Golden Pacific LABORATORIES

Determination of Pyrethrins and Piperonyl Butoxide (PBO) in Crops

GPL-MTH-074, Original
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1.0 INTRODUCTION

An analytical method was developed and validated for the determination of residues of pyrethrins and piperonyl butoxide (PBO) in lettuce, blueberry, peach and tomato crops.

Pyrethrins occur naturally and consist of PY I and PY II in various ratios. PY I and PY II consist of three esters each: pyrethrin I, jasmolin I and cinerin I in PY I, and pyrethrin II, jasmolin II and cinerin II in PY II. Residues of PY I are determined by analyzing for the three esters pyrethrin I (P I), jasmolin I (Jas I) and cinerin I (Cin I) by liquid chromatography-mass spectrometry/mass spectrometry (LC/MS/MS). The peak areas of P I, Jas I, and Cin I are summed to give total PY I. The concentration of PY I is then used to determine the residue of pyrethrins by using the following equation:

Residue of pyrethrins = Residue of PY I x (Ratio of pyrethrins:PY I)

Where the ratio of pyrethrins:PY I = $\frac{\text{\% pyrethrins in the reference substance}}{\text{\% PY I in the reference substance}}$

The target limit of quantitation (LOQ) for the method (GPL-MTH-074) is 0.02 µg/g for PY I and 0.01 µg/g for PBO.

2.0 REFERENCE SUBSTANCES

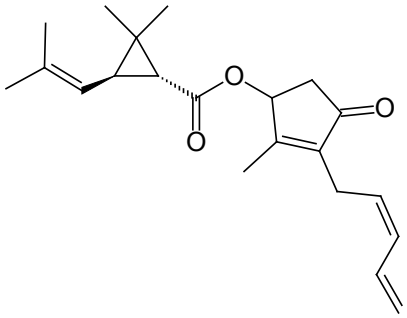
The reference substances, pyrethrins and PBO, are used to prepare calibration and fortification solutions.

2.1 Pyrethrins

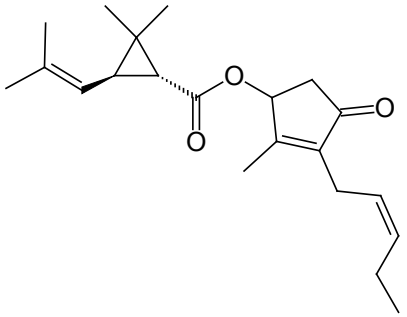
Common Name:	Premium PYROCIDE 175 Pyrethrum Standard
Chemical Name:	Pyrethrins
Supplier:	McLaughlin Gormley King Company (MGK)
Active Ingredients:	19.8% Total Pyrethrins PY I (10.7%) and PY II (9.1%)
CAS No.:	8003-34-7
Storage:	Refrigerator

Structures:

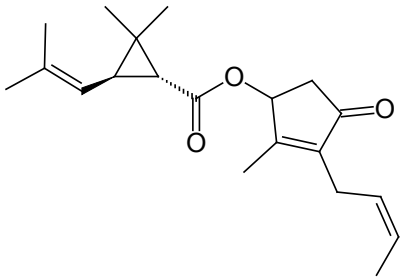
Pyrethrin I



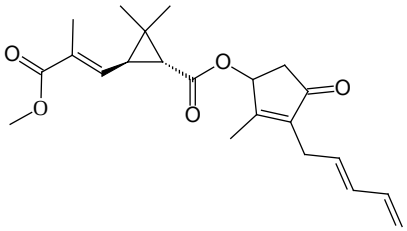
Jasmolin I



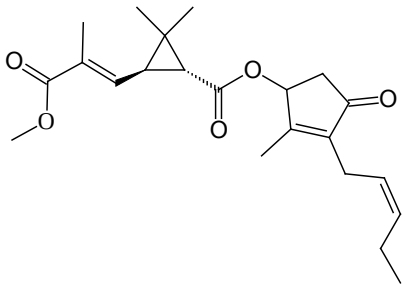
Cinerin I



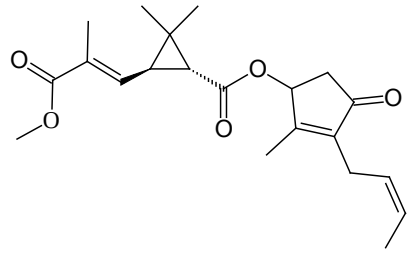
Pyrethrin II



Jasmolin II

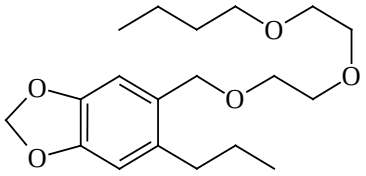


Cinerin II



2.2 PBO

Common Name:	Technical Piperonyl Butoxide (PBO)
Chemical Name:	[Alpha-(2-(2-butoxyethoxy)ethoxy)-4,5-methylenedioxy-2-propyltoluene]
Supplier:	McLaughlin Gormley King Company (MGK)
CAS No.:	51-03-6
Storage:	Refrigerator
Structure:	



2.3 PBO Internal Standard (IS)

Common Name:	ALB 115952
Chemical Name:	Deuterated ($^2H_4$)-Piperonyl Butoxide
Molecular Formula:	$C_{19}H_{26}D_4O_5$
Supplier:	Albany Molecular Research
CAS No.:	Not Applicable
Storage:	Refrigerator

3.0 PRINCIPLES OF THE METHOD

Residues of pyrethrins and PBO are extracted from crops with 100% acetonitrile. Samples are homogenized using an Omni mixer, and then centrifuged. An aliquot is filtered and diluted as necessary with acetonitrile. The final extract is diluted 50:50 with the PBO IS and analyzed using LC/MS/MS for residues of P I, Jas I, Cin I and PBO.

