# DPPH Antioxidant Assay Kit

Cat# AOX-3

## INSTRUCTION MANUAL ZBM0087.00

## STORAGE CONDITIONS

All orders are delivered via Federal Express Priority courier at 4°C.
All orders must be processed immediately upon arrival.

**DPPH**

Store at 4°C.

**Trolox standard reagent**

Store at -20°C

**Trolox Dilution Buffer and Assay Plate**

Store at room temperature

**Long-term storage:**

Remove the DPPH from the box and place at 4°C, store the Trolox solution at -20°C. Reagents are good for at least 3 months after arrival if stored properly.

**For *in vitro* Use Only**

### LIMITED PRODUCT WARRANTY

This warranty limits our liability to replacement of this product. No other warranties of any kind, expressed or implied, including without limitation, implied warranties of merchantability or fitness for a particular purpose, are provided by Zen-Bio, Inc. Zen-Bio, Inc. shall have no liability for any direct, indirect, consequential, or incidental damages arising out of the use, the results of use, or the inability to use this product.

### ORDERING INFORMATION AND TECHNICAL SERVICES


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# INTRODUCTION

Free radicals and reactive oxygen species (ROS) are highly reactive molecules that are generated by normal cellular processes, environmental stresses, and UV irradiation. ROS react with cellular components, damaging DNA, carbohydrates, proteins, and lipids causing cellular and tissue injury. Excess production of reactive oxygen species can also lead to inflammation, premature aging disorders, and several disease states, including cancer, diabetes, and atherosclerosis. Organisms have developed complex antioxidant systems to protect themselves from oxidative stress; however, excess ROS can overwhelm the systems and cause severe damage.

The Zen-Bio DPPH Antioxidant Assay Kit can be used to determine the antioxidant capacity of biological fluids, cells, and tissue. It can also be used to assay the antioxidant activity of naturally occurring or synthetic compounds for use as dietary supplements, topical protection, and therapeutics. The assay measures the reduction of the stable DPPH radical by electron transfer. Trolox [6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid], a water soluble vitamin E analog, serves as a positive control reducing the DPPH radical in a dose dependent manner. The antioxidant activity in the test samples can be normalized to equivalent Trolox units to quantify the composite antioxidant activity present. This assay measures radical scavenging by electron donation and when combined with Zen-Bio's other antioxidant assay kits, provides a comprehensive analysis of a test sample's antioxidant activity.

