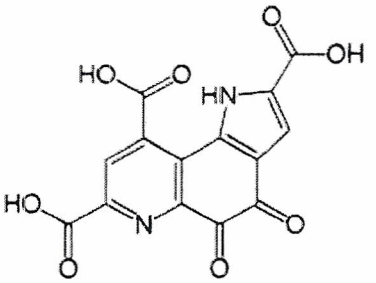
TITLE: **Assay for PQQ Pyrroloquinonline Quinone by HPLC**

1. **OBJECTIVE:**
To determine Pyrroloquinonline Quinone (PQQ) content in Raw materials using Reverse Phase HPLC

2. **INSTRUMENTATION:**
WATERS Alliance 2690 Separation Module
Detector: Waters PDA 996

3. **PRINCIPLE/METHOD DESCRIPTION:**
Standard and sample solutions in the concentration of 0.08 mg/ml are prepared in DI Water. Aliquots of the standard and sample solution are treated with 0.1M sodium carbonate buffer pH 9.20 and reacted with Acetone to give a stable adduct which is then quantitated on Reverse Phases HPLC using a C18 column with UV Detector at 254 nm.

4. **CHEMICAL STRUCTURES/CHEMISTRY:**
Molecular nature of PQQ: Because there are three carboxyl groups, PQQ is a water-soluble compound. It has a quinone skeleton as well as vitamin B2 (riboflavin), and the part of left side contains a similar chemical property to that of vitamin B6.



Pyrroloquinoline quinone

Synonym:	Methoxatin, PQQ, 4,5-Dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid
CAS Number:	72909-34-3
Linear Formula:	$C_{14}H_6O_68N_2O_8$
Molecular Weight:	330.206 g/mol

5. **REFERENCE STANDARD:**

5.1. Standard Pyrroloquinoline quinone ~98% (HPLC) Catalog # D-7783 from Sigma Aldrich
http://www.sigmaaldrich.com/catalog/product/sigma/d7783?lang=en&region=US

5.2. Alternately the Standard for Pyrroloquinoline quinone can also be purchased from Santa Cruz Biotechnology Catalog # sc-210178
http://www.scbt.com/datasheet-210178-Pyrroloquinoline-quinone.html

6. **SOLVENTS & REAGENTS:**
6.1. Water, HPLC grade
6.2. Acetonitrile, HPLC Grade
6.3. Acetone, ACS Reagent Grade
6.4. Glacial Acetic Acid
6.5. Sodium carbonate

6.6. Concentrated Nitric acid
6.7. **16 % Acetone:** Prepare by pipetting 16ml Acetone and transferring to 100 ml volumetric flask. QS to volume with DI Water. Mix well.
6.8. **0.1 M Sodium Carbonate Buffer (pH 9.2):** Prepare by weighing 1.2 g of sodium carbonate and transfer to 100 ml volumetric flask, add 30 ml DI Water and stir to dissolve. Cautiously pipette in 0.6 ml of Concentrated Nitric Acid using micropipette. QS to volume with DI Water, Check pH and adjust to 9.2 with Diluted Nitric acid or 0.1 N NaOH. Do not keep more than a week, check pH before use.

7. STANDARD PREPARATION:

7.1. **STOCK SOLUTION:** Transfer 10 mg accurately weighed reference standard into a 25ml volumetric flask.
7.2. Dissolve in 10 ml DI Water by sonication for 5 minutes.
7.3. Cool to room temperature and QS to volume with DI Water.
7.4. Refrigerate prepared standard till use.
7.5. **WORKING STANDARD:** Quantatively pipette and transfer 1 ml from stock to a 5 ml volumetric flask.
7.6. Pipette 1ml of the 0.1 M Sodium carbonate buffer solution.
7.7. QS to volume with 3 ml of 16 % Acetone solution and mix well.
7.8. Place in warm water bath (37° C) for 30 minutes,
7.9. Cool to room temperature.
7.10. Filter through 0.45Micron filter into HPLC vial.
7.11. Final standard concentration is 0.08 mg/ml

NOTE: If using 1 mg Reference standard, prepare accordingly to have working concentration of 0.08 mg/ml

8. SAMPLE PREPARATION:

8.1. Weigh accurately a powdered sample equivalent to 40 mg of PQQ into a 100ml volumetric flask.
8.2. Dissolve in 60ml DI Water by sonication for 10 minutes
8.3. Cool to RT and QS to volume with DI Water.
8.4. Quantatively pipette and transfer 1 ml to a 5 ml volumetric flask.
8.5. Pipette 1ml of the 0.1 M Sodium carbonate buffer solution.
8.6. QS to volume with 3 ml of 16 % Acetone solution and mix well.
8.7. Place in warm water bath (37° C) for 30 minutes,
8.8. Cool to room temperature.
8.9. Filter through 0.45Micron filter into HPLC vial.