Six concentrations of the reference substances in acetonitrile are used for standards. For quantitation purposes, calibration plots for PY I are drawn for peak area of analyte versus concentration of analyte using at least a five-point curve computed by Microsoft® Excel. PY I is determined from the sum of the

peak areas of P I, Jas I and Cin I. Pyrethrins are calculated from the measured residues found of PY I using the ratio of pyrethrins versus PY I in the reference standard or formulation used. For quantitation purposes, calibration plots for PBO are drawn from peak area ratio (PBO/IS) versus concentration ratio (PBO/IS).

4.0 EQUIPMENT

Unless otherwise indicated, the equipment listed below may be substituted with functionally equivalent equipment.

- Balance, Analytical: Mettler model AB 204-S (Columbus, OH, USA)
- Top Loading Balance: Mettler model PB3002-S
- Disposable Pasteur pipettes, glass
- Volumetric flasks, glass: 10, 50 and 100 mL
- Test Tube, glass: 7 mL, 15 mL
- Bottles, amber glass with Teflon lined cap: 30, 60, 125, 250 and 500 mL
- Disposable graduated pipettes, glass: various sizes
- Graduated Cylinders: 25, 50, 100, 250, 500 and 1000 mL
- Micropipette Drummond Wiretrol® 100 µL disposable micropipettes (Broomall, PA, USA)
- Adjustable micropipettor, 100 – 1000 µL, Lambda™ Single-Channel Pipettor (Corning, NY, USA)
- Centrifuge tubes - 50 mL
- Homogenizer - Omni mixer, variable speed
- Luer-slip Plastic Syringe, 5 mL
- Whatman Syringe Driven Filter, .45µm PTFE 13mm,
- Centrifuge - Eppendorf 5810
- HPLC vials, clear glass: 1.5 mL
- Sciex API3200QTrap LC/MS/MS with Shimadzu LC-20AD HPLC Pumps,
- Shimadzu SCL-10A VP Controller, Shimadzu SIL-20AC Autosampler (Applied Biosystems, Foster City, CA, USA) and Analyst Chromatography Data System version 1.4

5.0 CHEMICALS/REAGENTS/SUPPLIES

Alternate suppliers of reagents having comparable specifications may be used.

- Acetonitrile, Optima Grade, Fisher #A996-4 (Fair Lawn, NJ, USA)
- Water, Optima Grade, Fisher #W7-4 (Fair Lawn, NJ, USA)
- Formic Acid 88%, Certified A.C.S, Fisher #A118^{P}-500 (Fair Lawn, NJ, USA)

6.0 STANDARD SOLUTIONS

The reference substances are used in the preparation of the fortification and calibration solutions.

The primary PY I stock solution (Solution A) is prepared by weighing approximately 0.45 g of the Premium PYROCIDE® 175 reference substance into a 50-mL volumetric flask and bringing up to volume with acetonitrile. The target final concentration of primary stock solution is 1.8 mg/mL of pyrethrins and 1.0 mg/mL of PY I after being corrected for purity.

The primary PBO stock solution (Solution B) is prepared by weighing approximately 0.10 g of PBO reference substance into a 100-mL volumetric flask and bringing up to volume with acetonitrile. The target final concentration of the PBO primary stock solution is 1.0 mg/mL PBO after being corrected for purity.

6.1 Fortification Solutions

Solution C is prepared by aliquoting 2.0 mL of Solution A and 2.0 mL of Solution B into a 100-mL volumetric flask and diluting to 100 mL with acetonitrile. Solution C has final solution concentrations of 20 µg/mL PY I and 20 µg/mL PBO.

Solution D is prepared by aliquoting 4.0 mL of Solution A and 2.0 mL of Solution B into a 100-mL volumetric flask and diluting to 100 mL with acetonitrile. Solution D has final solution concentrations of 40 µg/mL PY I and 20 µg/mL PBO.

Solution E is prepared by aliquoting 5.0 mL of Solution D into a 50-mL volumetric flask and diluting to 50 mL with acetonitrile. Solution E has final solution concentrations of 4.00 µg/mL PY I and 2.00 µg/mL PBO.

Solution F is prepared by aliquoting 1 mL of Solution E into a 100-mL volumetric flask and diluting to 100 mL with acetonitrile. Solution F has final solution concentrations of 400 ng/mL PY I and 200 ng/mL PBO.

Solutions C and F are fortification solutions used for fortifying untreated crop samples. The fortification solutions are stored in the refrigerator in amber bottles and renewed every 3 months or as needed.

6.2 Calibration Solutions

Solution F is used to prepare a mixed set of calibration solutions containing both PY I and PBO.

Aliquots of Solution F are taken using Wiretrol® micropipettes or glass graduated pipettes to make calibration standards. Calibration standards for PY I and PBO are diluted with acetonitrile. Typical concentrations of calibration standards are shown below:

Volume Used (mL)	Solution Used	Final Volume (mL)	Concentration (ng/mL)	
			PY I	PBO
5	F	50	40	20
5	F	100	20	10
2.5	F	100	10	5
1.25	F	100	5	2.5
0.5	F	100	2	1
0.25	F	100	1	0.5

All calibration solutions are stored in amber bottles in the refrigerator (5°C). Solutions are prepared every three months or as required.

6.3 IS Calibration Solutions

The PBO IS stock solution (Solution G) is prepared by weighing approximately 0.0010 g of Deuterated ($^{2}H_{4}$)-Piperonyl Butoxide reference substance into a 10-mL volumetric flask and bringing up to volume with acetonitrile. The final concentration of the PBO IS primary stock solution is 100 µg/mL deuterated PBO after being corrected for purity.

Solution H is prepared by aliquoting 0.1 mL of Solution G into a 100-mL volumetric flask and diluting to 100 mL with acetonitrile. Solution H has a final solution concentration of 100 ng/mL deuterated PBO.

