Analytical Methods

for the Soup Industry

EDITED AND ISSUED BY THE
TECHNICAL COMMISSION OF THE INTERNATIONAL ASSOCIATION
OF THE BOUILLON AND
SOUP INDUSTRY (AIIBP)

Published on behalf of the
International Association of the Bouillon and Soup Industry (AIIBP)
by the
Association of Swiss Soup and Sauce Manufacturers
Elfenstrasse 19, CH-3000 Berne 16

Internet Edition January 2006

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GLOSSARY

This glossary was compiled to facilitate use of the manual. Consequently, the reader need no longer refer to texts and documents which are not always at hand.
The definitions are given in table form, which eliminates the need for some of them to be repeated in each method.
English, French and German equivalents of the terms are given.
The glossary contains the following tables:
- definitions of sampling and samples
- definitions of accuracy and precision
- definitions of repeatability
- definitions of reproducibility
- equivalent terms for solutions of defined concentrations
- prefixes and symbols for naming decimal multiples and decimal submultiples of units
- units and symbols

GLOSSAIRE

Ce glossaire a été préparé pour rendre l'utilisation du manuel plus commode. Ainsi le lecteur n'a plus besoin de se reporter à des textes et à des documents qui ne sont pas toujours sous la main.
Les définitions sont présentées sous forme de tableaux, ce qui évite la répétition de certaines d'entre elles dans chaque méthode.
L'équivalence des termes est donnée en anglais, en français et en allemand.
Le glossaire comprend les tableaux suivants:
- définitions de l'échantillonnage et des échantillons
- définitions de la justesse et de la fidélité
- définition de la répétabilité
- définition de la reproductibilité
- equivalence des termes pour les solutions de concentrations définies
- préfixes et symboles pour désigner multiples et sous-multiples décimaux d'unités
- unités et symboles

GLOSSAR

Dieser Glossar wurde erstellt, um den Gebrauch des Handbuches zu erleichtern. Zum Verständnis bedarf es infolgedessen nicht mehr eines Nachschlagewerkes, das oftmals nicht zur Verfügung steht.
Die Definitionen sind in Tabellen gefasst, wodurch ihre Wiederholung innerhalb der einzelnen Methoden vermieden wird.
Die Begriffe sind in englischer, französischer und deutscher Sprache angegeben.
Der Glossar enthält folgende Tabellen:
- Definition der Probenahme und Probe
- Definition der Richtigkeit und Genauigkeit
- Definition der Wiederholbarkeit
- Definition der Reproduzierbarkeit
- Begriffe für Lösungen von definierten Konzentrationen
- Vorsatzzeichen und Symbole zur Bezeichnung von dezimalen Vielfachen und dezimalen Teilen von Einheiten
- Einheiten und Symbole

GENERAL TERMS RELATING TO METHODS OF SAMPLING

Sampling:

The procedure used to draw or constitute a sample.

Sample:

One or more items taken from a population and intended to provide information on the population and possibly to serve as a basis for a decision on the population or on the process which had produced it.

Laboratory sample:

A sample intended for laboratory inspection or testing.

Test sample:

A sample as prepared for testing.

Test portion:

The quantity of product actually drawn from the test sample (or, if both are the same, from the laboratory sample) and on which the test is actually carried out.

TERMES GÉNÉRAUX RELATIFS AUX MÉTHODES D'ÉCHANTILLONNAGE

Echantillonnage:

Procédure utilisée pour tirer ou constituer un échantillon.

Echantillon:

Un ou plusieurs individus prélevés dans une population et destinés à fournir une information sur la population et, éventuellement, à servir de base à une décision concernant la population ou le procédé qui l'a produite.

Echantillon pour laboratoire:

Echantillon destiné à être utilisé pour un contrôle ou pour des essais en laboratoire.

Echantillon pour essai:

Echantillon dans l'état de préparation où il est soumis à l'essai.

Prise d'essai:

Quantité de produit effectivement prélevée de l'échantillon pour essai (ou, s'il est identique, de l'échantillon pour laboratoire) et sur laquelle sera effectivement effectuée l'analyse.

GENERELLE BEGRIFFE FÜR PROBENAHMEVERFAHREN

Probenahme:

Verfahren, das zur Entnahme einer Probe oder zur Bildung derselben angewendet wird.

Probe:

Eine oder mehrere aus einer Gesamtheit gezogenen Einheiten, die dazu dienen sollen, Informationen über die Gesamtheit zu erhalten; die Information soll die Grundlage zu Entscheidungen über die Gesamtheit oder über das zu ihrer Herstellung angewandte Verfahren sein.

Laboratoriumsprobe:

Eine Probe, die zur Untersuchung oder Bestimmung im Laboratorium bestimmt ist.

Analysenprobe:

Die zur Durchführung der Analyse vorbereitete Probe.

Probemenge:

Diejenige Substanzmenge der Analysenprobe (oder der Laboratoriumsprobe, falls diese mit der Analysenprobe identisch ist), die zur Durchführung der Analyse tatsächlich verwendet wird.

ACCURACY OF THE MEAN

Closeness of agreement between the true value and the mean result which would be obtained by applying the experimental procedure a very large number of times.
The procedure is the more accurate as the systematic part of the experimental errors which affect the results is smaller.

JUSTESSE

Etroitesse de l'accord entre la valeur vraie et le résultat moyen qui serait obtenu en appliquant le procédé expérimental un grand nombre de fois.
Le procédé est d'autant plus juste que la partie systématique des erreurs expérimentales qui affectent les résultats est moindre.

RICHTIGKEIT

Grad der Übereinstimmung des wahren Wertes mit dem Durchschnittswert bei oftmaliger Anwendung der Verfahrensweise.
Die Verfahrensweise ist um so richtiger (akkurater), je kleiner der systematische Anteil an experimentellen, die Ergebnisse beeinflussenden Fehlern ist.

PRECISION

Closeness of agreement between the results obtained by applying the experimental procedure several times under prescribed conditions.
The smaller the random part of the experimental errors, which affect the results, the more precise is the procedure.

FIDÉLITÉ

Etroitesse de l'accord entre les résultats obtenus en appliquant le procédé expérimental à plusieurs reprises dans des conditions déterminées.
Le procédé est d'autant plus fidèle que la partie aléatoire des erreurs expérimentales qui affectent les résultats est moindre.

GENAUIGKEIT

Grad der Übereinstimmung von Ergebnissen, welche bei wiederholter Anwendung der Verfahrensweise unter den vorgeschriebenen Bedingungen erhalten wurden.
Je kleiner der zufällige Anteil an experimentellen Fehlern ist, um so genauer ist die Verfahrensweise.

REPEATABILITY

Qualitatively:

Closeness of agreement between successive results obtained with the same method on identical test material and under the same conditions (same operator, same apparatus, same laboratory and short intervals of time).

Quantitatively:

The value below which the absolute difference between two single test results obtained in the above conditions may be expected to lie with a specified probability.
In the absence of other indications, the probability is 95%.

Expression:

The difference between the results obtained from two or more determinations carried out simultaneously or immediately one after the other should not exceed .....g/100 g test sample.

ISO 3534-1977 (E/F)

RÉPÉTABILITÉ

Qualitativement:

Etroitesse de l'accord entre les résultats successifs obtenus avec la même méthode sur une matière identique soumise à l'essai et dans les mêmes conditions (même opérateur, même appareil, même laboratoire et court intervalle de temps).

Quantitativement:

Valeur au-dessous de laquelle est située, avec une probabilité spécifiée, la valeur absolue de la différence entre deux résultats individuels obtenus dans les conditions ci-dessus.
En l'absence d'indication, la probabilité est de 95%.

Expression:

La différence entre les résultats de deux ou plusieurs dosages effectués simultanément ou immédiatement l'un après l'autre ne doit pas dépasser .....g/100 g d'échantillon pour essai.

WIEDERHOLBARKEIT

Qualitativ:

Der Grad der Übereinstimmung aufeinanderfolgender Ergebnisse, welche mit derselben Methode an identischem Prüfmaterial und unter gleichen Bedingungen erhalten wurden (derselbe Sachbearbeiter, dasselbe Gerät, dasselbe Laboratorium, kurze Zeitspanne).

Quantitativ:

Derjenige Wert, unterhalb dessen man die absolute Differenz zwischen zwei einzelnen Prüfergebnissen, die man unter obigen Bedingungen erhalten hat, mit einer vorgegebenen Wahrscheinlichkeit erwarten kann.
Wenn nichts anderes angegeben ist, so ist diese Wahrscheinlichkeit 95%.

Angabe:

Die Differenz zwischen den Ergebnissen von gleichzeitig oder unmittelbar nacheinander durchgeführten Bestimmungen soll nicht grösser sein als .....g/100 g Analysenprobe.

Entwurf DIN/ISO 5725

REPRODUCIBILITY

Qualitatively:

Closeness of agreement between individual results obtained with the same method on identical test material but under different conditions (different operators, different apparatuses, different laboratories and/or different times).

Quantitatively:

The value below which the absolute difference between two single test results obtained in the above conditions may be expected to lie with a specified probability.
In the absence of other indications, the probability is 95%.

Expression:

The difference between the results obtained by different laboratories should not exceed .....g/100 g test sample.

ISO 3534-1977 (E/F)

Entwurf DIN/ISO 5725

REPRODUCTIBILITÉ

Qualitativement:

Etroitesse de l'accord entre les résultats individuels obtenus avec la même méthode sur une matière identique soumise à l'essai mais dans des conditions différentes (opérateurs différents, appareils différents, laboratoires différents et/ou époques différentes).

Quantitativement:

Valeur au-dessous de laquelle est située, avec une probabilité spécifiée, la valeur absolue de la différence entre deux résultats individuels obtenus dans les conditions ci-dessus.
En l'absence d'indication, la probabilité est de 95%.

Expression:

La différence entre les résultats obtenus dans des laboratoires différents ne doit pas dépasser .....g/100 g d'échantillon pour essai.

REPRODUZIERBARKEIT (VERGLEICHBARKEIT)

Qualitativ:

Grad der Übereinstimmung von Einzelergebnissen, welche mit derselben Methode an identischem Prüfmaterial, aber unter verschiedenen Bedingungen erhalten wurden (verschiedene Sachbearbeiter, verschiedene Geräte, verschiedene Laboratorien und/oder zu verschiedenen Zeiten).

Quantitativ:

Derjenige Wert, unterhalb dessen man die absolute Differenz zwischen zwei einzelnen Prüfergebnissen, die man unter obigen Bedingungen erhalten hat, mit einer vorgegebenen Wahrscheinlichkeit erwarten kann.
Wenn nichts anderes angegeben ist, so ist diese Wahrscheinlichkeit 95%.

Angabe:

Die Differenz zwischen den Ergebnissen verschiedener Laboratorien soll nicht grösser sein als .....g/100 g Analysenprobe.

SOLUTION WITH DEFINED CONCENTRATION

SOLUTION DE CONCENTRATION DÉFINIE

LÖSUNG DEFINIERTER KONZENTRATION

(English – Français – Deutsch)

Equivalent Terms	Definitions	Expression of concentration
Standard volumetric solution Solution titrée Masslösung	Solution the concentration of which is defined accurately. Solution de concentration exactement définie. Lösung, deren Konzentration genau definiert ist.	N (M)
Standard reference solution Solution étalon de référence Kontrollösung	Solution used as a reference solution for standardizing other solutions. Solution utilisée comme solution de référence pour l'étalonnage des autres solutions. Lösung, die als Kontrollösung verwendet wird, um andere Lösungen zu standardisieren.	N (M)
Standard solution Solution étalon Standardlösung	Solution of accurately known concentration of an element, an ion, a compound or a group derived from the product used for its preparation. Solution de concentration connue avec précision, en un élément particulier, un ion, un composé ou un groupement dérivant du produit utilisé pour sa préparation. Lösung einer exakt bekannten Konzentration eines Elementes, eines Ions, einer Verbindung oder Gruppe, die von dem Erzeugnis stammt, das für die Herstellung dieser Lösung verwendet wurde.	m/v
Standard *matching** solution Solution témoin Standard-Vergleichslösung	Solution of which the relevant characteristic is exactly known or defined (for example, colour, turbidity, etc.) and is used to assess the test solution in relation to that characteristic. * This English term is used solely as a generic term for these solutions and each solution should be defined more precisely by the appropriate adjective; for example, "Standard *colorimetric* solution", "Standard *turbidimetric* solution", etc. Solution dont la caractéristique indiquée est exactement connue ou définie (p. ex. coloration, turbidité, etc.) et qui est utilisée dans un test comparatif pour juger la solution en essai par rapport à cette caractéristique. Lösung, deren charakteristisches Merkmal genau bekannt oder definiert ist (z. B. Farbe, Trübung usw.) und die benutzt wird, um die Probelösung im Hinblick auf eben diese Charakteristik zu beurteilen.	N (M) or/ou/oder m/v

PREFIXES AND SYMBOLS FOR NAMING DECIMAL MULTIPLES AND DECIMAL SUBMULTIPLES OF UNITS

PREFIXES ET SYMBOLES POUR DÉSIGNER MULTIPLES ET SOUS-MULTIPLES DÉCIMAUX D'UNITÉS

VORSATZZEICHEN UND SYMBOLE ZUR BEZEICHNUNG VON DEZIMALEN VIELFACHEN UND DEZIMALEN TEILEN VON EINHEITEN

1 000 000 000 000 000 000 = 10^{18}	exa	E
1 000 000 000 000 000 = 10^{15}	peta	P
1 000 000 000 000 = 10^{12}	tera	T
1 000 000 000 = 10^{9}	giga	G
1 000 000 = 10^{6}	mega	M
1 000 = 10^{3}	kilo	k
100 = 10^{2}	hecto/hekto	h
10 = 10^{1}	deca/deka	da
1 = 10^{0}		
0,1 = 10^{-1}	deci	d
0,01 = 10^{-2}	centi	c
0,001 = 10^{-3}	milli	m
0,000 001 = 10^{-6}	micro/mikro	µ
0,000 000 001 = 10^{-9}	nano	n
0,000 000 000 001 = 10^{-12}	pico/piko	p
0,000 000 000 000 001 = 10^{-15}	femto	f
0,000 000 000 000 000 001 = 10^{-18}	atto	a

CONVERSION TABLES

TABLES DE CONVERSION

UMRECHNUNGSTABELLEN

ppm = 10^{-4}%
g / t
mg / kg
µg / g
ng / mg
pg / µg

ppb = 10^{-7}%
g / 1000 t
mg / t
µg / kg
ng / g
pg / mg

UNITS – UNITÉS – EINHEITEN

Units of mass Unités de masse Masseeinheiten	kg	g	mg	µg	ng	pg
kg	1	10^{3}	10^{6}	10^{9}	10^{12}	10^{15}
g	10^{-3}	1	10^{3}	10^{6}	10^{9}	10^{12}
mg	10^{-6}	10^{-3}	1	10^{3}	10^{6}	10^{9}
µg	10^{-9}	10^{-6}	10^{-3}	1	10^{3}	10^{6}
ng	10^{-12}	10^{-9}	10^{-6}	10^{-3}	1	10^{3}
pg	10^{-15}	10^{-12}	10^{-9}	10^{-6}	10^{-3}	1

Units of volume Unités de volume Volumeneinheiten	m^3	hl	dm^3=l	dl	cl	cm^3=ml	mm^3
m^3	1	10^{1}	10^{3}	10^{4}	10^{5}	10^{6}	10^{9}
hl	10^{-1}	1	10^{2}	10^{3}	10^{4}	10^{5}	10^{8}
dm^3=l	10^{-3}	10^{-2}	1	10^{1}	10^{2}	10^{3}	10^{6}
dl	10^{-4}	10^{-3}	10^{-1}	1	10^{1}	10^{2}	10^{5}
cl	10^{-5}	10^{-4}	10^{-2}	10^{-1}	1	10^{1}	10^{4}
cm^3=ml	10^{-6}	10^{-5}	10^{-3}	10^{-2}	10^{-1}	1	10^{3}
mm^3	10^{-9}	10^{-8}	10^{-6}	10^{-5}	10^{-4}	10^{-3}	1

Units of length Unités de longueur Längeneinheiten	m	cm	mm	µm	nm
m	1	10^{2}	10^{3}	10^{6}	10^{9}
cm	10^{-2}	1	10^{1}	10^{4}	10^{7}
mm	10^{-3}	10^{-1}	1	10^{3}	10^{6}
µm	10^{-6}	10^{-4}	10^{-3}	1	10^{3}
nm	10^{-9}	10^{-7}	10^{-6}	10^{-3}	1

Units of pressure Unités de pression Druckeinheiten	Pa	bar	$\frac{kp}{cm^2}$ =at	atm	mm Hg =Torr
Pa	1	10^{-5}	$1{,}02 \cdot 10^{-5}$	$0{,}987 \cdot 10^{-5}$	$0{,}75 \cdot 10^{-2}$
bar	10^{5}	1	1,02	0,987	750
$\frac{kp}{cm^2}$ =at	$9{,}81 \cdot 10^{4}$	0,981	1	0,968	736
atm	$1{,}01 \cdot 10^{5}$	1,01	1,03	1	760
mm Hg=Torr	133	$1{,}33 \cdot 10^{-3}$	$1{,}36 \cdot 10^{-3}$	$1{,}32 \cdot 10^{-3}$	1

SENSORY ASSESSMENT

2.5 g of meat extract is dissolved in 250 ml of boiling drinking water in a white porcelain dish.

