



African Food Tradition rEvisited by Research
FP7 n°245025

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Organisation name of lead contractor for this deliverable: CIRAD

Authors: are indicated on the appropriate SOPs.

This document has been send to:

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| The coordinator by WP Leader | Date: September 2011 |
| To the Commission by the Coordinator | Date: October 2011 |

* PU: Public; PP: Restricted to other programme participants (including the Commission Services); RE: Restricted to a group specified by the consortium (including the Commission Services); CO: Confidential, only for members of the consortium (including the Commission Services)

Methodology for the development of SOPs for this deliverable.

This deliverable consists of several SOPs related to the chemical analysis for products of Group 1.

The SOP's come from four sources:

1. The literature that gives procedures on products similar to the African fermented products of the project. In this case several articles can be combined according to their precisions.
2. Standard procedures from the International Standardization Organization (ISO) or the AACC (American Association of Cereal Chemists). In this case, the method is used like it or after minor modifications. Only a brief summary and the modifications (if any) are detailed in the document, with the reference procedure (ISO or AACC) annexed to the SOP. To be in agreement with intellectual property rules, the project coordination purchased and distributed to partners all hard copies of the reference procedures annexed in the SOPs
3. The SOPs can come from the laboratory that developed the methods for the specific analysis.
4. Kit enzymatic procedures developed by the supplier of the kit.

After writing, the SOP's are approved by the Work Package Leader (WPL) related to the group of product concerned (Group 1: WP2; Group 2: WP3; Group 3: WP4).

The WPL is in charge to send the SOP's to the concerned partners for validation. Each partner, according to his laboratory facilities, validates the method and informs one of the following alternatives in his laboratory:

- The laboratory makes the analysis in routine (coded "R" in the joined table)
- The laboratory is able to make this analysis (coded "P", possible)
- The laboratory can make the analysis after buying equipment (coded "B")
- The laboratory can't make the analysis (coded "blank").



When several procedures are given for one character (for example moisture), the specific methods is selected according to partner facility, language and principle. The specific procedure can be directly viewed by clicking on the SOP number (link).

The WPL decides the end of the validation step. He accepts the final SOP version. He completes the table with the last revision date.

| Character | CIRAD | UAC | FRI | NRC | FAAU | Principle | SOP- fr | SOP- en |
|-----------------------------------|-------|-----|-----|-----|------|--|------------------|------------------|
| Moisture | P | R | R | R | R | Drying at 103-105°C for 16-48 h | Chem-Cere-025-fr | Chem-Cere-025-en |
| | R | | R | R | R | Semi rapid: drying at 130-133°C for 2 hours | Chem-Cere-007-fr | Chem-Cere-007-en |
| | P | P | B | | | Rapid: IR drying | Chem-Cere-24-fr | |
| Total starch | | P | P | P | | Enzymatic method: Kit | | Chem-Cere-11-en |
| | R | P | P | P | P | Enzymatic after alcali dissolution | Chem-Cere-10-fr | |
| Amylose | R | P | | P | R | Blue value with iodine | Chem-Cere-14-fr | Chem-Cere-14-en |
| | R | | | P | | DSC with LPL | Chem-Cere-12-fr | Chem-Cere-12-en |
| Crude protein in cereal products | R | R | R | P | R | Kjeldahl procedure | | Chem-Cere-22-en |
| Total Nitrogen in whole milk | R | R | R | P | R | Kjeldahl procedure | | Chem-Cere-21-en |
| Nonprotein Nitrogen in whole milk | P | | | | R | Kjeldahl procedure after precipitation of proteins | | Chem-Cere-026-en |
| Protein in milk | P | | | | R | Difference between total nitrogen and non protein nitrogen | | Chem-Cere-027-en |
| Total fibre | P | R | R | R | P | Acid and alcali hydrolysis, subtraction of ash (550°C/1hr) | | Chem-Cere-28-en |
| Crude fat | | R | R | | P | Extraction by boiling petroleum ether using Soxhlet | Chem-Cere-23-fr | |
| | R | | P | R | P | Extraction by boiling ethyl ether using Soxtec | Chem-Cere-13-fr | Chem-Cere-13-en |
| Total Fatty Acids (FFA) | | | | P | R | Gas chromatography of methyl esters | | Chem-Cere-029-en |
| Fat Acidity | R | P | P | | R | The acids are dissolved in ethanol solution followed by titration with sodium hydroxyde 0,05M. | Chem-Cere-16-fr | Chem-Cere-16-en |
| Crude ash | | R | R | | | Incineration at 550°C | | Chem-Cere-17-en |
| pH & Acidity | R | R | R | R | R | pH measurment & acidity by titration with NaOH | Chem-Cere-009-fr | |

AFTER (G.A n°245025) – Deliverable D. 1.2.3.8
Title of deliverable: Chemical analysis for Group 1

| Character | CIRAD | UAC | FRI | NRC | FAAU | Principle | SOP- fr | SOP- en |
|--|--------------|------------|------------|------------|-------------|---|------------------|------------------|
| Organic acids & simple sugars | R | R | P/B | | P | Extraction with diluted sulfuric acid followed by Aminex-based HPLC | Chem-Cere-002-fr | Chem-Cere-002-en |
| Simple sugars | | | | P | P | Extraction with water followed by NH2-based HPLC | | Chem-Cere-20-en |
| Dextrins | P | | | | | Size exclusion chromatography | Chem-Cere-001-fr | |
| β-amylase activity | R | P | P | | | Enzymatic methods using commercial kits provided by Megazyme | Chem-Cere-005-fr | Chem-Cere-005-en |
| α-amylase activity | R | P | P | | | Enzymatic methods using commercial kits provided by Megazyme | Chem-Cere-006-fr | Chem-Cere-006-en |

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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination de la teneur en eau dans les produits céréaliers fermentés : méthode à 103-105°C | | |
| SOP : Chem-Cere-025-fr | | |
| Date: 16/09/2011 | Version : 1 | |
| <p>Ecrit par : Noël AKISSOE</p> <p>Pour plus d'information sur ce SOP, contactez :</p> <ul style="list-style-type: none"> • Noël AKISSOE (noelyolande@yahoo.fr) • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader | | |
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| FRI NRC | Wis AMOA | 16/09/2011 |

**Détermination de la teneur en eau dans les produits céréaliers fermentés : méthode à
103-105°C**

SOP : Chem-Cere-025-fr

Date : 16/09/2011

Version: 1

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Détermination de la teneur en eau dans les produits céréaliers fermentés : méthode à 103-105°C

SOP : Chem-Cere-025-fr

Date : 16/09/2011

Version: 1

1 DOMAINE ET APPLICATION

Le présent document décrit une méthode de référence pratique pour la détermination de la teneur en eau dans les céréales et les produits céréaliers, à l'état de grains, grains broyés, semoule ou farine. Elle est aussi applicable aux produits dérivés humides : pâtes, bouillies etc.

Elle est applicable aux produits suivants : blé, riz (paddy, décortiqué et usiné), orge, millet (*Panicum miliaccum*), seigle, avoine, triticale, sorgho et kaffir (*Sorghum vulgare caffrorum*),

2 REFERENCES

AACC Method 44-15A

3 DEFINITIONS

L'eau libre et liée sont dosés par cette méthode. La teneur en eau est exprimée en pourcentage, base humide.

4 PRINCIPE

On mesure la perte d'eau par évaporation à une température supérieure au point d'ébullition.

- Broyage de l'échantillon, si sa granulométrie excède 1,7 mm.
- Séchage d'une prise d'essai de 5 g durant :
 - 16 heures à une température de 105°C, ou bien
 - 48 heures à une température de 103°C
- Le broyage est effectué après pré-conditionnement, si la teneur en eau du produit excède 17%.

5 REACTIFS

6 APPAREILLAGE

Cf AACC Method 44-15A

Détermination de la teneur en eau dans les produits céréaliers fermentés : méthode à 103-105°C

SOP : Chem-Cere-025-fr

Date : 16/09/2011

Version: 1

7 PROCÉDURE

Cf AACC Method 44-15A

8 EXPRESSION DES RÉSULTATS

Cf AACC Method 44-15A

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- La granulométrie de l'échantillon doit être inférieure à 1,7 mm ; sinon, un broyage préalable est nécessaire.
- Il ne faut pas ouvrir la porte de l'étuve durant le séchage.
- À la fin de la période de séchage, retirer les prises d'essai séchées avant d'y introduire des produits humides, car cela aurait pour conséquence de réhydrater partiellement ces dernières.
- Il est conseillé de vérifier la déshydratation complète de tout nouveau type d'échantillon (en particulier pour les produits très humides –pâtes, bouillies) : placer à nouveau quelques heures à 103 ou 105°C après la mesure de matière sèche, et peser à nouveau.
- La différence absolue entre deux répétitions réalisées dans un court intervalle de temps ne dépassera 0,15 g d'eau pour 100 g d'échantillon (0,15 % base humide)

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

Détermination de la teneur en eau dans les produits céréaliers fermentés : méthode à 103-105°C

SOP : Chem-Cere-025-fr

Date : 16/09/2011

Version: 1

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------|----------------------|--------------------------------|
| | | |
| | | |

12 ANNEXE

Texte de AACC Method 44-15A

MOISTURE—AIR-OVEN METHODS

Final approval 10-30-75; revised 10-28-81

Definition

These methods det moisture content as loss in wt of a sample when heated under specified conditions.

Scope

Applicable to flour, farina, semolina, bread, grain, soybeans, rice, beans, peas, lentils, cornmeal, corn grits, rolled oats, bulgur, rolled wheat, breakfast cereals (except those that are sugar-coated). This method gives results closely approximating those obtained by Method 44-40 (vacuum-oven). This method is not recommended for feeds and feedstuffs when fat detn is to be made on dried samples.

Apparatus

1. Wiley laboratory mill, intermediate model, equipped with 18- or 20-mesh screen and 4-oz receiving bottle; or any other mill that will grind to same degree of fineness without undue exposure to atmosphere and without appreciable heating.
2. Oven (either gravity-convection or mechanical-convection), capable of being maintained at 130° ($\pm 1^\circ$) uniformly thruout oven and provided with good ventilation. Thermometer shall be so situated in oven that tip of bulb is level with top of moisture dishes but not directly over any dish.
3. Oven for drying corn and beans, meeting requirements of oven in item 2 except that it is maintained at 103° ($\pm 1^\circ$).
4. Moisture dishes having diam of ca 55 mm and height of ca 15 mm. They should be of heavy-gauge aluminum with slightly tapered sides and provided with tightly fitting slip-in covers that are designed to fit snugly under dishes when they are placed in oven. Both dish and cover should be identified by same number. Before using, dry for 1 hr at 130°, cool in desiccator, and obtain tare wt. (See Note 1.)
5. Airtight desiccator contg activated alumina, molecular sieves (type 4A or 4A X W), or other equally suitable desiccant. (See Note 2.)
6. Balance, accurate to at least 1 mg.

Procedure**One-stage**

For samples contg less than 16% moisture, except soybeans and rice for which moisture values of 10 and 13%, respectively, apply; also for flour, cornmeal, farina, and semolina except for grinding.

1. Grind a 30- to 40-g sample in mill, leaving minimum possible amt in mill. Mix rapidly with spoon or spatula and transfer immediately a 2- to 3-g portion to each of two or more tared moisture dishes. Cover and weigh dishes at once.

Moisture—Air-Oven Methods (continued)

Subtract tare wts and record wt of sample. Dismantle and clean mill between samples.

2. Uncover dishes and place them on shelf of oven and place covers under dishes. Insert shelf in oven at level of thermometer bulb. Heat for exactly 60 min after oven recovers its temp (See Note 3.)

3. Remove shelf and dishes from oven, cover rapidly (using rubber finger insulators), and transfer to desiccator as quickly as possible. Weigh dishes after they reach room temp (45–60 min usually). Det loss in wt as moisture (see eq 1). Replicate detns must check within 0.2% moisture; otherwise repeat detn.

Two-stage

For samples contg 16% or more moisture (10 and 13% for soybeans and rice), loss of moisture incident to grinding is likely to be excessive; hence, following two-stage procedure should be used.

1. Fill two or more tared moisture dishes nearly full with representative portions of unground sample. Cover and weigh dishes. Subtract tare wts and record wt of sample.

2. Uncover dishes and place covers under dishes. Place tins in well-ventilated place (preferably on top of heated oven protected from dust) so that sample will dry reasonably fast and reach approx air-dry condition. This will usually be accomplished in 14–16 hr when top of heated oven is used, or approx 60 hr when room temp is used for this preliminary drying. In all cases, except for soybeans and rice, moisture content must be reduced to 16% or less (10% for soybeans and 13% for rice).

3. Cover dishes contg air-dried samples and weigh them soon after they cool to room temp. Det loss in wt and record it as moisture loss due to air-drying.

4. Using air-dried sample, follow one-stage procedure descr above, starting with grinding step. Calc total moisture loss by using eq 2.

Air-oven, for corn and beans, at 103°

Place approx 15 g of representative portion of unground sample in each of two or more tared moisture dishes. Weigh covered dishes and contents. Subtract wt of each dish from total wt and record result as wt of sample. Put covers under dishes and heat for 72 hr in oven regulated at $103 \pm 1^\circ$. Dishes should be placed on single shelf with bulb of oven thermometer as close as possible to them. At end of heating period, remove shelf contg dishes, cover dishes immediately, and place in desiccator. Weigh dishes when they reach room temp. Det loss in wt as moisture by using eq 1.

Replicate detns should check within 0.2% moisture.

Moisture—Air-Oven Methods (continued)*Air-oven, for flax, at 103°*

Proceed as above for corn and beans, except use 5- to 7-g sample and 4-hr oven time.

Bread, two-stage

Air-dry and grind sample as directed in Method 62-05. Using 2- to 3-g portion of ground air-dried sample, follow one-stage procedure descr above. Calc total moisture loss by using eq 2. To obtain % of total solids in fresh loaf, subtract % total moisture from 100.

Calculation

Eq 1 (one-stage and 103° air oven):

$$\% \text{ Moisture} = \frac{A}{B} \times 100$$

in which A = moisture loss in g; B = original wt of sample.

Eq 2 (two-stage):

$$\% \text{ Total solids} = \frac{Y \times Z}{X}$$

where X = wt of original sample used for air-drying, Y = wt of sample after air-drying, Z = % total solids in prepd ground sample (total solids = 100% - % moisture at assay).

Notes



1. Tare wt will usually remain const within few tenths of mg for approx 1 yr if dishes are dumped and then carefully wiped clean with soft cloth.
2. Silica gel and anhydrous CaCl_2 are not suitable desiccants.
3. Oven should regain temp within 15-20 min after insertion of full load (24 moisture dishes). If oven requires longer time to recover, it should not be used.

References

1. Anderson, J. E. 1936. Some factors concerning vacuum-oven moisture determination. *Cereal Chem.* 13:437.
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4. Hart, J. R., Fernsten, L., and Golumbic, C. 1959. Oven methods for precise measurement of moisture content of seeds. U.S. Department of Agriculture, Marketing Research Report 304.

Moisture—Air-Oven Methods (continued)

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8. Oxley, T. A., Pixton, S. W., and Howe, R. W. 1960. Determination of moisture content in cereals. I. Interaction of type of cereal and oven method. *J. Sci. Food Agric.* 11:18.
9. U.S. Department of Agriculture. 1971. *Federal Grain Inspection Serv. Equipment Manual*, GR Instruction 916-6, chapt. XII. (Nov.)

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|  | <u>African Food Tradition rEvisited by Research FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Determination of moisture content in fermented cereal foods: Procedure at 103-105°C | | |
| SOP: chem-cere-25-en | | |
| Date : 16/09/2011 | Release: 1 | |
| <p>Written by : Noël AKISSOE</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Noël AKISSOE (noelyolande@yahoo.fr) • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader | | |
| Approved by : | | |
| Partners | Name of the person who approved | Date DD/MM/YY |
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| CSIR | | |
| FAAU | | |
| ESB | | |
| FRI | Wis AMOA | 16/09/2011 |
| NRC | | |

Determination of moisture content in fermented cereal foods:

Procedure at 103-105°C

SOP: Chem-Cere-25-en

Date: 16/09/2011

Release: 1

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Determination of moisture content in fermented cereal foods:

Procedure at 103-105°C

SOP: Chem-Cere-25-en

Date: 16/09/2011

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1 SCOPE AND APPLICATION

This document specifies a routine reference method for the determination of moisture content in cereals and cereal products. It is also applicable to wet derivatives: pastes, porridges etc

It is applicable to the following products: wheat, durum wheat, rice (paddy, husked and milled rice), barley, millet (*Panicum miliaceum*), oats, sorghum and kaffir (*Sorghum vulgare caffrorum*), in the form of grains, milled grains, semolina or flour.

2 REFERENCES

AACC Method 44-15A

3 DEFINITIONS

Moisture content here refers to the amount of water in the fermented cereal foods. Bound water and free water are assessed together as moisture by this procedure.

4 PRINCIPLE

This method involves the measurement of the weight lost due to evaporation of water at or near boiling point.

- If the particle size is more than 1.7 mm, then it is necessary to grind the sample.
- 5 g sample is dried for:
 - 16 hours at a temperature of 105°C, or
 - 48 hours at a temperature of 103°C
- Grinding is performed after pre-conditioning, when required (water content over 17%).

5 REAGENTS

6 APPARATUS

- Galvanized pans with lids
- Ventilated hot-air oven (103 or 105°C)

Determination of moisture content in fermented cereal foods:

Procedure at 103-105°C

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- Analytical balance (accuracy: 1 mg)
- Spatula
- Desiccator, containing an efficient dehydrating agent

7 PROCEDURE

See AACC 44-15A procedure

8 EXPRESSION OF RESULTS

See AACC 44-15A procedure

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Sample particle size should be lower than 1.7 mm, otherwise grinding is necessary.
- Do not open the door of the oven during drying.
- At the end of the drying period, remove the dried test portions before placing moist products in the oven, otherwise partial rehydration of the dried test portions may result
- It is advisable to check the complete dehydration of any new type of sample (especially for very wet products – dough, paste, porridge): place again a few hours to 103 or 105° C after the measurement of dry matter, and weigh again.
- The difference between duplications performed in short time interval will not exceed 0.15 g water for 100 g sample (0.15 % wet basis)

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

Determination of moisture content in fermented cereal foods:

Procedure at 103-105°C

SOP: Chem-Cere-25-en

Date: 16/09/2011

Release: 1

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
| | | |
| | | |
| | | |

12 APPENDIX

AACC 44-15A procedure.

MOISTURE—AIR-OVEN METHODS

Final approval 10-30-75; revised 10-28-81

Definition

These methods det moisture content as loss in wt of a sample when heated under specified conditions.

Scope

Applicable to flour, farina, semolina, bread, grain, soybeans, rice, beans, peas, lentils, cornmeal, corn grits, rolled oats, bulgur, rolled wheat, breakfast cereals (except those that are sugar-coated). This method gives results closely approximating those obtained by Method 44-40 (vacuum-oven). This method is not recommended for feeds and feedstuffs when fat detn is to be made on dried samples.

Apparatus

1. Wiley laboratory mill, intermediate model, equipped with 18- or 20-mesh screen and 4-oz receiving bottle; or any other mill that will grind to same degree of fineness without undue exposure to atmosphere and without appreciable heating.
2. Oven (either gravity-convection or mechanical-convection), capable of being maintained at 130° ($\pm 1^\circ$) uniformly thruout oven and provided with good ventilation. Thermometer shall be so situated in oven that tip of bulb is level with top of moisture dishes but not directly over any dish.
3. Oven for drying corn and beans, meeting requirements of oven in item 2 except that it is maintained at 103° ($\pm 1^\circ$).
4. Moisture dishes having diam of ca 55 mm and height of ca 15 mm. They should be of heavy-gauge aluminum with slightly tapered sides and provided with tightly fitting slip-in covers that are designed to fit snugly under dishes when they are placed in oven. Both dish and cover should be identified by same number. Before using, dry for 1 hr at 130°, cool in desiccator, and obtain tare wt. (See Note 1.)
5. Airtight desiccator contg activated alumina, molecular sieves (type 4A or 4A X W), or other equally suitable desiccant. (See Note 2.)
6. Balance, accurate to at least 1 mg.

Procedure**One-stage**

For samples contg less than 16% moisture, except soybeans and rice for which moisture values of 10 and 13%, respectively, apply; also for flour, cornmeal, farina, and semolina except for grinding.

1. Grind a 30- to 40-g sample in mill, leaving minimum possible amt in mill. Mix rapidly with spoon or spatula and transfer immediately a 2- to 3-g portion to each of two or more tared moisture dishes. Cover and weigh dishes at once.

Moisture—Air-Oven Methods (continued)

Subtract tare wts and record wt of sample. Dismantle and clean mill between samples.

2. Uncover dishes and place them on shelf of oven and place covers under dishes. Insert shelf in oven at level of thermometer bulb. Heat for exactly 60 min after oven recovers its temp (See Note 3.)

3. Remove shelf and dishes from oven, cover rapidly (using rubber finger insulators), and transfer to desiccator as quickly as possible. Weigh dishes after they reach room temp (45–60 min usually). Det loss in wt as moisture (see eq 1). Replicate detns must check within 0.2% moisture; otherwise repeat detn.

Two-stage

For samples contg 16% or more moisture (10 and 13% for soybeans and rice), loss of moisture incident to grinding is likely to be excessive; hence, following two-stage procedure should be used.

1. Fill two or more tared moisture dishes nearly full with representative portions of unground sample. Cover and weigh dishes. Subtract tare wts and record wt of sample.

2. Uncover dishes and place covers under dishes. Place tins in well-ventilated place (preferably on top of heated oven protected from dust) so that sample will dry reasonably fast and reach approx air-dry condition. This will usually be accomplished in 14–16 hr when top of heated oven is used, or approx 60 hr when room temp is used for this preliminary drying. In all cases, except for soybeans and rice, moisture content must be reduced to 16% or less (10% for soybeans and 13% for rice).

3. Cover dishes contg air-dried samples and weigh them soon after they cool to room temp. Det loss in wt and record it as moisture loss due to air-drying.

4. Using air-dried sample, follow one-stage procedure descr above, starting with grinding step. Calc total moisture loss by using eq 2.

Air-oven, for corn and beans, at 103°

Place approx 15 g of representative portion of unground sample in each of two or more tared moisture dishes. Weigh covered dishes and contents. Subtract wt of each dish from total wt and record result as wt of sample. Put covers under dishes and heat for 72 hr in oven regulated at $103 \pm 1^\circ$. Dishes should be placed on single shelf with bulb of oven thermometer as close as possible to them. At end of heating period, remove shelf contg dishes, cover dishes immediately, and place in desiccator. Weigh dishes when they reach room temp. Det loss in wt as moisture by using eq 1.

Replicate detns should check within 0.2% moisture.

Moisture—Air-Oven Methods (continued)*Air-oven, for flax, at 103°*

Proceed as above for corn and beans, except use 5- to 7-g sample and 4-hr oven time.

Bread, two-stage

Air-dry and grind sample as directed in Method 62-05. Using 2- to 3-g portion of ground air-dried sample, follow one-stage procedure descr above. Calc total moisture loss by using eq 2. To obtain % of total solids in fresh loaf, subtract % total moisture from 100.

Calculation

Eq 1 (one-stage and 103° air oven):

$$\% \text{ Moisture} = \frac{A}{B} \times 100$$

in which A = moisture loss in g; B = original wt of sample.

Eq 2 (two-stage):

$$\% \text{ Total solids} = \frac{Y \times Z}{X}$$

where X = wt of original sample used for air-drying, Y = wt of sample after air-drying, Z = % total solids in prepd ground sample (total solids = 100% - % moisture at assay).

Notes



1. Tare wt will usually remain const within few tenths of mg for approx 1 yr if dishes are dumped and then carefully wiped clean with soft cloth.
2. Silica gel and anhydrous CaCl_2 are not suitable desiccants.
3. Oven should regain temp within 15-20 min after insertion of full load (24 moisture dishes). If oven requires longer time to recover, it should not be used.

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|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination de la teneur en eau dans les produits céréaliers fermentés : séchage à 130-133°C, 2 heures | | |
| SOP : Chem-Cere-007-fr | | |
| Date: 30/03/2011 | Version : 2 | |
| <p> Ecrit par : Christian MESTRES Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • • </p> | | |
| Ce document a été approuvé par : | | |
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**Détermination de la teneur en eau dans les produits céréaliers fermentés fermentés :
séchage à 130-133°C, 2 heures**

SOP : Chem-Cere-007-fr

Date : 31/03/2011

Version: 2

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Détermination de la teneur en eau dans les produits céréaliers fermentés fermentés : séchage à 130-133°C, 2 heures

SOP : Chem-Cere-007-fr

Date : 31/03/2011

Version: 2

1 DOMAINE ET APPLICATION

Le présent document décrit une méthode de référence pratique pour la détermination de la teneur en eau dans les céréales et les produits céréaliers, à l'état de grains, grains broyés, semoule ou farine.

Elle est applicable aux produits suivants : blé, riz (paddy, décortiqué et usiné), orge, millet (*Panicum miliaccum*), seigle, avoine, triticale, sorgho et kaffir (*Sorghum vulgare caffrorum*),

2 REFERENCES

NF EN ISO 712 : 2010

3 DEFINITIONS

L'eau libre et liée sont dosés par cette méthode. La teneur en eau est exprimée en pourcentage, base humide.

4 PRINCIPE

On mesure la perte d'eau par évaporation à une température supérieure au point d'ébullition.

- Broyage de l'échantillon, si sa granulométrie excède 1,7 mm.
- Séchage durant 2 heures d'une prise d'essai de 5 g à une température comprise entre 130°C et 133°C
- Le broyage est effectué après pré-conditionnement, si la teneur en eau du produit excède 17%.

5 REACTIFS

6 APPAREILLAGE

Cf procédure ISO 712 :1998

7 PROCÉDURE

Selon la procédure ISO 712 :1998

Détermination de la teneur en eau dans les produits céréaliers fermentés fermentés : séchage à 130-133°C, 2 heures

SOP : Chem-Cere-007-fr

Date : 31/03/2011

Version: 2

8 EXPRESSION DES RÉSULTATS

Selon la procédure ISO 712 :1998

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- La granulométrie de l'échantillon doit être inférieure à 1,7 mm ; sinon, un broyage préalable est nécessaire.
- Il ne faut pas ouvrir la porte de l'étuve durant le séchage.
- À la fin de la période de séchage, retirer les prises d'essai séchées avant d'y introduire des produits humides, car cela aurait pour conséquence de réhydrater partiellement ces dernières.
- La différence absolue entre deux répétitions réalisées dans un court intervalle de temps ne dépassera 0,15 g d'eau pour 100 g d'échantillon (0,15 % base humide)

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

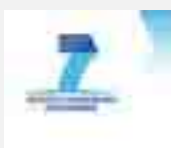
Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------------|----------------------|--------------------------------------|
| 30/03/2011 | Christian MESTRES | Edition, conformité version anglaise |
| | | |

12 ANNEXE

Le texte de la norme NF EN ISO 712 : 2010 sera annexé au document



**African Food Tradition rEvisited by
Research**
FP7 n°245025



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

**Determination of Moisture Content in Fermented Cereal Foods:
Drying at 130-133°C for 2 hours**

SOP: CHEM-CERE-EN-007

Date : **19/03/2011**

Release: **2**

Written by : Christian MESTRES

For information on this SOP please contact :

- Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader
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-

Approved by :

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| FRI | Wis AMOA | 13/03/2011 |
| NRC | | |

**Determination of Moisture Content in Fermented Cereal Foods:
Drying at 130-133°C for 2 hours
SOP: CHEM-CERE-EN-007**

Date: 19/03/2011

Release: 2

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Determination of Moisture Content in Fermented Cereal Foods:

Drying at 130-133°C for 2 hours

SOP: CHEM-CERE-EN-007

Date: 19/03/2011

Release: 2

1 SCOPE AND APPLICATION

This document specifies a routine reference method for the determination of moisture content in cereals and cereal products. It is applicable to the following products: wheat, durum wheat, rice (paddy, husked and milled rice), barley, millet (*Panicum miliaceum*), oats, sorghum and kaffir (*Sorghum vulgare caffrorum*), in the form of grains, milled grains, semolina or flour.

2 REFERENCES

NF EN ISO 712: 2010

3 DEFINITIONS

Moisture content here refers to the amount of water in the fermented cereal foods. Bound water and free water are assessed together as moisture by this procedure.

4 PRINCIPLE

This method involves the measurement of the weight lost due to evaporation of water at or near boiling point.

- If the particle size is more than 1.7 mm, then it is necessary to ground the sample.
- 5 g sample is dried at a temperature of 130°C ± 3 °C for 2 hours.
- Grinding is performed after pre-conditioning, when required (water content over 17%).

5 REAGENTS

6 APPARATUS

- Galvanized pans with lids
- Ventilated hot-air oven (130-133°C)
- Analytical balance (accuracy: 1 mg)
- Spatula
- Desiccator, containing an efficient dehydrating agent (Silica gel for example)

Determination of Moisture Content in Fermented Cereal Foods:

Drying at 130-133°C for 2 hours

SOP: CHEM-CERE-EN-007

Date: 19/03/2011

Release: 2

7 PROCEDURE

- Put oven on and allow it to stabilised at 130-133°C.
- Weigh the empty galvanised pan and lid and record the weight.
- Weigh 5 g of the sample into the galvanised pan and record the initial weight of the full pan and lid.
- Put the full pan and lid into the oven. Firmly close the door of the oven and wait for 2 hours.
- After the two (2) hour period, put the full pan in the desiccator and rapidly close it with the lid. Allow to cool in the dessicator (preferably in partial vacuum).
- Weigh the full pan and lid again after it has cooled (normally 30-45 minutes) and record the final weight.

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

$$\text{Moisture content (\% wet basis)} = 100 \times \frac{(W_i - W_f)}{(W_i - W_0)}$$

Where :
W_i is the initial weight of full pan and lid
W_f is the final weight of full sample and lid
W₀ is the weight of the empty pan and lid

8.2 Repeatability

The difference between duplications performed in short time interval will not exceed 0.15 g water for 100 g sample (0.15 % wet basis)

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Sample particle size should be lower than 1.7 mm, otherwise grinding is necessary.
- Do not open the door of the oven during drying.
- At the end of the drying period, remove the dried test portions before placing moist products in the oven, otherwise partial rehydration of the dried test portions may result

**Determination of Moisture Content in Fermented Cereal Foods:
Drying at 130-133°C for 2 hours
SOP: CHEM-CERE-EN-007**

Date: 19/03/2011

Release: 2

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|-------------|---------------------------|------------------------------|
| 13/03/2011 | Wis AMOA | Edition |
| 14/03/2011 | Christian MESTRES | Edition |
| | | |

12 APPENDIX

The NF EN ISO 712: 2010 procedure will be annexed to the document.



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|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination rapide de la teneur en eau dans les produits céréaliers fermentés | | |
| SOP : Chem-Cere-024-fr | | |
| Date : 23/08/2011 | Version : 1 | |
| <p> Ecrit par : Noel AKISSOE Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • Noël AKISSOE (noelyolande@yahoo.fr) • </p> | | |
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| NRC | | |

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Détermination rapide de la teneur en eau dans les produits céréaliers fermentés

SOP : Chem-Cere-024-fr

Date : 26/05/2011

Version : 1

1 DOMAINE ET APPLICATION

Le présent document décrit une méthode pour la détermination de la teneur en eau dans les céréales et les produits céréaliers, à l'état de grains, grains broyés, semoule ou farine, pâtes fermentées de céréales.

2 REFERENCES

3 DEFINITIONS

4 PRINCIPE

Séchage par infrarouge d'une prise d'essai de 3-5 g (durant 20-30 min en fonction de la nature du produit) jusqu'à poids constant à une température de $150\text{ }^{\circ}\text{C}\pm 1^{\circ}\text{C}$.

- Pour les grains, broyage de l'échantillon, si sa granulométrie excède 1,7 mm.
- Pour les grains humides (trempés ou germés), broyage effectué après pré-conditionnement, si la teneur en eau du produit excède 17%.
- Pour les pâtes (gowé, mawè, akpan nature), étaler le produit, avec épaisseur maximum de 3 mm

5 REACTIFS

6 APPAREILLAGE

Analyseur d'humidité comportant une balance analytique (précision 0.1 g) et une lampe infrarouge.
Par exemple : MB35 (Ohaus Corporation)

Détermination rapide de la teneur en eau dans les produits céréaliers fermentés

SOP : Chem-Cere-024-fr

Date : 26/05/2011

Version : 1

7 PROCEDURE

Le présent protocole est basé sur la NF-V03-707 (cf annexe). Les éléments modifiés sont signalés aux points 4 et 6, 8, 9 et 10 de ce document.

8 EXPRESSION DES RESULTATS

8.1 Mode de calcul et formules

La teneur en eau (% base humide) est directement lue sur l'appareil

8.2 Répétabilité

L'écart type entre mesures réalisées dans un court intervalle de temps ne dépassera pas 5% (coefficient de variation). Un exemple de séries d'analyses effectuées sur 2 échantillons (une farine sèche et un akpan humide) est donné dans le tableau ci-dessous.

| PARAMETRES | Echantillon | |
|--|-------------|-------|
| | Farine | Akpan |
| Nombre de techniciens | 3 | 2 |
| Valeur moyenne (% bh)* | 10.3 | 86.9 |
| Ecart type de répétabilité (ET) | 0.4 | 1.0 |
| Coefficient de variation (%) de répétabilité | 3.7 % | 1.1 % |
| Ecart type de reproductibilité (sr) | 0.08 | 0.2 |
| Coefficient de variation (%) de reproductibilité | 0.8 % | 0.2 % |
| Limite de repetabilite (ET*2√2) | 1.0 | 2.7 |
| Limite de reproductibilite (sr*2√2) | 0.2 | 0.6 |

*6 répétitions par techniciens

Détermination rapide de la teneur en eau dans les produits céréaliers fermentés

SOP : Chem-Cere-024-fr

Date : 26/05/2011

Version : 1

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- La granulométrie de l'échantillon doit être inférieure à 1,7 mm ; sinon, un broyage préalable est nécessaire.
- Il ne faut pas ouvrir le bouclier thermique durant le séchage.
- Il faut une répartition homogène (bien étaler) des produits, en particulier les pâtes (gowé, mawè, ogui, akpan)
- À la fin de la période de séchage, attendre quelques minutes pour retirer les prises d'essai séchées pour éviter la brulure.

10 RAPPORT D'ESSAI

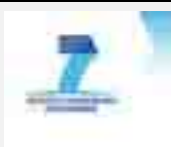
Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------------|----------------------|--------------------------------|
| 26/05/2011 | Christian MESTRES | Edition |
| | | |
| | | |

12 ANNEXE



**African Food Tradition rEvisited by
Research
FP7 n°245025**



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

Determination of total starch content in cereal fermented foods by using an enzymatic method

SOP: Chem-Cere-11-en

Date : **21/03/2011**

Release: **2**

Written by : Zahra AHMED

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| FAAU | | |
| ESB | | |
| FRI | | |
| NRC | Zahra AHMED | 21/03/2011 |

Determination of total starch content in cereal fermented foods by using an enzymatic method

SOP: Chem-Cere-11-en

Date: 21/03/2011

Release: 2

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Determination of total starch content in cereal fermented foods by using an enzymatic method

SOP: Chem-Cere-11-en

Date: 21/03/2011

Release: 2

1 SCOPE AND APPLICATION

This document describes a routine reference method for the determination of total starch content of cereals (raw and processed) following AACC International Method 76-13.01 and using total starch assay kit (K-TSTA 04/2009, Megazyme).

This procedure is quantitative for a wide range of cereals and cereal products using. It is applicable to the following products: wheat, durum wheat, rice (paddy, husked and milled rice), barley, millet (*Panicum miliaceum*), oats, sorghum and kaffir (*Sorghum vulgare caffrorum*), in the form of grains, milled grains, semolina or flour.

2 REFERENCES

AACC International Method 76-13.01

Total starch assay kit (K-TSTA 04/2009, Megazyme).

3 DEFINITIONS

For the purpose of this sop the following definition applies: Total starch is the mixture of two different polysaccharides comprising both the linear polymer amylose and the branched form, amylopectin.

4 PRINCIPLE

AACC International Method 76-13.01 analysis procedure allows the measurement of total starch in most cereal products (natural or processed). In the assay format described, starch hydrolysis proceeds in two phases. In phase I, starch is partially hydrolysed and totally solubilised. In phase II, the starch dextrins are quantitatively hydrolysed to D-glucose by amyloglucosidase. For most samples (*e.g.* wheat flour), complete solubilisation of starch can be achieved by cooking the sample in the presence of thermostable α -amylase. However, for samples containing high levels of resistant starch (*e.g.* high amylose maize starch), complete solubilisation and dextrinisation requires pre-treatment with dimethyl sulphoxide (DMSO) at 100°C. Samples should be milled to pass a 0.5 mm screen.

5 REAGENTS

1. Supplied with kit

1. Thermostable α -amylase, 10 ml, 3000 U/ml; stabilized solution. Dilute aliquot (1.0 ml) to 30 ml with 50 mM MOPS buffer, pH 7.0 (reagent 7). Store frozen between uses.
2. Amyloglucosidase, 10 ml, 200 U/ml; stabilized solution. Use directly with-out dilution. Dispense with positive displacement dispenser. Store concentrate at 4°C.

Determination of total starch content in cereal fermented foods by using an enzymatic method

SOP: Chem-Cere-11-en

Date: 21/03/2011

Release: 2

3. Glucose determination reagent (GOPOD), for 1 liter. Reagent concentrations after dissolution in buffer:
 - a. Glucose oxidase, >2000 U/liter
 - b. Peroxidase, >650 U/liter
 - c. 4-Aminoantipyrine, 0.4 mM OPS buffer, pH 7.0 (reagent 7). Store frozen between uses.
 4. Glucose reagent buffer (concentrate), 50 ml. Dilute entire contents to 1 liter with distilled water and use to dissolve GOPOD reagent. Divide GOPOD reagent into aliquots of desired volume for storage.
 5. Glucose standard solution (100 µg/0.1 ml in 0.2% benzoic acid).
 6. Regular maize starch (starch content ~98% dry weight).
- 2. Not supplied with kit**
7. 3-[N-morpholino]propanesulfonic acid (MOPS) buffer (50 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide (0.02%).
 - a. Add 11.55 g MOPS to 900 ml distilled water. Adjust solution to pH 7.0 by addition of 1M (10%) HCl. Approximately 17 ml is required.
 - b. Add 0.74 g calcium chloride dihydrate and 0.2 g sodium azide and dissolve. Adjust volume to 1 liter, and store at room temperature. *Caution:* Do not add sodium azide until pH is adjusted. Acidification of sodium azide releases a poisonous gas.
 8. Sodium acetate buffer, 200 mM (pH 4.5) plus sodium azide, 0.02%.
 - a. Add 11.8 ml glacial acetic acid (1.05 g/ml) to 900 ml of distilled water. Adjust solution to pH 4.5 by addition of 4 g/100 ml 1M sodium hydroxide solution. Approximately 60 ml is required.
 - b. Add 0.2 g sodium azide and adjust volume to 1 liter. Store at room temperature.
 9. Dimethyl sulfoxide (DMSO), laboratory grade.
 10. Ethanol, aqueous, 80% (v/v).

6 APPARATUS

1. Laboratory mill capable of reducing sample to pass 0.6-mm screen.
2. Glass test tubes, round-bottomed, 16 × 120 mm or 18 × 150 mm.
3. Micropipetters, 100-µl,
4. Positive displacement pipetter
 - a. with 50-ml tip (to dispense 3-ml aliquots of bacterial α-amylase solution)
 - b. with 5.0-ml tip (to dispense 0.1-ml aliquots of amyloglucosidase solution).
5. Bench centrifuge, required speed 3000 rpm.
6. Filter paper, Whatman no. 1, or equivalent.
7. Analytical balance.
8. Spectrophotometer set at 510 nm.
9. Vortex mixer.
10. Thermostatted water bath set at 50.0°.
11. Boiling water bath with tube rack.

Determination of total starch content in cereal fermented foods by using an enzymatic method

SOP: Chem-Cere-11-en

Date: 21/03/2011

Release: 2

12. Stop clock.

7 PROCEDURE

Preparation of standards and blanks

1. With each set of determinations, reagent blanks and glucose standards (100 µg quadruplicate) should be included.
 - a. Reagent blank consists of 0.1 ml distilled water + 3.0 ml GOPOD reagent (reagent 3).
 - b. Glucose standard consists of 0.1 ml glucose standard (reagent 5, 100 µg/0.1 ml) + 3.0 ml GOPOD reagent.
2. With each set of determinations, a standard flour or starch sample should be included.
3. Sample blanks can be determined using the standard assay method with the modifications that in step 4, 3 ml of distilled water is used and in step 5, amyloglucosidase is replaced by water. Alternatively, the need to perform sample blank analysis can be avoided by preextraction of samples with aqueous ethanol (see below).

Pretreatment of samples containing glucose and maltosaccharides

1. Add sample (~100 mg; weighed accurately) to glass centrifuge tube (16 × 120 mm; 17 ml capacity). Weigh and record weight of tube plus sample.
2. Add 5.0 ml of aqueous ethanol (80% v/v), and incubate tube at 80–85°C for 5 min. Mix contents on vortex stirrer and add another 5 ml of 80% aqueous ethanol.
3. Centrifuge the tube for 10 min at 1000 × g (about 3,000 rpm) on bench centrifuge. Discard supernatant.
4. Resuspend pellet in 10 ml 80% aqueous ethanol, and stir on vortex mixer. Centrifuge as above, and carefully pour off supernatant.
5. Proceed from step 4 of standard assay method or from step 4 of modified assay method if DMSO treatment is required.

Standard assay method (Method A)

1. Mill cereal product to pass 0.5-mm screen.
2. Add sample (~100 mg; weighed accurately) to glass tube (16 × 120 mm). Tap tube to ensure that all samples falls to bottom of tube. Weigh and record weight of tube plus sample.
3. Wet with 0.2 ml aqueous ethanol (80% v/v) to aid dispersion, and stir tube on vortex mixer.
4. Immediately add 3 ml thermostable α-amylase (300 U) in 50 mM MOPS buffer (pH 7.0) (reagent 1), and vigorously stir on vortex mixer. Incubate tube in boiling water bath for 5 min.
5. Place tube in bath at 50°; add 4 ml acetate buffer (200 mM, pH 4.5) (reagent 8), followed by 0.1 ml amyloglucosidase (20 U) (reagent 2). Stir tube on vortex mixer and incubate at 50° for 30 min.
6. Remove tube from water bath and dry with tissue.

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7. Weigh tube and contents and adjust total weight to 10.0 g plus weight recorded in step 2 (i.e., weight of tube plus sample). (Note that 1.0 ml of water weighs 1.0 g and contribution to weight from buffer salts is negligible.)
8. Centrifuge the tube at 3000 rpm for 10 min, or filter contents through filter paper.
9. Remove aliquots (1.0 ml) of supernatant and add to 9.0 ml of distilled water (i.e., 10-fold dilution); mix thoroughly.
10. Transfer aliquots (0.1 ml) of diluted solution to bottom of glass test tubes (16 × 100 mm) in duplicate.
11. Add 3.0 ml of GOPOD reagent (reagent 3) to each tube (including glucose standards and reagent blanks), and incubate tubes at 50° for 20 min.
12. Read absorbance at 510 nm for each sample, and read glucose standards against reagent blank.

Modified assay method (Method B, for samples containing resistant starch)

1. Mill cereal product to pass 0.5-mm screen.
2. Add sample (~100 mg, weighed accurately) to glass tube (16 × 120 mm). Weigh and record weight of tube plus sample.
3. Wet with 0.2 ml aqueous ethanol (80% v/v) to aid dispersion, and stir tube on vortex mixer.
4. Immediately add 2 ml DMSO and stir tube on vortex mixer. Place tube in vigorously boiling water bath, and remove after 5 min.
5. Proceed according to standard assay method from step 4.

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Starch content is:

$$\text{Starch (\%, as is)} = \Delta E/G \times 100 \times 10 \times 1/100 \times 100/W \times 162/180$$

where: ΔE = absorbance (reaction) read against the reagent blank, G = absorbance for 100 μg glucose standard solution, 100 = volume correction (0.1 ml taken from 10 ml), 10 = dilution factor, 1/1000 = conversion from μg to mg, $100/W$ = factor to express “starch” as a percentage of flour weight, W = weight in mg (“as is” basis) of flour analyzed, and 162/180 = adjustment from free glucose to anhydro glucose (as occurs in starch).

Starch content on dry basis is:

$$\text{Starch (\%, dry basis)} = \text{Starch (\%, as is)} \times 100 / (100 - \text{moisture content (\%, wet basis)})$$

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8.2 Repeatability

Standard errors of $\pm 2\%$ are achieved routinely.

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- **Sodium azide is a toxic chemical and should be treated accordingly. It is added to buffers solely as a preservative. It can be deleted from buffer recipes, but buffers should then be stored at 4°C.**
- **DMSO is a skin irritant and thus should be used with caution.**
- With each new batch of GOPOD reagent, check the time for maximum color formation with 100 μg of glucose standard. This is usually about 15 min. The time of incubation with GOPOD reagent is not critical but should be at least 20 min. The color formed should be measured within 60 min.
- The stability of the GOPOD reagent is 2–3 months at 4°C and 12 months at –20°C.
- Since 2 ml of DMSO weighs 2.2 g, when using the modified method, the sample weight should be adjusted to 10.2 g (i.e., 10.0 ml).
- Alternatively, at step 6, transfer the entire contents of the test tube to a 100-ml volumetric flask and adjust to volume with distilled water. Mix thoroughly. Centrifuge (3000 rpm, 10 min) an aliquot of this solution, and proceed from step 10 of the method.
- This approach gives relatively large errors for samples containing low levels of resistant starch because it involves subtraction of one high absorbance value from another.

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results. The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------------|--------------------|-----------------------|
| 19/03/2011 | MESTRES Christian | Edition |

Determination of total starch content in cereal fermented foods by using an enzymatic method

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12 APPENDIX

Total starch assay kit (K-TSTA 04/2009, Megazyme) procedure.

Megazyme

TOTAL STARCH ASSAY PROCEDURE

(AMYLOGLUCOSIDASE/
 α -AMYLASE METHOD)

K-TSTA 04/2009

(100 Assays per Kit)

AOAC Method 996.11

AACC Method 76.13

(and improvements)



INTRODUCTION:

Starch determination methods are broadly grouped into acid hydrolysis or enzymic procedures.¹ Acid hydrolysis procedures can only be applied to pure starch samples and thus have limited application. Enzymic procedures vary in pre-treatment steps,² starch gelatinisation, liquefaction and dextrinisation, hydrolysis of dextrans to glucose and glucose measurement³. AACC Method 76-11⁴ specifies starch gelatinisation under aqueous conditions in an autoclave, followed by starch conversion to glucose with amyloglucosidase and glucose measurement. AACC Method 76-11 underestimates starch content in a range of samples and materials, including high amylose maize starches and many processed cereal products. Most methods in use today incorporate treatment with thermostable α -amylase either during or immediately following the starch gelatinisation step.^{5,6} For samples which are difficult to gelatinise (such as high amylose maize starch) solvents such as sodium hydroxide or dimethyl sulphoxide (DMSO)^{7,8} have been employed. In a procedure to ensure complete solubilisation of starch in dietary fibre determination, Englyst and Cummings (1988)⁷ included treatment with the starch debranching enzyme, pullulanase.

To satisfy the need for an extremely simple, yet quantitative and reliable, procedure for the measurement of total starch, Megazyme produces, and offers a total starch assay kit based on the use of thermostable α -amylase and amyloglucosidase (McCleary *et al.*⁹). This method has been adopted by AOAC (Official Method 996.11) and AACC (Method 76.13).

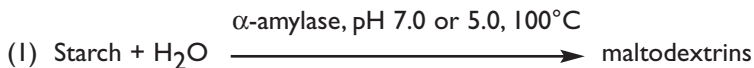
More recently, thermostable α -amylases that are active and stable at lower pH values have become available. Consequently, we have updated our total starch methodology to incorporate such an enzyme¹⁰. The major advantage of this improvement is to allow both the thermostable α -amylase and amyloglucosidase incubation steps to be performed at the same pH (pH 5.0), which in turn, simplifies the assay and minimises the possibility of production of maltulose (4- α -glucopyranosyl-D-fructose)¹¹, which is resistant to hydrolysis by amyloglucosidase and α -amylase. A further modification of this method involving D-glucose using the hexokinase/glucose-6-phosphate dehydrogenase/NADP⁺ based format is now also offered (K-TSHK5).

The Megazyme total starch analysis procedure allows the measurement of total starch in a wide range of food, feed, plant and cereal products (natural or processed). For most samples (e.g. wheat flour), starch is completely solubilized on incubating the sample at approx. 100°C in the presence of thermostable α -amylase. Samples

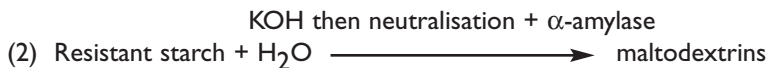
containing high levels of resistant starch (e.g. high amylose maize starch), require pre-dissolution in cold 2 M KOH or hot DMSO. For samples containing soluble starch or maltodextrins, cooking with thermostable α -amylase is not required.

PRINCIPLE:

Thermostable α -amylase hydrolyses starch into soluble branched and unbranched maltodextrins (1).



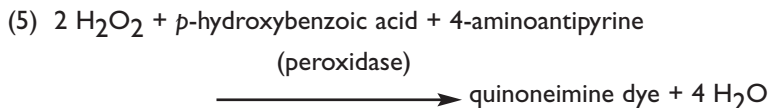
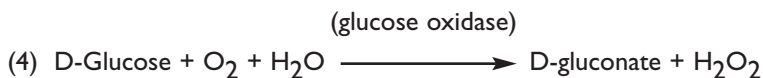
Where necessary, resistant starch in the sample is pre-dissolved by stirring the sample with 2 M KOH at approx. 4°C, followed by neutralisation with sodium acetate buffer and hydrolysis with α -amylase (2). Alternatively, dissolution in DMSO at 100°C is effective.



Amyloglucosidase (AMG) quantitatively hydrolyses maltodextrins to D-glucose (3).



D-Glucose is oxidised to D-gluconate with the release of one mole of hydrogen peroxide (H_2O_2) which is quantitatively measured in a colourimetric reaction employing peroxidase and the production of a quinoneimine dye.



Samples containing high levels of D-glucose and maltodextrins are washed with aqueous ethanol (80 % v/v) before analysis.