Solution I is prepared by aliquoting 50 mL of Solution H into 100-mL flask and adding 50 mL of acetonitrile for a total volume of 100 mL. Solution I has a final solution concentration of 50 ng/mL deuterated PBO

Solution J is prepared by aliquoting 100 mL of Solution I into a 200-mL flask and adding 100 mL of water for a total volume of 200 mL. Solution J has a final solution concentration of 25.0 ng/mL deuterated PBO. Solution J is used to vial samples 1:1 with IS during the final step in the method.

All IS solutions are stored in amber bottles in the refrigerator (5°C). Internal standard solutions are prepared as required.

7.0 REAGENT PREPARATION

7.1 Liquid Chromatography (LC) Mobile Phases

0.2% Formic Acid in water: To a 1 liter graduated cylinder, 900 mL of Optima grade water is added. Using a 2.0 mL graduated pipette, 2.0 mL of formic acid is added. The volume is adjusted to 1000 mL using Optima grade water and mixed thoroughly.

0.2% Formic Acid in Acetonitrile: To a 1 liter graduated cylinder, 900 mL of acetonitrile is added. Using a 2.0 mL graduated pipette 2.0 mL of formic acid is added. The volume is adjusted to 1000 mL using Acetonitrile and mixed thoroughly.

8.0 ANALYTICAL PROCEDURE

The analytical methods for analysis of PY I and PBO residues are described below and also presented schematically in Appendix 1.

8.1 Analytical Procedure for Lettuce, Blueberry, Peach and Tomato

Field-harvested samples are prepared for extraction by transferring approximately 2.00 g of homogenized sample into separate, labeled 50-mL disposable plastic centrifuge tubes. Procedural recovery samples are fortified with 100 µL of Solution C or F, providing the following concentrations of PY I and PBO:

Volume Used (mL)	Solution Used	Solution Conc. PY 1/PBO (µg/mL)	Sample Amount (g)	Fortification Amount (µg/g)	
				PY I	PBO
100 µL	C	20/20	2.00	1.0	1.0
100 µL	F	0.4/0.2	2.00	0.02	0.01

Samples are extracted with 20 mL of acetonitrile. After the acetonitrile is added, samples are blended at high speed for approximately one minute using an Omni Mixer Homogenizer. Samples are then centrifuged for four minutes at 3000 revolutions per minute (rpm). Following centrifugation, each sample is syringe filtered through a 13 mm, 0.45 µm PTFE filter into an 8-mL sample vial. No further clean-up of the samples is needed. If necessary, samples are diluted with acetonitrile.

An aliquot of each sample is transferred to an HPLC vial and diluted with an equal volume of Solution J (25.0 ng/mL IS). The samples are then submitted for analysis by LC/MS/MS.

9.0 QUANTITATION

The following parameters are suggested, and may be altered in order to optimize analyte sensitivity and resolution.

9.1 Instrumentation

Sciex API3200 QTrap LC/MS/MS with Shimadzu LC-20AD HPLC Pumps, Shimadzu SCL-10A VP Controller, Shimadzu 20-AC Autosampler with Analyst Chromatography Data System version 1.4, Applied Biosystems

9.2 LC Conditions

HPLC Column: Agilent ZORBAX Eclipse XDB-C18 (2.1 x 50 mm, 1.8 µ) Catalog No. 922700-902

Guard Column: Phenomenex Security Guard C18 Catalog No. KJ0-4282

Column Temperature: Ambient

Mobile Phase: **Gradient:**
A = 0.2% formic acid in acetonitrile
B = 0.2% formic acid in water

Time (min)	A (%)	B (%)
0.0	70	30
4.5	85	15
5.5	85	15
5.6	70	30
6.0	70	30

Flow Rate: 0.3 mL/min

Injector: autosampler

Injection Volume: 20 µL

Approximate Retention Times:

P I	3.80 minutes
Jas I	4.70 minutes
Cin I	3.70 minutes
PBO and IS	2.60 minutes

9.3 Mass Spectrometer Parameters

Interface: Turbo Ion Spray (ESI)

Polarity: Positive

Scan Type: MRM Monitoring with Unit resolution

Ions Monitored (Q1/Q3):		**Dwell Time (msec)**	**CEP (Start/Stop)**
(P I)	m/z 329.20/161.00	500	19.36/19.36
(Jas I)	m/z 331.20/163.00	500	19.42/19.42
(Cin I)	m/z 317.20/149.00	500	19.02/19.02
(PBO)	m/z 356.26/177.30	1000	16.00/16.00
(PBO IS)	m/z 360.26/181.30	1000	20.23/20.23

Parameter	**Period 1 (PBO)**	**Period 2 (Pyrethrins)**
Collision Energy (CE):	48	13
Collision Cell Exit Potential (CXP):	4	4
GS1:	60	40
GS2:	50	20
Source Temperature (TEM):	400	400
Curtain Gas (CUR):	10	10
Ion Spray Voltage (IS):	4500	5500
Collisionally Activated Dissociation (CAD):	Medium	Low
Declustering Potential (DP):	26	19
Interface Heater (Ihe):	On	On
Entrance Potential (EP):	4	4

9.4 LC/MS/MS Detector Response Calibration

The LC/MS/MS responses (peak areas) are determined for a series of calibration standards. Through the Analyst Software, version 1.4, the concentrations of the standards (and IS for PBO) and their corresponding peak area responses are compiled. From this, Analyst calculates a standard calibration curve using linear regression and a correlation coefficient (r) based on the standard (and IS for PBO) concentrations and their respective peak area responses (peak response ratio for PBO).

For each analytical set, the calibration data is used to perform a linear regression analysis.