To enhance the organoleptic characteristics of the extract, 0.5 g salt/100 ml water can be added.

The test is performed in regard to

- appearance
- odour
- taste

at a temperature of 60–65 °C.

Details of methodology: see chapter 8.

DETERMINATION OF DRY MATTER

For method see 2/2

7.2. Test portion

Weigh approximately 10 g homogenized meat extract to the nearest mg, dissolve in distilled water in a 100 ml measuring flask and fill to the mark.

20 ml of this solution (=1–2 g dry matter) are used for the determination carried out according to paragraph 8.2. of method 2.2.

DETERMINATION OF THE ASH

For method see 2/3

Preparation of the sample solution:

10 g of meat extract are dissolved in distilled water in a 100 ml measuring flask, and the solution is made up to volume.

20 ml of this solution (equivalent to 1–2 g dry matter) is used for the determination.

SENSORY ASSESSMENT

The product is prepared according to the recipe.

1 plate = 250 ml
1 cup = 150 ml

The test is performed in regard to

- appearance
- odour
- taste

at a temperature of 60–65 °C.

Details of methodology: see chapter 8.

DETERMINATION OF DRY MATTER

January 1980 – Original text in German

1. SCOPE

Determination of dry matter.

2. FIELD OF APPLICATION

Meat extract
Bouillon and meat bouillon
Seasonings
Soups and related products
Raw materials

3. DEFINITION

Dry matter is the portion remaining after the sample has been heated at 102 ±2 °C, expressed as %m/m.

4. PRINCIPLE

Volatile components are removed by heating for 4 hours at 102 ±2 °C.
Samples of dry products are directly heated at 102 °C.
Liquid products are mixed with sand, predried on a water bath, and subsequently dried in the oven.

5. REAGENTS

5.1. Seasand, cleaned with acid and heated in a muffle furnace.

6. APPARATUS

6.1. Drying oven (with maximum temperature fluctuations of ±1 °C)

6.2. Water bath or controlled heater.

6.3. Dessicator

6.4. Dish 7–8 cm diameter
- porcelain, nickel, stainless steel. Aluminium (for dry products only).

6.5. Dish with glass rod and sand (for liquid products).

6.6. Milling equipment (see 7.1.).

7. SAMPLING AND SAMPLES

7.1. Sample preparation

The laboratory sample is carefully homogenised. Dependent upon the state of the hardness of the sample (in case of tablets or cubes), this is done either:

- by grinding with a mill or
- by grinding in a mortar or
- by mincing

For very heterogenous materials a blender (with knives) is recommended.
The preparation of the sample should be carried out as quickly as possible in a room with little humidity, particularly in the case of hygroscopie material.
Store the samples in hermetically closed containers.
In case of liquid products the entire contents of the container should be homogenised and analysed immediately thereafter.

7.2. Test portion
The test portion should contain 1–2 g of dry matter.

8. PROCEDURE

8.1. Dry products

8.1.1. The test portion 7.2. is weighed to the nearest milligram into a tared dish (6.4.), which has been predried for 30 minutes at 102 ± 2 °C.

8.1.2. The dish with test portion is dried in the oven (6.1.) at a temperature of 102 ± 2 °C.
After 4 hours put the dish in a desiccator (6.3.) and, as soon as it has cooled to room temperature, weigh it to the nearest milligram.

8.2. Liquid products

8.2.1. A dish containing sand and a glass rod (6.5.) is dried for 30 minutes at 102 ± 2 °C. The test portion (7.2.) is weighed into this tared dish to the nearest milligram and carefully mixed with the sand.
The dish is put onto a water bath or a controlled heater (6.2.) and the mass is boiled down to dryness, while stirring repeatedly.

8.2.2. The procedure of 8.1.2. is then followed.

9. EXPRESSION OF RESULTS

9.1. Method of calculation: (expressed as %m/m to one decimal place)

$$\text{Dry matter} = \frac{M_3 - M_1}{M_2 - M_1} \times 100\%$$

M_1 = mass of dish + sand + rod, in g
M_2 = M_1 + test portion before drying, in g
M_3 = M_1 + test portion after drying, in g

9.2. Repeatability: 0,2 g/100 g test sample

10. NOTES

To samples of dry products and pasty products containing

- ingredients with water of crystallisation e.g. monosodium glutamate monohydrate, citric acid monohydrate,
- protein hydrolysate and/or amino acids,
- carbohydrate, particularly saccharides,

no water should be added before drying, in order to avoid chemical reactions during the drying which may give false results (up to several times the real water content).

DETERMINATION OF THE ASH

March 1975 – Original text in German

1. SCOPE

Determination of the inorganic substance.

2. FIELD OF APPLICATION

Meat extract
Bouillon based products
Seasonings
Soups

3. DEFINITION

The ash is the residue after combustion of the organic substance at a temperature of a maximum of 550 °C.

4. PRINCIPLE

Complete destruction of the organic substance without incineration aids, avoiding additional contamination with foreign substances, under conditions which exclude significant losses of minerals. For this purpose the test portion is first carbonised and then incinerated in a muffle furnace at 550 °C.

5. REAGENTS

5.1. Distilled or demineralised water.

6. APPARATUS

6.1. Platinum or quartz-glass bowls, suitable dimensions: diameter 7–8 cm, height 1–2 cm

6.2. Crucible tongs, with platinum-coated points when platinum bowls are used for 6.1

6.3. Pipettes

6.4. Water-bath

6.5. Surface evaporator (for instance infrared radiator), or Bunsen burner with refractory disc

6.6. Muffle furnace

6.7. Desiccator

7. PROCEDURE

The test portion, which should contain 1–2 g dry matter is weighed into a tared bowl to an accuracy of 1 mg. Alternatively an aliquot part of a prepared solution, with the same dry matter content is pipetted into the bowl. It is then dried under the surface evaporator and carbonised without inflammation. When a Bunsen burner with refractory disc is used liquid samples are vaporised on a water bath until dry, the flame during subsequent carbonisation having to be adjusted so as to avoid local heating to more than 550 °C.

The test portion is then incinerated in the muffle furnace at 550 °C, unti, a light grey white residue is obtained. If, after three hours, the ash is not free from black carbon particles, it is moistened with water after cooling to room temperature, and then crushed with a glass rod flattened on one side. After the glass rod has been rinsed the sample is vaporised on a water bath or under a surface evaporator until dry. The bowl is again ignited in the muffle furnace at 550 °C, until the ash is of an evenl light colour and free from carbon particles. The moistening of the ash may have to be repeated several times.

After cooling in the desiccator, the sample is weighed to an accuracy of 1 mg.

8. EXPRESSION OF RESULTS

8.1. Method of calculation:

(Specification in percentage by weight with one decimal place)

$$\text{Ash content} = \frac{\text{mg ash} \times 100}{\text{mg test portion}} \%$$

8.2. Repeatability:

0.1 g/100 g test sample with homogeneous products and
0.5 g/100 g test sample with heterogeneous products

9. BIBLIOGRAPHICAL REFERENCE

«Handbuch der Lebensmittelchemie», vol. II/2nd part (1967), p. 50, Springer-Verlag.

Salt Concentration

(Chloride expressed as

Sodium chloride)

August 1998

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1 PURPOSE OF DETERMINATION

Determination of salt (sodium chloride) concentration in savoury products by means of the chemical determination of chloride and consecutive stoichiometric calculations.

2 SCOPE

Applicable to bouillons, soups and seasonings with salt concentrations >1 %.

3 PRINCIPLE

The salt concentration of a product is calculated from the chloride concentration in a solution as determined by potentiometric titration with silver nitrate.

4 REFERENCE METHOD

AOAC Official Methods of Analysis, 15th Ed.(1990) Methods 32.034 - 32.039

5 VALIDATION

The method has been validated in a collaborative study with 15 laboratories participating. The results from 12 laboratories could be evaluated. The method has been applied to 12 samples from commercial production with salt concentrations between 70 g/kg to 500 g/kg. The 12 samples represent 6 different products. For each product, two different production batches were analysed.
The statistical preevaluation of the submitted results suggested a clustering into three different data sets for the validation calculations:
Data Set 1: 4 different samples (one production batch of each product) with salt concentrations between 70g/kg and 440g / kg.
Data Set 2: 5 different samples (the second production batch of each product) with salt concentrations between 70g/kg and 440g/kg.
Data Set 3: 2 samples (two batches of one product) with salt concentrations of around 500g / kg and high fat content.

Results:

	Repeatability	**Reproducibility**
Data Set 1	0.20	0.30
Data Set 2	0.27	0.32
Data Set 3	0.88	1.25

The complete description of samples and the data evaluation is given in Appendix I.

6 QUALITY ASSURANCE

6.1 REFERENCE MATERIALS

see section „CHEMICALS“

6.2 TESTS DURING ANALYSIS

The determination is verified by the following tests during the analytical procedure.

6.3 GENERAL

Per sample two determinations are performed.

6.4 QUALITY CONTROL

6.4.1 Molarity of silver nitrate solution Interval: weekly

The molarity of the 0.1M titration solution should be determined periodically. The molarity must not be determined if it is the first use of a freshly prepared titration solution. The test is performed with sodium chloride.

6.5 DOCUMENTATION

6.5.1 Calibration Data: Molarity of silver nitrate solution

(see page 5, **MEASUREMENT OF THE SILVER NITRATE MOLARITY**; for formula see page 6, **CALCULATION OF SILVER NITRATE MOLARITY**)

Weight of sodium chloride	Volume of $AgNO_3$ Solution	Molarity of $AgNO_3$ Solution	Date of Calibration	Remarks
$\mathbf{W_{NaCl}}$ [g]	$\mathbf{V_{st}}$ [ml]	**M** [mol / l]		

7 APPARATUS AND CHEMICALS

7.1 INSTRUMENTATION AND AUXILIARY EQUIPMENT

- Laboratory blender
- High Speed Blender with optimal dispersion tool (e.g. Polytron with min. tool diameter of 25 mm)
- Analytical balance (accuracy: 0.001g)
- Titration unit for potentiometric titration consisting of :
  Silver reference electrode,
  pH glass electrode or
  Silver combination electrode,
  Automatic or manual dosing unit,
  Magnetic stirrer,
  Potentiometric recorder
  (alternatively an automatic or semiautomatic titration unit may be used)

7.2 GLASSWARE

- Erlenmeyer flasks: 250 ml / 300 ml
- Measuring cylinder: 500 ml
- Volumetric flasks: 250 ml, 100 ml
- Beakers: 250 ml
- Pipettes: 10 ml and 25 ml
- Funnel

7.3 EXPENDABLES

- Filter paper for quantitative filtration *or*
- Clean glasswool

7.4 CHEMICALS

- Deionised Water
- Nitric acid, 65%
- Silver Nitrate p.a. (Merck Titrisol 0.1 M)
- Sodium chloride p.a. Merck 6404

7.5 SOLUTIONS

- Nitric Acid 2M:
  Dilute approximately 120ml nitric acid to 1L (2M)
- Silver Nitrate 0.1 M: prepare from stock solution according to instructions

8 SAMPLE PREPARATION

8.1 HOMOGENISATION

Homogeneous Samples:
- Use without any further pre-treatment

Inhomogeneous Samples / Whole Consumer Packages:
- Homogenise a sufficiently large sample of a product with coarse pieces (e.g. Minestrone) or the whole content of the consumer package with a clean and dry blender.

8.2 SAMPLE SIZE

Select approximately 5 g, determined accurately to 0.01g, of this homogenised sample in order to obtain reproducible results.

8.3 PROCEDURE

- Weigh the sample into a 250 ml Erlenmeyer flask (SW)
- Add approximately 150 ml deionised water
- Homogenise the sample for 1 - 1½ minutes with the High Speed Blender at medium to high speed
- Quantitatively transfer the solution into a 250 ml volumetric flask.
- If necessary, filter the sample trough a filter paper or glass wool that an efficient filtration is reached. Use a vacuum sucking device if gravity filtration is not effective.

Remark:
Products with a high fat content (e.g. bouillons) do not require filtration after homogenisation. Fat accumulates in lumps during the preparation of the solution. These lumps must be discarded.
Alternatively, 2 to 4 ml of hexane may be added to dissolve all the fat. This solution can then be pipetted off and discarded.

- For a quantitative transfer of the sample, thoroughly wash the homogenising tool and filters. Add the washings to the volumetric flask.
- Fill to the mark with cold water.
- Clean the High Speed Blender carefully after each use to avoid errors through cross contamination.

9 ANALYTICAL PROCEDURE

9.1 INSTRUMENT CONTROL:

Check all parts of the analytical instrumentation (dosing unit, electrodes, recording unit) according to the manufacturers guidelines. If necessary clean silver electrodes carefully with scouring powder, hot water and wipe clean with paper tissue.

9.2 MEASUREMENT OF THE SILVER NITRATE MOLARITY

- Weigh approximately 0.060g sodium chloride to an accuracy of 1mg (W_{NaCl}) into a 250 ml beaker,
- Add 10 ml nitric acid solution and fill to ~200ml with deionised water,
- Position the electrodes in the solution
- Titrate with 0.1 M silver nitrate solution to obtain a complete curve on the Potentiometric recorder.
  (Alternatively operate your instrument according to the manufacturers guidelines).

  Remark:
  Make sure to stir at constant rate producing vigorous agitation without

8 SAMPLE PREPARATION

8.1 HOMOGENISATION

Homogeneous Samples:
- Use without any further pre-treatment

Inhomogeneous Samples / Whole Consumer Packages:
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- Position the electrodes in the solution
- Titrate with 0.1 M silver nitrate solution to obtain a complete curve on the Potentiometric recorder.
  (Alternatively operate your instrument according to the manufacturers guidelines).

  Remark:
  Make sure to stir at constant rate producing vigorous agitation without

10.2 CALCULATION OF THE CONCENTRATION IN SAMPLES

$$\text{Salt concentration (in \%)} = \frac{V * M * MW * D_f}{SW * 10} \left[\frac{ml * mol / l * g / mol}{g} \right]$$

V:	Volume of silver nitrate used for titration (ml)
M:	Calculated molarity of silver nitrate solution (recommended: 0.1M)
MW	Molecular Weight of sodium chloride (58.443)
D_f	Dilution factor
SW	Sample weight (g)
10	Correction factor to give percentage value

10.3 RESULTS

The result should be expressed in % with one digit after the decimal point.