Analysis of a single sample can be performed within 70 min. Twenty samples can be analysed within 2 h.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

The assay is specific for α -glucans (including starch, glycogen, phytyglycogen and non-resistant maltodextrins).

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to 1.0 mg of D-glucose (or 0.9 mg starch)/L of sample solution at the maximum sample volume of 1.00 mL. The detection limit is 2.0 mg D-glucose (or 1.8 mg starch)/L, which is derived from an absorbance difference of 0.020 with the maximum sample volume of 1.00 mL.

The assay is linear over the range of 5 to 100 μ g of D-glucose per assay. In duplicate determinations using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 1.00 mL, this corresponds to a D-glucose concentration of approx. 0.05 to 1.0 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

If the conversion of D-glucose has been completed within the time specified in the assay (approx. 5 min), it can be generally concluded that no interference has occurred. However, this can be further checked by adding D-glucose (approx. 50 μ g in 0.1 mL) to the cuvette on completion of the reaction. A significant increase in the absorbance should be observed.

Interfering substances in the sample being analysed can be identified by including an internal standard. Quantitative recovery of this standard would be expected. Losses in sample handling and extraction are identified by performing recovery experiments i.e. by adding D-glucose to the sample in the initial extraction steps.

SAFETY:

The reagents used in the determination of D-glucose are not hazardous materials in the sense of the Hazardous Substances Regulations. However, the buffer concentrate contains sodium azide (0.02 % w/v) as a preservative. The general safety measures that apply to all chemical substances should be adhered to.

KITS:

Kits suitable for performing 100 determinations of total starch are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1:** Thermostable α -amylase (10 mL, 3,000 U/mL on Ceralpha reagent* at pH 6.5 and 40°C or 1600 U/mL on Ceralpha reagent at pH 5.0 and 40°C). Stable for > 4 years at 4°C.
- Bottle 2:** Amyloglucosidase (10 mL, 3300 U/mL on soluble starch (or 200 U/mL on *p*-nitrophenyl β -maltoside*) at pH 4.5 and 40°C; Stable for > 4 years at 4°C.

*Full assay procedure is available at “www.megazyme.com”.

- Bottle 3:** **GOPOD Reagent Buffer.** Potassium phosphate buffer (0.26 M, pH 7.4), *p*-hydroxybenzoic acid (0.22 M) and sodium azide (0.4 % w/v). Stable for > 4 years at 4°C.

NOTE:

1. If GOPOD Reagent Buffer is stored at -20°C, it will form salt crystals that must be completely dissolved when this buffer is diluted to 1 L with distilled water.
2. This concentrated buffer contains 0.4 % (w/v) sodium azide. This is a poisonous chemical and should be treated accordingly.

- Bottle 4:** **GOPOD Reagent Enzymes.** Glucose oxidase (> 12,000 U) plus peroxidase (> 650 U) and 4-aminoantipyrine (80 mg). Freeze-dried powder. Stable for > 4 years at -20°C.

- Bottle 5:** D-Glucose standard solution (5 mL, 1.0 mg/mL) in 0.2 % (w/v) benzoic acid. Stable for > 4 years at room temperature.

- Bottle 6:** Standardised regular maize starch control. Starch content shown on vial label. Stable for > 4 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

- Solution 1.** Dilute 1.0 mL of the contents of bottle 1 to 30 mL with Reagent 1 (100 mM sodium acetate buffer, pH 5.0; not supplied). Store the diluted enzyme frozen between use. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use and keep cool during use if possible. Stable for > 3 years at -20°C.

NOTE:

If the sample is to be analysed according to AOAC Official Method 996.11 (example b), the enzyme is diluted in MOPS buffer (50 mM, pH 7.0; Reagent 4)

- Solution 2.** Use the contents of bottle 2 as supplied. This solution is viscous and thus should be dispensed with a positive displacement dispenser e.g. Eppendorf Multipipette® with 5.0 mL Combitip® (to dispense 0.1 mL aliquots). Stable for > 3 years at 4°C
- Solution 3.** Dilute the contents of bottle 3 (GOPOD Reagent Buffer) to 1 L with distilled water. Use immediately.
- Solution 4.** Dissolve the contents of bottle 4 in 20 mL of solution 3 and quantitatively transfer this to the bottle containing the remainder of solution 3. Cover this bottle with aluminium foil to protect the enclosed reagent from light. This is Glucose Determination Reagent (**GOPOD Reagent**). Stable for ~ 3 months at 2-5°C or > 12 months at - 20°C.
- If this reagent is to be stored in the frozen state, preferably it should be divide into aliquots that should be freeze/thawed only once during use.
- When the reagent is freshly prepared it may be light yellow or light pink in colour. It will develop a stronger pink colour over 2-3 months at 4°C. The absorbance of this solution should be less than 0.05 when read against distilled water.
- Solutions 5 & 6.** Use the contents of bottles 5 and 6 as supplied. Stable for > 5 years at room temperature.

REAGENTS (NOT SUPPLIED):

- 1. Sodium acetate buffer** (100 mM, pH 5.0) plus calcium chloride (5 mM).
Add 5.8 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 5.0 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 30 mL is required). Stable for approx. 2 months at 4°C.
- Add 0.74 g of calcium chloride dihydrate and dissolve. Adjust the volume to 1 litre and store the buffer at 4°C. Stable for > 6 months at 4°C.

The stability of this buffer can be increased by adding sodium azide (0.2 g of sodium azide/L buffer).

Stable for approx. 2 years at room temperature.

NOTE: Sodium azide should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

2. Sodium acetate buffer (1.2 M, pH 3.8).

Add 69.6 mL of glacial acetic acid (1.05 g/mL) to 800 mL of distilled water and adjust to pH 3.8 using 4 M sodium hydroxide.

Adjust the volume to 1 litre with distilled water.

Stable for 12 months at room temperature .

3. Potassium hydroxide solution (2 M).

Add 112.2 g KOH to 900 mL of deionised water and dissolve by stirring. Adjust volume to 1 litre. Store in a sealed container.

Stable for > 2 years at room temperature.

4. MOPS buffer (50 mM, pH 7.0) plus calcium chloride (5 mM) and sodium azide (0.02 % w/v). **Optional: Only required if samples are analysed according to example (b).**

Dissolve 11.55 g of MOPS (sodium salt, Sigma cat. no. M-9381) in 900 mL of distilled water and adjust the pH to pH 7.0 by the addition of 1 M (10 % v/v) HCl (approx. 17 mL is required).

Add 0.74 g of calcium chloride dihydrate and 0.2 g of sodium azide and dissolve. Adjust the volume to 1 L.

Stable for 6 months at 4°C.

5. Sodium acetate buffer (200 mM, pH 4.5) plus sodium azide (0.02 % w/v). **Optional: Only required if samples are analysed according to example (b).**

Add 11.6 mL of glacial acetic acid (1.05 g/mL) to 900 mL of distilled water. Adjust the pH to 4.5 by the addition of 1 M (4 g/100 mL) sodium hydroxide solution (approx. 60 mL is required). Add 0.2 g of sodium azide and dissolve. Adjust the volume to 1 L. Stable for 6 months at 4°C.

NOTE: Sodium azide should not be added until the pH is adjusted. Acidification of sodium azide releases a poisonous gas.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (round bottomed; 16 x 120 mm or 18 x 150 mm).
2. Micro-pipettors, 100 μL (e.g. Gilson Pipetman[®] or Rainin EDP-2[®] motorised dispenser).
3. Positive displacement pipettor e.g. Eppendorf Multipette[®]
 - with 50 mL Combitip[®] (to dispense 3 mL aliquots of bacterial α -amylase solution).
 - with 5.0 mL Combitip[®] (to dispense 0.1 mL aliquots of amyloglucosidase solution).
4. Bench centrifuge (required speed 3,000 rpm; approx. 1,800 g).
5. Analytical balance.
6. Spectrophotometer set at 510 nm.
7. Vortex mixer (e.g. IKA[®] Yellowline Test Tube Shaker TTS2).
8. Thermostatted water bath set at 50.0°C.
9. Boiling water bath with tube rack.
10. Stop clock.

CONTROLS AND PRECAUTIONS:

1. The time of incubation with GOPOD reagent is not critical, but should be at least 20 min. The colour formed should be measured within 60 min.
2. With each set of determinations, reagent blanks and glucose controls (100 μg , quadruplicate) should be included.
 - a) The reagent blank consists of 0.1 mL distilled water + 3.0 mL GOPOD Reagent.
 - b) The glucose control consists of 0.1 mL glucose standard solution (100 $\mu\text{g}/0.1$ mL) + 3.0 mL GOPOD Reagent. The Factor "F" (pages 13 and 14) is calculated by dividing the amount of D-glucose analysed (100 μg) by the absorbance obtained for this amount of D-glucose in the standard assay (e.g. $100/1.038 = 96.386$). **The absorbance value will vary.**
3. With each set of determinations, a standard flour or starch sample should be included.
4. With each new batch of GOPOD Reagent, the time for maximum colour formation with 100 μg of glucose standard should be checked. This is usually approximately 15 min.

SAMPLE BLANKS:

Sample blanks can be determined using the Standard Assay Procedure [example (a)] with the modifications that in Step 4, three (3) mL of distilled water is used and in Step 5, amyloglucosidase is replaced by water. Alternatively, the need to perform sample blank analysis can be avoided by pre-extraction of samples with aqueous ethanol (80 % v/v) [see example (e)].

SAMPLE PREPARATION EXAMPLES:

(a) Determination of starch in cereal and food products not containing resistant starch, D-glucose and/or maltodextrins. (Recommended Procedure; all incubations at pH 5.0).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~100 mg; weighed accurately) to a glass test tube (16 x 120 mm). Tap the tube to ensure that all of the sample drops to the bottom of the tube.
3. Add 0.2 mL of aqueous ethanol (80 % v/v) to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
4. Immediately add 3 mL of thermostable α -amylase (contents of bottle 1 diluted 1:30 in Reagent 1; 100 mM sodium acetate buffer, pH 5.0). Incubate the tube in a boiling water bath for 6 min. (Stir the tube vigorously after 2, 4 and 6 min).

NOTE: In this step it is essential that the tube is stirred vigorously to ensure complete homogeneity of the slurry (removal of lumps). Also, stirring after 2 min prevents the possibility of some of the sample expelling from the top of the tube when the alcohol is evaporating.

If polypropylene tubes are used, increase the incubation time to 12 min, with stirring after 4, 8 and 12 min.

5. Place the tube in a bath at 50°C; add 0.1 mL of the contents of bottle 2 (amyloglucosidase, 330 U on starch). Stir the tube on a vortex mixer and incubate at 50°C for 30 min.
6. Transfer the entire contents of the test tube to a 100 mL volumetric flask (with a funnel to assist transfer). Use a wash bottle to rinse the tube contents thoroughly. Adjust to volume with distilled water. Mix thoroughly. Centrifuge an aliquot of this solution at 3,000 rpm for 10 min. Use the clear, undiluted filtrate for the assay.

ALTERNATIVELY, at Step 6, adjust the volume to 10 mL with distilled water and then centrifuge the tubes at 3,000 rpm for 10 min. For samples containing 1-10% starch content, this solution is used directly in Step 7. For samples containing 10-100% starch, an aliquot (1.0 mL) is diluted to 10 mL with distilled water before proceeding to Step 7.

7. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).
8. Add 3.0 mL of GOPOD Reagent to each tube (including the D-glucose controls and reagent blanks), and incubate the tubes at 50°C for 20 min.
9. **D-Glucose controls** consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. **Reagent Blank solutions** consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
10. Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.

(b) Determination of starch in cereal and food products not containing resistant starch, D-glucose and/or maltodextrins. (AOAC Official Method 996.11).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~100 mg; weighed accurately) to a glass test tube (16 x 120 mm). Tap the tube to ensure that all of the sample drops to the bottom of the tube.
3. Add 0.2 mL of aqueous ethanol (80 % v/v) to wet the sample and aid dispersion. Stir the tube on a vortex mixer.
4. Immediately add 3 mL of thermostable α -amylase (contents of bottle 1 diluted 1:30 in Reagent 4; 50 mM MOPS buffer, pH 7.0). Incubate the tube in a boiling water bath for 6 min. (Stir the tube vigorously after 2, 4 and 6 min).

NOTE: In this step it is essential that the tube is stirred vigorously to ensure complete homogeneity of the slurry (removal of lumps). Also, stirring after 2 min prevents the possibility of some of the sample expelling from the top of the tube when the alcohol is evaporating.

If polypropylene tubes are used, increase the incubation time to 12 min, with stirring after 4, 8 and 12 min.

5. Place the tube in a bath at 50°C; add sodium acetate buffer (4 mL, 200 mM, pH 4.5), followed by amyloglucosidase (0.1 mL, 20 U). Stir the tube on a vortex mixer and incubate at 50°C for 30 min.
6. Proceed according to **Step 6 of example (a)**.

(c) Determination of total starch content of samples containing resistant starch, but no D-glucose and/or maltodextrins (KOH Format - Recommended).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~100 mg, weighed accurately) to a glass tube (16 x 120 mm).
3. Wet with 0.2 mL of aqueous ethanol (80 % v/v) to aid dispersion, and stir the tube on a vortex mixer.
4. Add a magnetic stirrer bar (5 x 15 mm) and 2 mL of 2 M KOH to each tube and re-suspend the pellets (and dissolve the RS) by stirring for approx. 20 min in an ice/water bath over a magnetic stirrer (Figure 1).

NOTE:

1. Do not mix on a vortex mixer as this may cause the starch to emulsify.
 2. Ensure that the tube contents are vigorously stirring as the KOH solution is added. This will avoid the formation of a lump of starch material that will then be difficult to dissolve.
5. Add 8 mL of 1.2 M sodium acetate buffer (pH 3.8) to each tube with stirring on the magnetic stirrer. Immediately add 0.1 mL of thermostable α -amylase (bottle 1) and 0.1 mL of AMG (bottle 2), mix well and place the tubes in a water bath at 50°C.
 6. Incubate the tubes for 30 min with intermittent mixing on a vortex mixer.
 7. **For samples containing > 10 % total starch content;** quantitatively transfer the contents of the tube to a 100 mL volumetric flask (using a water wash bottle). Use an external magnet to retain the stirrer bar in the tube while washing the solution from the tube with a water wash bottle. Adjust to 100 mL with distilled water and mix well. Centrifuge an aliquot of the solution at 1,800 g for 10 min.
 8. **For samples containing < 10 % total starch content;** directly centrifuge the tubes at 1,800 g for 10 min (no dilution). For

such samples, the final volume in the tube is approx. 10.4 mL (however, this volume will vary particularly if wet samples are analyzed, and appropriate allowance for volume should be made in the calculations).

9. Proceed from **Step 7 of example (a)** .

(d) Determination of total starch content of samples containing resistant starch, but no D-glucose and/or maltodextrins (DMSO Format- AOAC Official Method 996.11).

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~100 mg, weighed accurately) to a glass tube (16 x 120 mm).
3. Wet with 0.2 mL of aqueous ethanol (80 % v/v) to aid dispersion, and stir the tube on a vortex mixer.
4. Immediately add 2 mL of dimethyl sulphoxide (DMSO) and stir the tube on a vortex mixer. Place the tube in a vigorously boiling water bath and remove after 5 min.
5. Proceed from **Step 4 of examples (a) or (b)**.

(e) Determination of starch in samples which also contain D-glucose and/or maltodextrins.

1. Mill cereal, plant or food product to pass a 0.5 mm screen.
2. Add milled sample (~100 mg, weighed accurately) to a glass centrifuge tube (16 x 120 mm; 17 mL capacity).
3. Add 5.0 mL of aqueous ethanol (80 % v/v), and incubate the tube at 80-85°C for 5 min. Mix the contents on a vortex stirrer and add another 5 mL of 80% v/v aqueous ethanol.
4. Centrifuge the tube for 10 min at 1,800 g (approx. 3,000 rpm) on a bench centrifuge. Discard the supernatant.
5. Resuspend the pellet in 10 mL of 80 % v/v aqueous ethanol and stir on a vortex mixer. Centrifuge as above and carefully pour off the supernatant.
6. Proceed from **Step 4 of example (a) or (b)**.

Alternatively:

Proceed from **Step 4 of example (c)** if the sample contains resistant starch.

(f) Determination of starch in samples in which the starch is present in a soluble form and D-glucose and maltodextrins are not present.

1. Filter an aliquot of the sample solution through Whatman No 1 filter paper (or Whatman GF/A Glass fibre filter paper if necessary). Use the clear, filtrate for the assay.
2. Add 10 mL of this filtrate to a glass tube. Add 2 mL of Reagent 1 (100 mM acetate buffer, pH 5.0) plus 0.1 mL of AMG (bottle 2) diluted 10-fold in Reagent 1 (i.e 33 U of AMG on starch) and incubate in a water bath at 50°C for 30 min. Adjust volume to 20 mL (or 20 g) with distilled water.
3. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).
4. Add 3.0 mL of GOPOD Reagent to each tube (including D-glucose controls and reagent blanks), and incubate the tubes at 50°C for 20 min.
5. **D-Glucose controls** consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. **Reagent Blank solutions** consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
6. Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.

(g) Determination of starch in samples in which the starch is present in a soluble form and D-glucose and maltodextrins are present.

1. Filter an aliquot of the sample solution through Whatman No 1 filter paper (or Whatman GF/A Glass fibre filter paper if necessary). Use the clear, filtrate for the assay.
2. Add 2 mL of the solution to be analysed to a 17 mL glass test tube. To this, add 8 mL of 95 % v/v ethanol and mix vigorously on a vortex mixer. Allow to stand at room temperature for 30 min and centrifuge at 1,800 g for 10 min.
3. Decant the supernatant solution and redissolve the starch containing pellet in 1 mL of water. If necessary, heat the tube and contents in a boiling water bath to aid redissolution. Adjust the volume to 3.9 mL (3.9 g) with Reagent 1 (100 mM acetate buffer, pH 5.0), taking account of the original weight of the tube.
4. If necessary, repeat the ethanol precipitation and centrifugation steps (e.g. for samples containing high levels of free D-glucose and/or maltodextrins). Decant the supernatant solution and

redissolve the starch containing pellet in 1 mL of water. If necessary, heat the tube and contents at 100°C to aid redissolution. Adjust the volume to 3.9 mL (3.9 g) with water, taking account of the original weight of the tube.

5. Add 0.1 mL of AMG (bottle 2) diluted 50-fold in Reagent I (i.e. 6.6 U of AMG on starch) and incubate in a water bath at 50°C for 30 min.
6. Transfer duplicate aliquots (0.1 mL) of the diluted solution to the bottom of glass test tubes (16 x 100 mm).
7. Add 3.0 mL of GOPOD Reagent to each tube (including D-glucose controls and reagent blanks), and incubate the tubes at 50°C for 20 min.
8. **D-Glucose controls** consist of 0.1 mL of D-glucose standard solution (1 mg/mL) and 3.0 mL of GOPOD Reagent. **Reagent Blank solutions** consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent.
9. Read the absorbance for each sample, and the D-glucose control at 510 nm against the reagent blank.

(h) Determination of enzyme resistant starch.

This can be accurately measured using the Resistant Starch assay kit (K-RSTAR) supplied by Megazyme. Results obtained closely simulate those obtained under *in vivo* conditions. Details of this method can be obtained from the Megazyme website (www.megazyme.com; K-RSTAR). This method has been successfully subjected to interlaboratory evaluation (37 labs, 16 samples) under the auspices of AOAC INTERNATIONAL (AOAC Official Method 2002.02)^{12, 13} and AACC International (Recommended Method 32-40)

CALCULATIONS (Solid samples):

$$\begin{aligned} \text{Starch, \%} &= \Delta_A \times F \times \frac{FV}{0.1} \times \frac{1}{1000} \times \frac{100}{W} \times \frac{162}{180} \\ &= \Delta_A \times \frac{F}{W} \times FV \times 0.9 \end{aligned}$$

where:

Δ_A = Absorbance (reaction) read against the reagent blank.

F = $\frac{100 \text{ (}\mu\text{g of D-glucose)}}{\text{absorbance for } 100 \mu\text{g of glucose}}$ (conversion from absorbance to μg)

FV = Final volume (i.e. equals 100 mL or 10 mL in examples (a) and (b); 100 or 10.4 mL in example (c); 100 mL or 10.0 mL in example (d); and 100 mL or 10 mL in example (e).

0.1 = volume of sample analysed.

$\frac{1}{1000}$ = Conversion from μg to mg.

$\frac{100}{W}$ = Factor to express “starch” as a percentage of flour weight.

W = The weight in milligrams (“as is” basis) of the flour analysed.

$\frac{162}{180}$ = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

Starch % w/w (dry wt. basis):

$$= \text{Starch \% w/w (as is)} \times \frac{100}{100 - \text{moisture content (\% w/w)}}$$

CALCULATIONS (Liquid samples; mg/100 mL):

$$\begin{aligned} \text{Starch} &= \Delta_A \times F \times \frac{100}{0.1} \times \frac{1}{1000} \times \frac{162}{180} \times 2 \times D \\ &= \Delta_A \times F \times D \times 1.8 \end{aligned}$$

where:

Δ_A = Absorbance (reaction) read against the reagent blank.

F = $\frac{100 \text{ (\mu g of D-glucose)}}{\text{absorbance for 100 } \mu\text{g of glucose}}$ (conversion from absorbance to μg)

100 = conversion to 100 mL sample volume.

0.1 = volume of sample analysed.

$\frac{1}{1000}$ = Conversion from μg to mg.

$\frac{162}{180}$ = Adjustment from free D-glucose to anhydro D-glucose (as occurs in starch).

2 = Dilution of the sample solution on incubation with AMG.

D = Further dilution of the incubation mixture (if required).

NOTE: These calculations can be simplified by using the Megazyme **Mega-Calc™**, for solid or liquid samples, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

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Table 1. Comparison of total starch values determined with AOAC Method 996.11 and the current method in which α -amylase and amyloglucosidase incubations were performed at pH 5.

| Sample | Total starch (<i>as is basis</i>) ^a | |
|--|--|-----------------------|
| | AOAC Method 996.11 | Current Method (pH 5) |
| Regular maize starch | 85.0 | 84.8 |
| Wheat flour | 69.1 | 68.7 |
| High amylose maize starch ^b | 76.8 | 77.2 |
| ACS soluble starch | 83.5 | 83.4 |
| Chemically modified starch | 81.7 | 82.7 |
| Oat bran | 37.2 | 37.0 |

^a The average of duplicate analyses by two separate analysts.

^b high amylose starch (total starch value is underestimated).

Table 2. Comparison of total starch values determined with AOAC Method 996.11 (DMSO modification) and the current modification in which starch is dissolved in 2 N KOH, pH adjusted and α -amylase and amyloglucosidase incubations performed at pH 5.

| Sample | Total starch (<i>as is basis</i>) ^a | |
|--|--|-----------------------------|
| | AOAC Method 996.11 | Current Method (KOH / pH 5) |
| Regular maize starch | 84.3 | 85.2 |
| High amylose maize starch ^b | 83.2 | 83.4 |
| Potato amylose ^b | 86.1 | 86.7 |
| Novelose 240 ^c | 84.5 | 84.7 |
| Hylon VII ^b | 85.1 | 84.9 |

^a The average of duplicate analyses by two separate analysts.

^b native high amylose starch.

^c retrograded high amylose starch.

Table 3. Results of an interlaboratory evaluation of the total starch assay procedure (AOAC Official Method 996.11; examples “b” and modification with DMSO).

| Sample | Chicken Feed Pellets | White Bread | Green Pea | High Amylose Maize Starch* | White Wheat Flour |
|--------------|----------------------|-------------|----------------|----------------------------|-------------------|
| Moisture % | 11.4 | 10.7 | 12.4 | 13.4 | 12.8 |
| No. of labs. | 32 | 32 | 31 | 25 | 31 |
| Outliers | 0 | 0 | 1 ^G | 1 ^C | 1 ^C |
| Average % | 50.7 | 68.1 | 44.0 | 86.3 | 78.0 |
| S_r | 1.6 | 1.8 | 1.5 | 2.5 | 2.2 |
| RSD_r | 3.1 | 2.7 | 3.4 | 2.9 | 2.9 |
| r | 4.4 | 5.2 | 4.2 | 7.0 | 6.3 |
| S_R | 2.4 | 3.4 | 2.1 | 4.1 | 3.3 |
| RSD_R | 4.6 | 5.0 | 4.8 | 4.8 | 4.2 |
| R | 6.6 | 9.5 | 6.0 | 11.6 | 9.2 |
| HORRAT | 2.1 | 2.4 | 2.1 | 2.8 | 2.0 |
| Range | 45.4-55.3 | 62.0-74.9 | 39.4-47.4 | 78.7-96.8 | 71.6-85.8 |

Table 3. (cont.)

| Sample | Wheat Starch* | Oat Bran | Spaghetti | High Amylose Maize Starch DMSO procedure | Wheat Starch DMSO procedure |
|--------------|---------------|----------------|----------------|--|-----------------------------|
| Moisture % | 12.3 | 8.8 | 11.8 | 13.4 | 12.3 |
| No. of labs. | 26 | 31 | 31 | 31 | 31 |
| Outliers | 0 | 1 ^C | 1 ^C | 1 ^C | 1 ^C |
| Average % | 97.2 | 42.2 | 76.6 | 97.2 | 96.5 |
| S_r | 3.2 | 1.6 | 3.0 | 2.0 | 3.0 |
| RSD_r | 3.3 | 3.8 | 3.9 | 2.1 | 3.1 |
| r | 9.0 | 4.5 | 8.4 | 5.7 | 8.4 |
| S_R | 3.7 | 2.1 | 3.7 | 2.8 | 4.4 |
| RSD_R | 3.8 | 5.0 | 4.8 | 2.9 | 4.6 |
| R | 10.4 | 6.0 | 10.3 | 7.8 | 12.4 |
| HORRAT | 1.9 | 2.2 | 2.3 | 1.4 | 2.3 |
| Range | 91.8-105.0 | 38.7-46.8 | 70.1-81.8 | 91.6-101.9 | 86.0-104.0 |

Number of Labs. = Number of laboratories included in calculations

Outliers = Number of outlier laboratories, not included in calculations (C=Cochran, G=Grubbs outlier)

S_r = Repeatability standard deviation

RSD_r = Repeatability relative standard deviation

r = Repeatability value ($2.8 \times S_r$)

S_R = Reproducibility standard deviation

RSD_R = Reproducibility relative standard deviation

R = Reproducibility value ($2.8 \times S_R$)

HORRAT = Horwitz ratio, an indication of the precision of the method.

* = With these samples, only 26 sets of results were supplied due to a misinterpretation of instructions.

In this table the statistical evaluation of results from an interlaboratory evaluation of the methods is shown. Thirty-two laboratories (worldwide) were involved and sixteen samples (eight blind duplicates) were analysed.



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| | | |
|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination de la teneur en amidon des produits céréaliers fermentés : dosage enzymatique après solubilisation à la soude | | |
| SOP Number: Chem-Cere-10-fr | | |
| Date : 04/11/2011 | Version : 2 | |
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| CIRAD UAC CSIR FAAU ESB FRI NRC | Christian MESTRES Laetitia MESTRES Noel AKISSOE | 30/03/2011 04/11/2011 25/08/2011 |

Détermination de la teneur en amidon des produits céréaliers fermentés fermentés : dosage enzymatique après solubilisation à la soude

SOP Number: **Chem-Cere-10-fr**

Date : 29/8/2011

Version : 2

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Détermination de la teneur en amidon des produits céréaliers fermentés fermentés : dosage enzymatique après solubilisation à la soude

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1 DOMAINE ET APPLICATION

Ce document décrit une méthode de quantification de la teneur en amidon des produits céréaliers (ou amyliacés) natifs ou fermentés. Il s'agit d'une modification de la norme NF V03-606.

2 REFERENCES

Amidons et féculés ; dosage de l'amidon. NF V03-606 (1996)

3 DEFINITIONS

L'amidon constitue la substance de réserve de nombreux végétaux. Présent sous forme de **granules**, il est composé d'amylose et d'amylopectine. Ces deux composés sont des polymères d'anhydro-D-glucose, dont les molécules sont liées par des liaisons glucosidiques essentiellement α -1,4- pour l'amylose (structure linéaire) et avec des branchements (liaisons α -1,6) reliant de courtes chaînes linéaires (15 à 35 monomères) pour l'amylopectine (structure ramifiée).

4 PRINCIPE

L'amidon est dispersé à l'aide d'hydroxyde de sodium, hydrolysé en glucose par action de l'amyloglucosidase, et le glucose libéré dosé par un système oxydoréducteur enzyme-colorant. On dose par ailleurs le glucose soluble par le même système oxydoréducteur enzyme-colorant. La teneur en amidon est obtenue en soustrayant cette valeur au glucose total libéré après action de l'amyloglucosidase.

5 REACTIFS

- Eau bi-distillée
- Ethanol absolu
- Solution de soude (hydroxyde de sodium) 1 N
- Solution d'acide sulfurique diluée (5 mM)
- amyloglucosidase (ref 10113, 120 U/mg ou 10115, 70 U/mg, Sigma)
- GOD : glucose oxidase (G 6125, Sigma)
- POD : peroxydase (P 8125, Sigma)
- 4-amino-antipyrine (A4382, Sigma)
- Acide 4-hydroxy-benzoïque (H2, 005-9, Aldrich)
- Amidon natif (de même nature que les échantillons)

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6 APPAREILLAGE

- Agitateurs type Vortex et rotatif
- Pipettes automatiques
- Centrifugeuse de paillasse
- Spectro-colorimètre visible

7 PROCEDURE

7.1 Préparation des solutions

7.1.1 Tampon acétate (pH 4.8 – 2M) (A)

- Dissoudre 164 d'acétate de sodium anhydre (NaOOCCH₃) dans 800 ml d'eau distillée, environ,
- Ajouter environ 120 mL d'acide acétique glacial en ajustant le pH à 4,8
- Compléter à 1000 mL.
- Peut se conserver environ 1 mois à 4°C

7.1.2 Solution d'Amylogucosidase (B)

- Peser environ 50 mg d'amylogucosidase 10113, ou 107 mg, de 10115 contenant 7500 U (125 µKat) d'activité amylogucosidase à 60°C
- Dissoudre dans 4 mL d'eau distillée, puis ajouter 1 mL de solution A
- Solution à préparer chaque jour

7.1.3 Tampon phosphate (pH 7,4 ; 1M) et acide hydroxybenzoïque (C)

- Dissoudre 8,4 g de soude en pastilles dans environ 100 mL d'eau distillée et 27,2 g de phosphate de potassium (KH₂PO₄) dans environ 50 mL d'eau distillée
- Mélanger les deux solutions en ajustant le pH à 7,4
- Ajouter 1 g d'acide hydroxybenzoïque, et ajuster à 200 mL
- Peut se conserver une semaine à température ambiante

7.1.4 Solution GOD-POD, -4-amino-antipyrine (D)

- Peser environ 20 mg de 4-amino-antipyrine, 100 mg de GOD et 3 mg de POD
- Ajouter environ 20 mL d'H₂O, puis 20 mL de la solution C, et compléter à 100 mL avec de l'H₂O
- Solution à préparer chaque jour

Détermination de la teneur en amidon des produits céréaliers fermentés fermentés : dosage enzymatique après solubilisation à la soude

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7.1.5 Solution de glucose

- Préparer une solution mère de glucose à 0,5 mg/mL dans de l'eau distillée
- Solution pouvant se conserver 2-3 jours à 4°C ; une aliquote peut être congelée pour dosages ultérieurs.

7.2 Dosage du glucose total

1. Dans une fiole de 100 mL, peser environ exactement 100 mg d'échantillon. Ajouter 1 mL d'éthanol absolu, en rinçant les particules de la prise d'essai adhérant à la paroi de la fiole.
2. Ajouter 9 mL de la solution d'hydroxyde de sodium (1N).
3. Laisser reposer à température ambiante durant 15 à 24 h.
4. Compléter au trait de jauge avec de l'eau et homogénéiser vigoureusement.
5. Prélever 1 mL de solution et filtrer à l'aide d'un filtre seringue 5µm
6. Dans un tube à hémolyse de 5 mL, placer un volume de filtrat adapté ($\leq 100 \mu\text{L}$) contenant au maximum 50 µg d'amidon. Compléter avec de l'eau distillée jusqu'à un volume total de 100 µL.
7. Ajouter 200 µL de la solution d'amyloglucosidase (solution B)
8. Laisser agir l'enzyme pendant 30 min à température ambiante. Agiter doucement (par mouvement latéral du porte tube de manière en évitant de mettre de la solution sur les parois des tubes) à T0' et à T15'
9. Après 30 min, ajouter 2,5 mL de GOD-POD (solution D)
10. Agiter modérément au Vortex, ou en retournant les tubes bouchés par du Parafilm
11. Laisser agir 20 min à température ambiante
12. Mesurer la DO à 510 nm (le zéro du spectro-colorimètre est effectué sur le blanc de la gamme d'étalonnage)

Remarque :

Pendant le dosage il est très recommandé de faire en même temps le dosage sur de l'amidon pur dans les mêmes conditions que pour l'échantillon. Prélever 100mg de poudre d'amidon pur et suivre les étapes n°1 au n°12 mais en prenant soin de pipeter 50µL seulement au n°6.

Le résultat doit approcher 98% de teneur en amidon (base sèche) ; il permet de valider de votre dosage

7.3 Etalonnage

Dans des tubes à hémolyse, déposer respectivement 100 µL d'eau (pour le zéro de la gamme), 50 µL d'eau + 50 µL de solution mère de glucose (en double), et 100 µL de solution mère de glucose (en double).

Ensuite chaque tube est soumis au dosage du glucose total en commençant par l'étape n°7 du paragraphe 7.2, jusqu'à la fin (n°12 du §7.2).

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7.4 Dosage du glucose libre

- Dans un tube à centrifuger jetable (type Eppendorf) de 2 mL, peser 100 mg d'échantillon
- Ajouter 1 mL de la solution d'acide sulfurique 5 mM. Boucher hermétiquement et agiter vigoureusement au Vortex
- Placer les tubes d'extraction sur l'agitateur rotatif et laisser extraire pendant 1 h à température ambiante
- Centrifuger le mélange à ~ 5000 g pendant 5 min.
- Prélever environ 0,5 mL de surnageant avec une seringue et filtrer avec un filtre seringue (0,45µm)
- Prélever 50 ou 100 µL (en fonction de la richesse du surnageant) dans un tube à hémolyse (en double)
- Pour doser le glucose total ajouter 200 µL d'eau distillée (**à la place de 200 µL d'amyloglucosidase**)
- Continuer le dosage du glucose total en commençant par l'étape n°8 jusqu'à la fin (n°12 du §7.2).

8 EXPRESSION DES RESULTATS

8.1 Methode de calcul et formules

Calculer la pente de la régression (P_e , µg/ml) de la courbe d'étalonnage en glucose

Calculer la teneur en glucose libre (Gl) :

$$Gl (\% bs) = \left(\frac{DOI \times P_e \times D \times 10}{Masse \times MS} \right)$$

Où :

DOI, est la DO mesurée pour le glucose libre

D, le facteur de dilution (1, si 100 µL d'extrait filtré sont dosés),

Masse, la masse d'échantillon analysée (mg)

MS, la teneur en matière sèche de l'échantillon (%)

Calculer la teneur en amidon (Amid) :

$$Amid (\% bs) = \left(\left(\frac{Dot \times P_e \times D \times 1000}{Masse \times MS} \right) - Gl \right) \times 0,9$$

Où :

Dot, est la DO mesurée pour le glucose total

D, le facteur de dilution (1, si 100 µL d'extrait filtré sont dosés),

Masse, la masse d'échantillon analysée (mg)

Détermination de la teneur en amidon des produits céréaliers fermentés fermentés : dosage enzymatique après solubilisation à la soude

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MS, la teneur en matière sèche de l'échantillon (%)

8.2 Répétabilité

L'écart type entre des répétitions réalisées dans un court intervalle de temps ne dépassera pas 5% de la moyenne (coefficient de variation)

9 POINTS CRITIQUES ET NOTES SUR LA PROCEDURE

- L'échantillon doit avoir une **granulométrie inférieure à 200 µm**
- Bien respecter les temps de réactions, en particulier avec la GOD-POD ; un décalage de temps (30 s ou 1 min par exemple) entre deux tubes doit être fixé et respecté tout au long de la séquence d'analyse.
- Il est conseillé d'analyser un amidon témoin en même temps que chaque série d'analyses afin de valider l'ensemble du dosage.

10 RAPPORT D'ESSAIS

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Il est conseillé d'indiquer en particulier la pente de la courbe d'étalonnage et la valeur obtenue pour l'amidon témoin.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Responsible person | Description of change |
|------------|--------------------|-----------------------|
| 04/11/2011 | MESTRES Christian | Etalonnage |
| | | |

**Détermination de la teneur en amidon des produits céréaliers fermentés fermentés :
dosage enzymatique après solubilisation à la soude**



SOP Number: **Chem-Cere-10-fr**

Date : **29/8/2011**

Version : **2**

12 ANNEXE

Le texte imprimé de la norme (Amidons et féculés ; dosage de l'amidon. NF V03-606) sera annexé à ce document

| | | |
|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP Chemical analysis for Group 1 | | |
| Détermination de l'amylose dans les produits céréaliers fermentés par complexation à l'iode | | |
| SOP : Chem-Cere-14-fr | | |
| Date : 16/09/2011 | Version : 1 | |
| <p> Ecrit par : Brigitte PONS Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • </p> | | |
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**Procédure pour la détermination de l'amylose dans les produits céréaliers fermentés
par complexation à l'iode**

SOP : Chem-Cere-14-fr

Date : 16/09/2011

Version : 1

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Procédure pour la détermination de l'amylose dans les produits céréaliers fermentés par complexation à l'iode

SOP : Chem-Cere-14-fr

Date : 16/09/2011

Version : 1

1 DOMAINE ET APPLICATION

Cette procédure décrit une méthode pour l'évaluation de la teneur en amylose des produits céréaliers fermentés à l'état de grains, grains broyés ou farine. Cette procédure s'appuie sur une norme mise au point pour le riz usiné (ISO 6647). Elle peut s'appliquer à d'autres produits amylacés, en supposant une teneur en amidon de 90% (base sèche), comme pour le riz blanchi.

2 REFERENCES

Norme NF EN ISO 6647-1 : 2007 (F) - Riz – Détermination de la teneur en amylose.

Nous allons uniquement renseigner ici les paragraphes 3 (définition), 4 (principe), 8 (répétabilité) et 9 (points critiques et notes). Pour plus de détails, se référer à la procédure ISO en annexe (12).

3 DEFINITIONS

L'amylose est un polymère essentiellement linéaire dont les unités de D-glucose sont reliées entre elles par des liaisons glycosidiques de type α -(1-4). Chaque chaîne linéaire est constituée de plus de 1000 résidus glucose.

L'amylopectine est aussi un polymère de glucose, formé de courtes chaînes linéaires de liaisons glucose liées par des liaisons glycosidiques de type α -(1-4), liées entre elles par des branchements (liaisons glycosidiques de type α -(1-6)). On distingue les chaînes B constituées par des enchaînements de l'ordre de 40 à 45 résidus de glucose, et les chaînes A qui viennent se greffer sur les chaînes B, plus courtes et renfermant de l'ordre de 15 à 20 résidus de glucose. Le ratio entre chaînes courtes et longues est de l'ordre de 9-10 pour les céréales.

4 PRINCIPE

Du fait de son caractère linéaire, l'amylose est susceptible de complexer des petites molécules hydrophobes, en particulier l'iode, entraînant un réarrangement de la macromolécule en une structure hélicoïdale (6 unités de glucose/tour). Les groupements hydrophobes sont tournés vers l'intérieur de l'hélice où s'alignent les molécules d'iode : la longueur du chapelet de molécules d'iode complexées détermine la longueur d'onde et l'intensité d'absorption du système.

Procédure pour la détermination de l'amylose dans les produits céréaliers fermentés par complexation à l'iode

SOP : Chem-Cere-14-fr

Date : 16/09/2011

Version : 1

L'amylose forme avec l'iode un complexe **coloré bleu** caractérisé par un maximum d'absorption à 620 nm.

L'amylopectine forme un complexe **coloré rouge** caractérisé par un maximum d'absorption à 545 nm.

La densité optique du complexe coloré global est évaluée et comparée à une gamme étalon préparée avec des mélanges d'amylose et d'amylopectine.

5 REACTIFS

6 APPAREILLAGE

7 PROCÉDURE

8 EXPRESSION DES RÉSULTATS

8.1 Mode de calcul et formules

8.2 Répétabilité

L'écart type de variation admissible entre deux déterminations réalisées simultanément par le même analyste pour un même échantillon doit être inférieur à **0,8 %** (base sèche).

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- La vaisselle utilisée doit être parfaitement propre et exempte en particulier de toute trace d'amidon insoluble et d'électrolytes (rinçage à l'eau desionisée).
- Utiliser de l'amylopectine de céréale (extraite de riz cireux, ou « waxy ») et non de tubercule (comme la pomme de terre). L'amylopectine de pomme de terre a une plus forte proportion de chaînes B que les céréales ; leur longueur permet une interaction plus forte avec l'iode induisant une sous-estimation de la teneur en amylose.
- A la fin de la phase solubilisation, vérifier l'absence de grumeaux gélifiés (particulièrement si la solubilisation se fait à chaud). Si le cas se présente, disperser

Procédure pour la détermination de l'amylose dans les produits céréaliers fermentés par complexation à l'iode

SOP : Chem-Cere-14-fr

Date : 16/09/2011

Version : 1

les grumeaux en exerçant un mouvement circulaire de la fiole tout en la maintenant à plat sur la paillasse ; puis compléter progressivement avec l'eau.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre, seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------|----------------------|--------------------------------|
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12 ANNEXE

Le texte de la Norme NF EN ISO 6647-1 : 2007 (Riz – Détermination de la teneur en amylose) sera annexé au document



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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP Chemical analysis for Group 1 | | |
| Amylose content determination in cereal fermented food using iodine complexation | | |
| SOP: Chem-Cere-14-en | | |
| Date: 18/04/2011 | Release: 1 | |
| <p>Written by : Brigitte PONS</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • • | | |
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1 SCOPE AND APPLICATION

This procedure describes a method for the evaluation of amylose content in cereal fermented products (grains, meals or flours). This procedure relies on a standard procedure developed for milled rice (ISO 6647). It can be applied to other starchy products, assuming a starch content of 90% (dry basis), as for milled rice.

2 REFERENCES

NF EN ISO 6647-1 : 2007 procedure.

Please refer to the international standard procedure for details (see appendix). Only definitions (3), principle (4), repeatability (8.2) and critical points (9) will be detailed in this form.

3 DEFINITIONS

Amylose is an essentially linear polymer of D-glucose units, linked by α -(1-4) glycosidic bonds. Each linear chain consists of more than 1000 glucose residues

Amylopectin is also a polymer of glucose, but consisting of short linear chains linked by α -(1-6) connections. One distinguishes the B chains consisting of chains of 40 to 45 glucose residues, and A chains just linked on the B chains, shorter and containing 15 to 20 glucose residues. The ratio between short and long strings is by 9-10 for cereals.

4 PRINCIPLE

Because of its linear nature, Amylose is capable of complexing small hydrophobic molecules, especially iodine, resulting in a rearrangement of the macromolecule in a helical structure (6 units of glucose/turn). The hydrophobic groups turned to the interior of the Helix where iodine molecules align: the length of the complexed iodine molecules determines the wavelength and intensity of the absorption of the system.

Amylose content determination in cereal fermented food using iodine complexation

SOP Number: **Chem-Cere-14-en**

Date: **16/09/2011**

Release: **1**

Amylose forms a blue colored complex characterized by a maximum absorption at 620 nm with iodine.

Amylopectin forms a red coloured complex characterized by a maximum absorption at 545 nm.

The optical density of the global colored complex is evaluated and compared to a standard range prepared with mixtures of amylose and amylopectin.

5 REAGENTS

6 APPARATUS

7 PROCEDURE

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

8.2 Repeatability

The standard deviation between two determinations carried out simultaneously by the same analyst for a same sample shall be less than **0.8%** (dry basis).

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Dishware must be perfectly clean and free of insoluble starch and electrolytes (rinse with deionised water).
- Use amylopectin from cereal (extracted from waxy rice, for example) but not from-tuber (such as potato). Potato Amylopectin has a higher proportion of B

Amylose content determination in cereal fermented food using iodine complexation

SOP Number: **Chem-Cere-14-en**

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Release: **1**

chains that allow a stronger interaction with iodine, thus inducing an underestimation of the amylose content.

- At the end of the solubilization phase, check the absence of gelled lumps (particularly if hot solubilization is performed). If the case arises, disperse lumps by gently agitation of the flask with a circular movement then gradually fill with water.

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
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12 APPENDIX

NF EN ISO 6647-1 : 2007 will be annexed to the document



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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination de la teneur en amylose dans les produits céréaliers fermentés par AED | | |
| SOP : Chem-Cere-12-fr | | |
| Date : 16/09/2011 | Version : 1 | |
| <p> Ecrit par : Christian MESTRES Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • • </p> | | |
| Ce document a été approuvé par : | | |
| Partenaire | Noms des personnes l'ayant approuvé | Date DD/MM/YY |
| CIRAD UAC CSIR FAAU ESB FRI NRC | Christian MESTRES Zahra AHMED | 28/03/2011 16/09/2011 |

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1 DOMAINE ET APPLICATION

Cette méthode vise à déterminer la teneur en amylose des aliments secs riches en amidon, en particulier les produits céréaliers, fermentés ou non.

2 REFERENCES

Cette méthode s'appuie sur la publication suivante : Mestres, C., Matencio, F., Pons, B., Yajid, M., & Fliedel, G. (1996). A rapid method for the determination of amylose content by using Differential Scanning Calorimetry. *Starch*, 48(1), 2-6.

3 DEFINITIONS

L'amidon constitue la substance de réserve de nombreux végétaux. Il est présent sous forme de **granules semi-cristallins**. Chauffé en présence de l'eau, l'amidon va perdre sa structure cristalline : c'est la **gélatinisation**.

On observe souvent au-delà de la gélatinisation (à une température supérieure de 20 à 30°C) un ou deux autres endothermes de **fusion des complexes** amylose-lipides. Cette transition est réversible, et les complexes amylose-lipides se reforment au cours du refroidissement. Ces complexes se forment entre l'amylose et les composés lipidiques amphiphiles : acides gras libres, monoglycérides ou **phospholipides**. La **lysophosphatidyl-choline** est un phospholipide.

On mesure les transitions thermiques par Analyse Enthalpique Différentielle (**AED**), « Differential Scanning Calorimetry » (DSC), en anglais. On mesure en particulier l'**exotherme** (la chaleur) générée par la formation des complexes amylose-lipides au cours du refroidissement.

4 PRINCIPE

L'échantillon amylicé est chauffé, puis refroidi à vitesse constante en excès d'eau et en présence de lysophosphatidyl-choline. Au cours du chauffage, les structures cristallines de l'amidon sont totalement détruites (gélatinisation, puis fusion des complexes). Au cours du refroidissement, les complexes entre amylose et lipides se reforment. Les lipides complexant (lysophosphatidyl-choline) sont en excès par rapport à l'amylose présente dans l'échantillon ; l'intensité de la complexation est ainsi directement proportionnelle à la teneur en amylose de l'échantillon.

On mesure la différence d'énergie entre une capsule de référence (généralement vide) et une capsule contenant l'échantillon. Le surplus d'énergie générée par l'échantillon au cours du refroidissement représente l'exotherme de formation des complexes amylose-lipides.

On mesure de manière identique un standard d'amylose pure. Le rapport des énergies pour l'échantillon et le standard permet de calculer la teneur en amylose de l'échantillon.

Il existe deux types d'appareils AED (Figure 1) :

- à compensation de puissance. Ils comportent deux fours indépendants, un pour la capsule échantillon, un pour la capsule de référence. On mesure directement les énergies apportées à chacun des fours, et on calcule la différence d'énergie entre ceux-ci,
- à compensation de température. Muni d'un seul four, dans lequel on place les deux capsules. La différence de température entre les deux capsules permet de calculer la différence d'énergie.

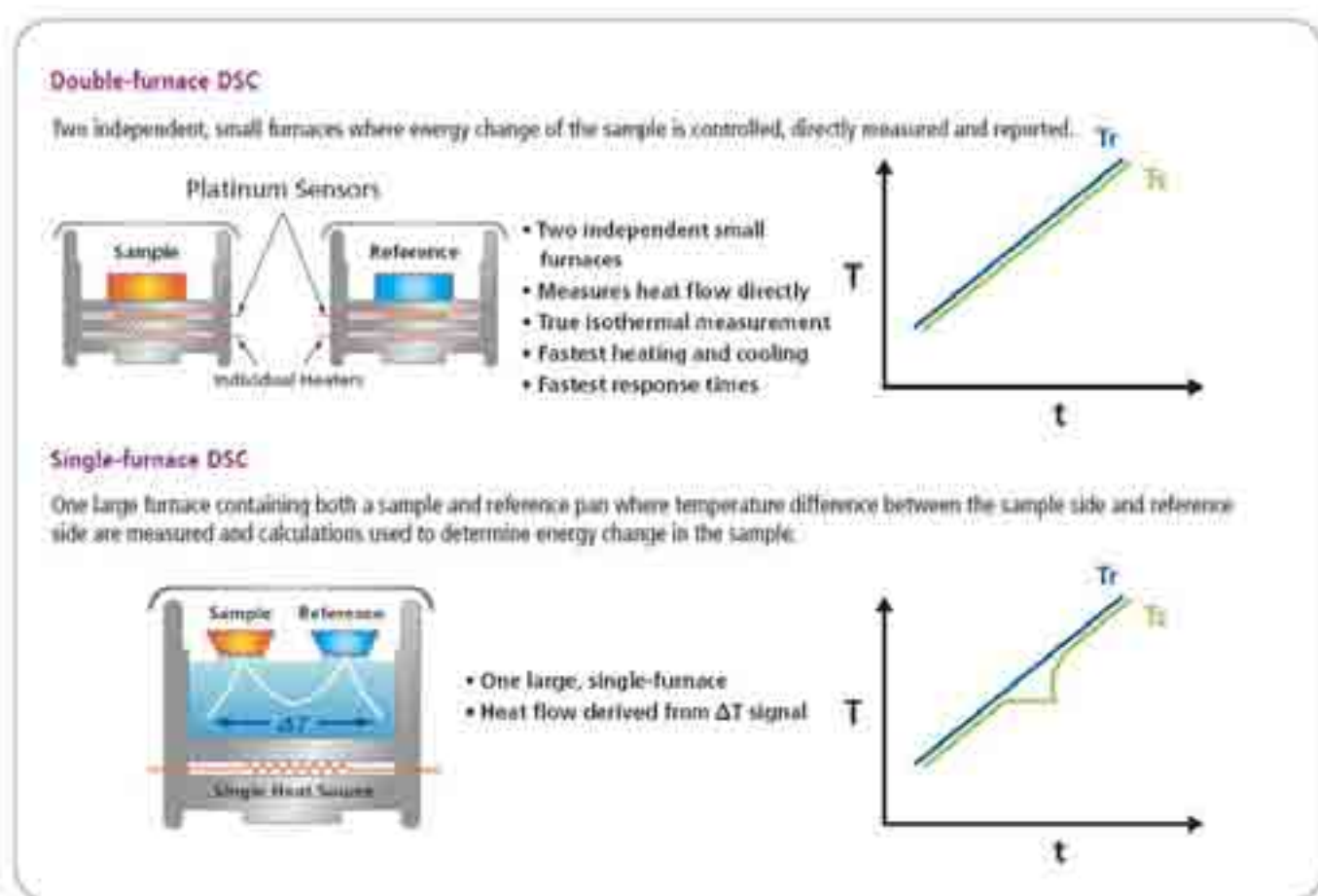


Figure 1. Principe des deux type d'AED (DSC)

5 REACTIFS

- Eau distillée
- Indium
- Lysophosphatidyl-choline (L4129 Sigma)
- Capsules à sertir pouvant supporter une pression supérieure à **8 bars**.