The peak area responses for the three PY I esters (P I, Jas I and

Cin I) are computed using the Analyst software, version 1.4. The peak area responses of the three esters are summed using Microsoft® Excel. This summed value represents the peak area response for total PY I. See Equation 1.

For the standards, a curve using the peak area response for total PY I and the concentrations of the calibration standards is generated using Microsoft® Excel. The concentrations of the standards in ng/mL are plotted as the X-axis. The summed peak area responses are plotted as the Y-axis to give Equation 2.

Peak area of total PY I =
peak area of P I + peak area of Jas I + peak area of Cin I [Eq. 1]

$y = ax + b$ [Eq. 2]

where:
y = summed peak area response (peak area of total PY I)
a = slope of the regression line
x = amount (ng/mL) of analyte found in the standard
b = intercept of the regression line

peak area = a(ng/mL in the standard) + b [Eq. 2]

For PBO, the analyte versus IS injected ratio (concentration analyte injected/concentration IS injected) is plotted as the X-axis (concentrations in ng/mL), and the detector response ratio (peak area analyte/peak area IS) is plotted as the Y-axis to give Equation 3.

$y = ax + b$ [Eq. 3]

where:
y = peak area response ratio (analyte peak area/IS peak area)
a = slope of the regression line
x = analyte versus IS injected ratio (analyte conc./IS conc.)
b = intercept of the regression line

peak area response ratio = a(analyte/IS conc. ratio) + b [Eq. 3]

9.5 Sample Analysis

In a given sample, the peak area responses for the three PY I esters (P I, Jas I and Cin I) are computed using the Analyst software, version 1.4. The peak area responses of the three esters are summed using Microsoft® Excel to generate the peak area response for total PY I (Equation 1). The amount of PY I in a sample is determined from the corresponding standard calibration plot.

Concentration of PY I in ng/mL is calculated from the summed peak area using Equation 4.

Peak area of PY I total =
peak area of P I + peak area of Jas I + peak area of Cin I [Eq. 1]

$$x \text{ (ng PY I/mL)} = \frac{\text{peak area of PY I total - } b}{a} \quad \text{[Eq. 4]}$$

In a given sample, the peak area response ratio of PBO versus IS is computed using the Analyst software, version 1.4. The amount of PBO in a sample is determined from the corresponding standard calibration plot. Concentration of PBO in ng/mL is calculated from the observed peak area response ratio (PBO/IS) and IS concentration using Equation 5.

$$x \text{ (ng PBO/mL)} = \frac{[(\text{peak area ratio PBO/IS}) - b] * \text{ng IS/mL}}{a} \quad \text{[Eq. 5]}$$

Both samples and standards are analyzed under the same LC/MS/MS conditions and within the same analytical sequence.

10.0 CALCULATION OF RESIDUES

From the standard calibration curve, PY I and PBO concentrations (µg/g) in unknown samples and fortified samples are determined using Equation 6 shown below:

$$\mu g/g = \frac{\text{(ng/mL from curve) (final volume in mL) (1 µg)}}{\text{(Sample amount in grams) (1000 ng)}} \quad \text{[Eq. 6]}$$

The concentration of pyrethrins in a given sample is calculated from the measured residues of PY I using the ratio of % pyrethrins to % PY I in the reference substance. The ratio of pyrethrins to PY I is determined using Equation 7. Equation 8 is used to calculate pyrethrins from the measured residues of PY I.

$$\text{Ratio of pyrethrins:PY I} = \frac{\text{\% pyrethrins in reference substance}}{\text{\% PY I in reference substance}} \quad \text{[Eq. 7]}$$

µg/g of pyrethrins = (PY I in µg/g) x (Ratio of pyrethrins:PY I) [Eq. 8]

For fortified samples, the percent recovery is calculated using the following equation:

$$\text{\% Recovery} = \frac{\text{measured residue in µg/g}}{\text{fortification amount in µg/g}} \times 100 \quad \text{[Eq. 9]}$$

11.0 QUALITY CONTROL PROCEDURES

11.1 LC/MS/MS Analysis

To verify the stability of the response, a standard plot is drawn for the levels of interest. Calibration standards are injected every two to four sample injections, as well as at the beginning and end of an analytical set. The lowest level analytical standard corresponds to 70% or less of the limit of quantitation. Residue results must not be determined by extrapolation outside of the concentration range of the calibration standards. Samples with area counts greater than the calibrated range must be diluted and re-injected, in a timely manner, so that they fall within the calibrated range.

The recovery of laboratory fortified controls should fall in the range of 70% to 120%. The analyte signal should be $\geq$ 3 times the background signal. The intra-laboratory reproducibility as indicated by the relative standard deviation (n >3) obtained from replicated analyses should fall within 20% (rel.) of the averaged result.

12.0 DETECTION LIMITS

The LOQ is 0.02 µg/g for PY I in lettuce, blueberry, peach and tomato. The LOQ is 0.01 µg/g for PBO in lettuce, blueberry, peach and tomato.

13.0 DISCUSSION

This method was previously validated in lettuce, blueberry, peach and tomato at Golden Pacific Laboratories (GPL) as part of individual magnitude of residue studies. Method validation results are shown in Tables 1 through 4.

