent R^2 values (>0.99). The linearity study resulted in a linear regression

Table 1. Detection limits, accuracy, and precision studies for 1,3-DMAA for all sample analysis.

Analysis Set	Check standard (µg/L)	USEPA MDL (µg/L)	Unc. MDL (µg/L)	Mean % recovery	% RSD	MDL factor	Equation of linear regression	r^2
Analysis Set 1	3.0	1.1	0.4	126	9	2.8	y = 195.81x – 19.049	0.999
	8.0	1.8	3.4	73	10	4.5	y = 148.84x + 420.8	0.999
Analysis Set 2	3.0	2.3	0.5	71	35	1.3	y = 121.94x + 72.43	0.998
	3.0	1.8	0.8	95	20	1.7	y = 90.587x – 43.177	0.994
	8.0	2.5	1.5	62	16	3.2	y = 114.66x + 199.46	0.999
	8.0	3.2	1.4	60	21	2.5	y = 78.45x + 131.18	0.999
Analysis Set 3	2.0	1.4	2.6	103	21	1.5	y = 111.83x + 60.259	0.996
	3.0	0.6	1.4	63	10	4.9	y = 135.07x + 190.33	0.999

Table 2. Detection limits, accuracy, and precision studies for 1,4-DMAA for all sample analyses.

Analysis Set	Check standard (µg/L)	USEPA MDL (µg/L)	Unc. MDL (µg/L)	Mean % recovery	% RSD	MDL factor	Equation of linear regression	r^2
Analysis Set 1	3.0	1.4	0.7	127	12	2.1	y = 201.78x – 78.268	0.996
	8.0	2.7	4.6	60	18	2.9	y = 147.57x + 552.07	0.998
Analysis Set 2	3.0	2.0	0.6	73	30	1.5	y = 130.39x + 31.787	0.996
	3.0	0.9	0.6	93	10	3.4	y = 85.06x – 38.131	0.997
	8.0	2.4	2.9	48	20	3.3	y = 109.18x + 340.17	0.995
	8.0	2.1	0.9	81	10	3.9	y = 79.501x – 7.9734	0.999
Analysis Set 3	2.0	0.8	2.6	98	13	2.4	y = 95.314x + 76.382	0.996
	3.0	0.8	1.6	76	11	3.7	y = 121.63x + 118.7	0.999

equation for 1,3-DMAA of y = 149.08x + 380.91 and for 1,4-DMAA of y = 148.05x + 473.94.

DMAA concentrations in the plant material

The reported concentration of the DMAA species in the geranium herb was determined using the calculated concentration from the calibration curve, final extraction volume, and mass of geranium (Equation 1, below). The MDL, accuracy, and precision studies (Tables 1 and 2) were conducted with prepared standards in solution (no extraction). However, the MDLs in the analyzed plant would vary with the amount of plant mass used and the final extracted volume. For Analysis Set 1, the amount of plant material used was 50 g extracted into 25.00 mL. This resulted in MDLs that ranged from 0.6 to 1.4 ng DMAA/g geranium. In Analysis Sets 2 and 3, 10 g of plant material were extracted into 100.00 mL, which resulted in MDLs ranging from 6 to 32 ng DMAA/g geranium. While the MDLs increased for the second extraction method, the percent recovery of DMAA analysis also increased for all samples. The increase in percent recovery is likely due to the hexane clean-up step as well as a more practical increase in the extraction solvent volume. If the mass of plant material were doubled, the MDLs of the optimized extraction protocol would likely increase by a factor of two.

$$DMAA_{geranium}(ng/g) = [[DMAA_{cal.curve}\ (\mu g/L) \times \text{Extraction volume (L)}] \div \text{Geranium mass (g)}] \times 1000 \quad (1)$$

Authenticated *Pelargonium graveolens* samples

The *Pelargonium graveolens* (geranium) samples were collected and authenticated as all belonging to the genus and species *Pelargonium graveolens* by Xu YouKai of the Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences. Samples were collected from three regions in China: Changzhou, Guiyang, and Kunming, during three different harvest seasons. The Chinese Academy received the geranium herbs as potted plants originally grown in the field. Multiple plants (ranging from two to ten in number) were collected from each location. The plants from each location were combined prior to shipment to The University of Memphis. Therefore, concentrations of 1,3-DMAA and 1,4-DMAA in individual plants and variations thereof are not reported here. The samples were sent by express airmail from Dr. Yi Jin of Yunnan University directly to the University of Memphis where the samples were immediately stored at –20 °C. Analysis Sets 1 and 2 consisted of a Changzhou sample collected on June 9, 2011 (Changzhou S11-1 and Changzhou S11-2), a Kunming, China, sample collected March 20, 2012 (Kunming 1 and 2); a Guiyang, China, sample collected March 16, 2012 (Guiyang 1 and 2); and an additional Changzhou, China, sample collected on March 10, 2012 (Changzhou 1). Analysis Set 3 consisted of a Changzhou sample collected on May 18, 2012 (Changzhou 3), a Guiyang sample collected May 20, 2012 (Guiyang 3), and a Kunming sample collected May 23, 2012 (Kunming 3). The Changzhou S11 sample was received from Intertek Labs (Detroit, MI, USA) and frozen upon arrival. The Changzhou S11 sample is an identical sample previously analyzed and reported by Li,[6] providing an inter-laboratory analysis of a sample. The numbers for each region identifier signify the various Analysis Sets.

Sample Analysis set 1: preliminary extraction protocol

The concentrations of 1,3-DMAA and 1,4-DMAA in the three winter geranium samples and Changzhou S11 sample are presented in Table 3. The Changzhou S11-1 analysis was conducted in duplicate and the winter samples were analyzed in singlet. A spike sample was analyzed to determine the percent recovery for that particular plant sample. There is no reported spike analysis for Changzhou 1 due to a sample loss during analysis. No additional sample was available. The percent recovery of the spike was calculated using equation 2:[13]

$$\text{Percent Recovery} = [[\text{Spike conc. } (\mu gL) - \text{Unspiked conc. } (\mu gL)] \div 10(\mu gL)] \times 100\% \quad (2)$$

Of the four samples in Analysis Set 1, only the Changzhou S11-1 and Changzhou 1 sample contained 1,3-DMAA and 1,4-DMAA above the MDLs of the method (Table 3). Figures 3 and 4 present an MRM chromatogram of Changzhou S11-1 and Changzhou 1 samples, respectively. Additional sample and spike chromatograms are presented in the supplementary materials (Figs. S4–S8). The average concentration of 1,3-DMAA in the Changzhou S11-1 sample was 94.7 ± 15.1 ng/g geranium, with a percent recovery of 19% on the 10 µg/L spike. The average concentration of 1,4-DMAA in Changzhou S11-1 was 13.5 ± 1.8 µg/L with a 65% recovery on a 10 µg/L spike. The concentrations of 1,3-DMAA and 1,4-DMAA in Changzhou 1 samples were 213 and 52 ng/g respectively. The reported 1,3-DMAA concentrations for Changzhou S11-1 and Changzhou 1 samples were outside the calibration range but within the linearity of the analyzer. A 1:1 dilution of both samples was analyzed and resulted in calculated concentrations within 9% of the original concentration reported in Table 3.

While the percent recovery of the DMAA species is not ideal, the relative concentrations should be considered for the spike. For Changzhou S11-1 sample, the concentrations of 1,3-DMAA in volumetric flask after extraction averaged 190 µg/L. The % RSD error of analysis from the MDL study was of 9% to 10% for Analysis Set 1 and translates to ~18 µg/L error. This is more than twice the 10 µg/L spike and thus a likely contributor to the low percent recovery (high error). When 1,4-DMAA was examined, the 10 µg/L spike addition was outside the error of analysis (2.7 µg/L) and gave a more reasonable 65% recovery. Additionally, the low percent recoveries across all samples indicated the presence of a matrix effect. Previous reports[6] have suggested that extraction protocols are likely to be extracting lipids from the cell membranes and contributing to ion suppression in the ESI source.

Analysis Set 2: optimized extraction protocol analysis of Changzhou S11 and winter geranium samples

The matrix effect identified in Analysis Set 1 was minimized by the addition of a hexane clean-up step. Additionally, the optimized method was more efficient as it used less plant sample mass per extraction. This efficiency provided an opportunity to re-analyze Changzhou S11, Kunming, and Guiyang winter samples. Each sample was extracted and analyzed with two different spike concentrations (15.0 µg/L and 25.0 µg/L) for both 1,3-DMAA and 1,4-DMAA and in duplicate. The spiked samples were analyzed concurrently with the unspiked ones, and the percent

Table 3. Analysis Set 1: preliminary extraction protocol results of geranium samples from Changzhou, Kunming, and Guiyang.

	1,3-DMAA			1,4-DMAA		
	Sample (ng/g)	Spike level (µg/L)	Percent recovery (%)	Sample (ng/g)	Spike level (µg/L)	Percent recovery (%)
Changzhou S11-1	94.7 ± 15.1	10.0	19	13.5 ± 1.8	10.0	65
Kunming 1	<0.5*	10.0	44	<0.7*	10.0	32
GuiYang 1	<0.5*	10.0	36	<0.7*	10.0	23
Changzhou 1	213	N/A	N/A	52.0	N/A	N/A

Note: *The results are less than the MDL values.

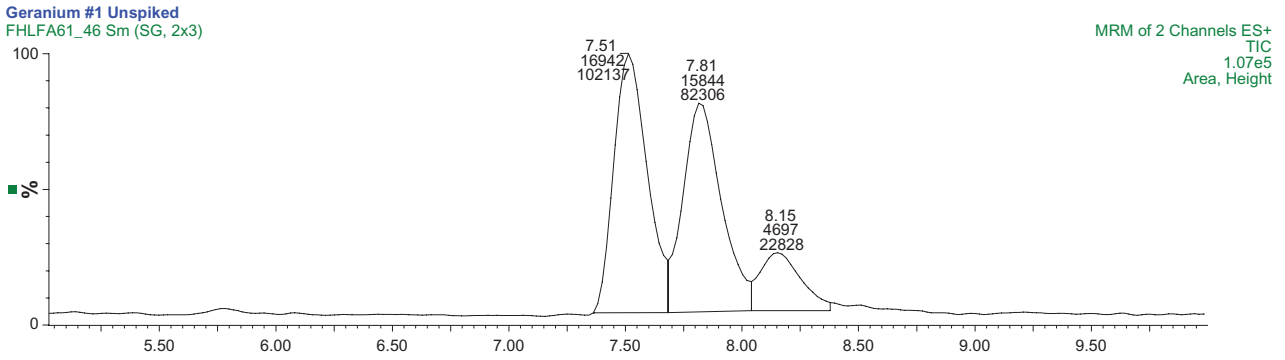


Figure 3. A MRM chromatogram of Changzhou S11-1 sample.
Notes: The first two peaks are 1,3-DMAA diastereomer pairs with retention times of 7.51 minutes and 7.81 minutes. The 1,4-DMAA peak retention time is 8.15 minutes. The chromatogram is produced using two mass transitions 116/99.7 m/z and 116/57 m/z.

recovery was subsequently calculated.[13] Detailed analysis results are presented in Table 4.

Changzhou S11 concentrations were expected to be high and thus analyzed on the high range calibration of 3 to 100 µg/L of both DMAA species. The concentrations of 1,3-DMAA and 1,4-DMAA were 254 ng/g and 39.8 ng/g, respectively, and an optimized extraction chromatogram of Changzhou S11-2 is presented in Figure 5. The percent recovery[13] for 1,3-DMAA was approximately 55% for both spike levels. Both Kunming and Guiyang (Fig. 6) samples were analyzed using the low range calibration curves (1 to 20 µg/L of each DMAA species). The concentrations of 1,3-DMAA and 1,4-DMAA are reported in Table 4. All are less than the MDL of the analysis. The percent recovery for all remaining samples ranged from 63% to 107%, indicating that the matrix effect previously identified was substantially mitigated by the optimized extraction protocol. The chromatograms for samples and 15.0 µg/L spikes of Analysis Set 2 are also presented in the supplementary materials (Figs. S9–S14).

A comparison of the two extraction protocols using Changzhou S11 geranium sample demonstrates that the preliminary extraction protocol underestimated the concentrations of both DMAA species as indicated by the percent recovery results. However, it is clear that Changzhou S11 geranium samples contain 1,3-DMAA species and the concentrations are well above the MDL of both analysis. In contrast, Kunming and Guiyang samples did not contain 1,3-DMAA or 1,4-DMAA species at significant concentrations above the MDL of analysis (20 ng/g).

Analysis set 3: optimized extraction protocol of summer geranium samples

An additional round of samples was collected from a summer harvest of geranium plants and analyzed using the same protocols from Analysis Set 2 (with two spike levels, in duplicate). The Changzhou 3 sample (Fig. 7)

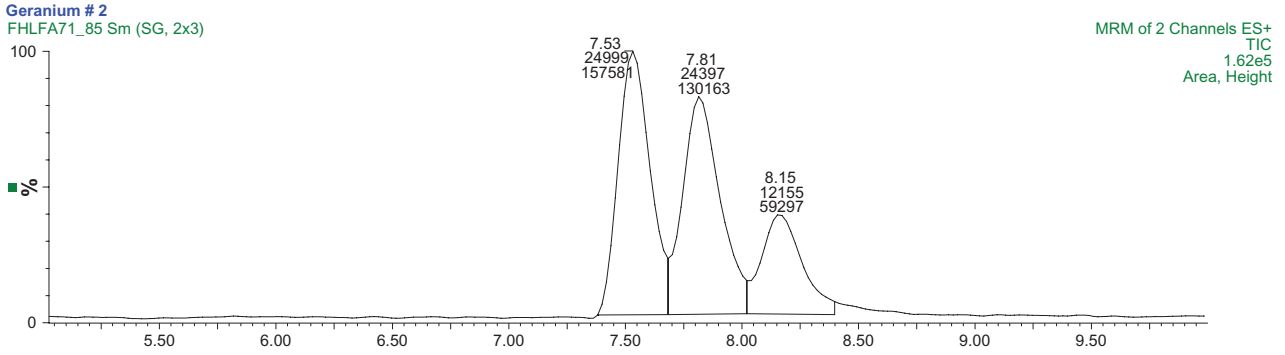


Figure 4. A MRM chromatogram of the Changzhou 1 sample.
Notes: The first two peaks are 1,3-DMAA diastereomer pairs with retention times of 7.51 minutes and 7.81 minutes. The 1,4-DMAA peak retention time is 8.15 minutes. The mass transitions used are 116/99.7 m/z and 116/57 m/z.