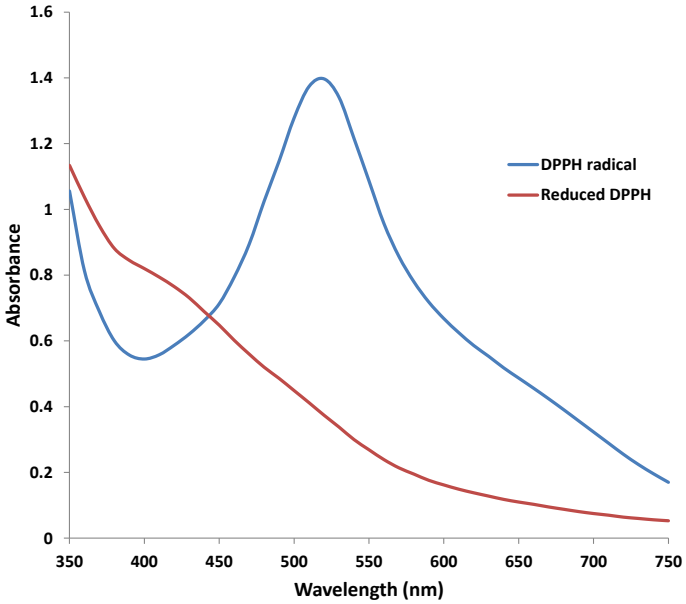


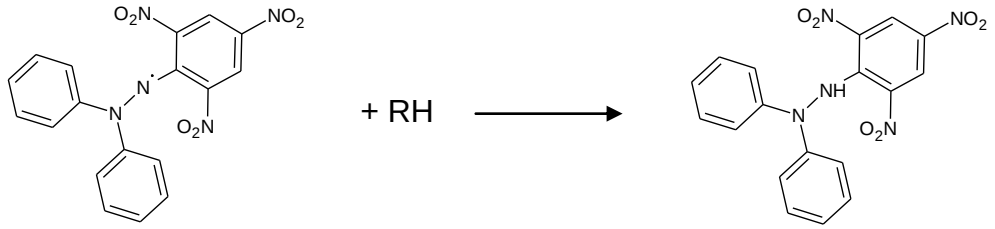

**Figure 1. DPPH radical and reduced DPPH absorbance specta**

500 uM DPPH radical and its reduced form were scanned from 350 nm to 750 nm for their absorbance spectra. The DPPH radical has a strong absorption peak at 517nm that is greatly diminished in the reduced form.

# PRINCIPLE OF THE ASSAY

DPPH (1,1-diphenyl-2-picryl-hydrazyl) is considered a stable radical and has a strong absorbance at 517nm due to an odd electron destabilized across the molecule. In the presence of a hydrogen donor (free radical scavenger), the odd electron is paired to form 1,1-diphenyl-2-picryl-hydrazine and the absorbance at 517nm is reduced in a 1:1 reaction. In this assay, we use trolox as the standard radical scavenger. The deep purple color of the DPPH free radical is decreased in the presence of increasing concentrations of trolox reducing the

absorbance at 517nm. Antioxidants reduce the DPPH radical color by hydrogen donation in a concentration dependent manner and the relative strengths of these activities can be determined by comparison to the trolox standard.




# ITEMS INCLUDED IN THE KIT

| ITEM | DESCRIPTION | Color | UNIT | QTY | STORAGE |
|---|---|---|---|---|---|
| Blank Assay Plates | 96-well assay plates, blank | --- | PLATE | 1 | ----- |
| Trolox Dilution Buffer | 2 ml | AMBER | BOTTLE | 1 | RT |
| DPPH | 5 mg | AMBER | BOTTLE | 1 | 4°C |
| Trolox Standard | 5.0 mM in Dilution Buffer | AMBER | 110 µl /VIAL | 1 | -20°C |
| Tray | For multi-channel pipettes | --- | EACH | 2 | RT |

**Other equipment/reagents required but not provided with the kit**:

- Methanol
- Multi-channel Pipet , single channel pipet and pipet tips
- Tube for preparing 12ml DPPH working solution (e.g. 15 ml conical tube).
- Small tubes for preparing Trolox standard curve (Optional – can use a microplate).
- Plate reader with a filter of 517 nm
- Optional – plate reader capable of kinetic measurement at 517 nm

**Reagents that might interfere with the assay results:**

| | |
|---|---|
| > 1% TWEEN 20 | 2-mercaptoethanol |
| > 1% TRITON X-100 | Tris |
| IGEPAL CA-630 (Nonidet P-40) | Borate |
| > 0.2% CHAPS | DTT |

# SAMPLE PREPARATION

## Cell Lysate Preparation

1. Scrape ~1 x$10^6$ cells and centrifuge at 1,000xg to prepare a cell pellet. DO NOT use proteolytic enzymes such as trypsin but scrape using a rubber policeman or cell scraper tool.
2. Homogenize or sonicate the cell pellet on ice in 1ml cold AOX Assay buffer
3. Centrifuge at 10,000 x g for 15 minutes at 4°C.
4. Remove the supernatant and keep on ice until ready to use in the assay.
5. If not using the same day, store the samples at -80°C.
6. Data is expressed as Trolox equivalents (TE) per cell number (i.e. µmole TE/$10^6$ cells)

## Tissue Lysate Preparation

1. Homogenize tissue samples on ice in cold buffer at ~200mg tissue per ml cold buffer
2. Centrifuge at 10,000 x g for 15 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as Trolox equivalents (TE) per gram of starting sample (i.e. µM TE/g)

## Plasma Preparation

1. Collect the blood in a tube containing heparin or other anticoagulant.
2. Centrifuge at 1,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

