Method: FSG Issue: 003
Created: 18 February 2010
Revision date: 18 February 2011

Spectrophotometric method for the determination of the cyanogenic potential of foods and beverages

Author: M.Scotter
Issued by: C. Crews (Accreditation Co-ordinator)

FERA

Food and Environment Research Agency
Sand Hutton
York YO41 1LZ

1. SAFETY

This method involves the use of several hazardous chemicals and procedures likely to produce an increased risk to the operator. A COSHH assessment should be written to identify the risks associated with this method.

1.1. General precautions

1.1.1. Protective clothing including laboratory coat (buttoned), safety spectacles and gloves should be worn at all times.

1.1.2.1 Samples should be regarded as a biological hazard. Direct contact with skin is best avoided and proper attention to hygiene must be maintained.

1.2. COSHH

UNDER THE CONTROL OF SUBSTANCES HAZARDOUS TO HEALTH (COSHH) REGULATIONS THIS METHOD HAS BEEN ASSESSED FOR ANY RISK TO HEALTH TO OPERATORS THROUGH ITS USE. A FULL ASSESSMENT HAS BEEN COMPLETED AND IS KEPT ON FILE (x). THIS ASSESSMENT IS ACCESSIBLE TO STAFF AND BEFORE STARTING THIS METHOD ANYONE USING THIS PROCEDURE MUST HAVE A COPY OF THE COSHH ASSESSMENT.

Particular attention is drawn to the following toxic chemical(s). Contact by inhalation, skin absorption or ingestion must be avoided. Work in a fume cupboard where designated and wear protective eyewear and gloves. Apparatus should be set up in a tray which will retain the reagent(s) should the apparatus break. Every precaution must be taken to avoid spillage of reagents and a sufficient supply of sodium hypochlorite solution (7.1.11) must be at hand.

Chemical	**Classification**
Potassium cyanide	T+ VERY TOXIC

There is no implied safety for any other compounds excluded from this list

1.3. First Aid

Any injury must be reported, in the first instance, to a qualified First Aider and recorded. The First Aider will decide on further action. All accidents, incidents and near misses should be reported to the Health and Safety team.

Emergency Procedures

a) Persons in the vicinity must summon assistance from Occupational Health and Safety Team.
b) In the event of an uncontrolled release of cyanide, the area must be evacuated.
c) If it is suspected that a person has been exposed to cyanide the following actions will be taken:
(i) The person will be removed from the area by a member of the Health and Safety Team (using Breathing Apparatus if necessary).
(ii) Contaminated clothing will be removed and affected areas copiously washed with water.
(iii) Emergency medical treatment will be administered by trained individuals and/or Occupational Health Staff.

2. INTRODUCTION

Cyanogenic glucosides present a potential problem in certain foods and beverages, due to the release of toxic cyanide upon hydrolysis by indigenous enzymes or in the gut. Cyanide is one of several biologically active principles (BAPs), which are undesirable chemical substances that are present naturally in some flavourings and food. A new EU Regulations has recently been published (EC 1334/2008) that focuses on the foods and beverages through which BAPs contribute most to the diet. The new Regulation sets the following numerical limits on hydrogen cyanide (hydrocyanic acid) in food and beverages:

Nougat, marzipan, or its substitutes or similar products	50 mg/kg
Canned stone fruits	5 mg/kg
Alcoholic beverages	35 mg/kg

3. SCOPE

This method is applicable to marzipan, or its substitutes or similar products; canned stone fruits; alcoholic beverages. The limit of quantitation is 1 mg/kg HCN equivalents for marzipan and canned stone fruits, and 0.1 mg/kg for alcoholic beverages.

4. PRINCIPLE

Samples are homogenised under alkaline conditions and extracted in acidic medium, centrifuged and an aliquot of the supernatant hydrolysed enzymatically to convert glycosides to cyanohydrins. The cyanohydrins are in turn hydrolysed to free cyanide in strong alkaline solution and determined spectrophotometrically after chemical conjugation.

5. SAMPLING

Samples are homogenised and sub-sample taken and stored at an appropriate temperature.

6. APPARATUS

General laboratory glassware to be used except where stipulated. Unless otherwise stated volumetric glassware should be of grade ‘A’ quality. Laboratory equipment may be generic, e.g., balances and homogenization equipment.