6 APPAREILLAGE

- AED à compensation de puissance ou à compensation de température
- Balance de haute précision : 0,01 mg

7 PROCÉDURE

Les conditions d'analyse sont : chauffage de 25 à 160°C, maintien à 160°C durant 2 minutes, puis refroidissement à 50°C. Les cinétiques de chauffage et refroidissement sont respectivement de 50°C/min et 10°C/min pour une AED à compensation de puissance, mais de 20 et 3 à 5°C/min pour une AED à compensation de température.

Vérifier la calibration de l'appareil avant toute séquence d'analyse à l'aide d'un standard comme l'Indium. L'enthalpie de fusion doit être proche de la valeur théorique (28,45 J/g pour l'Indium). Re-calibrer l'appareil si les valeurs observées s'écartent de plus de 1% de la valeur théorique.

Préparer une solution aqueuse de lysophosphatidyl-choline à 2 % (m/v) ; peser 20 mg de lysophosphatidyl-choline % (m/v). Y ajouter 1 mL d'eau bidistillée, boucher et agiter vigoureusement. Chauffer légèrement (vers 60°C) pour solubiliser totalement la lysophosphatidyl-choline : la solution doit être totalement limpide. Cette solution peut se conserver quelques jours à 4°C, mais doit être chauffée à nouveau pour re-solubiliser la lysophosphatidyl-choline avant son utilisation.

Peser une quantité d'échantillon représentant 15% de la masse d'eau totale que peut contenir une capsule (par exemple 9 mg pour une capsule de 60 µL)

Ajouter 5 fois la masse d'une solution aqueuse de lysophosphatidyl-choline à 2 % (m/v), soit 45 mg pour une capsule de 60 µL, puis sertir la capsule

Laisser équilibrer une heure minimum.

Réaliser chaque jour une ligne de base en plaçant une capsule vide dans le four échantillon et une autre dans le four de référence. Vérifier qu'aucun artefact n'apparaît sur la courbe obtenue.

Placer la capsule dans le four échantillon. Une capsule sertie vide est laissée dans le four de référence. Enregistrer la courbe de chauffage et refroidissement en soustrayant la ligne de base.

Calculer l'aire de l'exotherme de formation des complexes amylose-lipides; le début ($\approx 90^\circ\text{C}$, à 10°C/min) et la fin (70-75°C, à 10°C/min) de l'exotherme sont repérables par un changement de pente de la ligne de base du thermogramme (Figure 2).

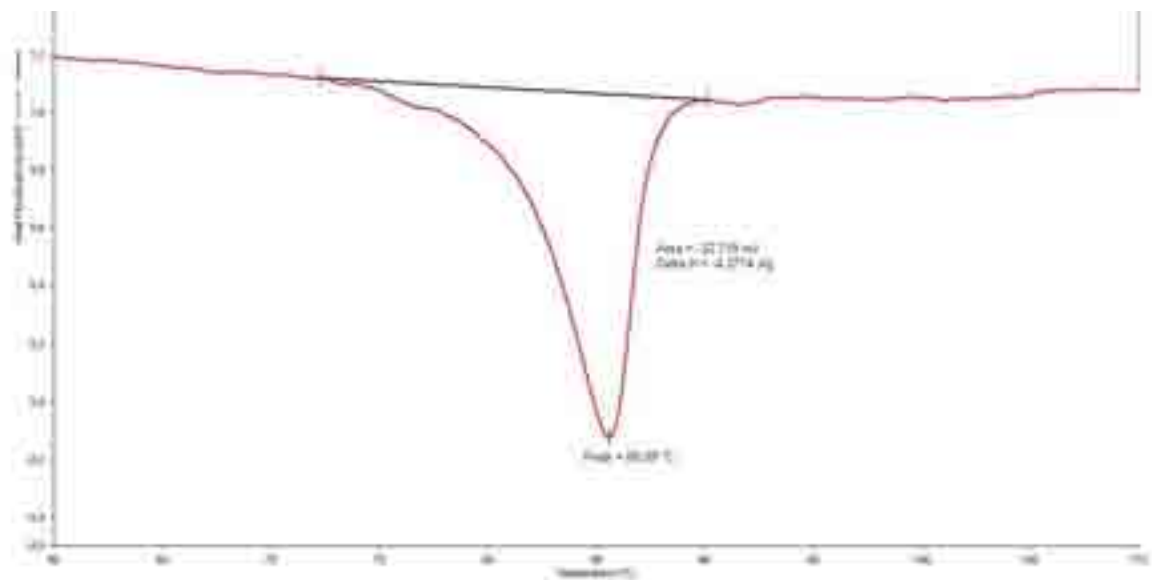


Figure 2. Thermogramme d'un produit amyacé montrant l'aire de formation des complexes amylose/lysophosphatidyl-choline ($\Delta H=4.2714$ J/g)

Procéder de même pour le standard d'amylose, excepté que la masse d'échantillon doit dans ce cas être proche de **5 mg**, pour une quantité de lysophosphatidyl-choline à 2 % (m/v) inchangée, soit 45 mg. Effectuer **1 à 2 mesures** pour le standard **chaque jour** ; la valeur de l'exotherme doit être proche de **28,5 J/g base sèche**. Calculer la moyenne des standards journaliers pour chaque série de mesure réalisée dans un bref intervalle de temps (1 mois).

8 EXPRESSION DES RESULTATS

8.1 Mode de calcul et formules

La teneur en amylose (Amy) est :

$$Amy (\%) = 100 \times \frac{Eech}{Estand}$$

Où :

- Ech (J/g base sèche) est l'exotherme mesuré pour l'échantillon,
- Estand (J/g base sèche) est l'exotherme moyen mesuré pour l'amylose standard

8.2 Répétabilité

L'écart type des exothermes mesurés dans un intervalle de temps court sera inférieur à **0.2 J/g (bs)**.

L'écart type des teneurs en amylose déterminées dans un intervalle de temps court sera inférieur à **0.8 %** (bs).

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

L'échantillon analysé doit avoir une granulométrie inférieure à **250 µm**

L'utilisation d'une balance précise au **centième de mg** est impérative pour garantir une bonne précision de la mesure.

La **calibration** doit être effectuée dans les conditions exactement identiques à celles de l'analyse ; en particulier, même type de capsule, même cinétique de chauffe, et énergie de transition proche de celle observée pour les échantillons (pour l'Indium, peser environ exactement 4 à 6 mg).

La **ligne de base** ne doit pas présenter d'artefact ; pour le valider, effectuer une deuxième ligne de base en la soustrayant à la première. Celle-ci doit alors être rigoureusement linéaire, avec un bruit de fond proche des spécifications de l'appareil : pente inférieure à 0,1 mW sur l'ensemble du domaine et bruit de fond inférieur à 0,05 mW (Figure 3).

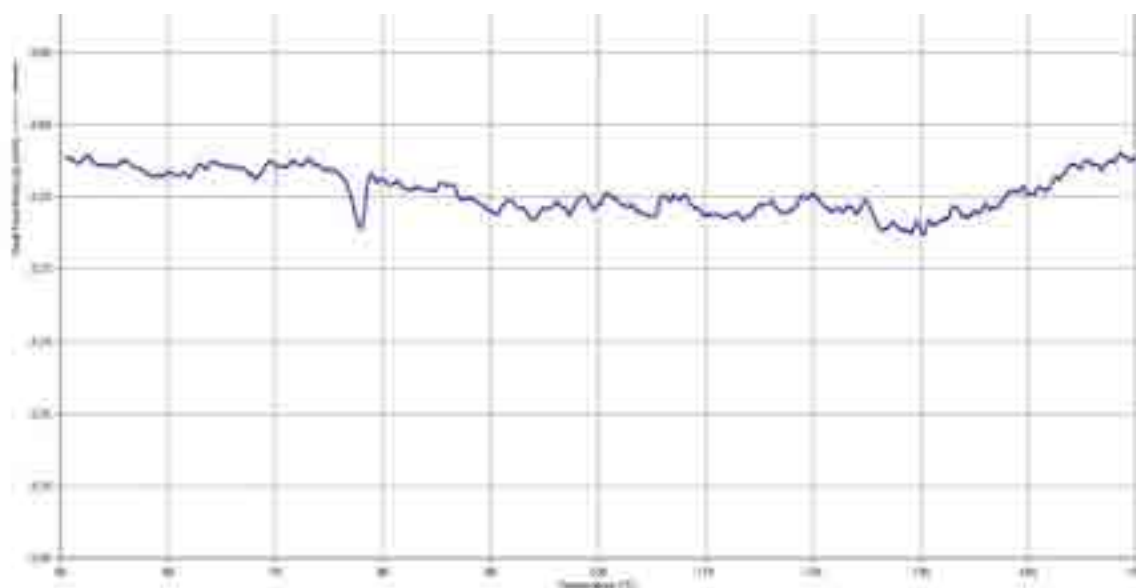


Figure 3. Thermogramme d'une capsule vide après soustraction d'une ligne de base

L'amylose standard doit être de **haute pureté** ; la valeur moyenne de l'exotherme doit être proche de 28,5 J/g (bs).

Les valeurs journalières mesurées pour l'amylose **standard** doivent être proches de 28,5 J/g (bs) ; en cas **d'écart supérieur à 2%**, préparer deux nouvelles capsules de référence (contrôler que la solution de lysophosphatidyl-choline à 2 % (m/v) est **limpide**) et recommencer la mesure.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Il est recommandé en particulier d'inclure le type d'appareil utilisé, et la valeur **moyenne mesurée pour le standard** d'amylose, utilisée pour le calcul de la teneur en amylose.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------|----------------------|--------------------------------|
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12 ANNEXE



| | | |
|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Amylose content determination using DSC in cereal fermented products | | |
| SOP: Chem-Cere-012-en | | |
| Date: 16/09/2011 | Release: 1 | |
| <p>Written by : Christian MESTRES</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • | | |
| This document has been approved by : | | |
| Partner | Name of the person who approved | Date DD/MM/YY |
| CIRAD UAC CSIR FAAU ESB FRI NRC | Christian MESTRES Zahra AHMED | 16/09/2011 |

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Amylose content determination using DSC in cereal fermented products

SOP: Chem-Cere-012-en

Date: 16/09/2011

Release: 1

1 SCOPE AND APPLICATION

This method seeks to determine the amylose content of dry foods rich in starch, especially cereal products, fermented or not.

2 REFERENCES

This method relies on the following reference: Mestres, v., Matencio, f., Pons, b., Yajid, M., & Fliedel, g. (1996). A rapid method for the determination of amylose content by using Differential Scanning Calorimetry. Starch, 48 (1), 2-6.

3 DEFINITIONS

Starch is the reserve substance of many plants. It is found in semi-crystalline granules. Heated in the presence of water, starch will lose its crystalline structure: it is the **gelatinization**.

There is often beyond the gelatinization (at a higher temperature of 20 to 30 ° C) one or two other endothermic peaks, due to the **fusion of amylose-lipid complexes**. This transition is reversible and lipids complex amylose again during cooling. These complexes are formed between the amylose and amphiphilic lipids: free fatty acids, monoglycerides and phospholipids. Lysophosphatidyl-choline is a phospholipid.

Thermal transitions are measured using **Differential Scanning Calorimetry(DSC)**. One measures in particular exotherm (heat) generated by the formation of amylose-lipids complexes during cooling.

4 PRINCIPLE

The starchy sample is heated at constant speed in excess of water in a sealed pan in the presence of Lysophosphatidyl-choline. During heating, the crystalline structure of starch is totally destroyed (gelatinization, then fusion of complexes). During cooling, lipids complex amylose again. Complexing lipids (lysophosphatidyl-choline) are in excess and complexation intensity is directly proportional sample amylose content.

The difference of energy between a reference (usually empty) pan and the pan containing the sample is measured. The surplus of energy generated by the sample during cooling represents the exotherm of formation of amylose-lipid complexes.

Amylose content determination using DSC in cereal fermented products

SOP: Chem-Cere-012-en

Date: 16/09/2011

Release: 1

A standard of pure amylose is measured with the same procedure. The ratio of energy of the sample to that of the amylose standard is used to calculate the amylose content.

There are two types of DSC devices (Figure 1):

- Power compensation devices. They have two independent ovens, one for the sample pan, one for the reference pan. They directly measure energy furnished to each of the ovens, and calculate the energy difference between them,
- Temperature compensation devices. They are equipped with a single furnace, in which we place two pans. The difference in temperature between the two pans is used to calculate the energy difference.

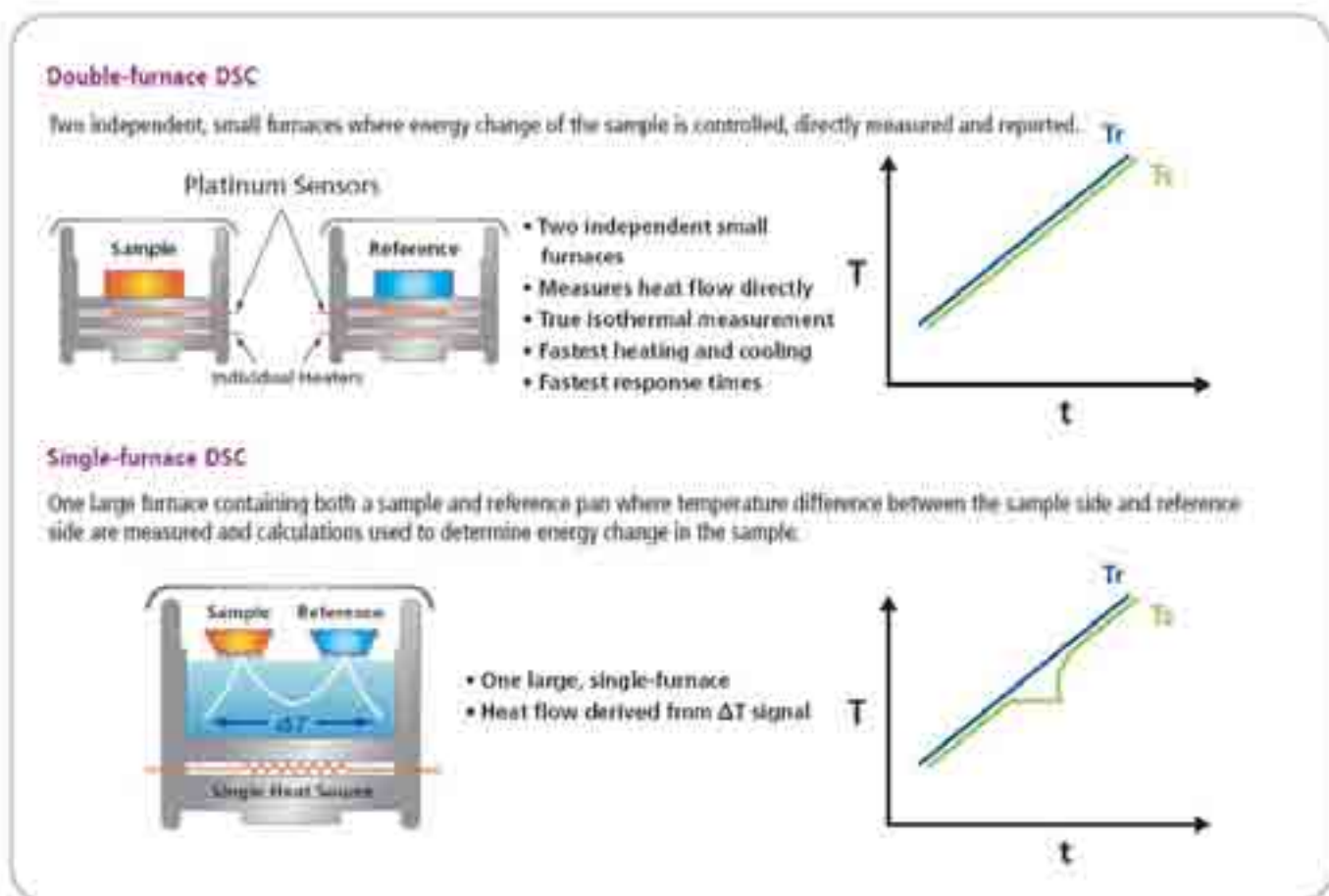


Figure 1. Principle of the two types of DSC

5 REAGENTS

- Ultrapure water
- Indium
- Lysophosphatidyl-choline (L4129 Sigma)
- High pressure (5 bars) pans

6 APPARATUS

- Power compensation or temperature compensation DSC
- High precision (0.01 mg) balance

7 PROCEDURE

Analysis conditions are: heating from 25 to 160°C, a plateau at 160°C for 2 min, then cooling to 50°C. Heating and cooling speed are 50°C/min and 10°C/min, respectively, for a power compensation DSC, but 20 and 3-5 °C/min for a temperature compensation DSC.

Check the calibration of the device before any sequence analysis using a standard such as Indium. The enthalpy of melting temperature must be close to the theoretical values (28.45 J/g for Indium). Re-calibrate the apparatus if the observed value differs from more than 1% of the theoretical value.

Prepare an aqueous solution of 2% (m/v) lysophosphatidyl-choline; weigh 20 mg of lysophosphatidyl-choline, add 1 mL of distilled water, stopper and shake vigorously. Heat slightly (to 60°C) to completely solubilize lysophosphatidyl-choline: the solution must be completely clear. This can be stored a few days at 4°C, but it must be heated again for re - solubilize lysophosphatidyl-choline before use.

Weigh a quantity of sample representing 15% of total water that may contain a pan (for example 9 mg for a pan of 60 µL).

Add 5 times the mass of 2% (m/v) lysophosphatidyl-choline (45 mg for a pan of 60 µL) and then crimp the pan.

Leave for balancing a minimum of one hour.

Perform every day a base line by placing an empty pan in the sample furnace and another in the furnace of reference. Verify that no artefact appears on the obtained curve.

Amylose content determination using DSC in cereal fermented products

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Place the sample pan in sample oven. An empty pan is left in the reference oven. Save heating curve by subtracting the base line.

Calculate the area of the exotherm of formation of the complexes; the beginning and the end of the exotherm are identifiable by a change in slope of the base line of the thermogram (Figure 2).

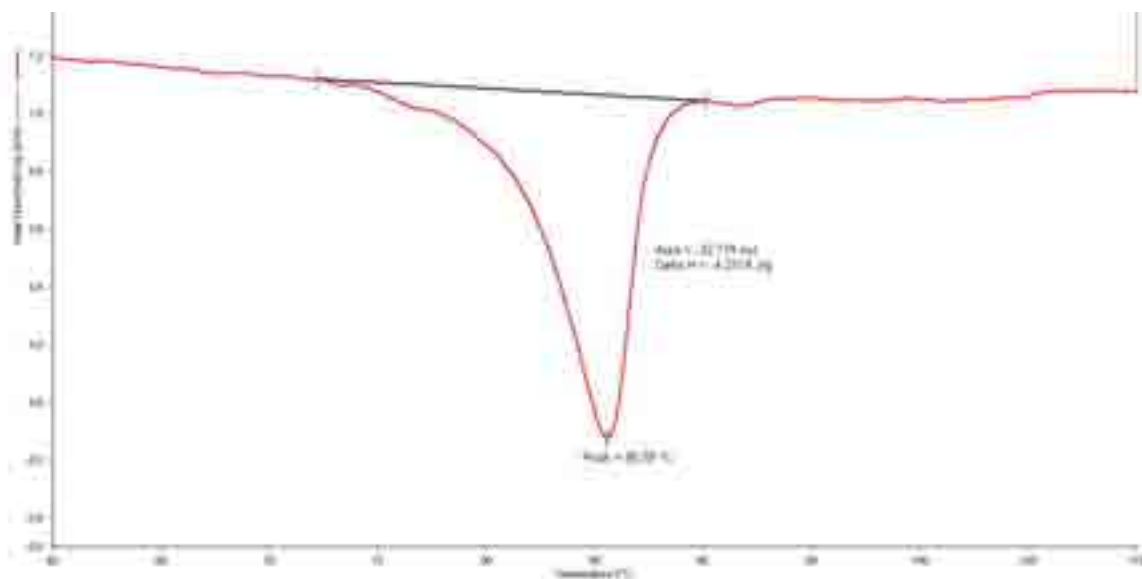


Figure 2. Thermogram of a starchy product with the area of formation of amylose/lysophosphatidyl-choline complexes ($\Delta H=4.2714$ J/g)

Proceed similarly with standard amylose, except that the mass of sample must be in this case close to 5 mg, for the same quantity (45 mg) of 2% (m/v) lysophosphatidyl-choline. Perform 1 to 2 determinations for the standard every day; the value of the exotherm must be close to 28.5 J/g dry basis. Calculate the average of the daily standards for each series of measurement carried out in a short interval of time (1 month).

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Amylose content (Amy) is:

$$Amy(\%) = 100 \times \frac{E_{ech}}{E_{stand}}$$

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Where:

- Ech (J/g dry basis) is the exotherm measured on the sample,
- Enat (J/g dry basis) is the exotherm mean calculated for amylose standard

8.2 Repeatability

The standard deviation of the exotherm between measurements performed in a short time interval will be less than **0.2 J/g (db)**.

The standard deviation of the amylose content between measurements performed in a short time interval will be less than **0.8 % (db)**.

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

The sample must have a particle size less than **250 µm**

The use of a high precision balance (**0.01 mg**) is imperative to ensure a good accuracy of measurement.

The calibration be carried out under the conditions exactly identical to those of analysis; in particular, same type of pan, same kinetics of heat, and energy of transition close to that of samples (for Indium, weigh about exactly **4 to 6 mg**).

The base line must not show any artefact; to check this, perform a second base line by subtracting the first one. Observed curve must then be strictly linear, with a noise level close to the specifications of the device: for example, slope less than **0.1 mW** across the measurement domain and noise of less than **0.05 mW** for a power compensation device (Figure 3).

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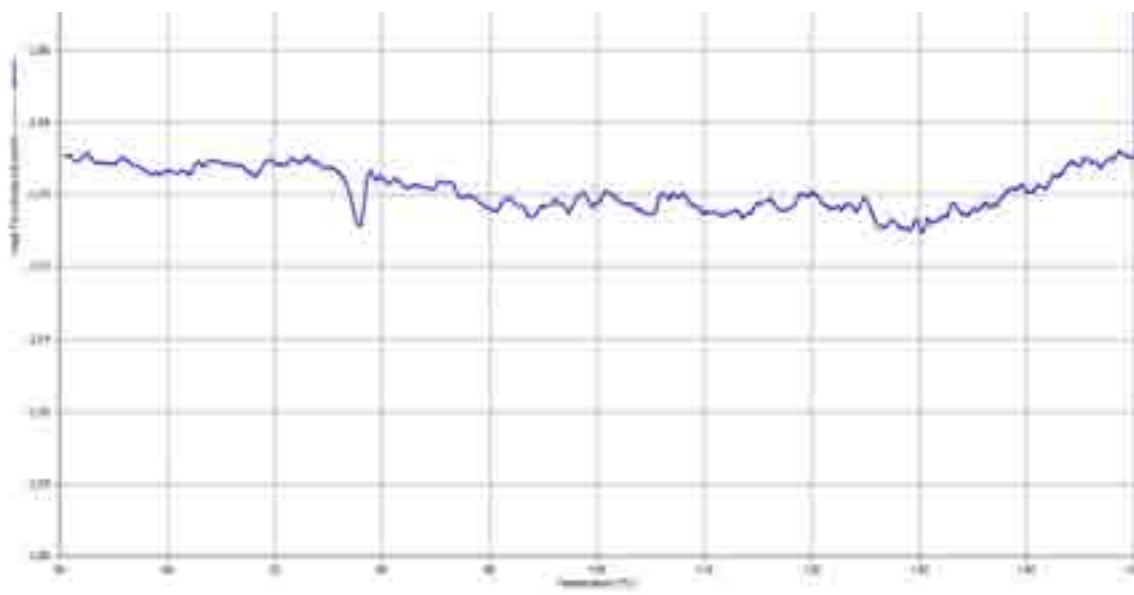


Figure 3. Thermogram of an empty pan after subtraction of the baseline

Amylose standard must be high purity; the average value of exotherm must be close to 28.5 J/g (bs).

Daily values measured for standard Amylose must be close to 28.5 J/g (bs); if the value differs for more than 2%, prepare two new reference pans (check that the solution of 2% (m/v) lysophosphatidyl-choline is clear) and start the measurement again.

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

It is recommended to specify the type of apparatus, the heating rate, and the mean value measured for standard amylose.

The test report shall include all details necessary for the complete identification for the sample.

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SOP: Chem-Cere-012-en

Date: 16/09/2011

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11 REVISION RECORD

| Date | Responsible person | Description of change |
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12 APPENDIX



**African Food Tradition rEvisited by
Research**
FP7 n°245025



Deliverable D.1.2.3.1: SOP for sensory: Physical and Textural analysis for Group 1

Crude protein: Nitrogen (Total) in Cereal products- Kjeldahl Method

SOP: Chem-Cere-22-en

Date: **16/09/2011**

Release: **1**

Written by : Christian MESTRES

For information on this SOP please contact :

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| FAAU | Sameh AWAD | 16/09/2011 |
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| FRI | | |
| NRC | Zahra AHMED | 16/09/2011 |

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Crude protein: Nitrogen (Total) in Cereal products - Kjeldahl Method

SOP: Chem-Cere-22-en

Date: 16/09/2011

Release: 1

1 SCOPE AND APPLICATION

Determination of crude protein in cereal products

2 REFERENCES

NF EN ISO 20483

3 DEFINITIONS

4 PRINCIPLE

Destruction of organic matter by sulphuric acid in the presence of a catalyst, alkalization of the reaction products, distillation of the liberated ammonia and collection in a boric acid solution, followed by titration with a standard volumetric hydrochloric acid solution.

5 REAGENTS

- a. **Sulfuric acid**. 95-98% H₂SO₄. Nitrogen free.
- b. **Copper catalyist (CuSO₄, 0.15g)**, **Potassium sulfate (K₂SO₄, 5g)**, **Titane oxyde (TiO₂, 0.15g) tablets**
- c. **Sodium hydroxide solution**. 32 to 50% w/v nitrate-free NaOH.
- d. **Titration solution**. Dissolve 50 g H₃BO₃ and dilute to 1 L in water (it may necessary to gently heat the mixture to achieve perfect solubilization). Add 3.95 l of water and 50 ml of coloured indicator (Tecator). Homogenize and let stabilize for at least one night.
- e. **Sulfuric standard (0.05 M, 0.1 N) solution**.
- f. **Ammonium sulfate solution**. Weigh exactly 0.6611 g sulfate ammonium in 100 g water.

6 APPARATUS

- a. **Digestion and distillation flasks**. Hard, moderately thick, well annealed glass. Total capacity is ca 300 mL (30 cm height).
- b. **Digestion system**. Traditional apparatus (electric block heater, vapor aspiration and neutralization system) with adjustable temperature and duration controls for several (6 or 12) flasks.

Crude protein: Nitrogen (Total) in Cereal products - Kjeldahl Method

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- c. **Distillation and Titration system**. Distillator and automatic titration system with colorimetric endpoint detection. The system will deliver sodium hydroxide solution in the distillation flask (a minimum of 30 ml for example for 32% sodium hydroxide solution) and titration solution in the titration vessel. During distillation, it will add standard sulfuric solution to maintain the colour of the titration vessel solution to end point. Distillation volume will be of 130 ml.

7 PROCEDURE

7.1 Sampling

Weigh sample (1 ± 0.1 g, containing a maximum of 25 mg N)

Add 1 tablet catalyst and 12 ml of concentrated H₂SO₄

7.2 Digestion

Turn on digestion system at least 30 minutes before starting digestion: temperature should be 420°C.

Start digestion. It will last 60 minutes. At the end, digest clears (clear with light blue-green color).

Cool acid digest to room temperature (ca 25 min.). Cooled digest should be liquid or liquid with few small crystals. (Large amount of crystallization before addition of water indicates too little residual H₂SO₄ at end of digestion and can result in low test values.) After digest is cooled to room temperature, add 50 mL H₂O to flask. When room temperature water is added some crystals may form and then go into solution; this is normal. Let mixture cool to room temperature before distillation. Flasks can be stoppered for distillation at a later time.

7.3 Distillation/titration

- Turn on distillation and titration system.
- Place a distillation flask with 50 ml water and perform distillation/titration until the volume value for blank is stable.
- Test with 5 ml of Ammonium sulfate solution, with 50 ml water: titration volume should be close to 5 ml (± 0.1 ml).
- Perform distillation/titration for each sample flask.

Crude protein: Nitrogen (Total) in Cereal products - Kjeldahl Method

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8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Calculate results as follows:

$$\text{Protein (\% dry basis)} = \frac{140 * K * (V_{\text{samp}} - V_{\text{blank}}) * \text{Norm}}{\text{Weight} * \text{MS}}$$

Where: Vsamp = mL H₂SO₄ for sample
Vblank = ml H₂SO₄ for blank
Norm = normality HCl (0.1)
Weight = Sample mass (g)
MS = dry matter content of sample (% wet basis)
K = conversion factor: 5.70 for wheat, 5.95 for rice, 6.25 for maize and sorghum

8.2 Repeatability

Maximum standard deviation between duplicates is 0.03% "protein."

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

9.1 Safety Precautions :

- ✓ **Sulfuric acid**. Sulfuric acid can cause severe burns. Wear eye protection and acid resistant gloves. If acid is spilled on skin, wash immediately with large amounts of cool water. **NEVER NEUTRALIZE ACID ON SKIN WITH BAKING SODA.**
- ✓ **Sodium Hydroxide**. Alkalies can cause severe burns. Violent boiling can occur when sodium hydroxide is added to the digest (especially if there is too much residual acid). Wear eye protection and heavy rubber gloves when working with sodium hydroxide. If sodium hydroxide is spilled on skin, wash immediately with large amounts of cool water.
- ✓ **Digestion**. Check the bottom of the Kjeldahl flasks for star cracks prior to adding the sample and reagents. Discard cracked flasks. Acid fumes are generated during digestion. Make sure the digestion apparatus (traditional or block) effectively removes fumes. Wear eye protection and heavy rubber gloves when handling Kjeldahl flasks.
- ✓ **Distillation**. Distillation involves the heating of an acid digest plus sodium hydroxide with the release of ammonia gas. Care should be taken under conditions such as

Crude protein: Nitrogen (Total) in Cereal products - Kjeldahl Method

SOP: Chem-Cere-22-en

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these. Always wear eye protection and heavy rubber gloves. Most Kjeldahl distillation units have a protective glass (or plastic) barrier that is used as protection for the operator during distillation. If your unit has this, use it!

9.2 General procedure

- Prepare titration solution at least **one night** before use.
- Begin sample titration, only after perfect stabilization of **blank value** and getting correct value (5 ml) for **ammonium sulfate control**.
- Regularly **check delivered volumes** of sulfuric acid (distillation) and sodium hydroxide solution: sodium hydroxide equivalent should be higher than that of sulfuric acid.
- Regularly perform determination on **test material** to check the validity of the process.

10 TEST REPORT

| Sample | Sample weight | H2SO4 (ml) | Protein percent | Notes |
|--------|---------------|------------|-----------------|-------|
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11 REVISION RECORD

| Date | Responsible person | Description of change |
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12 APPENDIX

The text of NF EN ISO 20483 is annexed to the document



**African Food Tradition rEvisited by
Research**
FP7 n°245025



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

Crude protein: Nitrogen (Total) in Milk - Kjeldahl Method

SOP Number: **Chem-Cere-21-en**

Date: **22/08/2011**

Release: **1**

Written by : Sameh Awad

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| FAAU | Sameh AWAD | 22/08/2011 |
| ESB | | |
| FRI | | |
| NRC | | |

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SOP Number: **Chem-Cere-21-en**

Date: **22/08/2011**

Release: **1**

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Crude protein: Nitrogen (Total) in Milk - Kjeldahl Method

SOP Number: **Chem-Cere-21-en**

Date: **22/08/2011**

Release: **1**

1 SCOPE AND APPLICATION

Determination of crude protein in milk and milky products

2 REFERENCES

AOAC Officials methods 967.12; 975.17; 975.18 (in AOAC International 2007 18th edition Edited by Horwitz W. and Latimer G. W)

ISO 8968-1 / IDF 20-1:2001. Milk determination of Nitrogen content - Part 1: Kjeldahl Method. International Dairy Federation, Brussels

3 DÉFINITIONS

4 PRINCIPLE

Destruction of organic matter by sulphuric acid in the presence of a catalyst, alkalization of the reaction products, distillation of the liberated ammonia and collection in a boric acid solution, followed by titration with a standard volumetric hydrochloric acid solution.

5 REAGENTS

- a. **Sulfuric acid.** 95-98% H₂SO₄. Nitrogen free.
- b. **Copper catalyst solution.** CuSO₄ · 5H₂O. Nitrogen free. Prepare solution 0.05 g/mL H₂O.
- c. **Potassium sulfate.** K₂SO₄. Nitrogen free.
- d. **Sodium hydroxide solution.** 50% w/w nitrate-free NaOH.
- e. **Boiling chips.** Mesh size 10 suggested. High purity, amphoteric alundum granules, plain.
- f. **Methyl red/bromocresol green indicator solution.** Dissolve 0.2 g methyl red and dilute to 100 mL in 95% ethanol. Dissolve 1.0 g bromocresol green and dilute to 500 mL in 95% ethanol. Mix 1 part methyl red solution with 5 parts bromocresol green solution (combine all of both solutions).
- g. **Boric acid solution.** 4%, with indicator. Dissolve 40 g H₃BO₃ and dilute to 1 L in water and add 3 mL methyl red/bromocresol green indicator solution, (f). Solution will be light orange color.
- h. **Hydrochloric acid standard solution.** 0.1000N. Prepare as in Association of Official Analytical Chemists method number 936.15 or use pre-made solution of certified specification range 0.0995-0.1005N and use 0.1000N for calculation.

Crude protein: Nitrogen (Total) in Milk - Kjeldahl Method

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6 APPARATUS

- a. **Digestion flasks.** Kjeldahl. Hard, moderately thick, well annealed glass. Total capacity is ca 500 or 800 mL.
- b. **Distillation flasks.** Same as Kjeldahl flask as in (a), fitted with rubber stopper through which passes lower end of efficient connecting bulb or trap to prevent mechanical carryover of NaOH during distillation. Connect upper end of bulb to condenser tube with rubber tubing. Use graduated 500 mL Erlenmeyer titration flask to collect distillate. Trap outlet of condenser in manner to ensure complete absorption of NH_3 distilled into boric acid solution.
- c. **Digestion/distillation system.** Traditional apparatus with adjustable controls for individual flasks.
- d. **Titration buret.** 50 mL. Class A or equivalent.

7 PROCEDURE

7.1 Sampling

Add 15 g K_2SO_4 , 1 mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ catalyst solution and 8-10 boiling chips to digestion flask.

Warm milk to $38 \pm 1^\circ\text{C}$ and mix thoroughly. Weigh warm sample (5 ± 0.1 mL) and immediately place in digestion flask. (Note: Weights must be recorded to nearest 0.0001 g.)

Add 25 mL H_2SO_4 , rinsing any milk on neck of flask down into bulb. Flask may be stoppered and held for digestion at later time. Digest and distill a blank (all reagents and no sample) each day.

7.2 Digestion burner setting

Conduct digestion over heating device that can be adjusted to bring 250 mL H_2O at 25°C to rolling boil in ca 5-6 min. To determine maximum heater setting to be used during digestion, preheat 10 minutes (gas) or 30 minutes (electric) at burner setting to be evaluated. Add 3 or 4 boiling chips to 250 mL water at 25°C and place flask on preheated burner. Determine heater setting that brings water from 25°C to rolling boil in 5-6 minutes on each burner. This is maximum burner setting to be used during digestion.

Crude protein: Nitrogen (Total) in Milk - Kjeldahl Method

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7.3 Digestion

Place flask in inclined position with fume ejection system on:

- Start with setting low enough so that sample does not foam up into neck of Kjeldahl flask. Digest at least 20 minutes or until white fumes appear in flask.
- Next, increase burner setting half way to maximum setting determined in (a) and heat for 15 minutes.
- Next, increase heat to maximum setting determined in (a). When digest clears (clear with light blue-green color), continue to boil 1-1.5 hr at maximum setting (total time ca 1.8-2.25 hr).

To determine specific boil time needed for analysis condition in your laboratory, select a high protein, high fat milk sample and determine protein content using different boil times (1-1.5 hr) after clearing. Mean protein test increases with increasing (0-1.5 hr) boil time, becomes constant, and then decreases when boil time is too long. Select boiling time that yields maximum protein test.

At end of digestion, digest should be clear and free of undigested material. Cool acid digest to room temperature (ca 25 min.). Cooled digest should be liquid or liquid with few small crystals. (Large amount of crystallization before addition of water indicates too little residual H_2SO_4 at end of digestion and can result in low test values.) After digest is cooled to room temperature, add 300 mL H_2O to flask and swirl to mix (for 800 mL flasks add 400 mL H_2O). When room temperature water is added some crystals may form and then go into solution; this is normal. Let mixture cool to room temperature before distillation. Flasks can be stoppered for distillation at a later time.

7.4 Distillation

- Turn on condenser water.
- Add 50 mL H_3BO_3 solution with indicator to graduated 500 mL Erlenmeyer titration flask and place flask under condenser tip so that tip is well below H_3BO_3 solution surface.
- To room temperature diluted digest, carefully add 75 mL 50% NaOH down sidewall of Kjeldahl flask with no agitation. NaOH forms clear layer under the diluted digest. Immediately connect flask to distillation bulb on condenser. Vigorously swirl flask to mix contents thoroughly; heat until all NH_3 has been distilled (>150 mL distillate; >200 mL total volume). Do not leave distillation unattended. Flasks (500 mL) may bump at this point (ca 150 mL distillate; 200 mL total volume).
- Lower receiving flask and let liquid drain from condenser tip. Turn off distillation heater.

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SOP Number: **Chem-Cere-21-en**

Date: **22/08/2011**

Release: **1**

- Titrate H_3BO_3 receiving solution with standard 0.1000N HCL solution to first trace of pink. Lighted stir plate may aid visualization of end point. Record mL HCL to at least nearest 0.05 mL.

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Calculate results as follows:

$$\text{Nitrogen (\%)} = \frac{1.4007 * (V_{\text{samp}} - V_{\text{blank}}) * \text{Norm}}{\text{Weight}}$$

Where: V_{samp} = mL HCl for sample
 V_{blank} = ml HCl for blank
Norm = normality HCl (0.1)
Weight = Sample mass (g)

Multiply percent nitrogen by factor 6.38, to calculate percent "protein." This is "protein" content on a total nitrogen basis.

8.2 Repeatability

Maximum recommended difference between duplicates is 0.03% "protein."

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

Safety Precautions :

- ✓ **Sulfuric acid.** Sulfuric acid can cause severe burns. Wear eye protection and acid resistant gloves. If acid is spilled on skin, wash immediately with large amounts of cool water. **NEVER NEUTRALIZE ACID ON SKIN WITH BAKING SODA.**
- ✓ **Sodium Hydroxide.** Alkalies can cause severe burns. Violent boiling can occur when sodium hydroxide is added to the digest (especially if there is too much residual acid). Wear eye protection and heavy rubber gloves when working with sodium hydroxide. If sodium hydroxide is spilled on skin, wash immediately with large amounts of cool water.
- ✓ **0.01N Hydrochloric acid (HCL).** HCL solutions can cause burns. Wear eye protection. Wearing acid resistant gloves when handling HCL is recommended but

Crude protein: Nitrogen (Total) in Milk - Kjeldahl Method

SOP Number: **Chem-Cere-21-en**

Date: **22/08/2011**

Release: **1**

may not be practical during titration. If HCL is spilled on the skin, wash immediately with large amounts of cool water.

- ✓ **Digestion**. Check the bottom of the Kjeldahl flasks for star cracks prior to adding the sample and reagents. Discard cracked flasks. Acid fumes are generated during digestion. Make sure the digestion apparatus (traditional or block) effectively removes fumes. Wear eye protection and heavy rubber gloves when handling Kjeldahl flasks.
- ✓ **Distillation**. Distillation involves the heating of an acid digest plus sodium hydroxide with the release of ammonia gas. Care should be taken under conditions such as these. Always wear eye protection and heavy rubber gloves. Most Kjeldahl distillation units have a protective glass (or plastic) barrier that is used as protection for the operator during distillation. If your unit has this, use it!

10 TEST REPORT

| Sample | Sample weight | HCL (ml) | HCL normality | Protein percent | Notes |
|--------|---------------|----------|---------------|-----------------|-------|
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11 REVISION RECORD

| Date | Responsible person | Description of change |
|------------|--------------------|-----------------------|
| 22/08/2011 | Christian MESTRES | Edition |
| | | |
| | | |

12 APPENDIX



**African Food Tradition rEvisited by
Research**
FP7 n°245025



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

Date: **07/10/2011**

Release: **1**

Written by : Sameh Awad

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| FAAU | Sameh AWAD | 30/07/2011 |
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| FRI | | |
| NRC | | |

Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

Date: 7/10/2011

Release: 1

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Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

Date: 7/10/2011

Release: 1

1 SCOPE AND APPLICATION

Determination of non protein nitrogen in milk and milky products

2 REFERENCES

AOAC Officials methods 967.12; 975.17; 975.18 (in AOAC International 2007 18th edition Edited by Horwitz W. and Latimer G. W)

ISO 8968-1 / IDF 20-1:2001. Milk determination of Nitrogen content - Part 1: Kjeldahl Method. International Dairy Federation, Brussels

3 DEFINITIONS

4 PRINCIPLE

Protein is precipitated from milk by addition of trichloroacetic acid (TCA) solution. Final concentration of TCA in the mixture is about 12%. Precipitated milk protein is removed by filtration. Filtrate contains nonprotein nitrogen components of milk. Nitrogen content of filtrate is determined as in method titled **Nitrogen (Total) in Milk - Kjeldahl Methods** (SOP : Chem-Cere-21-en).

5 REAGENTS

- a. **Sulfuric acid**. 95-98% H₂SO₄. Nitrogen free.
- b. **Copper catalyst solution**. CuSO₄ · 5H₂O. Nitrogen free. Prepare solution 0.05 g/mL H₂O.
- c. **Potassium sulfate**. K₂SO₄. Nitrogen free.
- d. **Sodium hydroxide solution**. 50% w/w nitrate-free NaOH.
- e. **Boiling chips**. Mesh size 10 suggested. High purity, amphoteric alundum granules, plain.
- f. **Methyl red/bromocresol green indicator solution**. Dissolve 0.2 g methyl red and dilute to 100 mL in 95% ethanol. Dissolve 1.0 g bromocresol green and dilute to 500 mL in 95% ethanol. Mix 1 part methyl red solution with 5 parts bromocresol green solution (combine all of both solutions).
- g. **Boric acid solution**. 4%, with indicator. Dissolve 40 g H₃BO₃ and dilute to 1 L in water and add 3 mL methyl red/bromocresol green indicator solution, (f). Solution will be light orange color.

Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

Date: **7/10/2011**

Release: **1**

- h. **Hydrochloric acid standard solution.** 0.1000N. Prepare as in Association of Official Analytical Chemists method number 936.15 or use pre-made solution of certified specification range 0.0995-0.1005N and use 0.1000N for calculation.
- i. **Trichloroacetic acid solution.** 15% w/v, analytical grade CCl_3COOH . (Caution: See safety note on trichloroacetic acid.) TCA is a soft, white, deliquescent crystal, which should be stored in a container protected from light and moisture.

6 APPARATUS

- a. **Digestion flasks.** Kjeldahl. Hard, moderately thick, well annealed glass. Total capacity is ca 500 or 800 mL.
- b. **Distillation flasks.** Same as Kjeldahl flask as in (a), fitted with rubber stopper through which passes lower end of efficient connecting bulb or trap to prevent mechanical carryover of NaOH during distillation. Connect upper end of bulb to condenser tube with rubber tubing. Use graduated 500 mL Erlenmeyer titration flask to collect distillate. Trap outlet of condenser in manner to ensure complete absorption of NH_3 distilled into boric acid solution.
- c. **Digestion/distillation system.** Traditional apparatus with adjustable controls for individual flasks.
- d. **Titration buret.** 50 mL. Class A or equivalent.

7 PROCEDURE

7.1 Preparation of Sample

- Warm milk to $38 \pm 1^\circ\text{C}$ and mix thoroughly. Immediately pipet milk (10 ± 1 mL) into preweighed 125 mL Erlenmeyer flasks and weigh.
- Add 40 ± 0.5 mL 15% TCA solution to flask. Weigh flask and contents, swirl to mix.
- Let precipitate settle (ca 5 min).
- Filter (Whatman No. 1 paper, 15 cm, N-free; or equivalent) and collect entire filtrate. Filtrate should be clear and free of particulate matter; if it is not, repeat sample preparation.
- Swirl filtrate to mix. Pipet 20 ± 0.2 mL filtrate into a 50 mL beaker and weigh. Pour filtrate from beaker into Kjeldahl digestion flask that contains boiling chips, Cu_2SO_4 , and $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ catalyst solution, and then add H_2SO_4 as in method titled **Nitrogen (Total) in Milk - Kjeldahl Methods**. Flask may be stoppered and held for digestion at a later time.
- Digest and distill sample and blank solution (using distilled water instead of milk in the blank) as in method titled **Nitrogen (Total) in Milk - Kjeldahl Methods**.

Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

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Release: **1**

7.2 Determination

Proceed as in method titled **Nitrogen (Total) in Milk - Kjeldahl Methods (Chem-Cere-21-en)**, substituting 0.01 N HCL solution for 0.1 N HCL solution as a titrant.

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Calculate as follows:

$$\text{Nitrogen \%} = \frac{1.4007 \times (V_s - V_b) \times N}{(W_f \times W_m) / [W_t - (W_m \times 0.065)]}$$

Where:

- V_s = mL titrant used for sample
- V_b = mL titrant used for blank
- N = normality of HCL solution
- W_f = weight, g, of 20 mL filtrate
- W_m = weight, g, of milk
- W_t = weight, g, of milk plus 40 mL 15% TCA solution

Note: Factor 0.065 in denominator assumes that milk contains about 3.5% fat and 3.0% true protein (i.e., 0.035 + 0.030). Factor may need to be adjusted if liquid dairy products of different composition are analyzed (i.e., concentrated or fractionated skim or whole milk products, etc.).

"protein equivalent, " % = nitrogen x 6.38 which is nonprotein nitrogen expressed as protein equivalent.

8.2 Repeatability

For method performance parameters obtained in collaborative study of this method, $S_r = 0.0006$, $S_R = 0.012$, $RSD_r = 2.817\%$, $RSD_R = 5.707\%$, r value = 0.016, and R value = 0.033.

Reference: D.M> Barbano, J.M. Lynch, and J.R. Fleming. 1991. Direct and Indirect Determination of True Protein Content of Milk by Kjeldahl Analysis: Collaborative Study. Journal of Association of Official Analytical Chemists 74:281-288.

Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

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9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

9.1 Safety Precautions :

- ✓ **Sulfuric acid**. Sulfuric acid can cause severe burns. Wear eye protection and acid resistant gloves. If acid is spilled on skin, wash immediately with large amounts of cool water. **NEVER NEUTRALIZE ACID ON SKIN WITH BAKING SODA.**
- ✓ **Sodium Hydroxide**. Alkalies can cause severe burns. Violent boiling can occur when sodium hydroxide is added to the digest (especially if there is too much residual acid). Wear eye protection and heavy rubber gloves when working with sodium hydroxide. If sodium hydroxide is spilled on skin, wash immediately with large amounts of cool water.
- ✓ **0.01N Hydrochloric acid (HCL)**. HCL solutions can cause burns. Wear eye protection. Wearing acid resistant gloves when handling HCL is recommended but may not be practical during titration. If HCL is spilled on the skin, wash immediately with large amounts of cool water.
- ✓ **Digestion**. Check the bottom of the Kjeldahl flasks for star cracks prior to adding the sample and reagents. Discard cracked flasks. Acid fumes are generated during digestion. Make sure the digestion apparatus (traditional or block) effectively removes fumes. Wear eye protection and heavy rubber gloves when handling Kjeldahl flasks.
- ✓ **Distillation**. Distillation involves the heating of an acid digest plus sodium hydroxide with the release of ammonia gas. Care should be taken under conditions such as these. Always wear eye protection and heavy rubber gloves. Most Kjeldahl distillation units have a protective glass (or plastic) barrier that is used as protection for the operator during distillation. If your unit has this, use it!

9.2 General procedure

- ✓ Record all weights to nearest 0.0001g
- ✓ Keep record of blank values. If a blank value changes, identify the cause.