11 LITERATURE

- CPC Europe, Quality Assurance
  Report "Determination of salt content" (Sample Preparation/Comparison of Chloride Meter and Potentiograph)
- AOAC Official Methods of Analysis, 15th Ed.(1990)
  Methods 32.034 - 32.039

12 VALIDATION PROCEDURE

Validation of the method has been performed by the collaborative study. New validation is only required if significant changes in sample preparation are introduced. The decision for additional validation studies must be based on reliable comparisons to the original procedure.

APPENDIX I:
COLLABORATIVE STUDY: DATA EVALUATION

A collaborative study with 15 laboratories located in 9 countries of Europe has been performed in June 1995.
These laboratories received 12 samples from regular production runs. Of six commercial products, two different batches were supplied for analysis. The theoretical concentrations for salt, sodium and fat was calculated from the recipe. The real salt concentration as obtained during manufacturing, was unknown.

Sample	Theoretical Salt Concentration: [g / 100 g]	Theoretical Sodium Concentration: [g / 100 g]	Fat Concentration: [g / 100 g]	Batch Identification
Beef Bouillon Paste (High Fat)	48.6	21.4	23.1	9911/9503
Beef Bouillon Granulate (Low Fat)	43.5	19.3	2.0	2631/4957
Standard Cream Soup (Asparagus)	12.2	5.5	8.1	7454/5971
Standard Gravy (Demi Glace)	11.4	5.6	13.8	7471/1532
Cream Sauce (Béarnaise)	14.2	6.7	9.6	3556/4312
Instant Cream Soup (Tomato)	7.3	3.4	8.3	736/7780

The initial statistical data analysis could be performed on the results supplied by 12 laboratories.
The statistical preevaluation of the submitted results allowed for the acceptance of data from 12 laboratories and suggested a clustering into three different data sets for the validation calculations:

Data Set 1: 4 different samples (one production batch of each product) with salt concentrations between 70g / kg and 440g / kg. Sample identification: 2631, 7454, 1532, 3556
Data Set 2: 5 different samples (the second production batch of each product) with salt concentrations between 70g / kg and 440g / kg. Sample identification: 4957, 5971, 7471, 4312, 7780
Data Set 3: 2 samples (two batches of one product) with salt concentrations of around 500g / kg and high fat content. Sample identification: 9503, 9911

STATISTICAL ANALYSIS

Results:

	Repeatability	**Reproducibility**
Data Set 1	**0.20**	**0.30**
Data Set 2	**0.27**	**0.32**
Data Set 3	**0.88**	**1.25**

Result Sheet

Calibration Data:

	Molarity of AgNO3 Solution	Weight of sodium chloride (g)	Volume ofAgNO3 Solution (ml)	Date of Calibration / Analysis:
Use for Samples:	**M:**	W(NaCl)	V st:	
Example	**0.102**	0.58	97	

Molarity of silver nitrate, $M = \frac{W_{NaCl} * 1000}{MW * V_{ST}} \left[\frac{g}{g / Mol * ml} \right]$

W_{NaCl}: Weight of sodium chloride (g)

MW : Molecular weight of sodium chloride (58.443)

V_{st} : Volume of silver nitrate used for titration of standard solution (ml)

Results of Analyses

Correction Factor (for percent output) **10** (should remain unchanged) Molecular Weight of Sodium Chloride (MW) **58.443**

			Subsample 1						**Subsample 2**					
Sample ID	**Fill Weight (g)**	**Salt Concen-tration:**	Sample Weight 1 (g)	Dilution Factor	Titration 1 Volume of AgNO3 Solution	Titration 2 Volume of AgNO3 Solution	Average (of 2 Titrations)	**Salt Concen-tration:**	Sample Weight 2 (g)	Dilution Factor	Titration 1 Volume of AgNO3 Solution	Titration 2 Volume of AgNO3 Solution	Average (of 2 Titrations)
			SW	Df	V:	V:	V:		SW	Df	V:	V:	V:
Example		**6.58**	5	10	5	6	5.5	**0.15**	22	29	19	20	19.5

Determination of

Creatinine

by HPLC

Revision 2000

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1 PURPOSE OF DETERMINATION

Creatine, creatinine and creatine phosphate are present in muscles of mammalian species and thus in meat of bovine origin.

In the Worldwide Codex Standard for Bouillons and Consommés[1] the minimum levels of creatinine in meat extract and products containing meat (beef) are fixed either by direct reference to creatinine levels or the amount of beef added to such products.

The concentration of total creatinine (derived from creatine phosphate, free creatine and creatinine) in bouillons, soups and gravies (sauces) with beef serves as an indicator for the addition of meat or meat extract to such products.

The method allows for the control of products containing meat of bovine origin.

2 SCOPE

Determination of (total) creatinine in meat extract and bouillons.

Creatinine levels in soups and gravies, although not tested in a collaborative study, may also be determined with this method. The analyst has to show adherence to the quality requirements outlined in this method.

3 PRINCIPLE

Creatine phosphate is hydrolysed and creatine converted to creatinine in hot hydrochloric acid solution[2]. After addition of an internal standard (cytosine) the neutralised extract is cleaned on alumina- and C_{18}-cartridges. Separation is achieved by reversed phase HPLC with detection of the constituent by UV - spectroscopy. Calculations are performed on the peak heights using the internal standard method.

4 VALIDATION

4.1 Basic principles

Method validation was performed under the guidance of AIIBP with a test protocol and data evaluation based on principles defined by AOAC*. Two different sample types covering the range of interest of the soup industry were used:

- Commercial quality beef extract which should contain 8.5% total creatinine on salt free dry matter [3]. Only one figure has to be monitored.
- End products which contain creatinine in a range of concentrations reflecting product category and product composition. This necessitated the use of five pairs of split-level samples in the range of 0.1 to 0.35 g Creatinine / 100 g of product before preparation.

Samples were dispatched to 10 laboratories willing to participate in this collaborative study. All laboratories reported their data. One laboratory reported only figures for peak areas.

4.2 Repeatability and reproducibility of the method

Statistical evaluation of the test results using peak height and peak area satisfied the requirements to a large extend. However, the shape of the creatinine peak in the HPLC determination is usually not perfect. Calculations using peak height consequently proved to have a better precision. Therefore only data for peak height are reported.

4.2.1 Creatinine in meat extract

The test was based on blind duplicate design for one single matrix (82% dry matter) with a target level of 7% creatinine. The test was restricted to 3 batches, analysed as blind duplicates (6 samples) on day A.

Samples T/Y, S/U and X/Z were from the same batches and evaluated as pairs.

Results from 2 participants were partially eliminated from the evaluation due to failure in the Grubb or Cochran tests.

---

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Sample pairs	Number of participants†	Target Values	mean concen-tration	s(r)	s(R)	r	R	Recovery
		[g/100g]	[g/100g]					
TY	9 (0)	6.7	6.76	0.05	0.15	0.15	0.43	100.90%
TY	7 (2)	6.7	6.69	0.06	0.07	0.16	0.18	99.90%
SU	9 (0)	7.0	7.02	0.02	0.11	0.05	0.30	100.30%
XZ	9 (0)	7.3	7.28	0.07	0.11	0.19	0.32	99.70%

4.2.2 Creatinine in Bouillon

Ten samples of products containing meat extract in different concentrations were dispatched as blind duplicates (labelled A to J). The split-level pair's consist of samples A/B; D/C; G/I; E/H; F/J.

Sample pairs	Number of participants*	Target Values	mean concen-tration	s(r)	s(R)	r	R	Recovery
		[g/100g]	[g/100g]					
AB	9 (0)	0.105	0.102	0.003	0.005	0.009	0.013	97.1%
DC	9 (0)	0.1825	0.180	0.005	0.005	0.013	0.013	98.6%
GI	9 (0)	0.225	0.224	0.006	0.008	0.016	0.023	99.6%
EH	9 (0)	0.28	0.279	0.005	0.008	0.014	0.023	99.6%
FJ	9 (0)	0.35	0.347	0.008	0.011	0.023	0.032	99.1%

5 MEASUREMENT UNCERTAINTY

5.1 Confidence (95%) level

The 95% confidence levels are calculated from the calculated mean concentration +/- R. The respective figures are summarized in the following tables.

5.1.1 Creatinine in meat extract

Sample pairs	Lower limit	Measured value (mean from statistical evaluation)	Upper limit	
	[g/100g]	[g/100g]	[g/100g]	
TY [9 (0)] *	6.33	6.76	7.19	+/- 6.4%
TY [7 (2)] *	6.51	6.69	6.87	+/- 2.7%
SU	6.72	7.02	7.32	+/- 4.3%
XZ	6.96	7.28	7.60	+/- 4.4%

5.1.2 Creatinine in Bouillon

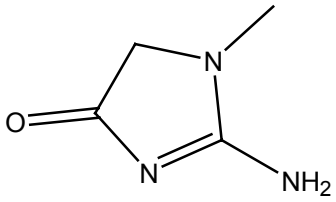
Sample pairs	Lower limit	Measured value (mean from statistical evaluation)	Upper limit	
	[g/100g]	[g/100g]	[g/100g]	
AB	0.089	0.102	0.115	+/-12.7%
DC	0.167	0.180	0.193	+/- 7.2%
GI	0.201	0.224	0.247	+/- 10.3%
EH	0.256	0.279	0.302	+/- 8.2%
FJ	0.315	0.347	0.379	+/- 9.2%

† 9 (0) or 7 (2) : results from number of participants evaluated (results from number of participants rejected)

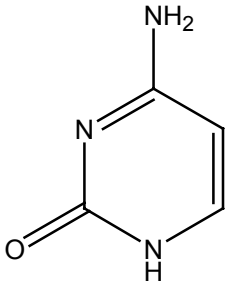
6 QUALITY ASSURANCE

6.1 Reference Chemicals **

Creatinine	$C_4H_7N_3O$	CAS 60-27-5	Fluka 27910
Cytosine	$C_4H_5N_3O$	CAS 71-30-7	Fluka 30430

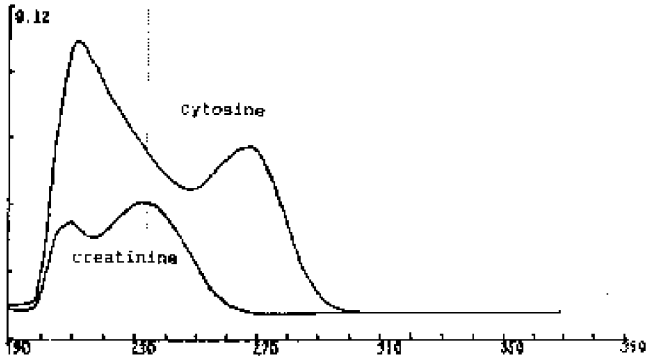


Creatinine



Cystine

6.1.1 UV Absorption Spectra of Reference Chemicals



6.2 Tests during Analysis

6.2.1 General

It is recommended to verify the HPLC-system in regular intervals by performing tests of the chromatographic system (see chapter 12.1) and the analysis of sample material free from creatinine to which a standard creatinine solution is added (see chapter 12.2).
For routine use, include the analysis of a reference sample with a known concentration of creatinine. Stored at –20° C, this sample can be kept for at least 18 months.

6.2.2 Quality Control

The calibration solution C2 (section 9.1.3, Calibration solutions) has to be injected in triplicate at the beginning of the day, once after each fourth sample and in duplicate at the end of a test series. The result (in peak area or height) should remain stable. Use the average response of the first three runs for the initial calculation of the results and adjust during operation as needed. The individual response shall never exceed the average response by more than ±2%.

6.3 Verification of the System

The verification procedure tests the performance of the chromatographic system and the quantitative recovery of the sample preparation procedure.
Verification of the system has to be performed at regular intervals. If the method is used only occasionally an extended verification procedure has to be applied before each set of analysis.

** see chapter 13, REMARKS, last indent

6.3.1 Chromatographic Resolution

Chromatographic resolution shall be determined by visual inspection of the calibration and sample chromatograms (example see 10.3 "Sample Chromatograms").
The internal standard, cytosine, must be properly separated from creatinine. Some tailing of the internal standard and the sample peak is tolerable and will not significantly degrade the required performance of the analytical system. It is recommended to determine the resolution and peak shape.

6.3.2 Performance test for the chromatographic system

The performance of the chromatographic system shall be checked according to the procedure "Chromatographic system" (chapter 12.1). This test provides among other data, information on the linearity of the chromatographic detection system.

6.3.3 Performance test of complete procedure

A performance test with samples that do not contain creatinine or its precursors should be run according to the procedure "Creatinine Addition (Food Sample without Creatinine)", (chapter 12.2). This test determines the overall performance of the method and provides recovery information.

6.4 Documentation

All activities and data from the verification procedure must be properly recorded. The verification data must be unambiguously assignable to the test results of samples.

7 APPARATUS **

7.1 Instrumentation

- Autosampler: recommended
- HPLC system Isocratic
- Detector: Variable Wavelength UV detector at 234 nm or Diode Array detector at 234 nm

7.2 Glassware

- Pipettes: 2 ml, 5 ml, 10ml, 20 ml
- Volumetric flasks: 500 ml, 250 ml, 100 ml
- Beakers: 50 ml, 500 ml

7.3 Auxiliary Equipment

- Water bath
- Hot plate
- pH-Meter, table top model
- Adjustable pipette, 5 ml: 0.5 – 5 ml (e.g. Socorex, model 831)
- Water aspirator
- Woulff bottle

8 SUPPLIES **

8.1 Expendables

Disposable polyethylene beakers 30 ml
Disposable syringe 10 ml
PP tips for adjustable pipette
Pasteur pipettes 150 mm
Sep-Pak plus cartridges Alumina B WATERS 20505
Sep-Pak plus cartridges C18 WATERS 20515
Disposable syringe filter: WATERS 85996
0.45 µm pore size, 25 mm or 13 mm o.d.
Millipore membrane Type GS: Millipore GSWP04700
0.22 µm pore size, 47 mm o.d.

** see chapter 13, REMARKS, last indent

8.2 Chemicals

Safety Warning
Before using chemicals refer to adequate manuals or safety data sheets approved by the local authorities.

Methanol (gradient grade)	Merck 6007
ortho-Phosphoric acid	Merck 564
Hydrochloric acid 32 %	Merck 319
Sodium hydroxide	Fluka 71692
di-Potassium hydrogen phosphate anhydrous ($K_2HPO_{4)}$	Merck 5104
Petroleum ether (boiling range 50 - 75°C)	Merck 1773

Sodium Azide NaN_3 [optional, see Appendix I; Mobile Phase M1 (Stability)]
Ultra pure water: Milli Q+ Waters purification system

8.3 Solutions

- Hydrochloric acid 21 %
  Add 200 ml hydrochloric acid (32 %) to 100 ml water
- Sodium hydroxide solution ~6M (ca. 25 %):
  Dissolve 36 g sodium hydroxide in 150 ml water

8.4 Chromatographic Columns

Choose from one of two column systems that were identified to perform properly

8.4.1 System A:

Separation column

TSK - Gel ODS80 TM, 5 µm, 150 mm x 4.6 mm ID	TOSO HAAS 08148

Pre-column:

TSK - Gel Guard Cartridge ODS80 TM	TOSO HAAS 14123
TSK - Gel Cartridge Holder	TOSO HAAS 14100

8.4.2 System B:

Separation column

Spherisorb ODS 2, 5 µm, 250 mm x 4.6 mm ID	Metrohm CH-Herisau

Pre-column:

Cartridge: Spherisorb ODS 2, 5 pm, 20 x 4 mm ID
Cartridge Holder Metrohm CH-Herisau

8.5 Mobile phases for HPLC

- M1 (K_2HPO_4 solution 0.1 M):
  Dissolve 17.4 g K_2HPO_4 in 1000 ml water and add phosphoric acid until a pH of 7.0 is reached. Filter through a 0.45 µm or 0.22 µm Millipore membrane (see also comments in " Appendix I:, Mobile Phase M1 (Stability)").
- M2 (Methanol-water 1:1):
  Mix 500 ml methanol with 500 ml purified water (This mobile phase is used during prolonged non-use of the chromatographic column)

9 PREPARATION

9.1 Equipment Calibration

Always prepare and test your equipment according to the manufacturers guidelines and recommendations before starting the method calibration and analytical work

9.1.1 Calibration Substances

see 6.1, Reference Chemicals

9.1.2 Stock solutions

S1: Creatinine 1 mg/ml; **Storage time: 1 week at 4°C**

- Weigh 100 mg creatinine in a 100 ml volumetric flask.
- Add 2 ml hydrochloric acid 21 %.
- Dissolve and fill to the mark with water.