## Serum Preparation

1. Collect the blood in a tube WITHOUT any anticoagulant. Allow the blood to clot.
2. Centrifuge at 2,000 x g for 10 minutes at 4°C.
3. Remove the supernatant and keep on ice until ready to use in the assay.
4. If not using the same day, store the samples in small aliquots at -80°C.
5. Data is expressed as micromoles Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

## Saliva Collection

1. Collect whole saliva for a defined period of time (i.e. 1-5 minutes) into polypropylene tubes.
2. Immediately place on ice or store at -80°C for later analysis.
3. Data is expressed as micromoles Trolox equivalents (TE) per volume sample (i.e. µmole TE/L)

## Food Extract Preparation

1. Weigh the starting material.
2. Homogenize in a small volume ice cold buffer or water.
3. Store small aliquots at -80°C for analysis.
4. When ready to assay, keep thawed samples on ice.
5. Data is expressed as Trolox equivalents (TE) per gram of starting sample (i.e. µM TE/g)

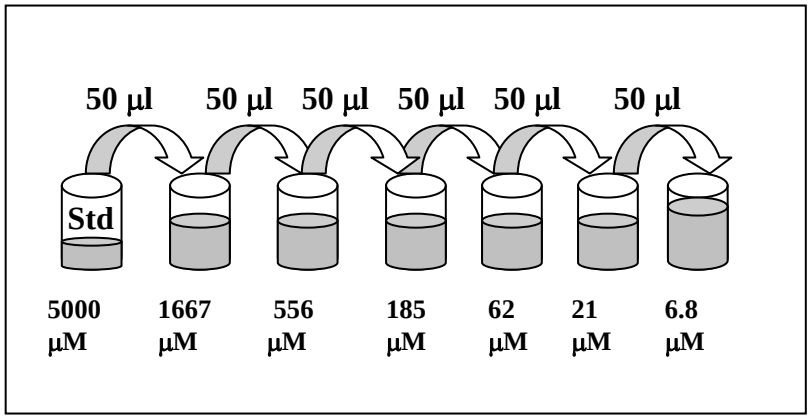
# ASSAY PROCEDURE

1. Remove the DPPH from the refrigerator and add 12.7 ml of methanol (not provided) to make a 1 mM DPPH stock solution.

2. Prepare a working 250 uM DPPH solution by diluting 3.0 ml of the 1 mM DPPH stock solution into an additional 9 ml of methanol. Keep this solution protected from light prior to use.

3. Prepare Trolox standards as follows:

   Briefly spin down the contents of the 5000 μM Trolox standard tube after thawing. Pipette 100 μl of Trolox Dilution Buffer into 6 tubes (not provided). Prepare a dilution series as depicted below. Mix each the new dilution thoroughly before proceeding to the next. The 5000 μM stock serves as the highest standard, and the Trolox Dilution Buffer serves as the zero standard.




4. Add 20 μl of samples or Trolox standards to duplicate wells of the assay plate provided, add 20 μl of Trolox Dilution Buffer to duplicate wells as a negative control.

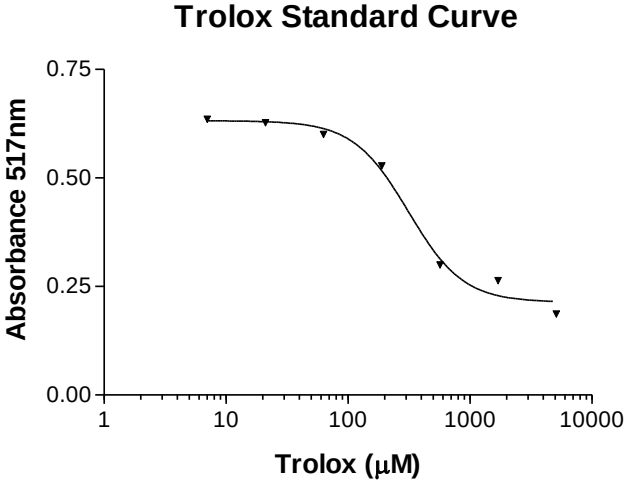
5. To begin the assay, add 80 μl of the DPPH working solution per well and place on plate shaker at room temperature protected from light. Allow the reaction to proceed for 10 minutes.
   **OPTIONAL: if performing a kinetic assay, do not pre-incubate for 10 minutes, but directly proceed to kinetic absorbance reading (See Page 8).**