Summaries of the mean recoveries, standard deviations and coefficients of variation (CV) from the method validations are shown in following tables:

Analyte	Matrix	Fortification Level	Mean Recovery ± Standard Deviation (CV)
PY I	Lettuce	LOQ	77.6 ± 1.70 (2.19)
		50 xLOQ	90.1 ± 0.462 (0.512)
	Blueberry	LOQ	77.3 ± 1.35 (1.75)
		50 xLOQ	88.8 ± 2.11 (2.37)
	Peach	LOQ	79.6 ± 3.84 (4.83)
		50 xLOQ	93.2 ± 2.00 (2.15)
	Tomato	LOQ	82.1 ± 0.493 (0.601)
		50 xLOQ	95.9 ± 1.70 (1.77)

PY I LOQ = 0.02 µg/g

Analyte	Matrix	Fortification Level	Mean Recovery ± Standard Deviation (CV)
PBO	Lettuce	LOQ	85.7 ± 5.87 (6.86)
		100 xLOQ	93.3 ± 0.306 (0.327)
	Blueberry	LOQ	83.4 ± 2.44 (2.93)
		100 xLOQ	93.9 ± 1.86 (1.98)
	Peach	LOQ	86.0 ± 4.01 (4.66)
		100 xLOQ	97.9 ± 2.73 (2.79)
	Tomato	LOQ	89.7 ± 1.95 (2.17)
		100 xLOQ	95.2 ± 1.03 (1.08)

PBO LOQ = 0.01 µg/g

14.0 REFERENCES

"Raw Agricultural Commodity (RAC) Decline of Pyrethrins/Piperonyl Butoxide Applied to Lettuce," Study Number 060231, Golden Pacific Laboratories, April 24, 2007.

"Raw Agricultural Commodity (RAC) Decline of Pyrethrins/Piperonyl Butoxide Applied to Blueberry," Study Number 060233, Golden Pacific Laboratories, April 24, 2007.

"Raw Agricultural Commodity (RAC) Decline of Pyrethrins/Piperonyl Butoxide Applied to Peach," Study Number 060232, Golden Pacific Laboratories, April 24, 2007.

"Raw Agricultural Commodity (RAC) Decline of Pyrethrins/Piperonyl Butoxide Applied to Tomato," Study Number 060230, Golden Pacific Laboratories, April 24, 2007.

Table 1 Method Validation Results of PY I and PBO in/on Lettuce

Lab ID	Measured Residues (µg/g)	Fortification Amount (µg/g)	Percent Recovery (%)
PY I			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.0151	0.0199	75.9
Low Spike B	0.0157	0.0202	77.7
Low Spike C	0.0157	0.0198	79.3
		Mean (n=3)	**77.6**
		Standard Deviation	**1.70**
		CV	**2.19**
High Spike A	0.922	1.02	90.4
High Spike B	0.904	1.00	90.4
High Spike C	0.878	0.980	89.6
		Mean (n=3)	**90.1**
		Standard Deviation	**0.462**
		CV	**0.512**
OVERALL		**Mean (n=6)**	**83.9**
		Standard Deviation	**6.94**
		CV	**8.27**
PBO			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.00800	0.00980	81.6
Low Spike B	0.00826	0.00995	83.0
Low Spike C	0.00901	0.00975	92.4
		Mean (n=3)	**85.7**
		Standard Deviation	**5.87**
		CV	**6.86**
High Spike A	0.936	1.00	93.6
High Spike B	0.916	0.985	93.0
High Spike C	0.902	0.966	93.4
		Mean (n=3)	**93.3**
		Standard Deviation	**0.306**
		CV	**0.327**
OVERALL		**Mean (n=6)**	**89.5**
		Standard Deviation	**5.61**
		CV	**6.27**

LOQ = 0.02 µg/g for PY I and 0.01 µg/g for PBO

Table 2 Method Validation Results of PY I and PBO in/on Blueberry

Lab ID	Measured Residues (µg/g)	Fortification Amount (µg/g)	Percent Recovery (%)
PY I			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.0158	0.0201	78.6
Low Spike B	0.0154	0.0199	77.4
Low Spike C	0.0154	0.0203	75.9
		Mean (n=3)	**77.3**
		Standard Deviation	**1.35**
		CV	**1.75**
High Spike A	0.910	1.00	91.0
High Spike B	0.885	1.02	86.8
High Spike C	0.882	0.995	88.6
		Mean (n=3)	**88.8**
		Standard Deviation	**2.11**
		CV	**2.37**
OVERALL		**Mean (n=6)**	**83.1**
		Standard Deviation	**6.49**
		CV	**7.82**
PBO			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.00798	0.00990	80.6
Low Spike B	0.00834	0.00980	85.1
Low Spike C	0.00845	0.01	84.5
		Mean (n=3)	**83.4**
		Standard Deviation	**2.44**
		CV	**2.93**
High Spike A	0.946	0.985	96.0
High Spike B	0.924	1.00	92.4
High Spike C	0.915	0.980	93.4
		Mean (n=3)	**93.9**
		Standard Deviation	**1.86**
		CV	**1.98**
OVERALL		**Mean (n=6)**	**88.7**
		Standard Deviation	**6.09**
		CV	**6.87**

LOQ = 0.02 µg/g for PY I and 0.01 µg/g for PBO

Table 3 Method Validation Results of PY I and PBO in/on Peach

Lab ID	Measured Residues (µg/g)	Fortification Amount (µg/g)	Percent Recovery (%)
PY I			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.0147	0.0195	75.4
Low Spike B	0.0165	0.0199	82.9
Low Spike C	0.0162	0.0201	80.6
		Mean (n=3)	**79.6**
		Standard Deviation	**3.84**
		CV	**4.83**
High Spike A	0.947	0.995	95.2
High Spike B	0.951	1.02	93.2
High Spike C	0.912	1.00	91.2
		Mean (n=3)	**93.2**
		Standard Deviation	**2.00**
		CV	**2.15**
OVERALL		**Mean (n=6)**	**86.4**
		Standard Deviation	**7.92**
		CV	**9.16**
PBO			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.00859	0.00961	89.4
Low Spike B	0.00800	0.00980	81.6
Low Spike C	0.00862	0.00990	87.1
		Mean (n=3)	**86.0**
		Standard Deviation	**4.01**
		CV	**4.66**
High Spike A	0.941	0.980	96.0
High Spike B	0.976	1.01	96.6
High Spike C	0.992	0.985	101
		Mean (n=3)	**97.9**
		Standard Deviation	**2.73**
		CV	**2.79**
OVERALL		**Mean (n=6)**	**92.0**
		Standard Deviation	**7.17**
		CV	**7.80**

