Ferric reducing ability of plasma (frap) experiment

Antioxidants are compounds that can protect the body or foods against the potentially harmful effects of free radicals or reactive oxygen species (ROS).

In this laboratory practical, you will use a method called the ferric reducing ability of plasma (FRAP) assay to measure the ‘ antioxidant power’ of a number of plasma and food samples.

The method measures the ability of antioxidants in plasma or in foods to reduce the ferric component (Fe3+) of a ferric tripyridyltriazine (Fe3+-TPTZ) complex (which is contained in the FRAP reagent) to the ferrous form (Fe2+). During this reaction which takes place at a low pH, the reduction of ferric iron (Fe3+) to the ferrous form (Fe2+) is accompanied by the formation of a blue colour which can be measured at an absorption maximum of 593nm using a spectrophotometer.

The human body is constantly under attack from free radicals and ROS which are produced endogenously in the body as by products of normal aerobic metabolism and enzyme systems as well as being supplied from sources outside the body from cigarette smoke, lipid peroxidation products in foods or from pollutants. In order to protect the body from inappropriate exposure to these free radicals and ROS, the human body has developed a powerful and complex antioxidant defence system. Enzymatic antioxidants within the cell such as superoxide dismutase function by inactivating or removing ROS from the cell before they can cause damage. Non-enzymatic protein antioxidants function by controlling the storage and release of metal ions which are needed for the enzymatic antioxidants to function but can also convert relatively unreactive radicals such as superoxide to the much more

reactive hydroxyl radical. However, antioxidants such as vitamin C, ï)¡-tocopherol, carotenoids and phenolic compounds such as flavonols act as hyrdrogen (and electron) atom donors, and in this way behave as reducing agents. It is this ability to act as a reducing agent that is used to measure ‘ antioxidant potential’ in the FRAP assay.

When the FRAP assay is carried out with foodstuffs, antioxidants contained in the foods that behave as reducing agents convert Fe3+ in the ferric complex (Fe3+-TPTZ) to Fe2+ions forming a blue colour in the same way as non-enzymatic antioxidants contained in plasma. However, whether antioxidants contained in foods can exhibit antioxidant potential when taken into the body will depend on their bioavailability i. e. absorption and incorporation into body tissues.

The FRAP assay offers a simple index of ‘ antioxidant or reducing power’ and as you will see the method can be used easily with plasma and foodstuffs. Furthermore, the results are reported to be highly reproducible and the procedure is straightforward and easy to carry out.

However, there are also a number of limitations associated with the use of this assay for measuring ‘ antioxidant power’. The most obvious limitation of the FRAP assay is that while it claims to measure ‘ antioxidant power’, it actually only measures ‘ antioxidant power’ of non-enzymatic antioxidants that act as reducing agents. While the FRAP assay can be used to measure the ‘ antioxidant power’ of foodstuffs, the results to not give us any information on the bioavailability of these antioxidants in the body and thus about their actual physiological ‘ antioxidant power’ in vivo. In addition,

substances that bind with either Fe3+ or Fe2+ could in theory interfere with the results of the assay.

Aim

The aim of this experiment is to use the FRAP assay to measure the ‘ antioxidant power’ of a number of plasma and food samples.

Objectives:

By the end of this practical session and the lecture on antioxidants you should be able to...

1. List the different type of antioxidants in the body.

2. Explain the mechanism of action of the different type of antioxidants.

3. List the food sources of antioxidants.

4. List examples of free radicals and reactive oxygen species and their sources.

5. Explain the principle on which the FRAP assay is based.

6. Explain the main limitations of the assay.

Part A

Experiment

This experiment involves constructing a standard curve with concentrations of 0-1. 0mM ferrous sulphate (FeSO4, Fe2+) and carrying out the FRAP assay on 6 food samples and 4 plasma samples.