9. ANALYTICAL METHOD:

9.1. **Diluting Solution:** DI Water, 16 % Acetone and 0.1 M Sodium Carbonate Buffer (pH 9.2)
9.2. **Mobile Phase A: 0.1% Glacial Acetic acid in Purified Water :**Prepare 1000 ml at a time, Pipette 1 ml of Glacial Acetic Acid to 999 ml of HPLC Grade Deionized water, Stir/Mix well for 20 minutes, Filer and degas before use
9.3. **Mobile Phase B: 0.1% Glacial acetic Acid in HPLC-Grade Acetonitrile:** Prepare 1000 ml at a time, Pipette 1 ml of Glacial Acetic Acid to 999 ml of HPLC Grade Acetonitrile, Stir/Mix well for 20 minutes, Filer and degas before use
9.4. **Mobile Phase Program**: 70: 30 (A:B)
9.5. **Column**: Agilent Zorbax Eclipse XDB C18, 5um, 4.6 X 150 mm, packing L1 Part # 993967

9.6. **Column Temperature** Ambient (Room Temperature)
9.7. **Detector:** UV @ 254 nm
9.8. **Flow Rate**: 0.5 ml/min
9.9. **Injection Volume:** 10 µl
9.10. **Run Time**:10 minutes
9.11. **Retention times:**

5.4.1	PQQ:	2.46 minutes
5.4.2	Solvent (Acetone):	3.3 minutes

6 CALCULATION:

Calculate the amount of PQQ in the sample by using the formula:

Ru/Rs X Cs/Cu X P X 100/Spl wt, where

Ru: Peak AREA response of the sample (Average 2 injections)
Rs: Peak AREA response of the standard (Average 5 injections)
Cs: Concentration of Standard (in mg/ml)
Cu: Concentration of sample
P: Purity of the Standard
100: To calculate in percentage
Spl wt: The weight of the sample taken in mg

7 CHROMATOGRAMS:

7.4 **System Suitability**: The Relative Standard Deviation for Replicate Injections is not more than 2.0 %

8 ATTACHMENT:

8.4 Chromatographic raw data for 5 standard injections with summary followed by sample injections with summary and a solvent injection.

Reported by User: System

Project Name: Phytochemicals4

Component Summary For Retention Time
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
1	PQQ Std	1	996	20	2.456	3.318
2	PQQ Std	2	996	20	2.462	3.303
3	PQQ Std	3	996	20	2.462	3.298
4	PQQ Std	4	996	20	2.467	3.315
5	PQQ Std	5	996	20	2.461	3.296
Mean					2.462	3.306
Std. Dev.					0.004	0.010
% RSD					0.2	0.3

Component Summary For Area
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
1	PQQ Std	1	996	20	4895746	5009246
2	PQQ Std	2	996	20	4851843	4825408
3	PQQ Std	3	996	20	4853486	4742426
4	PQQ Std	4	996	20	4881456	4779766
5	PQQ Std	5	996	20	4839748	4651885
Mean					4864456	4801746
Std. Dev.					23227	132360
% RSD					0.5	2.8

Component Summary For Amount
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
1	PQQ Std	1	996	20		
2	PQQ Std	2	996	20		
3	PQQ Std	3	996	20		
4	PQQ Std	4	996	20		
5	PQQ Std	5	996	20		

Reported by User: System

Project Name: Phytochemicals4

Component Summary For Amount
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
Mean						
Std. Dev.						
% RSD						