6.1 Homogeniser for sample preparation, Ultra-Turrax type T-25 or equivalent with 25mm dispersion probe or suitable blender/food processor. For sample extraction an 8-10mm Ultra-Turrax dispersion probe is used.
6.2 Centrifuge capable of 3,000 x *g.*
6.3 Centrifuge tubes, Nunc 50mL with screw cap or equivalent.
6.4 Oven/incubator set at 30 ± 1℃.
6.5 Positive displacement pipettes.
6.6 Shaker, orbital type.
6.7 Spectrophotometer capable of measuring absorbance at 605nm.
6.8 Spectrophotometer cuvettes, 1cm path length.
6.9 Vials, 10mL glass with screw cap

6.10 Syringe filters, 0.45µm PTFE.
6.11 Water/ice bath for chilling to ca 5℃.

7. REAGENTS

7.1 Chemicals

All chemicals should be of recognised analytical grade unless specified otherwise.

7.1.1 Amygdalin (trihydrate), ca. 99% e.g. Sigma-Aldrich #A6005-5G.
7.1.2 Chloramine-T, e.g. Sigma-Aldrich #31224-250G.
7.1.3 Cyanide test papers e.g. Merckoquant cyanide test kit, VWR #1.10044.0001.
7.1.4 1,3-Dimethylbarbituric acid, e.g. Sigma-Aldrich #39565-150G.
7.1.5 β-Glucosidase EC 3.2.1.21, e.g. Sigma-Aldrich #49290-250MG.
7.1.6 Hydrochloric acid.
7.1.7 Isonicotinic acid, e.g. Sigma-Aldrich #I17508-100G.
7.1.8 Orthophosphoric acid.
7.1.9 Potassium cyanide.
7.1.10 Starch-iodide test papers, e.g. Sigma-Aldrich #37215-1EA.
7.1.11 Sodium hydroxide.
7.1.12 Sodium hypochlorite solution 10-15%, general purpose reagent.
7.1.13 Trisodium phosphate.
7.1.14 Water, HPLC grade.

7.2 Solutions

7.2.1 Hydrochloric acid 1M.
7.2.2 Orthophosphoric acid 1M, 0.1M.
7.2.3 Sodium hydroxide 1M, 0.2M, 0.1M.
7.2.4 Trisodium phosphate 0.1M.

7.3 Buffer solutions

Prepare from mixtures of 0.1M H_3PO_4 and 0.1M Na_3PO_4 to give buffers at pH 4 ± 0.1, 6 ± 0.1 and 7 ± 0.1.

8. STANDARDS

8.1 Stock Standards

8.1.1 Amygdalin 500µg/mL: Dissolve 50mg in 100mL water.
8.1.2 Potassium cyanide 500µg/mL: Dissolve 50mg in 100mL 0.2M NaOH.
8.1.3 β-Glucosidase: Dissolve in pH 6.0 buffer to give an activity of 5EU/mL. Store refrigerated and check activity periodically to be at least 3 EU/mL.
8.1.4 Chloramine-T (1% w/v): Dissolve 0.1g in 10mL water. ***Make up fresh every day.***
8.1.5 Colour reagent: Dissolve 3.7g NaOH in 200mL water, add 7.0g 1,3-dimethylbarbituric acid and 5.7g isonicotinic acid and dissolve with stirring/agitation. Adjust to pH 7-8 using 1M HCl or 1M NaOH.

8.2 Standard dilutions for spectrophotometry

8.2.1 Prepare a KCN working standard at 50 µg/mL by diluting 5mL of the KCN stock standard (8.1.2) to 50mL with 0.2M NaOH. Prepare a series of calibration standards as shown in Table 1.

8.2.2 Prepare a series of amygdalin (AMG) calibration standards by diluting the 50µg/mL amygdalin stock standard (8.1.1) as shown in Table 2.

Table 1. KCN calibration standards preparation

Standard	Volume of KCN 50µg/mL (µL)	Dilution volume 0.1M NaOH (mL)	HCN equivalent (µg/mL)*
C1	0	25	0
C2	250	25	0.21
C3	1000	25	0.83
C4	2000	25	1.66
C5	4000	25	3.35
C6	8000	25	6.69
C7	16000	25	13.39

[*RMM HCN = 27.025 and RMM KCN = 65.116 therefore mass ratio HCN/KCN = 0.415]

Table 2. Amygdalin calibration standards preparation

Standard	Volume of AMG 500µg/mL (µL)	Dilution volume water (mL)	HCN equivalent (µg/mL)*
A1	0	25	0
A2	250	25	0.26
A3	1000	25	1.06
A4	2000	25	2.11
A5	4000	25	4.23
A6	8000	25	8.46
A7	16000	25	16.91

[*RMM HCN = 27.025 and RMM amygdalin trihydrate = 511.49 therefore mass ratio HCN/AMG = 0.0529]

9. PROCEDURE

9.1 Sample preparation

Samples must be prepared with minimum processing in order to prevent losses of HCN. Samples should be chilled to ca. 5℃ thoroughl y and homogenization should be carried out immediately using a Turrax probe or blender/food processor.