Non protein Nitrogen in Whole Milk

SOP: Chem-Cere-026-en

Date: **7/10/2011**

Release: **1**

10 TEST REPORT

| Sample | Non Protein nitrogen % | Notes |
|--------|------------------------|-------|
| | | |
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| | | |
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11 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
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12 APPENDIX



**African Food Tradition rEvisited by
Research**
FP7 n°245025



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

Determining the protein content in milk

SOP: Chem-Cere-027-en

Date: **30/7/2011**

Release: **1**

Written by : Sameh Awad

For information on this SOP please contact :

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Determining the protein content in milk

SOP: Chem-Cere-027-en

Date: 7/10/2011

Release: 1

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Determining the protein content in milk

SOP: Chem-Cere-027-en

Date: 7/10/2011

Release: 1

1 SCOPE AND APPLICATION

Determination of the protein content in milk and milky products

2 REFERENCES

ISO 8968-1 / IDF 20-1:2001. Milk determination of Nitrogen content - Part 1: Kjeldahl Method. International Dairy Federation, Brussels

3 DEFINITIONS

4 PRINCIPLE

Protein is precipitated from milk by trichloroacetic acid (TCA) solution. . The TCA solution, which contains nonprotein nitrogen components of a sample, is separated from protein precipitate by filtration.

Total nitrogen and nonprotein nitrogen contents of milk sample are determined separately. Difference between results of these 2 determinations is protein nitrogen content of milk.

5 REAGENTS

See methods of total nitrogen and non protein nitrogen determinations (Chem-Cere-21-en and Chem-Cere-26-en)

6 APPARATUS

See methods of total nitrogen and non protein nitrogen determinations (Chem-Cere-21-en and Chem-Cere-26-en)

7 PROCEDURE

See methods of total nitrogen and non protein nitrogen determinations (Chem-Cere-21-en and Chem-Cere-26-en)



**African Food Tradition rEvisited by
Research**
FP7 n°245025



Deliverable D.1.2.3.8: SOP for Chemical Analysis for Group 1

Determination of Crude fiber

SOP: Chem-Cere-28-en

Date: **16/11/2011**

Release: **1**

Written by : Zahra AHMED

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Determination of Crude fiber

SOP: Chem-Cere-28-en

Date: 16/11/2011

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Determination of Crude fiber

SOP: Chem-Cere-28-en

Date: 16/11/2011

Release: 1

1 SCOPE AND APPLICATION

This procedure describes a method for the determination of crude fibre in cereals and cereal products.

2 REFERENCES

AACC-32-10: Crude fiber in flours, feeds, and feedstuffs.

3 DEFINITIONS

By the term "crude fibre" is understood a mixture of largely undigestible substances of vegetable origin obtained as the residue after sequential digestion of a sample with H₂SO₄ and NaOH solutions under specific conditions. "Crude fibre" consists chiefly of cellulose and other vegetable cell wall substances. The crude fibre value does not represent the absolute content of these components.

4 PRINCIPLE

Cereal products are exposed to a series of sulfuric acid and sodium hydroxide digestions. The insoluble residue is dried, weighed then ignited. The crude fibre value is calculated from the ignition loss.

The compounds removed are predominantly protein, sugar, starch, lipids and portions of both the structural carbohydrates and lignin. The residue (containing cellulose, hemicellulose, lignin, ash and tannins) is indigestible substances, and often called structural carbohydrates.

5 REAGENTS

See Appendix

6 APPARATUS

See Appendix.

Determination of Crude fiber

SOP: Chem-Cere-28-en

Date: 16/11/2011

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7 PROCEDURE

See Appendix

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

$$\text{Crude fibre percent by wt} = \frac{(W_1 - W_2) \times 100}{W}$$

Where :

W1 = wt in g of crucible and insoluble residues before ashing

W2 = wt in g of crucible containing ash

W = Wt in g of the material taken for the test

Calculate crude fibre on dry wt basis by giving correction for the moisture content

8.2 Repeatability

If the coefficient of variation (standard deviation over mean value) between duplications performed in short time interval exceed 5%, rerun the analysis.

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Acid and base solubilize some of the true fibers (particularly hemicellulose, pectin and lignin). Cellulose too is partially lost. Hence, crude fiber underestimates true fiber.
- The rate of heating should be gently boiled and must be controlled.
- Filtering after each digestion must be completed within a given time; delays in filtering after acid or alkali digestion generally lower the result.
- Particle size is important; the finer the material is grounded the lower the crude fiber content.

Determination of Crude fiber

SOP: Chem-Cere-28-en

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10-Test report

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

11-REVISION RECORD

| Date | Responsible person | Description of change |
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12 APPENDIX

AACC-32-10: Crude fiber in flours, feeds, and feedstuffs

CRUDE FIBER IN FLOURS, FEEDS, AND FEEDSTUFFS

First approval 10-28-81, revised 10-27-82

Definition

Crude fiber is loss on ignition of dried residue remaining after digestion of sample with 1.25% H₂SO₄ and 1.25% NaOH solutions under specific conditions. This method utilizes the Oklahoma State filter screen or the Modified California State Büchner funnel for filtration and uses prepd ceramic fiber as a filtration aid instead of asbestos.

Scope

Applicable to grains, meals, flours, feeds, and fiber-bearing material from which fat can be extd to leave a workable residue; including soy, flour, and meal.

Apparatus

1. Digestion app with condenser to fit 600-ml beaker and hot plate adjustable to temp that will bring 200 ml water at 25° to rolling boil in 15 ± 2 min. (Available from Labconco Corp., 8811 Prospect Ave., Kansas City, MO 64132.)

2. Ashing dishes, Silica, Vitreosil 70 × 15 mm; or porcelain, Coors, No. 450, size 1, or equiv.

3. Desiccator with efficient desiccant such as 4-8 mesh Drierite (CaCl₂ is not satisfactory).

4. Filtering device with No. 200 type 304 or 316 stainless-steel screen (W. S. Tyler Co., 8200 Tyler Blvd., Mentor, OH 44060), easily washed free of digested residue. Either Oklahoma State filter screen (see Fig. 1; available from Labconco Corp.) or modified California plastic Büchner (see Fig. 2; consists of 2-piece polypropylene plastic funnel manufactured by Halge Co., 75 Panorama Creek Dr., Rochester, NY 14602, Cat. No. 4280), 70 mm, without No. 200 screen, or equiv (also available from Labconco Corp.). Seal screen to filtering surface of funnel, using small tip soldering iron.

5. Suction filter to accommodate filtering devices. Attach suction flask to trap in line with aspirator or other source of vacuum with valve to break vacuum.

6. Liquid preheater for preheating water, 1.25% H₂SO₄, and 1.25% NaOH solns to bp of water. Convenient system, shown in Fig. 3, consists of sheet copper tank with three cuts of 3/8-in (10 mm) o.d. copper tubing, 12 1/2 ft (3.8 m) long. Solder inlets and outlets where tubing passes thru tank walls. Connect to reflux condenser and fill with water. Keep water boiling with two 750-W thermostatically controlled hot plates. Use Tygon for inlet leads to reservoirs of water, acid, and alk; use gum rubber tubing for outlets. Capacity of preheater is adequate for 60 analyses in 8 hr.

Crude Fiber in Flours, Feeds, and Feedstuffs (continued)

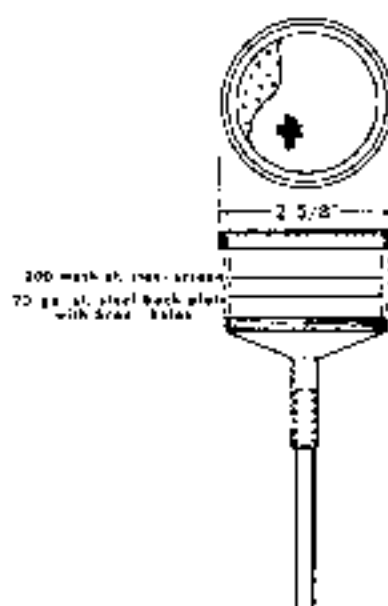


Fig. 1. Oklahoma State filter screen. (Reprinted, by permission, from the AACC Methods.)



Fig. 2. Modified California State Böhner funnel, 2-piece polypropylene plastic, covered with 200-mesh screen. A, heat-sealed to edge of filtering surface. (Reprinted, by permission, from the AACC Methods.)

Reagents

1. H_2SO_4 soln, 0.255 \pm 0.005N, contg 1.25 H_2SO_4 · 100 ml. Concn must be checked by titration.
2. NaOH soln, 0.313 \pm 0.005N, contg 1.25 g NaOH, 100 ml free, or nearly so, from Na_2CO_3 . Concn must be checked by titration.
3. Prepd ceramic fiber. Place 60 g ceramic fiber (Cat. No. 1740M, Lab Safety Supply Co., P.O. Box 1368, Janesville, WI 53545) in blender, add 400 ml water, and blend 1 min at low speed. Det blank by treating ca 2 g (dry wt) of prepd ceramic fiber with acid and alk as in detn. Corr crude fiber results for any blank, which should be negligible (ca 2 mg).
4. Alc 95% or reagent alc, methyl alc, or isopropanol.
5. Antifoam—Dow Corning Corp. Antifoam A compound dild 1 + 4 with mineral spirits or pet ether, or water dild Antifoam B Emulsion (1 + 4). Do not use Antifoxan Spray.
6. Bumping chips or granules. Broken Alundum crucibles or equiv granules (RR Alundum 90 mesh, Norton Co., 1 New Bond St., Worcester, MA 01606) are satisfactory.

Crude Fiber in Flours, Feeds, and Feedstuffs (continued)

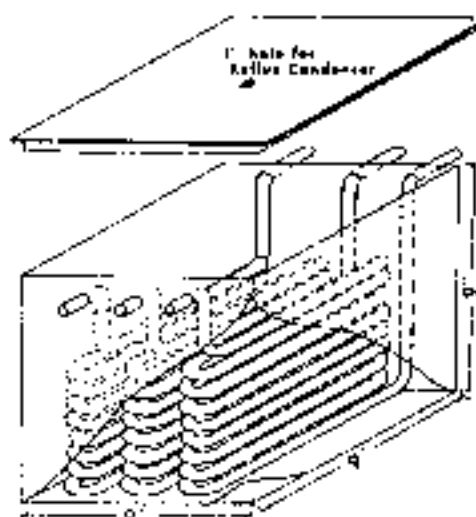


Fig. 3. Continuous heater for distilled water, 1.25% H₂SO₄, and 1.25% acid. (Reprinted, by permission, from the AOAC Methods.)

Preparation of Sample

Reduce sample (if feasible) to 100 g and place portion in sealed container for moisture detn. Immediately det moisture. Grind remainder to uniform fineness. Weber mill (Sargent-Welch Scientific Co. S-60870) with screen 0.033–0.040 in. (No. 18 or 20), Micro mill (Pulverizing Machinery, Div. Mikropul Corp., 10 Clatham Rd., Summit, N.J. 07901) with screen 3:25–1, 16 in. (No. 18–No. 12), and Wiley mill with 1-mm (No. 18) screen give comparable fineness. Since most materials lose moisture during grinding, det moisture on ground sample at same time sample is taken for crude fiber detn.

Procedure

1. Ext 2 g ground material with ether or pet ether. If fat is less than 1%, extn may be omitted. Transfer to 600-ml beaker, avoiding fiber contamination from paper or brush. Add ca 1.5–2.0 g dry wt prepd ceramic fiber, 200 ml boiling 1.25% H₂SO₄, and 1 drop dild antifoam. (Excess antifoam may give high results; use only if necessary to control foaming.) Bumping chips or granules may also be added.
2. Place beaker on digestion app with preadjusted hot plate and boil exactly 30 min, rotating beaker periodically to keep solids from adhering to sides. Remove beaker, and filter as in a or b below.
 - a. Using Oklahoma filter screen, turn on suction and insert screen into beaker, keeping face of screen just under surface of liquid until all

Crude Fiber in Flours, Feeds, and Feedstuffs (continued)

liquid is removed. Without breaking suction or raising filter, add 50-75 ml boiling water. After wash is removed, repeat with three 50-ml washings. (Work rapidly to keep mat from becoming dry.) Remove filter from beaker and drain all water from line by raising above trap level. Return mat and residue to beaker by breaking suction and blowing back. Add 200 ml boiling 1.25% NaOH and boil exactly 30 min. Remove beaker and filter as above. Without breaking suction, wash with 25 ml boiling 1.25% H₂SO₄ and three 50-ml portions boiling water. Drain free of excess water by raising filter. Lower filter into beaker and wash with 25 ml alcohol. Drain line, break suction, and remove mat by blowing back thru filter screen into ashing dish. Proceed as in 3.

- b. Using California Büchner. Filter contents of beaker thru Büchner, rinse beaker with 50-75 ml boiling water, and wash thru Büchner. Repeat with three 50-ml portions water, and suck dry. Remove mat and residue by snapping bottom of Büchner against top while covering stem with thumb or forefinger and replace in beaker. Add 200 ml boiling 1.25% NaOH and boil exactly 30 min. Remove beaker and filter as above. Wash with 25 ml boiling H₂SO₄, three 50-ml portions water, and 25 ml alcohol. Remove mat and residue; transfer to ashing dish.

3. Treatment of residue: Dry mat and residue 2 hr at 100 ± 2°. Cool in desiccator and weigh. Ignite 30 min at 600 ± 15°. Cool in desiccator and reweigh.

Calculation

$$\% \text{ Crude fiber} = \frac{\text{loss in wt on ignition} - \text{loss in wt residue fiber blank}}{\text{wt of sample}} \times 100$$

Reference

1. American Oil Chemists Society, 1973. Official and Tentative Methods, 3rd ed. Method Cc-49.
2. Association of Official Analytical Chemists, 1980. Official Methods of Analysis, 13th ed. Sec. 7.061-7.065, pp. 132-134.

Determining the protein content in milk

SOP: Chem-Cere-027-en

Date: 7/10/2011

Release: 1

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Subtract nonprotein nitrogen content from nitrogen content of milk sample and multiply result by 6.38.

8.2 Repeatability

For method performance parameters obtained in collaborative study of this method $S_r = 0.014$, $S_R = 0.017$, $RSD_r = 0.385\%$, $RSD_R = 0.504\%$, r value = 0.038 and R value = 0.049.

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE



10 TEST REPORT

| Samples | Total Nitrogen % | Non Protein Nitrogen | Protein % | Comments |
|---------|------------------|----------------------|-----------|----------|
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| | | | | |

11 REVISION RECORD

| Date | Responsible person | Description of change |
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12 APPENDIX

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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination de la teneur en lipides libres dans les produits céréaliers fermentés – Méthode au Soxhlet | | |
| SOP : Chem-Cere-023-fr | | |
| Date : : 23/08/2011 | Version : 2 | |
| <p> Ecrit par : Noël AKISSOE Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • Noël AKISSOE (noel.akis@yahoo.fr) • • </p> | | |
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| FAAU | | |
| ESB | | |
| FRI | | |
| NRC | | |

**Détermination de la teneur en lipides totaux dans les produits céréaliers fermentés –
Méthode au Soxhlet**

SOP : Chem-Cere-023-fr

Date : 23/08/2011

Version: 2

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Détermination de la teneur en lipides totaux dans les produits céréaliers fermentés – Méthode au Soxhlet

SOP : Chem-Cere-023-fr

Date : 23/08/2011

Version: 2

1 DOMAINE ET APPLICATION

Le présent document décrit une méthode de référence pratique pour la détermination de la teneur en lipides libres dans les céréales et les produits céréaliers, à l'état de grains, grains broyés, semoule ou farine.

2 REFERENCES

AACC International Method 30-25.01 (cf annexe)

3 DEFINITIONS

On appelle lipides libres, les lipides directement extractibles par un solvant organique.

4 PRINCIPE

- Mouture de l'échantillon pour le transformer en farine (particules de taille inférieure à 0,5 mm).
- Séchage préalable de l'échantillon dans l'étude à 95-100°C
- Extraction complète durant 4 heures d'une prise d'essai de 2- 5 g (selon la teneur en lipides) au moyen d'éther de pétrole, à une température comprise entre 50-60°C

5 REACTIFS

Cf annexe

6 APPAREILLAGE

Cf annexe

7 PROCÉDURE

Cf annexe

Détermination de la teneur en lipides totaux dans les produits céréaliers fermentés – Méthode au Soxhlet

SOP : **Chem-Cere-023-fr**

Date : 23/08/2011

Version: 2

8 EXPRESSION DES RÉSULTATS

8.1 Mode de calcul et formules

Pour le mode de calcul, se référer à la méthode AACC International Method 30-25.01

8.2 Répétabilité

L'écart type entre mesures réalisées dans un court intervalle de temps ne dépassera pas **5%** (coefficient de variation).

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

La manipulation et le stockage de l'Ether de pétrole présente des dangers, il est essentiel de le manipuler sous sorbonne, et de ne pas en stocker un grand volume dans le laboratoire.

- La température du réfrigérant (eau) doit être inférieure à 30°C, sinon utiliser un système de réfrigération approprié.
- Bien contrôler la vitesse de distillation/condensation de l'éther de pétrole
- Si l'échantillon humide, bien respecter la phase de séchage avant extraction.
- Utiliser du coton délipidé.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

Détermination de la teneur en lipides totaux dans les produits céréaliers fermentés – Méthode au Soxhlet

SOP : **Chem-Cere-023-fr**

Date : **23/08/2011**

Version: **2**

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------------|----------------------|--------------------------------|
| 23/08/2011 | Christian MESTRES | Edition et ajouts de notes |
| | | |
| | | |

12 ANNEXE

Texte de la norme AACC International Method 30-25.01

Crude Fat in Wheat, Corn, and Soy Flour, Feeds, and Mixed Feeds

First approval April 13, 1961; Reapproval November 3, 1999

Objective

Dried sample is exhaustively extracted by Soxhlet or continuous (Goldfisch) extraction, utilizing petroleum ether as solvent. Solvent is evaporated, and residue is dried to constant weight at 100°. Residue is expressed as % crude fat or ether extract. This method is applicable to flours of grains (wheat, corn, soybean, etc.) and mixed feeds that have not been heat-treated, baked, or expanded.

Apparatus

1. Extractor.
2. Filter paper.
3. Extraction thimbles 22 × 80 mm.
4. Cotton, free from ether extract, or Pyrex glass wool.
5. Vacuum oven or vacuum desiccator for nonoxidative drying of sample.

Reagent

Petroleum ether, redistilled, boiling point 30–60°. *Caution.* See Note.

Procedure

1. Weigh 2–5 g (depending on fat content) of sample that has been previously dried in vacuum oven at 95–100°, under pressure not to exceed 100 mm Hg (about 5 hr). Vacuum desiccator over concentrated H₂SO₄ for about 24 hr, under pressure not exceeding 10 mm Hg, can also be used to dry sample.
2. Quantitatively transfer sample to extractor and extract with petroleum ether for 4 hr at condensation rate of 5–6 drops/sec to 16 hr at 2–3 drops/sec rate.
3. Remove ether from collection flask or beaker at low temperature (below autoignition temperature of solvent used) volatilization before oven drying.
4. Dry fat remaining in previously dried and tared fat beaker or flask in oven at 100° for 30 min. Desiccate and cool.
5. Weigh, and repeat step 4 to constant weight.

Calculation

$$\text{Crude fat or ether extract, \%} = \frac{\text{weight of extract} - \text{blank}}{\text{weight of sample}} \times 100$$

Note



Caution. Petroleum ether is an extremely flammable solvent. Do not let vapors concentrate to a flammable level in the work area, since it is nearly impossible to eliminate all chance of sparks from static electricity even though

Crude Fat in Wheat, Corn, and Soy Flour, Feeds, and Mixed Feeds (continued)

the electrical equipment is grounded. Use an effective fume removal device to remove these vapors when released.

References

1. American Oil Chemists' Society. 1998. Official Methods and Recommended Practices, 5th ed. Method Ba 3-38.
2. AOAC International. 1995. Official Methods of Analysis of AOAC International, 16th ed. Method 920.39C. The Association, Arlington, VA.

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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination des lipides libres dans les produits céréaliers fermentés – Méthode rapide au Soxtec | | |
| SOP : Chem-Cere-13-fr | | |
| Date : 16/09/2011 | Version : 1 | |
| <p> Ecrit par : Brigitte PONS Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • </p> | | |
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**Détermination des lipides libres dans les produits céréaliers fermentés –
Méthode rapide au Soxtec
SOP : Chem-Cere-13-fr**

Date : 16/09/2011

Version : 1

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Détermination des lipides libres dans les produits céréaliers fermentés –

Méthode rapide au Soxtec

SOP : Chem-Cere-13-fr

Date : 16/09/2011

Version : 1

1 DOMAINE ET APPLICATION

La méthode décrit une méthode rapide pour la détermination de la teneur en lipides libres (triglycérides, acides gras, ...), extractibles à l'éther éthylique, dans des produits céréaliers fermentés. Elle s'applique à des produits secs ; teneur en eau inférieure à 14% (base humide).

2 REFERENCES

Cette procédure s'inspire d'une méthode AOAC (2003.05: Crude Fat in Feeds, Cereal Grains, and Forages).

3 DEFINITIONS

On appelle lipides libres, les lipides directement extractibles par un solvant organique.

Ether diéthylique : $(\text{CH}_3\text{CH}_2)_2\text{O}$

4 PRINCIPE

Il s'agit d'une méthode gravimétrique. Les lipides libres sont extraits par l'éther diéthylique bouillant (34,6°C) sous reflux. Après élimination du solvant, le résidu huileux est séché puis pesé.

5 REACTIFS

Ether diéthylique pour analyse

6 APPAREILLAGE

- Broyeur mécanique de laboratoire
- Appareillage d'extraction sous-reflux, type Soxtec (1043), muni d'un bain d'huile thermostaté pour le chauffage et d'un système de condensation des vapeurs utilisant l'eau du robinet, ou un bain réfrigéré si celle-ci a une température supérieure à 20°C.
- Capsules d'extraction aluminium à fond plat (volume 75 mL)
- Cartouches d'extraction en cellulose (diamètre 28 mm, volume 25 ml, Whatman D26 H60 2800266)
- Bagues en inox aimantées (diamètre 30 mm)
- Etuve réglée à 100°C

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- Dessiccateur
- Coton hydrophile dégraissé
- Balance de précision (0,1 mg)

7 PROCÉDURE

- Broyer l'échantillon, si nécessaire, afin de le réduire en fine particules dont 95% passent au travers du tamis de 250 µm.
- Bien homogénéiser avant la prise d'essai
- Allumer le bain d'huile de l'extracteur. Vérifier et/ou régler la température de consigne à 100°C. Attendre la mise en équilibre environ 2 heures.
- Mettre les capsules en aluminium à l'étuve à 100°C pendant 15 mn, puis les laisser refroidir dans un dessiccateur sous vide avant de les tarer avec la balance de précision.
- Peser environ exactement 5g d'échantillon broyé dans une cartouche de cellulose. Recouvrir de coton (aide à la dispersion de l'éther dans tout le produit et évite la mise en suspension de la farine). Enfiler la bague en inox sur le sommet de la cartouche, et stocker la cartouche sur le portoir prévu à cet effet.
- Verser 50 ml d'éther diéthylique dans les capsules en aluminium préalablement tarées (opération à réaliser sous sorbonne)
- Ouvrir le robinet d'eau alimentant le réfrigérant, ou allumer le bain réfrigéré.
- Vérifier que les robinets de recyclage du solvant soient en position ouverte
- Placer 6 cartouches dans le dispositif d'extraction, et mettre les capsules d'extraction sur la plaque chauffante. Plonger rapidement les cartouches dans l'éther contenu dans les capsules, en amenant la manette sur la position « **boiling** ». Vérifier la vitesse de condensation de l'éther en fermant temporairement le robinet de recyclage de chaque poste d'extraction : on doit voir le niveau de condensat monter assez rapidement. La phase d'extraction par immersion démarre et dure **20 mn**.
- Après 20 mn, relever les cartouches en positionnant la manette sur « **rinsing** ». La phase de rinçage débute ; elle dure **40 mn**.
- Après 40 mn, fermer les robinets de recyclage du solvant et laisser évaporer pendant environ 7 à 8 mn

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- Le résidu d'éther est ensuite éliminé des capsules par un balayage d'air, en positionnant la manette sur évaporation pendant 5 mn environ
- Les capsules en aluminium sont récupérées et mise à l'étuve à 100°C pendant 15 mn
- Les mettre dans un dessiccateur sous vide jusqu'à refroidissement (20 mn minimum) et ensuite les peser à la balance de précision ; les capsules doivent être rigoureusement froide au toucher au moment de la pesée.

8 EXPRESSION DES RESULTATS

8.1 Mode de calcul et formules

La teneur en lipides éthersolubles est exprimée en pourcentage base sèche :

$$\text{Teneur en lipides (\% bs)} = \frac{(\text{Cap} - \text{Cap}_0) \times 10000}{M \times (100 - H)}$$

Cap₀ = masse en grammes de la capsule en aluminium avant extraction (soit la tare)

Cap = masse en grammes de la capsule en aluminium après extraction

M = masse de la prise d'essai (g)

H = teneur en eau de l'échantillon (% base humide)

8.2 Répétabilité

Le coefficient de variation entre deux mesures réalisées sur un même échantillon dans un délai court ne doit pas dépasser **5%**.

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- La manipulation et le stockage de l'Ether diéthylique présente des dangers, il est essentiel de le manipuler sous sorbonne, et de ne pas en stocker un grand volume dans le laboratoire.
- Le réseau d'alimentation en eau pour le réfrigérant doit être constant et à une température **inférieure à 20°C**.
- **Bien vérifier la vitesse de condensation** de l'éther en début d'extraction

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- Positionner le coton uniformément sur toute la surface de la farine de manière à ce que la dispersion de l'éther sur le coton soit homogène.
- Vérifier que tout l'éther soit évaporé avant de retirer les capsules aluminium
- Lavage des capsules : éliminer les résidus gras avec du papier jetable puis laver les capsules avec de l'eau bien chaude et du produit dégraissant.
- Les cartouches d'extraction peuvent être réutilisées tant qu'elles n'apparaissent pas endommagées ou souillées.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------|----------------------|--------------------------------|
| | | |
| | | |
| | | |

12 ANNEXE

AOAC Official Method 2003.05 (Crude Fat in Feeds, Cereal Grains, and Forages)

4.5.05

AOAC Official Method 2003.05
Crude Fat in Feeds, Cereal Grains, and Forages
Randall/Soxtec/Diethylether Extraction-Submersion Method
First Action 2003
Final Action 2006

[Applicable to the analysis of forages, cereal grains, and animal feeds other than baked or expanded products, dried milk or milk products, fishmeal, or oilseeds at concentrations from 0.5 to 100% fat. It is applicable to the same matrixes as **920.39** (see 4.5.01) and **930.09** (see 3.5.07).]

Caution: Store solvents in metal containers in solvent cabinet or solvent room that conforms to applicable safety legislation. Ethers are extremely flammable. Have no open flames in the laboratory where the analysis is being performed. Avoid inhaling vapors. Use solvents in a properly operating hood equipped with explosion-proof lighting, wiring, and fan. Diethylether has the potential to form shock-sensitive, explosive peroxides with age. Check each new container of ether for peroxides when it is opened. Also check partial containers of ether that have not been used for several months before using them again. *Do not* use ether that contains peroxides. Dispose as hazardous material. Stabilized ether may be used. Ground electrical equipment and maintain in proper working order. Follow manufacturer recommendations for installation, operation, and safety of all extraction equipment. Make sure all solvent is evaporated from cups before placing them in the oven to avoid a fire or explosion.

See Table **2003.05** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

The Randall modification of the standard Soxhlet extraction submerges the test portion in boiling solvent, reducing the time needed for extraction. The solvent dissolves fats, oils, pigments, and other soluble substances, collectively termed “crude fat.”

A dried, ground test portion is extracted by a 2-step process: In the first step, the thimble containing the test portion is immersed into the boiling solvent. The intermixing of matrix with hot solvent ensures rapid solubilization of extractables. The thimble is then raised above the solvent and the test portion is further extracted by a continuous flow of condensed solvent. The solvent is evaporated and recovered by condensation. The resulting crude fat residue is determined gravimetrically after drying.

The solubility characteristics of different solvents may result in slight differences in crude fat results. For this reason, the report should reflect the solvent used. Example: % Crude Fat, Ether Extraction.

B. Apparatus

(a) *Solvent extraction system.*—Multiple position extraction unit conducting 2-stage Randall extraction process with solvent recovery cycle, with Viton or Teflon™ seals compatible with ether.

(b) *Thimbles and stand.*—Cellulose thimbles and stand to hold thimbles.

(c) *Extraction cups.*—Aluminum or glass. (Extraction temperature settings may differ; consult manufacturer’s operating instructions.)

Items (a)–(c) are available as Soxtec systems from Foss or other Randall-type extraction systems.

C. Reagents

(a) *Anhydrous diethylether.*—Purified for fat extraction, Fisher E492 labeled “For Fat Extraction” is also stabilized, or E134-4, or equivalent. To prevent ether from absorbing water, purchase it in small containers and keep containers tightly closed. Petroleum ether cannot be substituted for diethylether because it does not dissolve all of the plant lipid material.

Table 2003.05. Interlaboratory results for crude fat in animal feed, cereal grain, and forage, diethylether extraction (submersion) method

| Material | Mean | No. of labs ^a | s _r | RSD _r , % | s _R | RSD _R , % |
|-----------------------------|-------|--------------------------|----------------|----------------------|----------------|----------------------|
| Dehydrated alfalfa | 4.44 | 10 | 0.21 | 4.62 | 0.23 | 5.1 |
| Corn silage | 2.09 | 9(1) | 0.02 | 1.02 | 0.06 | 3.06 |
| Mixed bird seed | 7.19 | 10 | 0.19 | 2.64 | 0.23 | 3.17 |
| Texturized feed | 3.20 | 9(1) | 0.1 | 3.22 | 0.22 | 6.77 |
| Fat supplement | 96.38 | 10 | 4.82 | 5.00 | 4.82 | 5.00 |
| Medicated goat feed | 1.89 | 10 | 0.04 | 2.09 | 1.08 | 4.5 |
| Feedlot concentrate pellets | 1.53 | 10 | 0.14 | 9.26 | 0.32 | 21.0 |
| Cellulose (blank) | 0.17 | 10 | 0.05 | 26.1 | 0.08 | 48.3 |
| Calf starter medicated | 2.96 | 8(2) | 0.05 | 1.58 | 0.06 | 1.9 |
| Calf feed medicated | 3.42 | 9(1) | 0.05 | 1.44 | 0.12 | 3.50 |
| Meat meal/hulls mix | 4.99 | 10 | 0.22 | 4.33 | 10.55 | 11.08 |
| Swine feed | 2.77 | 10 | 0.07 | 2.61 | 0.15 | 5.5 |
| Broiler starter | 7.05 | 10 | 0.1 | 1.35 | 0.16 | 2.33 |
| High oil corn | 7.69 | 10 | 0.1 | 1.31 | 0.22 | 2.93 |

^a Number of laboratories retained after eliminating the number of laboratories in parentheses.

(b) *Cotton*.—Defatted. Soak medical grade cotton in diethylether or hexanes for 24 h, agitating several times during this period. Remove and air dry.

(c) *Sand*.—Ashed (for ignition boats). EM SX0075-3, or equivalent (CAS 14808-60-7).

(d) *Celite 545*.

D. Preparation of Analytical Sample

Reduce particle size of samples to fineness of 0.75–1 mm.

E. Determination

Weigh 1–5 g test portions containing ca 100–200 mg fat directly into tared cellulose thimbles, according to following scheme:

| Crude fat, % | Test portion weight, g |
|--------------|------------------------|
| <2 | 5 |
| 5 | 2–4 |
| 10 | 1–2 |
| >20 | 1 |

Record weight to nearest 0.1 mg (S) and thimble number. *Note:* If test sample contains large amounts of urea salts (>5%) or soluble carbohydrates (>15%), glycerol, lactic acid, amino acid salts (>10%) or other water-soluble components, remove by water extraction. Weigh test portion onto filter paper, extract with five 20 mL portions of water, allowing each portion to drain. Place filter paper containing washed test portion into thimble and dry at 102 °C for 2 h. To facilitate filtration, add 1–2 g ashed, acid-washed sand, or Celite to bottom of filter or mix in with test portion before water extraction.

Dry thimbles containing test portions at 102 °C for 2 h. If dried test portions will not be extracted immediately, store in desiccator. Both solvent and test materials must be free of moisture to avoid extraction of water-soluble components such as carbohydrates, urea, lactic acid, and glycerol, which will result in false high values.

An absorbent, such as diatomaceous earth (Celite or Super-Cel), can be added to the test portion when high fat materials, which melt through the thimble during the predry step, are present. Alternatively, defatted cotton can be added before the predry step to absorb the melted fat. If the material melts at 102 °C, place a pretared extraction cup under the thimble during the drying step to catch any melted fat that was unabsorbed and escaped the thimble.

Place defatted (with same solvent to be used for extraction) cotton plug on top of test portion to keep material immersed during the boiling step and prevent any loss of test portion from top of thimble. Prepare cotton plug large enough to hold materials in place, yet as small as possible to minimize absorption of solvent. Adding the cotton plug before the 102 °C, 2 h drying step is acceptable.

Place three or four 5 mm glass boiling beads into each cup, and dry cups for at least 30 min at 102 °C. Transfer to desiccator and cool to room temperature. Weigh extraction cups and record weight to nearest 0.1 mg (T).

Extract, following manufacturer's instructions for operation of extractor. Preheat extractor and turn on condenser cooling water. Attach thimbles containing dried test portions to extraction columns. Put sufficient amount of solvent into each extraction cup to cover test portion when thimbles are in boiling position. Place cups under extraction columns and secure in place. Make sure that cups are matched to their corresponding thimble. Lower thimbles into solvent and boil for 20 min. Verify proper reflux rate which is critical to the complete extraction of fat. This rate depends upon the equipment and should be supplied by the manufacturer. A reflux rate of ca 3–5 drops/s applies to many extraction systems.

Raise thimbles out of solvent and extract in this position for 40 min. Then evaporate as much solvent as possible from cups to reclaim solvent and attain apparent dryness.

Remove extraction cups from extractor and place in operating fume hood to finish evaporating solvent at low temperature. (*Note:* Take care not to pick up any debris on bottom of extraction cup while in hood. Let cups remain in hood until all traces of solvent are gone.)



Dry extraction cups in a 102 °C oven for 30 min to remove moisture. Excessive drying may oxidize fat and give high results. Cool in desiccator to room temperature and weigh to nearest 0.1 mg (F).

F. Calculations

$$\% \text{ Crude fat, diethylether extract} = \frac{F - T}{S} \times 100$$

where F = weight of cup + fat residue, g; T = weight of empty cup, g; S = test portion weight, g.

References: *J. AOAC Int.* **86**, 888(2003); 899(2003).

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| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Determination of crude fat in cereal fermented products – Method using Soxtec | | |
| SOP: Chem-Cere-13-en | | |
| Date: 16/09/2011 | Release: 1 | |
| <p>Written by : Brigitte PONS</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • | | |
| This document has been approved by : | | |
| Partner | Name of the person who approved | Date DD/MM/YY |
| CIRAD UAC CSIR FAAU ESB FRI NRC | Christian MESTRES Sameh AWAD Wis AMOA Zahra AHMED | 18/04/2011 16/09/2011 16/09/2011 16/09/2011 |

**Determination of crude fat in cereal fermented products –
Method using Soxtec
SOP: Chem-Cere-13-en**

Date: 16/09/2011

Release: 1

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Determination of crude fat in cereal fermented products –

Method using Soxtec

SOP: **Chem-Cere-13-en**

Date: **16/09/2011**

Release: **1**

1 SCOPE AND APPLICATION

The procedure describes a rapid method for the determination of crude fats (triglycerides, fatty acids, pigments, etc...), extractable with ethyl ether, in fermented grain products. It applies to dry products; with less than 14% moisture content (wet basis).

2 REFERENCES

The procedure is based on AOAC Official Method 2003.05 (Crude Fat in Feeds, Cereal Grains, and Forages), and we will only detail in this procedure definitions (3), principles (4), repeatability (8.2) and critical points and notes (9). For details, see AOAC official method in appendix (12).

3 DÉFINITIONS

The solvent dissolves fats, oils, pigments, and other soluble substances, collectively termed “crude fat.”

Ethyl ether: $(\text{CH}_3\text{CH}_2)_2\text{O}$

4 PRINCIPLE

It is a gravimetric method. A dried, ground test portion is extracted by a 2-step process: extraction into the boiling solvent (20 min), then rinsing by a continuous flow of condensed solvent (40 min). After removal of the solvent, the oily residue is dried and then weighed.

5 REAGENTS

See appendix

6 APPARATUS

See appendix

Determination of crude fat in cereal fermented products –

Method using Soxtec

SOP: Chem-Cere-13-en

Date: 16/09/2011

Release: 1

7 PROCEDURE

See appendix

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

8.2 Repeatability

Coefficient of variation (standard deviation over mean value) between replicates performed in short time interval will not exceed **5%**.

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Handling and storage of diethyl Ether is hazardous. It is essential to manipulate under hood, and not to store a large volume in the laboratory.
- The water supply network for the refrigerant must be constant and at a temperature **below 20°C**.
- **Verify proper reflux rate at the beginning of extraction.**
- Position the cotton uniformly on the surface of flour so that the dispersion of ether on cotton is homogeneous.
- Verify that the ether is completely evaporated before removing aluminum capsules
- Washing the capsules: eliminate fatty residues with disposable paper and then wash the capsules with hot water and degreaser product.
- Thimbles can be reused as long as they are not damaged or dirty.

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

**Determination of crude fat in cereal fermented products –
Method using Soxtec
SOP: Chem-Cere-13-en**

Date: 16/09/2011

Release: 1

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
| | | |
| | | |
| | | |

12 APPENDIX

AOAC Official Method 2003.05 (Crude Fat in Feeds, Cereal Grains, and Forages)

4.5.05

AOAC Official Method 2003.05
Crude Fat in Feeds, Cereal Grains, and Forages
Randall/Soxtec/Diethylether Extraction-Submersion Method
First Action 2003
Final Action 2006

[Applicable to the analysis of forages, cereal grains, and animal feeds other than baked or expanded products, dried milk or milk products, fishmeal, or oilseeds at concentrations from 0.5 to 100% fat. It is applicable to the same matrixes as **920.39** (see 4.5.01) and **930.09** (see 3.5.07).]

Caution: Store solvents in metal containers in solvent cabinet or solvent room that conforms to applicable safety legislation. Ethers are extremely flammable. Have no open flames in the laboratory where the analysis is being performed. Avoid inhaling vapors. Use solvents in a properly operating hood equipped with explosion-proof lighting, wiring, and fan. Diethylether has the potential to form shock-sensitive, explosive peroxides with age. Check each new container of ether for peroxides when it is opened. Also check partial containers of ether that have not been used for several months before using them again. *Do not* use ether that contains peroxides. Dispose as hazardous material. Stabilized ether may be used. Ground electrical equipment and maintain in proper working order. Follow manufacturer recommendations for installation, operation, and safety of all extraction equipment. Make sure all solvent is evaporated from cups before placing them in the oven to avoid a fire or explosion.

See Table **2003.05** for results of the interlaboratory study supporting acceptance of the method.

A. Principle

The Randall modification of the standard Soxhlet extraction submerges the test portion in boiling solvent, reducing the time needed for extraction. The solvent dissolves fats, oils, pigments, and other soluble substances, collectively termed “crude fat.”

A dried, ground test portion is extracted by a 2-step process: In the first step, the thimble containing the test portion is immersed into the boiling solvent. The intermixing of matrix with hot solvent ensures rapid solubilization of extractables. The thimble is then raised above the solvent and the test portion is further extracted by a continuous flow of condensed solvent. The solvent is evaporated and recovered by condensation. The resulting crude fat residue is determined gravimetrically after drying.

The solubility characteristics of different solvents may result in slight differences in crude fat results. For this reason, the report should reflect the solvent used. Example: % Crude Fat, Ether Extraction.

B. Apparatus

(a) *Solvent extraction system.*—Multiple position extraction unit conducting 2-stage Randall extraction process with solvent recovery cycle, with Viton or Teflon™ seals compatible with ether.

(b) *Thimbles and stand.*—Cellulose thimbles and stand to hold thimbles.

(c) *Extraction cups.*—Aluminum or glass. (Extraction temperature settings may differ; consult manufacturer’s operating instructions.)

Items (a)–(c) are available as Soxtec systems from Foss or other Randall-type extraction systems.

C. Reagents

(a) *Anhydrous diethylether.*—Purified for fat extraction, Fisher E492 labeled “For Fat Extraction” is also stabilized, or E134-4, or equivalent. To prevent ether from absorbing water, purchase it in small containers and keep containers tightly closed. Petroleum ether cannot be substituted for diethylether because it does not dissolve all of the plant lipid material.

Table 2003.05. Interlaboratory results for crude fat in animal feed, cereal grain, and forage, diethylether extraction (submersion) method

| Material | Mean | No. of labs ^a | s _r | RSD _r , % | s _R | RSD _R , % |
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| Dehydrated alfalfa | 4.44 | 10 | 0.21 | 4.62 | 0.23 | 5.1 |
| Corn silage | 2.09 | 9(1) | 0.02 | 1.02 | 0.06 | 3.06 |
| Mixed bird seed | 7.19 | 10 | 0.19 | 2.64 | 0.23 | 3.17 |
| Texturized feed | 3.20 | 9(1) | 0.1 | 3.22 | 0.22 | 6.77 |
| Fat supplement | 96.38 | 10 | 4.82 | 5.00 | 4.82 | 5.00 |
| Medicated goat feed | 1.89 | 10 | 0.04 | 2.09 | 1.08 | 4.5 |
| Feedlot concentrate pellets | 1.53 | 10 | 0.14 | 9.26 | 0.32 | 21.0 |
| Cellulose (blank) | 0.17 | 10 | 0.05 | 26.1 | 0.08 | 48.3 |
| Calf starter medicated | 2.96 | 8(2) | 0.05 | 1.58 | 0.06 | 1.9 |
| Calf feed medicated | 3.42 | 9(1) | 0.05 | 1.44 | 0.12 | 3.50 |
| Meat meal/hulls mix | 4.99 | 10 | 0.22 | 4.33 | 10.55 | 11.08 |
| Swine feed | 2.77 | 10 | 0.07 | 2.61 | 0.15 | 5.5 |
| Broiler starter | 7.05 | 10 | 0.1 | 1.35 | 0.16 | 2.33 |
| High oil corn | 7.69 | 10 | 0.1 | 1.31 | 0.22 | 2.93 |

^a Number of laboratories retained after eliminating the number of laboratories in parentheses.

(b) *Cotton*.—Defatted. Soak medical grade cotton in diethylether or hexanes for 24 h, agitating several times during this period. Remove and air dry.

(c) *Sand*.—Ashed (for ignition boats). EM SX0075-3, or equivalent (CAS 14808-60-7).

(d) *Celite 545*.

D. Preparation of Analytical Sample

Reduce particle size of samples to fineness of 0.75–1 mm.

E. Determination

Weigh 1–5 g test portions containing ca 100–200 mg fat directly into tared cellulose thimbles, according to following scheme:

| Crude fat, % | Test portion weight, g |
|--------------|------------------------|
| <2 | 5 |
| 5 | 2–4 |
| 10 | 1–2 |
| >20 | 1 |

Record weight to nearest 0.1 mg (S) and thimble number. *Note:* If test sample contains large amounts of urea salts (>5%) or soluble carbohydrates (>15%), glycerol, lactic acid, amino acid salts (>10%) or other water-soluble components, remove by water extraction. Weigh test portion onto filter paper, extract with five 20 mL portions of water, allowing each portion to drain. Place filter paper containing washed test portion into thimble and dry at 102 °C for 2 h. To facilitate filtration, add 1–2 g ashed, acid-washed sand, or Celite to bottom of filter or mix in with test portion before water extraction.

Dry thimbles containing test portions at 102 °C for 2 h. If dried test portions will not be extracted immediately, store in desiccator. Both solvent and test materials must be free of moisture to avoid extraction of water-soluble components such as carbohydrates, urea, lactic acid, and glycerol, which will result in false high values.

An absorbent, such as diatomaceous earth (Celite or Super-Cel), can be added to the test portion when high fat materials, which melt through the thimble during the predry step, are present. Alternatively, defatted cotton can be added before the predry step to absorb the melted fat. If the material melts at 102 °C, place a pretared extraction cup under the thimble during the drying step to catch any melted fat that was unabsorbed and escaped the thimble.

Place defatted (with same solvent to be used for extraction) cotton plug on top of test portion to keep material immersed during the boiling step and prevent any loss of test portion from top of thimble. Prepare cotton plug large enough to hold materials in place, yet as small as possible to minimize absorption of solvent. Adding the cotton plug before the 102 °C, 2 h drying step is acceptable.

Place three or four 5 mm glass boiling beads into each cup, and dry cups for at least 30 min at 102 °C. Transfer to desiccator and cool to room temperature. Weigh extraction cups and record weight to nearest 0.1 mg (T).

Extract, following manufacturer's instructions for operation of extractor. Preheat extractor and turn on condenser cooling water. Attach thimbles containing dried test portions to extraction columns. Put sufficient amount of solvent into each extraction cup to cover test portion when thimbles are in boiling position. Place cups under extraction columns and secure in place. Make sure that cups are matched to their corresponding thimble. Lower thimbles into solvent and boil for 20 min. Verify proper reflux rate which is critical to the complete extraction of fat. This rate depends upon the equipment and should be supplied by the manufacturer. A reflux rate of ca 3–5 drops/s applies to many extraction systems.

Raise thimbles out of solvent and extract in this position for 40 min. Then evaporate as much solvent as possible from cups to reclaim solvent and attain apparent dryness.

Remove extraction cups from extractor and place in operating fume hood to finish evaporating solvent at low temperature. (*Note:* Take care not to pick up any debris on bottom of extraction cup while in hood. Let cups remain in hood until all traces of solvent are gone.)

Dry extraction cups in a 102 °C oven for 30 min to remove moisture. Excessive drying may oxidize fat and give high results. Cool in desiccator to room temperature and weigh to nearest 0.1 mg (F).

F. Calculations

$$\% \text{ Crude fat, diethylether extract} = \frac{F - T}{S} \times 100$$

where F = weight of cup + fat residue, g; T = weight of empty cup, g; S = test portion weight, g.

References: *J. AOAC Int.* **86**, 888(2003); 899(2003).



**African Food Tradition rEvisited by
Research
FP7 n°245025**



Deliverable D.1.2.3.8: SOP for chemical analysis for Group 1

Fatty acids profile by Gas chromatography

SOP: Chem-Cere-029-en

Date: **8/09/2011**

Release: **1**

Written by : Sameh Awad

For information on this SOP please contact :

- Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader
- Sameh Awad (sameh11leg@yahoo.com) FAAU

This document has been approved by :

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| FAAU | Sameh Awad | 8/09/2011 |
| ESB | | |
| FRI | | |
| NRC | | |

Fatty acids profile by Gas chromatography

SOP: Chem-Cere-029-en

Date: 10/10/2011

Release: 1

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Fatty acids profile by Gas chromatography

SOP: Chem-Cere-029-en

Date: 10/10/2011

Release: 1

1 SCOPE AND APPLICATION

This method provides means for preparing methyl esters of fatty acids for analysis by gas-liquid chromatography. It is applicable to common fats, oils, and fatty acids. This procedure can be applied to Group 1: Cereal based products (Akpan, Kenkey, Gowé, Kishk Sa'eedi).

2 REFERENCES

1. Preparation of methyl esters and long-chain fatty acids. AACC International Method 58-17.01.
2. American Oil Chemists' Society. 1998. Official Methods and Recommended Practices, 5th ed. Methods Ce 1-62; Ce 2-66. The Society, Champaign, IL.
3. American Society for Testing and Materials. 1971. Method D-1983-64T.
4. AOAC International. 1997. Official Methods of Analysis of AOAC International, 16th ed., 3rd rev. Method 969.33. The Association, Gaithersburg, MD.
5. Wiener, N. D., and Felmeister, A. 1966. Thermometric titration of cetylpyridinium chloride. Anal. Chem. 38:514.

3 DÉFINITIONS

4 PRINCIPLE

Unsaponifiables are not removed and, if present in large amounts, may interfere with subsequent analyses. Procedure results in partial or complete destruction of epoxy, hydroperoxy, cyclopropenyl, cyclopropyl, and possibly hydroxyl groups, and is not suitable for preparation of methyl esters of fatty acids containing these groups. This method is equivalent to Official Methods Ce 1-62 and Ce 2-66 of the American Oil Chemists' Society (AOCS). The methyl esters of fatty acids will be separated and determined using Gas Chromatography (GC).

5 REAGENTS

1. Boron trifluoride (BF₃)-Methanol reagent (125 g/liter of methanol). Available commercially or may be prepared using gas and methanol (see section 3(d) of Ref. 2, or Ref. 4).
2. NaOH, 0.5N in methanol.
3. NaCl, saturated solution in water.

Fatty acids profile by Gas chromatography

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4. Petroleum ether, redistilled, boiling point 30–60°. *Caution.* See Note 1.
5. Heptane, gas-chromatographically clean.
6. Na₂SO₄, anhydrous, analytical reagent, ACS grade.
7. Methyl red indicator, 0.1% in 60% ethanol.
8. Nitrogen gas, high purity.

6 APPARATUS

1. Flat-bottom boiling flasks or Erlenmeyer flasks with ST 19/38 or 24/40 outer necks, 50- and 125-ml.
2. Water-cooled condensers, with ST 19/38 or 24/40 interjoint.
3. Separatory funnels, 250-ml.
4. Boiling flask for solvent removal, 200-ml.
5. Boiling chips, free of fat.
6. Gas chromatographic system fitted with a split/splitless injector suitable flame ionisation detector (FID).
7. Capillary GC column

7 PROCEDURE

7.1 Methyl ester preparation

Accurate weighing is not required. Sample size need be known only to determine size of flask and amounts of reagents that should be used according to Table I.

- For fatty acids. Introduce fatty acids into 50- or 125-ml reaction flask. Add specified amount of BF₃-methanol reagent, attach a condenser, and boil 2 min. See Note 2. Add 2–5 ml heptane through condenser and boil 1 min longer. Remove from heat, remove condenser, and add enough saturated salt solution to float heptane solution of methyl esters into neck of flask. Transfer about 1 ml heptane solution into test tube and add small amount of anhydrous Na₂SO₄. Dry heptane solution may then be injected directly in gas chromatograph. See Note 3. To recover dry esters, transfer solution and heptane phase to 250-ml separatory funnel. Extract twice with 50-ml portions redistilled petroleum (boiling point 30–60°). Wash combined extracts with 20-ml portions water until free of acids (test water with methyl red indicator), dry with Na₂SO₄, and evaporate solvent under stream of nitrogen on steam bath. See Notes 4 and 5.
- For fats and oils. Introduce fat into 50- or 125-ml reaction flask. Add specified amount of 0.5N methanolic NaOH and add boiling chip. Attach condenser, and heat mixture on steam bath until fat globules go into solution. This step should take 5–10 min. Add

Fatty acids profile by Gas chromatography

SOP: Chem-Cere-029-en

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specified amount of BF₃-methanol reagent through condenser and proceed as described under fatty acid section.