LOQ = 0.02 µg/g for PY I and 0.01 µg/g for PBO

Table 4 Method Validation Results of PY I and PBO in/on Tomato

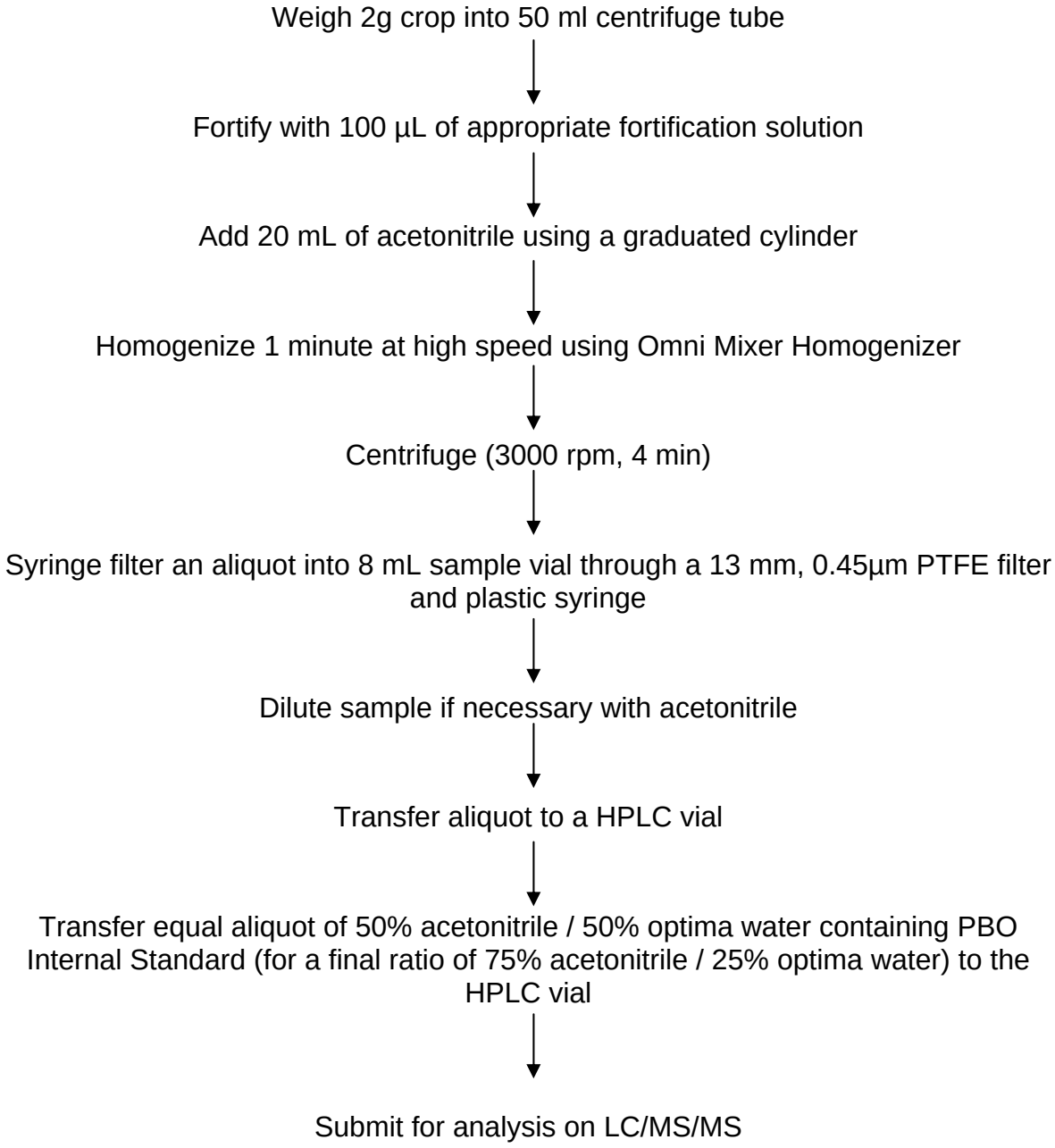
Lab ID	Measured Residues (µg/g)	Fortification Amount (µg/g)	Percent Recovery (%)
PY I			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.0163	0.0198	82.3
Low Spike B	0.0159	0.0195	81.5
Low Spike C	0.0164	0.0199	82.4
		Mean (n=3)	**82.1**
		Standard Deviation	**0.493**
		CV	**0.601**
High Spike A	0.951	1.01	94.2
High Spike B	0.949	0.990	95.9
High Spike C	0.966	0.990	97.6
		Mean (n=3)	**95.9**
		Standard Deviation	**1.70**
		CV	**1.77**
OVERALL		**Mean (n=6)**	**89.0**
		Standard Deviation	**7.66**
		CV	**8.61**
PBO			
Reagent Blank	<LOQ	NA	NA
Control	<LOQ	NA	NA
Low Spike A	0.00853	0.00975	87.5
Low Spike B	0.00876	0.00961	91.2
Low Spike C	0.00886	0.00980	90.4
		Mean (n=3)	**89.7**
		Standard Deviation	**1.95**
		CV	**2.17**
High Spike A	0.953	0.990	96.3
High Spike B	0.925	0.975	94.9
High Spike C	0.919	0.975	94.3
		Mean (n=3)	**95.2**
		Standard Deviation	**1.03**
		CV	**1.08**
OVERALL		**Mean (n=6)**	**92.4**
		Standard Deviation	**3.30**
		CV	**3.57**