1. For this practical you will need to assemble the following items on your bench space:

A tube containing the standard solution (2mM FeSO4)

Distilled H2O

6 food samples

4 plasma samples

Rack with 6 universal tubes (each containing 24 ml distilled H2O),

Rack with 6 plastic tubes for making up the standards,

Marker (for labelling)

Plastic cuvettes + cuvette box

200ïl pipette

5 ml pipette

1 ml pipette

Note: Before you start the experiment, you should switch on your spectrophotometer and set the wavelength to 593nm (you can ask your demonstrator for help).

1. Pipette the standards, food samples and plasma samples into the plastic cuvettes by following the instructions below:

Standard

Firstly, you need to prepare dilutions of the stock standard in distilled H2O so that you can produce a standard curve in the range of 0. 1-1. 0 mM.

You should have a rack with 6 plastic test tubes + lids on your bench.

You should label these tubes Blank, STD 0. 2, STD 0. 4, STD 0. 6, STD 0. 8 and STD 1. 0.

You should have a tube containing the standard solution (2. 0 mM FeSO4)

You should have a container of distilled H2O.

You should make the dilutions of the standard as follows (see table on next page):

Standard

(final Ferrous Sulphate Concentration, mM)

Volume (ml) of 2 mM Ferrous Sulphate to be added

Volume (ml) of distilled H2O to be added

0 (Blank)

0

10. 0

0. 2

1. 0

9. 0

0. 4

2. 0

8. 0

0. 6

3. 0

7. 0

0. 8

4. 0

6. 0

1. 0

5. 0

5. 0

Mix the contents of these tubes by inversion (with lid on!).

In your cuvette box, you should have 12 plastic cuvettes as you are going to prepare these standards in duplicate.

Label the cuvettes with marker up high on the cuvette on the frosted side.

Pipette 200ïl of each concentration of standard, in duplicate, into the appropriate cuvette.

Food samples

You should have 6 plastic containers of food samples / beverages.

You need to make a 1 in 25 dilution of the food samples and a 1 in 50 dilution of the coffee sample and with distilled H2O. [You should also have a rack containing 6 universal tubes each containing 24 ml of distilled H2O].

Pipette 1 ml of each of the food samples and 0. 5 ml coffee and into a labelled universal tube. Mix the contents using by inversion (with lid on!).

Pipette 200ïl of each of the diluted food samples into the appropriate cuvette.

Plasma samples

You should have plasma samples from 4 individuals namely, a smoker, a healthy adult, a type 2 diabetic patients and an individual with a diet rich in fruit and vegetables. The plasma samples are labelled P1, P2, P3 and P4 but you do not know which plasma sample belongs to which individual.

Pipette 200ïl of each plasma sample into an appropriate cuvette.

2. Add 3ml FRAP solution to each cuvette in the rack. The FRAP reagent has already been prepared for you and is in the 370C water bath back on the bench.

3. Place your cuvette box into the 370C incubator for 4 min (use your watch to time).

4. Read the absorbance of each standard, food and plasma sample (against the blank) at 593nm using the spectrophotometer and write the values in the table below.

Concentration of FeSO4 Standard (mM)

Absorbance @ 593 nm (reading 1)

Absorbance @ 593 nm (reading 2)

Average absorbance reading

0. 0 (blank)

0

0

0

0. 2

0. 262

0. 272

0. 267

0. 4

0. 507

0. 503

0. 505

0. 6

0. 823

0. 793

0. 808

0. 8

1. 084

1. 071

1. 0775

1. 0

1. 351

1. 305

1. 328

Food Samples (dilution factor)

Absorbance @ 593 nm

Coffee (1/50)

0. 786

Green Tea (1/25)

0. 468

Black Tea (1/25)

0. 624

Blackcurrant Juice (1/25)

0. 489

Red Wine (1/25)

1. 323

White Wine (1/25)

0. 122

POM (1/100)

1. 164

Pomegranate Juice (1/50)

0. 800

Plasma Samples

Absorbance @ 593 nm

P1

1. 041

P2

0. 772

P3

0. 566

P4

0. 274

Part B

Calculation of results

1) Using the graph paper provided:

If you did not “ blank” your spectrophotometer, remember to substract your blank reading from all other readings.

Draw your calibration curve.