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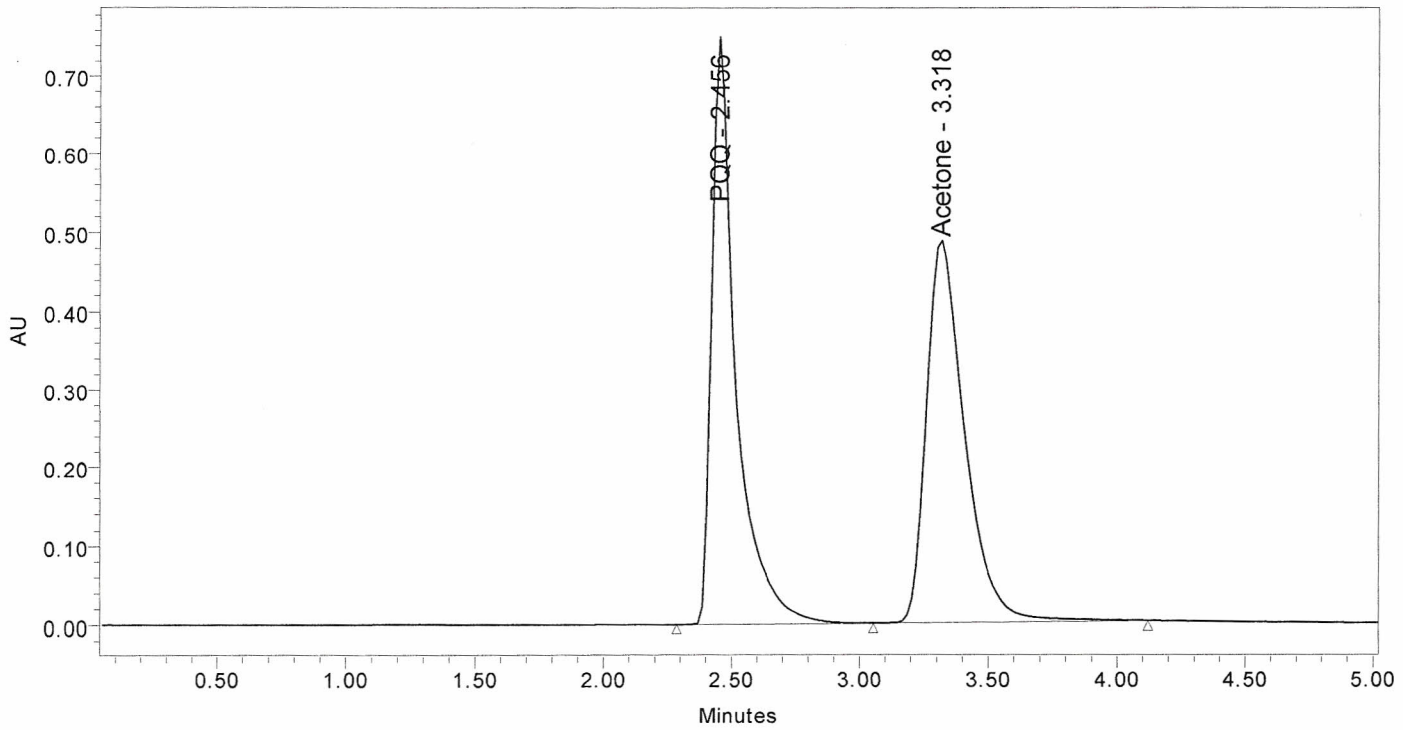
Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	PQQ Std	Acquired By:	System
Sample Type:	Standard	Date Acquired:	6/19/2012 12:30:05 PM
Vial:	20	Acq. Method Set:	PQQ
Injection #:	1	Date Processed:	6/19/2012 1:31:01 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.456	4895746	49.43	740909
2	Acetone	3.318	5009246	50.57	486581

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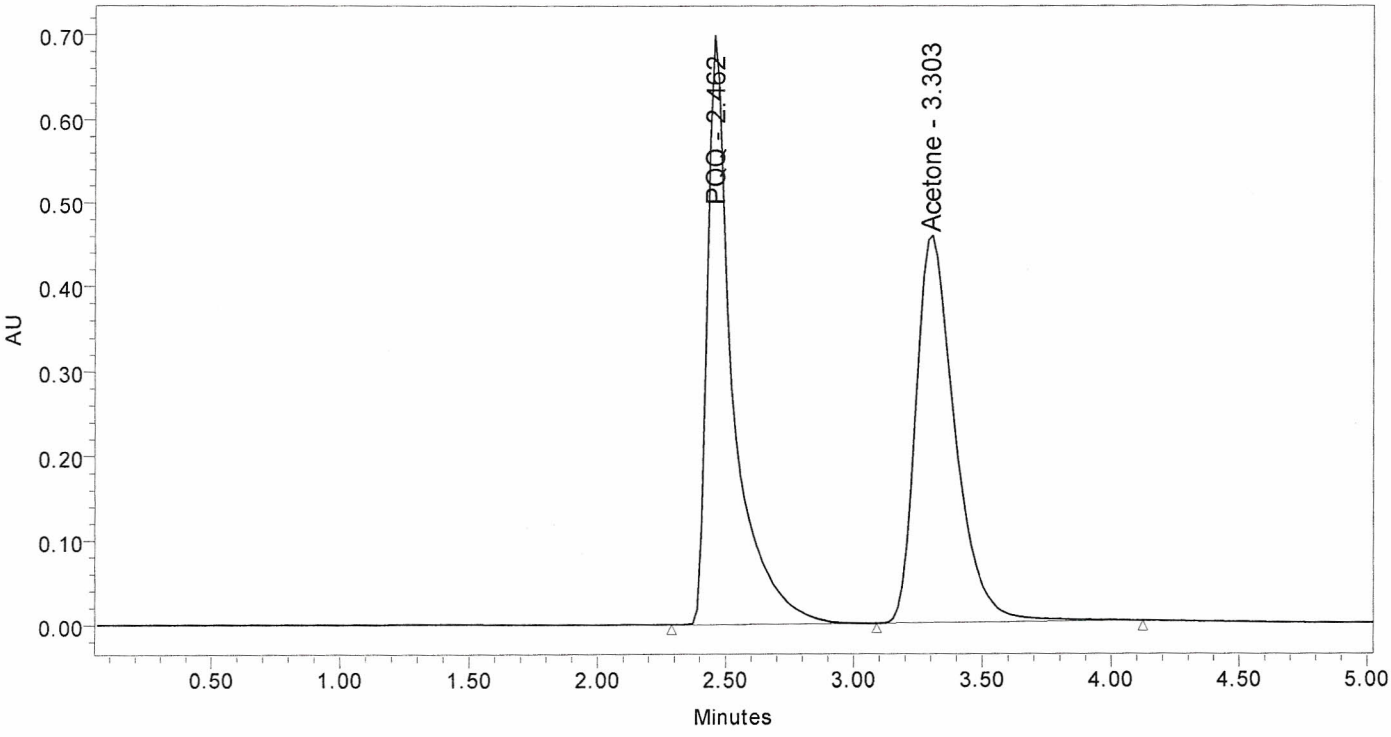
Default Individual Report

Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	PQQ Std	Acquired By:	System
Sample Type:	Standard	Date Acquired:	6/19/2012 12:36:01 PM
Vial:	20	Acq. Method Set:	PQQ
Injection #:	2	Date Processed:	6/19/2012 1:31:05 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.462	4851843	50.14	691956
2	Acetone	3.303	4825408	49.86	459462

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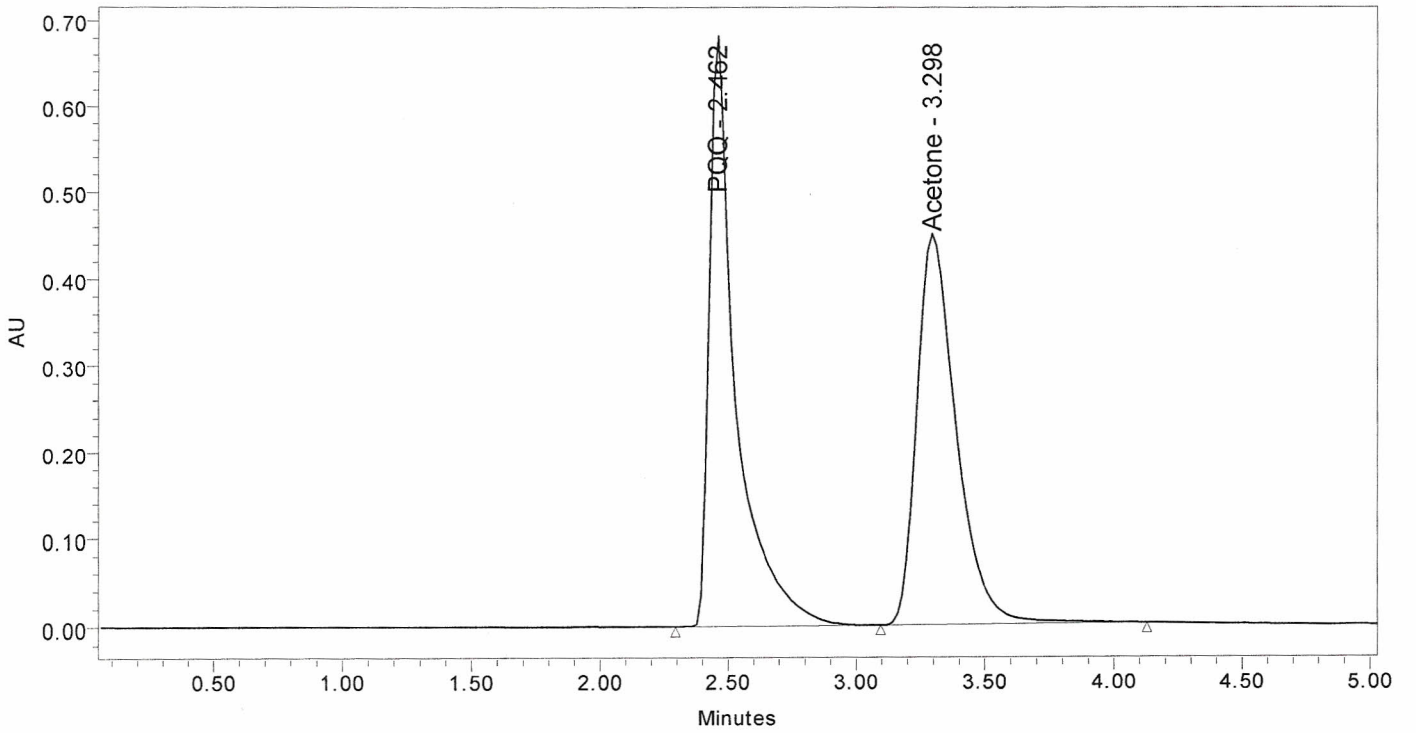
Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	PQQ Std	Acquired By:	System
Sample Type:	Standard	Date Acquired:	6/19/2012 12:41:44 PM
Vial:	20	Acq. Method Set:	PQQ
Injection #:	3	Date Processed:	6/19/2012 1:31:08 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.462	4853486	50.58	672119
2	Acetone	3.298	4742426	49.42	449927