TABLE I
Flasks and Reagents for Sample Size Ranges

| Sample (mg) | Flask (ml) | NaOH, 0.5N (ml) | BF ₃ -Methanol Reagent (ml) |
|-------------|------------|-----------------|--|
| 100–250 | 50 | 4 | 5 |
| 250–500 | 50 | 6 | 7 |
| 500–750 | 125 | 8 | 9 |
| 750–1000 | 125 | 10 | 12 |

7.2 GC-Chromatography

Separation and quantification of the methyl esters will be carried out using a gas chromatograph (Acme 6100 GC, Korea), equipped with a split/splitless injector and a Flame Ionization Detector (FID). The methyl ester separation will be carried out on capillary column (25 m × 0.25 mm i.d., 0.25 µm of phase; Supelco Inc., Bellefonte, PA) using nitrogen as the carrier gas (0.5 ml/ min flow rate). The injector temperature will be set at 220°C, while detector temperature will be set at 260°C. The injection will be done in split mode with 1:100 split-ratio. The temperature of the column will be initially at 140°C for 5 min, and then increased to 240°C at 4°C/min.

The results will be analyzed by GC software. Each fatty acid will be identified with reference to the retention time of the standards (Sigma-Aldrich, St. Louis, MO) and quantified with respect to the following internal standards: C5:0 (C4:0–C8:0), C13:0 (C10:0–C17:0), and C19:0 (C18:0–C18:3). The concentration of each internal standard added to the sample will be 170 mg/g of fat.

8 CRITICAL POINTS OR NOTE ON THE PROCEDURE

1. **Caution.** Petroleum ether, hexane, and heptane are extremely flammable. Use effective fume-removal device. Avoid static electricity.
2. The required reaction time appears to increase as both the carbon number and degree of unsaturation increase. For longer-chain and more-unsaturated fatty acids, a general observation is that the reaction should be allowed to proceed just until there is no visual evidence of oil globules in the reaction mixture.
3. It is recommended that all methyl esters be washed with water (to the disappearance of a methyl red end point) before injection. An alternative is to partition the hexane-methyl ester phase against water containing a small amount of mixed-bed (indicating resin).

Fatty acids profile by Gas chromatography

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4. There is danger of losing some of the more volatile esters if the solvent-removal step is prolonged, or if too vigorous a stream of nitrogen is used. For infrared spectroscopy, this step should be terminated as soon as all solvent is removed. For gas-liquid chromatography, the method may be extended to fatty acids with eight carbon atoms, if the solvent is not completely removed.

5. The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screw-cap vial at 2° for 24 hr. For longer storage, they should be sealed in a glass ampule, subjected first to a vacuum, and then backfilled with nitrogen and stored at -20° (in a freezer).

6. The methyl esters should be analysed by GC. The injector temperature should be set at 220°C and in split mode (split ratio:10:1). The column should be initially maintained at 140°C for 5 min, and the temperature should be subsequently increased to 240°C at a rate of 4°C/min (total program time 30 min).

9 TEST REPORT

| C-composition | Sample 1 Area % | Sample 2 Area % |
|---------------|--------------------|--------------------|
| C4:0 | | |
| C6:0 | | |
| C8:0 | | |
| C10:0 | | |
| C12:0 | | |
| C13:0 | | |
| C14:0 | | |
| C14:1n9c | | |
| C15:0 | | |
| C16:0 | | |
| C16:1n9c | | |
| C17:0 | | |
| C18:0 | | |
| C18:1n9t | | |
| C18:1n9c | | |
| C18:2n6c | | |
| C20:0 | | |
| C18:3n3 | | |
| C20:1 | | |
| C22:0 | | |
| C22:1n9 | | |
| Sum | | |

Fatty acids profile by Gas chromatography

SOP: **Chem-Cere-029-en**

Date: **10/10/2011**

Release: **1**

10 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
| | | |
| | | |
| | | |

11 APPENDIX

Preparation of methyl esters and long-chain fatty acids. AACC International Method 58-17.01

Preparation of Methyl Esters of Long-Chain Fatty Acids

Final approval October 24, 1974; Reapproval November 3, 1999

Objective

This method provides means for preparing methyl esters of long-chain fatty acids for further analysis by methods such as gas-liquid chromatography (**Method 58-18.01**). It is applicable to common fats, oils, and fatty acids. See Note 1. Unsaponifiables are not removed and, if present in large amounts, may interfere with subsequent analyses. Procedure results in partial or complete destruction of epoxy, hydroperoxy, cyclopropenyl, cyclopropyl, and possibly hydroxyl groups, and is not suitable for preparation of methyl esters of fatty acids containing these groups. This method is equivalent to Official Methods Ce 1-62 and Ce 2-66 of the American Oil Chemists' Society (AOCS).

Apparatus

1. Flat-bottom boiling flasks or Erlenmeyer flasks with ST 19/38 or 24/40 outer necks, 50- and 125-ml.
2. Water-cooled condensers, with ST 19/38 or 24/40 interjoint.
3. Separatory funnels, 250-ml.
4. Boiling flask for solvent removal, 200-ml.
5. Boiling chips, free of fat.

Reagents

1. Boron trifluoride (BF₃)-Methanol reagent (125 g/liter of methanol). Available commercially or may be prepared using gas and methanol (see section 3(d) of Ref. 2, or Ref. 4).
2. NaOH, 0.5N in methanol.
3. NaCl, saturated solution in water.
4. Petroleum ether, redistilled, boiling point 30–60°. *Caution.* See Note 1.
5. Heptane, gas-chromatographically clean.
6. Na₂SO₄, anhydrous, analytical reagent, ACS grade.
7. Methyl red indicator, 0.1% in 60% ethanol.
8. Nitrogen gas, high purity.

Procedure

Accurate weighing is not required. Sample size need be known only to determine size of flask and amounts of reagents that should be used according to Table I.

1. *For fatty acids.* Introduce fatty acids into 50- or 125-ml reaction flask. Add specified amount of BF₃-methanol reagent, attach a condenser, and boil 2 min. See Note 2. Add 2–5 ml heptane through condenser and boil 1 min longer. Remove from heat, remove condenser, and add enough saturated salt solution to

**Preparation of Methyl Esters of Long-Chain Fatty Acids
(continued)****TABLE I**
Flasks and Reagents for Sample Size Ranges

| Sample (mg) | Flask (ml) | NaOH, 0.5N (ml) | BF ₃ -Methanol Reagent (ml) |
|----------------|---------------|--------------------|--|
| 100–250 | 50 | 4 | 5 |
| 250–500 | 50 | 6 | 7 |
| 500–750 | 125 | 8 | 9 |
| 750–1000 | 125 | 10 | 12 |

float heptane solution of methyl esters into neck of flask. Transfer about 1 ml heptane solution into test tube and add small amount of anhydrous Na₂SO₄. Dry heptane solution may then be injected directly in gas chromatograph. See Note 3. To recover dry esters, transfer solution and heptane phase to 250-ml separatory funnel. Extract twice with 50-ml portions redistilled petroleum (boiling point 30–60°). Wash combined extracts with 20-ml portions water until free of acids (test water with methyl red indicator), dry with Na₂SO₄, and evaporate solvent under stream of nitrogen on steam bath. See Notes 4 and 5.

2. *For fats and oils.* Introduce fat into 50- or 125-ml reaction flask. Add specified amount of 0.5N methanolic NaOH and add boiling chip. Attach condenser, and heat mixture on steam bath until fat globules go into solution. This step should take 5–10 min. Add specified amount of BF₃-methanol reagent through condenser and proceed as described under fatty acid section.

Notes

1. *Caution.* Petroleum ether, hexane, and heptane are extremely flammable. Use effective fume-removal device. Avoid static electricity.

2. The required reaction time appears to increase as both the carbon number and degree of unsaturation increase. For longer-chain and more-unsaturated fatty acids, a general observation is that the reaction should be allowed to proceed just until there is no visual evidence of oil globules in the reaction mixture.

3. It is recommended that all methyl esters be washed with water (to the disappearance of a methyl red end point) before injection. An alternative is to partition the hexane-methyl ester phase against water containing a small amount of mixed-bed (indicating resin).



4. There is danger of losing some of the more volatile esters if the solvent-removal step is prolonged, or if too vigorous a stream of nitrogen is used. For infrared spectroscopy, this step should be terminated as soon as all solvent is removed. For gas-liquid chromatography, the method may be extended to fatty acids with eight carbon atoms, if the solvent is not completely removed.

Preparation of Methyl Esters of Long-Chain Fatty Acids (continued)

5. The methyl esters should be analyzed as soon as possible. They may be kept in an atmosphere of nitrogen in a screw-cap vial at 2° for 24 hr. For longer storage, they should be sealed in a glass ampule, subjected first to a vacuum, and then backfilled with nitrogen and stored at -20° (in a freezer).

References

1. American Oil Chemists' Society. 1998. Official Methods and Recommended Practices, 5th ed. Methods Ce 1-62; Ce 2-66. The Society, Champaign, IL.
2. American Society for Testing and Materials. 1971. Method D-1983-64T.
3. AOAC International. 1997. Official Methods of Analysis of AOAC International, 16th ed., 3rd rev. Method 969.33. The Association, Gaithersburg, MD.
4. Wiener, N. D., and Felmeister, A. 1966. Thermometric titration of cetylpyridinium chloride. Anal. Chem. 38:514.

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|--|--|---|
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Détermination de l'acidité grasse dans les produits céréaliers | | |
| SOP : Chem-Cere-16-fr | | |
| Date : 24/08/2011 | Version: 1 | |
| <p> Ecrit par : Christian MESTRES Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • • </p> | | |
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| FAAU | Sameh AWAD | 06/06/2011 |
| ESB | | |
| FRI | Wis AMOA | 17/06/2011 |
| NRC | | |

Détermination de l'acidité grasse dans les produits céréaliers

SOP : Chem-Cere-16-fr

Date: 24/08/2011

Version : 1

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Détermination de l'acidité grasse dans les produits céréaliers

SOP : Chem-Cere-16-fr

Date: 24/08/2011

Version : 1

1 DOMAINE ET APPLICATION

La présente norme spécifie une méthode de détermination de l'acidité dite «grasse» dans les produits de mouture des céréales.

2 REFERENCES

Cette procédure reprend la norme internationale (ISO 7305:1998(F)) ; seuls le principe (4) et les points critiques (9) seront détaillés dans ce formulaire.

3 DEFINITIONS

4 PRINCIPE

Mise en solution des acides dans l'éthanol à la température du laboratoire, suivie de centrifugation et titrage d'une partie aliquote du surnageant par l'hydroxyde de sodium.

5 REACTIFS

6 APPAREILLAGE

7 PROCÉDURE

8 EXPRESSION DES RÉSULTATS

Détermination de l'acidité grasse dans les produits céréaliers

SOP : Chem-Cere-16-fr

Date: 24/08/2011

Version : 1

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- ✓ Vérifier le titre de la solution de soude immédiatement avant chaque série de déterminations de l'acidité grasse.
- ✓ La granulométrie de l'échantillon doit être inférieure à 1 mm ; sinon, un broyage préalable est nécessaire

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.



Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------------|----------------------|--------------------------------|
| 24/08/2011 | Noël AKISSOE | Granulométrie des farines |
| | | |
| | | |

12 ANNEXE

Le texte de la norme ISO 7305:1998 est annexé au document

| | | |
|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Determination of fat acidity in cereal products | | |
| SOP: Chem-Cere-16-en | | |
| Date: 24/08/2011 | Release: 1 | |
| <p>Written by : Christian MESTRES</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • | | |
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| UAC | Noël AKISSOE | 24/08/2011 |
| CSIR | | |
| FAAU | Sameh AWAD | 06/06/2011 |
| ESB | | |
| FRI | Wis AMOA | 17/06/2011 |
| NRC | | |

Determination of fat acidity in cereal products

SOP Number: **Chem-Cere-16-en**

Date: 24/08/2011

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Determination of fat acidity in cereal products

SOP Number: **Chem-Cere-16-en**

Date: **24/08/2011**

Release: **1**

1 SCOPE AND APPLICATION

This standard procedure specifies a method for the determination of fat acidity in milling of cereals products.

2 REFERENCES

This procedure is based on the international standard ISO 7305: 1998 procedure; only principle (4) and (9) critical points will be detailed in this form.

3 DEFINITIONS

4 PRINCIPLE

Dissolution of the acids in ethanol at room temperature, followed by centrifuging and titration of an aliquot portion of the supernatant liquid against sodium hydroxide

5 REAGENTS

6 APPARATUS

7 PROCEDURE

8 EXPRESSION OF RESULTS

Determination of fat acidity in cereal products

SOP Number: **Chem-Cere-16-en**

Date: **24/08/2011**

Release: **1**

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- ✓ The exact concentration shall be known and checked immediately prior to each series of determinations of fat acidity.
- ✓ The sample size must be less than 1 mm; otherwise, a preliminary grinding is required

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------------|--------------------|-----------------------|
| 24/08/2011 | Noël AKISSOE | Sample granulometry |
| | | |
| | | |

12 APPENDIX

Text of ISO 7305:1988 will be annexed to the document



**African Food Tradition rEvisited by
Research
FP7 n°245025**



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

Determination of Total Ash in Fermented Cereal Foods

SOP: CHEM-CERE-17-EN

Date : **05/10/2011**

Release : **1**

Written by : Hayford Ofori & Wisdom Amoa-Awua

For information on this SOP please contact :

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Determination of Total Ash in Fermented Cereal Foods

SOP: CHEM-CERE-17-EN

Date : 05/10/2011

Release: 1

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Determination of Total Ash in Fermented Cereal Foods

SOP: CHEM-CERE-17-EN

Date : 05/10/2011

Release: 1

1 SCOPE AND APPLICATION

This document specifies a routine reference method for the determination of total ash in cereals and cereal products.

2 REFERENCES

Official Methods of Analysis of AOAC International, Ed.18, 2005, (USA) JAOAC 7,132(1923)

3 DEFINITIONS

Total ash here refers to the inorganic residue remaining after the organic matter has been burnt in the fermented cereal foods. The ash obtained may not be necessarily the same composition of the mineral matter present in the original food as there may be losses due to volatilisation or some interactions between constituents (Ronald S. Kirk, and Ronald Sawyer, "Pearson's composition and analysis of food," Longman Singapore, Ed 9,1991.)

4 PRINCIPLE

This method is based on vaporisation of water and volatiles and incineration of organic substances in the presence of oxygen in air and CO₂ and oxides of N₂. A muffle furnace capable of maintaining temperatures of 500-600 °C is used. Most minerals are converted to oxides, sulphates phosphates, chlorides and silicates while elements such as Fe, Se, Pb and Hg may partially volatilize with this procedure.

5 REAGENTS

Silica gel or reignited CaO as drying agent for the desiccators.

6 APPARATUS

- Platinum, Porcelaine, Ceramic or Alumina Crucible
- Muffle Furnace
- Analytical balance (0.0001g)
- Desiccators

Determination of Total Ash in Fermented Cereal Foods

SOP: CHEM-CERE-17-EN

Date : 05/10/2011

Release: 1

7 PROCEDURE

Weigh 3-5g well-mixed test portion into a shallow, relatively broad crucible that has been ignited, cooled in desiccator, and weighed soon after reaching room temperature. Ignite in furnace at 550°C (dull red) until light gray ash results, or to constant weight. Cool in desiccator and weigh soon after reaching room temperature. Reignited CaO is satisfactory drying agent for desiccators.

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Calculate the percentage total ash as:

$$\% \text{ total ash (wb)} = \frac{\text{weight of ash}}{\text{weight of original food}} * 100$$

$$\% \text{ total ash (wb)} = \frac{W_3 - W_1}{W_2 - W_1} * 100$$

Where W_1 = weight of empty crucible, W_2 = weight of crucible + food before ashing and W_3 = weight of crucible + ash.

Total ash can also be calculated on dry basis:

$$\% \text{ total ash (db)} = \frac{\% \text{ total ash (wb)}}{\% \text{ dry matter of original food}} * 100$$

8.1 Repeatability

The analyses is perform in duplicate with standard deviation of +/- 0.2%

Ash value is reported to the nearest 0.1 %

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Do not open the furnace during ashing.
- Care must be taken when moving crucibles containing fluffy ashes, which turn to blow away easily. Such ashes should be covered with Petri dish or watch glass after placing in the desiccator.

Determination of Total Ash in Fermented Cereal Foods

SOP: CHEM-CERE-17-EN

Date : **05/10/2011**

Release: **1**

- Retain the ash where you are to determine the individual minerals.

10 TEST REPORT

The test report shall indicate the method used and the results obtained.

The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
| | | |
| | | |
| | | |

12 APPENDIX

Official Methods of Analysis of AOAC International, Ed.18, 2005, (USA) JAOAC 7,132(1923)

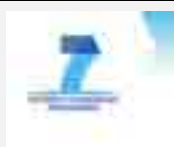
32.1.05

AOAC Official Method 923.03 Ash of Flour

Direct Method
First Action 1923
Final Action

Weigh 3–5 g well-mixed test portion into shallow, relatively broad ashing dish that has been ignited, cooled in desiccator, and weighed soon after reaching room temperature. Ignite in furnace at ca 550°C (dull red) until light gray ash results, or to constant weight. Cool in desiccator and weigh soon after reaching room temperature. Re ignited CaO is satisfactory drying agent for desiccator.

Reference: *AOAC* 7, 132(1923).



African Food Tradition rEvisited by Research

FP7 n°245025



Deliverable 1.2.3.8 Chemical analysis for group 1

Détermination du pH et de l'acidité titrable dans les produits céréaliers fermentés

SOP : Chem-Cere-009-fr

Date : **24/05/2011**

Version : **2**

Ecrit par : Noël AKISSOE

Pour plus d'information sur ce SOP, contactez :

- Noël AKISSOE (noelyolande@yahoo.fr)
-
-
-

Ce document a été approuvé par :

| Partenaire | Noms des personnes l'ayant approuvé | Date DD/MM/YY |
|------------|-------------------------------------|------------------|
| CIRAD | Christian MESTRES | 01/12/2010 |
| UAC | Noël AKISSOE | 16/05/2011 |
| CSIR | | |
| FAAU | | |
| ESB | | |
| FRI | | |
| NRC | | |

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Détermination du pH et de l'acidité titrable dans les produits céréaliers fermentés

SOP : Chem-Cere-009-fr

Date : 24/05/2011

Version : 2

1 DOMAINE ET APPLICATION

Le présent document décrit une méthode de référence pour la détermination du pH et acidité titrable applicable pour les pâtes fermentées, semoules ou farines de céréales après dispersion dans l'eau. Toutefois, elle peut être utilisée pour les surnageants de fermentation et dans les boissons.

2 REFERENCES

Cette procédure est une modification de la norme AACC 02-31.01 (Annexe, paragraphe 12).

3 DEFINITIONS

Le pH est l'activité des ions hydrogène, encore appelé acidité libre.

L'acidité titrable est l'acidité totale (acidité libre et liée) du produit.

4 PRINCIPE

Le pH est directement mesuré à l'électrode sur une suspension homogénéisée pendant 2 min. Cette détermination est basée sur la mesure de la différence de potentiel d'une cellule électrochimique.

L'acidité titrable est déterminée par neutralisation au moyen de la soude (0.1N) de toute l'acidité du produit jusqu'au virage complet (mesuré au pH 8.5), maintenu pendant 10 minutes.

5 REACTIFS

Solution standard de soude 0,1 N fraîchement ouverte. Si la solution n'est pas fraîchement ouverte le jour du dosage, réaliser une mesure de sa normalité à l'aide d'une solution d'acide sulfurique 0,1 N standard (virage à pH 5,0).

6 APPAREILLAGE

Le pH-mètre est préalablement étalonné avec les solutions tampon de pH 4 et pH 7 préalablement équilibrées à température ambiante.

Détermination du pH et de l'acidité titrable dans les produits céréaliers fermentés

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Burette avec graduations de 0,1 mL, dispenseur (graduations 0,02 mL) ou titrateur automatique.

7 PROCEDURE

- Disperser sous agitation magnétique 10 g de pâte ou de farine dans 20 mL d'eau pure. Homogénéiser pendant 2 min.
- Plonger l'électrode du pH-mètre dans la solution. Attendre 1 min et lire la valeur du pH.
- Compléter ensuite avec 70 mL d'eau pure.
- Ajouter, sous agitation magnétique, de la soude (0.1N) jusqu'au pH 8.5
- Attendre 5 min et continuer la neutralisation en ramenant au pH8.5.
- Attendre de nouveau 5 min et ajuster au pH 8.5. Lire la quantité finale (mL) de soude utilisée.

8 EXPRESSION DES RESULTATS

8.1 Mode de calcul et formules

L'acidité titrable est exprimée en équivalent d'acide lactique (% base sèche) :

$$\% \text{ ac. Lact} = V \times 9 \times N / (M \times ms)$$

Où :

V = volume de titrant (mL)

N = normalité de la soude

M = masse d'échantillon (g)

ms = matière sèche de l'échantillon (% base humide)

8.2 Répétabilité

Pour le pH, la différence absolue entre deux répétitions réalisées dans un court intervalle de temps ne dépassera **0.3**.

Pour l'acidité titrable, l'écart type entre des répétitions réalisées dans un court intervalle de temps ne dépassera pas **2%** de la moyenne (coefficient de variation)

Détermination du pH et de l'acidité titrable dans les produits céréaliers fermentés

SOP : Chem-Cere-009-fr

Date : 24/05/2011

Version : 2

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- Toujours mesurer la normalité de la soude si celle-ci n'est pas fraîchement ouverte.
- S'assurer de la bonne dispersion de l'échantillon avant la prise du pH,
- lorsqu'il s'agit d'un échantillon liquide, ne pas ajouter d'eau
- Respecter la durée d'attente

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------------|----------------------|--------------------------------|
| 16/05/2011 | Noël AKISSOE | Répétabilité |
| 01/12/2011 | Christian MESTRES | Edition |
| | | |

12 ANNEXE

Norme AACC 02-31.01

TITRATABLE ACIDITY

Final approval 4-13-81; reviewed 10-27-82

Scope

Applicable to NFDM.

Apparatus

1. Buret graduated in 0.1-ml divisions.
2. Casserole, white glazed porcelain, 50-ml capacity.
3. Pipet, vol. 17.6-ml.

Reagents

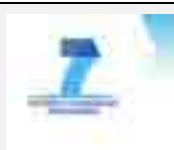
1. Phenolphthalein indicator, 1%. Prep by dissolving 1 g phenolphthalein in 50 ml 95% ethyl alc and dilg to 100 ml with water.
2. NaOH, 0.1N soln (Method 70-70).

Procedure

1. Dissolve and disperse 10 g of sample in 100 ml water and mix thoroly. Let stand approx 1 hr. Stir gently and then pipet 17.6 ml into porcelain casserole. Rinse out same pipet with 17.6 ml water and add this to sample in casserole.
2. Add 0.5 ml phenolphthalein indicator and titrate with stdzd 0.1N NaOH until faint pink color persists for 30 sec.

CalculationTitratable acidity (in terms of lactic acid) = ml required for titration \div 20.**References**

1. American Dry Milk Institute. 1948. Standard Methods of Analysis. Bull. 918.
2. Association of Official Analytical Chemists. 1980. Official Methods of Analysis, 13th ed. Sec. 16.023.



African Food Tradition rEvisited by Research
FP7 n°245025



Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1

**Détermination des acides organiques et sucres solubles dans les produits
céréaliers fermentés par HPLC avec une colonne type Aminex**

SOP Number: **Chem-Cere-002-fr**

Date : **23/05/2011**

Version : **3**

Ecrit par : Laetitia MESTRES

Pour plus d'information sur ce SOP, contactez :

- Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader
-
-

Ce document a été approuvé par :

| Partenaire | Noms des personnes l'ayant approuvé | Date DD/MM/YY |
|------------|-------------------------------------|------------------|
| CIRAD | Christian MESTRES | 23/05/2011 |
| UAC | Noël AKISSOE | 17/05/2011 |
| CSIR | | |
| FAAU | | |
| ESB | | |
| FRI | Wis AMOA | 13/03/2011 |
| NRC | | |

Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

SOP Number: **Chem-Cere-002-fr**

Date : 23/05/2011

Version : 3

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Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

SOP Number: **Chem-Cere-002-fr**

Date : **23/05/2011**

Version : **3**

1 DOMAINE ET APPLICATION

La méthode décrite permet l'extraction à température ambiante et le dosage des acides organiques et des sucres dans les végétaux, en particulier les produits fermentés préalablement lyophilisés, séchés ou ne contenant que leur humidité résiduelle par chromatographie haute performance. Elle permet aussi d'évaluer le taux d'alcool (éthanol et méthanol)

2 REFERENCES

MESTRES, C ROUAU, X. **1997**. Influence of natural fermentation and drying conditions on the physicochemical characteristics of cassava starch. *Journal of the science of food and agriculture* **74**, pp. 147-155.

3 DEFINITIONS

Un acide organique est un composé organique avec des propriétés acides : les acides organiques les plus courants sont les acides carboxyliques, dont l'acidité est liée à un groupement carboxyl $-COOH$. Les acides organiques rencontrés dans les produits céréaliers fermentés sont en premiers lieux issus des processus fermentaires (acide lactique, acétique, propionique, butyrique, valérique), mais aussi certains acides issus de la physiologie des plantes (oxalique, citrique etc)

Les glucides, ou hydrates de carbone, sont des molécules organiques contenant un groupement carbonyle (aldéhyde ou cétone) et plusieurs groupements hydroxyle. Les sucres solubles, ou glucides solubles, des produits céréaliers fermentés sont essentiellement constitués de d'oses (monosaccharides : glucose, fructose en particulier), certains di-holosides (saccharose et maltose) et le maltotriose.

4 PRINCIPE

Les sucres solubles et les acides organiques sont extraits à température ambiante dans un milieu aqueux dans des conditions inhibant les enzymes et limitant leur dégradation chimique ($pH < 3$). Ils sont ensuite séparés par HPLC sur colonne d'exclusion d'ionique (exemple : Aminex HPX87H-Biorad, Hercules, USA) **thermostatée à 37°C**. Ils sont élués avec de l'acide sulfurique 5mM à un débit de 0.6 ml/min. Les sucres solubles sont quantifiés par réfractométrie et les acides organiques sont détectés par spectrophotométrie UV à 210 nm. Les différents composés sont identifiés par leur temps de rétention (par comparaison à des standards). La double détection permet de valider l'identification des composés élués : un sucre ne présentera qu'un pic en réfractométrie, le rapport relatif des aires des pics entre

Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

SOP Number: **Chem-Cere-002-fr**

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réfractométrie et spectrophotométrie UV à 210 nm est connu pour chacun des acides organiques. Les résultats sont exprimés en mg/g, base sèche.

5 REACTIFS

- Acide sulfurique 0,5 M
- Eau ultrapure ou bi-distillée puis filtrée sur filtre 0.45 µm (**préparée le jour même**)
- Eluant acide sulfurique 5 mM : diluer 100 fois l'acide sulfurique 0.5 M avec de l'eau ultrapure. A préparer le jour même : **se conserve au maximum 48 H.**
- Les standards des sucres, acides organiques et alcool les plus couramment rencontrés sont présentés dans le tableau 1. Cette liste doit être adaptée selon la nature du produit analysé.

Tableau 1 : Standards de sucres, acides organiques et alcool

| | |
|-------------------|--|
| sucres | maltose saccharose glucose fructose |
| acides organiques | acide oxalique acide citrique acide lactique acide acétique |
| Alcool | éthanol |

6 APPAREILLAGE

- Système HPLC constitué d'un dégazeur en ligne, d'une pompe, d'un injecteur, d'un four de colonne et de deux détecteurs en série : spectrophotomètre ultra-violet, puis réfractomètre différentiel.
- Colonne exclusion d'ions pour analyse d'acides organiques et sucres : par exemple, Aminex HPX-87H colonne, 300 mm x 7,8 mm (Biorad, Hercules, USA).
- Pré-colonne de même nature .
- Boucle d'injection de 20 µL
- Tubes d'extraction/centrifugation à bouchon « caps lock » jetables de 2 mL (Ependorfou équivalent)

Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

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Date : **23/05/2011**

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- Agitateur rotatif (type agitest 34050, Bioblock)
- Centrifugeuse (type Genofuge, 24 D Legallais)
- Filtres seringues (pores de 0,45 µm)
- Seringues et vials de 1 mL
- Balance sensible au 0,1 mg
- Agitateur vortex

7 PROCEDURE

7.1 Extraction

- Préparer la solution d'extraction et d'élution (H₂SO₄, 5 mM)
- Peser 25 mg de poudre d'échantillon directement dans un tube 2 mL (selon la richesse en sucres et acides organiques du produit, cette quantité pourra être modifiée)
- Y ajouter 1 mL de la solution d'extraction et agiter vigoureusement à l'aide du Vortex
- Placer les tubes d'extraction sur l'agitateur rotatif et laisser extraire **pendant 1 h à température ambiante**
- Centrifuger le mélange à 10 000 g pendant 5 min
- Prélever environ 0,5 mL de surnageant avec une seringue et filtrer avec le filtre seringue (0,45µm) ; le filtrat (l'extrait) est recueilli directement dans un vial de 1 ml.
- Selon la concentration en sucres et acides organiques du matériel analysé, l'extrait peut être dilué (avec le solvant d'extraction/élution) avant analyse.

7.2 Réalisation de la solution des standards de sucres et acides organiques

En fonction des sucres et acides organiques attendus, il faut réaliser des injections des solutions de standards à des concentrations connues (en général entre 1 et 10 mg/ml). Ci-dessous (tableau 2) un exemple de sucres et d'acides organiques avec l'ordre d'élution et les coefficients de réponse pour les deux types de détecteurs. Plusieurs standards peuvent être injectés en mélange dès lors que leur temps de rétention diffère de 2 minutes environ. A noter que le méthanol et l'éthanol peuvent aussi être dosés par cette méthode.

Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

SOP Number: **Chem-Cere-002-fr**

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Tableau 2 : Ordre d'élution des anions et des sucres avec un ordre de grandeur des coefficients de réponse.

| Composés | | TR (min) | coefficient réponse [surface /concentration mg mL ⁻¹] | |
|-------------------|-------------|----------|--|-----------|
| | | | Réfractométrie | UV 210 nm |
| sucres | maltose | 8 | 23,2 | 0 |
| | saccharose | 8,2 | 24,4 | 0 |
| | glucose | 9,6 | 24,7 | 0 |
| | fructose | 10,7 | 24,8 | |
| Acides organiques | oxalic acid | 6,6 | | 414 |
| | citric acid | 8,4 | 21,6 | 35,3 |
| | lactic acid | 13,5 | 16,8 | 24,6 |
| | acetic acid | 15,4 | 11,3 | 17,1 |
| alcools | méthanol | | XX | 0 |
| | éthanol | 22,2 | 9,9 | 0 |

7.3 Conditions chromatographiques

Phase mobile : Acid Sulfurique 5mM

Flux : 0.6 mL/min

Volume d'injection : 20 µL

Température d'élution: 35°C

Longueur d'onde : 210nm

8 EXPRESSION DES RESULTATS

8.1 Mode de calcul et formules

Identifier chaque composé sur la base de son temps de rétention, par comparaison à celui obtenu pour le standard ; ce temps ne doit pas différer de 10% pour une identification fiable. Par ailleurs, cette identification peut être confirmée en observant les profils sur les deux voies de détection (UV210 nm et réfractométrie) ; un sucre ou un alcool détecté en réfractométrie ne devra pas présenter de pic sur la voie UV210 nm au même temps de rétention.

La teneur en un composé (mg/g de l'échantillon sec) = (SRe * F * 100000) / (CRs * PE * MS)

Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

SOP Number: **Chem-Cere-002-fr**

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Où

- SRe est la surface de réponse du sucre dans l'échantillon
- F est le facteur de dilution
- CRs est le coefficient de réponse du standard : surface du pic / concentration (mg/ml)
- PE prise d'essai en mg
- MS est le pourcentage de matière sèche, exprimé en base humide

Dans le cas des acides organiques, calculer la valeur moyenne des teneurs évaluées par les deux voies de détection (UV210 nm et réfractométrie). Si la valeur calculée par l'absorption à 210 nm est beaucoup plus élevée (**plus du double**) que celle calculée en réfractométrie, cela signifie qu'un autre composé absorbant fortement en UV masque l'acide organique ; celui-ci doit être considéré comme **absent**.

8.2 Répétabilité

L'écart type entre des répétitions réalisées dans un court intervalle de temps ne dépassera pas 5% de la moyenne (coefficient de variation)

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- ✓ Lors de la mise en route du système HPLC, le temps de stabilisation du réfractomètre est long (en général de plusieurs heures) ; il faut donc laisser le système **en élution** plusieurs heures avant de démarrer une séquence d'analyse, et rincer la cellule de référence du réfractomètre une à deux fois par jour (entre deux analyses) avec de l'éluant acide sulfurique 5mM. Attendre environ ¼ d'heure pour que la ligne de base se stabilise. Faire le zéro du réfractomètre.
- ✓ Bien vérifier l'identification des composés par l'observation conjointe des deux voies de détection (cf 8.1)

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

Détermination des acides organiques et sucres solubles dans les produits céréaliers fermentés par HPLC avec une colonne type Aminex

SOP Number: **Chem-Cere-002-fr**



Date : **23/05/2011**

Version : **3**

11 ENREGISTREMENT DES REVISIONS

| Date | Responsible person | Description of change |
|------------|--------------------|---|
| 13/03/2011 | Wis AMOA | Edition |
| 17/05/2011 | Noël AKISSOE | Edition |
| 23/05/2011 | Christian MESTRES | Edition, conformité avec version anglaise |

12 ANNEXE

| | | |
|---|--|---|
|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Determination of Organic Acids and Sugars in Fermented Cereal Foods: by HPLC using Aminex type column | | |
| SOP: Chem-Cere-002-en | | |
| Date: 23/05/2011 | Version: 3 | |
| <p>Written by : Laetitia MESTRES</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... | | |
| Approved by : | | |
| Partner | Name of the person who approved | Date DD/MM/YY |
| CIRAD | Christian MESTRES | 23/05/2011 |
| UAC | Noël AKISSOE | 17/05/2011 |
| CSIR | | |
| FAAU | | |
| ESB | | |
| FRI | Wis AMOA | 13/03/2011 |
| NRC | | |

**Determination of Organic Acids and Sugars in Fermented Cereal Foods:
by HPLC using Aminex type column
SOP: Chem-Cere-002-en**

Date: **23/05/2011**

Version: **3**

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Determination of Organic Acids and Sugars in Fermented Cereal Foods: by HPLC using Aminex type column SOP: Chem-Cere-002-en

Date: 23/05/2011

Version: 3

1 SCOPE AND APPLICATION

The procedure describes the extraction (at ambient temperature) and assessment of organic acids and sugars from plant by HPLC, in particular in fermented cereal products that have been previously freeze-dried, dried or only containing residual water. Alcohol and methanol contents can also be assessed with this procedure.

2 REFERENCES

MESTRES, C ROUAU, X.1997. Influence of natural fermentation and drying conditions on the physicochemical characteristics of cassava starch. *Journal of the science of food and agriculture* 74, pp. 147-155.

3 DÉFINITIONS

An **organic acid** is an organic compound with acidic properties. The most common organic acids are the carboxylic acids, whose acidity is associated with their carboxyl group –COOH. Organic acids generally encountered in fermented cereal foods are first those originating from fermentative processes (lactic, acetic, propionic, butyric, valeric), and also several from plant physiology (oxalic, citric etc)

Sugar is a term for a class of edible crystalline carbohydrates (with the empirical formula $C_m(H_2O)_n$) characterized by a sweet flavor. The procedure can be used to assess simple sugars (glucose, fructose for example), and several di-holosides (sucrose and maltose) and maltotriose, generally encountered in fermented cereal foods.

4 PRINCIPLE

Soluble sugars and organic acids are extracted at ambient temperature in aqueous medium in conditions inhibiting enzymic activities and limiting chemical degradation ($pH < 3$). Extracted metabolites are then separated by HPLC on ionic and exclusion column (for example: Aminex HPX87H-Biorad, Hercules, USA) **thermostated at 35°C**. They are eluted with 5mM sulfuric acid at 0.6 ml/min. Sugars are assessed by refractometry and organic acids by spectrophotometry at 210 nm. The different compounds are identified by their retention time (by comparison with standards). The double detection allows some validation of the identification: a sugar will only be detected by refractometry and the ratio between refractometric and spectrophotometric responses is known for each organic acid. Results are calculated in mg/g, dry basis.

**Determination of Organic Acids and Sugars in Fermented Cereal Foods:
by HPLC using Aminex type column
SOP: Chem-Cere-002-en**

Date: **23/05/2011**

Version: **3**

5 REAGENTS

- 0.5 mM sulfuric acid
- Ultrapure or bi-distilled water, filtrated through 0.45 µm (**freshly prepared**)
- Eluent: **5 mM sulfuric acid**. Dilute time 100 time 0.5 M sulfuric acid with ultrapure water. To be prepared just before use: **keep for a maximum of 48 H**.
- Standards of the sugars, organic acids and alcohol most commonly encountered are presented in table 1. This list should be adapted depending on the nature of the product to be analysed.

Table 1: Standards of sugars, organic acids and alcohol

| | |
|---------------|---|
| sugars | maltose sucrose glucose fructose |
| organic acids | oxalic citric lactic acetic |
| Alcohol | ethanol |

6 APPARATUS

- HPLC system consisting of a degasser, a pump, injector, a column and two detectors: ultraviolet spectrophotometer, and refractometer.
- Column exclusion of ions for analysis of sugars and organic acids: for example, Aminex HPX - 87 H column, 300 mm x 7.8 mm (Biorad, Hercules, USA).
- Guard-Column of the same nature.
- 20 µL injection loop
- 2 mL extraction/centrifugation tubes with « caps lock » caps (Ependorf for example)
- Rotary shaker (type agitest 34050, Bioblock)
- Centrifuge (type Genofuge, 24 D Legallais)
- filter syringes (pore size 0,45 µm)
- 1 mL Syringes and vials
- Balance (sensitivity : 0.1 mg)
- Vortex agitator

**Determination of Organic Acids and Sugars in Fermented Cereal Foods:
by HPLC using Aminex type column
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7 PROCEDURE

7.1 Extraction

- Prepare extraction and eluting solution (5 mM H₂SO₄.)
- Weigh 25 mg of powdery sample directly into a 2 mL tube (depending on the levels of organic acids and sugars in the product, this amount may be modified)
- Add 1 mL of extraction solution and agitate vigorously by placing on Vortex agitator
- Put extract on the Rotary Shaker for **1 h at room temperature**
- Centrifuge at 10 000 x g for 5 min
- Filter 0.5 ml of supernatant with filter syringe (0.45µm); filtrate (extract) directly into a 1 ml vial.
- Depending on the concentration of sugars and organic acids of the analyzed material, the filtrate can be diluted (with the extraction solvent) prior to analysis.

7.2 Solution of the standards of sugars and organic acids

Depending on the expected sugars and organic acids, injections of standard solutions (generally between 1 and 10 mg/ml) known should be performed. An example of sugars and organic acids with the elution order and coefficients of response for both types of detectors is given in Table 2. Several standards can be injected together once their retention time differs from 2 minutes approximately. Note that methanol and ethanol can be determined by this procedure.

Table 2: Order of Elution of anions and sugars with an order of magnitude of response factors.

| Compound | | RT (min) | Response coefficient [surface /concentration mg mL ⁻¹] | |
|---------------|-------------|----------|---|-----------|
| | | | Réfractometry | UV 210 nm |
| sugars | maltose | 8 | 23.2 | 0 |
| | saccharose | 8.2 | 24.4 | 0 |
| | glucose | 9.6 | 24.7 | 0 |
| | fructose | 10.7 | 24.8 | |
| organic acids | oxalic acid | 6.6 | | 414 |
| | citric acid | 8.4 | 21.6 | 35.3 |
| | lactic acid | 13.5 | 16.8 | 24.6 |
| | acetic acid | 15.4 | 11.3 | 17.1 |
| alcools | methanol | | XX | 0 |
| | éthanol | 22.2 | 9.9 | 0 |

**Determination of Organic Acids and Sugars in Fermented Cereal Foods:
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7.3 Chromatographic conditions

Mobile phase : 5mM Sulfuric acid

Flow rate : 0.6 mL/min

Injection volume : 20 μ L

Column oven : 35°C

UV Detector wavelength : 210nm

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Identify each compound on the basis of its retention time and compare it to that obtained for the standard; this time must not differ by 10 percent for reliable identification. On the other hand, this identification can be confirmed by observing the profiles on the two detectors (UV210 nm and refractometry); sugar or alcohol detected by refractometry should not attain a peak with similar retention time at UV210 nm.

Compound content (mg/g dry matter) = $(A * f * 100000) / (RC * W * DM)$

Where

- A is sample peak area
- f is the dilution factor
- RC is the response coefficient of the standard (mg/ml)
- W is sample weight in mg
- DM is the dry matter content (% wet basis) of the sample

In the case of organic acids, the average value of the levels assessed by the two detectors (UV210 nm and refractometry) is calculated. If the value calculated by absorbance at 210 nm is much higher (**more than double**) than that calculated by refractometry, it means that another compound is highly absorbing in UV and masking the organic acid. It must thus be considered to be **absent**

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8.2 Repeatability

The standard deviation between repetitions in a short time interval shall not exceed 5% of the average (coefficient of variation).

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- ✓ When the HPLC system is switched on, it takes several hours for the refractometer to stabilize ; Therefore, allow the elution system several hours before starting a sequence of analysis, and rinse the cell reference of the refractometer one to two times per day (between two analyses) with the eluent (5 mM sulphuric acid). When doing so, wait approximately ¼ hour to stabilize the base line before auto-zero the refractometer.
- ✓ Check well the identification of compounds by joint observation of the two detectors (see 8.1)



10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification of the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|-------------|---------------------------|---|
| 13/03/2011 | Wis AMOA | Edition |
| 17/05/2011 | Noël AKISSOE | Edition |
| 23/05/2011 | Christian MESTRES | Editing, conformity between French and English versions |

| | | |
|--|---|---|
|  | <u>African Food Tradition rEvisited by Research FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical analysis for Group 1 | | |
| Determination of simple sugars in Cereal Products – HPLC NH2-based column Method | | |
| SOP: Chem-Cere-20-en | | |
| Date: 16/08/2011 | Release: 1 | |
| <p>Written by : Zahra AHMED</p> <p>For information on this SOP please contact :</p> <ul style="list-style-type: none"> • Zahra AHMED (zahra3010@hotmail.com) / NRC-Egypt. • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader | | |
| This document has been approved by : | | |
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| CIRAD UAC CSIR FAAU ESB FRI NRC | Christian MESTRES Zahra AHMED | 16/08/2011 04/08/2011 |

**Determination of simple sugars in Cereal Products—
HPLC NH₂-based column Method
SOP: Chem-Cere-20-en**

Date: 01/07/2011

Release: 1

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Determination of simple sugars in Cereal Products—

HPLC NH₂-based column Method

SOP: **Chem-Cere-20-en**

Date: **01/07/2011**

Release: **1**

1 SCOPE AND APPLICATION

This procedure aims to separately measure simple sugars using chromatographic method. This method is applicable to all cereal products

2 REFERENCES

AACC International Method 80-04.01

3 DEFINITIONS

Carbohydrates, the main nutrient in grain products, can be classified into: Complex and simple carbohydrate. Complex carbohydrates often supply energy and other nutrients and fiber that the body needs, whereas, Simple carbohydrates are quick energy sources, but they do not usually supply any other nutrients. Simple carbohydrates include:

- Glucose, the major kind of simple sugar. Nearly all plant foods contain glucose.
- Sucrose: commonly known as table sugar, beet sugar, or cane sugar. Sucrose occurs in many fruits and some vegetables.
- Fructose: known as fruit sugar. Most plants contain fructose, especially fruits and saps.
- Lactose: commonly known as milk sugar. It is the principal carbohydrate found in milk.

4 PRINCIPLE

Fructose, glucose, sucrose, maltose, and lactose are separated and quantitated by high-pressure liquid chromatography (HPLC) after aqueous extraction from sample matrix and dilution with water-miscible mobile-phase modifier.

5 REAGENTS

See Appendix

**Determination of simple sugars in Cereal Products—
HPLC NH₂-based column Method
SOP: Chem-Cere-20-en**

Date: 01/07/2011

Release: 1

6 APPARATUS

See Appendix.

7 PROCEDURE

See Appendix

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

Measure peak heights and peak area of sugars in standards and respective peak heights in samples. Calculate percent fructose as follows:

$$\% \text{ Fructose} = \frac{\text{PHSAM} \times C \times \text{DF} \times 100}{\text{PHSTD} \times W}$$

Where PHSAM = peak height of fructose in sample,

PHSTD = peak height of fructose in standard,

C = concentration of fructose in standard, in mg/ml,

W = weight of sample, in mg,

DF = dilution factor for sample, usually 250, 100 = conversion to percent.

Similar formula can be applied for peak area instead of peak height.

Calculate quantity of other sugars in same manner.

8.2 Repeatability

- Every 10 injections one control standard has to be injected. If the area of the standard deviates for more than $\pm 5\%$ from the area found in the calibration performed in the first part of the analysis, the possible source of failure has to be identified and fixed and all samples injected after the previous control standard have to be re-injected.
- The retention time in the test solution is not expected to be deviate more than $\pm 2.5\%$ from the reference retention time.

Determination of simple sugars in Cereal Products—

HPLC NH₂-based column Method

SOP: Chem-Cere-20-en

Date: 01/07/2011

Release: 1

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

- Due to the status of the HPLC apparatus, or due to sensitivity reason, it is recommended to keep the injection volume constant.
- The extraction duration will be strictly fixed, as there is a hazard of modifications due to enzymes (glucosidases, invertases etc) present in the material.

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------------|--------------------|-----------------------------|
| 16/08/2011 | Mestres Christian | Note on extraction duration |
| | | |
| | | |

12 APPENDIX

AACC International Method 80-04.01

Determination of Simple Sugars in Cereal Products—HPLC Method

First approval 9-25-85; reviewed 10-26-94

Objective

Fructose, glucose, sucrose, maltose, and lactose are separated and quantitated by high-pressure liquid chromatography (HPLC) after aqueous extraction from sample matrix and dilution with water-miscible mobile-phase modifier. This method is applicable to all cereal products.

Apparatus

1. HPLC system, including pump, autosampler, differential refractometer, and data acquisition interface.
2. Chromatograph software.
3. Carbohydrate column, normal-phase NH_2 -based column.
4. Filter paper, Whatman no. 4.
5. Syringe filters.
6. Aqueous and organic membrane filters.
7. Autosampler vials, septa, and cups.

Reagents

1. Fructose.
2. Glucose.
3. Sucrose.
4. Maltose.
5. Lactose.
6. Water, HPLC grade.
7. Acetonitrile, HPLC grade.
8. Stock solution. Weigh 0.5 g fructose, 0.5 g glucose, 1.0 g sucrose, 1.0 g maltose, and 1.0 g lactose into 100-ml volumetric flask.
9. Working sugar standard. Pipet 10 ml stock solution into 25-ml volumetric flask. Dilute to volume with acetonitrile, mix, allow to warm to room temperature. (Endothermic cooling results from mixing acetonitrile and water.) Adjust volume with acetonitrile if necessary and remix. Standard contains 2 mg/ml fructose and glucose and 4 mg/ml sucrose, maltose, and lactose.
10. Mobile phase. Begin by combining acetonitrile and distilled water (75:25, v/v). Sonicate mobile phase with vacuum attached to top of bottle until air bubbles are gone (20 min).
11. NaCl-interference test solution. On occasion, bonded propylamino phase columns are purchased that give retention time for NaCl that is same as for one of sugars being analyzed. Dissolve 0.5 g NaCl in 100 ml distilled water. Pipet 10 ml aqueous solution into 25-ml volumetric flask and dilute to volume with acetonitrile. Allow to warm to room temperature and adjust with acetonitrile if necessary.

Determination of Simple Sugars in Cereal Products—HPLC Method (continued)

Procedure

Preparation of sample

1. Grind all dry samples to pass 40-mesh sieve. Homogenize all high-moisture samples thoroughly. Store all high-moisture samples in refrigerator until analyzed.

2. Extraction procedure for low-fat samples: Weigh appropriate size sample (Table I) into 250-ml disposable plastic cup. Pipet in 100 ml distilled water and stir for 20 min. Remove and allow solids to settle. Sonicate for 5 min. Proceed to step 1 of *Method* below.

3. Extraction procedure for high-fat samples. Weigh appropriate size sample (Table I) into 250-ml beaker. Record weight of beaker and its contents. Add approximately 90 ml boiling water to beaker, add magnetic stirring bar, and stir for 15 min. Remove and rinse stirring bar. Reweigh beaker and add water until total weight of water added to sample equals 100 g. Sonicate for 5 min. Proceed to step 1 of *Method* below.

4. Dilution procedure for liquid samples: Weigh appropriate size sample (Table I) into 100-ml volumetric flask. Dissolve in and dilute to volume with distilled water. Proceed to step 1 of *Method* below.

Method

1. Pipet 10 ml aqueous extract into 25-ml volumetric flask. Dilute to volume with acetonitrile. Mix well, allow to warm to room temperature, and adjust volume with acetonitrile if necessary. Shake flask and pour some into 30-ml beaker. Using a disposable syringe, draw up 5 ml. Attach a filter to syringe and push down to 3-ml mark into a waste beaker. Add remaining 3 ml to a glass vial. Cap and label with sample number.

2. Repeat this for sugar standard and NaCl solution.

3. Run standard and samples according to chromatography software.

Calculation

1. Measure peak heights and peak area of sugars in standards and respective peak heights in samples.

2. Calculate percent fructose as follows:

$$\% \text{ Fructose} = \frac{PHSAM \times C \times DF \times 100}{PHSTD \times W}$$

where *PHSAM* = peak height of fructose in sample, *PHSTD* = peak height of fructose in standard, *C* = concentration of fructose in standard, in mg/ml, *W* = weight of sample, in mg, *DF* = dilution factor for sample, usually 250, 100 = conversion to percent. Similar formula can be applied for peak area instead of peak height.