S2: Cytosine 0.45 mg/ml (internal standard); **Storage time: 1 week at 4°C**

- Weigh 225 mg cytosine in a 500 ml beaker
- Dissolve in about 300 ml water and 10 ml hydrochloric acid 21 %.
- Transfer to a 500 ml volumetric flask and fill to the mark with water

9.1.3 Calibration solutions

C1: 20 µg/ml creatinine; 22.5 µg/ml cytosine; **Storage time: 1 day**

- Transfer exactly 2 ml creatinine solution (S1) to a 100 ml volumetric flask.
- Add 10 ml hydrochloric acid 21 %, 4 ml sodium hydroxide solution 6M and 5 ml cytosine stock solution (S2) (procedure as in sample preparation)
- Fill to the mark with water

C2: 2 µg/ml creatinine; 2.25 µg/ml cytosine; **Storage time: 1 day**

- Transfer 2 ml from calibration solution C1 to a flask and dilute with water to 20 ml.

9.1.4 Internal Standard

- Solution S2 is used as internal standard

9.1.5 Solutions for Additions

S3: 2.5 mg/ml creatinine; **Storage time: 1 day**

- Weigh 250 mg creatinine in a 100ml volumetric flask.
- Dissolve and fill to the mark with water.

9.2 Sample

9.2.1 General

Meat extracts and food samples require slightly different sample preparations due to differences in their physical state and composition.
Pasty meat extracts tend to form sediment of crystallised creatinine and mineral salts. To obtain a homogeneous test portion, heat the sample in a water bath to 45 – 50° C and mix well. Cool to room temperature before performing further steps.
Caution: prevent the evaporation of water from the sample since this leads to results being too high, thus not reflecting the true value.
Foods (bouillons, soups, gravies) shall be analysed after a representative sample is sufficiently homogenised.

9.2.2 Extraction and hydrolysis

Meat extract:

Extraction

- Weigh 2 g (+/- 0.01 g) meat extract in a 250 ml beaker; dissolve in approximately 100 ml hot water (ca. 60°C). Stir for better dissolution. Transfer the sample solution to a 250 ml volumetric flask.
- Shake vigorously, allow to cool and fill to the mark with water.

Hydrolysis and reaction to form Creatinine from Creatin

- Transfer a 5 ml aliquot into a 100 ml volumetric flask, add 10 ml hydrochloric acid 21 % and let react for one hour in a hot water bath at 95 -100°C.
- Allow to cool, add 4 ml sodium hydroxide solution 6M and 5 ml cytosine stock solution (S2), fill to the mark with water.
- Transfer 2 ml sample solution into a 20 ml volumetric flask, fill to the mark with water (This dilution actually is uncritical since the calculation is based on the internal standard which has been added in the previous step).
- Filter the sample through a disposable filter (0.45 µm pore size) before injection to the HPLC system.
- Proceed to step 10.4

Food products (bouillon cubes, gravies, soups):

Extraction

- Weigh 10 g (+/- 0.05 g) sample in a beaker, dissolve with approximately 100 ml hot water (ca. 60°C) and transfer into a 250 ml volumetric flask (If products are well soluble, they can be directly dissolved in a volumetric flask).

- Shake vigorously; reduce the temperature by adding 100 ml of cold water.
- If the fat content is less than about 7 %, fill the flask to the mark and proceed with the hydrolysis.

Defatting (apply to samples with high concentration of fat)

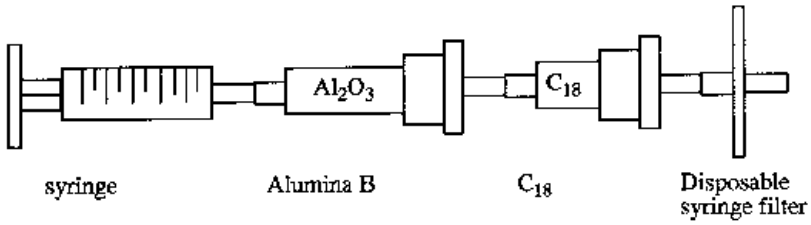
- Add 3 ml petroleum ether to extract any fat from the aqueous solution.
- Fill the flask to the mark with water (with the organic layer above the mark).
- Shake and let stand to allow phases to separate and to let settle insoluble components.
- Remove the organic layer by using a water aspirator with a Woulff bottle or pipette off with a pasteur pipette.

Hydrolysis and reaction to form Creatinine from Creatin

- Transfer a 20 ml aliquot into a 100 ml volumetric flask add 10 ml hydrochloric acid 21 % and let react for one hour in a hot water bath at 95 -100°C.
- Allow to cool, add 4 ml sodium hydroxide solution 6M and 5 ml cytosine stock solution (S2); fill to the mark with water.

9.2.3 Sample Cleanup

- Transfer 2 ml of sample solution into a 20 ml volumetric, fill to the mark with water and mix well (This dilution actually is uncritical since the calculation is based on the internal standard which has been added in the previous step).
- Condition the Sep-Pak cartridges
  Alumina B: flush with 10 ml water
  C_{18}: flush first with 5 ml methanol then 5 ml water
- Attach the Alumina-, C18-SepPAk cartridges and the 0.22µm pore size filter to a 10 ml syringe:



- Fill the 10 ml syringe with sample solution and connect to the cleanup system
- Elute with a flow of approximately 1 drop/sec
- Discard the first 3.5 ml
- Collect at least 2 ml sample solution and use for HPLC-Analysis
- Proceed to step 10.4

10 ANALYTICAL PROCEDURE

10.1 General

The chromatographic procedure described shall be applied to system verification, tests during analyses and analyte determination.
Prepare different calibration solutions, perform the chromatographic analyses and calculate statistically relevant factors for evaluation of chromatographic systems.

10.2 Operating Conditions

10.2.1 Chromatography

Separation columns

Choose one of two column systems that were identified to perform properly (see 8.4, "Chromatographic Columns")

10.2.2 Mobile Phase M1: (see 8.5 Mobile phases for HPLC)

di-Potassium hydrogen phosphate solution 0.1 M
Flow: 0.8 ml/min.

10.2.3 Detection:

UV at 234 nm, Bandwidth 10 nm

10.2.4 Chromatographic conditions

- Injection volume: 10 µl
- Acquisition time: 8.0 min.
- Column flush: 4.0 min.

10.2.5 Retention time:

TSK - Gel ODS80 TM

- Cytosine: approx. 4.6 min.
- Creatinine: approx. 5.7 min.

Spherisorb ODS 2

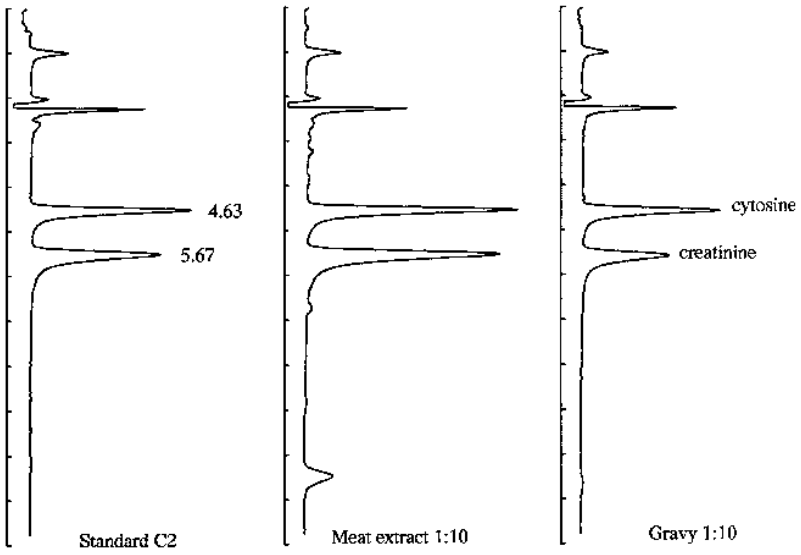
- Cytosine: approx. 5.2 min.
- Creatinine: approx. 6.3 min.

10.2.6 Detection Limits

- Standard Solutions: 0.2 µg/ml
- Meat extract: 0.5 g/100g sample
- Finished products**:** 0.025 g/100g sample

10.3 Sample Chromatograms

TSK - Gel ODS80TM 1



10.4 Analyte Determination

Allow the chromatographic system to equilibrate. Inject 10 µl sample solution with internal standard and perform the HPLC analysis with the conditions given in section 10.2 "Operating Conditions".
Identify creatinine and cytosine in the chromatographic curve by comparison with retention times of the corresponding peaks obtained with standard solution. Under the above-mentioned conditions cytosine and creatinine must be base-line separated.
A few minutes of flushing with mobile Phase M1 is recommended after each sample injection.

10.5 Documentation

All data shall be saved according to principles defined by the operating laboratory. Correlation of analyte data to performance and quality control data must be retained.

11 CALCULATIONS

All calculations are best performed on peak height as indicated in section 4,"Validation". All formulas therefore refer to peak height.

11.1 Concentration in the Sample

For calculations the internal standard method in conjunction with an integrating system capable of determining peak heights is recommended.
At least one set of chromatograms with the creatinine standard solution must be available.

$$\text{Creatinine [g/100g]} = \frac{Q_{sample}}{Q_{Std}} \bullet C_{std} \bullet \frac{1}{w} \bullet \frac{V_1}{V_2} \bullet \frac{a(=100)}{c(=1000) \bullet d(=10)} \quad \left[\mu g/ml * \frac{1}{g} * \frac{ml}{ml} * ml\right]$$

where :

$$Q_{Sample} = \frac{\text{PeakHeight}\langle\text{of Creatinine}\rangle}{\text{PeakHeight}\langle\text{of Cytosine}\rangle} \quad \text{(in Sample Solution)}$$

$$Q_{Std} = \frac{\text{PeakHeight}\langle\text{of Creatinine}\rangle}{\text{PeakHeight}\langle\text{of Cytosine}\rangle} \quad \text{(in Standard solution)}$$

C_{Std} = Concentration of Creatinine in the Standard Solution C1 (suggested 20 µg/ml, see Section " Calibration Solutions")

w = Weight of test portion in g

V_1 = Volume of initial solution of the test portion (mostly 250 ml)

V_2 = Volume of V_1 used for hydrolysis (5 ml for meat extract, 20 ml for finished products)

Fix Factors : Corrections for Dimensions in Solutions to Reporting Units

a = 100 : adjustement for 100 g in output

c = 1000 : adjustement for µg to g in output

d = 10 : adjustement for primary dilution of standard C1

11.2 Calibration Curve

The performance check of the chromatographic system provides enough data points for the calculation of a calibration curve. It is therefore advised to perform this calculation.

11.2.1 Definitions:

- c_i denominates the theoretical concentration of the calibration solution as calculated for CP1.1 to CP8.1
- Q_i denominates the ratio Q_{sample} / Q_{std}
- Take the ratio Q_i for each creatinine standard solution (CP1.1 to CP8.1) and the internal standard as calculated by your integrating system.
- Average the data from the two (2) injections of the same calibration solution.

11.2.2 Linear regression analysis

Perform the linear regression analysis (least square fit) for the pairs concentration/peak height ratio over the complete range of the calibration curve and calculate the regression coefficient r. Use an appropriate computer program.

12 PERFORMANCE TESTS

12.1 Chromatographic system

12.1.1 Stock solution (see 8.3 Solutions)

- S1: creatinine 1 mg/ml

12.1.2 Solutions for performance test

- Prepare eight (8) 100 ml volumetric flasks and label them CP1 to CP8.
- Transfer exactly the required volumes of stock solution (S1) into the corresponding volumetric flasks (CP1 to CP8).
- For each solution, perform the following steps:
- Add 10 ml hydrochloric acid 21%, 4 ml sodium hydroxide solution 6M and 5 ml cytosine stock solution (S2); fill to the mark with water.
- Prepare eight (8) 20 ml volumetric flasks and label them CP1.1 to CP8.1
- Transfer 2 ml of each calibration point solution (CP1 to CP8) into the corresponding flask (CP1.1 to CP8.1) and fill to the mark with water.

Label	Concen-tration	Aliquot S1	Int.Std. S2	Label	Aliquot	**Concen-tration c_i**
CP1:	5 µg/ml	0.5 ml	5 ml	CP1.1:	2 ml CP1	**0.5 µg/ml**
CP2:	10 µg/ml	1.0 ml	5 ml	CP2.1:	2 ml CP1	**1.0 µg/ml**
CP3:	15 µg/ml	1.5 ml	5 ml	CP3.1:	2 ml CP3	**1.5 µg/ml**
CP4:	20 µg/ml	2.0 ml	5 ml	CP4.1:	2 ml CP4	**2.0 µg/ml**
CP5:	25 µg/ml	2.5 ml	5 ml	CP5.1:	2 ml CP5	**2.5 µg/ml**
CP6:	30 µg/ml	3.0 ml	5 ml	CP6.1:	2 ml CP6	**3.0 µg/ml**
CP7:	40 µg/ml	4.0 ml	5 ml	CP7.1:	2 ml CP7	**4.0 µg/ml**
CP8:	50 µg/ml	5.0 ml	5 ml	CP8.1:	2 ml CP8	**5.0 µg/ml**

- Inject 10 µl of each calibration solution (CP1.1 to CP8.1) twice.
- Data acquisition must be performed with the procedure, which will be used for analyte determination (sample analysis).
- Calculate a calibration Curve as described in 11.2 "Calibration Curve"
- The result of this test shall be retained and used for performance documentation.

12.2 Creatinine Addition (Food Sample without Creatinine)

Perform the complete analysis procedure with a food sample known to be free of creatine/creatinine that is fortified at different levels.

12.2.1 Creatinine addition:

- Prepare three solutions, as described below, and call them: A, B, C.
- Weigh 10 g (+/-0.05 g) food sample in a 250 ml volumetric flask and dissolve with about 100 ml hot water (ca.60°C).
- Shake vigorously; reduce the temperature by adding 100 ml of cold water.
- Pipet the required volume creatinine solution S3 (see chapter 9.1.5, "Solutions for Additions"), as prescribed in the following table, to the sample solutions A, B, C

Sample solution	ml creatinine solution S3	g creatinine per 100 g product	mg creatinine per litre prepared soup
A	5.0 ml	0.125	25 mg
B	10.0 ml	0.25	50 mg
C	15.0 ml	0.375	75 mg

- Fill to the mark with water.

Hydrolysis and reaction to form Creatinine from Creatin

- Perform according to the procedure given in 9.2.2, "Hydrolysis and reaction to form Creatinine from Creatin".

13 REMARKS

- If the creatinine content of the sample is outside of the calibration curve it is recommended to adjust the sample weight.
- The cleanup step with Sep-Pak cartridges is necessary to remove matrix components which otherwise elute before and after the components of interest.
- The column flush of 4 min. is required to elute residual matrix components from the analytical system.
- Evaluation of extracts from various samples showed no interfering components in the region of the internal standard cytosine. Thus the internal standard technique may be applied.
- Sample preparation is based on WATERS Sep-Pak material. Other suppliers may provide similar or identical material. However the performance of other materials must be checked carefully and procedures may need adjustment.
- Products of certain suppliers are referenced because they performed properly. Products from other suppliers may also perform adequately. However, before they can be applied, their performance has to be demonstrated by applying relevant method checks.