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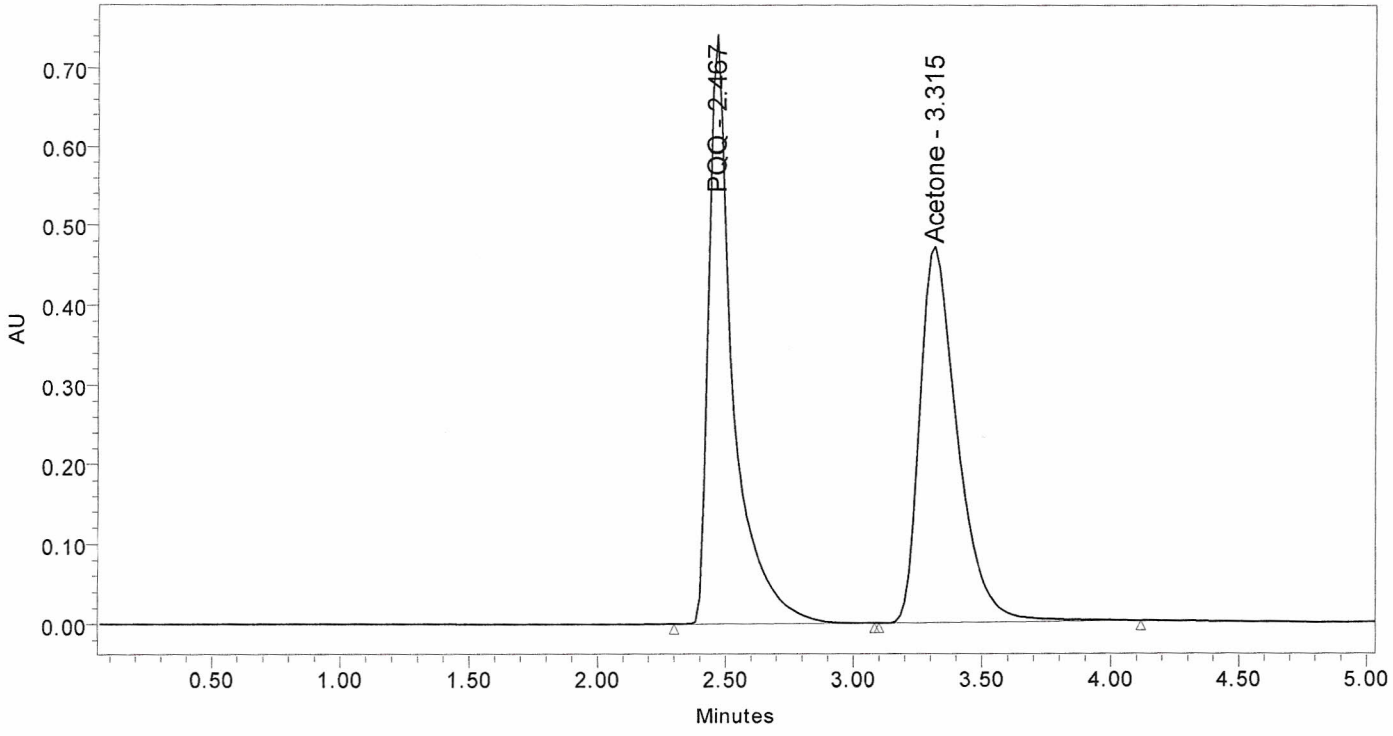
Default Individual Report

Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	PQQ Std	Acquired By:	System
Sample Type:	Standard	Date Acquired:	6/19/2012 12:47:30 PM
Vial:	20	Acq. Method Set:	PQQ
Injection #:	4	Date Processed:	6/19/2012 1:31:11 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.467	4881456	50.53	730122
2	Acetone	3.315	4779766	49.47	470538