6. Read absorbance using plate reader at a wavelength of 517 nm.
   **OPTIONAL: Kinetic absorbance readings (517 nm) may be acquired every minute for 10 minutes to determine Area Under the Curve (AUC) values.**

# TROLOX STANDARD CURVE

Generate standard curve: see example below
[DO NOT use this standard curve to generate your data. This is an example.]

| µM Trolox | Abs 1 | Abs 2 | Average | Stdev |
|---|---|---|---|---|
| 5000.00 | 0.187 | 0.189 | 0.188 | 0.001 |
| 1666.67 | 0.265 | 0.268 | 0.267 | 0.002 |
| 555.56 | 0.306 | 0.303 | 0.305 | 0.002 |
| 185.19 | 0.526 | 0.526 | 0.526 | 0.000 |
| 61.73 | 0.602 | 0.606 | 0.604 | 0.003 |
| 20.58 | 0.63 | 0.622 | 0.626 | 0.006 |
| 6.86 | 0.63 | 0.643 | 0.637 | 0.009 |
| 0 | 0.635 | 0.613 | 0.624 | 0.016 |




A sigmoidal dose curve fit can be used for the data.
Bottom = 0.662
Top = 0.1533
Log EC50 = 2.56

Y= Bottom + (Top-Bottom)/(1+ 10^((LogEC50-X))).

y = observed O.D.
x = log (concentration of Trolox in µM)
To calculate x for each y, (i.e. to change the observed O.D. into Trolox equivalent concentration) use the following equation:

X = LogEC50 – Log(((Top-Bottom)/(Y-Bottom)) – 1)
X = 2.56 – Log(((0.1533 – 0.662)/(Y – 0.662)) – 1)

To calculate the µM Trolox equivalent: calculate 10^X.

Antioxidant activity is expressed as µM Trolox equivalents (TE).

Alternatively, antioxidant activity can be expressed as µmole Trolox / gram of starting material. This can be calculated by using the µM Trolox determined above and the concentration of the test sample in mg/ml. Where mg/ml = g/L and µM Trolox = µmole Trolox / L:

**Example**: X mg sample / ml gave Y µM Trolox equivalents.
Is the same as: X g sample / L = Y µmole Trolox / L
The activity is: 1 g sample = (Y / X) µmole Trolox or (Y/X) µmole Trolox per gram sample

# *OPTIONAL KINETIC ASSAY PROCEDURE*

1. Prepare Trolox and DPPH reagents as described above.

2. Set-up plate reader for kinetic reading mode:
   Total time = 10 minutes
   Interval = 60 seconds
   Absorbance= 517 nm

3. Add 20 µl of samples or Trolox standards to duplicate wells of the assay plate provided, add 20 µl of Trolox Dilution Buffer to individual wells as a negative control.

4. To begin the assay, add 80 µl of the DPPH working solution per well and place in plate reader and begin reading absorbance using the kinetic mode.