Table 4. Analysis set 2: optimized extraction protocol results of geranium samples from Changzhou S11, Kunming, and Guiyang.

	1,3-DMAA			1,4-DMAA		
	Sample (ng/g)	Spike level (µg/L)	Percent recovery (%)	Sample (ng/g)	Spike level (µg/L)	Percent recovery (%)
Changzhou S11-2	254 ± 17	15.0	54 ± 5	39.8**	15.0	76 ± 2
		25.0	55 ± 8		25.0	65 ± 1
Kunming 2	<20 ± 4*	15.0	83 ± 11	<14 ± 8	15.0	78 ± 10
		25.0	67 ± 1		25.0	63 ± 5
Guiyang 2	<20 ± 4*	15.0	107 ± 23	<14 ± 8	15.0	82 ± 16
		25.0	81 ± 2		25.0	78 ± 6

Notes: *The results are less than the MDL values; **one duplicate was less than MDL for the sample (23.9 ng/g).

contained 1,3-DMAA and 1,4-DMAA concentrations of 68.8 ± 36.5 ng/g and 118 ± 45 ng/g, respectively (Table 5). Both Kunming 3 and Guiyang 3 had concentrations of 1,3-DMAA and 1,4-DMAA below the MDL (less than 10 ng/g). These results are consistent with the previous winter sample analysis. Both DMAA species were detected and quantified in the Changzhou samples, but no DMAA species were detected above the MDL in Kunming and Guiyang samples (See Supplementary materials Figs. S15–S20). The percent recovery for all samples was excellent and ranged between 64% and 86%.

Winter versus summer sample analysis

Previous research has shown that concentrations of chemical species in natural products can be highly variable.[18,19] A seasonal comparison is possible between the winter harvest (March 2012) and the summer harvest (May 2012) for Kunming, Guiyang and Changzhou samples. Neither the winter nor summer harvest samples of Kunming and Guiyang samples contained 1,3-DMAA or 1,4-DMAA species above the MDLs of the analysis. However, Changzhou sample resulted in similar concentrations of 1,3-DMAA and 1,4-DMAA in the June 2011 and March 2012 samples. From March 2012 to May 2012, 1,3-DMAA resulted in a factor of 3 decrease in concentration while 1,4-DMAA about doubled in concentration. These results indicate a potential seasonal effect of 1,3-DMAA and 1,4-DMAA concentrations in agreement with previously reported research discussing environmental effects on chemical composition.[18,19] It is also possible the concentrations of 1,3-DMAA in Changzhou winter samples were higher due to an apparent underestimation of 1,3-DMAA concentrations by the preliminary extraction protocol as evidenced by Changzhou S11 analysis.

Standard addition analysis of 1,3-DMAA and 1,4-DMAA

A standard addition analysis protocol was developed for sample analysis. Standard addition analysis

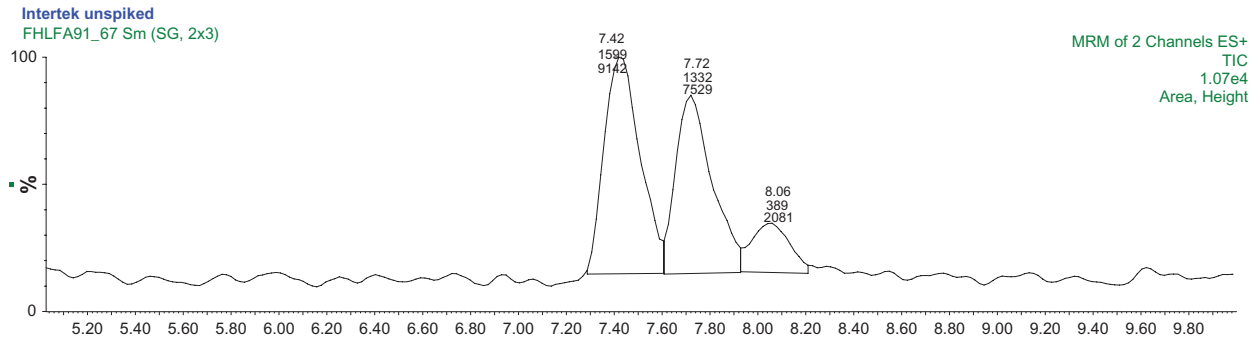


Figure 5. A MRM chromatogram of the optimized extraction protocol for Changzhou S11-2 showing the presence of 1,3-DMAA diastereomers (peaks 1 and 2) and 1,4-DMAA (peak 3).
Note: The mass transitions used are 116/99.7 m/z and 116/57 m/z.

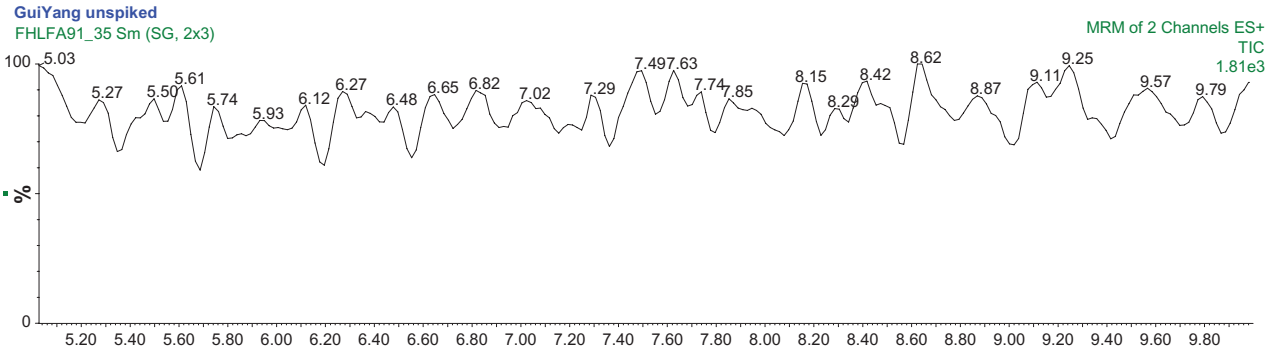


Figure 6. A typical MRM chromatogram of the Guiyang 2 sample demonstrating the absence of 1,3-DMAA and 1,4-DMAA in the geranium plant.
Note: The mass transitions used are 116/99.7 m/z and 116/57 m/z.

compensates for matrix effects found in geranium plants caused by chemical species other than DMAA affecting analytical signal (either positive or negative).[13] In the standard addition method, known quantities of the 1,3-DMAA and 1,4-DMAA standards are added to the sample extract. This is termed "spiking" the sample. The added standard is affected by matrix effects just as the analyte in the sample. The unknown concentration can then be derived from a plot of signal versus spike concentration as long as the analyte has been previously established to have a linear signal response. Thus, the standard addition method resolves matrix interferences present in the complex geranium sample composition.[13]

The standard addition protocol was applied to both Analysis Sets 2 and 3 (Changzhou S11, Kunming, and Guiyang winter samples and Changzhou, Kunming, and Guiyang summer samples). For this study, a three-point standard addition plot was constructed using the unspiked sample, a 15.0 µg/L spike each of 1,3-DMAA and 1,4-DMAA, and a 25.0 µg/L spike each of 1,3-DMAA and 1,4-DMAA. The signal was plotted against the spike concentration (0, 15, and 25 µg/L), and a linear regression analysis was performed. The slope (m) and y-intercept (b) of the calibration curve were used to calculate the concentration of analyte (x) in the sample.[13] The equation for determining the x-intercept is $x = -b/m$, and in standard addition, the negative of the x-intercept is the concentration present in the unspiked sample.

The standard addition analysis results showed some matrix effects were still present in the optimized procedure and the external calibration analysis likely underestimated DMAA concentrations. However, the standard addition analysis agreed overall with the external calibration results. Samples reported to contain 1,3-DMAA by external calibration also contained 1,3-DMAA by standard addition. Concentrations of 1,3-DMAA species were quantified in both Changzhou S11-2 and Changzhou 3 samples at 496 ± 46 ng/g and 97 ± 20 ng/g, respectively. The concentrations of 1,4-DMAA in Changzhou S11-2 and Changzhou 3 samples were 68 ± 7 ng/g and 162 ± 48 ng/g, respectively. All concentrations were

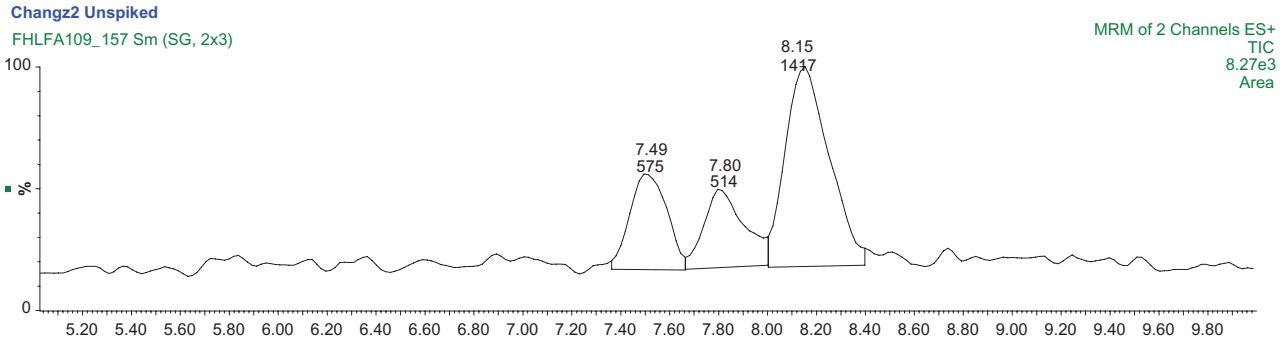


Figure 7. A MRM chromatogram of Changzhou 3 sample showing the presence of 1,3-DMAA at a lower concentration than 1,4-DMAA.
Note: Mass transitions are 116/99.7 m/z and 116/57 m/z.

Table 5. Analysis set 3: optimized extraction protocol results of geranium summer samples from Kunming, Guiyang, and Changzhou.

	1,3-DMAA			1,4-DMAA		
	Sample (ng/g)	**Spike level (µg/L)**	**Percent recovery (%)**	**Sample (ng/g)**	**Spike level (µg/L)**	**Percent recovery (%)**
Kunming 3	<10 ± 6*	15.0	68 ± 3	<8.2 ± 0.3*	15.0	64 ± 2
		25.0	74 ± 6		25.0	75 ± 9
Guiyang 3	<10 ± 6*	15.0	75 ± 4	<8.1 ± 0.2*	15.0	78 ± 1
		25.0	81 ± 8		25.0	84 ± 6
Changzhou 3	68.8 ± 36.5	15.0	76 ± 13	118 ± 45	15.0	86 ± 4
		25.0	79 ± 13		25.0	77 ± 7

Note: *The results are less than the MDL values.

well above the MDL of the analysis and clearly demonstrated 1,3-DMAA and 1,4-DMAA were present in geranium herbs from the Changzhou region.

The standard addition results for winter and summer samples of Kunming and Guiyang agreed with the external calibration results. Concentrations of 1,3-DMAA and 1,4-DMAA were all less than the MDL previously reported, or so close to the MDL that the confidence of analysis was extremely low. One of the Kunming 3 duplicates resulted in a 1,3-DMAA concentration of 21 ng/g, while the other duplicate was below the MDL of 14 ng/g. Similarly, one of the Kunming 2 duplicates resulted in a 1,4-DMAA concentration of 10 ng/g, whereas the other duplicate had concentrations less than the 20 ng/g MDL of that particular analysis.

Measurement of the diastereomer ratios of 1,3-DMAA in the Changzhou geranium samples

Zhang et al[5] measured the diastereomer ratios (reported as first peak/second peak) of synthetic standards and dietary supplements containing 1,3-DMAA using GC-FID analysis. The reported results showed the diastereomer ratio of a Sigma-Aldrich standard of 1,3-DMAA was 1.22 ± 0.06 and the ChromaDex standard ratio was 1.42 ± 0.09. The dietary supplements had identical ratios to those of the standards suggesting that both standards and supplements were of synthetic origin.

In this report, both pairs of diastereomers were detected in the Changzhou region samples as well as the synthetic calibration standards. By inspection of the chromatograms (Figs. 3, 4, 5, and 7), both standards and geranium samples present similar diastereomer ratios. Quantitatively, the average ratio of 1,3-DMAA diastereomers (first peak/second peak) in typical 20, 50 and 100 µg/L calibration standards is 1.14 ± 0.08. The diastereomer ratio of Changzhou S11-1 sample was 1.10 ± 0.01, Changzhou 1 was 1.02, Changzhou S11-2 was 1.25 ± 0.03, and Changzhou 3 was 1.16 ± 0.10. The results of the geranium plant diastereomer ratios are similar to the ratios of the synthetic standards presented here, as well as the standards and supplements analyzed by Zhang et al. This indicates that supplements containing both 1,3-DMAA diastereomer pairs could be naturally produced and extracted from geranium plants.