14 LITERATURE

[1] REVISED CODEX STANDARD FOR BOUILLONS AND CONSOMMÉS *CODEX STAN 117-1981, Rev. 2-2001,* ALINORM 01/29A, April 2001

[2] H.Müller, V.Siepe und W.Stadelmann: Quantitative Bestimmung von Kreatinin in Fleischextrakt und fleischextrakthaltigen Lebensmitteln durch Anwendung der HPLC auf die Methode von Carisano et al. Dtsch.Lebensm.-Rundsch. 79,(1983) 395-400.

[3] REVISED CODEX STANDARD FOR BOUILLONS AND CONSOMMÉS *CODEX STAN 117-1981, Rev. 2-2001*, section 2.1.3; ALINORM 01/29A, April 2001;

Internet Link (April 2003) to
REVISED CODEX STANDARD FOR BOUILLONS AND CONSOMMÉS:
http://www.codexalimentarius.net/more_info.asp?id_sta=286
Full text (english):
ftp://ftp.fao.org/codex/standard/en/CXS_117e.pdf

APPENDIX I:
COLUMN HANDLING AND MAINTENANCE

Overnight

- Operate the HPLC system with 0.05 to 0.1 ml/min. mobile phase M1 (buffer solution).

During extended period of non-use (e.g. weekends)

- Rinse column with pure water for about one hour (flow 0.8ml/min. = 50 ml) to wash out all buffer salts.
- Run the chromatographic system with mobile phase M2 (methanol-water 1:1) at 0.05 to 0.1 ml/min.

Restart for use

- Rinse column with pure water for about half an hour (flow 0.8ml/min. = 25 ml).
- Condition column with mobile phase M1 at 0.8 ml/min. for about half an hour. The baseline must be stable and standard solution must be injected to evaluate system performance.

Alternate Chromatographic Columns:

Since the collaborative study was peformed, alternate columns, better suited for chromatographic analyses in pure aqueous phases are available and may be used. Recommended is:

ProntoSIL 120-3-C18 AQ 3 µm, 250 mm x 4.6 mm ID Metrohm CH-Herisau

Mobile Phase M1 (Stability)

Some users of the method suggest to add a small amount of Sodium azide (NaN_3) as antimicrobial agent before the filtering step. Thus the stability of the mobile phase M1 is enhanced.

DETERMINATION OF TOTAL NITROGEN

February 1978 – Original text in English

1. SCOPE

Determination of total nitrogen in products of animal or vegetable origin. The results do *not* include nitrogen present in the form of nitrites or nitrates.

2. FIELD OF APPLICATION

Meat extract
Bouillons
Seasonings
Soups and related products
Raw materials

3. DEFINITION

Total nitrogen content is the quantity of nitrogen corresponding to the ammonium ions present and/or produced and determined under the conditions described.

4. PRINCIPLE

Digestion of organic matter and conversion of nitrogen to ammonium ions with sulphuric acid using a mixture of copper (II) sulphate and titanium dioxide as a catalyst. Liberation of ammonia by alkalinisation. Distillation and acidimetric determination of the ammonia.

5. REAGENTS

5.1. Water, distilled or demineralised.

5.2. Sulphuric acid – 98% m/m, analytical grade.

5.3. Catalyst mixture:
- 930 g potassium sulphate (K_2SO_4)
- 30 g copper (II) sulphate 5 aq. ($CuSO_4 \cdot 5H_2O$)
- 30 g titanium dioxide (TiO_2)
- 10 g stearic acid (antifoaming agent).

5.4. Sodium hydroxide solution – 40% m/v, analytical grade.

5.5. Sulphuric acid solution – 0,1 *N*, standard volumetric solution. Substances low in nitrogen content may be titrated with more dilute sulphuric acid.

5.6. Boric acid – Indicator-solution

5.6.1. Dissolve 20 g boric acid (H_3BO_3) in water and dilute to 1000 ml.

5.6.2. Mixed indicator solution prepared by dissolving:
- 20 mg methyl red and
- 100 mg bromocresol green in
- 120 ml 96% (v/v) ethanol.

5.6.3. Add 40 ml mixed indicator solution (5.6.2.) to 1000 ml boric acid solution (5.6.1.).

6. APPARATUS

6.1. Kjeldahl flasks at least 250 ml capacity and a suitable device for elimination of fumes and vapours.

6.2. Steam distillation apparatus.

6.3. Heating apparatus, on which the Kjeldahl flask can be heated in an inclined position, in such a way that the source of heat only touches that part of the flask wall which is below the liquid level. If gas heating is used, the equipment should be made of heat resistant material provided with a circular hole, so that only the lower part of the flask is exposed to the free flame.

6.4. Graduated cylinders or dispensers, for sulphuric acid (25 ml) and for sodium hydroxide solution (15 ml).

6.5. Volumetric flask, 250 ml.

6.6. Beaker or Erlenmeyer, 150 ml

6.7. Magnetic stirrer and magnetic rod.

6.8. Normal or piston burette, 20, 25 or 50 ml.

6.9. Pipettes 10, 15, 20 and 25 ml.

7. SAMPLING AND SAMPLES

Test portion	(mass of dry substance)
2–2,5 g	(low in fat content)
1,5–2 g	(high in fat content).

The test portion must be homogeneous and should be weighed to the nearest mg. For dry and pasty products the use of nitrogen-free paper boats is recommended.

8. PROCEDURE

8.1. Digestion

8.1.1. Introduce the test portion into the Kjeldahl flask.

8.1.2. Add 10 g catalyst mixture (5.3.).

8.1.3. Add 25 ml sulphuric acid 98% m/m (5.2.).

8.1.4. Place the flask on the heating device and heat until the solution is clear (green).

8.1.5. Continue heating for about 30 minutes (see 10).

8.1.6. Prepare the stock solution as follows: transfer the digest after cooling quantitatively into a 250 ml volumetric flask, using about 200 ml distilled water. Cool again, make up to the mark and mix.

8.2. Distillation

8.2.1. Pipet about 20 ml boric acid indicator solution (5.6.) into a 150 ml beaker or erlenmeyer (6.6.).

8.2.2. Place the beaker or erlenmeyer under the condenser of the distillation apparatus (tip at least 1 cm under liquid level).

8.2.3. Pipet an aliquot part of the stock solution (8.1.6.), 25 ml maximum, which should preferably contain 10–30 mg nitrogen into the distillation apparatus. If more than 25 ml stock solution is taken, the specified amount of sodium hydroxide (8.2.4.) solution is not sufficient to neutralize the sulphuric acid.

8.2.4. Add 15 ml sodium hydroxide solution (5.4.).

8.2.5. Distil the solution till the volume of the distillate is about 60 ml. The colour changes from red to blue.

8.2.6. Distil further for about 1 minute with the tip of the condenser above the liquid level.

8.2.7. Rinse off the tip with distilled water and remove the beaker.

8.2.8. Carry out a blank determination according to 8.1.2–8.2.7.

8.3. Titration (using a magnetic stirrer 6.7.)

8.3.1. Titrate the distillate of the blank with sulphuric acid (5.5.) till the colour changes from blue to red (a ml).

8.3.2. Titrate the distillate of the sample with sulphuric acid (5.5.) to the same colour as the blank after titration (b ml).

9. EXPRESSION OF RESULTS

9.1. Method of calculation:

(Data in percentage by weight to two decimal places)

$$\text{Nitrogen content} = \frac{(b-a)\times N\times 14\times V\times 100}{V_1\times m}\ \%$$

Where

a = sulphuric acid (ml) used for the blank (8.3.1.)
b = sulphuric acid (ml) used for the sample (8.3.2.)
N = normality of the sulphuric acid solution
V = ml of the stock solution derived from the digested test portion (8.1.6.)
V_1 = ml stock solution used for distillation
m = mass of test portion in mg

9.2. Repeatability:

0,02 g/100 g test sample with homogeneous products
0,1 g/100 g test sample with heterogeneous products

10. SPECIAL CASES

In the presence of compounds which are difficult to digest (like lysine) a longer digesting time is necessary.

11. BIBLIOGRAPHICAL REFERENCES

11.1. KJELDAHL J.: Medd. Carlsberg Lab. 2 (1883).

11.2. KJELDAHL J.: Neue Methode zur Bestimmung des Stickstoffs in organischen Körpern. Z. Anal. Chem. 22 (1883) 366–382.

11.3. BAKER P.R.W.: The micro-Kjeldahl determination of nitrogen. An investigation of the effects of added salt and catalysts. Talanta 8 (1961) 57–71.

11.4. WILLIAMS P.C.: The use of Titanium dioxide as a catalyst for large-scale Kjeldahl determination of the total nitrogen content of cereal grains. J. Sci. Fd. Agric. 24 (1973) 343–348.

11.5. STIRRUP J.E. and HARTLEY A.W.: The use of Titanium dioxide and Copper sulphate as a catalyst mixture for the Kjeldahl determination of nitrogen in feedingstuffs. J. Ass. Pub. Anal. 13 (1975) 72–75.

DETERMINATION OF THE α-AMINO NITROGEN CONTENT

September 1985 – Original Text in German

1. SCOPE

Determination of α-amino nitrogen as a measure of free α-amino acids in foods.

2. FIELDS OF APPLICATION

Bouillon products
Seasoning

3. DEFINITION

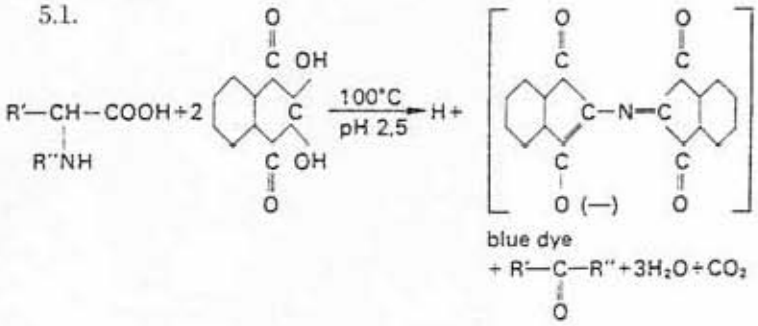
The α-amino nitrogen content is directly proportional to the amount of free and alkylated α-amino groups of free amino acids and is determined by means of the equivalent amount of carbon dioxide liberated under the conditions of test.

4. PRINCIPLE

The carbon dioxide liberated (5.1.) by ninhydrin decarboxylation is fed into a barium hydroxide solution (5.2.) by means of a nitrogen flow, the excess barium hydroxide is retitrated (5.3.) with hydrochloric acid and calculated according to the definition (3) as nitrogen.

5. REACTIONS

5.1.



5.2. $CO_2 + Ba(OH)_2 \rightarrow BaCO_3 + H_2O$

5.3. $Ba(OH)_2 + 2HCl \rightarrow BaCl_2 + 2H_2O$

6. REAGENTS

6.1. 0,1 *N* hydrochloric acid (HCl).

6.2. 0,1 *N* barium hydroxide solution [$Ba(OH)_2$] with 20 g of barium chloride per litre ($BaCl_2 \cdot 2H_2O$)
For determination of exact titer, see 9.8.

6.3. Ninhydrin, solid, A.R.

6.4. Citrate buffer pH 2,5.
In the mortar grind finely and mix thoroughly
2,06 g of trisodium citrate-dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$)
19,15 g of citric acid-monohydrate($C_6H_8O_7 \cdot H_2O$).

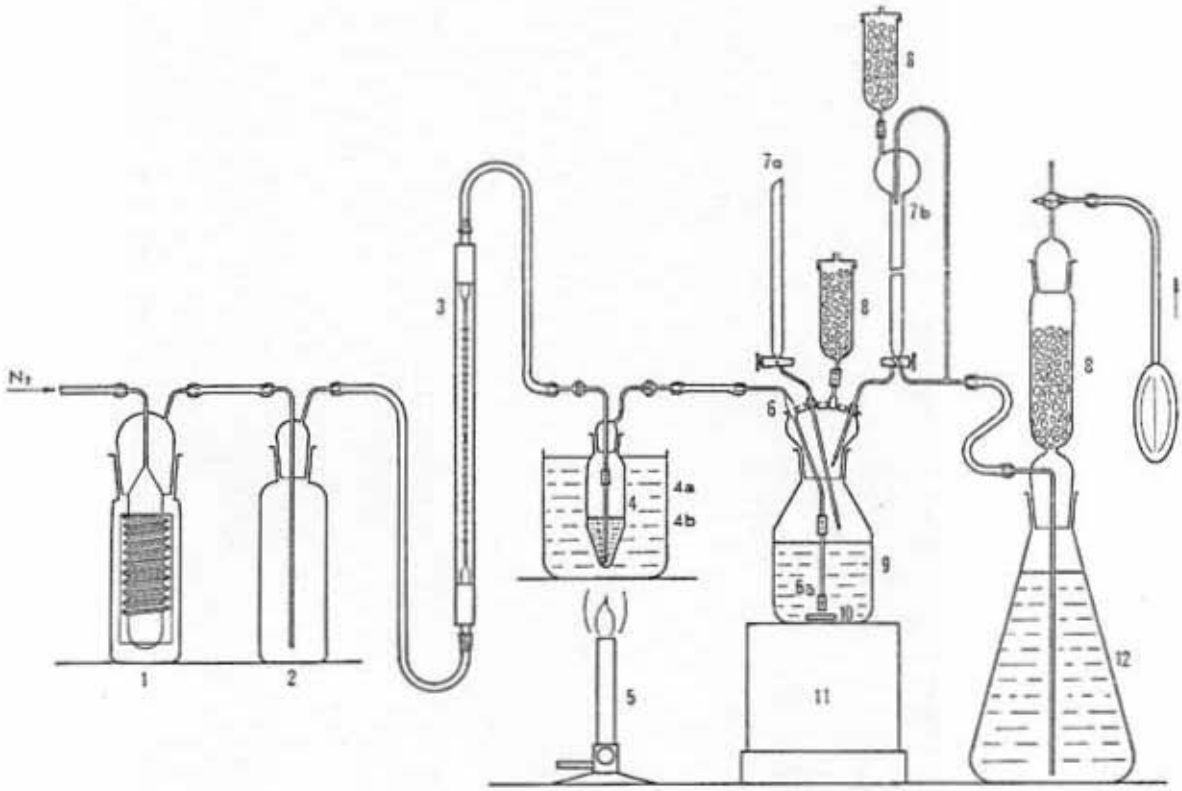
6.5. Phenolphthalein, dissolve 1 g in 100 ml 95% ethanol.

6.6. Sodium hydroxide (NaOH) 10%.

6.7. Nitrogen (CO_2-free).

6.8. Octyl Alcohol.

7. APPARATUS



7.1. Friedrichs wash bottle (1) containing sodium hydroxide (6.6.).

7.2. Wash bottle (2) (filled with glass wool).

7.3. Flow meter, measuring range 5–150 ml/min (3).

7.4. Reaction vessel (4), 30 ml and 4a – boiling water bath
4b – ice bath (0 °C).

7.5. Bunsen burner or hot plate (5).

7.6. Gas inlet tube (6).
a) plastic tube, inside diameter 2 mm (6a).

7.7. Burettes
a) 0,1 *N* HCl (7a).
b) 0,1 *N* $Ba(OH)_2$ (prevent contamination by CO_2 during filling) (7b).

7.8. Columns containing soda lime (8)

7.9. Titration vessel (9)

7.10. Bar magnet (10)

7.11. High/low speed magnetic stirrer (11)

7.12. Storage bottle for 0.1 *N* $Ba(OH)_2$-solution (12)

7.13. Stopwatch

Note: The distance between the gas inlet tube (7.6a.) and the bar magnet (7.10.) should be 2–3 mm in order that the gas can be dispersed in the absorption liquid in very small bubbles.

8. SAMPLE

The homogenised test portion (m) should be such that 100 ml (V) of stock solution contains 60–140 mg of α-amino nitrogen.
In the case of products ready to eat, a concentration by evaporation is necessary.

9. PROCEDURE

9.1. Heat 5 ml (V_1) stock solution (≙approximately 5 mg of α-amino nitrogen) together with 100 mg of citrate buffer (6.4.) and 1–2 drops of octyl alcohol, in the open reaction vessel in the boiling water bath for 2–3 minutes to eliminate any CO_2 present.