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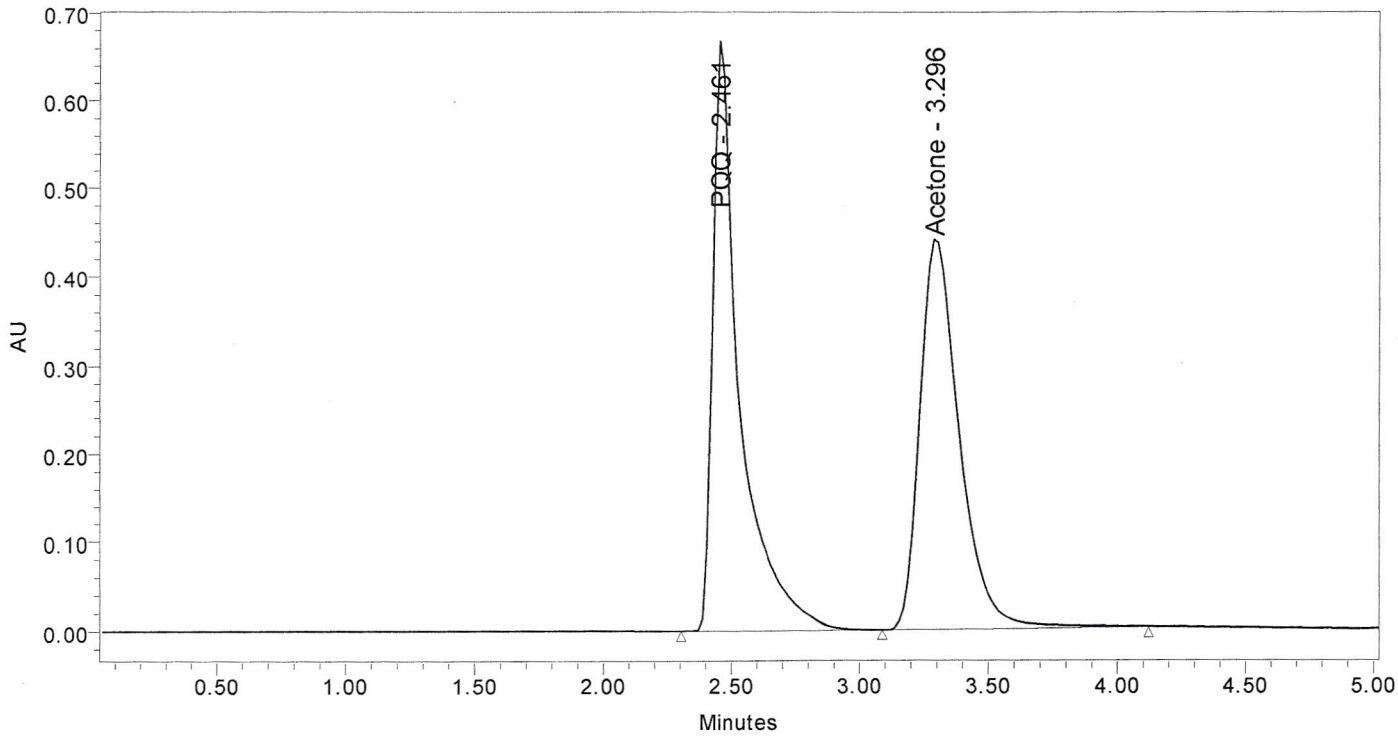
Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	PQQ Std	Acquired By:	System
Sample Type:	Standard	Date Acquired:	6/19/2012 12:53:13 PM
Vial:	20	Acq. Method Set:	PQQ
Injection #:	5	Date Processed:	6/19/2012 1:31:14 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.461	4839748	50.99	665152
2	Acetone	3.296	4651885	49.01	443247

Component Summary For Retention Time
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
1	CH 4120 12	1	996	21	2.459	3.305
2	CH 4120 12	2	996	21	2.474	3.311
Mean					2.466	3.308
Std. Dev.					0.011	0.005
% RSD					0.4	0.1

Component Summary For Area
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
1	CH 4120 12	1	996	21	4839630	4654785
2	CH 4120 12	2	996	21	4834157	4574726
Mean					4836893	4614756
Std. Dev.					3871	56610
% RSD					0.1	1.2

Component Summary For Amount
Channel: 996

	SampleName	Inj	Channel	Vial	PQQ	Acetone
1	CH 4120 12	1	996	21		
2	CH 4120 12	2	996	21		
Mean						
Std. Dev.						
% RSD						

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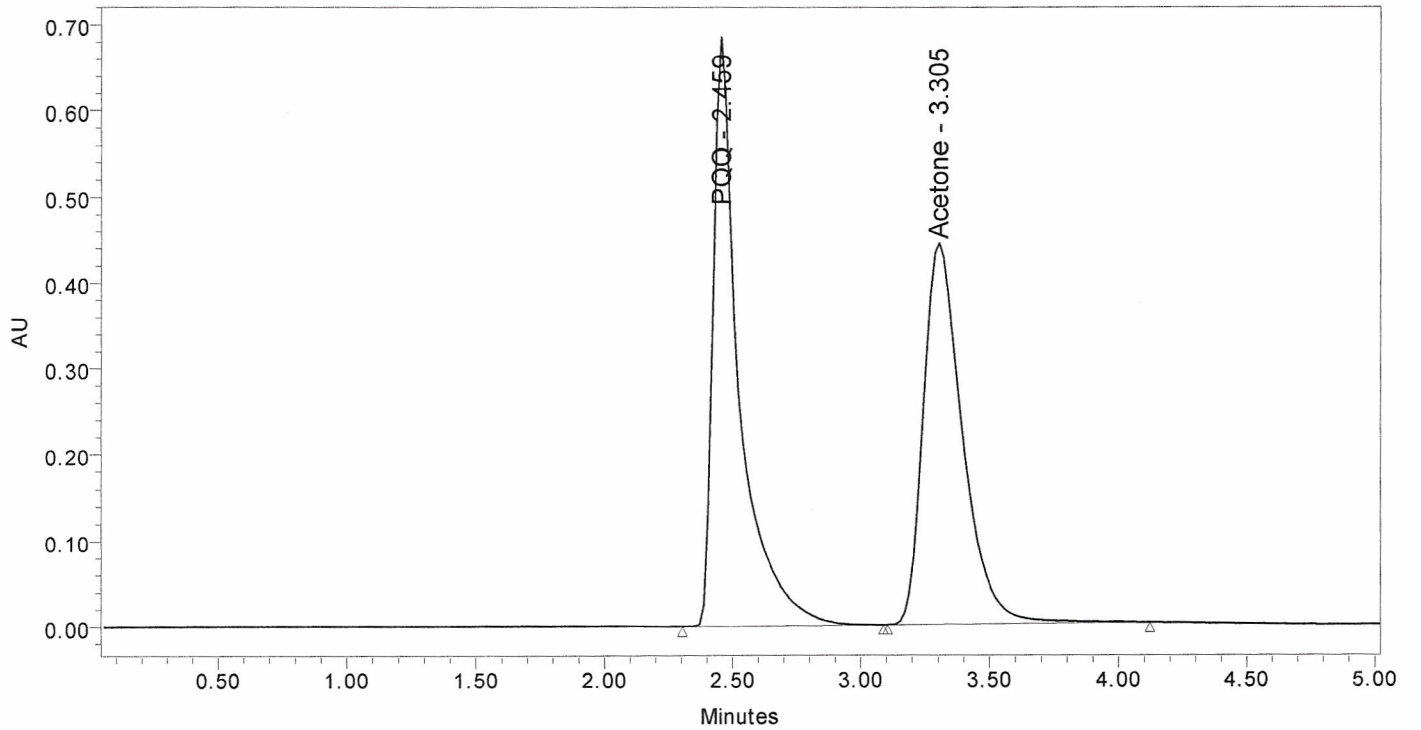
Default Individual Report

Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	CH 4120 12	Acquired By:	System
Sample Type:	Unknow n	Date Acquired:	6/19/2012 12:59:04 PM
Vial:	21	Acq. Method Set:	PQQ
Injection #:	1	Date Processed:	6/19/2012 1:31:18 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.459	4839630	50.97	678087
2	Acetone	3.305	4654785	49.03	443343

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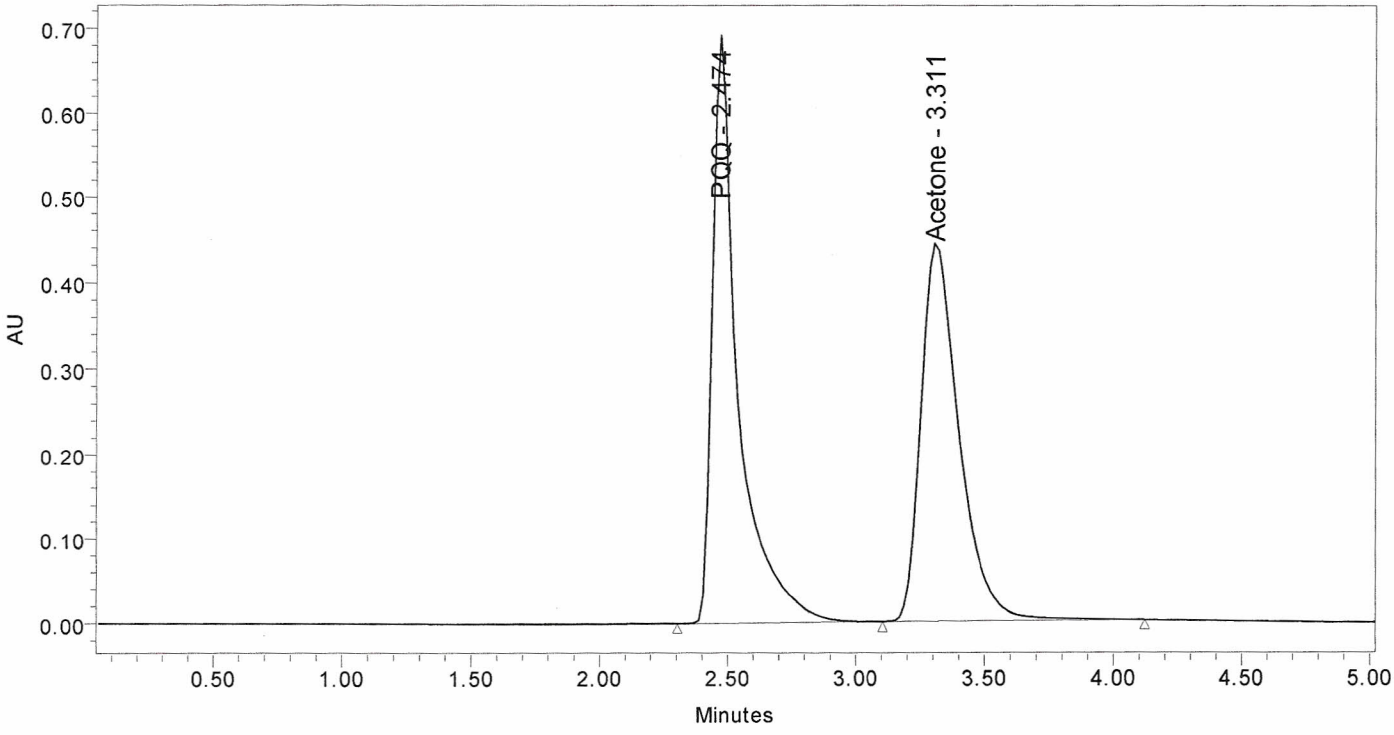
Default Individual Report

Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	CH 4120 12	Acquired By:	System
Sample Type:	Unknow n	Date Acquired:	6/19/2012 1:04:48 PM
Vial:	21	Acq. Method Set:	PQQ
Injection #:	2	Date Processed:	6/19/2012 1:31:21 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.474	4834157	51.38	682587
2	Acetone	3.311	4574726	48.62	444703

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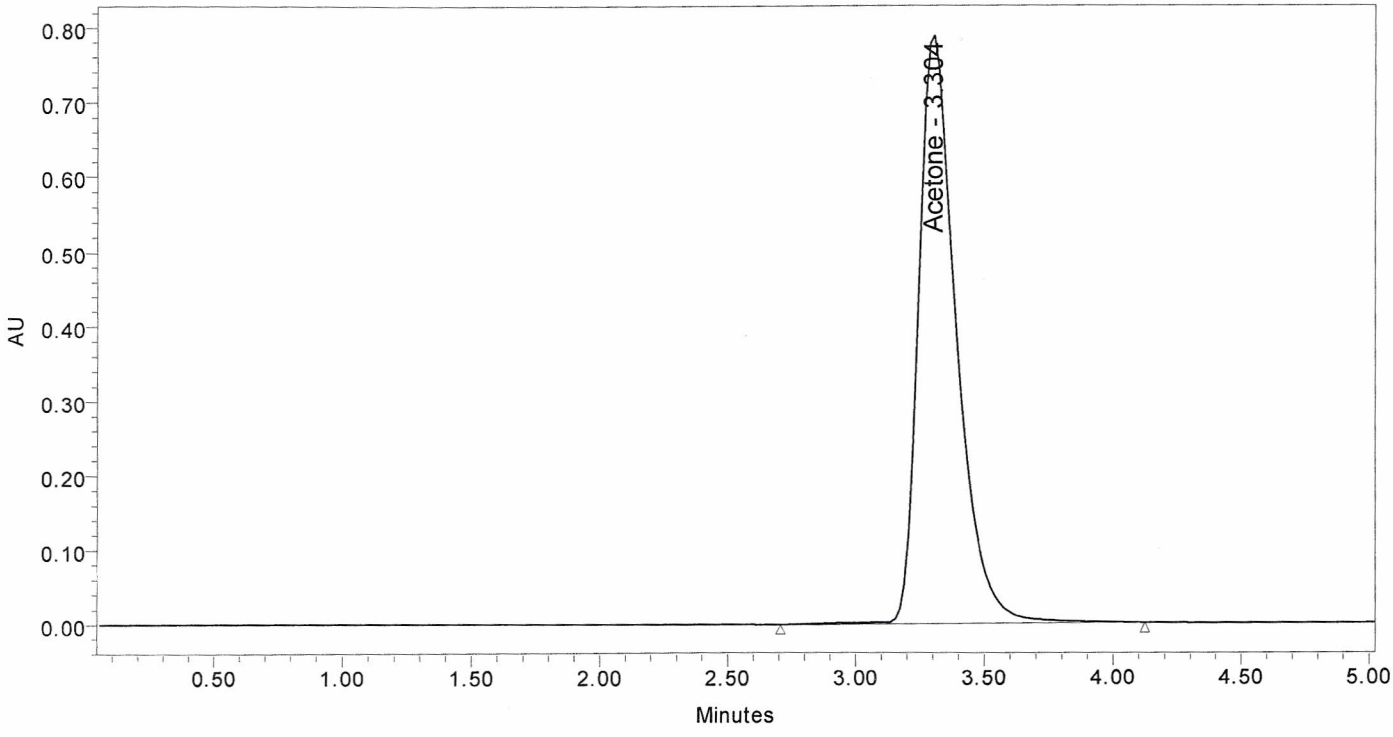
Default Individual Report

Reported by User: System

Project Name: Phytochemicals4

SAMPLE INFORMATION

Sample Name:	16% Acetone	Acquired By:	System
Sample Type:	Control	Date Acquired:	6/19/2012 1:16:26 PM
Vial:	23	Acq. Method Set:	PQQ
Injection #:	1	Date Processed:	6/19/2012 1:31:34 PM
Injection Volume:	10.00 ul	Processing Method:	PQQ
Run Time:	5.0 Minutes	Channel Name:	Extract 254.0
Sample Set Name:	061912 PQQ	Proc. Chnl. Descr.:	PDA 254.0 nm



	Peak Name	RT	Area	% Area	Height
1	PQQ	2.467			
2	Acetone	3.304	8005857	100.00	789533