LOQ = 0.02 µg/g for PY I and 0.01 µg/g for PBO

APPENDIX I

Analysis Flowchart

Flowchart for the analysis of PY I and PBO in/on Crop Samples (Lettuce, Blueberry, Peach and Tomato)



APPENDIX II

Example Chromatograms

Figure 1

Representative Chromatograms of the P I, Jas I and Cin I Esters of a 0.500 ng/mL PY I LC/MS/MS Standard

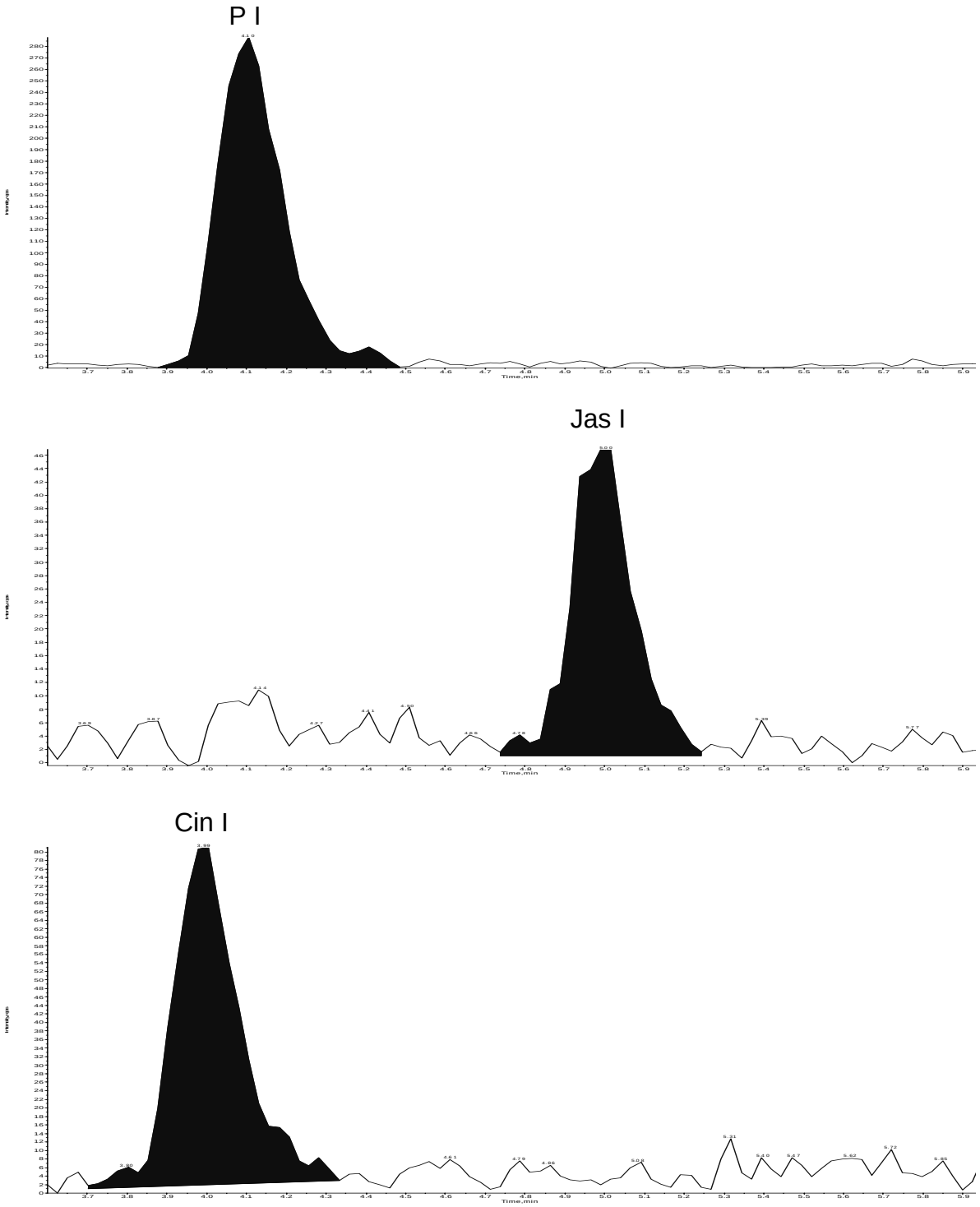


Figure 2

Representative Chromatogram of a 0.247 ng/mL PBO LC/MS/MS Standard and a 12.4 ng/mL Internal Standard LC/MS/MS Standard