Conclusion

In conclusion, geranium plants (*Pelargonium graveolens*) from three different regions of China (Kunming, Guiyang, and Changzhou) and three different harvests (June 2011, March 2012, and May 2012) were analyzed for 1,3-DMAA and 1,4-DMAA. An extraction and HPLC-MS/MS analysis method was used to determine concentrations of 1,3-DMAA and 1,4-DMAA with both external calibration and standard addition analysis. The extraction and external calibration analysis likely suffered from matrix effects and thus underestimated concentrations of 1,3-DMAA and 1,4-DMAA in geranium plants. The matrix effects were largely solved by the standard addition analysis, as expected. This demonstrates that future analysis should use standard addition to minimize matrix effects and increase confidence of analysis with little additional labor. All extraction and calibration protocols reported 1,3-DMAA and 1,4-DMAA concentrations in geranium plants from the Changzhou region

of China above the reported MDLs. The reported concentrations of 1,3-DMAA ranged from 68 to 496 ng/g and 1,4-DMAA ranged from 13 to 162 ng/g. Similarly, 1,3-DMAA and 1,4-DMAA were not detected above the MDL in samples from Guiyang and Kunming regions. To the best of the authors' knowledge, this is the first reported inter-laboratory analysis confirming the presence of 1,3-DMAA in a geranium plant (specifically Changzhou S11 sample). Finally, the diastereomer ratios of the 1,3-DMAA in geranium plants from Changzhou are similar to those of the synthetic standards. This indicates that 1,3-DMAA could be a natural product extract, fulfilling a requirement of the Dietary Supplement Health and Education Act.[20]

The results reported here provide evidence that 1,3-DMAA naturally occurs in geranium plants in agreement with Li et al,[6] but clearly in disagreement with other previously reported articles by well-respected chemists and organizations.[4,5] However, this may not be a case of right or wrong. In analytical chemistry, the critical review of data is important for explaining differences in reported results. These differences can also provide insight into why analysis of seemingly identical plant species can result in very different outcomes. Khan has published an extensive review showing that it is not uncommon for plants of different locations to exhibit variations in their chemical compositions.[18] For example, studies show that fluctuating geographical dynamics such as water stress and nutrient availability in the soil are associated with the variations in cyanide concentration in the cassava plant.[19]

The published research to date includes a substantial amount of geranium plant and oil analysis.[1–6] However, until now, none of the samples analyzed have been identical or reported as from the same region. Thus, regional environmental variations[18,19] could explain the presence of 1,3-DMAA in the Changzhou S11, Changzhou March 2012, and Changzhou May 2012 samples and the absence of 1,3-DMAA concentrations in Kunming and Guiyang geranium samples reported here; the Indian and Mississippi samples reported by ElSohly et al,[4] the France, Egypt, and New Zealand samples reported by Lisi et al,[3] and the China and Egypt samples reported by Zhang et al.[5] A possible solution to this discrepancy would be a multiple laboratory and blind analysis of identical samples expected to have 1,3-DMAA (such as Changzhou region samples) as well as samples that are not expected to contain 1,3-DMAA. Using this approach, a satisfactory answer for the national regulatory agencies as well as the commercial interests could be provided.

Acknowledgements
The authors would like to acknowledge and thank Dr. Yi Jin of Yunnan University for overseeing the geranium sample collection and shipment to The University of Memphis.

Author Contributions
Conceived and designed the experiments: PSS, HLF, PLR. Analysed the data: HLF, PLR, PSS. Wrote the first draft of the manuscript: HLF, PLR, PSS. Contributed to the writing of the manuscript: HLF, PLR, PSS. Agree with manuscript results and conclusions: HLF, PLR, PSS. Jointly developed the structure and arguments for the paper: HLF, PLR, PSS. Made critical revisions and approved final version: HLF, PLR, PSS. All authors reviewed and approved of the final manuscript.

Funding
The authors would like to acknowledge and thank USP Labs, LLC for funding portions of this work. Authors confirm that USP Labs, LLC had no influence over the content of this paper and contributing research.

Competing Interests
PSS has received consulting fees from CirQuest Labs for consulting work on analysis for pharmaceutical and implantable devices. All other authors disclose no competing interests.

Disclosures and Ethics
As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship

and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

References

1. Perrenoud L, Saugy M, Saudan C. Detection in urine of 4-methyl-2-hexaneamine, a doping agent. *J Chromatogr B*. 2009;877(1):3767–70.
2. Vorce SP, Holler JM, Cawrse BM, Magluilo J. Dimethylamylamine: A drug causing positive immunoassay results for amphetamines. *J Anal Toxicol*. 2011;35(3):183–7.
3. Lisi A, Hasick N, Kazlauskas R, Goebel C. Studies of methylhexaneamine in supplements and geranium oil. *Drug Test Anal*. 2011;3(11–2):873–6.
4. ElSohly MA, Gul WG, ElSohly KM, Murphy TP, et al. Pelargonium oil and methyl hexaneamine (MHA): Analytical approaches supporting the absence of MHA in authenticated *pelargonium graveolens* plant material and oil. *J Anal Toxicol*. 2012;36(7):457–71.
5. Zhang Y, Woods RM, Breitbach ZS, Armstrong DW. 1,3-Dimethylamylamine (DMAA) in supplements and geranium products: natural or synthetic? *Drug Test Anal*. Jul 12, 2012 [Epub ahead of print.]
6. Li JS, Chen M, Li ZC. Indentification and quantification of dimethylamylamine in geranium by liquid chromatography tandem mass spectrometry. *Anal Chem Insights*. 2012;7:47–58.
7. United States Food and Drug Administration. FDA challenges marketing of DMAA products for lack of safety evidence. *FDA News Release*. Apr 27, 2012. http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm302133.htm. Accessed Aug 10, 2012.
8. Skoog DA, Holler FJ, Crouch SR. *Principles of Instrumental Analysis*. 6th ed. Belmont, CA: Thomson Higher Education; 2007.
9. Li J. *Standard Analytical Method AACL-SAM 11044: 1,3- and 1,4-Dimethylpentylamines Geranium Plant by LC/MS/MS*. Champaign, IL: Intertek-AAC Labs; 2011.
10. Savitzky M, Golay JE. Smoothing and differentiation of data by simplified least squares procedures. *Anal Chem*. 1964;36(8):1627–39.
11. Glaser JA, Foerst DL, McKee GD, Quave SA, Budde WL. Trace Analysis for Wastewaters. *Environ Sci Tech*. 1981;15(12):1426–35.
12. United States Environmental Protection Agency. Code of Federal Regulations. Title 40—Protection of Environment. 40 CFR Appendix B to Part 136-Definition and Procedure for the Determination of the Method Detection Limit-Revision 1.11. Cincinnati, OH: US Environmental Protection Agency; 1996:303–6.
13. Harris DC. *Quantitative Chemical Analysis*. 8th ed. New York, NY: WH Freeman and Company; 2010.
14. United States Environmental Protection Agency. *DBP/ICR Analytical Methods Manual*. Cincinnati, OH: US Environmental Protection Agency, Office of Water; 1996. EPA 814-B-96-002.
15. Ranaivo PL, Henson CM, Simone PS, Emmert GL. Analysis of haloacetic acids in drinking water using post-column reaction-ion chromatography with on-line internal standardization. *Anal Methods*. 2011;3:2873–80.
16. Brown MA, Emmert GL. On-line purge and trap as chromatography for monitoring of THMs in drinking water distribution systems. *Anal Chim Acta*. 2007;592(2):154–61.
17. Simone PS Jr, Ranaivo PL, Geme G, Brown MA, Emmert GL. On-line monitoring of nine haloacetic acid species at the μg L^{-1} level using post-column reaction-ion chromatography with nicotinamide fluorescence. *Anal Chim Acta*. 2009;654(2):133–40.
18. Khan IA. Issues related to botanicals. *Life Sci*. 2006;78(18):2033–8.
19. Burns AE, Gleadow RM, Zacarias AM, Cuambe CE, Miller RE, Cavagnaro TR. Variations in the chemical compositin of cassava (*Manihot esculenta* Crantz) leaves and roots as affected by genotypic and environmental variation. *J Agric Food Chem*. 2012;60(19):4946–56.
20. National Institutes of Health. Dietary Supplement Health and Education Act of 1994. Bethesda, MD: National Institutes of Health, Office of Dietary Supplements; 1994.

Supplemental Materials

Chromatograms of typical blanks, standards, and sample spikes

Figure S1 is an example of a typical, original (bottom) and smoothed (top) chromatogram for a sample blank. Figures S2 and S3 are examples of 1,3 and 1,4-DMAA standard chromatograms. Two concentrations are shown: 3 (Fig. S2) and 20 (Fig. S3) µg/L each DMAA. 1,3-DMAA elutes as the first two peaks, followed by 1,4-DMAA as the third peak. Figures S4 to S20 are chromatograms for each geranium herb sample. Unspiked and spiked chromatograms are shown for each sample where possible. Each chromatogram is labeled by the corresponding table number found in the paper. As with the standards, 1,3-DMAA elutes as the first two peaks followed by 1,4-DMAA as the third peak. Figures S4 to S8 are typical chromatograms from Analysis set 1 and were used to determine the concentration of Guiyang 1, Kunming 1, and Changzhou S11-2 in Table 3. Figures S9 toS14 are typical chromatograms from Analysis set 2 and were used to determine the concentration of Guiyang 2, Kunming 2, and Changzhou S11-2 in Table 4. Figures S15 to S20 are typical chromatograms from Analysis set 3 and were used to determine the concentration of Guiyang 3, Kunming 3, and Changzhou 3 in Table 5.

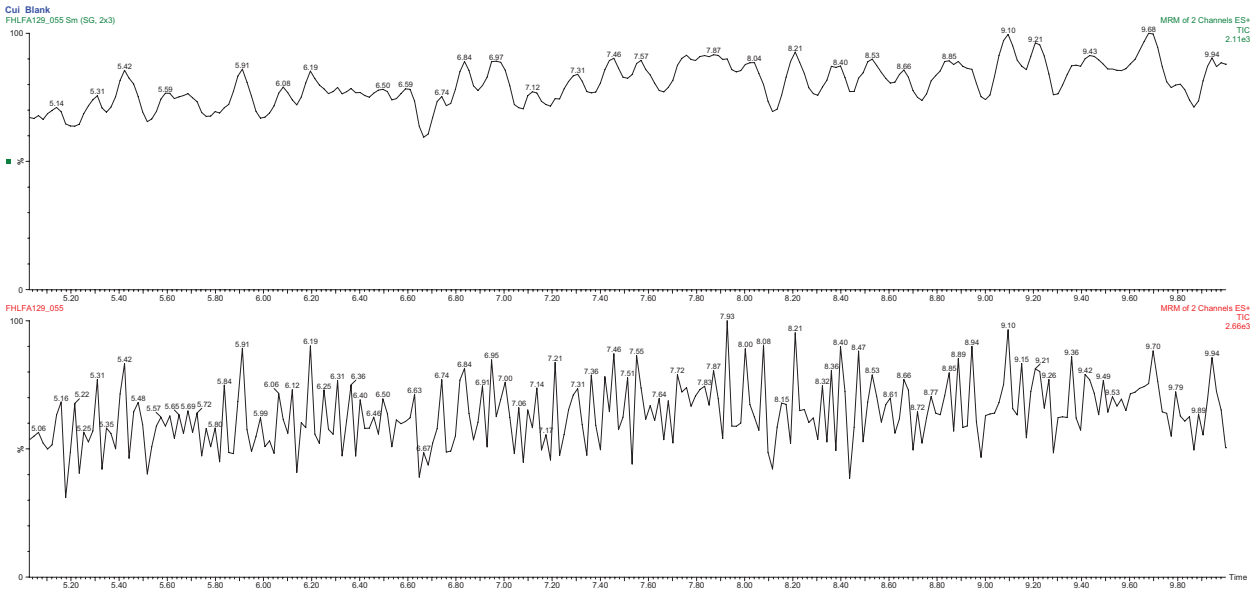


Figure S1. An example of a blank chromatogram.
Notes: The blank sample is prepared in the same way as an unspiked sample but there is no addition of geranium herb to the blender. The original chromatogram is presented on the bottom and the smoothed chromatogram is presented on the top.

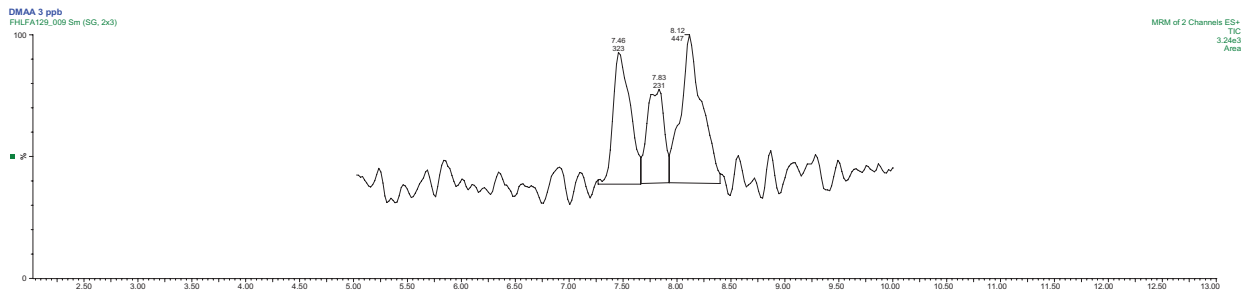


Figure S2. Typical MRM chromatogram of 3 µg/L 1,3-DMAA and 1,4-DMAA.