Determine the slope of the curve (“ a” in the equation y= ax)

Using the equation, work out the antioxidant power of each food sample, in Fe2+ mM equivalent.

For the food samples, you also need to adjust the final concentration for the initial dilution made.

Food sample

Antioxidant Power (mM)

Final Antioxidant Power (mM Fe2+ equivalent) adjusted for the initial dilution

Coffee

0. 587

29. 350

Green Tea

0. 350

8. 738

Black Tea

0. 466

11. 650

Blackcurrant Juice

0. 365

9. 130

Red Wine

0. 988

24. 701

White Wine

0. 091

2. 278

POM

0. 869

86. 931

Pomegranate Juice

0. 597

29. 873

Plasma sample

Antioxidant Power (mM Fe2+ equivalent)

P1

0. 777

P2

0. 577

P3

0. 423

P4

0. 205

Submit, as part of your report, a calibration curve created in Excel, showing the equation.

1. In excel, draw scatter plot with the absorbance reading on Y-axis and concentration of standards on the X-axis as follows:

Type the concentrations and absorbance values for your standards into an excel spread sheet as shown in the example below.

Example:

Concentration (mM FeSO4)

0

0. 2

0. 4

0. 6

0. 8

1. 0

Absorbance

Using your cursor, highlight the information you have typed and click on the chart wizard button on the bar at the top of the screen.

Choose xy (Scatter) which will draw a scatter plot of the absorbance and concentration values. Click Finish.

Hold down the control key and click on any of the points on your scatter plot.

On the menu bar, click on chart, and choose add trend line.

Under Type – choose a linear trend line

Under options and tick the following.

ïÐ¡ Set intercept = 0

ïÐ¡ Display equation on chart

ïÐ¡ Display R-square value on chart

2. This should produce a straight line through your points and through zero. There will be an equation displayed on the chart and an R-squared value. The R-squared value should be as close as possible to 0. 999.

Use the equation of the line to calculate Antioxidant Power of the samples.

1. You now have an equation of the line that you will use to calculate the concentrations for each of your samples.

For example:

Equation of line y = 0. 7543x

Remember y = absorbance

x = concentration

You have measured the absorbance values of your samples and you want to find the concentration.

x = y/0. 7543

2. Calculate the concentration for each sample and write the value in the table below. For the food samples, you also need to adjust the final concentration for the initial dilution made.

Food sample

Antioxidant Power (mM Fe2+ equivalent)

Final Antioxidant Power (mM Fe2+ equivalent) adjusted for the initial dilution

Red wine

0. 587

29. 350

White wine

0. 350

8. 738

Blackcurrant juice

0. 466

11. 650

Coffee

0. 365

9. 130

Black tea

0. 988

24. 701

Green tea

0. 091

2. 278

POM

0. 869

86. 931

Pomegranate Juice

0. 597

29. 873

Plasma sample

Antioxidant Power (mM Fe2+ equivalent)

P1

0. 777

P2

0. 577

P3

0. 423

P4

0. 205

Part C

Questions

1. What were the FRAP values or ‘ antioxidant power’ values (in Fe2+ mM equivalent) of the 4 plasma samples?

P1 =

P2 =

P3 =

P4 =

2. The plasma samples that you analysed were taken from 4 individuals, namely, a smoker, a type 2 diabetic, a healthy adult and a healthy adult with a diet rich in fruit and vegetables. Which plasma sample do you think belongs to each individual and why?

P1 –

P2 –

P3 –

P4 –

3. What were the FRAP or ‘ antioxidant power’ values (in mM Fe2+ equivalent) for each of your food samples?

4. List the antioxidants present in each of the foods, which could contribute towards the ‘ antioxidant power’ values that you have measured?

Sample number	Food name / type	Main antioxidants
1		
2		
3		
4		

5

6

5. Can you explain briefly how the FRAP assay claims to measure ‘ antioxidant power’?

6. What are the main limitations of measuring ‘ antioxidant power’ using the FRAP assay?

7. The ‘ antioxidant power’ that you have measured is greater for some foods compared to others. What factors would influence whether these foods could exert antioxidant potential in the body?