3. Calculate quantity of other sugars in same manner.

Determination of Simple Sugars in Cereal Products—HPLC Method (continued)

TABLE I
Determination of Sample Size

| Estimated Sugar Level, % | Suggested Sample Weight, g |
|---------------------------------|-----------------------------------|
| <10 | 10 |
| 10–25 | 5 |
| >25 | 3 |

References

1. AOAC International. 1995. Official Methods of Analysis of AOAC International, 16th ed. Methods 980.13 and 982.14. The Association, Arlington, VA.
2. DeVries, J. W., Chang, H. L., Heroff, J. C., and Johnson K. D. 1983. Elimination of sodium chloride interferences during high pressure liquid chromatographic determination of sugars. *J. Assoc. Off. Anal. Chem.* 66:197–198.
3. DeVries, J. W., Heroff, J. C., and Egberg, D. C. 1979. High pressure chromatographic determination of carbohydrates in food products. *J. Assoc. Off. Anal. Chem.* 62:1292–1296.



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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8: SOP for Chemical Analysis for Group 1 | | |
| Analyse des dextrines dans les produits céréaliers fermentés | | |
| SOP Number: Chem-Cere-001-fr | | |
| Date : 09/01/2012 | Version : 2 | |
| <p>Ecrit par : Laetitia MESTRES</p> <p>Pour plus d'information sur ce SOP, contactez :</p> <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader | | |
| This document has been approved by : | | |
| Partner | Name of the person who approved | Date DD/MM/YY |
| CIRAD UAC CSIR FAAU ESB FRI NRC NRC | Christian MESTRES | 09/01/2012 |

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Analyse des dextrines dans les produits céréaliers fermentés

SOP : Chem-Cere-001-fr

Date : 4-Jan-11

Version :2

1 DOMAINE ET APPLICATION

Cette méthode est adaptée spécialement pour la caractérisation des dextrines solubles dans l'eau et les alcalis et les maltodextrines. Elle est applicable aux produits amylacés. Les dextrines sont issues de l'hydrolyse acide ou enzymatique de l'amidon.

2 REFERENCES

3 DEFINITIONS

Les **dextrines** sont des glucides solubles et amorphes, de formule brute $(C_6H_{10}O_5)_n$. Ce sont des mélanges de gluco-oligosides (des oligosides de glucose) dont les unités de glucose sont liées par des liaisons osidiques du type α -(1,4) et α -(1,6). Les dextrines sont des produits incolores en solution et dextrogyres. Elles sont obtenues par hydrolyse acide ou enzymatique de l'amidon.

4 PRINCIPE

Les dextrines sont solubilisées en milieu alcalin puis séparées en chromatographie d'exclusion stérique sur une colonne Tricon Superdex TM peptide 10/300GL contenant du gel Sephadex, limite d'exclusion: 20 000 Da. Les gels de Séphadex sont des tamis moléculaires laissant sortir les constituants du mélange étudié par ordre de poids moléculaires décroissants. Les molécules de taille moléculaire supérieure à la limite d'exclusion du gel employé ne peuvent pénétrer dans les grains réticulés du gel de Séphadex : elles traversent le gel dans la phase libre, ou volume mort, et quittent le gel les premières. Les molécules plus petites diffusent dans les pores du gel plus ou moins selon leur taille moléculaire et sont éluées d'autant plus tard qu'elles sont plus petites. Le volume maximal d'élué est par définition le volume total de la colonne.

Volume total = volume mort + volume Interne + vol.de la matrice ; soit $V_t = V_o + V_i + V_m$.

Analyse des dextrines dans les produits céréaliers fermentés

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5 REACTIFS

Hydroxyde de sodium (impuretés 0,005%)

Eau ultra pure

6 APPAREILLAGE

- Système HPLC constitué d'un dégazeur en ligne, d'une pompe, d'un injecteur, d'un four de colonne et de deux détecteurs en série : spectrophotomètre ultra-violet, puis réfractomètre différentiel.
- Colonne Tricon Superdex TM peptide 10/300GL pour filtration sur gel des biomolécules de petite taille, matrice composée de dextrane et d'agarose, limite d'exclusion 20 000 (peptides), pression maximale 1,8 bar,
- Tubes en verre à vis de 10 mL
- Agitateur rotatif (exemple : agitest 34050, BIOBLOCK)
- Filtres seringues (pores 5 µm, exemple : Millex-Sv, Millipores)
- Filtres microfibre GF/F N° 1825047 ; 4,7 cm
- Support de filtre en verre
- Fiole à vide en verre
- Seringues et vials de 1 mL
- Balance
- Agitateur vortex

7 PROCEDURE

7.1 Préparation de l'éluant

- Préparer 200 mL de solution de NaOH 0,5M ; c'est la solution-mère.
- Filtrer sous vide cette solution : poser le filtre N°1825047 ; Ø 4,7cm sur le support de filtre et au dessus, déposer l'entonnoir, maintenir l'ensemble avec la pince (voir photo) et relier à la fiole à vide elle-même reliée à la pompe à vide. Mettre la pompe en marche et verser la solution dans l'entonnoir (filtration) et rincer avec de l'eau ultra pure.
- Mettre le filtrat dans une fiole d'un litre, le compléter au trait de jauge avec de l'eau ultrapure, puis ajouter 1 L d'eau mesurée avec une fiole jaugée : on obtient 2 L de solution NaOH, 0,05M : c'est l'éluant.



Support de filtre

7.2 Solubilisation

- Préparer la solution d'extraction (NaOH, 1M) en solubilisant la soude dans de l'eau ultrapure.
- Peser 120 mg de malt directement dans le tube en verre à vis et y ajouter sous agitation (vortex) 3 mL de la solution de solubilisation
- Placer le tube sur l'agitateur rotatif et laisser extraire pendant 2 h
- Prélever 0,5 mL de solution et y ajouter 2 mL d'eau extra pure ; agiter 1 h
- Prélever 2 mL et filtrer avec le filtre seringue (5,0 µm)
- Le filtrat (l'extrait) est recueilli directement dans le vial

7.3 Standards

Des standards de solutions de glucose (G1), et oligomères de glucose (G2, G3, G4, G5, G6, et G7) sont préparées à 1 mg/mL et injectées pour déterminer leur temps de rétention et coefficients de réponse (tableau 1)

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Tableau 1 : Ordre de grandeur des volumes d'éluion et des coefficients de réponse.

| Sucres | Volume (mL) | coefficient réponse [(concentration mg mL ⁻¹) / surface] |
|--------|-------------|--|
| G 7 | 11,7 | 28 |
| G 6 | 12,1 | |
| G 5 | 12,6 | |
| G 4 | 13,2 | |
| G 3 | 13,8 | |
| G 2 | 14,9 | |
| G 1 | 15,8 | |

7.4 Dosage

Une aliquote de 200 µL est injectée et éluee avec le NaOH 0,05M à un débit isocratique de 0,5 à 0,7 mL/min

Les oligomères de glucose sont identifiés par leur temps de rétention sur le tracé réfractométrique, en comparaison aux temps de rétention des standards ; seuls les pics n'absorbant pas en UV sont pris en compte (les sucres n'absorbent pas dans l'UV). La figure 1 présente un exemple de chromatogramme pour un malt de sorgho et des standards G1 à G7.

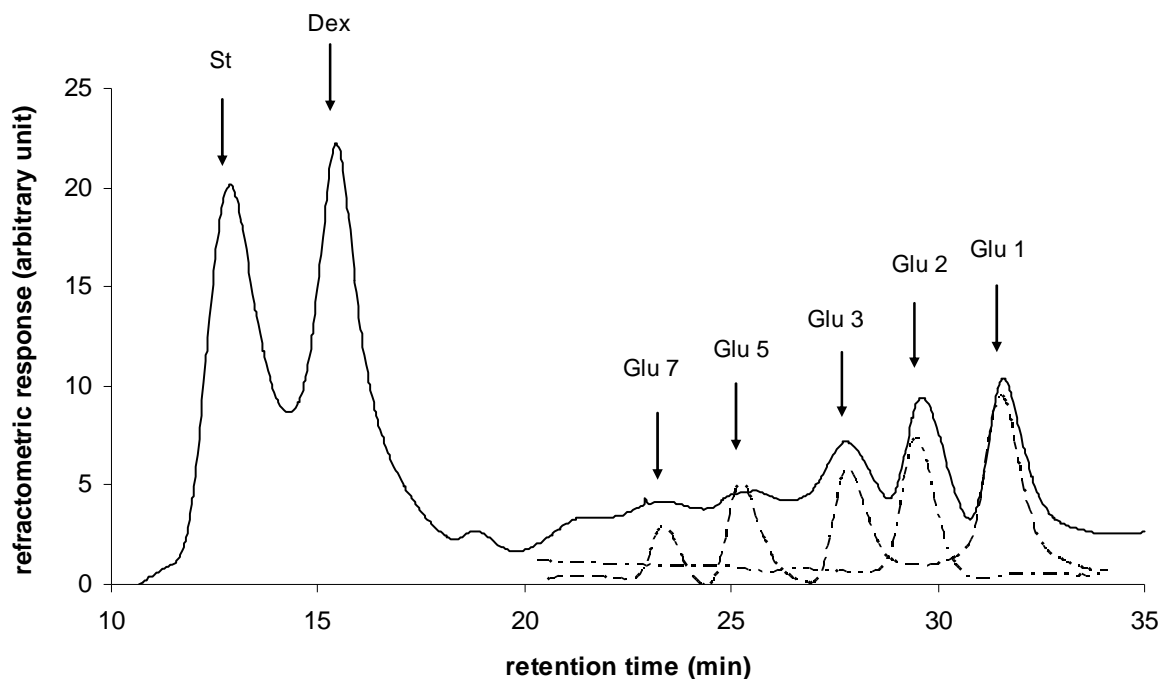


Figure 1. Exemple de chromatogramme

Analyse des dextrines dans les produits céréaliers fermentés

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8 EXPRESSION DES RÉSULTATS

8.1 Mode de calcul et formules

La teneur en chaque oligoside est calculé par la formule suivante :

$$[Gx] \text{ (g/100g de malt sec)} = (SRe * f * 10000) / (CRs * PE * MS)$$

Où

- SRe est la surface de réponse de l'oligoside dans l'échantillon
- f est le facteur de dilution
- CRs est le coefficient de réponse du standard Gx (Surface du pic / quantité injectée)
- PE prise d'essai en mg
- MS est le pourcentage matière sèche

8.2 Répétabilité

Indiquer ici la différence acceptable entre deux déterminations réalisées simultanément par le même analyste pour un échantillon. Cette valeur ne devra pas excéder une valeur limite spécifiée dans ce SOP

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

- ✓ Lors de la mise en route du système HPLC, le temps de stabilisation du réfractomètre est long (en général de plusieurs heures) ; il faut donc laisser le système **en élution** plusieurs heures avant de démarrer une séquence d'analyse, et rincer la cellule de référence du réfractomètre une à deux fois par jour (entre deux analyses) avec de l'éluant. Attendre environ ¼ d'heure pour que la ligne de base se stabilise. Faire le zéro du réfractomètre.
- ✓ Bien vérifier l'identification des composés par l'observation conjointe des deux voies de détection (cf 7.4)
- ✓ Bien rincer le système avec de l'eau (une journée) avant extinction et stockage de la colonne.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillés toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

Analyse des dextrines dans les produits céréaliers fermentés

SOP : Chem-Cere-001-fr

Date : 4-Jan-11

Version :2

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------|----------------------|--------------------------------|
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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
| Deliverable D.1.2.3.8 : SOP for Chemical analysis for Group 1 | | |
| Mesure de l'activité β-amylasique dans les produits céréaliers fermentés | | |
| SOP Number: Chem-Cere-005-fr | | |
| Date : 16/09/2011 | Version :2 | |
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Mesure de l'activité β -amylasique dans les produits céréaliers fermentés

SOP : Chem-Cere-005-fr

Date : 16/09/2011

Version : 2

1 DOMAINE ET APPLICATION

Cette méthode décrit le dosage de l'activité de la β -amylase dans les produits céréaliers.

2 REFERENCES

Megazyme International. 2010. Beta-amylase assay procedure (betamyl-3 method) K-Beta3 10/10 (100/200 assays per kit). *Megazyme International Ireland Limited*.

Ici nous allons uniquement renseigner les paragraphes 3 (définition) 4 (principe), 8.1 (mode de calcul et formules), 8.2 (répétabilité) et 9 (points critiques). Pour plus de détails, se référer à la procédure K-beta-3 en annexe.

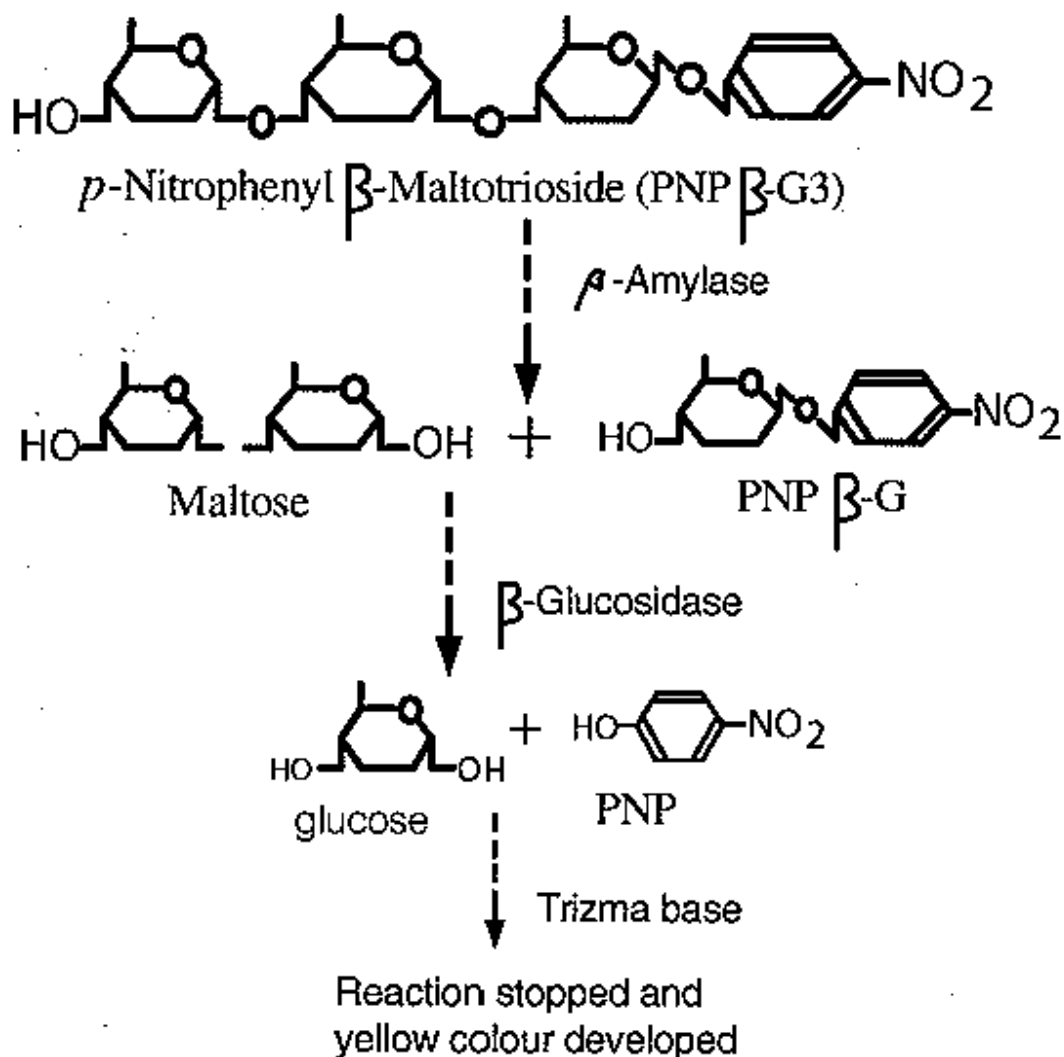
3 DEFINITIONS

La β -amylase est une enzyme qui hydrolyse les liaisons alpha-glucosidiques (α 1-4) présentes dans les polysaccharides comme l'amidon ou le glycogène. Elle catalyse l'hydrolyse de l'amidon en maltose et en dextrines : c'est une hydrolyse **saccharifiante**, dont l'action est stoppée au niveau des ramifications (α 1-6) de l'amylopectine.

4 PRINCIPE

La méthode proposée mesure l'activité spécifique de la β -amylase sur un maltotrioside ; cette mesure n'est pas influencée par la présence d' α -amylase qui ne peut dégrader ce substrat.

Le réactif Betamyl-3 comporte un mélange de *p*-nitrophenyl- β -D-maltotrioside (PNP β -G3) et de β -glucosidase en excès. Le PNP β -G3 est hydrolysé en maltose et *p*-nitrophényl- β -D-glucose par β -amylase. Le *p*-nitrophényl- β -D-glucose est immédiatement clivé en D-glucose et *p*-nitrophénol libre par la β -glucosidase (cf. **Schéma 1**). En fin de réaction, le *p*-nitrophénol développe une couleur jaune par l'addition de Trizma à pH élevé. L'intensité de cette couleur jaune, proportionnelle à la teneur en *p*-nitrophénol, traduit ainsi directement la teneur en maltose, et donc l'activité β -amylasique.



Scheme 1. Theoretical basis of the Betamyl-3[®] β -amylase assay procedure. When PNP β -G3 is cleaved to maltose and PNP β -G, the latter is rapidly cleaved to *p*-nitrophenol and glucose by the excess quantities of β -glucosidase which is an integral part of the reagent mixture.

5 REACTIFS

Cf Annexe

6 APPAREILLAGE

Cf Annexe

7 PROCEDURE

Cf Annexe

8 EXPRESSION DES RÉSULTATS

8.1 Mode de calcul et formules

L'activité peut être calculée en unités **Betamyl-3** ou convertie en unité **Betamyl** (utilisée par le précédent kit) :

$$\text{Unités Betamyl} = 58.6 * \text{Unités Betamyl-3}$$

8.2 Répétabilité

Le coefficient de variation peut atteindre 5%.

A noter que l'activité β -amylasique du malt de sorgho est plus faible (valeurs généralement inférieures à 5 Unités Betamyl-3/g de MS) que celle d'un malt d'orge (plus de 20 Unités Betamyl-3/g de MS).

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

Pour des malts de sorgho traditionnel où l'activité β -amylasique est faible, la DO mesurée est inférieure 0,3. **Mais**, une réduction du taux de dilution (21 dans le protocole original) pour augmenter la DO mesurée est à **proscrire**, car aboutissant à une **sous-estimation** de l'activité β -amylasique.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus ; **l'unité** utilisée (**Unités Betamyl** ou **Unités Betamyl-3**) doit être précisée.

En outre seront détaillées toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

Mesure de l'activité β -amylasique dans les produits céréaliers fermentés

SOP : Chem-Cere-005-fr

Date : 16/09/2011

Version : 2

11 ENREGISTREMENT DES REVISIONS

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
| | | |
| | | |

12 ANNEXES

Beta-amylase assay procedure (betamyl-3 method) K-Beta3 (Megazyme International). **2010.**

Megazyme

BETA-AMYLASE

ASSAY PROCEDURE

(BETAMYL-3 METHOD)

K-BETA3 04/09

(100 / 200 Assays per Kit)



INTRODUCTION:

β -Amylase plays a central role in the complete degradation of starch to metabolisable or fermentable sugars during the germination or malting of cereal grains. It also finds considerable application, together with starch debranching enzymes, in the production of high maltose syrups. β -Amylase is usually measured using non-specific reducing sugar assays with starch as substrate. In some methods, the α -amylase is first inactivated by treatment at low pH.

A major advance in the assay of β -amylase was introduced by Mathewson and Seabourn¹ who found that the Calbiochem Pantrak[®] serum α -amylase reagent could be used to measure β -amylase in the presence of cereal α -amylase. The reagent (Pantrak) consists of a mixture of *p*-nitrophenyl- α -D-maltopentaoside (PNPG5) and *p*-nitrophenyl- α -D-maltohexaoside (PNPG6). These substrates are rapidly hydrolysed by β -amylase, but are only slowly cleaved by cereal α -amylase, which requires a longer stretch of α -1,4-linked D-glucosyl residues to satisfy the substrate sub-site binding requirements. Subsequently, Megazyme offered a product, **Betamyl**[®] (β -Amylase Assay Reagent) that comprised just PNPG5 and α -glucosidase², which gave greater specificity. This reagent is now superseded by the Megazyme **Betamyl-3**[®], β -Amylase assay reagent, which is more specific and considerably more stable than the **Betamyl**[®] reagent.

The Megazyme **Betamyl-3**[®], β -amylase test reagent employs high purity β -glucosidase and *p*-nitrophenyl- β -D-maltotrioside (PNP β -G3). The level of β -glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of PNP β -G3 to maltose and *p*-nitrophenyl- β -D-glucose by β -amylase, the *p*-nitrophenyl- β -D-glucose is immediately cleaved to D-glucose and free *p*-nitrophenol by the β -glucosidase present in the substrate mixture (Scheme 1). Thus, the rate of release of *p*-nitrophenol relates directly to the rate of release of maltose by β -amylase. The reaction is stopped, and the phenolate colour is developed, on addition of a high pH Trizma base solution.

The PNP β -G3 plus β -glucosidase mixture contains stabilisers which significantly increase its stability. The blank absorbance value of the **Betamyl-3**[®] substrate solution, when stored at 4 or 20°C, increases much more slowly than similar increases for **Betamyl**[®] reagent

ACCURACY:

Standard errors of less than 5 % are readily achieved.

SPECIFICITY:

The assay is highly selective for β -amylase. The substrate is hydrolysed by α -glucosidase and amyloglucosidase.

KITS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1: (x2)** Each vial contains *p*-nitrophenyl- β -D-maltotriose (PNP β -G3) plus β -glucosidase (50 U) and stabilisers. Stable for > 4 years at -20°C.
- Bottle 2:** Tris/HCl buffer (25 mL, 1 M, pH 8.0) plus disodium EDTA (20 mM) and sodium azide (0.02 % w/v). Stable for approx. 4 years at 4°C.
- Bottle 3:** MES buffer (48 mL, 1 M, pH 6.2) plus disodium EDTA (20 mM), BSA 10 mg/mL and sodium azide (0.20 % w/v). Stable for approx. 4 years at 4°C.
- Bottle 4:** Cysteine hydrochloride (16 g). Stable for > 2 years at room temperature.
- Bottle 5:** Malt flour of standardised β -amylase activity (as specified on bottle label). Stable for > 4 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dissolve the contents of bottle 1 in 10 mL of boiled and cooled distilled water. This is **Betamyl-3[®] Substrate Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use. Store unused reagent on ice awaiting use. Do not dissolve the contents of the second bottle until required. Stable for > 2 years at -20°C.
2. Dilute 2.5 mL of the contents of bottle 2 to 50 mL with distilled water. Before use, add 0.88 g of cysteine HCl (bottle 4; Sigma chemical company, cat. no. C-7990) (final concentration of cysteine HCl approx. 100 mM) and adjust the pH to 8.0 with 4 M NaOH. Stable for 8 h at 4°C.
3. Dilute the entire contents of bottle 3 to 500 mL with distilled water. Stable for approx. 1 year at 4°C.
4. Use as supplied. Stable for > 4 years at room temperature.
5. Use the contents of bottle 5 as supplied. Stable for > 4 years at room temperature.

PREPARATION OF ADDITIONAL EXTRACTION BUFFER: 0.05 M Tris-HCl plus 1 mM EDTA

Dissolve 6.06 g of trizma base (Sigma cat. no. T-1503) and 0.37 g of disodium EDTA (Sigma cat. no. E4884-500G) in 700 mL of distilled water. Adjust the pH to 8.0 with 1 M HCl and the volume to one litre. Stable for approx. 3 months at 4°C.

Immediately before use, add 1.75 g of cysteine HCl (Sigma cat. no. C-7880 to 100 mL of the buffer (final concentration of cysteine HCl approx. 100 mM). Adjust pH to 8.0 with 4 M NaOH. Stable for 8 h at 4°C.

Cysteine is added to Buffer A (followed by pH adjustment) immediately prior to use of the buffer. Cysteine is required to extract the “insoluble” β -amylase present in ungerminated grain. This buffer has been changed from our original recommendation, based on research by Erdal (1993) and Santos and Riis (1996). Enzyme extracted without added cysteine is termed “Soluble” β -amylase; that extracted with cysteine is “Total” β -amylase.

PREPARATION OF ADDITIONAL DILUTION / ASSAY BUFFER:

0.1 M MES buffer plus 1 mM EDTA, 1.0 mg/mL of BSA and 0.02 % w/v sodium azide.

Dissolve 21.3 g of MES monohydrate (Sigma cat. no. 69892-500G) and 0.37 g of disodium EDTA (Sigma cat. no. ED2SS) in 700 mL of distilled. Adjust the pH to 6.2 with 4 M (16 g/100 mL) sodium hydroxide and the volume to 1 litre. Add 1.0 g of BSA (Sigma cat. no. A-2153) and 0.2 g of sodium azide as a preservative. Stable for > 2 years at 4°C.

NOTE:

1. Do not add the sodium azide to the buffer until it has been adjusted to pH 6.2. Adding sodium azide to an acidic solution results in the release of a poisonous gas.
2. If buffer is prepared without adding sodium azide as a preservative, then it should be used on the day of preparation.

STOPPING REAGENT:

1 % (w/v) Trizma base (approx. pH 8.5)

Dissolve 10 g of Trizma base (Sigma cat. no. T-1503) in 900 mL of distilled water. Adjust the pH to 8.5 (if necessary) and the volume to 1 litre. Stable for approx. 1 year at room temperature.

EQUIPMENT (RECOMMENDED):

1. Disposable 13 mL polypropylene tubes e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
2. Disposable 1.5 mL polypropylene screw cap microfuge tubes e.g. Sarstedt cat. no. 72.692 (www.sarstedt.com).
3. Disposable plastic micro-cuvettes (1 cm light path, 1.5 mL) e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 7591 15 (www.brand.de).
4. Micro-pipettors, e.g. Gilson Pipetman® (100 μ L and 200 μ L).
5. Positive displacement pipettor e.g. Eppendorf Multipette®
 - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of substrate solution).
 - with 25 mL Combitip® (to dispense extraction buffer and 3.0 mL of Stopping Reagent).
6. Analytical balance.
7. Spectrophotometer set at 400 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Stop clock.
10. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.
11. Eppendorf centrifuge 54XX (13,000 rpm; 15,000 g).
12. Laboratory mill e.g. Buhler Miag disc mill DLFU: Setting 0.2 mm (fine); Frisch Pulverisette 14® with 0.5 mm screen or Tecator Cyclotec® Mill.

CONTROLS AND PRECAUTIONS:

1. β -Amylase is extremely unstable when highly diluted in buffers not containing other proteins. It is thus **essential** that additional dilution/assay buffer is prepared **exactly** as described, and particularly that bovine serum albumin (BSA) is included.
2. For each set of assays, a **reagent blank** value should be determined. To obtain this value, add 3.0 mL of Stopping Reagent to 0.2 mL of pre-equilibrated **Betamyl-3**® reagent solution and then add 0.2 mL of diluted malt extract. A single reagent blank determination is sufficient for each batch of assays.
3. If **reagent blank** absorbance value exceeds **0.3**, then the **Betamyl-3**® substrate should be discarded.
4. If **reaction values** exceed **1.8**, then the enzyme extract should be diluted in Buffer B and re-assayed. Appropriate corrections to the calculations should then be made.
5. PNP β -G3 is very resistant to cleavage by cereal α -amylases, but some α -amylases, particularly those of fungal origin do cleave it. Thus, this assay cannot be used to specifically measure β -amylase

in materials which also contain substantial levels of fungal α -amylase activity e.g. wheat flours to which fungal α -amylase has been added. The substrate is rapidly hydrolysed by α -glucosidase and amyloglucosidase.

USEFUL HINTS:

1. The substrate should be stored frozen between use and on ice after thawing. In the lyophilised powder form (as supplied), the substrate mixture is stable for > 4 years at -20°C .
2. The number of assays which can be performed per kit can be doubled by halving the volumes of all the reagents used and by employing semi-micro spectrophotometer tubes. Do not alter the concentration of substrate in the final reaction mixture.

ASSAY PROCEDURE:

Enzyme Extraction:

1. Mill malt or barley to pass a 0.5 mm screen with a suitable laboratory mill (e.g. Buhler Miag disc mill DLFU; Setting fine, or Frisch Pulverisette 14[®] with 0.5 mm screen).
2. To exactly 0.5 g of flour in a 13 mL polypropylene tube (Sarstedt cat. no. 60.541.685 PP; www.sarstedt.com), **add** 5.0 mL of Extraction Buffer (Bottle 2).
3. Allow the enzyme to extract over a **1 hour** period at room temperature, with frequent vigorous stirring on a vortex mixer (approx. 5 times over the 1 hour period). Alternatively, place the tube into Stuart Blood Tube Rotator (<http://design.hileytech.com/fisher/Stuartblood.html>) which allows end-over-end mixing and run the machine for 1 hour.
4. Filter an aliquot of the enzyme preparation through Whatman GF/A glass fibre filter paper, or centrifuge in a bench or micro-centrifuge at a minimum of 2,000 g for 10 min.
5. Add 0.2 mL of filtrate to 4.0 mL of Dilution/Assay Buffer B (bottle 3), mix, and use this for the assay of β -amylase activity.

NOTE:

If the level of α -amylase is to be assayed in the same extract using the Ceralpha method, proceed as follows:

1. Add 0.2 mL of the diluted malt or barley extract (as used in the Betamyl-3[®] assay above) to 3 mL of Ceralpha Buffer **A** (prepared as shown on page 3 of the Ceralpha Booklet (K-CERA).
2. Proceed with assay of α -amylase (page 7 this booklet).

Assay of β -Amylase:

1. Dispense 0.2 mL aliquots of the diluted malt extract directly to the bottom of 13 mL polypropylene tubes and pre-incubate the tubes at 40°C for approx. 5 min.
2. Pre-incubate **Betamyl-3**[®] Substrate Solution at 40°C for approx. 5 min.
3. To each tube containing diluted malt extract add 0.2 mL of **Betamyl-3**[®] Substrate Solution, stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add 3.0 mL of Stopping Reagent and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent blank** at **400 nm** against distilled water.

CALCULATION OF ACTIVITY:

Units of β -Amylase / g of flour:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable β -glucosidase, required to release one micromole of *p*-nitrophenol from PNP β -G3 in one minute under the defined assay conditions, and is termed a **Betamyl-3**[®] **Unit**.

Units/g Flour:

$$= \frac{\Delta A_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\epsilon_{\text{mM}}} \times \frac{\text{Extraction Volume}}{\text{Sample Weight}} \times \text{Dilution}$$

$$= \frac{\Delta A_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{5}{0.5} \times 21$$
$$= A_{400} \times 19.72$$

where:

| | | |
|--|---|---|
| ΔA_{400} | = | Absorbance (sample) - Absorbance (blank) |
| Incubation time | = | 10 min |
| Total volume in cell | = | 3.4 mL (or 1.7 mL) |
| Incubation time | = | 10 min |
| Aliquot assayed | = | 0.2 mL (or 0.1 mL) |
| ϵ_{mM} <i>p</i> -nitrophenol | = | 18.1 (at 400 nm) in 1 % Trizma base |
| Extraction volume | = | 5 mL per 0.5 g of malt |
| Dilution | = | 0.2 to final volume of 4.2 mL (i.e. 21 fold). |

Assay of α -Amylase in malt extracts:

(Note: for complete details of Ceralpha method and required reagents, refer to the K-CERA booklet).

1. Dispense 0.2 mL aliquots of suitably diluted malt extract (see Note on page 5) directly to the bottom of 13 mL polypropylene tubes and pre-incubate the contents at 40°C for approx. 5 min.
2. Pre-incubate **Amylase HR Reagent**[®] at 40°C for approx. 5 min.
3. To each tube containing diluted malt extract add 0.2 mL of **Amylase HR Reagent**[®], stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add 3.0 mL of Stopping Reagent and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent blank** at **400 nm** against distilled water.

CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α -glucosidase, required to release one micromole of *p*-nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed a **Ceralpha**[®] **Unit**.

Units/g Flour:

$$= \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\epsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

$$= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{5}{0.5} \times 336$$
$$= \Delta E_{400} \times 315.6$$

where:

| | | |
|---------------------------------------|---|--|
| ΔA_{400} | = | Absorbance (sample) - Absorbance (blank) |
| Incubation time | = | 10 min |
| Total volume in cell | = | 3.4 mL (or 1.7 mL) |
| Aliquot assayed | = | 0.2 mL (or 0.1 mL) |
| ϵ_{mM} <i>p</i> -nitrophenol | = | 18.1 (at 400 nm) in 1 % Trizma base |
| Extraction volume | = | 5 mL per 0.5 g of malt |

Dilution = 0.2 to volume of 4.2 mL (i.e. 21 fold)
then 0.2 mL to 3.2 mL (a further 16-fold)
= $21 \times 16 = 336$ -fold.

DETERMINATION OF, AND ALLOWANCE FOR, THE DEGREE OF α -AMYLASE INTERFERENCE IN THE ASSAY:

Cereal α -amylase hydrolyses **Betamyl-3[®]** substrate at less than 1 % the rate of β -amylase.

RELATIONSHIP BETWEEN UNITS OF ACTIVITY ON **Betamyl** (based on PNP β G5) and **Betamyl-3[®]** (PNP β -G3) SUBSTRATES:

Units on **Betamyl[®]** substrate / **Betamyl-3[®]** substrate = 58.6

CONVERSION OF ACTIVITY ON PNP β -G3 TO INTERNATIONAL UNITS ON STARCH SUBSTRATE:

The activity of purified β -amylases (devoid of α -amylase, α -glucosidase and glucoamylase) on **Betamyl-3[®]** substrate and on soluble starch (assayed using the Nelson/Somogyi reducing sugar procedure) have been compared and conversion factors obtained. The ratios of activities on **Betamyl-3[®]** compared to starch for β -amylases from wheat, soybean, sweet potato and *Bacillus cereus* are 69.4, 57.2, 56.1 and 42.7, respectively.

REFERENCES:

1. Mathewson, P. R. and Seabourn, B. W. (1983). *Journal of Agriculture and Food Chemistry*. **31**, 1322-1326.
2. McCleary, B. V. and Codd, R. (1989). *Journal of Cereal Science*. **9**, 17-33.
3. Erdal, K., Jensen, M. O., Kristensen, M., Krogh, J. J., Riis, P. and Vaag, P., E. B. C. *Proceedings of the 24th European Brewery Convention Congress, Oslo, 1993*, 147.
4. Santos, M. M. and Riis, P. (1996). *Journal of the Institute of Brewing*. **102**, 271-275.

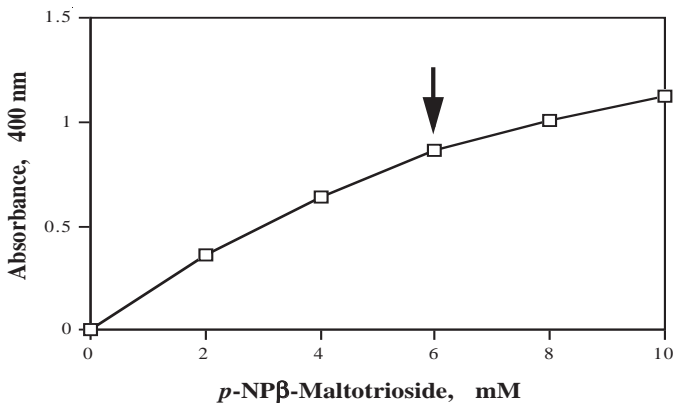


Figure 1. Effect of the substrate concentration (in the presence of excess β-glucosidase) on rate of hydrolysis of PNPβ-G3 by pure barley β-amylase. The arrow shows the final concentration in the Betamyl-3[®] reagent mixture.

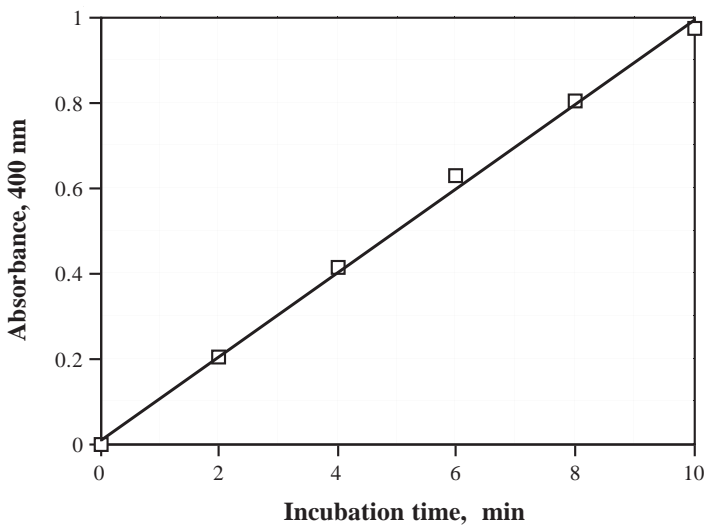


Figure 2. Time course of hydrolysis of PNPβ-G3 (in Betamyl-3[®] reagent) by pure barley β-amylase, as shown by increase in absorbance at 400 nm.

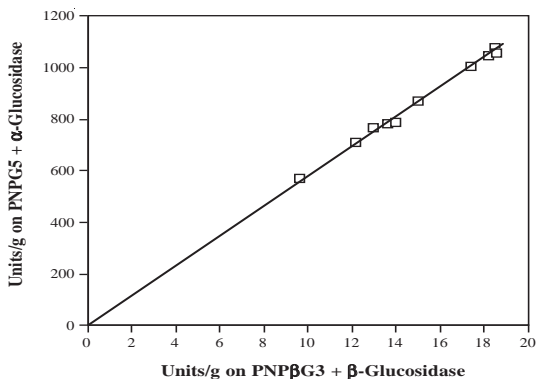
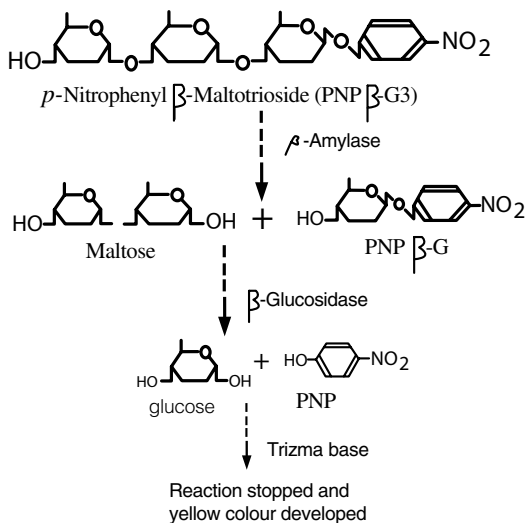


Figure 3. Comparison of activity of β -amylase in ten malt samples on Betamyl[®] substrate (PNPG5 plus α -glucosidase) and Betamyl-3[®] substrate (containing PNP β -G3 plus β -glucosidase).

Units on Betamyl[®] Reagent = 58.6 x Units on Betamyl-3[®] Reagent.

| Sample | Ceralpha (U/g) | Betamyl-3 (U/g) | Diastatic Power (EBC)WK (Units/100 g) | DP (°IoB) (Units/100 g) |
|--------|----------------|-----------------|---------------------------------------|-------------------------|
| A1 | 157.8 | 13.1 | 243 | 67.3 |
| A2 | 153.7 | 13.8 | 239 | 66.2 |
| A3 | 218.1 | 14.8 | 242 | 67.0 |
| A4 | 258.8 | 16.4 | 212 | 59.2 |
| A5 | 294.8 | 15.0 | 218 | 60.8 |
| A6 | 324.1 | 16.2 | 251 | 69.4 |
| A7 | 334.5 | 17.4 | 242 | 67.0 |
| A8 | 268.6 | 10.5 | 226 | 62.9 |
| A9 | 241.1 | 15.7 | 253 | 69.9 |
| A10 | 187.8 | 15.1 | 229 | 63.6 |
| B1 | 172.0 | 13.9 | 220 | 61.3 |
| B2 | 186.8 | 17.7 | 228 | 63.4 |
| B3 | 183.0 | 17.9 | 220 | 61.3 |
| B4 | 174.2 | 17.4 | 233 | 64.7 |
| B5 | 202.9 | 12.8 | 229 | 63.6 |
| B6 | 192.5 | 16.8 | 253 | 69.9 |
| B7 | 353.5 | 9.6 | 217 | 60.5 |
| B8 | 167.3 | 14.4 | 230 | 63.9 |
| B9 | 263.5 | 11.8 | 207 | 57.9 |
| B10 | 278.7 | 13.1 | 233 | 64.7 |

Table 1. Comparison of values obtained for α -amylase (Ceralpha), β -amylase (Betamyl-3[®]) and Diastatic Power for a number of malt samples. Diastatic Power was determined using Analytica-EBC Method 4.12 (Diastatic Power of Malt), and DP (°IoB) calculated as DP (°IoB) = [DP (WK) + 16] / 3.85. As can be seen from the data, there is no correlation between DP and either β -amylase or α -amylase activity.



Scheme 1. Theoretical basis of the Betamyl-3[®] β -amylase assay procedure. When PNP β -G3 is cleaved to maltose and PNP β -G, the latter is rapidly cleaved to *p*-nitrophenol and glucose by the excess quantities of β -glucosidase which is an integral part of the reagent mixture.

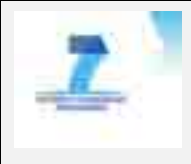



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Procedure for measuring β -amylase activity in cereal fermented foods

SOP: Chem-Cere-005-en

Date: 16/09/2011

Release: 2

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Procedure for measuring β -amylase activity in cereal fermented foods

SOP: Chem-Cere-005-en

Date: 16/09/2011

Release: 2

1 SCOPE AND APPLICATION

This method describes the determination of the β -amylase activity in cereal products.

2 REFERENCES

Megazyme International. **2010**. Beta-amylase assay procedure (betamyl-3 method) K-Beta3 10/10 (100/200 assays per kit). *Megazyme International Ireland Limited*.

This SOP only covers subsections 3 (definition), 4 (principle), 8.1 (Method of calculation and formulae), 8.2 (repeatability) and 9 (critical points). For details, see the “betamyl-3” procedure in appendix (12)

3 DEFINITIONS

β -amylase is an enzyme which hydrolyzes α 1-4 glycosidic bonds present in polysaccharides such as starch or glycogen. It hydrolyzes α -1-4 links from the non-reducing end, and is stopped by α -1-6 branching points. It produces maltose and dextrans: it is a saccharifying enzyme.

4 PRINCIPLE

The proposed method measures the specific activity of the β -amylase on a maltotriose; the measure is not influenced by the presence of α -amylase that cannot degrade this substrate.

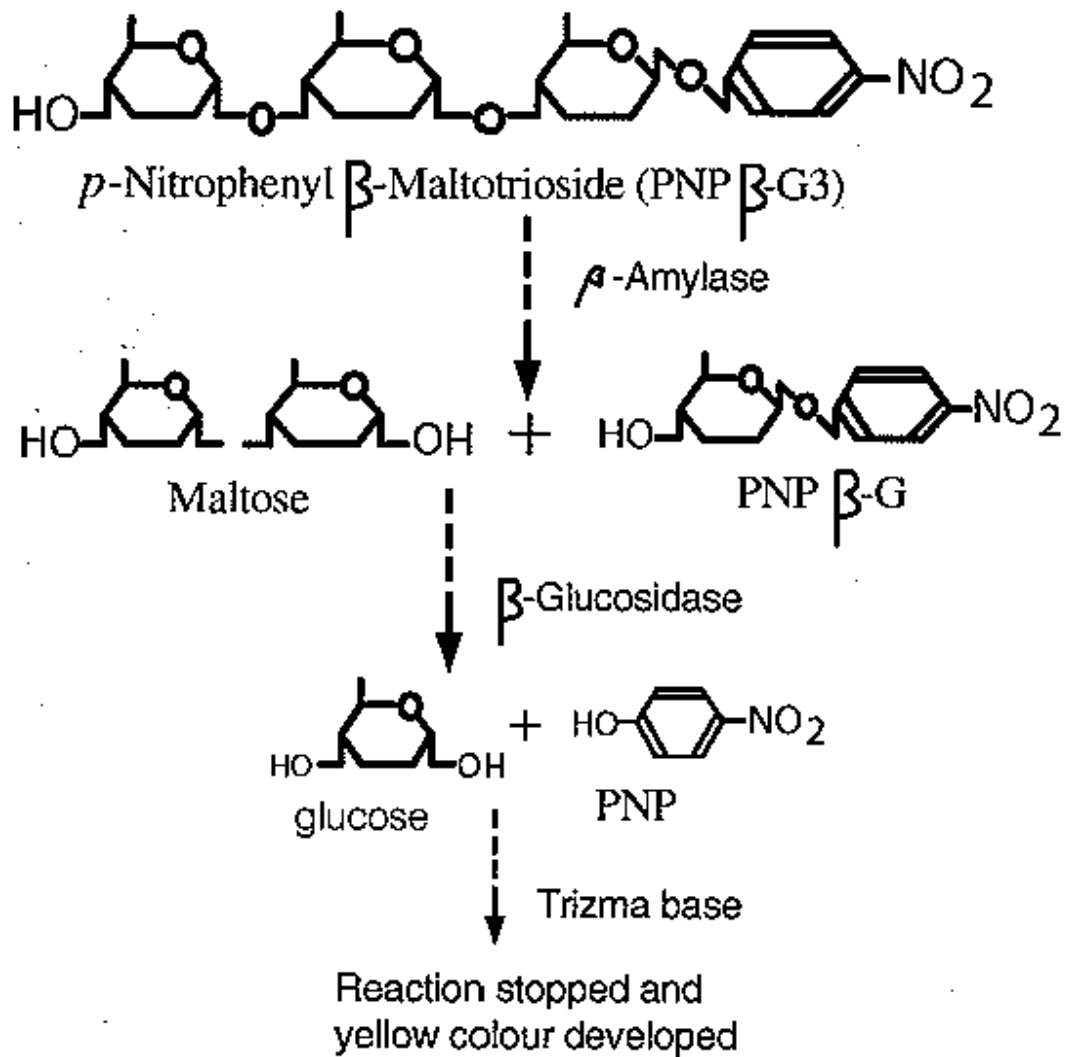
The Megazyme Betamyl-3®, β -amylase test reagent employs high purity β -glucosidase and p-nitrophenyl- β -D-maltotriose (PNP β -G3). The level of β -glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of PNP β -G3 to maltose and p-nitrophenyl- β -D-glucose by β -amylase, the p-nitrophenyl- β -D-glucose is immediately cleaved to D-glucose and free p-nitrophenol by the β -glucosidase present in the substrate mixture (Scheme 1). Thus, the rate of release of p-nitrophenol relates directly to the rate of release of maltose by β -amylase. The reaction is stopped, and the phenolate colour is developed, on addition of a high pH Trizma base solution

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Scheme 1. Theoretical basis of the Betamyl-3[®] β -amylase assay procedure. When PNP β -G3 is cleaved to maltose and PNP β -G, the latter is rapidly cleaved to p -nitrophenol and glucose by the excess quantities of β -glucosidase which is an integral part of the reagent mixture.

5 REAGENTS

Procedure for measuring β -amylase activity in cereal fermented foods

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6 APPARATUS

7 PROCEDURE

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

The activity can be calculated on **Betamyl-3 Units** or converted in former **Betamyl Units** :

$$\text{Betamyl Units} = 58.6 * \text{Betamyl-3 Units}$$

8.2 Repeatability

The coefficient of variation can reach **5%**.

Note that β -amylase activity of sorghum malt is much lower (usually **below 5 Betamyl-3 Units/g** dry basis) than that of barley malt (**more than 20 Betamyl-3 Units /g**)

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

For sorghum malt (with low β -amylase activity), the measured OD is less than 0.3. But, a reduction in the rate of dilution (21 in the original procedure) to increase the measured OD is not acceptable, because it leads to an underestimation of β -amylase activity.

10 TEST REPORT

The test report shall indicate the method used and the results obtained; the **type of units (Betamyl-3 or Betamyl Units)** must be precised.

In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

Procedure for measuring β -amylase activity in cereal fermented foods

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The test report shall include all details necessary for the complete identification for the sample.

11 REVISION RECORD

| Date | Responsible person | Description of change |
|------|--------------------|-----------------------|
| | | |
| | | |
| | | |

12 APPENDIX

Megazyme International. **2010**. Beta-amylase assay procedure (betamyl-3 method) K-Beta3 10/10 (100/200 assays per kit). *Megazyme International Ireland Limited*.

Megazyme

BETA-AMYLASE

ASSAY PROCEDURE

(BETAMYL-3 METHOD)

K-BETA3 04/09

(100 / 200 Assays per Kit)



INTRODUCTION:

β -Amylase plays a central role in the complete degradation of starch to metabolisable or fermentable sugars during the germination or malting of cereal grains. It also finds considerable application, together with starch debranching enzymes, in the production of high maltose syrups. β -Amylase is usually measured using non-specific reducing sugar assays with starch as substrate. In some methods, the α -amylase is first inactivated by treatment at low pH.

A major advance in the assay of β -amylase was introduced by Mathewson and Seabourn¹ who found that the Calbiochem Pantrak[®] serum α -amylase reagent could be used to measure β -amylase in the presence of cereal α -amylase. The reagent (Pantrak) consists of a mixture of *p*-nitrophenyl- α -D-maltopentaoside (PNPG5) and *p*-nitrophenyl- α -D-maltohexaoside (PNPG6). These substrates are rapidly hydrolysed by β -amylase, but are only slowly cleaved by cereal α -amylase, which requires a longer stretch of α -1,4-linked D-glucosyl residues to satisfy the substrate sub-site binding requirements. Subsequently, Megazyme offered a product, **Betamyl**[®] (β -Amylase Assay Reagent) that comprised just PNPG5 and α -glucosidase², which gave greater specificity. This reagent is now superseded by the Megazyme **Betamyl-3**[®], β -Amylase assay reagent, which is more specific and considerably more stable than the **Betamyl**[®] reagent.

The Megazyme **Betamyl-3**[®], β -amylase test reagent employs high purity β -glucosidase and *p*-nitrophenyl- β -D-maltotrioside (PNP β -G3). The level of β -glucosidase used ensures maximum sensitivity of the assay. On hydrolysis of PNP β -G3 to maltose and *p*-nitrophenyl- β -D-glucose by β -amylase, the *p*-nitrophenyl- β -D-glucose is immediately cleaved to D-glucose and free *p*-nitrophenol by the β -glucosidase present in the substrate mixture (Scheme 1). Thus, the rate of release of *p*-nitrophenol relates directly to the rate of release of maltose by β -amylase. The reaction is stopped, and the phenolate colour is developed, on addition of a high pH Trizma base solution.