9.2. Spiked samples

Spiked samples are prepared at the weighing out stage prior to solvent extraction. Add an appropriate amount of the amygdalin standard solution (8.1.1) to give the desired level of HCN equivalents (see Table 2), to the sample or to a reagent blank spike using a positive displacement pipette. At least one blank and spike of the appropriate sample must be extracted for each 10 unknown samples.

9.2 Extraction

Liquid samples

9.2.1 Pipette 5mL of sample into a 50mL centrifuge tube, add 15mL 0.1M NaOH chilled to ca. 5℃ and shake thoroughly.

9.2.2 Add 3mL 1M orthophosphoric acid, secure the cap well and place on an orbital shaker for 30 minutes.

9.2.3 Transfer to a 25mL volumetric flask, dilute to volume with water and mix well.

9.2.4 Proceed from step 9.2.14.

Marzipan, nougat and canned stone fruits

9.2.5 Using a balance accurate to 0.001g, weigh ca. 0.1 to 5g sample depending upon the expected cyanogen content, into a 50mL Falcon/centrifuge tube.

[***Note:*** *The presence of sugars, complex carbohydrates and/or proteins in the sample matrix may inhibit the extraction of amygdalin, so the sample size may need to be reduced. Typical sample weights are 1g for marzipan and 5g for canned stone fruits*].

9.2.6 Add 15mL 0.1M NaOH chilled to ca. 5℃ and hom ogenise using a Turrax probe for ca. 20 seconds.
9.2.7 Add 3mL 1M orthophosphoric acid, secure the cap well and place on an orbital shaker for 2 hours.
9.2.8 Centrifuge at 3,000 x g for 15 minutes.
9.2.9 Transfer the supernatant to a 50mL volumetric flask and stopper well.
9.2.10 To the residue contained in the Falcon/centrifuge tube, add 15mL 0.1M NaOH chilled to ca. 5℃ and shake vigorously to disperse .
9.2.11 Add 3mL 1M orthophosphoric acid, secure the cap well and place on an orbital shaker for 30 minutes.
9.2.12 Centrifuge at 3,000 x g for 15 minutes.
9.2.13 Pool the supernatant with that from the first extraction in the 50mL volumetric flask, dilute to volume with water and mix well.
9.2.14 If the extract is opaque/hazy, filter ca. 0.5mL through a 0.45µm syringe filter.

The following procedures should be carried out in a fume cupboard.

9.2.15 Using positive displacement pipettes, transfer 0.1mL (liquid sample extracts in 25mL volumetric) or 0.2mL (other sample extracts in 50mL volumetric) to a 10mL glass vial.
9.2.16 Add 0.4mL pH 7 buffer, 0.1mL β-glucosidase solution and mix well. Incubate at 30 ± 1℃ for 30 minutes.
9.2.17 Add 0.6mL 0.2M NaOH, mix well and allow to stand for 5 minutes.
9.2.18 Add 2.8mL (liquid sample extract) or 2.7mL (other sample extracts) of pH 6 buffer and mix.
9.2.19 Add 0.1mL Chloramine-T solution, mix well and stand for 5 minutes.
9.2.20 Add 0.6mL colour reagent, mix well and stand for 30 minutes.
9.2.21 Transfer the solution to a cuvette and measure the absorbance at 605nm against a reagent blank (i.e. replace sample extract with 0.1mL water in 9.2.15).

10. CALIBRATION

Hydrogen cyanide

10.1 Use the calibration standards prepared as shown in Table 1.
10.2 Using a displacement pipette, transfer 0.1mL of standard into a 10mL vial and add 3.9mL of pH 4 buffer. Chill in an ice/water bath for 5 minutes.
10.3 Proceed with steps 9.2.19 - 9.2.21 using standard C1 to zero the spectrophotometer.
10.4 Construct a calibration graph of HCN concentration (µg/mL) against absorbance at 605nm.