# *TROLOX KINETIC STANDARD CURVE*

**Trolox concentration (µM)**

| | 5000 | | 1666.67 | | 555.56 | | 185.19 | | 61.73 | | 20.58 | | 6.86 | | 0 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 | Rep 1 | Rep 2 |
| 0:00 | 0.221 | 0.219 | 0.333 | 0.322 | 0.452 | 0.449 | 0.567 | 0.574 | 0.611 | 0.632 | 0.644 | 0.644 | 0.65 | 0.674 | 0.648 | 0.647 |
| 1:00 | 0.198 | 0.196 | 0.293 | 0.285 | 0.414 | 0.407 | 0.547 | 0.544 | 0.605 | 0.631 | 0.639 | 0.637 | 0.641 | 0.663 | 0.639 | 0.64 |
| 2:00 | 0.187 | 0.183 | 0.271 | 0.266 | 0.385 | 0.38 | 0.527 | 0.527 | 0.593 | 0.619 | 0.626 | 0.634 | 0.626 | 0.658 | 0.634 | 0.626 |
| 3:00 | 0.182 | 0.18 | 0.259 | 0.256 | 0.358 | 0.359 | 0.516 | 0.515 | 0.578 | 0.607 | 0.616 | 0.621 | 0.624 | 0.651 | 0.624 | 0.609 |
| 4:00 | 0.181 | 0.181 | 0.257 | 0.255 | 0.343 | 0.349 | 0.507 | 0.51 | 0.579 | 0.609 | 0.612 | 0.61 | 0.616 | 0.647 | 0.611 | 0.606 |
| 5:00 | 0.18 | 0.181 | 0.254 | 0.255 | 0.351 | 0.34 | 0.51 | 0.508 | 0.569 | 0.613 | 0.605 | 0.599 | 0.612 | 0.646 | 0.611 | 0.617 |
| 6:00 | 0.18 | 0.18 | 0.262 | 0.256 | 0.339 | 0.329 | 0.497 | 0.504 | 0.573 | 0.61 | 0.597 | 0.619 | 0.608 | 0.655 | 0.598 | 0.597 |
| 7:00 | 0.182 | 0.184 | 0.253 | 0.254 | 0.329 | 0.322 | 0.503 | 0.503 | 0.571 | 0.608 | 0.592 | 0.629 | 0.6 | 0.662 | 0.615 | 0.628 |
| 8:00 | 0.184 | 0.188 | 0.257 | 0.257 | 0.321 | 0.318 | 0.5 | 0.523 | 0.551 | 0.618 | 0.598 | 0.64 | 0.608 | 0.661 | 0.632 | 0.633 |
| 9:00 | 0.19 | 0.189 | 0.264 | 0.266 | 0.313 | 0.311 | 0.52 | 0.529 | 0.586 | 0.614 | 0.614 | 0.628 | 0.619 | 0.648 | 0.647 | 0.621 |
| 10:00 | 0.187 | 0.189 | 0.265 | 0.268 | 0.306 | 0.303 | 0.526 | 0.526 | 0.602 | 0.606 | 0.63 | 0.622 | 0.63 | 0.643 | 0.635 | 0.613 |

| µM Trolox | AUC 1 | AUC 2 | Average | Stdev |
|---|---|---|---|---|
| 5000.00 | 112.08 | 111.96 | 112.020 | 0.085 |
| 1666.67 | 160.14 | 158.7 | 159.420 | 1.018 |
| 555.56 | 211.92 | 209.46 | 210.690 | 1.739 |
| 185.19 | 310.41 | 312.78 | 311.595 | 1.676 |
| 61.73 | 348.69 | 368.88 | 358.785 | 14.276 |
| 20.58 | 368.16 | 375 | 371.580 | 4.837 |
| 6.86 | 371.64 | 392.97 | 382.305 | 15.083 |
| 0 | 375.15 | 372.42 | 373.785 | 1.930 |

Area under the curve (AUC)
AUC= 0.5+ f0+f1+...+f20+(0.5*f21)
Where fx = Abs value at time points 0 – 10 min
**(most plate readers will calculate this for you)**