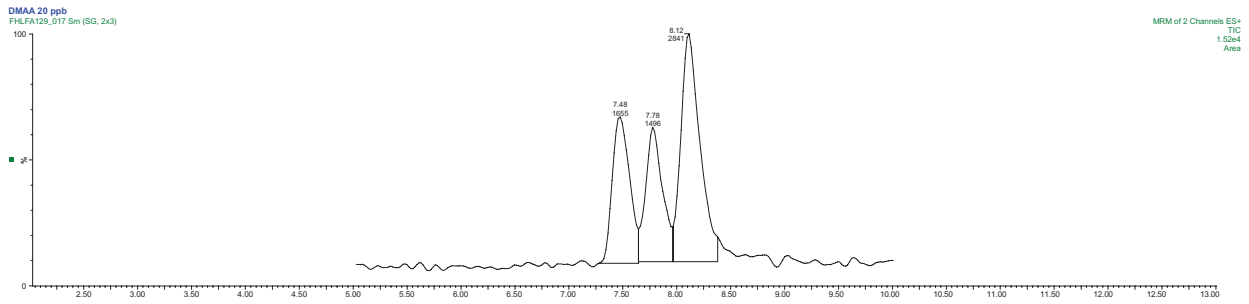


Figure S3. Typical MRM chromatogram of 20 μg/L 1,3-DMAA and 1,4-DMAA.

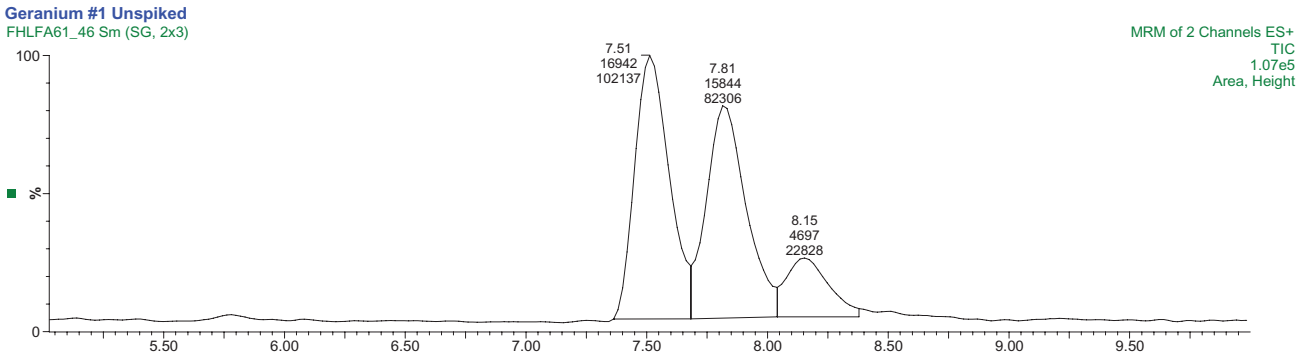


Figure S4. Analysis set 1—Changzhou S11-1, unspiked.

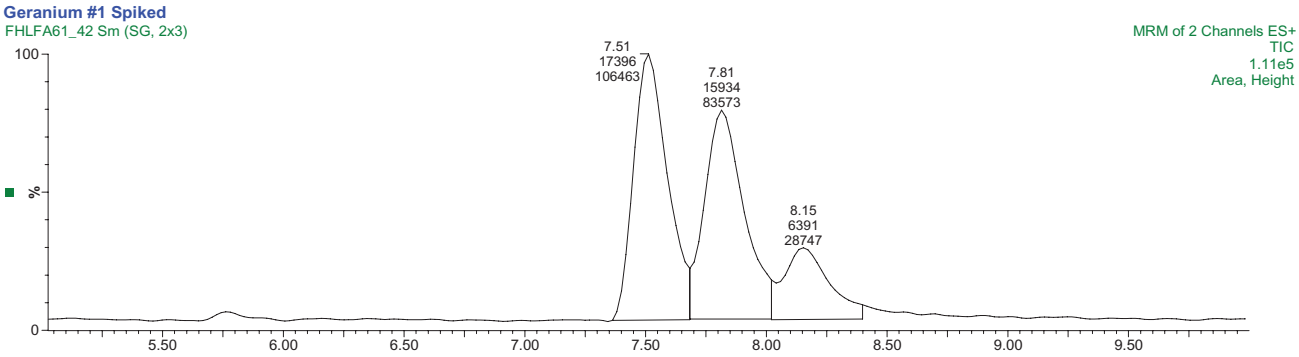


Figure S5. Analysis set 1—Changzhou S11-1, spike 10 μg/L.

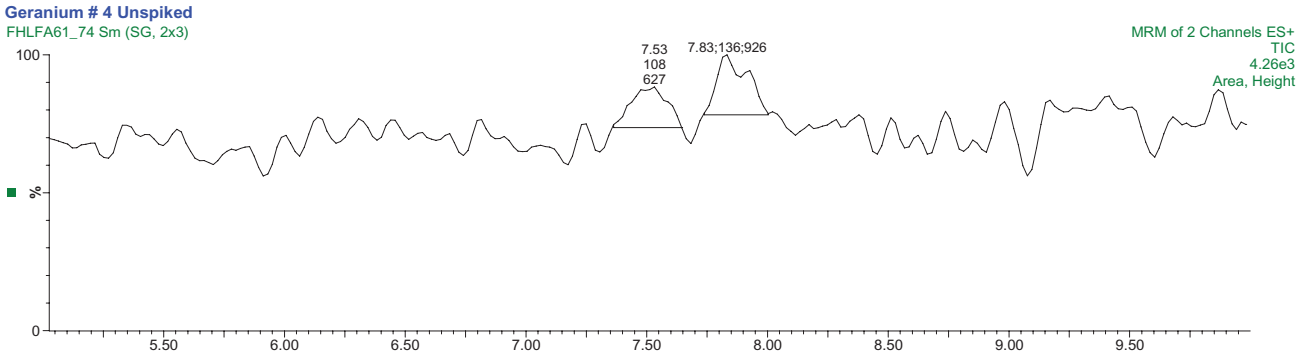


Figure S6. Analysis set 1—Guiyang 1, unspiked.

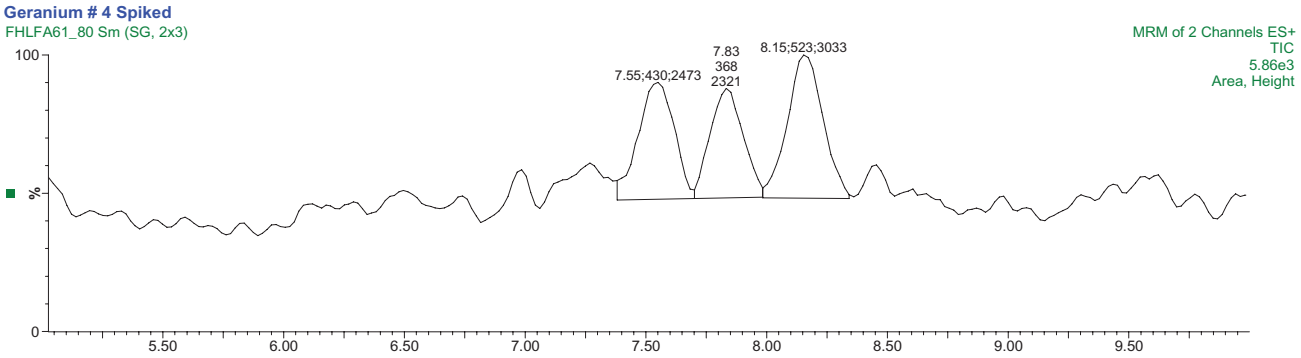


Figure S7. Analysis set 1—Guiyang 1, spike 10 μg/L.

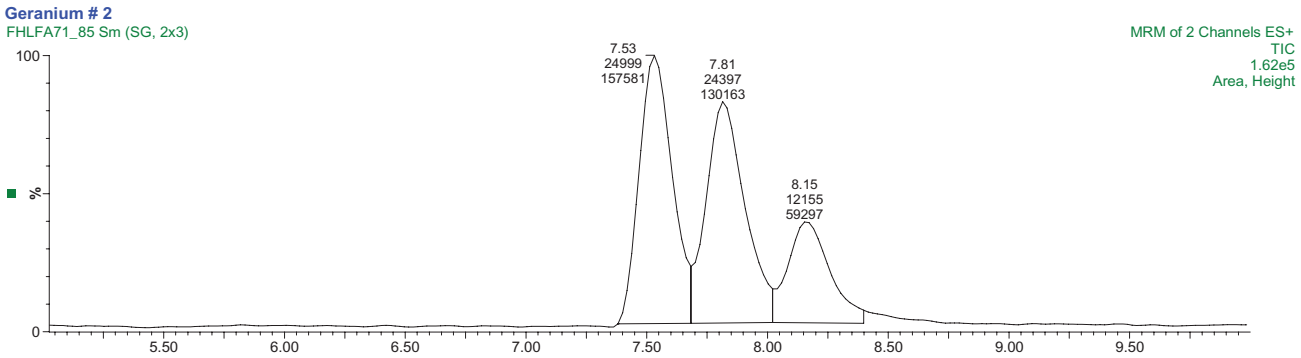


Figure S8. Analysis set 1—Changzhou 1, unspiked.

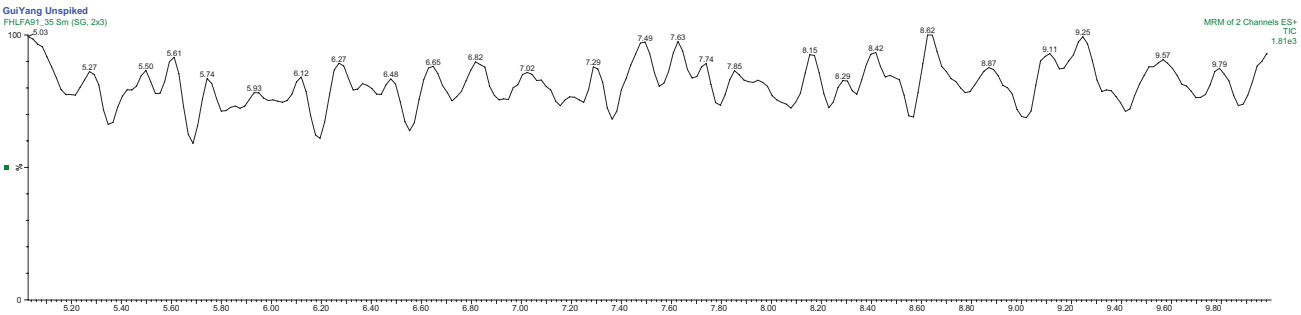


Figure S9. Analysis set 2—Guiyang 2 unspiked.

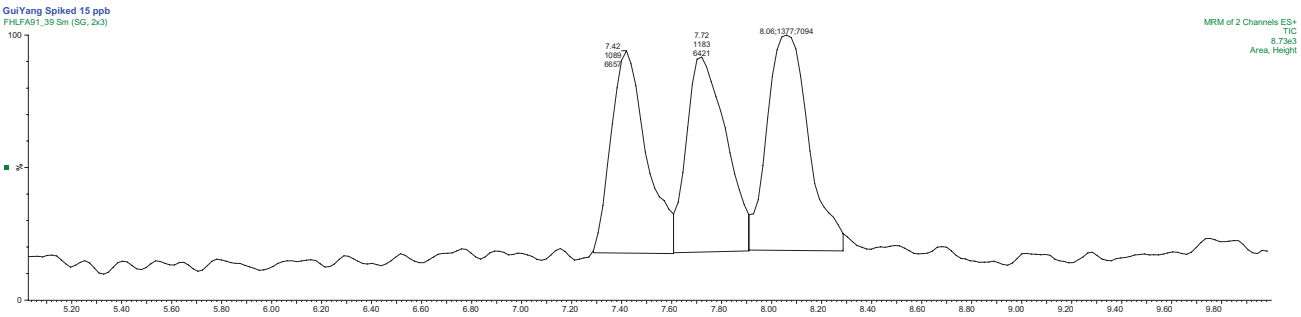


Figure S10. Analysis set 2—Guiyang 2, spike 15 μg/L.

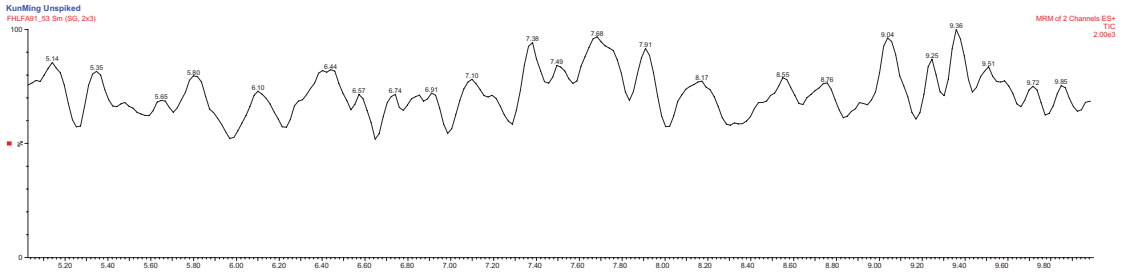


Figure S11. Analysis set 2—Kunming 2, unspiked.

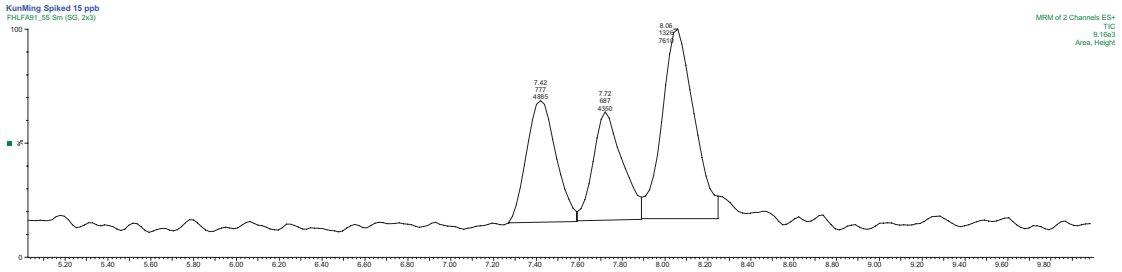


Figure S12. Analysis set 2—Kunming 2, spike 15 μg/L.