9.2. Cool the reaction vessel, by means of ice water (to less than 5° C); add 250 mg of ninhydrin (6.3.) and connect the reaction vessel to the apparatus immediately.

9.3. Add 2 drops each of phenolphthalein solution (6.5.) and octyl alcohol (6.8.) to the collecting (titration) vessel, make all connections as shown in the diagram and start the flow of nitrogen (150 ml/min).

9.4. Purge with CO_2-free nitrogen (6.7.) for 3 minutes and then add 20 ml of 0,1 *N* $Ba(OH)_2$-solution into the collecting (titration) vessel. Start the magnetic stirrer and immerse the reaction vessel in the boiling water bath. The magnetic bar should rotate as fast as possible but consistently in order to produce very small gas bubbles. The nitrogen flow is now reduced to 5 ml/min.

9.5. As soon as the solution has taken on a blue colour (5.1.), heat the reaction vessel for exactly 7 minutes.

Note: With longer boiling time the malonaldehyde, formed primarily from aspartic acid according to the reaction in 5.1., disintegrates releasing a further CO_2 molecule.

9.6. Subsequently the nitrogen flow to 50 ml/min to transfer the CO_2 quantitatively within 10 minutes.

9.7. Then titrate the contents of the collecting (titration) vessel with 0,1 *N* HCl (6.1.) until the solution is colourless.

9.8. The titer of the barium hydroxide solution, $Ba(OH)_2$ should be checked by extra titration with 0,1 *N* HCl once a day (b ml).
To ensure that the amount of $Ba(OH)_2$ solution is added and titrated under identical conditions, it is recommended to perform this step immediately after the first determination of a series (without opening or emptying the titration vessel).

10. EXPRESSION OF RESULTS

10.1. Method of calculation: (% m/m to two decimal places)

$$\text{Content of } \alpha\text{-amino nitrogen} = \frac{(b - a) \times 0{,}7 \times V \times 100}{V_1 \times m} \%$$

b = ml 0,1 *N* HCl equivalent to the amount of $Ba(OH)_2$ solution added (9.8.)
a = 0,1 *N* HCl consumption for retitration (9.7.), in ml
V = ml of stock solution produced from the test portion
V_1 = ml of stock solution used
m = mass of the test portion, in mg

10.2. Limit of determination: approximately 0,15 mg α-amino nitrogen per 5 ml of stock solution (V_1)

10.3. Repeatability: 0,05 g/100 g test sample

11. LITERATURE

11.1. SCHENK, P. and SCHERZ, R.: Bestimmung des α-Aminostickstoffs freier Aminosäure durch Decarboxylierung mit Ninhydrin. Mitt. Lebensmittelunters. Hyg. *56* (1965) 484–491.

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11.3. VAN SLYKE, D.D., McFADYEN, D.A. and HAMILTON, P.: Determination of free amino acids by titration of the carbon dioxide formed in the reaction with ninhydrin. J. biol. Chem. *141* (1941) 671–680.

DETERMINATION OF AMMONIACAL NITROGEN

October 1983 – Original text in English

1. SCOPE

Determination of ammoniacal nitrogen in products of animal or vegetable origin.

2. FIELD OF APPLICATION

Meat extract
Bouillons
Seasonings
Soups and related products
Raw materials

3. DEFINITION

The ammoniacal nitrogen content corresponds to the quantity of ammonium ions present in the sample at the time of determination, analysed under the conditions described.

4. PRINCIPLE

The ammonia liberated from the fat free sample solution by alkali treatment is distilled and determined acidimetrically.

5. REAGENTS

5.1 Water, distilled or demineralised

5.2 Sodium hydroxide solution – 40% m/v, analytical grade

5.3 Sulphuric acid solution – 0,01 *N*, standard volumetric solution

5.4 Boric acid – indicator solution

5.4.1 Dissolve 20 g boric acid (H_3BO_3) in water and dilute to 1000 ml

5.4.2 Mixed indicator solution prepared by dissolving:
- 20 mg methyl red and
- 100 mg bromocresol green in
- 120 ml 96 % (v/v) ethanol

5.4.3 Add 40 ml mixed indicator solution (5.4.2) to 1000 ml boric acid solution (5.4.1)

6. APPARATUS

6.1 Beaker, 100 ml

6.2 Conical flask (Erlenmeyer), 150 ml

6.3 Volumetric flask, 100 ml

6.4 Magnetic stirrer and magnetic rod

6.5 Normal or piston burettes 5, 10 or 20 ml

6.6 Pipettes

6.6.1 20 ml, volumetric
6.6.2 Graduated for measuring 0,5 ml

6.7 Funnels

6.8 Fluted filter paper

6.9 Water bath

6.10 Glass rods

6.11 Steam distillation apparatus

7. SAMPLING AND SAMPLE

The sample must be homogenised by suitable means so that the 10 g test portion is truly representative for the sample.

8. PROCEDURE

8.1 Preparation of stock solution

10 g of the homogenised sample, weighed with an accuracy of 1 mg, are dissolved in distilled water in a volumetric flask (6.3), filled up to the mark and filtered (=stock solution V).

In the case of products containing fat, such as clear soups and bouillons, the test portion is heated in a beaker (6.1) with distilled water in a water bath (6.9) until the fat is melted; subsequently the solution is cooled until the fat has solidified, then the layer of fat is pierced with a glass rod (6.10) and the solution is filtered through a fluted filter (6.8) into a 100 ml volumetric flask (6.3). The process (melting and solidification of fat) is then repeated with smaller water portions. Finally the solidified fat is put onto the filter and washed with distilled water. The collected filtrate in the volumetric flask is then mixed and made up to 100 ml with distilled water (=stock solution V).

Note:
The stock solution may be kept in the refrigerator for up to 48 hours.

8.2 Distillation

8.2.1 Pipette about 20 ml boric acid indicator solution (5.4.3) into a conical flash (6.2).

8.2.2 Place the conical flask under the condenser of the distillation apparatus (tip at least 1 cm under liquid level).

8.2.3 Pipette 20 ml (V_1) of the stock solution (8.1) into the distillation apparatus (6.11).

8.2.4 Add, by pipette (6.6.2) 0,5 ml sodium hydroxide solution (5.2); immediately close the tap.

8.2.5 Distill the mixture until the volume of distillate is about 60 ml.

8.2.6 Distill further for about 1 minute with the tip of the condenser 0,5–1 cm above the liquid level.

8.2.7 Then rinse off the tip with distilled water and remove the conical flash.

8.2.8 Carry out a blank determination according to 8.2.1–8.2.7 (using 20 ml of distilled water instead of stock solution).

8.3 Titration (using a magnetic stirrer 6.4)

8.3.1 Titrate the distillate of the blank with sulphuric acid (5.3) until the colour changes from blue to reddish tinted violet, which corresponds to a pH of 4,6±0,1 (a ml).

8.3.2 Titrate the distillate of the sample with sulphuric acid (5.3) to the same colour as the blank after titration (b ml).

9. EXPRESSION OF RESULTS

9.1 Method of calculation: (Results in percentage m/m to two decimals)

$$\text{Ammoniacal nitrogen content} = \frac{(b-a) \cdot N \cdot 14 \cdot V \cdot 100}{V_1 \cdot m} \%$$

Where
a = sulphuric acid (ml) used for the blank (8.3.1)
b = sulphuric acid (ml) used for the sample (8.3.2)
N = exact normality of the sulphuric acid solution
V = ml of the stock solution (=100 ml) derived from the test portion (8.1)
V_1 = ml stock solution (=20 ml) used for distillation (8.2.3)
m = mass of test portion in mg

Note:
If the content is to be expressed as ammonia (NH_3), rather than ammoniacal nitrogen, replace in the calculation the equivalent 14 by 17.

9.2 Repeatability:
0,01 g/100 g test sample

10. SPECIAL CASES

At the start of treatment with alkali the ammonia present is completely liberated; 'secondary' ammonia formation by splitting nitrogenous organic substances such as amino acids, acid amides, proteins and similar substances will appear only gradually. For this reason the distillation period should be as short as possible. Also the addition of sodium hydroxide (0.5 ml) must be accurate since too high a pH will accelerate the evolution of 'secondary' ammonia.
During distillation primary, secondary and tertiary aliphatic amines react like ammonia. To determine ammonia besides amines, NH_3 can be precipitated either as a molecular complex with mercuric oxides (11.3 and 11.4), or as ammoniumhexanitrilocobaltate (III) $(NH_4)_3[Co(NO_2)_6]$ (11.5) or as magnesiumammoniumphosphate $MgNH_4PO_4 \cdot 6H_2O$ (11.2). The precipitate is transferred into the distillation apparatus and distilled as described in paragraph 8.2.

11. BIBLIOGRAPHICAL REFERENCES

11.1 Handbuch der Lebensmittelchemie, Band II/2 (1967) 168 ff., Springer-Verlag

11.2 BAYER, A.: Eine neue quantitative Ammoniakbestimmung. Chemiker-Ztg. *27* (1903) 809–810

11.3 BRINER, E., and GAUDILLON, J.: Recherches sur l'obtention des methylamines par déshydratation catalytique du système ammoniac-alcool méthylique. Helv. chim. Acta *14* (1931) 1283–1307

11.4 FRANZEN, H., and SCHNEIDER, A.: Über die Trennung aliphatischer Amine voneinander und von Ammoniak. Biochem. Z. *116* (1921) 195–207

11.5 LEONE, P.: Nuovo processo di separazione delle ammine alifatiche dall'ammoniaca. Gazz. chim. ital. *55* (1925) 246–252

DETERMINATION OF FREE L-GLUTAMIC ACID CONTENT

December 1980 – Original text in English

1. SCOPE

Determination of free L-glutamic acid and its salts.

2. FIELD OF APPLICATION

Bouillons and meat bouillons
Soups and related products
Seasonings (protein hydrolysates)

3. DEFINITION

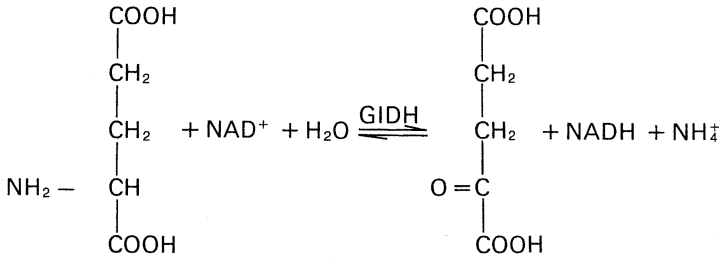
The free L-glutamic acid content is equivalent to the quantity of formazan formed under the test conditions.

4. PRINCIPLE

In the presence of the enzyme glutamic dehydrogenase (GlDH), L-glutamic acid is de-aminated oxidatively by nicotin-amide dinucleotide (NAD) to α-ketoglutaric acid (5.1).
The NADH formed simultaneously reduces iodonitrotetrazolium chloride (INT) in a reaction catalysed by diaphorase. The colour intensity of the formazan produced (5.2) is measured at 492 nm.

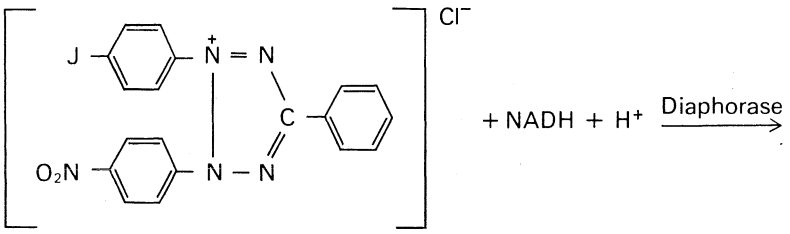
5. REACTIONS

5.1

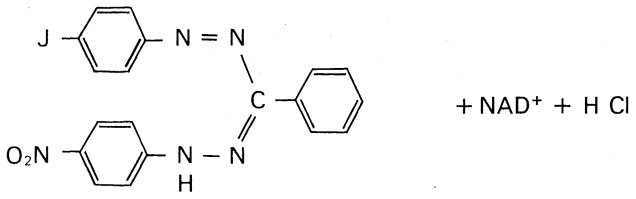


L-Glutamic Acid α-Ketoglutaric Acid

5.2



INT (colourless)



Formazan (red)

The equilibrium of reaction 5.1 lies far on the side of glutamic acid. Oxidation of the NADH to NAD^+ with INT (5.2) displaces the equilibrium in favour of α-ketoglutaric acid.
If the NH_4^+ content is high the reaction proceeds less rapidly.

6. REAGENTS

6.1 Test-Combination

Boehringer		Reagents	Quantity supplied	Add ml bidist. water	Stability of the solutions
6.1.1	Solution 1	Buffersolution pH 8,6: Potassiumphosphate/ Triethanolamine Triton X 100 – 0,5 ml	25 ml	–	+ 25 °C, 2 months
6.1.2	Solution 2	Lyophilisate consisting of diaphorase from pig heart – 12 Units β-NAD 40 mg Stabilizers	100 mg	8 ml	+ 4 °C, 3 weeks
6.1.3	Solution 3	Iodonitrotetrazolium chloride solution	2,5 ml	6 ml	+ 20 °C, 1 month (in the dark)
6.1.4	Solution 4	Glutamic acid dehydrogenase – 1000 Units	1 ml	–	+ 4 °C, 1 year

6.2 Bidistilled Water

6.3 Glutamic acid standard solution

6.3.1 Weigh approx. 500 mg (m_o) glutamic acid (Merck No. 291) to an accuracy of 0,1 mg, dissolve in 3 ml of 1 *N* NaOH (6.5) and dilute to 1000 ml with water (6.2) in a volumetric flask (7.1).

6.3.2 Pipette 10 ml (~ 5 mg glutamic acid) of solution 6.3.1 into a 1000 ml volumetric flask (7.1) and dilute with water (6.2) to the mark (~ 10 µg/2 ml).

6.4 Petroleum ether (BP 40–60°C or 50–70°C)

6.5 1 *N* sodium hydroxide solution.

7. APPARATUS

7.1 Volumetric flask, 1000 ml

7.2 Funnel, filter paper fast

7.3 Pipettes

7.3.1 Piston Pipettes, 30 µl, 200 µl and 600 µl

7.3.2 2 ml

7.3.3 10 ml

7.3.4 Graduated pipettes for measuring 3, 6, 8 ml

7.4 Plastic rods to mix the content of the cells (supplied by Boehringer)

7.5 Photometer

7.5.1. Wave length 492 nm

7.5.2 Cells optical path 1 cm

7.6 Waterbath

8. SAMPLING AND SAMPLES

The sample is homogenised, and kept in airtight containers.

8.1 Stock Solution

Weigh a test portion of approx. 2 g (see 8.3) to an accuracy of 1 mg and suspend in 100–150 ml water (6.2); heat the suspension in a waterbath (7.6) at 60°C, until the fat is melted.

Add 10 ml petroleum ether (6.4), cool and transfer quantitatively into a 1000 ml (V) volumetric flask (7.1). Dilute the aqueous phase to the mark with water (6.2) and shake.

After separation draw off with a capillary the ether-fat-solution and filter the aqueous phase. (The first 10 ml of the filtrate is discarded).

8.2 Sample Solution
A volume (V_1) of the stock solution filtrate (8.1) containing about 5 mg glutamic acid is withdrawn and made up with water (6.2) to 1000 ml in a volumetric flask (7.1). This solution must be clear and, if necessary should be filtered again.

8.3 Note:
The glutamic acid concentrations of the sample solution and of the glutamic acid standard solution (6.3) should be nearly the same (~ 10 µg/2 ml).
To achieve this, the test portions (8.1) of substances containing less than 0,5% glutamic acid need to be increased accordingly, while test portions of substances containing more than 25% glutamic acid will have to be reduced as required.

9. PROCEDURE

9.1 The following solutions are pipetted into the cells and the extinctions are measured against air (without cell in the light path).