**Figure 2. Absorbance over time for Trolox standards**

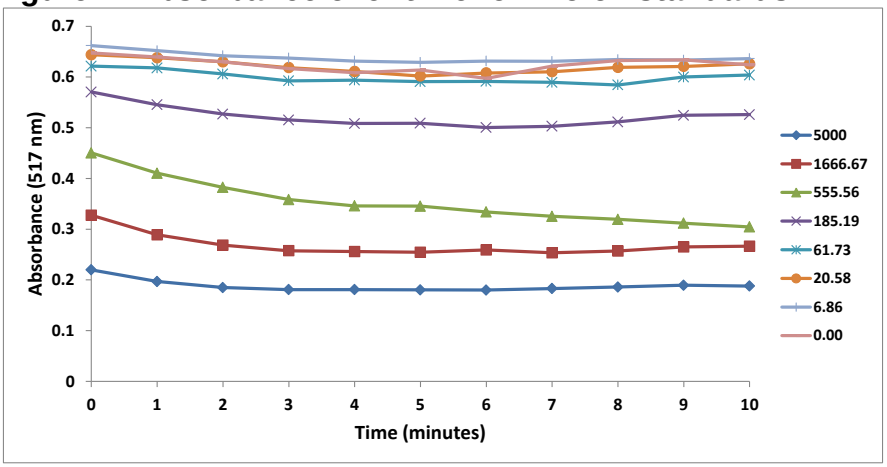
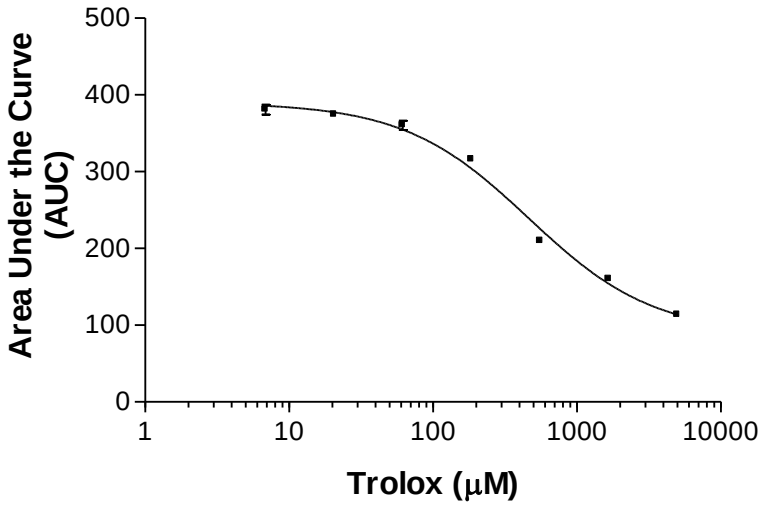


**Figure 3. AUC values for increasing Trolox concentrations**




Analysis of test samples can be carried out as described above to determine μM Trolox equivalent or μmole Trolox per gram of starting material.

# APPENDIX A: Plate layout _____

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 5000 uM T | 5000 uM T | | | | | | | | | | |
| B | 1667 uM T | 1667 uM T | | | | | | | | | | |
| C | 555 uM T | 555 uM T | | | | | | | | | | |
| D | 185 uM T | 185 uM T | | | | | | | | | | |
| E | 62 uM T | 62 uM T | | | | | | | | | | |
| F | 21 uM T | 21 uM T | | | | | | | | | | |
| G | 7 uM T | 7 uM T | | | | | | | | | | |
| H | 0 uM T | 0 uM T | | | | | | | | | | |

# APPENDIX B: Protocol Flowchart

**DPPH ASSAY**

Make necessary test compound dilutions in Trolox Dilution Buffer.

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Prior to assay, prepare DPPH working solution and Trolox standards.

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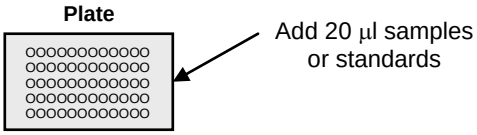
Add 20 µl/well samples or standards to blank assay plate.

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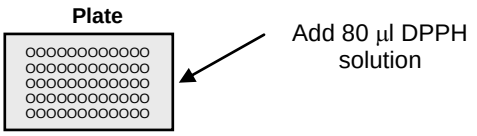
Start assay by adding 80 µl/well DPPH solution.




↓

Incubate at 25°C (room temperature) for 10 minutes away from light.

*Optionally, perform kinetic assay by placing in plate reader and measure absorbance at 517 nm every 60 seconds for 10 minutes.*

↓

Endpoint Assay: Measure the optical density of each well at 517 nm using a spectrophotometer plate reader.

## REFERENCES


1. J. Food Sci. Technol., 48(4):412-422, 2011.
2. Chem. Pap., 61(3): 214-216, 2007.
3. Indian J Biochem & Biophy. 40: 416-422, 2003.