The PNP β -G3 plus β -glucosidase mixture contains stabilisers which significantly increase its stability. The blank absorbance value of the **Betamyl-3**[®] substrate solution, when stored at 4 or 20°C, increases much more slowly than similar increases for **Betamyl**[®] reagent

ACCURACY:

Standard errors of less than 5 % are readily achieved.

SPECIFICITY:

The assay is highly selective for β -amylase. The substrate is hydrolysed by α -glucosidase and amyloglucosidase.

KITS:

Kits suitable for performing 100 assays are available from Megazyme. The kits contain the full assay method plus:

- Bottle 1: (x2)** Each vial contains *p*-nitrophenyl- β -D-maltotriose (PNP β -G3) plus β -glucosidase (50 U) and stabilisers. Stable for > 4 years at -20°C.
- Bottle 2:** Tris/HCl buffer (25 mL, 1 M, pH 8.0) plus disodium EDTA (20 mM) and sodium azide (0.02 % w/v). Stable for approx. 4 years at 4°C.
- Bottle 3:** MES buffer (48 mL, 1 M, pH 6.2) plus disodium EDTA (20 mM), BSA 10 mg/mL and sodium azide (0.20 % w/v). Stable for approx. 4 years at 4°C.
- Bottle 4:** Cysteine hydrochloride (16 g). Stable for > 2 years at room temperature.
- Bottle 5:** Malt flour of standardised β -amylase activity (as specified on bottle label). Stable for > 4 years at room temperature.

PREPARATION OF REAGENT SOLUTIONS/SUSPENSIONS:

1. Dissolve the contents of bottle 1 in 10 mL of boiled and cooled distilled water. This is **Betamyl-3[®] Substrate Solution**. Divide into appropriately sized aliquots and store in polypropylene tubes at -20°C between use. Store unused reagent on ice awaiting use. Do not dissolve the contents of the second bottle until required. Stable for > 2 years at -20°C.
2. Dilute 2.5 mL of the contents of bottle 2 to 50 mL with distilled water. Before use, add 0.88 g of cysteine HCl (bottle 4; Sigma chemical company, cat. no. C-7990) (final concentration of cysteine HCl approx. 100 mM) and adjust the pH to 8.0 with 4 M NaOH. Stable for 8 h at 4°C.
3. Dilute the entire contents of bottle 3 to 500 mL with distilled water. Stable for approx. 1 year at 4°C.
4. Use as supplied. Stable for > 4 years at room temperature.
5. Use the contents of bottle 5 as supplied. Stable for > 4 years at room temperature.

PREPARATION OF ADDITIONAL EXTRACTION BUFFER: 0.05 M Tris-HCl plus 1 mM EDTA

Dissolve 6.06 g of trizma base (Sigma cat. no. T-1503) and 0.37 g of disodium EDTA (Sigma cat. no. E4884-500G) in 700 mL of distilled water. Adjust the pH to 8.0 with 1 M HCl and the volume to one litre. Stable for approx. 3 months at 4°C.

Immediately before use, add 1.75 g of cysteine HCl (Sigma cat. no. C-7880 to 100 mL of the buffer (final concentration of cysteine HCl approx. 100 mM). Adjust pH to 8.0 with 4 M NaOH. Stable for 8 h at 4°C.

Cysteine is added to Buffer A (followed by pH adjustment) immediately prior to use of the buffer. Cysteine is required to extract the “insoluble” β -amylase present in ungerminated grain. This buffer has been changed from our original recommendation, based on research by Erdal (1993) and Santos and Riis (1996). Enzyme extracted without added cysteine is termed “Soluble” β -amylase; that extracted with cysteine is “Total” β -amylase.

PREPARATION OF ADDITIONAL DILUTION / ASSAY BUFFER:

0.1 M MES buffer plus 1 mM EDTA, 1.0 mg/mL of BSA and 0.02 % w/v sodium azide.

Dissolve 21.3 g of MES monohydrate (Sigma cat. no. 69892-500G) and 0.37 g of disodium EDTA (Sigma cat. no. ED2SS) in 700 mL of distilled. Adjust the pH to 6.2 with 4 M (16 g/100 mL) sodium hydroxide and the volume to 1 litre. Add 1.0 g of BSA (Sigma cat. no. A-2153) and 0.2 g of sodium azide as a preservative. Stable for > 2 years at 4°C.

NOTE:

1. Do not add the sodium azide to the buffer until it has been adjusted to pH 6.2. Adding sodium azide to an acidic solution results in the release of a poisonous gas.
2. If buffer is prepared without adding sodium azide as a preservative, then it should be used on the day of preparation.

STOPPING REAGENT:

1 % (w/v) Trizma base (approx. pH 8.5)

Dissolve 10 g of Trizma base (Sigma cat. no. T-1503) in 900 mL of distilled water. Adjust the pH to 8.5 (if necessary) and the volume to 1 litre. Stable for approx. 1 year at room temperature.

EQUIPMENT (RECOMMENDED):

1. Disposable 13 mL polypropylene tubes e.g. Sarstedt cat. no. 60.541.685 PP (www.sarstedt.com).
2. Disposable 1.5 mL polypropylene screw cap microfuge tubes e.g. Sarstedt cat. no. 72.692 (www.sarstedt.com).
3. Disposable plastic micro-cuvettes (1 cm light path, 1.5 mL) e.g. Plastibrand®, semi-micro, PMMA; Brand cat. no. 7591 15 (www.brand.de).
4. Micro-pipettors, e.g. Gilson Pipetman® (100 μ L and 200 μ L).
5. Positive displacement pipettor e.g. Eppendorf Multipette®
 - with 5.0 mL Combitip® (to dispense 0.2 mL aliquots of substrate solution).
 - with 25 mL Combitip® (to dispense extraction buffer and 3.0 mL of Stopping Reagent).
6. Analytical balance.
7. Spectrophotometer set at 400 nm.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Stop clock.
10. Bench centrifuge or Whatman GF/A glass fibre filter paper circles.
11. Eppendorf centrifuge 54XX (13,000 rpm; 15,000 g).
12. Laboratory mill e.g. Buhler Miag disc mill DLFU: Setting 0.2 mm (fine); Frisch Pulverisette 14® with 0.5 mm screen or Tecator Cyclotec® Mill.

CONTROLS AND PRECAUTIONS:

1. β -Amylase is extremely unstable when highly diluted in buffers not containing other proteins. It is thus **essential** that additional dilution/assay buffer is prepared **exactly** as described, and particularly that bovine serum albumin (BSA) is included.
2. For each set of assays, a **reagent blank** value should be determined. To obtain this value, add 3.0 mL of Stopping Reagent to 0.2 mL of pre-equilibrated **Betamyl-3**® reagent solution and then add 0.2 mL of diluted malt extract. A single reagent blank determination is sufficient for each batch of assays.
3. If **reagent blank** absorbance value exceeds **0.3**, then the **Betamyl-3**® substrate should be discarded.
4. If **reaction values** exceed **1.8**, then the enzyme extract should be diluted in Buffer B and re-assayed. Appropriate corrections to the calculations should then be made.
5. PNP β -G3 is very resistant to cleavage by cereal α -amylases, but some α -amylases, particularly those of fungal origin do cleave it. Thus, this assay cannot be used to specifically measure β -amylase

in materials which also contain substantial levels of fungal α -amylase activity e.g. wheat flours to which fungal α -amylase has been added. The substrate is rapidly hydrolysed by α -glucosidase and amyloglucosidase.

USEFUL HINTS:

1. The substrate should be stored frozen between use and on ice after thawing. In the lyophilised powder form (as supplied), the substrate mixture is stable for > 4 years at -20°C .
2. The number of assays which can be performed per kit can be doubled by halving the volumes of all the reagents used and by employing semi-micro spectrophotometer tubes. Do not alter the concentration of substrate in the final reaction mixture.

ASSAY PROCEDURE:

Enzyme Extraction:

1. Mill malt or barley to pass a 0.5 mm screen with a suitable laboratory mill (e.g. Buhler Miag disc mill DLFU; Setting fine, or Frisch Pulverisette 14[®] with 0.5 mm screen).
2. To exactly 0.5 g of flour in a 13 mL polypropylene tube (Sarstedt cat. no. 60.541.685 PP; www.sarstedt.com), **add** 5.0 mL of Extraction Buffer (Bottle 2).
3. Allow the enzyme to extract over a **1 hour** period at room temperature, with frequent vigorous stirring on a vortex mixer (approx. 5 times over the 1 hour period). Alternatively, place the tube into Stuart Blood Tube Rotator (<http://design.hileytech.com/fisher/Stuartblood.html>) which allows end-over-end mixing and run the machine for 1 hour.
4. Filter an aliquot of the enzyme preparation through Whatman GF/A glass fibre filter paper, or centrifuge in a bench or micro-centrifuge at a minimum of 2,000 g for 10 min.
5. Add 0.2 mL of filtrate to 4.0 mL of Dilution/Assay Buffer B (bottle 3), mix, and use this for the assay of β -amylase activity.

NOTE:

If the level of α -amylase is to be assayed in the same extract using the Ceralpha method, proceed as follows:

1. Add 0.2 mL of the diluted malt or barley extract (as used in the Betamyl-3[®] assay above) to 3 mL of Ceralpha Buffer **A** (prepared as shown on page 3 of the Ceralpha Booklet (K-CERA).
2. Proceed with assay of α -amylase (page 7 this booklet).

Assay of β -Amylase:

1. Dispense 0.2 mL aliquots of the diluted malt extract directly to the bottom of 13 mL polypropylene tubes and pre-incubate the tubes at 40°C for approx. 5 min.
2. Pre-incubate **Betamyl-3®** Substrate Solution at 40°C for approx. 5 min.
3. To each tube containing diluted malt extract add 0.2 mL of **Betamyl-3®** Substrate Solution, stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add 3.0 mL of Stopping Reagent and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent blank** at **400 nm** against distilled water.

CALCULATION OF ACTIVITY:

Units of β -Amylase / g of flour:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable β -glucosidase, required to release one micromole of *p*-nitrophenol from PNP β -G3 in one minute under the defined assay conditions, and is termed a **Betamyl-3® Unit**.

Units/g Flour:

$$= \frac{\Delta A_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\epsilon_{\text{mM}}} \times \frac{\text{Extraction Volume}}{\text{Sample Weight}} \times \text{Dilution}$$

$$= \frac{\Delta A_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{5}{0.5} \times 21$$
$$= A_{400} \times 19.72$$

where:

| | | |
|--|---|---|
| ΔA_{400} | = | Absorbance (sample) - Absorbance (blank) |
| Incubation time | = | 10 min |
| Total volume in cell | = | 3.4 mL (or 1.7 mL) |
| Incubation time | = | 10 min |
| Aliquot assayed | = | 0.2 mL (or 0.1 mL) |
| ϵ_{mM} <i>p</i> -nitrophenol | = | 18.1 (at 400 nm) in 1 % Trizma base |
| Extraction volume | = | 5 mL per 0.5 g of malt |
| Dilution | = | 0.2 to final volume of 4.2 mL (i.e. 21 fold). |

Assay of α -Amylase in malt extracts:

(Note: for complete details of Ceralpha method and required reagents, refer to the K-CERA booklet).

1. Dispense 0.2 mL aliquots of suitably diluted malt extract (see Note on page 5) directly to the bottom of 13 mL polypropylene tubes and pre-incubate the contents at 40°C for approx. 5 min.
2. Pre-incubate **Amylase HR Reagent**[®] at 40°C for approx. 5 min.
3. To each tube containing diluted malt extract add 0.2 mL of **Amylase HR Reagent**[®], stir on a vortex mixer and incubate tubes at 40°C for exactly 10 min (from time of addition).
4. At the end of the 10 min incubation period, add 3.0 mL of Stopping Reagent and stir the tube contents.
5. Read the absorbance of the **reaction solutions** and the **reagent blank** at **400 nm** against distilled water.

CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α -glucosidase, required to release one micromole of *p*-nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed a **Ceralpha**[®] **Unit**.

Units/g Flour:

$$= \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{\epsilon_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

$$= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{5}{0.5} \times 336$$
$$= \Delta E_{400} \times 315.6$$

where:

| | | |
|---------------------------------------|---|--|
| ΔA_{400} | = | Absorbance (sample) - Absorbance (blank) |
| Incubation time | = | 10 min |
| Total volume in cell | = | 3.4 mL (or 1.7 mL) |
| Aliquot assayed | = | 0.2 mL (or 0.1 mL) |
| ϵ_{mM} <i>p</i> -nitrophenol | = | 18.1 (at 400 nm) in 1 % Trizma base |
| Extraction volume | = | 5 mL per 0.5 g of malt |

Dilution = 0.2 to volume of 4.2 mL (i.e. 21 fold)
then 0.2 mL to 3.2 mL (a further 16-fold)
= $21 \times 16 = 336$ -fold.

DETERMINATION OF, AND ALLOWANCE FOR, THE DEGREE OF α -AMYLASE INTERFERENCE IN THE ASSAY:

Cereal α -amylase hydrolyses **Betamyl-3**[®] substrate at less than 1 % the rate of β -amylase.

RELATIONSHIP BETWEEN UNITS OF ACTIVITY ON **Betamyl** (based on PNP β G5) and **Betamyl-3**[®] (PNP β -G3) SUBSTRATES:

Units on **Betamyl**[®] substrate / **Betamyl-3**[®] substrate = 58.6

CONVERSION OF ACTIVITY ON PNP β -G3 TO INTERNATIONAL UNITS ON STARCH SUBSTRATE:

The activity of purified β -amylases (devoid of α -amylase, α -glucosidase and glucoamylase) on **Betamyl-3**[®] substrate and on soluble starch (assayed using the Nelson/Somogyi reducing sugar procedure) have been compared and conversion factors obtained. The ratios of activities on **Betamyl-3**[®] compared to starch for β -amylases from wheat, soybean, sweet potato and *Bacillus cereus* are 69.4, 57.2, 56.1 and 42.7, respectively.

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2. McCleary, B.V. and Codd, R. (1989). *Journal of Cereal Science*. **9**, 17-33.
3. Erdal, K., Jensen, M. O., Kristensen, M., Krogh, J. J., Riis, P. and Vaag, P., E. B. C. *Proceedings of the 24th European Brewery Convention Congress, Oslo, 1993*, 147.
4. Santos, M. M. and Riis, P. (1996). *Journal of the Institute of Brewing*. **102**, 271-275.

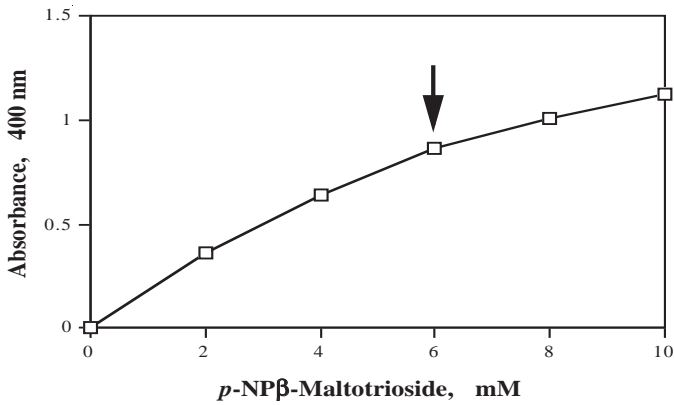


Figure 1. Effect of the substrate concentration (in the presence of excess β-glucosidase) on rate of hydrolysis of PNPβ-G3 by pure barley β-amylase. The arrow shows the final concentration in the Betamyl-3® reagent mixture.

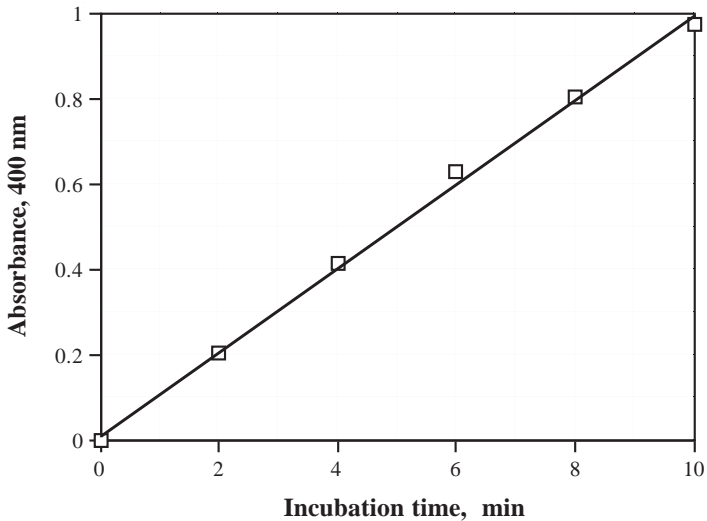


Figure 2. Time course of hydrolysis of PNPβ-G3 (in Betamyl-3® reagent) by pure barley β-amylase, as shown by increase in absorbance at 400 nm.

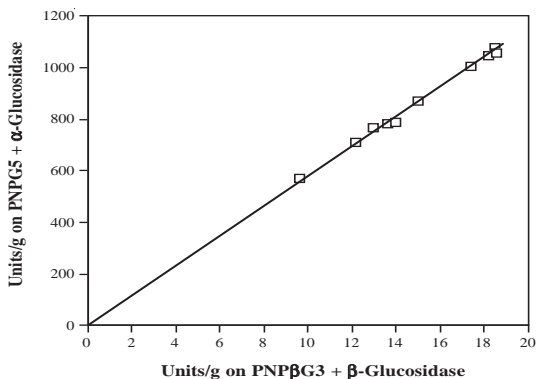
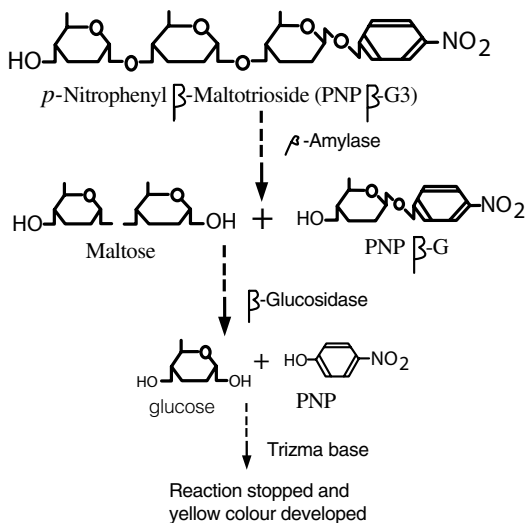


Figure 3. Comparison of activity of β -amylase in ten malt samples on Betamyl[®] substrate (PNPG5 plus α -glucosidase) and Betamyl-3[®] substrate (containing PNP β -G3 plus β -glucosidase).

Units on Betamyl[®] Reagent = 58.6 x Units on Betamyl-3[®] Reagent.

| Sample | Ceralpha (U/g) | Betamyl-3 (U/g) | Diastatic Power (EBC)WK (Units/100 g) | DP (°IoB) (Units/100 g) |
|--------|----------------|-----------------|---------------------------------------|-------------------------|
| A1 | 157.8 | 13.1 | 243 | 67.3 |
| A2 | 153.7 | 13.8 | 239 | 66.2 |
| A3 | 218.1 | 14.8 | 242 | 67.0 |
| A4 | 258.8 | 16.4 | 212 | 59.2 |
| A5 | 294.8 | 15.0 | 218 | 60.8 |
| A6 | 324.1 | 16.2 | 251 | 69.4 |
| A7 | 334.5 | 17.4 | 242 | 67.0 |
| A8 | 268.6 | 10.5 | 226 | 62.9 |
| A9 | 241.1 | 15.7 | 253 | 69.9 |
| A10 | 187.8 | 15.1 | 229 | 63.6 |
| B1 | 172.0 | 13.9 | 220 | 61.3 |
| B2 | 186.8 | 17.7 | 228 | 63.4 |
| B3 | 183.0 | 17.9 | 220 | 61.3 |
| B4 | 174.2 | 17.4 | 233 | 64.7 |
| B5 | 202.9 | 12.8 | 229 | 63.6 |
| B6 | 192.5 | 16.8 | 253 | 69.9 |
| B7 | 353.5 | 9.6 | 217 | 60.5 |
| B8 | 167.3 | 14.4 | 230 | 63.9 |
| B9 | 263.5 | 11.8 | 207 | 57.9 |
| B10 | 278.7 | 13.1 | 233 | 64.7 |

Table 1. Comparison of values obtained for α -amylase (Ceralpha), β -amylase (Betamyl-3[®]) and Diastatic Power for a number of malt samples. Diastatic Power was determined using Analytica-EBC Method 4.12 (Diastatic Power of Malt), and DP (°IoB) calculated as DP (°IoB) = [DP (WK) + 16] / 3.85. As can be seen from the data, there is no correlation between DP and either β -amylase or α -amylase activity.



Scheme 1. Theoretical basis of the Betamyl-3[®] β -amylase assay procedure. When PNP β -G3 is cleaved to maltose and PNP β -G, the latter is rapidly cleaved to *p*-nitrophenol and glucose by the excess quantities of β -glucosidase which is an integral part of the reagent mixture.



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|  | <u>African Food Tradition rEvisited by Research</u> <u>FP7 n°245025</u> |  |
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| Deliverable D.1.2.3.8: SOP for chemical analysis for Group 1 | | |
| Mesure de l'activité α-amylase dans les produits céréaliers fermentés | | |
| SOP Number: Chem-Cere-006-fr | | |
| Date: 16/09/2011 | Version : 2 | |
| <p> Ecrit par : Laetitia MESTRES Pour plus d'information sur ce SOP, contactez : <ul style="list-style-type: none"> • Christian MESTRES (christian.mestres@cirad.fr) / WP2 Leader • ... • • </p> | | |
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Mesure de l'activité α -amylase dans les produits céréaliers fermentés

SOP Number: **Chem-Cere-006-fr**

Date : **16/09/2011**

Version:**2**

1 DOMAINE ET APPLICATION

Cette méthode permet de mesurer l'activité de l' α -amylase dans les produits céréaliers.

2 REFERENCES

Megazyme International. **2004**. Alpha-amylase assay procedure (Cerealpha method) K-CERA 08/05 for the measurement of plant and microbial alpha-amylases. *Megazyme International Ireland Limited*.

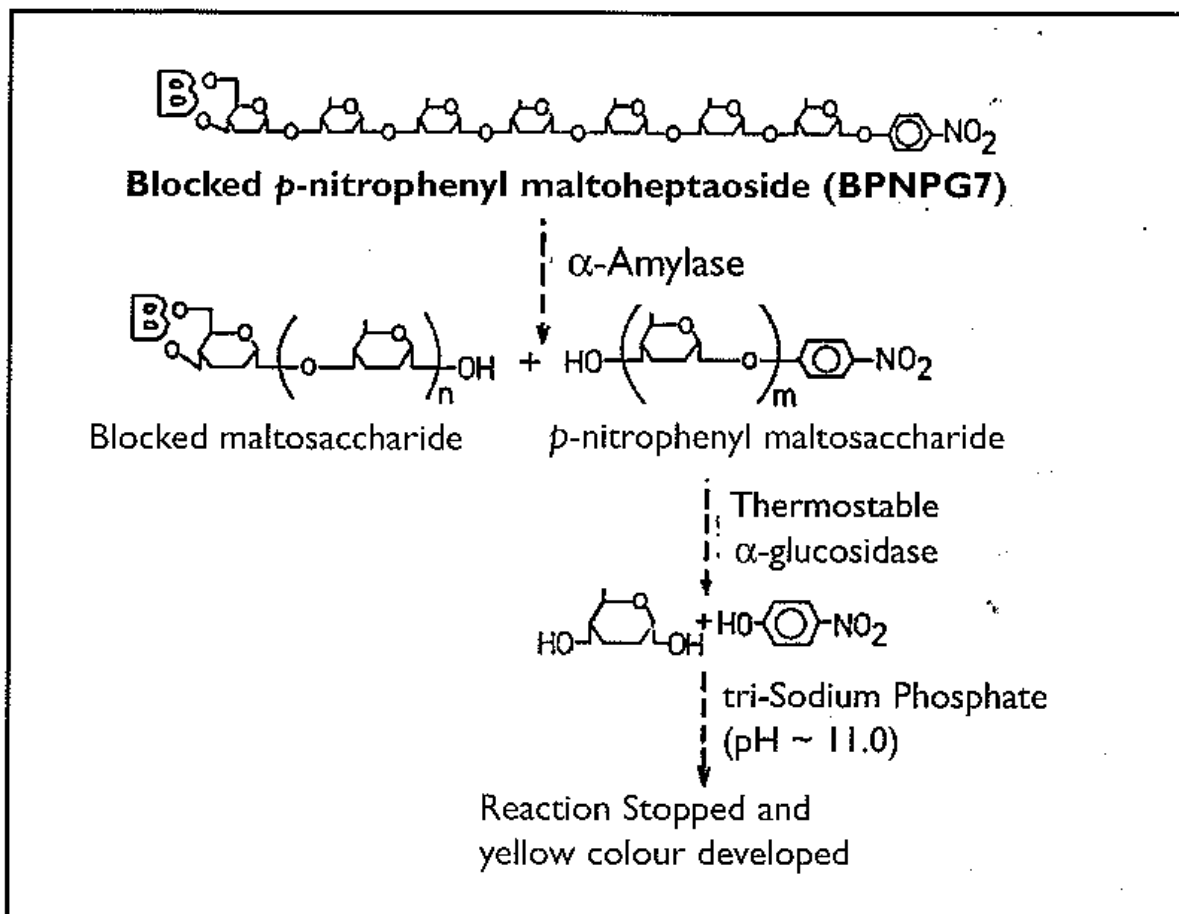
Ici nous allons uniquement renseigner les paragraphes 3 (définition) 4 (principe), 8.2 (répétabilité) et 9 (points critiques). Pour plus de détails, se référer à la procédure K-CERA en annexe.

3 DEFINITIONS

L' α -amylase est une enzyme qui hydrolyse les liaisons alpha-glucosidiques (α 1-4) présentes dans les polysaccharides comme l'amidon ou le glycogène. L' α -amylase catalyse l'hydrolyse de l'amidon en maltose et en dextrines. Elle coupe les liaisons α 1-4, au hasard : c'est une enzyme **liquéfiante** On trouve une α -amylase dans la salive (ptyaline) et une α -amylase dans le suc pancréatique.

4 PRINCIPE

La procédure mesure l'activité de l' α -amylase à l'aide d'un substrat spécifique (*p*-nitrophényl maltoheptaoside, BPNPG7) en présence d'un excès d' α -glucosidase thermostable. L'excès de d' α -glucosidase entraîne l'hydrolyse instantanée et quantitative des fragments de *p*-nitrophényl maltosaccharide en glucose et *p*-nitrophénol libre. Le schéma 1 résume la réaction d'hydrolyse. La réaction est stoppée (et la couleur jaune révélée) par addition d'une solution faiblement alcaline (Trizma) et l'absorbance mesurée à 400 nm ; elle est directement proportionnelle à l'activité α -amylasique de l'échantillon analysé.



Scheme 1. Theoretical basis of the Ceralpha α -amylase assay procedure. Immediately α -amylase cleaves a bond within the blocked p-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the p-nitrophenyl substituent is instantly cleaved to glucose and free p-nitrophenol by the excess quantities of thermostable α -glucosidase which is an integral part of the substrate mixture, and free p-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of tri-sodium phosphate (pH ~ 11.0).

5 REACTIFS

6 APPAREILLAGE

7 PROCEDURE

8 EXPRESSION DES RESULTATS

8.1 Mode de calcul et formules

8.2 Répétabilité

Le coefficient de variation peut atteindre 5%.

L'activité alpha-amylasique du malt de sorgho est plus faible (valeurs généralement inférieures à 100 UC/g de MS) que celle d'un malt d'orge (plus de 300 UC/g de MS).

9 POINTS CRITIQUES ET NOTE SUR LA PROCEDURE

Cette procédure est absolument sélective pour l' α -amylase.

Il est théoriquement recommandé d'obtenir une DO proche de 0,8 (cf kit). Pour des malts de sorgho traditionnel où l'activité alpha-amylasique est faible, la DO mesurée est inférieure 0,2. **Mais**, une réduction du taux de dilution (20 dans le protocole original) pour augmenter la DO mesurée est à **proscrire**, car aboutissant à une **sous-estimation** de l'activité alpha-amylasique.

10 RAPPORT D'ESSAI

Le rapport d'essai doit indiquer la méthode utilisée et les résultats obtenus. En outre seront détaillées toute condition opératoire non indiquée dans le SOP, ou optionnelle, et les circonstances particulières qui auraient pu affecter les résultats.

Le rapport d'essai doit inclure tous les détails nécessaires à une identification précise de l'échantillon.

Mesure de l'activité α -amylase dans les produits céréaliers fermentés

SOP Number: **Chem-Cere-006-fr**

Date : **16/09/2011**

Version:**2**

11 ENREGISTREMENT DES REVISIONS

| Date | Personne responsable | Description de la modification |
|------------|----------------------|--------------------------------|
| 16/05/2011 | Noël AKISSOE | Edition |
| | | |
| | | |

12 ANNEXES

Alpha-amylase assay procedure (Cerealalpha method, K-CERA 08/05, Megazyme International). **2004**

Megazyme

ALPHA-AMYLASE ASSAY PROCEDURE (CERALPHA METHOD)

K-CERA 08/05

FOR THE MEASUREMENT OF PLANT AND MICROBIAL ALPHA-AMYLASES

AOAC Method 2002.01
AACC Method 22-02
ICC Standard No. 303



INTRODUCTION:

Microbial α -amylases find widespread application in the modification of starch in cereal products and in cereal processing. The level of endogenous α -amylase in cereal grains and products significantly affects the industrial exploitation of these commodities. In bread-making, the level of α -amylase must be sufficient to produce saccharides which can be absorbed and utilised by yeast, but not so high as to cause excessive starch dextrinisation, which can lead to sticky crumb and problems in processing. In the brewing industry, the level of malt α -amylase is a key quality parameter. α -Amylase also finds application as a silage additive, to assist in the degradation of starch and thus to provide fermentable sugars for bacterial growth. Bacterial, fungal and cereal α -amylases can all be measured with Amylase HR reagent, however, assay conditions (specifically pH) need to be modified to suit each particular enzyme. Amylase HR Reagent is specific for α -amylase. The substrate is absolutely resistant to hydrolysis by *exo*-enzymes such as β -amylase, amyloglucosidase and α -glucosidase.

PRINCIPLE:

The Ceralpha procedure (employing Amylase HR reagent) for the assay of α -amylase, employs as substrate, the defined oligosaccharide “non-reducing-end blocked *p*-nitrophenyl maltoheptaoside” (BPNPG7) in the presence of excess levels of a thermostable α -glucosidase (which has no action on the native substrate due to the presence of the “blocking group”). On hydrolysis of the oligosaccharide by *endo*-acting α -amylase, the excess quantities of α -glucosidase present in the mixture give instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. The assay format is shown in Scheme 1 (page 17) and the linearity of the assay is shown in Figure 1 (page 11).

Essentially, an aliquot of a cereal flour extract or fermentation broth is incubated with substrate mixture under defined conditions, and the reaction is terminated (and colour developed) by the addition of a weak alkaline solution. The absorbance at 400 nm is measured (previously, absorbance values were measured at 410 nm in line with literature values, however, the true absorption peak is at 400 nm) (see Figure 4, page 12) and this relates directly to the level of α -amylase in the sample analysed.

Amylase HR Reagent mixture can be used to quantitatively assay cereal, fungal and bacterial α -amylases. With the replacement of amyloglucosidase and yeast α -glucosidase (as present in the original Ceralpha Reagent mixture) by thermostable α -glucosidase, the assay can now be used over a broader pH range (5.2 to 7.0) and at temperatures of up to 60°C. With this new reagent, the optimal pH

for activity of cereal α -amylases is 5.2-5.4 (see Figure 7). Furthermore, in this pH range, the activity values obtained for cereal α -amylases with Amylase HR reagent, are essentially the same as those obtained with Ceralpha reagent (containing amyloglucosidase and α -glucosidase) at pH 5.2. Reagent mixtures employing blocked *p*-nitrophenyl maltoheptaoside as substrate do not distinguish between fungal, cereal and bacterial α -amylases.

ACCURACY:

Standard errors of less than 5 % are achieved routinely.

KITS:

Kits suitable for performing 100/200 assays are available from Megazyme International Ireland Limited, and consist of:-

1. Full assay method;
2. Freeze dried BPNPG7 plus thermostable α -glucosidase;
3. Concentrated Extraction Buffer;
4. Concentrated Stopping Reagent;
5. Control Malt Flour.

SPECIFICITY:

The assay is absolutely specific for α -amylase.

Table 1: Reproducibility of the Ceralpha assay for the measurement of wheat-flour α -amylase^a

| Sample | Absorbance (400 nm) | | | | | | | | | |
|---------------------|---------------------|-------|--------|-------|--------|-------|--------|-------|---------|--|
| | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Average | |
| A | 0.365 | 0.390 | 0.365 | 0.354 | 0.384 | 0.379 | 0.385 | 0.398 | 0.378 | |
| B | 0.486 | 0.534 | 0.463 | 0.502 | 0.502 | 0.507 | 0.514 | 0.486 | 0.499 | |
| C | 0.255 | 0.259 | 0.270 | 0.286 | 0.265 | 0.287 | 0.264 | 0.284 | 0.271 | |
| D | 0.142 | 0.146 | 0.143 | 0.150 | 0.142 | 0.137 | 0.135 | 0.153 | 0.143 | |
| S.E.M. ^b | 0.0134 | | 0.0134 | | 0.0134 | | 0.0134 | | 0.0067 | |

^aDuplicate analyses of single extracts made on four separate days.

^bBased on a pooled estimate of the variance for each sample mean.

s.d. of single observation (for comparisons on same and different days) = 0.0189.

c.v. (%) = 4.05.

ENCLOSED SUBSTRATE:

Blocked *p*-nitrophenyl maltoheptaoside (BPNPG7, 54.5 mg)
Thermostable α -glucosidase (125 U at pH 6.0), per vial.

Dissolve the entire contents of one vial in 10.0 mL of distilled water. Divide into 2-3 mL aliquots and store frozen between use. At 0-5°C the dissolved substrate is stable for seven days; in the frozen state it is stable for at least 12 months.

ENCLOSED MALT FLOUR:

Malt flour of standardised α -amylase activity (as specified on the vial label). It is recommended that the user standardises at least one batch of user's own wheat or malt flour to be employed as a secondary reference flour.

ENCLOSED SOLUTIONS:

(1) Concentrated Extraction Buffer: 1 M sodium malate
(Buffer A) 1 M sodium chloride
40 mM calcium chloride
0.1 % sodium azide

Dilute the entire contents (50 mL) (plus a crystalline precipitate which may be present) to 1000 mL with distilled water before use. Stable at 0-5°C for 12 months. The pH should be 5.4; adjust if necessary.

(2) Concentrated Stopping Reagent: [20% (w/v) tri-sodium phosphate solution, pH ~11]

Dilute the entire contents (25 mL) to 500 mL with distilled water. Stable at room temperature for three months.

PREPARATION OF ADDITIONAL EXTRACTION BUFFERS:

A. Buffer A (for cereal and fungal α -amylase)

| | |
|--|-------------------|
| Malic acid (Sigma M-0875; 1 M) | 134.1 grams/litre |
| Sodium hydroxide | 70 grams/litre |
| Sodium chloride | 58.4 grams/litre |
| Calcium chloride.2H ₂ O (40 mM) | 5.9 grams/litre |
| Sodium azide (Sigma S2002; 0.1 %) | 1.0 grams/litre |

Add malic acid, sodium chloride and sodium hydroxide to 800 mL of distilled water, allow to cool to room temperature and add the calcium chloride. Adjust the pH to 5.4 by dropwise addition of sodium hydroxide (4 M) or HCl (4 M). **Then** add the sodium azide. Adjust volume to 1 litre. Store at room temperature. **For use, dilute 50 mL of this concentrated buffer solution to 1 litre with dist. water.**

CAUTION

Dissolve the reagents and adjust the pH to 5.4 before adding the sodium azide. Adding sodium azide to an acidic solution results in the release of a poisonous gas.

Powdered malic and maleic acids are irritants, and thus should be handled with due care.

B. Buffer B (for *Bacillus* sp. α -amylase).

| | |
|--|---------------------|
| Maleic acid (Sigma M-0375; 0.1 M) | 23.2 grams/2 litres |
| Sodium chloride | 11.6 grams/2 litres |
| Calcium chloride. 2H ₂ O (2 mM) | 0.6 grams/2 litres |
| Sodium azide (Sigma S-2002; 0.01 % w/v) | 0.2 grams/2 litres |

Add the maleic acid and sodium chloride to 1600 mL of distilled water and adjust the pH to 6.5 with 4 M (160 g/litre) sodium hydroxide. Add the calcium chloride and sodium azide and adjust the volume to 2 litres. Store at room temperature between use.

Use this buffer directly without further dilution.

Some bacterial α -amylases are unstable on dilution. This problem is usually resolved by inclusion of BSA (0.5 mg/mL) in the buffer.

PREPARATION OF ADDITIONAL STOPPING REAGENT:

Dissolve 10 g of tri-sodium phosphate (anhydrous) in 1 litre of distilled water and adjust the pH to approx. 11.0. Stable at room temperature for at least three months.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL and 20 mL capacity).
2. Pipettors, 0.1 and/or 0.2 mL (e.g. Gilson Pipetman®) to dispense enzyme extract and substrate.
3. Adjustable-volume dispenser:
 - 0-10 mL (for Extraction Buffer)
 - 0-5 mL (for Stopping Reagent).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
 - with 5.0 mL Combitip® (to dispense 0.5 mL aliquots of concentrated enzyme solutions), and
 - with 25 mL Combitip® (to dispense various aliquots of dilution buffers).

5. Top-pan balance
6. Spectrophotometer set at 400 nm.
7. Vortex mixer (optional).
8. Thermostated water bath set at 40.0°C.
9. Stop Clock.
10. Bench centrifuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).

CONTROLS AND PRECAUTIONS:

1. α -Amylase is an enzyme present at high levels in all body fluids. It is thus recommended that disposable gloves are used when handling and dispensing the substrate mixture.
2. It is essential that the water used to dissolve the Ceralpha substrate mixture is high purity. If freshly distilled water is not available, heat the water to boiling and cool it to less than 30°C before using. Algal growth in water in wash bottles can produce sufficient α -amylase to significantly reduce the long-term stability of the reagent dissolved in such water.
3. The freeze-dried substrate is extremely stable at room temperature, however, when dissolved it should be stored at 0-5°C during use and at -20°C between use. If the number of assays performed at any one time is limited, it is recommended that the substrate be divided into 2-3 mL aliquots and stored in the frozen state.
4. On storage at 0-5°C, the blank absorbance values will increase from 0.03 to about 0.05 in 5 days, this does not affect the performance of the substrate, but obviously these values must be determined at the same time as the assay is performed. Blank absorbance values as high as 0.50 do not affect the reliability or accuracy of the assay.

NOTE

A single Reaction Blank only is normally sufficient for each batch of samples being analysed. To obtain this blank value, 3.0 mL of **stopping reagent** should be added to 0.2 mL of **substrate** solution. Then add 0.2 mL of **enzyme** preparation.

5. The spectrophotometer employed should be standardised with a *p*-nitrophenol standard in 1% tri-sodium phosphate ($E_{mM} = 18.1$). *p*-Nitrophenol solution (10 μ moles per mL) can be obtained from Sigma Chemical Company (cat no. 104-1). An aliquot of this solution when diluted 200-fold in 1 % tri-sodium phosphate gives an absorbance of 0.905 at 400 nm.
6. The assay format should be standardised with the enclosed malt flour. The activity of this flour is shown on the enclosed vial. **A wheat flour standard can be provided on request.**
7. The time of extraction of **wheat flours** should be carefully controlled (15-20 min). With **malt flour** samples, the optimal extraction time is also 15-20 min.

USEFUL HINTS:

1. If the absorbance values for a particular assay are greater than 1.20, the enzyme extract should be diluted with the appropriate buffer and re-assayed.
2. The number of assays which can be performed per kit can be doubled by halving the volumes of all reagents used and employing semi-micro spectrophotometer cuvettes.

ENZYME EXTRACTION:

A. Wheat and Barley Flours:

1. Mill wheat, barley or other grain (approximately 10-50 g sample) to pass a 0.5 mm screen (e.g. with a Fritsch centrifugal mill).
2. Accurately weigh 3.0 g of flour into a flask of 50 mL capacity.
3. To each flask add 20.0 mL of Extraction Buffer solution (pH 5.4) and stir the flask contents vigorously.
4. Allow the enzyme to extract over 15-20 min at 40°C, with occasional mixing.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge an aliquot at 1,000 g for ten minutes. **Assay enzyme activity** within two hours.

NOTE

The control wheat flour from Megazyme is very homogeneous. Consequently, a smaller sample weight can be extracted (e.g. 1.0 g per 6.0 mL of extraction buffer).

B. Malt Flours:

1. Mill malt (20 g sample) to pass a 0.5 mm screen.
2. Accurately weigh 0.5 g malt flour into a 100 mL volumetric flask.
3. To the volumetric flask add a solution of 1% sodium chloride plus 0.02% calcium chloride plus 0.02% sodium azide; adjust to volume.
4. Allow the enzyme to extract for 15-20 min at room temperature, with occasional stirring.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge at 1,000 g for ten minutes.
6. Dilute 0.5 mL of the filtrate with 9.5 mL of Extraction Buffer Solution. Assay activity within two hours.

C. Microbial Preparations:

Liquid preparations:

1. Add 1 mL of liquid enzyme preparation (using a positive displacement dispenser) to **buffer A or B** (49 mL, pH 5.4 or 6.5) and mix thoroughly. This is termed the **Original Extract**.
2. Dilute 1.0 mL of **original extract** 10-fold by addition to 9.0 mL of appropriate **buffer (A or B)** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.
For example, for the industrial enzyme preparation, Bacterial Alpha-Amylase (from Kerry Ingredients, Ireland) a dilution of the **original extract** of approximately 4,000-fold is required.

Powder preparations:

1. Add 1 g of enzyme powder preparation to 50 mL of **buffer A or B** (pH 5.4 or 6.5) and gently stir the slurry over a period of about 15 min or until the sample is completely dispersed or dissolved.
2. Clarify this solution (the **Original Extract**) by centrifugation (1,000 g, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles.
3. Dilute 1.0 mL of this solution 10-fold by addition to 9.0 mL of appropriate **extraction/ dilution buffer** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.

ASSAY PROCEDURE:

A. Wheat and barley flours:

1. Dispense 0.2 mL aliquots of **Amylase HR Reagent** Solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate cereal extract at 40°C for 5 min.
3. To each tube containing **Amylase HR Reagent** solution (0.2 mL), add 0.2 mL of pre-equilibrated wheat or barley extract directly to the bottom of the tube. Incubate at 40°C for exactly twenty (20) min (from time of addition).
4. At the end of the **20 min** incubation period, add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

B. Malt and microbial preparations:

1. Dispense 0.2 mL aliquots of **Amylase HR Reagent** solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate buffered malt or microbial preparation extract at 40°C for 5 min.
3. To each tube containing **Amylase HR Reagent** Solution (0.2 mL), add 0.2 mL of pre-equilibrated (and suitably diluted) microbial enzyme or malt extract directly to the bottom of the tube. Incubate at 40°C for exactly **10 min** (from time of addition).
4. At the end of the **10 min** incubation period, add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α -glucosidase, required to release one micromole of *p*-nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed a **Ceralpha Unit**.

Units/g Flour:

$$= \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{E_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

Where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min (malt and microbial preparation extracts)
= 20 min (wheat and barley extracts)

Total Volume in Cell = 3.4 mL

Aliquot Assayed = 0.2 mL

E_{mM} of *p*-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1

Extraction volume = 20 mL per 3 gram (wheat and barley)
100 mL per 0.5 gram (malt)
50mL per 1g or 1mL of microbial preparation.

Dilution = Dilution of the original extract
(= 20-fold for malt extracts).

Thus:

A. For Wheat and Barley:

Units (CU)/g flour:

$$\begin{aligned} &= \frac{\Delta E_{400}}{20} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{20}{3.0} \\ &= \Delta \Delta E_{400} \times 0.313 \end{aligned}$$

B. For Malt:

Units (CU)/gram of milled malt:

$$\begin{aligned} &= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{100}{0.5} \times 20 \\ &= \Delta E_{400} \times 376 \end{aligned}$$

C. For microbial preparations:

Units (CU)/ml or gram of original preparation:

$$\begin{aligned} &= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{50}{1.0} \times \text{Dilution} \\ &= \Delta E_{400} \times 4.7 \times \text{Dilution} \end{aligned}$$

where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total volume in cell = 3.4 mL

Aliquot Assayed = 0.2 mL

E_{mM} of *p*-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1

Extraction vol = 50mL/1.0g (or 49mL plus 1mL of enzyme concentrate).

Dilution = further dilution of the **Original Extract**.

APPENDIX:

A. Linearity of Ceralpha assay with enzyme concentration and incubation time.

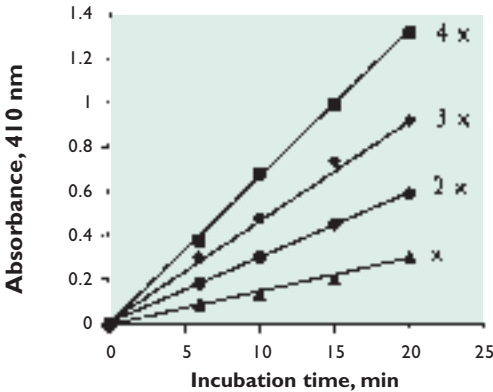


Figure 1. Linearity of the the Ceralpha assay with malt α -amylase in sodium malate buffer (pH 5.4). The assay was performed with four concentrations of enzyme (1x, 2x, 3x and 4x). Reaction was terminated at various times by adding tri-sodium phosphate (3.0 mL, 1% w/v).

B. Effect of the concentration of thermostable α -glucosidase in the reagent solution on determined α -amylase values.

From the results shown in Figure 2, it is evident that the concentration of α -glucosidase required to saturate the reaction is 12 Units/mL in the substrate solution.

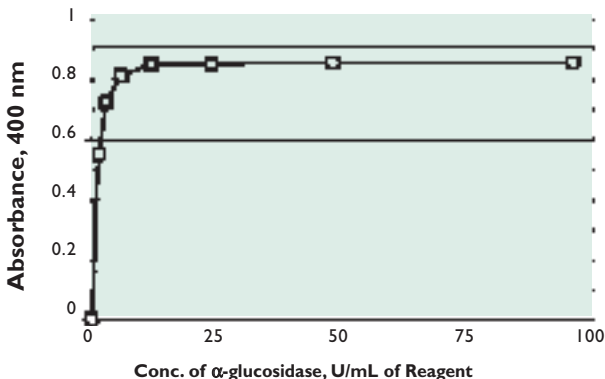


Figure 2. The effect of the concentration of α -glucosidase in the substrate reagent solution on the determined absorbance values.

C. Stability of reagent mixture at 60°C.

The stability of the reagent solution was determined by incubating aliquots of this solution at 60°C for 0-20 min. These solutions were then used to assay the activity of fungal α -amylase (at 40°C). From the data shown in Figure 3, it is evident that the reagent is very stable at 60°C. Over a 20 min incubation period, blank absorbance values increased by less than 0.01 absorbance units, and the determined activity decreased by less than 3% (of the non pre-incubated reagent).

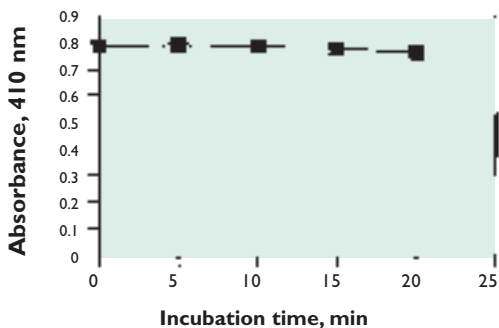


Figure 3. Temperature stability of Amylase HR assay reagent. Aliquots of the reagent were stored at 60°C for 0-20 min, cooled to room temperature, and used to assay fungal α -amylase at 40°C.

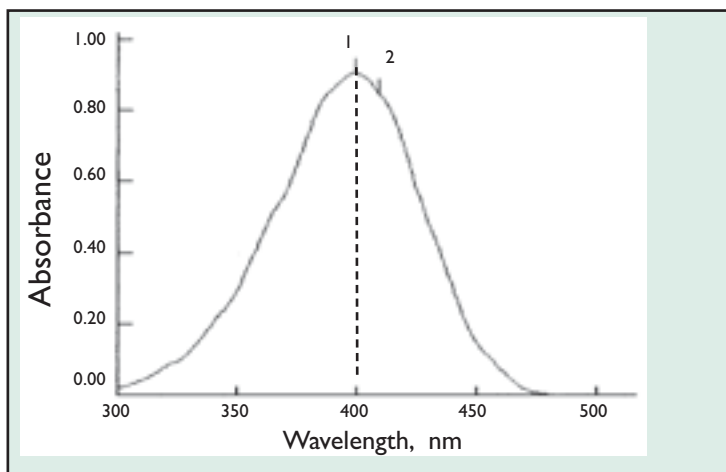


Figure 4. Absorbance curve for *p*-nitrophenol in 1% tri-sodium phosphate at pH 11.0

D. Conversion of Ceralpha Units (CU) to International Units (IU) on Starch Substrate.

The activity of pure *Bacillus subtilis*, *Aspergillus niger* and barley malt α -amylases on Amylase HR Reagent and on ACS soluble starch (1 % w/v; assayed using the Nelson-Somogyi reducing sugar procedure; International Units) were determined and the conversion factors are:

A. niger (both assays performed at pH 5.4)
International Units on Starch = 0.94 x Ceralpha Units.

B. subtilis (both assays performed at pH 6.5)
International Units on Starch = 4.6 x Ceralpha Units.

Barley malt (both assays performed at pH 5.4)
International Units on Starch = 4.1 x Ceralpha Units.

One International Unit (IU) of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar equivalents per minute under defined conditions of temperature and pH.

E. Comparison of the Ceralpha and Farrand Methods for the Measurement of Wheat and Fungal α -Amylase.

The Farrand method employs a β -limit dextrin of starch as substrate, and measures the decrease in the colour of the starch/iodine complex on depolymerisation of the substrate. The assay is performed in the presence of excess quantities of β -amylase, which for cereal samples, originate from the flour extract. For fungal samples, a pure β -amylase has to be added. The Farrand method was commonly used in the United Kingdom and employed a β -limit dextrin preparation supplied by Rank Hovis. [A purified β -limit dextrin (maltose removed) is now available from Megazyme International].