Solutions	Cells		
	Blank	Standard	Sample
Solution 1 (6.1.1)	600 µl	600 µl	600 µl
Solution 2 (6.1.2)	200 µl	200 µl	200 µl
Solution 3 (6.1.3)	200 µl	200 µl	200 µl
Water bidist. (6.2)	2,0 ml	–	–
Glutamic acid standard solution (6.3.2)	–	2,0 ml	–
Sample solution (8.2)	–	–	2,0 ml
Mix and read the extinction E_1 after 2 minutes			
Solution 4 (6.1.4)	30 µl	30 µl	30 µl
Mix and read the extinction E_2 after 30 minutes			

9.2 Repeat the procedure to obtain a second series of values.

10. EXPRESSION OF RESULTS

10.1 Calculation
(Data as percentages m/m to one decimal)

10.1.1 Results expressed as glutamic acid (GA)

$$\text{Glutamic acid content} = \frac{(A - C) \cdot m_o \cdot V}{(B - C) \cdot m \cdot V_1} \%$$

A = mean value ($E_2 - E_1$) for the sample solution
B = mean value ($E_2 - E_1$) for the glutamic acid standard solution
C = mean value of ($E_2 - E_1$) for the blank
m_o = mass of glutamic acid in mg (6.3.1)
m = test portion in mg (8.1)
V = stock solution in ml (8.1) [1000 ml]
V_1 = ml stock solution ($\sim$ 5 mg GA) used for preparing 1000 ml sample solution (8.2)

10.1.2 Results expressed as monosodium glutamate (MSG)

MSG content = glutamic acid content × F

glutamic acid content: see 10.1.1

$$F = \frac{\text{Molecular weight MSG} \cdot 1\,H_2O}{\text{Molecular weight glutamic acid}} = \frac{187{,}14}{147{,}13} = 1{,}272$$

10.2 Repeatabilty

0,1 g/100 g sample

11. BIBLIOGRAPHICAL REFERENCES

11.1 BEUTLER, H.O. and MICHAL, G. in H.U. BERGMEYER: Methoden der enzymatischen Analyse, 3rd Edition (1974) 1753, Verlag Chemie Weinheim

11.2 Boehringer, Mannheim GmbH: *Food Analysis,* Colour Test for the determination of L-glutamic acid in foodstuffs. (Cat. No. 15 264)

Enzymatic Determination of L-Glutamic Acid by Warburg's Manometric Method

The technique of the Warburg method is not dealt with in the procedure described below.

The quantitative determination of L-glutamic acid is carried out by means of specific decarboxylases. The evolved CO_2 is measured quantitatively in a Warburg apparatus.

The following decarboxylases have been used successfully:

Decarboxylase from radishes (Raphanus Sativus) (1) (2).
Decarboxylase from Clostridium welchii (3).
Decarboxylase from Escherichia coli (4).

Preparation of the solution for analysis

The amount of all products for these determinations must be chosen so that 1 ml of the final solution will contain 0.4–1.5 mg of glutamic acid. On the other hand there should not be more than 10 g of the product in a volume of to 100 ml.

Very fatty products must first be freed from fats by dissolving the weighed substance in about 50 ml of water and heating the product on a water-bath until the fat is melted. After cooling, the solidified fat layer is pierced and the solution is directly filtered through a folded filter into a volumetric flask. This must be performed twice. Finally, the solid fat is transferred to the filter and thoroughly washed.

Products containing starch should not be dissolved hot.

1 ml of the solution thus prepared is introduced into the reaction vessel with a calibrated pipette. To it is added 1 ml of the buffer solution specific for the enzyme to be used. The enzyme solution itself is put into the side bulb of the reaction vessel.

It is recommended that at least two determinations be made for each sample.

The calculation is done according to the following equation:

$$\text{mg L glutamic acid per ml of test solution:} = \frac{D \times K \times 0.147}{22.4}$$

D = corrected manometric difference in mm.
K = constant for the vessel.

The value expressed in terms of sodium glutamate is as follows:

$$\text{mg L sodium glutamate per 1 ml of test solution} = \frac{D \times K \times 0.187}{22.4}$$

Limits of error: ± 0.3% to ± 0.5%.

Literature:

(1) Bestimmung der L-Glutaminsäure, K. Hasse and H. W. Schumacher, Z. anal. Chem. *137*, 433 (1953).

(2) Bestimmung der L-Glutaminsäure in Zuckerfabrikprodukten, Z. für Zuckerindustrie *8*, 583 (1958).

(3) Quantitative Determination of Glutamine and Glutamic Acid, H. A. Krebs, Biochem. *43*, 51 (1948).

(4) Quantative Determination of L-Glutamic Acid by Decarboxylase (from E. Coli), M. Seidmann and M. J. Blish. J. Agr. and Food Chem. *5*, 448 (1957).

Determination of Amino Acids by Paper Electrophoresis with Special Attention to Glutamic Acid

Reagents:

Buffer solution pH 3.8 (0.2 n-acetate buffer).

Ninhydrin solution: 1% in 90% (vol.) ethyl alcohol. To this solution is added 0.1% of crystallized stannous chloride and filtered. Some commercial ninhydrin preparations do not react with amino acids. In most cases, however, it is sufficient to re-crystallize such a sample from water or hydrochloric acid. The reagent should always be freshly prepared before use.

Acetone solution 80% (by volume).

The solutions to be analyzed are prepared in the same way as described for the Warburg method. It is, however, recommended to prepare the solution for analysis so that it will contain, as nearly as possible, 2 mg of glutamic acid in 1 ml. It is furthermore necessary to prepare a standard solution of pure glutamic acid, or of sodium glutamate, to be used simultaneously. The concentration of the standard solution should, as nearly as possible, be the same as that of the solution to be analyzed.

Procedure:

The solution to be analyzed is applied to the centre of paper strips for electrophoresis (e.g. Schleicher and Schüll 2043a or Whatmann 1) perpendicular to the direction of migration. Margins of a few mm on both sides of the paper strips should remain free of the test solution. The solution is applied with a 0.01 ml micropipette, the contents being distributed evenly. The applied solutions are dried in a current of warm air and then the strips are sprayed evenly with a pH 3.8 buffer solution (0.2 n acetate buffer). The paper strips are then placed in the electrophoresis cell containing the same buffer solution. When a potential of 270 volts direct current (about 10 V/cm) is applied, separation takes place in about 2 hours. With a lower voltage separation takes correspondingly longer.

After electrophoretic separation is completed, the electropherograms are first dried for a few minutes at room temperature, and then in a drier at 90° C. Afterwards, the strips are passed through a 1% ninhydrin solution and heated for 10 minutes in a oven saturated with water vapour (an oven with a flat dish containing water). For drying and developing of the ninhydrin colour, the paper strips are spread out horizontally and kept taut.

The glutamic acid bands are cut out, reduced to small pieces and eluted with 10 ml of 80% acetone in a test tube provided with a glass stopper. For this purpose the acetone solution is allowed to remain with the paper fragments for 30 minutes, then the whole is vigorously shaken and the solution measured in a 1 cm or 0.5 cm cell at 550 mμ against the blank extract of the paper.

Calculation of the glutamic acid content is effected by comparison of absorbance with that of a standard solution.

It is necessary to make from 3 to 5 determinations for each sample, and for each standard solution, and to take the mean value of these.

Limits of error: ± 0.5 to ± 1%.

Literature:

Papierelektrophoretische Bestimmung von Glutamin-Asparagin- und γ-Aminobuttersäure, F. Schneider, E. Reinefeld and H. Müller, Biochem. Z. *327*, 189 (1955).

Über die stickstoffhaltigen Nichtzuckerstoffe, F. Schneider, E. Reinefeld and H. Müller, Zucker Beihefte 1957, Heft 3, 78–85.

DETERMINATION OF TOTAL FAT CONTENT BASED ON WEIBULL-STOLDT METHOD

July 1989 – Original text in English

1. SCOPE

This method specifies a reference method for the determination of the total fat content.

2. FIELD OF APPLICATION

Broths
Bouillons and Meat bouillons,
Soups and related products

containing fat that cannot be isolated by direct extraction techniques. It can also be used for samples having an unknown composition.

3. DEFINITION

The percentage by weight of total fat in the sample analysed is the content of fat determined according to the procedure described in this standard.

4. PRINCIPLE

A suitable sample portion is boiled with hydrochloric acid in order to release the fat from any substances present. The fatty mass is isolated by filtration, washed and dried.

Subsequently, the fat is separated from the mass by a Soxhlet extraction with petroleum ether. The solvent is removed by distillation and the isolated fat is heated in a stove to remove the last traces of solvent residue. The resulting fatty mass is weighed.

5. REACTIONS

Not applicable.

6. REAGENTS

All reagents must be of a sufficiently pure quality and be tested for interferences.

6.1 Diatomaceous earth (for instance Celite).

6.2 Hydrochloric acid 4 mol/l. Add 330 ml concentrated hydrochloric acid to 670 ml demineralised or distilled water and mix.

6.3 Extraction solvent: petroleum ether, BP 40–60 °C, with a maximal residue after distillation of 2 mg/100 ml.
The same purity limit applies to distilled solvent which is reused after extraction.

6.4 pH paper, wide range, blue litmus paper or the like.

6.5 Wash bottle with demineralised or distilled hot water.

6.6 Supply of nitrogen gas or air, fat free.

7. APPARATUS AND MATERIALS

7.1 Beaker, tall form with spout, 600 ml, provided with a watch glass.

7.2 Glass stirring rod for 7.1.

7.3 Desiccator containing dry silica gel with moisture indicator.

7.4 Glass funnel, diameter at least 100 mm.

7.5 Graduated cylinder, 250 ml.

7.6 Extraction flask, RN 29/32, 250 ml.

7.7 Soxhlet extraction apparatus for 7.6.

7.8 Condenser, socket RN 29/32, jacket length 300 mm. The cooler should not be a wall cooler but have a cooling system (coil) in the middle of the unit. The solvent must drip into the thimble and not run along the glass into the extraction chamber (use for instance a Twisselman cooler).

7.9 Analytical balance.

7.10 Rotavapor system.

7.11 Electrical heating system for Soxhlet extraction (sand bath or heating mantle or water bath set at 70 °C).

7.12 Heating oven, 103±2 °C, with air circulation (preferably fan assisted).

7.13 Defatted cotton-wool.

7.14 Extraction thimble, fat free and 5 mm higher than the siphon of 7.7. Use for instance Schleicher & Schull no. 603 or Macherey, Nagel & Co. no. 645 F.

7.15 Filter paper, sheet, fat free.

7.16 Fluted filters, diameter 150–200 mm, fat free, pore size maximal 5 µm, for instance Schleicher & Schull no. 597 1/2 or Macherey, Nagel & Co. no. 616 1/4 or 614 1/4.

7.17 Pumice stone heated, fat free, small size.

7.18 Pair of pincers.

7.19 Conical flask, RN 29/32, 500 ml.

Remark

Adapt the form of the flask (7.6) to the heating device used (use a round bottom flask where a Rotavapor system is used).

8. SAMPLE

8.1 Start with a representative sample of at least 200 g of the product to be examined.

8.2 Store the sample in such a way, that spoilage and changes in its composition are prevented.

9. PROCEDURE

9.1 Preparation of the material.

Allow the sample to reach ambient temperature. Mix well.

Put some pieces of pumice stone into the extraction flask and dry the flask for one hour at 103 °C. Allow to cool for 45 min in a desiccator and weigh to the nearest 0.1 mg.

9.2 Isolation of the fat (acid hydrolysis).

Weigh a sample portion containing 3–10 g of total solids to the nearest 0,1 mg into a 600 ml beaker. The quantity taken depends on the expected fat content: the isolated fatty mass should be less than 3 g.

Add some pieces of pumice stone and 150 ml diluted hydrochloric acid (6.2).

Put a stirring rod into the beaker and cover the latter with a watch glass. Boil gently for one hour with frequent stirring. Start slowly while stirring until the solution boils very gently and the sample is well suspended in the acid solution (keep the watch glass vertically above the beaker during stirring to avoid losses). Add water during the boiling when needed.

Put two fluted filters, one inside the other, into a funnel and moisten them thoroughly with hot water. Place the funnel on an Erlenmeyer flask. Add 150 ml hot water to the contents of the beaker and mix. Add also 3 g diatomaceous earth to the beaker when insoluble material seems to be absent and mix again.

Filter the hot mixture, including all substances adhering to the glass (pumice stone). Wash the glassware three times with 100 ml hot water and filter the washings through the same fluted filters. Continue the washing procedure until the water used reacts almost neutral (pH paper). Use as little water as possible. Place the filters into the beaker used for the acid hydrolysis and dry for 3 hours at 103 °C. Dry the watch glass and the stirring rod as well.

Remarks

– The filters used should be kept moistened at all times until the final drying before extraction.

– The filters may also be dried inside the thimble when little residue besides fat and diatomaceous earth is present. Residual water however influences the subsequent extraction with petroleum ether

9.2.1 Important

The analysis should be discontinued if strong creeping of the fat is observed when boiling with acid. In this case repeat the analysis in a 500 ml conical flask under reflux boiling. Rinse the condenser subsequently with hot water. Collect the water into the conical flask before it is removed from the cooler. Rinse the socket also.

9.3 Extraction of the fat

Wrap the filters into a slightly larger piece of filter paper and put the parcel into the extraction thimble. Wipe the beaker with a piece of cotton-wool slightly moistened with extraction solvent and held between a pair of pincers. Put this cotton-wool into the thimble in such a way that it comes underneath the solvent when extracting.

Place the thimble into the Soxhlet extractor. Rinse the beaker and other glassware (watch glass, stirring rod) at least twice with the extraction solvent and pour the washings into the extraction flask in order to recover any fat residue still adhering to the glassware used.

Fill the flask with more solvent so that the total volume is at least twice the volume of the extractor.

Extract for 4 hours, using a water bath or other suitable heating system (place the flask for the greatest part inside the water when a waterbath is used). The number of extraction cycles should be at least 30. Remove the extraction solvent by distillation and flush the flask, while still warm, with a gentle stream of nitrogen or air.

9.4 Drying of the fat

Dry the flask in the heating oven for two hours in an almost horizontal – inclined – position 103 °C. Flush the flask, while still hot, with a stream of nitrogen or air and cool in a desiccator for at least 45 min. Weigh the cooled flask to the nearest 0.1 mg. Continue drying in the oven for 30 min periods until the difference between subsequent weighings is less than 0.1 percent of the mass of the test portion. The lowest result obtained should be taken for the calculation if the mass appears to be rising again.

9.5 Remarks

– The use of a Rotavapor system or a Twisselman cooler for the distillation of the extraction solvent is recommended. The Twisselman cooler can be used by closing the stopcock no. 4 (see figure).

– Use a slightly larger extraction flask if difficulties are met when distilling, especially with a Rotavapor system.

– Inspect the extraction flask after the last contact with water for the presence of any deposit adhering to the glass. Clean the surface before drying.

- The temperature of the waterbath or other heating device used must be adapted if irregular or too slow/rapid boiling of the solvent is observed.
- A second extraction with fresh solvent is recommended if an incomplete extraction of the fat is suspected.

Important:

Execute the hydrolysis, extraction, drying and weighing of the fatty mass without interruption.

10. EXPRESSION OF RESULTS

10.1 Calculation

(Results in per cent by weight, expressed to one decimal place)

$$\text{Total fat content} = \frac{(A-B)\times 100}{C}\%$$

A = mass of the extraction flask with fat after drying in g

B = mass of the dried extraction flask without fat in g

C = mass of the test portion taken in g.