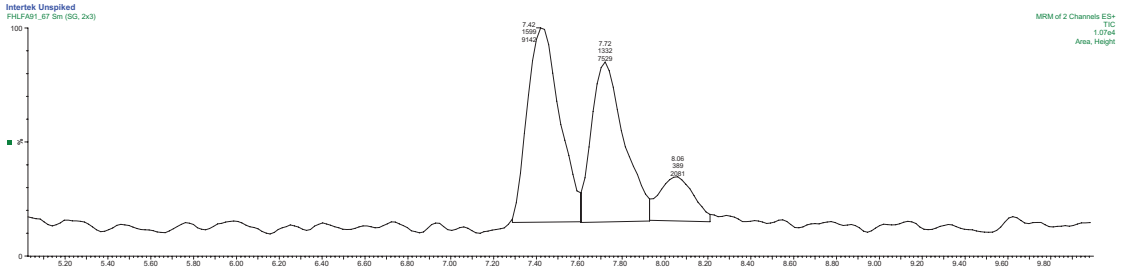


Figure S13. Analysis set 2—Changzhou S11-2 unspiked.

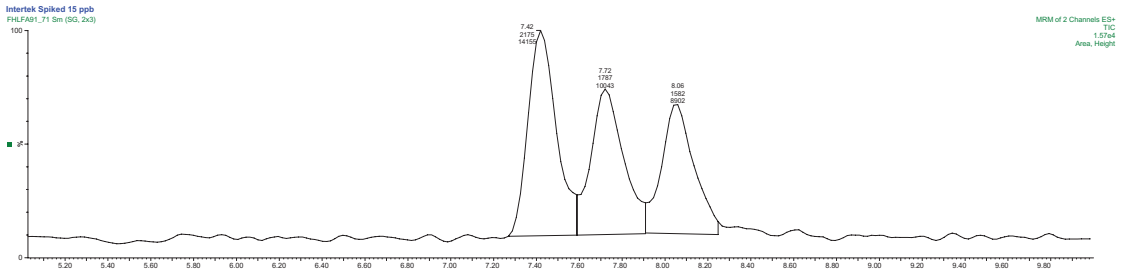


Figure S14. Analysis set 2—Changzhou S11-2, spike 15 μg/L.

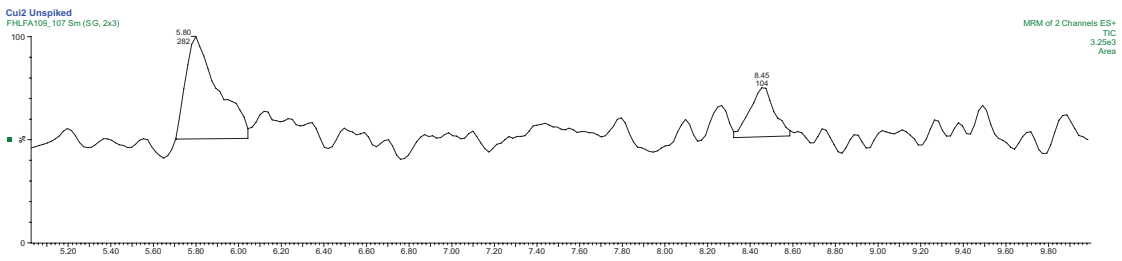


Figure S15. Analysis set 3—Guiyang 3, unspiked.

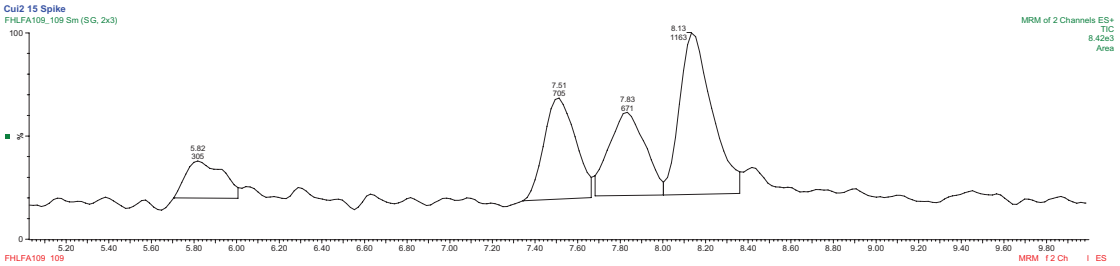


Figure S16. Analysis set 3—Guiyang 3, spiked 15 μg/L.

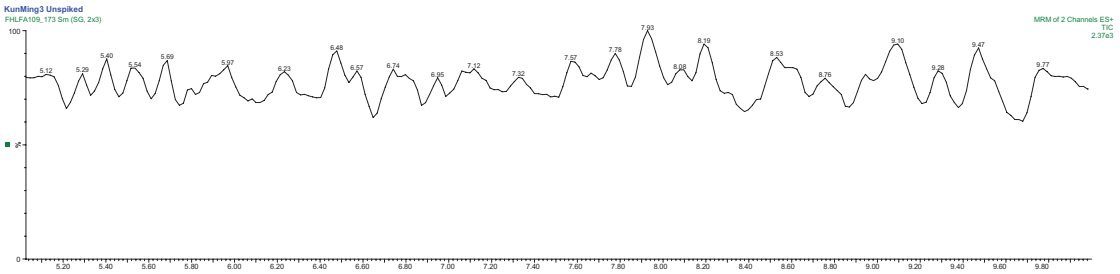


Figure S17. Analysis set 3—Kunming 3, unspiked.

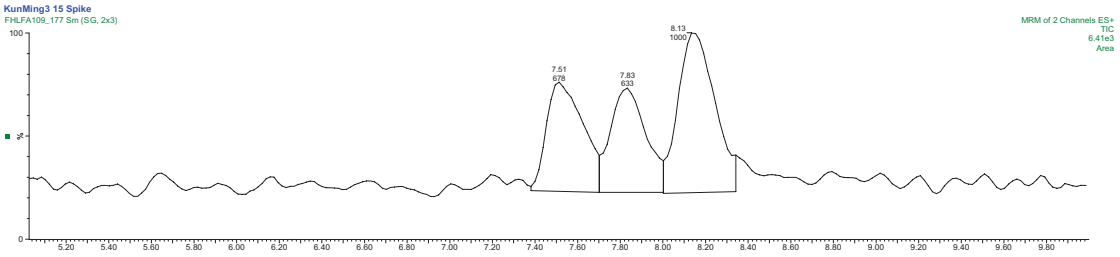


Figure S18. Analysis set 3—Kunming 3, spiked 15 μg/L.

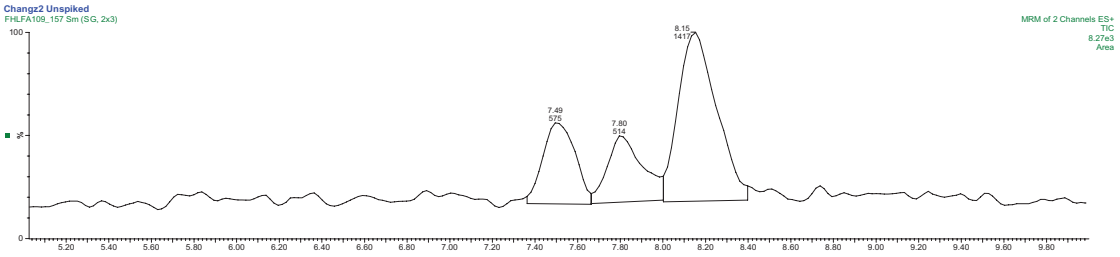


Figure S19. Analysis set 3—Changzhou 3, unspiked.

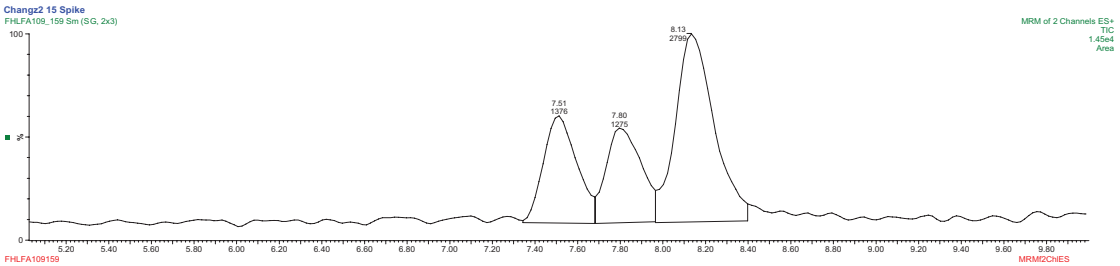


Figure S20. Analysis set 3—Changzhou 3, spiked 15 μg/L.

Supplementary data

A video abstract by the authors of this paper is available. video-abstract10445.mov

Analytical Chemistry Insights

RAPID COMMUNICATION

Identification and Quantification of Dimethylamylamine in Geranium by Liquid Chromatography Tandem Mass Spectrometry

J.S. Li, M. Chen and Z.C. Li

Intertek-AAC Labs, 711 Parkland Court, Champaign, Illinois, 61821.
Corresponding author email: z.charlie.li@intertek.com

Abstract: A sensitive and reliable method of liquid chromatography–electrospray ionization/tandem mass spectrometry (LC-ESI/MS/MS) was developed and validated for determining 1,3-dimethylamylamine (1,3-DMAA) and 1,4-dimethylamylamine (1,4-DMAA) in geranium plants (*Pelargonium graveolens*). The sample was extracted with 0.5 M HCl and purified by liquid-liquid partition with hexane. The parameters for reverse-phase (C18) LC and positive ESI/MS/MS were optimized. The matrix effect, specificity, linearity, precision, accuracy and reproducibility of the method were determined and evaluated. The method was linear over a range of 0.10–10.00 ng/mL examined, with R^2 of 0.99 for both 1,3-DMAA and 1,4-DMAA. The recoveries from spiked concentrations between 5.00–40.00 ng/g were 85.1%–104.9% for 1,3-DMAA, with relative standard deviation (RSD) of 2.9%–11.0%, and 82.9%–101.8% for 1,4-DMAA, with RSD of 3.2%–11.7%. The instrument detection limit was 1–2 pg for both DMAAs. The quantification limit was estimated to be 1–2 ng/g for the plant sample. This method was successfully applied to the quantitative determination of 1,3- and 1,4-DMAA in both geranium plant and geranium oil.

Keywords: 1,3-dimethylamylamine, 1,4-dimethylamylamine, geranium (*Pelargonium graveolens*), liquid chromatography-tandem mass spectrometry (LC/MS/MS)

Analytical Chemistry Insights 2012:7 47–58

doi: 10.4137/ACI.S9969

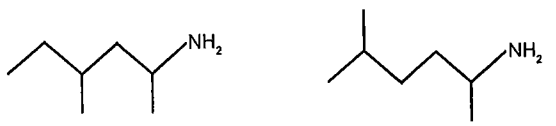
This article is available from http://www.la-press.com.

Introduction

Dimethylamylamine (DMAA), also known as methylhexaneamine, or 1,3-dimethylpentylamine, is a simple aliphatic amine (Fig. 1). In recent years, it has appeared as an ingredient in various dietary supplements and sports nutrition products. This use of the compound has given rise to inquiries as to whether DMAA is a naturally occurring constituent of the geranium (*Pelargonium graveolens*) plant and its oil. Consequently, an investigation was undertaken to make an identification and determination of DMAA in the plant and its oil, if present.

The result of a GC/MS analysis of geranium oil, which indicated the presence of 1,3- DMAA (1) and 1,4-DMAA (2) was published in a journal that has not been widely circulated internationally.[1] Aside from that publication, geranium essential oil has been the subject of numerous other investigations seeking to identify and quantify all of the compounds present, generally employing the use of GC/FID and GC/MS; these other investigations have failed to identify 1,3-DMAA (1) or 1,4-DMAA (2).[2–12] However, because geranium oil presents such a complex sample matrix and contains many volatile compounds, a possible shortcoming of the GC/FID method is interference by the sample matrix.[6] Although the use of GC/MS is an improvement, the complex nature of the sample matrix still presents issues when seeking to identify all components of the oil;[6] in fact, we are not aware of any published data demonstrating an identification of components comprising 100% of the oil. These factors, along with differences in sample origin, processing and composition, may explain why these other investigations failed to identify DMAA as a component of geranium oil. Regarding 1,3-DMAA (1), it has been noted that although it is amenable to GC/MS analysis,[13] great care must be taken during method development because of DMAA's strong polarity, volatility and low molecular mass, making it a challenge for GC-column retention and separation.

DMAA has been successfully determined in urine using LC/MS/MS.[13,14] In those investigations, 1,3-DMAA (1) was detected as a pair of peaks with identical MS/MS spectra. However, the determination of DMAA in plant and especially in geranium oil has yet to be published. In the present study, we have developed and validated a sensitive and simple LC/MS/MS method to identify 1,3-DMAA (1) and 1,4-DMAA (2) and determine their concentrations as naturally occurring compounds in geranium plants.



1,3-dimethylamylamine (1) 1,4-dimethylamylamine (2)

Figure 1. Structures of 1,3-Dimethylamylamine (1) and 1,4-Dimethylamylamine (2).

Experimental

Chemicals and reagents

1,3-Dimethylamylamine (1) (CAS No. 105-41-9, >99%) and 1,4-dimethylamylamine (2) (CAS No. 28292-43-5, >99%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Formic acid (88%), methanol (HPLC grade), hexane (pesticide residue grade) and hydrochloric acid (37% HCl) were all obtained from Fisher Scientific (Waltham, MA, USA). All other chemicals are analytical grades. Water (18.2 MΩ-cm) was prepared with a Barnstead NanoPure Diamond System (Lake Balboa, CA, USA).

Apparatus and instruments

Agilent 1100 HPLC system with quaternary pump (Santa Clara, CA, USA) was coupled to a Micromass Quattro Ultima mass spectrometer with electrospray ionization (ESI) source (Manchester, England). Masslynx (version 4.1) were used to control the system of LC-triple quadrupole mass spectrometer and for data acquisition and processing. The analytical column is a Phenomenex Kinetex C18 column (4.6 × 150 mm, 2.6 μm) (Torrance, CA, USA). High Speed Grinder DFY-200 was from Gaoyi In. (Wenzhou, China). Commercial Blender 200G was from Waring Co. (Torrington, CT, USA).