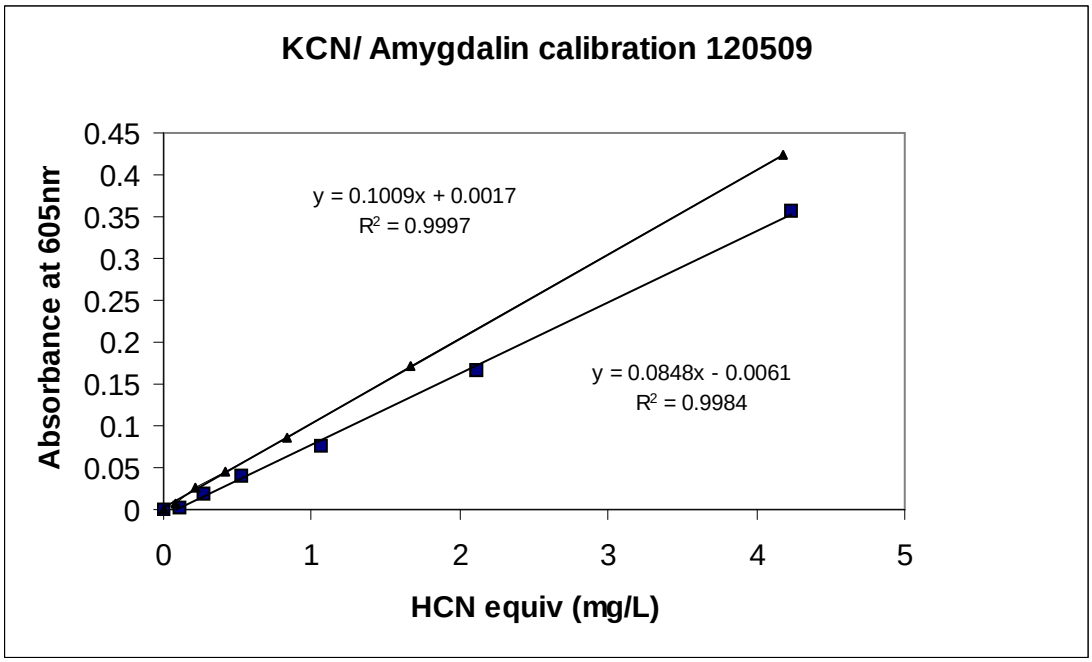
Amygdalin

10.5 Use the calibration standards prepared as shown in Table 2.

10.6 Using a displacement pipette, transfer 0.1mL of standard into a 10mL vial.

10.7 Proceed with steps 9.2.16 - 9.2.21 using standard A1 to zero the spectrophotometer

Typical calibration lines for KCN and Amygdalin



[KCN= ▲; Amygdalin = ■]

11. CALCULATION OF RESULTS

Calculate the concentration of HCN in the amygdalin standard or in the sample extract (X) by interpolation from the calibration graph:

X (µg/mL)= (A_{605} – intercept)/gradient

Calculate the amount of HCN equivalents in the sample from:

$$HCN_{equiv}\ (mg/kg) = \frac{X * 25}{\text{sample weight (g)}}$$

12. QUALITY ASSURANCE

Recovery of spiked samples within the batch should be in the range of 60 – 120% but ideally in the range 70-110%.

Reagent blank samples should be prepared with each batch. Any analyte in the blank should be subtracted from the spiked samples.

12. WORKING WITH CYANIDE

Before any work is carried out using cyanides, THE Section Head or appointed Senior Scientist must authorise the use of cyanide.
Cyanides must be kept in a locked cupboard in a ventilated room in a chemical store and can only be withdrawn after authorisation has been obtained. Cyanide must be returned to the store immediately after the required amount has been weighed out. A record must be kept of the user, the date of withdrawal and return of the cyanide, the amount used and cross-reference to the experiment or lab notebook/page number.

Waste cyanide disposal

All procedures must be carried out in a fume cupboard and personal protective clothing must be worn at all times i.e. safety glasses and disposable gloves.

Waste cyanide solutions are extremely toxic and must be disposed of in an appropriate way. Cyanide salts form hydrogen cyanide gas upon contact with acids so this must be avoided. It must not be disposed to drain or into waste bins.

Aqueous solutions of cyanide should be made strongly alkaline using 0.1M sodium hydroxide. The solutions must then be treated with excess sodium hypochlorite solution (10-15%) for 24 hours. Starch-iodide paper should be used to test for the presence of excess hypochlorite (turns blue).

The absence of cyanide should be confirmed using the Merckoquant test kit.

Glass and plastic apparatus must be decontaminated by soaking in 0.1M NaOH for 24hrs after which the solutions must then be treated with excess sodium hypochlorite solution (10-15%) for 24 hours.

Treated solutions must be stored in a vented container and not allowed to drain. They must be treated as hazardous waste, be kept separated from other aqueous waste and disposed of in a separately marked container.