In the standard Farrand method, the extract is unbuffered and the pH of the flour extracts is approx. 5.8. In an interlaboratory comparison of the Farrand and Ceralpha methods, coordinated by Campden-Chorleywood Food Research Association, the correlation between Farrand and Ceralpha Units for **wheat flour** α -amylase was found to be:

Farrand Units = Ceralpha Units x 57 - 1.9.

A very similar correlation for wheat α -amylase was previously reported by McCleary and Sheehan (1987), namely:

$$\text{Farrand Units} = \text{Ceralpha Units} \times 57.1.$$

For fungal preparations, the regression equation obtained by McCleary *et al.* was:

$$\text{Farrand Units} = \text{Ceralpha Units} \times 69.$$

The measurement of α -amylase in wheat flour supplemented with fungal α -amylase is complicated by the problems associated with the blending of two components, one of which has a low level of enzyme, and the other of which has a level several thousand-fold higher than the first component. To try to minimise the errors associated with possible incomplete mixing of these components, duplicate samples should be assayed, and larger samples should be extracted (~6g/40mL).

F. Comparison of the Ceralpha Method (CU), the ASBC Method (DU) and AACC Method 22-01 (SKB Units) for the Measurement of α -Amylase:

AACC method 22-01 (SKB Units) uses a β -limit dextrin prepared from a “special” lintner starch supplied by AACC/ASBC. The method measures the time to reach a particular colour with iodine, on incubation of α -amylase with the β -limit dextrin at 30°C.

The ASBC/EBC/International Method (Dextrinising Units, DU) uses the same substrate and the same concentration of enzyme as employed in AACC Method 22-01, and the Units of activity are calculated the same way. However, since the assay is performed at 20°C, DU values for a particular malt sample are approximately half the SKB value for the same malt.

The correlation between AACC Method 22-01 (SKB Units) and Ceralpha Units (CU) for malt flours is shown in Figure 5. The correlation between the ASBC (International Method) for α -amylase (DU) and the Ceralpha method (CU) is shown in Figure 6.

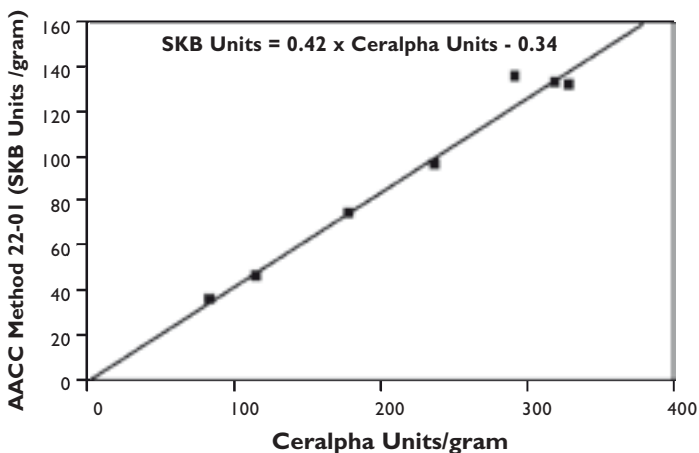


Figure 5. Comparison of the Ceralpha method and AACC Method 22-01 (SKB) for the measurement of α -amylase in malt flours. Seven malt samples were analysed in duplicate by the two methods.

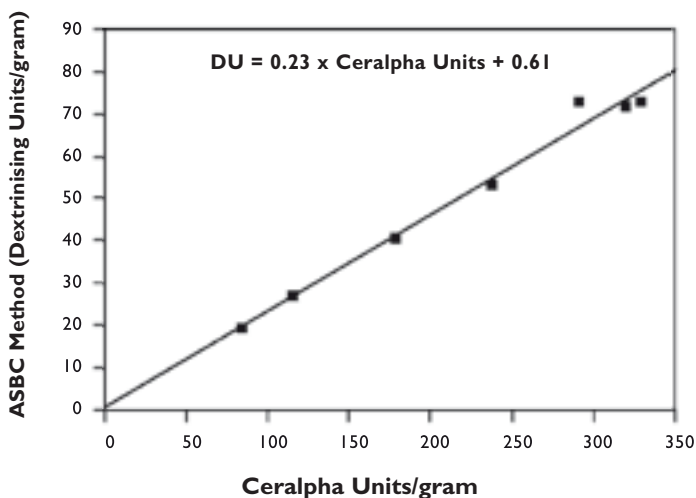


Figure 6. Comparison of the Ceralpha and the ASBC (International) methods for the measurement of α -amylase in malt flours. Seven malt samples were analysed in duplicate by the two methods.

The conversion factors relating SKB Units to Ceralpha Units for malt, fungal and bacterial α -amylases are:

Malt α -amylase:

$$\text{SKB Units} = 0.42 \times \text{Ceralpha Units (CU)} - 0.34.$$

(SKB performed at pH 4.7; Ceralpha performed at pH 5.4).

Fungal α -amylase:

$$\text{SKB Units} = 0.60 \times \text{Ceralpha Units (CU)}.$$

(SKB performed at pH 5.4; Ceralpha performed at pH 5.4).

Bacterial α -amylase:

$$\text{SKB Units} = 1.8 \times \text{Ceralpha Units (CU)}.$$

(SKB performed at pH 6.5; Ceralpha performed at pH 6.5).

G. pH Activity Curves for Cereal, Fungal and Bacterial α -Amylases.

pH Activity curves for malted wheat, malted barley, fungal (*A. niger*) and bacterial (*B. subtilis*) α -amylases were determined using the Ceralpha method with Amylase HR Reagent. For cereal and fungal α -amylases, the pH curve was prepared using malate and maleate buffers (100 mM, pH 5.0-6.4); for bacterial α -amylase, maleate and Bis-Tris Propane buffers were employed (pH 5.6-9.0). All buffers contained 10 mM calcium chloride. The curves for purified malted wheat and fungal α -amylases are shown in Figure 7. Malted barley α -amylases gave the same curve as malted wheat. The pH activity curve for *B. subtilis* α -amylase is shown in Figure 8.

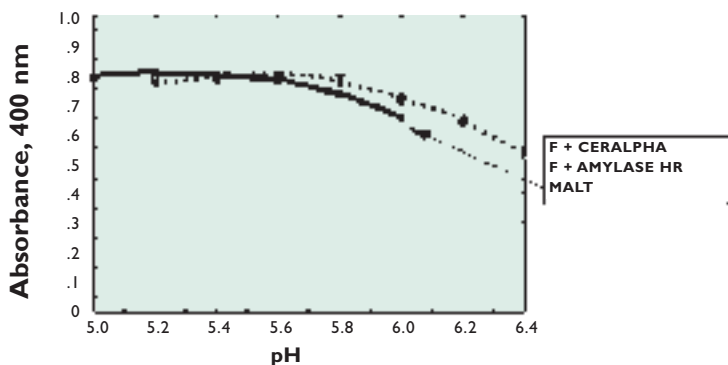


Figure 7. pH activity curves for fungal (*A.niger*) and malted wheat α -amylases. Fungal α -amylase was assayed with both Amylase HR Reagent and Cereal α -Amylase assay reagent (containing amyloglucosidase and yeast α -glucosidase). The curves with the two reagents were the same.

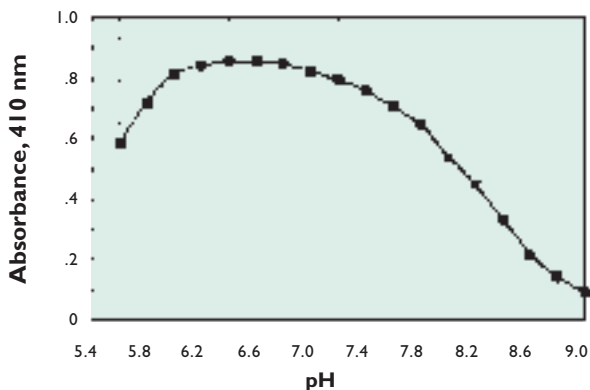
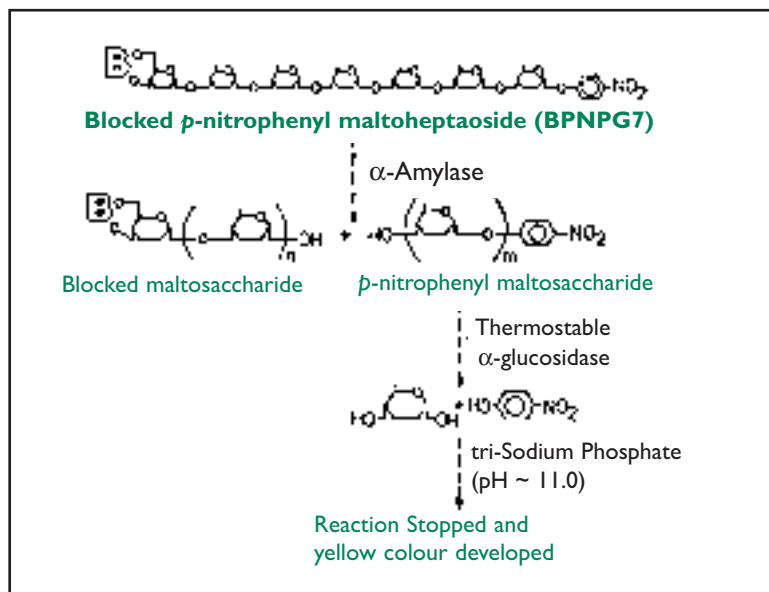


Figure 8. pH activity curves for *B. subtilis* α -amylase.



Scheme 1. Theoretical basis of the Ceralpha α -amylase assay procedure. Immediately α -amylase cleaves a bond within the blocked *p*-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the *p*-nitrophenyl substituent is instantly cleaved to glucose and free *p*-nitrophenol by the excess quantities of thermostable α -glucosidase which is an integral part of the substrate mixture, and free *p*-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of tri-sodium phosphate (pH ~ 11.0).

References:

McCleary, B.V. & Sheehan, H. (1987). Measurement of Cereal α -Amylase: A New Assay Procedure. *Journal of Cereal Science*, **6**, 237-251.

Sheehan, H. & McCleary, B.V. (1988). A New Procedure for the Measurement of Fungal and Bacterial α -Amylase. *Biotechnology Techniques*, **2**, 289-292.

McCleary, B.V., McNally, M., Monaghan, D. & Mugford, D.C (2002) Measurement of α -amylase activity in white wheat flour, milled malt, and microbial enzyme preparations using the Ceralpha Assay. collaborative study. *J. AOAC International*, **85**, 1096-1102.



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Procedure for measuring α -amylase activity in cereal fermented foods

SOP: Chem-Cere-006--en

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Procedure for measuring α -amylase activity in cereal fermented foods

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Date: **16/09/2011**

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1 SCOPE AND APPLICATION

This method is for the determination of α -amylase activity in cereal products.

2 REFERENCES

Megazyme International. **2004**. Alpha-amylase assay procedure (Cerealpha method) K-CERA 08/05 for the measurement of plant and microbial alpha-amylases. *Megazyme International Ireland Limited*.

This SOP only covers subsections 3 (definition), 4 (principle), 8.2 (repeatability) and 9 (critical points). For details, see the “Ceralpha” procedure in appendix

3 DEFINITIONS

Alpha-amylase is an enzyme which hydrolyzes α 1-4 glycosidic bonds present in polysaccharides such as starch or glycogen. Alpha-amylase catalyzes the hydrolysis of starch into maltose and dextrans. It hydrolyzes α -1-4 links at random (endo-enzyme): it is a liquefying enzyme. An α -amylase is found in saliva (ptyaline), and an- α amylase is also found in pancreatic juice..

4 PRINCIPLE

The proposed method measures the activity of α -amylase, based on the transformation of the defined oligosaccharide “non-reducing-end blocked p-nitrophenyl maltoheptaoside” (BPNPG7) into glucose and free p-nitrophenol (in excess of α -glucosidase, Figure 1).

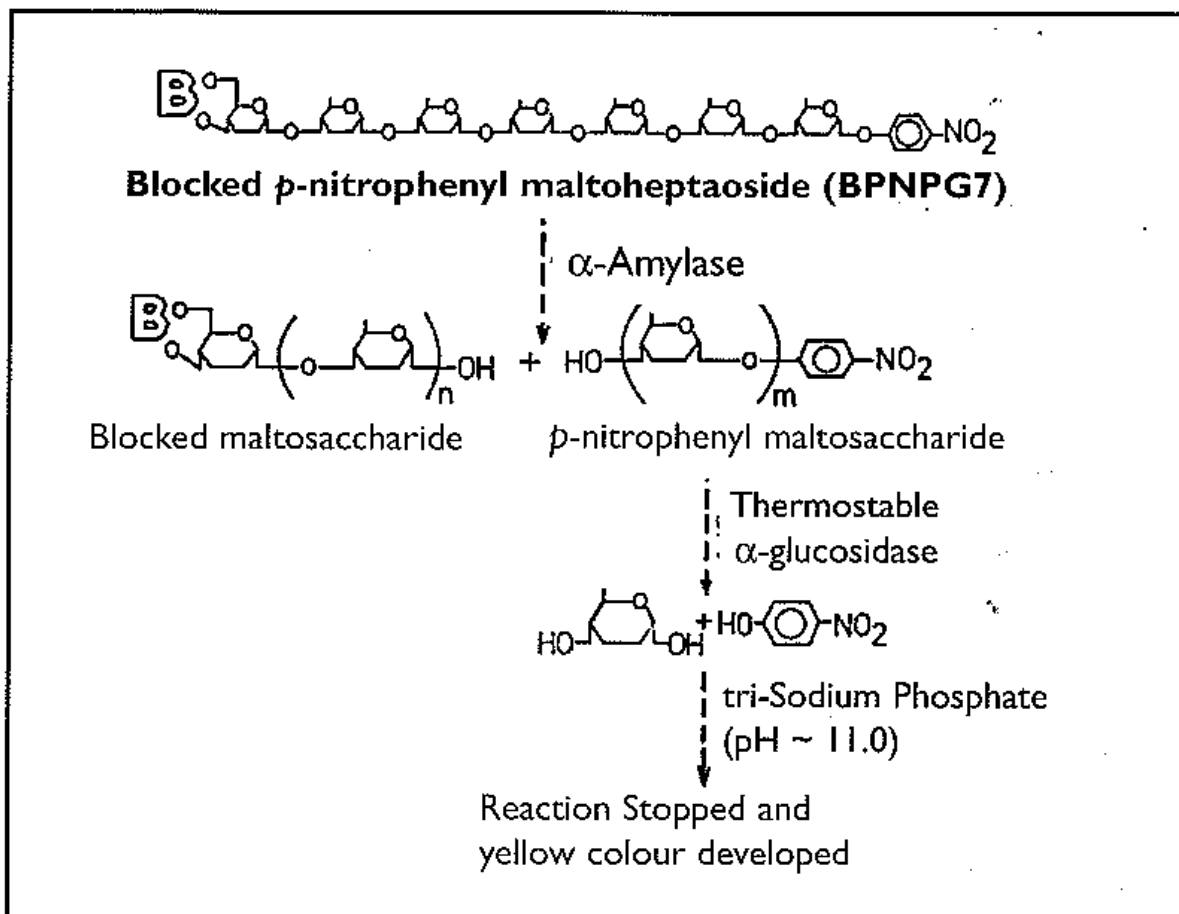
The reaction is terminated (and colour developed) by the addition of a weak alkaline solution (Trizma) and the absorbance measured at 400 nm; it relates directly to the level of α -amylase in the analysed sample.

Procedure for measuring α -amylase activity in cereal fermented foods

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Scheme 1. Theoretical basis of the Ceralpha α -amylase assay procedure. Immediately α -amylase cleaves a bond within the blocked p-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the p-nitrophenyl substituent is instantly cleaved to glucose and free p-nitrophenol by the excess quantities of thermostable α -glucosidase which is an integral part of the substrate mixture, and free p-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of tri-sodium phosphate (pH ~ 11.0).

5 REAGENTS

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6 APPARATUS

7 PROCEDURE

8 EXPRESSION OF RESULTS

8.1 Method of calculation and formulae

8.2 Repeatability

The coefficient of variation can reach 5%.

Alpha-amylase activity of sorghum malt is lower (usually below 100 MS UC/g dry basis) than that of barley malt (more than 300 MS UC/g).

9 CRITICAL POINTS OR NOTE ON THE PROCEDURE

This procedure is selective for only α -amylase.

It is theoretically recommended to obtain an OD near 0.8 (cf kit). For a traditional sorghum malt where alpha-amylase activity is low, the measured OD is less than 0.2. But, a reduction in the rate of dilution (20 in the original procedure) to increase the OD is **not acceptable**, because it leads to an **underestimation** of alpha-amylase activity.

10 TEST REPORT

The test report shall indicate the method used and the results obtained. In addition, it shall mention all operating conditions not specified in the international procedure, or regarded as optional, as well as any circumstances that may have influenced the results.

The test report shall include all details necessary for the complete identification for the sample.

Procedure for measuring α -amylase activity in cereal fermented foods

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11 REVISION RECORD

| Date | Responsible person | Description of change |
|------------|--------------------|-----------------------|
| 16/05/2011 | Noël AKISSOE | Editing |
| | | |
| | | |

12 APPENDIX

Alpha-amylase assay procedure (Cerealpha method, K-CERA 08/05, Megazyme International). **2004**

Megazyme

ALPHA-AMYLASE ASSAY PROCEDURE (CERALPHA METHOD)

K-CERA 08/05

FOR THE MEASUREMENT OF PLANT AND MICROBIAL ALPHA-AMYLASES

AOAC Method 2002.01
AACC Method 22-02
ICC Standard No. 303



INTRODUCTION:

Microbial α -amylases find widespread application in the modification of starch in cereal products and in cereal processing. The level of endogenous α -amylase in cereal grains and products significantly affects the industrial exploitation of these commodities. In bread-making, the level of α -amylase must be sufficient to produce saccharides which can be absorbed and utilised by yeast, but not so high as to cause excessive starch dextrinisation, which can lead to sticky crumb and problems in processing. In the brewing industry, the level of malt α -amylase is a key quality parameter. α -Amylase also finds application as a silage additive, to assist in the degradation of starch and thus to provide fermentable sugars for bacterial growth. Bacterial, fungal and cereal α -amylases can all be measured with Amylase HR reagent, however, assay conditions (specifically pH) need to be modified to suit each particular enzyme. Amylase HR Reagent is specific for α -amylase. The substrate is absolutely resistant to hydrolysis by *exo*-enzymes such as β -amylase, amyloglucosidase and α -glucosidase.

PRINCIPLE:

The Ceralpha procedure (employing Amylase HR reagent) for the assay of α -amylase, employs as substrate, the defined oligosaccharide “non-reducing-end blocked *p*-nitrophenyl maltoheptaoside” (BPNPG7) in the presence of excess levels of a thermostable α -glucosidase (which has no action on the native substrate due to the presence of the “blocking group”). On hydrolysis of the oligosaccharide by *endo*-acting α -amylase, the excess quantities of α -glucosidase present in the mixture give instantaneous and quantitative hydrolysis of the *p*-nitrophenyl maltosaccharide fragment to glucose and free *p*-nitrophenol. The assay format is shown in Scheme 1 (page 17) and the linearity of the assay is shown in Figure 1 (page 11).

Essentially, an aliquot of a cereal flour extract or fermentation broth is incubated with substrate mixture under defined conditions, and the reaction is terminated (and colour developed) by the addition of a weak alkaline solution. The absorbance at 400 nm is measured (previously, absorbance values were measured at 410 nm in line with literature values, however, the true absorption peak is at 400 nm) (see Figure 4, page 12) and this relates directly to the level of α -amylase in the sample analysed.

Amylase HR Reagent mixture can be used to quantitatively assay cereal, fungal and bacterial α -amylases. With the replacement of amyloglucosidase and yeast α -glucosidase (as present in the original Ceralpha Reagent mixture) by thermostable α -glucosidase, the assay can now be used over a broader pH range (5.2 to 7.0) and at temperatures of up to 60°C. With this new reagent, the optimal pH

for activity of cereal α -amylases is 5.2-5.4 (see Figure 7). Furthermore, in this pH range, the activity values obtained for cereal α -amylases with Amylase HR reagent, are essentially the same as those obtained with Ceralpha reagent (containing amyloglucosidase and α -glucosidase) at pH 5.2. Reagent mixtures employing blocked *p*-nitrophenyl maltoheptaoside as substrate do not distinguish between fungal, cereal and bacterial α -amylases.

ACCURACY:

Standard errors of less than 5 % are achieved routinely.

KITS:

Kits suitable for performing 100/200 assays are available from Megazyme International Ireland Limited, and consist of:-

1. Full assay method;
2. Freeze dried BPNPG7 plus thermostable α -glucosidase;
3. Concentrated Extraction Buffer;
4. Concentrated Stopping Reagent;
5. Control Malt Flour.

SPECIFICITY:

The assay is absolutely specific for α -amylase.

Table 1: Reproducibility of the Ceralpha assay for the measurement of wheat-flour α -amylase^a

| Sample | Absorbance (400 nm) | | | | | | | | | |
|---------------------|---------------------|-------|--------|-------|--------|-------|--------|-------|---------|--|
| | Day 1 | | Day 2 | | Day 3 | | Day 4 | | Average | |
| A | 0.365 | 0.390 | 0.365 | 0.354 | 0.384 | 0.379 | 0.385 | 0.398 | 0.378 | |
| B | 0.486 | 0.534 | 0.463 | 0.502 | 0.502 | 0.507 | 0.514 | 0.486 | 0.499 | |
| C | 0.255 | 0.259 | 0.270 | 0.286 | 0.265 | 0.287 | 0.264 | 0.284 | 0.271 | |
| D | 0.142 | 0.146 | 0.143 | 0.150 | 0.142 | 0.137 | 0.135 | 0.153 | 0.143 | |
| S.E.M. ^b | 0.0134 | | 0.0134 | | 0.0134 | | 0.0134 | | 0.0067 | |

^aDuplicate analyses of single extracts made on four separate days.

^bBased on a pooled estimate of the variance for each sample mean.

s.d. of single observation (for comparisons on same and different days) = 0.0189.

c.v. (%) = 4.05.

ENCLOSED SUBSTRATE:

Blocked *p*-nitrophenyl maltoheptaoside (BPNPG7, 54.5 mg)
Thermostable α -glucosidase (125 U at pH 6.0), per vial.

Dissolve the entire contents of one vial in 10.0 mL of distilled water. Divide into 2-3 mL aliquots and store frozen between use. At 0-5°C the dissolved substrate is stable for seven days; in the frozen state it is stable for at least 12 months.

ENCLOSED MALT FLOUR:

Malt flour of standardised α -amylase activity (as specified on the vial label). It is recommended that the user standardises at least one batch of user's own wheat or malt flour to be employed as a secondary reference flour.

ENCLOSED SOLUTIONS:

(1) Concentrated Extraction Buffer: 1 M sodium malate
(Buffer A) 1 M sodium chloride
40 mM calcium chloride
0.1 % sodium azide

Dilute the entire contents (50 mL) (plus a crystalline precipitate which may be present) to 1000 mL with distilled water before use. Stable at 0-5°C for 12 months. The pH should be 5.4; adjust if necessary.

(2) Concentrated Stopping Reagent: [20% (w/v) tri-sodium phosphate solution, pH ~11]

Dilute the entire contents (25 mL) to 500 mL with distilled water. Stable at room temperature for three months.

PREPARATION OF ADDITIONAL EXTRACTION BUFFERS:

A. Buffer A (for cereal and fungal α -amylase)

| | |
|--|-------------------|
| Malic acid (Sigma M-0875; 1 M) | 134.1 grams/litre |
| Sodium hydroxide | 70 grams/litre |
| Sodium chloride | 58.4 grams/litre |
| Calcium chloride.2H ₂ O (40 mM) | 5.9 grams/litre |
| Sodium azide (Sigma S2002; 0.1 %) | 1.0 grams/litre |

Add malic acid, sodium chloride and sodium hydroxide to 800 mL of distilled water, allow to cool to room temperature and add the calcium chloride. Adjust the pH to 5.4 by dropwise addition of sodium hydroxide (4 M) or HCl (4 M). **Then** add the sodium azide. Adjust volume to 1 litre. Store at room temperature. **For use, dilute 50 mL of this concentrated buffer solution to 1 litre with dist. water.**

CAUTION

Dissolve the reagents and adjust the pH to 5.4 before adding the sodium azide. Adding sodium azide to an acidic solution results in the release of a poisonous gas.

Powdered malic and maleic acids are irritants, and thus should be handled with due care.

B. Buffer B (for *Bacillus* sp. α -amylase).

| | |
|--|---------------------|
| Maleic acid (Sigma M-0375; 0.1 M) | 23.2 grams/2 litres |
| Sodium chloride | 11.6 grams/2 litres |
| Calcium chloride. 2H ₂ O (2 mM) | 0.6 grams/2 litres |
| Sodium azide (Sigma S-2002; 0.01 % w/v) | 0.2 grams/2 litres |

Add the maleic acid and sodium chloride to 1600 mL of distilled water and adjust the pH to 6.5 with 4 M (160 g/litre) sodium hydroxide. Add the calcium chloride and sodium azide and adjust the volume to 2 litres. Store at room temperature between use.

Use this buffer directly without further dilution.

Some bacterial α -amylases are unstable on dilution. This problem is usually resolved by inclusion of BSA (0.5 mg/mL) in the buffer.

PREPARATION OF ADDITIONAL STOPPING REAGENT:

Dissolve 10 g of tri-sodium phosphate (anhydrous) in 1 litre of distilled water and adjust the pH to approx. 11.0. Stable at room temperature for at least three months.

EQUIPMENT (RECOMMENDED):

1. Glass test tubes (12 mL and 20 mL capacity).
2. Pipettors, 0.1 and/or 0.2 mL (e.g. Gilson Pipetman®) to dispense enzyme extract and substrate.
3. Adjustable-volume dispenser:
 - 0-10 mL (for Extraction Buffer)
 - 0-5 mL (for Stopping Reagent).
4. Positive displacement pipettor e.g. Eppendorf Multipette®
 - with 5.0 mL Combitip® (to dispense 0.5 mL aliquots of concentrated enzyme solutions), and
 - with 25 mL Combitip® (to dispense various aliquots of dilution buffers).

5. Top-pan balance
6. Spectrophotometer set at 400 nm.
7. Vortex mixer (optional).
8. Thermostated water bath set at 40.0°C.
9. Stop Clock.
10. Bench centrifuge or Whatman GF/A glass fibre filter paper circles (9 cm diameter).

CONTROLS AND PRECAUTIONS:

1. α -Amylase is an enzyme present at high levels in all body fluids. It is thus recommended that disposable gloves are used when handling and dispensing the substrate mixture.
2. It is essential that the water used to dissolve the Ceralpha substrate mixture is high purity. If freshly distilled water is not available, heat the water to boiling and cool it to less than 30°C before using. Algal growth in water in wash bottles can produce sufficient α -amylase to significantly reduce the long-term stability of the reagent dissolved in such water.
3. The freeze-dried substrate is extremely stable at room temperature, however, when dissolved it should be stored at 0-5°C during use and at -20°C between use. If the number of assays performed at any one time is limited, it is recommended that the substrate be divided into 2-3 mL aliquots and stored in the frozen state.
4. On storage at 0-5°C, the blank absorbance values will increase from 0.03 to about 0.05 in 5 days, this does not affect the performance of the substrate, but obviously these values must be determined at the same time as the assay is performed. Blank absorbance values as high as 0.50 do not affect the reliability or accuracy of the assay.

NOTE

A single Reaction Blank only is normally sufficient for each batch of samples being analysed. To obtain this blank value, 3.0 mL of **stopping reagent** should be added to 0.2 mL of **substrate** solution. Then add 0.2 mL of **enzyme** preparation.

5. The spectrophotometer employed should be standardised with a *p*-nitrophenol standard in 1% tri-sodium phosphate ($E_{mM} = 18.1$). *p*-Nitrophenol solution (10 μ moles per mL) can be obtained from Sigma Chemical Company (cat no. 104-1). An aliquot of this solution when diluted 200-fold in 1 % tri-sodium phosphate gives an absorbance of 0.905 at 400 nm.
6. The assay format should be standardised with the enclosed malt flour. The activity of this flour is shown on the enclosed vial. **A wheat flour standard can be provided on request.**
7. The time of extraction of **wheat flours** should be carefully controlled (15-20 min). With **malt flour** samples, the optimal extraction time is also 15-20 min.

USEFUL HINTS:

1. If the absorbance values for a particular assay are greater than 1.20, the enzyme extract should be diluted with the appropriate buffer and re-assayed.
2. The number of assays which can be performed per kit can be doubled by halving the volumes of all reagents used and employing semi-micro spectrophotometer cuvettes.

ENZYME EXTRACTION:

A. Wheat and Barley Flours:

1. Mill wheat, barley or other grain (approximately 10-50 g sample) to pass a 0.5 mm screen (e.g. with a Fritsch centrifugal mill).
2. Accurately weigh 3.0 g of flour into a flask of 50 mL capacity.
3. To each flask add 20.0 mL of Extraction Buffer solution (pH 5.4) and stir the flask contents vigorously.
4. Allow the enzyme to extract over 15-20 min at 40°C, with occasional mixing.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge an aliquot at 1,000 g for ten minutes. **Assay enzyme activity** within two hours.

NOTE

The control wheat flour from Megazyme is very homogeneous. Consequently, a smaller sample weight can be extracted (e.g. 1.0 g per 6.0 mL of extraction buffer).

B. Malt Flours:

1. Mill malt (20 g sample) to pass a 0.5 mm screen.
2. Accurately weigh 0.5 g malt flour into a 100 mL volumetric flask.
3. To the volumetric flask add a solution of 1% sodium chloride plus 0.02% calcium chloride plus 0.02% sodium azide; adjust to volume.
4. Allow the enzyme to extract for 15-20 min at room temperature, with occasional stirring.
5. Filter an aliquot of the solution through a Whatman GF/A glass fibre filter paper, or centrifuge at 1,000 g for ten minutes.
6. Dilute 0.5 mL of the filtrate with 9.5 mL of Extraction Buffer Solution. Assay activity within two hours.

C. Microbial Preparations:

Liquid preparations:

1. Add 1 mL of liquid enzyme preparation (using a positive displacement dispenser) to **buffer A or B** (49 mL, pH 5.4 or 6.5) and mix thoroughly. This is termed the **Original Extract**.
2. Dilute 1.0 mL of **original extract** 10-fold by addition to 9.0 mL of appropriate **buffer (A or B)** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.
For example, for the industrial enzyme preparation, Bacterial Alpha-Amylase (from Kerry Ingredients, Ireland) a dilution of the **original extract** of approximately 4,000-fold is required.

Powder preparations:

1. Add 1 g of enzyme powder preparation to 50 mL of **buffer A or B** (pH 5.4 or 6.5) and gently stir the slurry over a period of about 15 min or until the sample is completely dispersed or dissolved.
2. Clarify this solution (the **Original Extract**) by centrifugation (1,000 g, 10 min) or filtration through Whatman No. 1 (9 cm) filter circles.
3. Dilute 1.0 mL of this solution 10-fold by addition to 9.0 mL of appropriate **extraction/ dilution buffer** and mix thoroughly. Repeat this step until a dilution suitable for assay is obtained.

ASSAY PROCEDURE:

A. Wheat and barley flours:

1. Dispense 0.2 mL aliquots of **Amylase HR Reagent** Solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate cereal extract at 40°C for 5 min.
3. To each tube containing **Amylase HR Reagent** solution (0.2 mL), add 0.2 mL of pre-equilibrated wheat or barley extract directly to the bottom of the tube. Incubate at 40°C for exactly twenty (20) min (from time of addition).
4. At the end of the **20 min** incubation period, add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

B. Malt and microbial preparations:

1. Dispense 0.2 mL aliquots of **Amylase HR Reagent** solution (unbuffered) into test tubes and pre-incubate the tubes and contents at 40°C for 5 min.
2. Pre-incubate buffered malt or microbial preparation extract at 40°C for 5 min.
3. To each tube containing **Amylase HR Reagent** Solution (0.2 mL), add 0.2 mL of pre-equilibrated (and suitably diluted) microbial enzyme or malt extract directly to the bottom of the tube. Incubate at 40°C for exactly **10 min** (from time of addition).
4. At the end of the **10 min** incubation period, add exactly 3.0 mL of Stopping Reagent and stir the tube contents vigorously.
5. Read the absorbance of the solutions and the reaction blank at 400 nm against distilled water.

CALCULATION OF ACTIVITY:

One Unit of activity is defined as the amount of enzyme, in the presence of excess thermostable α -glucosidase, required to release one micromole of *p*-nitrophenol from BPNPG7 in one minute under the defined assay conditions, and is termed a **Ceralpha Unit**.

Units/g Flour:

$$= \frac{\Delta E_{400}}{\text{Incubation Time}} \times \frac{\text{Total Volume in Cell}}{\text{Aliquot Assayed}} \times \frac{1}{E_{mM}} \times \frac{\text{Extraction Vol.}}{\text{Sample Weight}} \times \text{Dilution}$$

Where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min (malt and microbial preparation extracts)
= 20 min (wheat and barley extracts)

Total Volume in Cell = 3.4 mL

Aliquot Assayed = 0.2 mL

E_{mM} of *p*-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1

Extraction volume = 20 mL per 3 gram (wheat and barley)
100 mL per 0.5 gram (malt)
50mL per 1g or 1mL of microbial preparation.

Dilution = Dilution of the original extract
(= 20-fold for malt extracts).

Thus:

A. For Wheat and Barley:

Units (CU)/g flour:

$$\begin{aligned} &= \frac{\Delta E_{400}}{20} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{20}{3.0} \\ &= \Delta \Delta E_{400} \times 0.313 \end{aligned}$$

B. For Malt:

Units (CU)/gram of milled malt:

$$\begin{aligned} &= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{100}{0.5} \times 20 \\ &= \Delta E_{400} \times 376 \end{aligned}$$

C. For microbial preparations:

Units (CU)/ml or gram of original preparation:

$$\begin{aligned} &= \frac{\Delta E_{400}}{10} \times \frac{3.4}{0.2} \times \frac{1}{18.1} \times \frac{50}{1.0} \times \text{Dilution} \\ &= \Delta E_{400} \times 4.7 \times \text{Dilution} \end{aligned}$$

where:

ΔE_{400} = Absorbance (reaction) - Absorbance (blank)

Incubation Time = 10 min

Total volume in cell = 3.4 mL

Aliquot Assayed = 0.2 mL

E_{mM} of *p*-nitrophenol (at 400 nm) in 1% tri-sodium phosphate = 18.1

Extraction vol = 50mL/1.0g (or 49mL plus 1mL of enzyme concentrate).

Dilution = further dilution of the **Original Extract**.

APPENDIX:

A. Linearity of Ceralpha assay with enzyme concentration and incubation time.

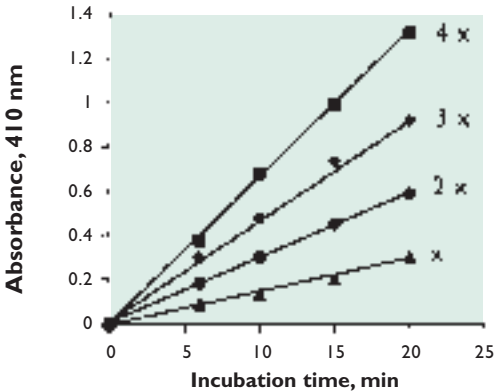


Figure 1. Linearity of the the Ceralpha assay with malt α -amylase in sodium malate buffer (pH 5.4). The assay was performed with four concentrations of enzyme (1x, 2x, 3x and 4x). Reaction was terminated at various times by adding tri-sodium phosphate (3.0 mL, 1% w/v).

B. Effect of the concentration of thermostable α -glucosidase in the reagent solution on determined α -amylase values.

From the results shown in Figure 2, it is evident that the concentration of α -glucosidase required to saturate the reaction is 12 Units/mL in the substrate solution.

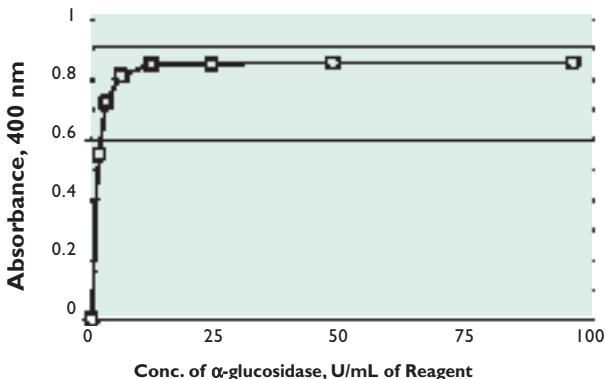


Figure 2. The effect of the concentration of α -glucosidase in the substrate reagent solution on the determined absorbance values.

C. Stability of reagent mixture at 60°C.

The stability of the reagent solution was determined by incubating aliquots of this solution at 60°C for 0-20 min. These solutions were then used to assay the activity of fungal α -amylase (at 40°C). From the data shown in Figure 3, it is evident that the reagent is very stable at 60°C. Over a 20 min incubation period, blank absorbance values increased by less than 0.01 absorbance units, and the determined activity decreased by less than 3% (of the non pre-incubated reagent).

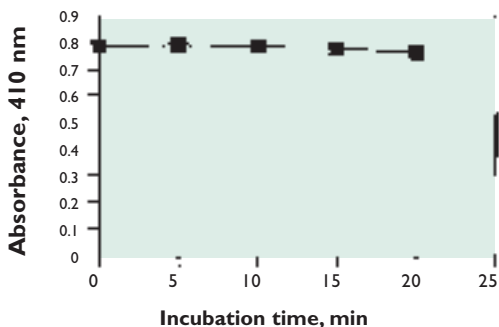


Figure 3. Temperature stability of Amylase HR assay reagent. Aliquots of the reagent were stored at 60°C for 0-20 min, cooled to room temperature, and used to assay fungal α -amylase at 40°C.

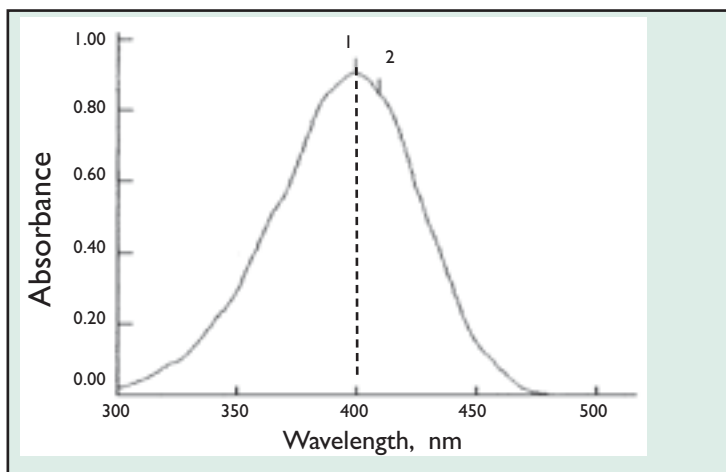


Figure 4. Absorbance curve for *p*-nitrophenol in 1% tri-sodium phosphate at pH 11.0

D. Conversion of Ceralpha Units (CU) to International Units (IU) on Starch Substrate.

The activity of pure *Bacillus subtilis*, *Aspergillus niger* and barley malt α -amylases on Amylase HR Reagent and on ACS soluble starch (1 % w/v; assayed using the Nelson-Somogyi reducing sugar procedure; International Units) were determined and the conversion factors are:

A. niger (both assays performed at pH 5.4)
International Units on Starch = 0.94 x Ceralpha Units.

B. subtilis (both assays performed at pH 6.5)
International Units on Starch = 4.6 x Ceralpha Units.

Barley malt (both assays performed at pH 5.4)
International Units on Starch = 4.1 x Ceralpha Units.

One International Unit (IU) of activity is defined as the amount of enzyme required to release one micromole of glucose reducing-sugar equivalents per minute under defined conditions of temperature and pH.

E. Comparison of the Ceralpha and Farrand Methods for the Measurement of Wheat and Fungal α -Amylase.

The Farrand method employs a β -limit dextrin of starch as substrate, and measures the decrease in the colour of the starch/iodine complex on depolymerisation of the substrate. The assay is performed in the presence of excess quantities of β -amylase, which for cereal samples, originate from the flour extract. For fungal samples, a pure β -amylase has to be added. The Farrand method was commonly used in the United Kingdom and employed a β -limit dextrin preparation supplied by Rank Hovis. [A purified β -limit dextrin (maltose removed) is now available from Megazyme International].

In the standard Farrand method, the extract is unbuffered and the pH of the flour extracts is approx. 5.8. In an interlaboratory comparison of the Farrand and Ceralpha methods, coordinated by Campden-Chorleywood Food Research Association, the correlation between Farrand and Ceralpha Units for **wheat flour** α -amylase was found to be:

Farrand Units = Ceralpha Units x 57 - 1.9.

A very similar correlation for wheat α -amylase was previously reported by McCleary and Sheehan (1987), namely:

$$\text{Farrand Units} = \text{Ceralpha Units} \times 57.1.$$

For fungal preparations, the regression equation obtained by McCleary *et al.* was:

$$\text{Farrand Units} = \text{Ceralpha Units} \times 69.$$

The measurement of α -amylase in wheat flour supplemented with fungal α -amylase is complicated by the problems associated with the blending of two components, one of which has a low level of enzyme, and the other of which has a level several thousand-fold higher than the first component. To try to minimise the errors associated with possible incomplete mixing of these components, duplicate samples should be assayed, and larger samples should be extracted (~6g/40mL).

F. Comparison of the Ceralpha Method (CU), the ASBC Method (DU) and AACC Method 22-01 (SKB Units) for the Measurement of α -Amylase:

AACC method 22-01 (SKB Units) uses a β -limit dextrin prepared from a “special” lintner starch supplied by AACC/ASBC. The method measures the time to reach a particular colour with iodine, on incubation of α -amylase with the β -limit dextrin at 30°C.

The ASBC/EBC/International Method (Dextrinising Units, DU) uses the same substrate and the same concentration of enzyme as employed in AACC Method 22-01, and the Units of activity are calculated the same way. However, since the assay is performed at 20°C, DU values for a particular malt sample are approximately half the SKB value for the same malt.

The correlation between AACC Method 22-01 (SKB Units) and Ceralpha Units (CU) for malt flours is shown in Figure 5. The correlation between the ASBC (International Method) for α -amylase (DU) and the Ceralpha method (CU) is shown in Figure 6.

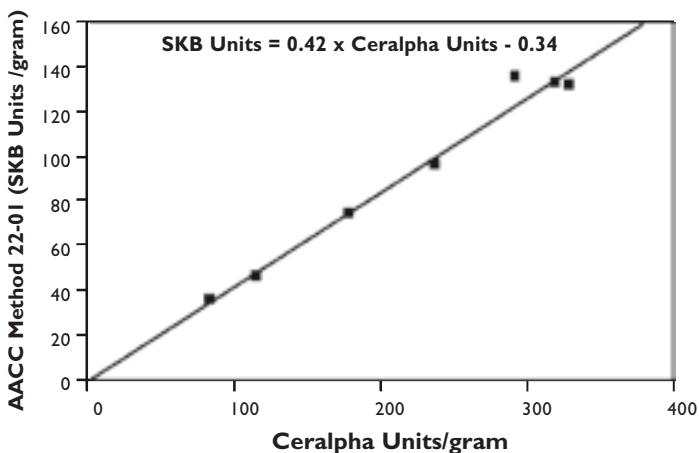


Figure 5. Comparison of the Ceralpha method and AACC Method 22-01 (SKB) for the measurement of α -amylase in malt flours. Seven malt samples were analysed in duplicate by the two methods.

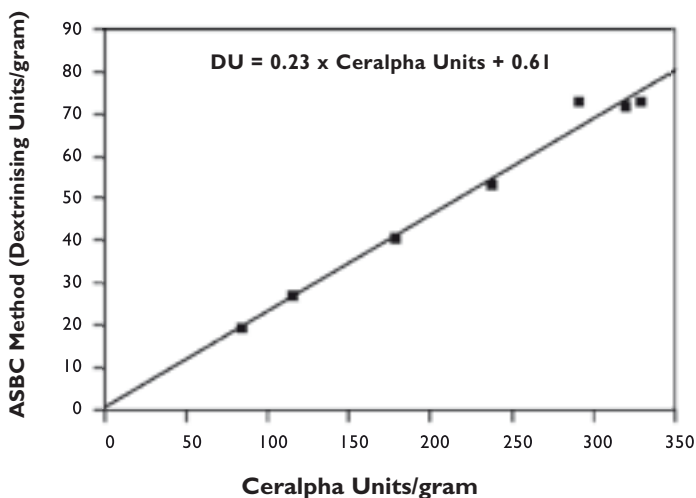


Figure 6. Comparison of the Ceralpha and the ASBC (International) methods for the measurement of α -amylase in malt flours. Seven malt samples were analysed in duplicate by the two methods.

The conversion factors relating SKB Units to Ceralpha Units for malt, fungal and bacterial α -amylases are:

Malt α -amylase:

$$\text{SKB Units} = 0.42 \times \text{Ceralpha Units (CU)} - 0.34.$$

(SKB performed at pH 4.7; Ceralpha performed at pH 5.4).

Fungal α -amylase:

$$\text{SKB Units} = 0.60 \times \text{Ceralpha Units (CU)}.$$

(SKB performed at pH 5.4; Ceralpha performed at pH 5.4).

Bacterial α -amylase:

$$\text{SKB Units} = 1.8 \times \text{Ceralpha Units (CU)}.$$

(SKB performed at pH 6.5; Ceralpha performed at pH 6.5).

G. pH Activity Curves for Cereal, Fungal and Bacterial α -Amylases.

pH Activity curves for malted wheat, malted barley, fungal (*A. niger*) and bacterial (*B. subtilis*) α -amylases were determined using the Ceralpha method with Amylase HR Reagent. For cereal and fungal α -amylases, the pH curve was prepared using malate and maleate buffers (100 mM, pH 5.0-6.4); for bacterial α -amylase, maleate and Bis-Tris Propane buffers were employed (pH 5.6-9.0). All buffers contained 10 mM calcium chloride. The curves for purified malted wheat and fungal α -amylases are shown in Figure 7. Malted barley α -amylases gave the same curve as malted wheat. The pH activity curve for *B. subtilis* α -amylase is shown in Figure 8.

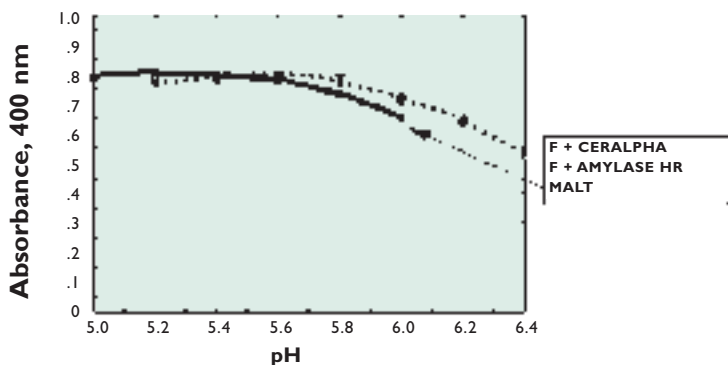


Figure 7. pH activity curves for fungal (*A.niger*) and malted wheat α -amylases. Fungal α -amylase was assayed with both Amylase HR Reagent and Cereal α -Amylase assay reagent (containing amyloglucosidase and yeast α -glucosidase). The curves with the two reagents were the same.

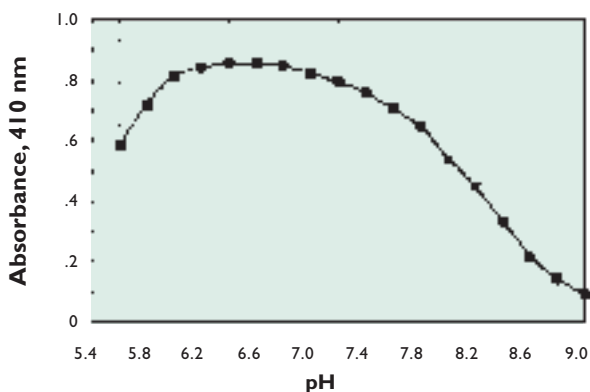
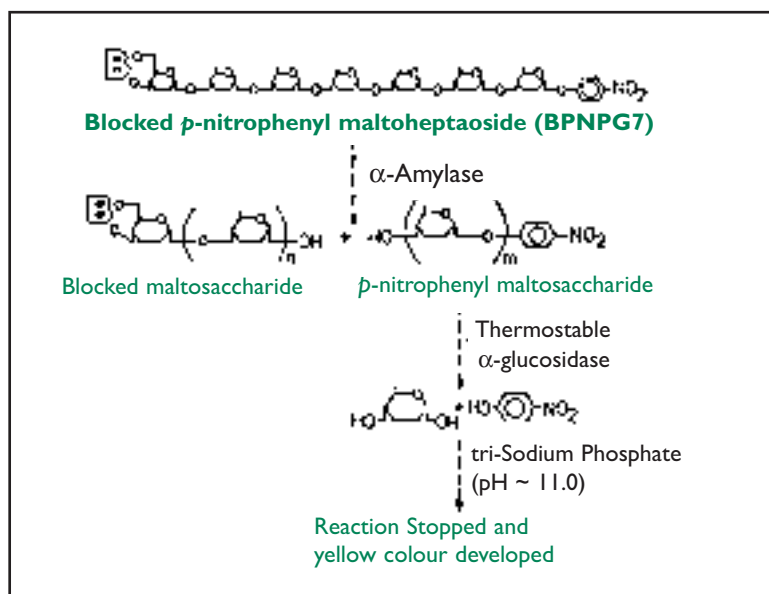


Figure 8. pH activity curves for *B. subtilis* α -amylase.



Scheme 1. Theoretical basis of the Ceralpha α -amylase assay procedure. Immediately α -amylase cleaves a bond within the blocked *p*-nitrophenyl maltosaccharide substrate, the non-blocked reaction product containing the *p*-nitrophenyl substituent is instantly cleaved to glucose and free *p*-nitrophenol by the excess quantities of thermostable α -glucosidase which is an integral part of the substrate mixture, and free *p*-nitrophenol is released. The reaction is terminated and the phenolate colour is developed on addition of tri-sodium phosphate (pH ~ 11.0).

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