Geranium plants and geranium oils

The geranium plants were procured by and obtained from Dr. Yi Jin of Yunnan University (Kunming, Yunnan Province, China) and were authenticated by Professor Xu Youkai of the Xishuangbanna Tropical Botanical Garden-Chinese Academy of Sciences (Mengla, Yunnan Province, China). The plant samples were collected from different areas of China

(*see* Table 5), shipped to this lab fresh and stored at −20 °C immediately upon receipt in the lab. All geranium oil samples were obtained from Jiangxi Ji'an Hengcheng Flavor Oil Factory (Ji'an, Jiangxi Province, China). The geranium oil was stored at room temperature.

Standard preparation

The stock solutions of 1,3-DMAA (1) and 1,4-DMAA (2) were prepared separately in methanol at a concentration of 1.00 mg/mL and 1.068 mg/mL, respectively. Working solutions composed of the two DMAAs were prepared by serial dilution of the stock solution with 0.5 M HCl to obtain a set of standard concentrations of 0.10–10.00 ng/mL of 1,3- DMAA (1), and 0.11–10.68 ng/mL of 1,4-DMAA (2). All working standard solutions were stored at 4 °C and used within one week after preparation, although no significant degradation was observed in one month of storage.

Sample preparation and extraction

Geranium plant (wet leaves and stems) was thawed at room temperature and cut into pieces at about 1–2 cm and mixed well. After about five hundred grams of the plant was ground into fine pieces with a high speed grinder, 10 g of sample was weighed into a stainless steel blender cup. To this cup, 80 mL of 0.5 M HCl was added and mixed. The mixture was homogenized at high speed for 2 min. The homogenate was then transferred into a 100-mL volumetric flask. The blade and cup were washed with additional 15 mL of 0.5 M HCl. The solution was collected into the flask and extracted by sonication at 50 °C for 1 hour. After being cooled to room temperature, the volume was adjusted to the mark with 0.5 M HCl. This solution was centrifuged at 4000 × g for 10 min, and the supernatant was further purified as below.

For geranium oil, 1 mL of sample was mixed with 1 mL of hexane in a 10-mL glass tube with screw cap. Five mL of 0.5 M HCl was added and shaken with a vortex shaker for 5 min at high speed. The aqueous layer (lower) was diluted with 0.5 M HCl as necessary, filtered with a 0.45-μm nylon filter and applied to LC/MS/MS without further purification.

Purification

Four mL of supernatant and 2 mL of hexane were added to a 10-mL glass tube with screw cap. The mixture was shaken by a vortex shaker for 30 sec. The mixture was centrifuged at 2000 × g for 5 min. The aqueous layer was diluted as necessary and filtered for LC/MS/MS analysis.

LC/MS/MS conditions

The mobile phase of HPLC is composed of Water:acetonitrile (85:15) containing 0.1% formic acid. Flow rate was 0.5 mL/min; column temperature was 35 °C; the flow was diverted 0.2 mL/min to MS. Injection volume was 50 μL.

The mass spectrometer was operated in positive ESI and multiple reaction monitoring (MRM) mode. Nitrogen was used as the nebulizer, heater, and cone gas. Argon was used as the collision induced dissociation (CID) gas. The precursor-to-product ion transitions were monitored at m/z 116 [M + H] → 57 (quantification) and m/z 116→99 (qualification) for both 1,3-DMAA (1) and 1,4-DMAA (2). ESI parameters were optimized for maximizing the generation and stability of the precursor and fragment ions by infusion as follows: Capillary 2.5 kV, Cone 20V, Source temperature 120 °C, Desolvation temperature 360 °C, Cone gas 120 L/hour, Desolvation gas 850 L/hour, CID 11 eV, collision cell pressure 2 × e-3 mbar.

Method validation

The analytical method was validated according to guidelines for United States Pharmacopeia (USP). The parameters validated include linearity, specificity, limit of detection, limit of quantification, accuracy, precision and reproducibility.

Linearity

To evaluate the linearity, calibration curves of 1,3-DMAA (1) and 1,4-DMAA (2) were established using concentrations in the range from 0.1 to 10 ng/mL. The responses of each compound against its respective concentration were plotted. Linear regression analysis was performed to obtain calibration equation and correlation coefficients (R^2).

Specificity

Two ion transitions coupled with a high resolution column were used to enhance the method selectivity. Specificity was assessed by comparing the chromatograms of blanks (glassware and reagent blanks), standard, spiked samples and their peak purity (peak

shape and relative intensity of transitions). The peak was identified by retention time and relative intensity of transitions against the reference standard.

Matrix effects

The matrix effects (ion suppression or enhancement) were evaluated by comparing peak area of the standard, sample extract and the extract directly spiked at corresponding concentrations of DMAAs, which were set at the medium spiked concentration (20 ng/g).

Accuracy

Accuracy of the method was determined by assaying spiked samples of geranium plants at four different levels: about 5, 10, 20 and 40 ng/g for both 1,3-DMAA (1) and 1,4-DMAA (2) (ie, adding 50, 100, 200 or 400 ng DMAA in solution to about 10 g of sample). Each concentration level had three replicates. All samples were extracted, purified and determined as described above. For the blank, 10 mL of water instead of sample was included and carried through the same procedures of sample preparation.

Precision and reproducibility

Precision was performed by assaying a geranium sample in six subsamples. The concentration of each subsample, and average and RSD of the analyses were calculated to access the precision of the method.

Reproducibility of this method was evaluated by a second chemist beside the primary chemist in this laboratory. The geranium sample was assayed in six subsamples. The concentrations, average and RSD of the six analyses were compared with the results obtained by the primary chemist to assess the reproducibility of the method by a different chemist.

Data analysis

The concentrations of 1,3-DMAA (1) and 1,4-DMAA (2) in the sample preparations were obtained from their corresponding standard curves. The mean, standard deviation (SD) and relative standard deviation (RSD) of spike recoveries were calculated for assessing the accuracy of the method. Mean and RSD of repeated analyses of the geranium samples were used for precision evaluation. The mean concentration of DMAAs from the precision experiments was used as the original value to calculate recoveries of DMAAs. The recoveries from spiked samples were calculated by the following formula:

$$\text{Recovery, \%} = 100\ (Fc - Bc)/Sc$$

where, Fc is the concentration (ng/g) found in the spiked sample; Bc is the original value of the sample (ng/g) prior to spiking; Sc (ng/g) is the concentration spiked to the sample.

Results and Discussion

Analytical conditions

Because DMAA is a simple aliphatic amine with no chromatic group, the HPLC-UV method is not suitable for its detection unless the compound is derivatized with a chromophore prior to analysis. Therefore, LC/MS/MS was selected for the current method. Two solvent systems, methanol-water and acetonitrile-water were tested for optimization on the C18 column and tuning of ESI/MS. Both solvent systems produce similar ESI (+) signals. Further investigations showed that formic acid (0.1%) in either mobile phase enhances the signal significantly, with little effect on retention time and shape of the peaks. As a result, acetonitrile:water (15:85) containing 0.1% formic acid was selected and used routinely as mobile phase in the current experiment. Under these conditions, 1,3-DMAA (1) and 1,4- DMAA (2) were well separated with good peak shape (tailing factor = 1.1–1.3) and retention (K′ = 2.5–2.8) (Fig. 2).

When a standard solution of 1,3-DMAA (1) was analyzed under the current conditions, double peaks with similar intensity were observed. Similar results were observed by Vorce et al[13] and Perenoud et al.[14] Both peaks show identical CID mass spectra (data not shown), suggesting that these peaks are stereo isomers (Fig. 2A). In contrast, 1,4-DMAA (2) shows only a single peak (Fig. 2B). 1,3-DMAA (1) has two chiral centers (carbon-1 and carbon-3) in its structure, and thus it theoretically has four stereo isomers. The double peaks observed are likely formed by its diastereomers, (1*S*,3*S*)/(1*R*,3*R*) and (1*S*,3*R*)/(1*R*,3*S*). These diastereomers have different molecular shapes or stereo-hindrance effects when they interact with the stationary phase. For example, they may be resolved on a reverse-phase column.[15] In contrast, 1,4-DMAA (2) has only one chiral center at carbon-1.

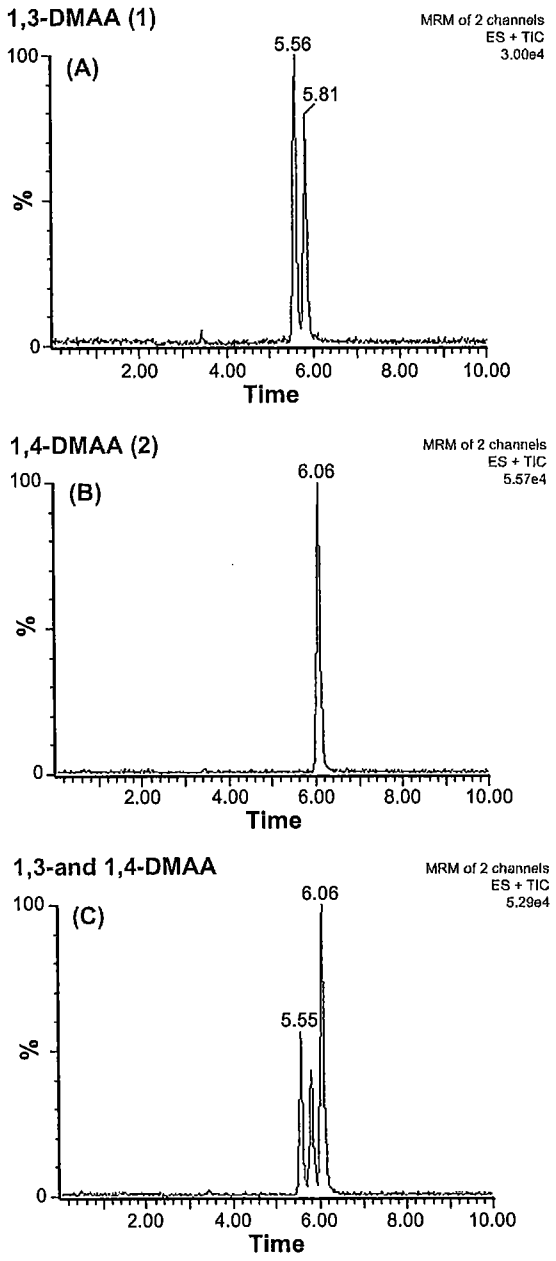


Figure 2. MRM chromatograms of (A) 2 ng/mL 1,3-DMAA (1) (the peaks of a pair of diastereomers at 5.5 min and 5.8, respectively); (B) 2.67 ng/mL 1,4-DMAA (2) (6.1 min); (C) the mixture.

Its enantiomers [(1*S*,1*R*) and (1*R*,1*S*)] have the same chemical and physical properties, and therefore show a single peak under the current analytical conditions. Perrenoud et al[14] further confirmed that the double peaks were diastereomers by their identical chemical shifts of ^{13}C NMR and ^{1}H NMR.

Figure 3 shows that 1,3-DMAA (1) and 1,4-DMAA (2) produced similar CID mass spectra for the same precursor ion m/z 116 $[M+H]^+$). Their CID spectra had a strong and stable product ion m/z 57 $[C_4H_9]^+$ that was used for quantification. Other product ions with relatively higher abundance were m/z 43 $[C_3H_7]^+$, m/z 75 and m/z 99 $[M+H-NH3]^+$.

Sample preparation, matrix effect and specificity

DMAA is slightly soluble in water, but soluble in diluted HCl and many polar solvents. Both 0.5 M HCl and methanol were examined for their extraction of the geranium samples. It was found that both solvents had similar extraction efficiency. However, methanol extracts much more of the matrix components from the geranium plant than 0.5 N HCl. The final extract prepared using methanol was found to interfere with LC/MS analysis (data not shown). In contrast, the extract prepared using 0.5 N HCl carries less matrix components, especially fat-soluble compounds, which was later found to be important for the accuracy of determination. Therefore, the

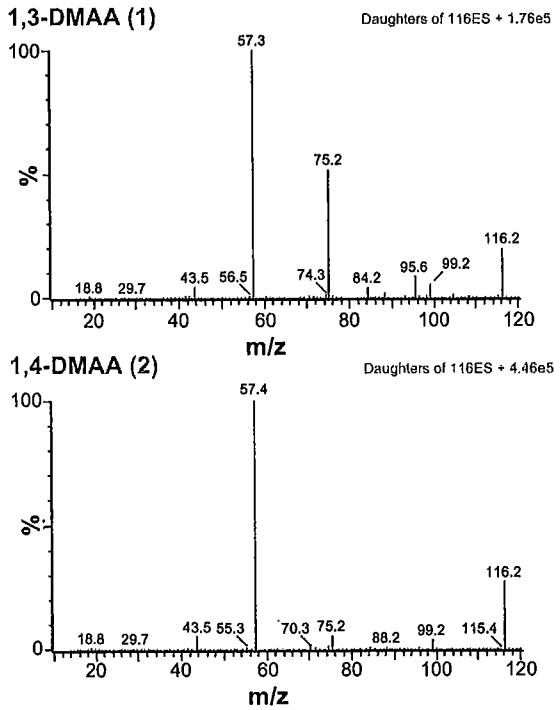


Figure 3. The CID spectra (11 eV) of 1,3-DMAA (1) (Top); 1,4-DMAA (2) (Bottom).

diluted HCl solution was used for routine sample extraction. Furthermore, sonication for 60 minutes of the geranium sample was found to be necessary for reproducible results.