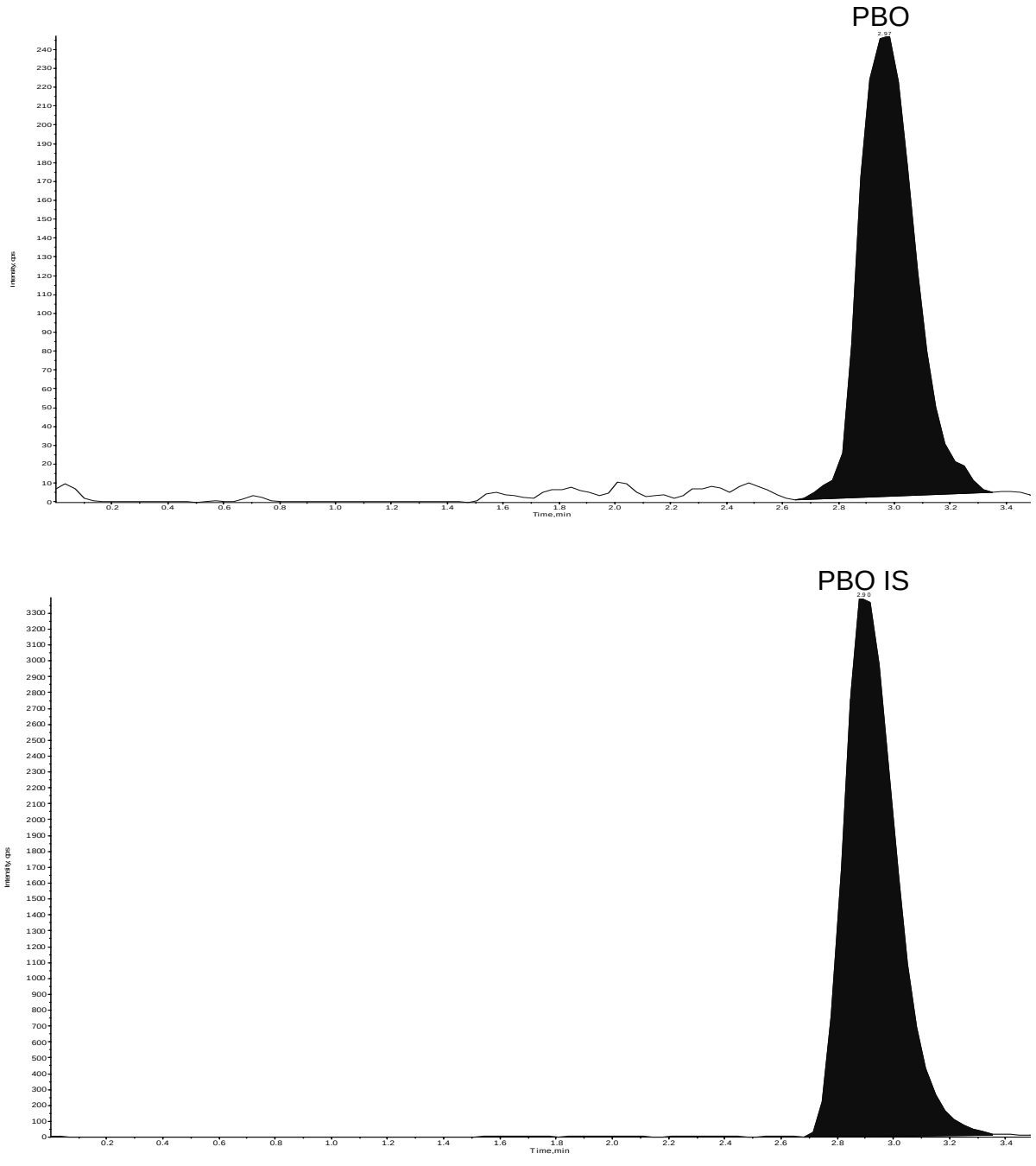


Figure 3

Representative Calibration Curve for PY I Total (r = 0.9999)

Curve Equation: y = 8201.8 x + 906.88

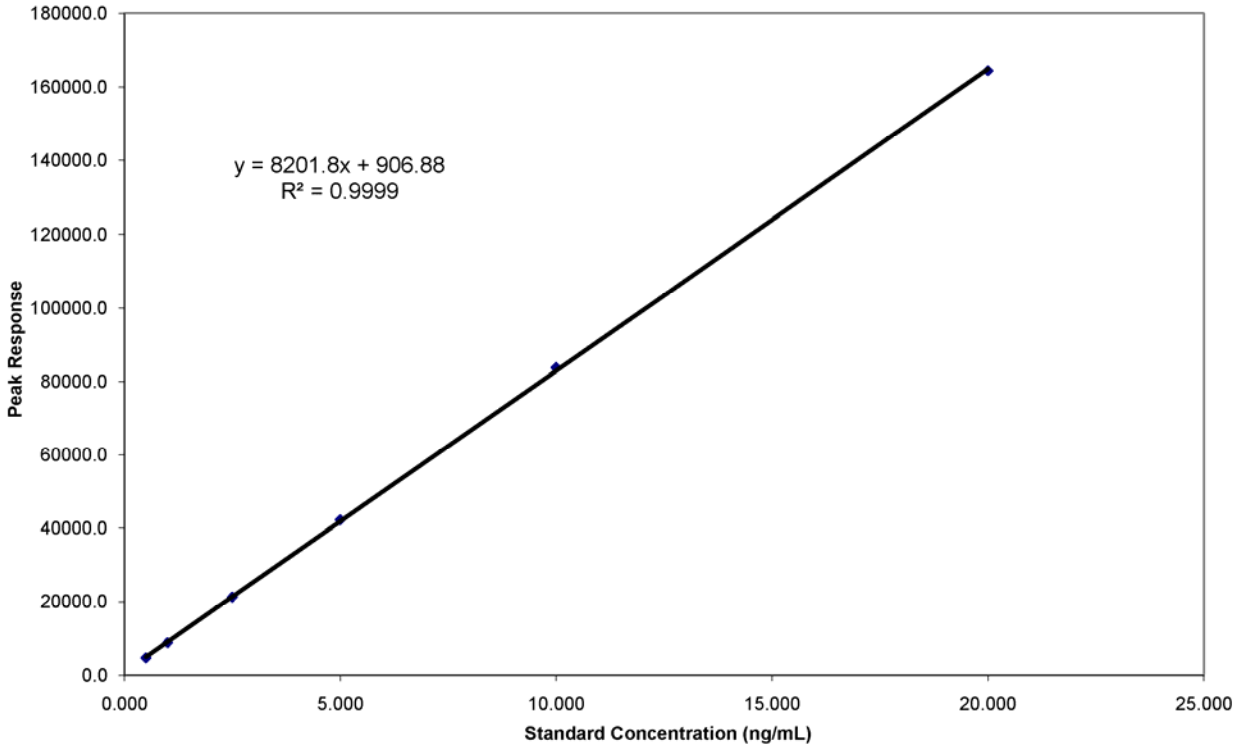


Figure 4

Representative Calibration Curve for PBO (r = 0.9999)

Curve Equation: y = 2.98 x + 0.0205