The recovery of DMAAs in initial experiments was found to be low (about 60%–70%) when the HCl-extract was directly applied to LC/MS/MS without further purification. To investigate the cause of this low recovery, our focus was on the effect of the sample matrix, since the ESI signal is more susceptible to matrix-induced suppression. Results of the investigation clearly showed that the matrix of the sample had suppressed the signal of DMAAs in the sample extract or spiked sample extract (Fig. 4). For example, after subtracting the background, the ESI signal of 1,3-DMAA (1) in the spiked extract with purification was about 35% greater than that without purification (Fig. 4B and C). These results suggested that ion suppression had occurred in the raw extract. It is also noted that ion-suppression is slightly less at the elution window for 1,4-DMAA (2), which was only 20% lower in the spiked extract without purification.

It has been known that ion suppression occurs in many ESI/MS-based methods for biological samples.[16] Methanol is a strong protic solvent and can solubilize considerable amounts of lipid components, such as fatty acids and phospholipids. Considering the ESI mechanism, these nonvolatile or less volatile compounds were the potential solutes suppressing the ESI signal.[17] The diluted HCl solution used in the current method provides strong solubility for DMAAs but solubilizes considerably less of these fat-soluble components. Furthermore, the HCl-extract is readily purified by partitioning with hexane to remove the lipid-soluble components. This simple purification step was found to be effective in reducing sample matrix and ion suppression, and improved recovery significantly (Fig. 4B and C).

No interfering peaks were observed from glassware/reagent blank samples. The 1,3-DMAA (1) (its diastereomers) and 1,4-DMAA (2) are well-resolved (Fig. 5) in the purified extracts. The DMAAs between the standards and the samples have identical retention time and similar peak relative intensity (See Table 6). These results provide evidence that the method is specific for analysis of DMAAs since

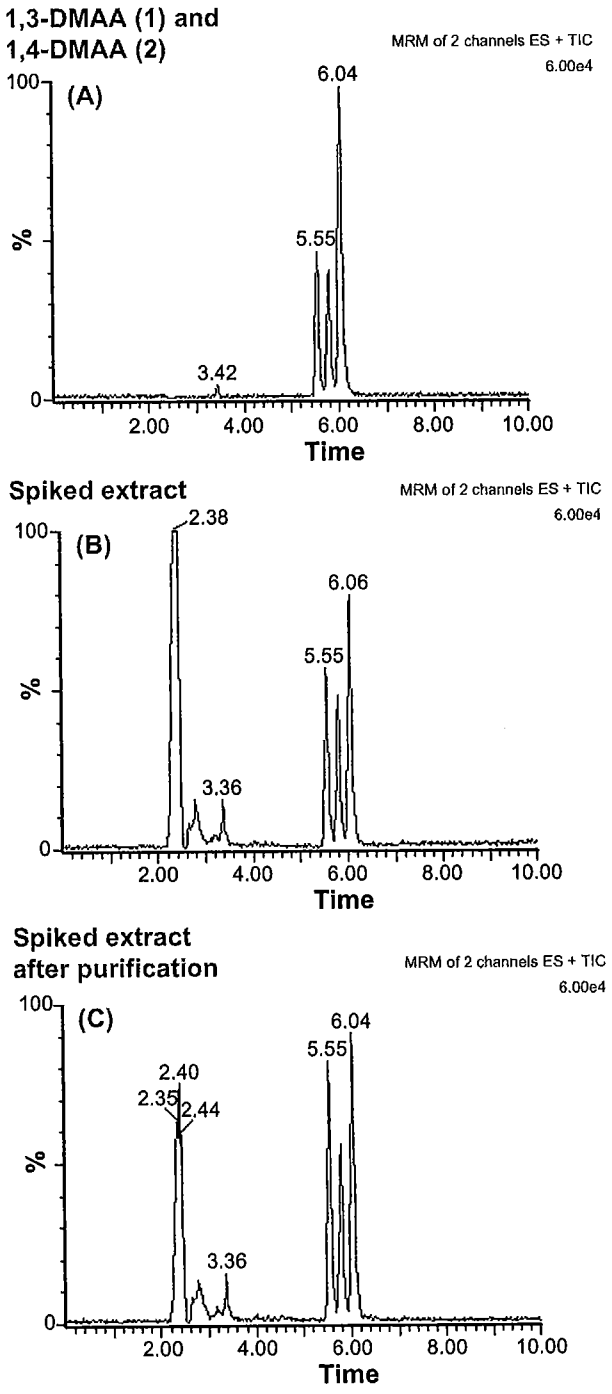


Figure 4. Matrix effect on determination of DMAAs in geranium (*Pelargonium graveolens*) plant: (**A**) 2.50 ng/mL 1,3-DMAAs (1) and 2.67 ng/mL 1,4-DMAA (2) in 0.5 HCl; (**B**) 2.5 ng/mL 1,3-DMAA (1) and 2.67 ng/mL 1,4-DMAA (2) -spiked extract before purification; (**C**) the spiked extract after purification.
Note: The ESI signals in hexane-purified extracts are significantly higher than that of extracts without purification.

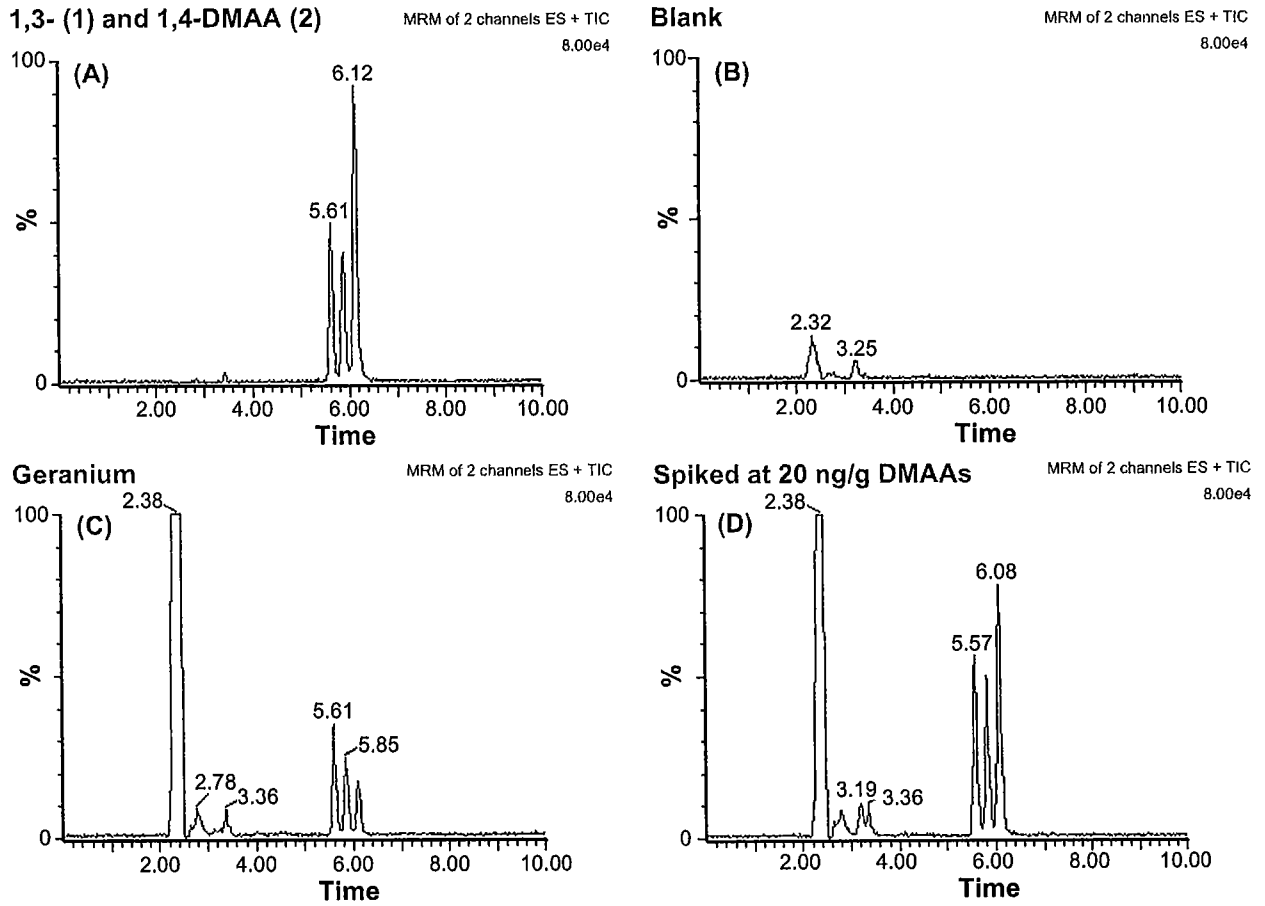


Figure 5. MRM chromatogram of (**A**) 2.50 ng/mL 1,3-DMAA (1) and 2.67 ng/mL 1,4-DMAA (2), (**B**) blank, (**C**) geranium (*Pelargonium graveolens*) sample and (**D**) 20.00 ng/g 1,3-DMAA (1) and 21.26 ng/g-spiked sample.

the matrix interference in the purified extract was not observed.

Linearity

Standard solutions of 1,3-DMAA (1) and 1,4-DMAA (2) were analyzed with concentration range at about 0.1 to 10 ng/mL. The two isomer peaks of 1,3-DMAA (1) was summed prior to regression analysis. The results were linear with R^2 of 0.998 for 1,3-DMAA (1) (Y = 2537X + 497.6, Y-peak area, X-ng/mL), and 0.999 for 1,4-DMAA (2) DMAA (Y = 2705X + 311.3).

Precision

The geranium sample showing the presence of DMAAs in initial investigation was selected for precision analysis. All the test procedures were carried out according to the method described. The results are shown in Table 1. The average amounts of 1,3-DMAA (1) and 1,4-DMAA (2) in the sample were 13.6 and 3.56 ng/g, respectively, in this geranium sample on a wet weight basis. The RSD of 1,3-DMAA (1) and 1,4-DMAA (2) are 8.3% and 5.4%, respectively. A typical MRM chromatogram (scan) of the sample is shown in Figure 5C.

Accuracy

The sample used in the precision experiment was spiked with known amounts of 1,3-DMAA (1) and 1,4-DMAA (2) for evaluating the accuracy of the method. The amounts of 1,3-DMAA (1) and 1,4-DMAA (2) (Tables 2 and 3) were used as original

Table 1. Precision for determination of 1,3-DMAA (1) and 1,4-DMAA (2) in geranium (*Pelargonium graveolens*).*

Sample no.	Weight (g)	Volume (mL)	1,3-DMAA		1,4-DMAA	
			Read (ng/mL)	Found (ng/g)	Read (ng/mL)	Found (ng/g)
1	10.59	100	1.51	14.24	0.40	3.76
2	10.66	100	1.52	14.26	0.37	3.47
3	10.88	100	1.59	14.63	0.41	3.77
4	10.62	100	1.31	12.33	0.35	3.31
5	10.11	100	1.22	12.03	0.37	3.63
6	10.34	100	1.48	14.28	0.35	3.40
Mean (ng/g)				13.63		3.56
S.D.				1.13		0.19
RSD (%)				8.3		5.4

Note: *Sample ID# 070611-0164.

Table 2. Accuracy for determination of 1,3-DMAA (1) in geranium (*Pelargonium graveolens*).

Spiked (ng)	Weight (g)	Spiked (ng/g)	Volume (mL)	Read (ng/mL)	Found (ng/g)	Recovery (%)*	Mean ± S.D.	RSD (%)
400	11.65	34.33	100	5.71	48.99	103.0	100.8 ± 2.90	2.88
	10.41	38.42	100	5.32	51.10	97.5		
	10.68	37.45	100	5.53	51.79	101.9		
200	10.11	19.78	100	3.07	30.40	84.8	86.5 ± 1.78	2.05
	10.61	18.85	100	3.18	29.94	86.5		
	10.01	19.98	100	3.13	31.28	88.3		
100	11.83	8.45	100	2.61	22.09	100.1	104.9 ± 7.52	7.16
	10.83	9.23	100	2.61	24.12	113.6		
	11.26	8.88	100	2.55	22.61	101.1		
50	10.02	4.99	100	1.77	17.71	81.8	85.1 ± 9.38	11.02
	11.05	4.52	100	1.98	17.96	95.7		
	10.06	4.97	100	1.76	17.50	77.9		

Note: *Recovery (%) = (Found-13.63)/Spiked × 100 (13.63 ng/g is the background value, See Table 1).

Table 3. Accuracy for determination of 1,4-DMAA (2) in geranium (*Pelargonium graveolens*).

Spiked (ng)	Weight (g)	Spiked (ng/g)	Volume (mL)	Read (ng/mL)	Found (ng/g)	Recovery (%)*	Mean ± S.D.	RSD (%)
427	11.65	36.67	100	5.02	43.06	107.7	101.8 ± 5.16	5.07
	10.41	41.04	100	4.62	44.42	99.6		
	10.68	40.00	100	4.57	42.82	98.1		
214	10.11	21.13	100	2.18	20.71	81.2	82.9 ± 2.66	3.21
	10.61	20.13	100	2.09	19.96	81.5		
	10.01	21.34	100	2.12	21.90	85.9		
107	11.83	9.03	100	1.42	12.04	93.9	88.6 ± 5.02	5.67
	10.83	9.86	100	1.33	12.24	88.0		
	11.26	9.48	100	1.30	11.52	83.9		
53	10.02	5.33	100	0.92	9.20	105.9	95.8 ± 10.29	10.7
	11.05	4.83	100	0.85	7.68	85.3		
	10.06	5.31	100	0.87	8.67	96.3		

Note: *Recovery (%) = (Found-3.56)/Spiked × 100 (3.56 ng/g is the background value, See Table 1).

values and taken into consideration in calculation of the recoveries (*See* "4.8.6 Data analysis"). The results are shown in Table 2 and Table 3. The average recoveries of each spiking level are 85.1%–104.9% for 1,3-DMAA (1) and 82.9%–101.8% for 1,4-DMAA (2). The RSD are 2.9%–11.0% for 1,3-DMAA and 3.2%–11.7% for 1,4-DMAA. A typical MRM chromatogram (scan) of spiked sample is shown in Figure 5D.

Detection limit and quantification limit

The instrument detection limit was estimated by analyzing a standard at a concentration of 0.2 ng/mL with injection volume of 50 µL. The chromatogram is shown in Figure 6. The detection limit was estimated to be 1–2 pg, based on the signal-to-noise ratio of 3:1.

To evaluate the method quantification limit (MQL), the signal to noise ratio of 5:1 is used for calculation. Taking into consideration of the sample weight of 10 g and the final volume of sample preparation in 100 mL, the MQL of 1,3-DMAA (1) and 1,4-DMAA (2) is estimated to be 1–2 ng/g.

Reproducibility

When the method was performed by a second chemist, similar results were obtained (Table 4). The RSD for 1,3-DMAA (1) and 1,4-DMAA (2) are 2.5% and 4.1%, respectively. These results indicate that the method is reproducible between analysts.

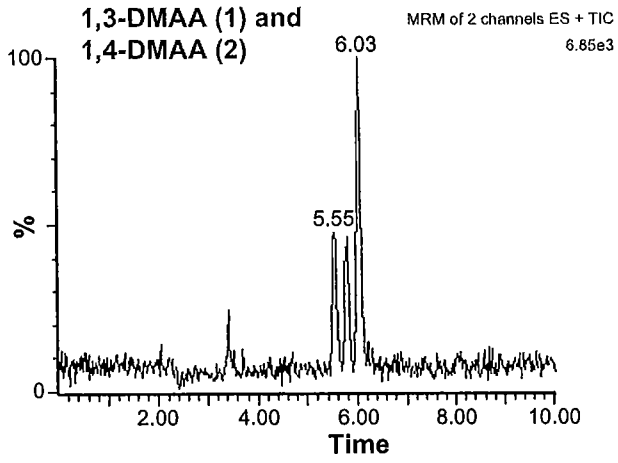


Figure 6. MRM chromatogram for estimating the instrument detection limit: 0.20 ng/mL 1,3-DMAA (1) (5.5 min and 5.8 min) and 0.11 ng/mL 1,4-DMAA (2) (6.0 min).

Table 4. Reproducibility of the procedure for 1,3-DMAA (1) and 1,4-DMAA (2) in geranium (*Pelargonium graveolens*).

Compounds	Sample (g)	Fund (ng/g)	Mean ± SD	RSD (%)
1,3-DMAA	10.51	14.24	14.00 ± 0.35	2.5
	10.80	13.81		
	10.91	13.52		
	10.76	13.84		
	10.81	14.06		
	10.78	14.51		
1,4-DMAA	10.51	4.94	4.74 ± 0.19	4.1
	10.80	5.02		
	10.91	4.56		
	10.76	4.62		
	10.81	4.58		
	10.78	4.75		

Application of the method to investigating geranium plants and geranium oils

The current method was applied to analyze geranium plants and geranium oils from different sources. The results are shown in Table 5. As expected, 1,3-DMAA (1) was further confirmed by multi-ion transition and the product ion ratios (Table 6). These results provide strong evidence that 1,3-DMAA (1) and 1,4-DMAA (2) are naturally present in both geranium plant and geranium oil.

A major advantage of the currently described method enabling detection of DMAAs in geranium plants and oils is its extreme sensitivity. The instrumental detection limit is approximate 1 pg. Another advantage is its simplicity. The method involves simple sample extraction and sample partition with hexane. There are no extended purification and derivatization steps involved, which should be necessary to GC/MS. The accuracy and precision of the method at ppb levels is easily achievable with a conventional LC column and mobile phase.

While the presence of DMAA has been reported in geranium in one investigation, those data were not considered conclusive due to issues regarding the experimental design and data analysis.[1] Therefore, to our knowledge, the present study is the first to show

Table 5. Levels of 1,3-DMAA (1) and 1,4-DMAA (2) in geranium (*Pelargonium graveolens*) plant and geranium oil from different sources.

Sample ID	Source	Date of collection	1,3-DMAA, ng/g	1,4-DMAA, ng/g
070611-0164 (plant)	Yunnan, China	June 9, 2011	13.60	3.56
072811-1026 (plant)	Jiangsu, China	June 9, 2011	165.0	35.30
072811-1027 (plant)	Guizhou, China	June 5, 2011	365.0	9.12
051911-0588 (oil)	Jiangxi, China	–	13271	220.0
042911-0988 (oil)	Jiangxi, China	–	167.0	Not detected
042911-0989 (oil)	Jiangxi, China	–	377.0	Not detected

conclusively that DMAA is naturally occurring in geranium plants.

It is well-documented that the variations of environmental conditions and geographical locations have great effect on the chemical profiles of the geranium plant.[5,8] The results in the current study showing various amounts of DMAA in geranium plants from different regions appear to be consistent with these observations (Table 5). Although the proportion of 1,3-DMAA (1) to 1,4-DMAA (2) varied considerably from sample to sample, in general, the concentration of 1,3-DMAA is much higher than that of 1,4-DMAA (2), suggesting that 1,3-DMAA (1) is the predominant form naturally occurring in geranium plants. The fact that 1,3-DMAA (1) is highly concentrated in one geranium oil sample when compared to the other two geranium oils could have been a result of either different geranium plants used for oil processing or from different manufacturing processes.

Another intriguing observation is that 1,4-DMAA (2) was not detected (below quantification limits) in two geranium oil samples where 1,3-DMAA (1) was present although at relatively lower concentrations. This discrepancy was unexpected. One explanation might be that 1,4-DMAA (2) is not stable at low concentrations. However, based on the structure of 1,3-DMAA (1) and 1,4-DMAA (2), they appear to be stable molecules with relaxed structure and no labile parts under various storage temperature conditions. Thus, an alternative explanation is that these two samples contained a higher ratio of 1,3-DMAA (1) to 1,4-DMAA (2). We have noted in the other samples, varying 1,3-DMAA (1):1,4-DMAA (2) ratios of approximately 5:1, 40:1 and 60:1, thus it is possible that with a combination of an even higher ratio and a smaller amount of 1,3-DMAA (1) present, the 1,4-DMAA (2) would have been below our quantification limits.

The results from the present study show that 1,3-DMAA (1) has two isomer peaks which are present in equal amounts and which are identical in all tested samples, including the standard reference. The reference standard of 1,3-DMAA (1) is synthetic and produced via chemical reaction. However, most compounds present in plants should be made through an enzymatic process. Therefore, most likely only one chiral configuration would be present in plants (often referred to as natural form). The results in the current study show that 1,3-DMAA (1) in geranium plants and geranium oils appears to be an exception to this notion. Indeed, this is not the first report demonstrating the presence of a racemate in a plant tissue.[18–20] In fact, the presence of a racemate (ie, nerol oxide) has been demonstrated once before in

Table 6. The relative intensity (%) of transitions for qualifying DMAAs in geranium (*Pelargonium graveolens*).

Detection ion (m/z) (precursor → product ion)		116 → 43	116 → 57	116 → 99
1,3-DMAA (1)	10 ng/mL standard	2.7	100	5.7
	072811-1026 (plant)	2.7	100	6.9
	051911-588 (oil)	3.0	100	3.7
1,4-DMAA (2)	10.68 ng/mL standard	4.1	100	4.5
	072811-1026 (plant)	3.7	100	6.1
	051911-588 (oil)	–	–	–

the geranium plant as well.[19] Further study is needed to elucidate the biosynthetic pathway of DMAAs in the geranium plant.

Conclusion

DMAA, which is used in some nutritional supplements, has led some to question whether it is actually a constituent of the geranium plant and its oils. A validated method for quantification of DMAA in geranium plants has been established in the present investigation and has confirmed the presence of 1,3-DMAA (1) and 1,4-DMAA (2) in the plant tissue and essential oil. The conditions of LC and ESI positive MS/MS have been optimized. A simple and rapid procedure for sample extraction and purification has been developed. This LC/MS/MS method is sensitive and reliable and has been used successfully for the simple and rapid analysis of DMAA in the geranium plant and its oils.

Author Contributions

J.S. Li and M Chen were responsible for data collection/analysis; J.S. Li was primarily responsible for manuscript preparation. Z.C. Li was responsible for study design and revision of manuscripts. All authors have read and approved the final manuscript.

Funding

This research was financially supported by USPlabs LLC.

Competing Interests

JSL, MC and ZCL disclose that funding for analytical research and manuscript preparation was provided by USPlabs, LLC. ZCL served as an expert witness in 2011 for the case: DeRosier v. USPlabs. Assistance with English grammar in preparation of the manuscript was provided as a courtesy to the authors by The Brewer Law Group, PLLC.

The sponsor initiated a request to this laboratory to investigate the presence of DMAA in geranium plant and geranium oil. All experimental design, method of extraction and method of quantification were carried out independently by Intertek-AAC Labs. Data analysis and the manuscript preparation were performed by the authors of the manuscript, while the sponsor provided grammatical review and assistance. The submission of the paper for publication was suggested by Intertek-AAC Labs to the study sponsor and the sponsor agreed.

Disclosures and Ethics

As a requirement of publication author(s) have provided to the publisher signed confirmation of compliance with legal and ethical obligations including but not limited to the following: authorship and contributorship, conflicts of interest, privacy and confidentiality and (where applicable) protection of human and animal research subjects. The authors have read and confirmed their agreement with the ICMJE authorship and conflict of interest criteria. The authors have also confirmed that this article is unique and not under consideration or published in any other publication, and that they have permission from rights holders to reproduce any copyrighted material. Any disclosures are made in this section. The external blind peer reviewers report no conflicts of interest.

References

1. Ping Z, Jun Q. A study on the chemical constituents of geranium Oil. *J Guizhou Inst Technol.* 1996;25:82–5.
2. Babu KGD, Kaul VK. Variation in essential oil composition of rose-scented geranium (*Pelargonium* sp.) distilled by different distillation techniques. *Flavour Fragr J.* 2005;20:222–31.
3. Fayed SA. Antioxidant and Anticancer Activities of *Citrus reticulate* (*Petitgrain mandarin*) and *Pelargonium graveolens* (Geranium) Essential Oils. *Res J Agric Bio Sci.* 2009;5:740–7.
4. Gomes PB, Mata VG, Rodrigues AE. Characterization of Portuguese-grown geranium oil (*Pelargonium sp.*). *J Essent Oil Res.* 2004;16:490–5.
5. Jain N, Aggarwal KK, Syamasundar KV, Srivastava SK, Kumar S. Essential oil composition of geranium (*Pelargonium sp.*) from the plains of northern India. *Flavour Fragr J.* 2001;16:44–6.
6. Jalali-Heravi M, Zekavat B, Sereshti H. Characterization of essential oil components of Iranian geranium oil using gas chromatography-mass spectrometry combined with chemometric resolution techniques. *J Chromatogr A.* 2006;1114:154–63.
7. Kulkarni RN, Mallavarapu GR, Baskaran K, Ramesh S, Kumar S. Composition of the essential oils of two isomenthone-rich variants of geranium (*Pelargonium sp.*). *Flavour Fragr J.* 1998;13:389–92.
8. Lalli JYY, Viljoen AM, Baser KHC, Demirci B, Ozek T. The essential oil composition and chemotaxonomical appraisal of South African *Pelargoniums* (*Geraniaceae*). *J Essent Oil Res.* 2006;18: 89–105.
9. Peterson A, Machmudah S, Roy BC, Goto M, Sasaki M, Hirose T. Extraction of essential oil from geranium (*Pelargonium graveolens*) with supercritical carbon dioxide. *J Chem Technol Biotechnol.* 2006;81:167–72.
10. Prakasa EVS, Rao RS, Rao G, Ramesh S. Seasonal variation in oil content and its composition in two chemotypes of scented geranium (*Pelargonium* sp.). *J Essent Oil Res.* 1995;7:159–63.
11. Shellie RA, Marriott PJ. Comprehensive two-dimensional gas chromatography-mass spectrometry analysis of *Pelargonium graveolens* essential oil using rapid scanning quadrupole mass spectrometry. *Analyst.* 2003;128: 879–83.

12. Vermia RS, Verma RK, Yadav AK, Chauhan A. Changes in the essencial oil composition of rose-scented geranium (*Pelargonium graveolens* L' Herit. Ex. Ait) due to date of transplanting under hill conditions of Uttarakhand. *Indian J Nat Prod Resour.* 2010;1:367–70.
13. Vorce SP, Holler JM, Cawrse BM, Magluilo Jr J. Dimethylamylamine: a drug causing positive immunoassay results for amphetamines. *J Anal Toxicol.* 2011;35:183–7.
14. Perrenoud L, Saugy M, Saudan C. Detection in urine of 4-methyl-2-hexaneamine, a doping agent. *J Chromatogr B.* 2009;877:3767–70.
15. Dorsey JD, Dill KA. The molecular mechanism of retention in reversed-phase liquid chromatography. *Chem Rev.* 1989;89:331–4.
16. Mallet CR, Lu Z, Mazzeo JR. A study of ion suppression effects in electrospray ionization from mobile phase additives and solid-phase extracts. *Rapid Commun Mass Spectrom.* 2004;18:49–58.
17. Annesley TM. Ion Suppression in Mass Spectrometry. *Clin Chem.* 2003;49:1041–3.
18. Batista Jr JM, Lopez SN, Mota JS, et al. Resolution and absolute configuration assignment of a natural racemic chromane from *Peperomia obtusifolia* (*Piperaceae*). *Chirality.* 2009;21:799–801.
19. Jung DJ, Porzel A, Huneck S. Gigasol and other coumarins from *Angelica gigas*, *Phytochem.* 1991;30:710–2.
20. Wust M, Reindl J, Fuchs S, Beck T, Mosandl A. Structure elucidation, enantioselective analysis, and biogenesis of nerol oxide in Pelargonium species. *J Agric Food Chem.* 1999;47:3145–50.