10.2 Report with the result also the method and the solvent used.

10.3 Repeatability:

$r = 0.5$ g/100 g Sample $-s_r = 0.18$ g/100 g

Reproducibility

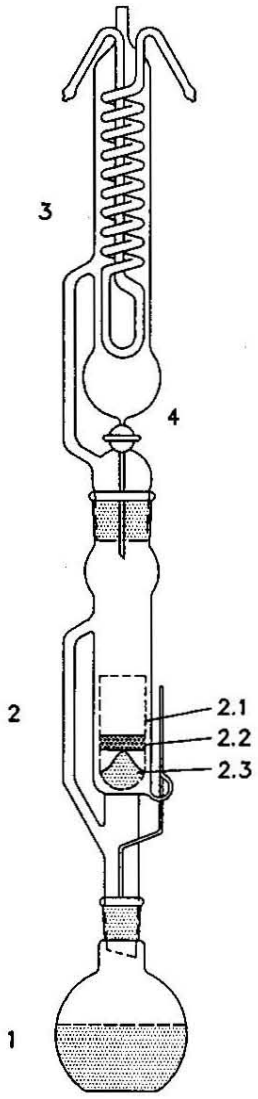
$R = 0.7$ g/100 g Sample $-s_R = 0.25$ g/100 g

(s = standard deviation)

10.4 Repeatability and reproducibility are based on figures:

1) Dutch degree for analysis methods for gravy and sauces, Staatsblad 1985
2) Bestimmung des Gesamtfettgehaltes in Fleisch und Fleischerzeugnissen. Amtliche Sammlung von Untersuchungsverfahren nach §35 LMBG, September 1980 (BRD)
3) Results AIIBP ring test 1987

DETERMINATION OF TOTAL FAT CONTENT



Apparatus

1. Extraction flask with solvent
2. Soxhlet extractor
   - 2.1 Extraction thimble
   - 2.2 Cotton wool, defatted
   - 2.3 Sample according to point 9.3
3. Twisselmann condenser
4. Stopcock

Important: The place of the cooler outlet and the height difference between thimble and siphon (5 mm).

DETERMINATION OF FREE FAT CONTENT BY DIRECT SOXHLET EXTRACTION

July 1989 – Original text in English

1. SCOPE

The method specifies a reference method for the determination of the free fat content.

2. FIELD OF APPLICATION

Broths
Bouillons and Meat bouillons
Soups and related products

containing easily extractable fat.

3. DEFINITION

The percentage by weight of free fat in the sample analyzed is the content of fat determined according to the procedure described in this standard.

4. PRINCIPLE

Free fat in a suitable sample portion is isolated from the mass by a Soxhlet extraction with petroleum ether.

The solvent is subsequently removed by distillation and the fat is weighed after heating in a stove to remove traces of solvent left.

5. REACTIONS

Not applicable.

6. REAGENTS

All reagents must be of a sufficiently pure quality and be tested for interferences.

6.1 Extraction solvent: petroleum ether, BP 40–60 °C, with a maximal residue after distillation of 2 mg/100 ml. The same purity limit applies to distilled solvent which is reused after extraction.

6.2 Supply of nitrogen gas or air, fat free.

7. APPARATUS AND MATERIALS

7.1 Desiccator containing dry silica gel with moisture indicator.

7.2 Extraction flask, RN 29/32, 250 ml.

7.3 Soxhlet extraction apparatus for 7.2.

7.4 Condenser, socket RN 29/32, jacket length 300 mm. The cooler should not be a wall cooler but have a cooling system (coil) in the middle of the unit. The solvent must drip into the thimble and not run along the glass into the extraction chamber (use for instance a Twisselman cooler).

7.5 Analytical balance.

7.6 Rotavapor system.

7.7 Electrical heating system for Soxhlet extraction (sand bath or heating mantle or water bath set at 70 °C).

7.8 Heating oven, 103±2 °C, with air circulation (preferably fan assisted).

7.9 Defatted cotton-wool.

7.10 Filter paper, sheet, fat free.

7.11 Extraction thimble, fat free and 5 mm higher than the siphon of 7.3. Use for instance Schleicher & Schull no. 603 or Macherey, Nagel & Co. no. 645 F.

7.12 Pumice stone heated, fat free, small size.

7.13 Pair of pincers.

Remark

Adapt the form of the flask (7.2) to the heating device used (use a round bottom flask where a Rotavapor system is used).

8. SAMPLE

8.1 Start with a representative sample of at least 200 g of the product to be examined.

8.2 Store the sample in such a way, that spoilage and changes in its composition are prevented.

9. PROCEDURE

9.1 Preparation of the material

Allow the sample to reach ambient temperature. Mix well.

Put some pieces of pumice stone into the extraction flask and dry the flask for one hour at 103 °C. Allow to cool for 45 min in a desiccator and weigh to the nearest 0.1 mg.

9.2 Preparation of the extraction

a) Sample in dry form, containing little water.

Weigh upto 10 g of the product to the nearest 0.1 mg into a thimble. The quantity taken should be adapted so that less than 3 g fat is obtained after extraction. Close the thimble with a piece of cotton-wool to keep the sample in the thimble during the extraction. Place the thimble into the Soxhlet extractor in such a way that the cotton-wool is underneath the solvent when extracting.

b) Liquid sample

A suitable sample portion of liquid samples should first be dried on sea sand according to method 2/2: 'Bouillon and Meat Bouillon'.

Bring the dried residue quantitatively onto a piece of filter paper. Wrap the paper together and put it into a thimble. Clean all utensils and the drying scale + stirring rod used with cotton-wool containing solvent (use pincers!).

Put this cotton-wool into the thimble in such a way that it is underneath the solvent when extracting. Place the thimble into the Soxhlet extractor. Rinse subsequently the materials used at least twice with solvent to remove the last traces of fat and pour the solvent into the extraction flask.

Remark

Take care that any residue remaining in the drying scale is not flushed into the extraction flask. Too high results will be obtained otherwise.

9.3 Extraction of the fat

Fill the flask with more solvent so that the total volume is at least twice the volume of the extractor.

Extract for 4 hours, using a water bath or other suitable heating system (place the flask for the greatest part inside the water when a waterbath is used). The number of extraction cycles should be at least 30. Remove the extraction solvent by distillation and flush the flask, while still warm, with a gentle stream of nitrogen or air.

9.4 Drying of the fat

Dry the flask in the heating oven for two hours in an almost horizontal (inclined) position at 103 °C. Flush the flask, while still hot, with a stream of nitrogen or air and cool in a desiccator for at least 45 min. Weigh the cooled flask to the nearest 0.1 mg. Continue drying in the oven for 30 min periods until the difference between subsequent weighings is less than 0.1 percent of the mass of the test portion. The lowest result obtained should be taken for the calculation if the mass appears to be increasing again.

9.5 Remarks

– The use of a Rotavapor system or a Twisselman cooler for the distillation of the extraction solvent is recommended. The Twisselman cooler can be used by closing the stopcock no. 4 (see figure).

- Use a slightly larger extraction flask if difficulties are met when distilling, especially with a Rotavapor system.
- Inspect the extraction flask after the last contact with water for the presence of any deposit adhering to the glass. Clean the surface before drying.
- The temperature of the waterbath or other heating device used must be adapted if irregular or too slow/rapid boiling of the solvent is observed.
- A second extraction with fresh solvent is recommended if an incomplete extraction of the fat is suspected.

Important

Execute the extraction, drying and weighing of the fat without interruption.

10. EXPRESSION OF RESULTS

10.1 Calculation

(Results in per cent by weight, expressed to one decimal place)

$$\text{Free fat content} = \frac{(A-B)\times 100}{C}\%$$

A = mass of the extraction flask with fat after drying in g

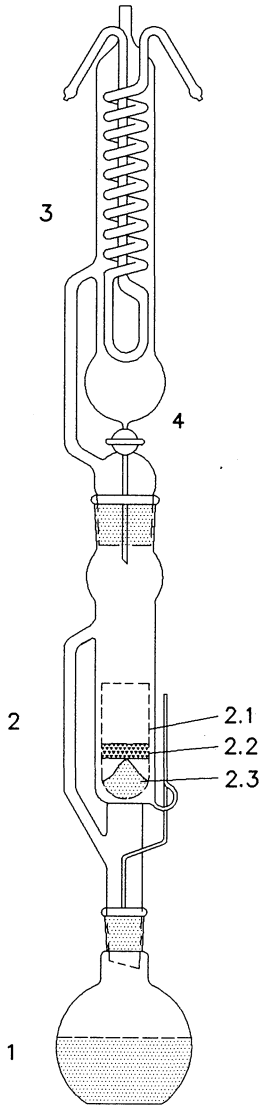
B = mass of the dried extraction flask without fat in g

C = mass of the test portion taken in g

10.2 The repeatability between two determinations should be within 0.5 g/100 g.

10.3 Report with the result also the method and the solvent used.

DETERMINATION OF FREE FAT CONTENT



Apparatus

1. Extraction flask with solvent
2. Soxhlet extractor
   - 2.1 Extraction thimble
   - 2.2 Cotton-wool, defatted
   - 2.3 Sample according to point 9.2a respectively 9.2b.
3. Twisselmann condenser
4. Stopcock

Important: The place of the cooler outlet and the height difference between thimble and siphon (5 mm).

Residue from evaporation of 25 ml of fat solution	Total amount of fat per gr at a sp. gr. of											Interval values	
	0.90	0.91	0.92	0.93	0.94	0.95	0.96	0.97	0.98	0.99	1.00		
0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0	04
0.010	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.040	0.040	1	04
0.020	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	0.080	2	08
0.030	0.121	0.121	0.121	0.121	0.121	0.121	0.120	0.120	0.120	0.120	0.120	3	12
0.040	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.161	0.161	4	16
0.050	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	0.201	5	20
0.060	0.241	0.241	0.241	0.241	0.241	0.241	0.241	0.241	0.241	0.241	0.241	6	24
0.070	0.281	0.281	0.281	0.281	0.281	0.281	0.281	0.281	0.281	0.281	0.281	7	28
0.080	0.321	0.321	0.321	0.321	0.321	0.321	0.321	0.321	0.321	0.321	0.321	8	32
0.090	0.362	0.362	0.362	0.362	0.362	0.362	0.361	0.361	0.361	0.361	0.361	9	36
0.100	0.402	0.402	0.402	0.402	0.402	0.402	0.402	0.402	0.402	0.402	0.402	0	00
0.110	0.442	0.442	0.442	0.442	0.442	0.442	0.442	0.442	0.442	0.442	0.442	1	04
0.120	0.483	0.483	0.483	0.483	0.483	0.483	0.483	0.483	0.483	0.483	0.483	2	08
0.130	0.523	0.523	0.523	0.523	0.523	0.523	0.523	0.523	0.523	0.523	0.523	3	12
0.140	0.564	0.563	0.563	0.563	0.563	0.563	0.563	0.563	0.563	0.563	0.563	4	16
0.150	0.604	0.604	0.604	0.604	0.604	0.604	0.604	0.604	0.604	0.604	0.604	5	20
0.160	0.645	0.645	0.645	0.645	0.644	0.644	0.644	0.644	0.644	0.644	0.644	6	24
0.170	0.685	0.685	0.685	0.685	0.685	0.685	0.685	0.685	0.685	0.685	0.685	7	28
0.180	0.726	0.726	0.726	0.726	0.726	0.725	0.725	0.725	0.725	0.725	0.725	8	32
0.190	0.767	0.766	0.766	0.766	0.766	0.766	0.766	0.766	0.766	0.766	0.766	9	36
0.200	0.807	0.807	0.807	0.807	0.807	0.807	0.807	0.807	0.807	0.807	0.806	0	00
0.210	0.848	0.848	0.848	0.848	0.848	0.847	0.847	0.847	0.847	0.847	0.847	1	04
0.220	0.889	0.889	0.889	0.888	0.888	0.888	0.888	0.888	0.888	0.888	0.888	2	08
0.230	0.930	0.930	0.930	0.929	0.929	0.929	0.929	0.929	0.929	0.929	0.929	3	12
0.240	0.971	0.971	0.971	0.970	0.970	0.970	0.970	0.970	0.970	0.969	0.969	4	16
0.250	1.012	1.012	1.012	1.011	1.011	1.011	1.011	1.011	1.011	1.010	1.010	5	20
0.260	1.052	1.052	1.052	1.052	1.052	1.052	1.052	1.051	1.051	1.051	1.051	6	25
0.270	1.093	1.093	1.093	1.093	1.093	1.092	1.092	1.092	1.092	1.092	1.092	7	29
0.230	1.134	1.134	1.134	1.134	1.133	1.133	1.133	1.133	1.133	1.133	1.133	8	33
0.290	1.175	1.175	1.175	1.175	1.175	1.174	1.174	1.174	1.174	1.174	1.174	9	37
0.300	1.216	1.216	1.216	1.216	1.216	1.215	1.215	1.215	1.215	1.215	1.215	0	00
0.310	1.257	1.257	1.257	1.257	1.257	1.256	1.256	1.256	1.256	1.256	1.256	1	04
0.320	1.298	1.298	1.298	1.298	1.298	1.298	1.297	1.297	1.297	1.297	1.297	2	08
0.330	1.340	1.340	1.340	1.339	1.339	1.339	1.339	1.338	1.338	1.338	1.338	3	12
0.340	1.381	1.381	1.381	1.380	1.380	1.380	1.380	1.379	1.379	1.379	1.379	4	16
0.350	1.423	1.422	1.422	1.422	1.422	1.421	1.421	1.421	1.420	1.420	1.420	5	21
0.360	1.464	1.464	1.463	1.463	1.463	1.462	1.462	1.462	1.462	1.461	1.461	6	25
0.370	1.505	1.505	1.505	1.504	1.504	1.504	1.503	1.503	1.503	1.503	1.502	7	29
0.380	1.546	1.546	1.546	1.546	1.545	1.545	1.545	1.544	1.544	1.544	1.543	8	33
0.390	1.588	1.587	1.587	1.587	1.586	1.586	1.586	1.586	1.585	1.585	1.585	9	37
0.400	1.629	1.629	1.628	1.628	1.628	1.627	1.627	1.627	1.627	1.626	1.626	0	00
0.410	1.670	1.670	1.670	1.669	1.669	1.669	1.669	1.668	1.668	1.668	1.667	1	04
0.420	1.712	1.711	1.711	1.711	1.711	1.710	1.710	1.710	1.709	1.709	1.709	2	09
0.430	1.753	1.753	1.753	1.752	1.752	1.752	1.751	1.751	1.751	1.750	1.750	3	13
0.440	1.795	1.795	1.795	1.794	1.794	1.793	1.793	1.793	1.792	1.792	1.791	4	17
0.450	1.837	1.837	1.836	1.836	1.835	1.835	1.835	1.834	1.834	1.833	1.833	5	21
0.460	1.878	1.878	1.878	1.877	1.877	1.877	1.876	1.876	1.875	1.875	1.875	6	25
0.470	1.920	1.920	1.919	1.919	1.918	1.918	1.918	1.917	1.917	1.916	1.916	7	29
0.480	1.962	1.961	1.961	1.960	1.960	1.960	1.959	1.959	1.958	1.958	1.958	8	33
0.490	2.004	2.004	2.003	2.003	2.002	2.002	2.001	2.001	2.000	2.000	1.999	9	38

Residue from evaporation of 25 ml of fat solution	Total amount of fat per gr at a sp. gr. of											Interval values	
	0.90	0.91	0.92	0.93	0.94	0.95	0.96	0.97	0.98	0.99	1.00		
0.500	2.046	2.045	2.045	2.044	2.044	2.043	2.043	2.042	2.042	2.041	2.041	0	00
0.510	2.087	2.087	2.086	2.086	2.085	2.085	2.084	2.084	2.083	2.083	2.082	1	04
0.520	2.129	2.129	2.128	2.128	2.127	2.127	2.126	2.126	2.125	2.125	2.124	2	08
0.530	2.171	2.170	2.170	2.169	2.169	2.168	2.168	2.167	2.167	2.166	2.166	3	13
0.540	2.213	2.213	2.212	2.211	2.211	2.210	2.210	2.209	2.209	2.208	2.208	4	17
0.550	2.255	2.255	2.254	2.254	2.253	2.252	2.252	2.251	2.251	2.250	2.249	5	21
0.560	2.297	2.297	2.296	2.295	2.295	2.294	2.294	2.293	2.292	2.292	2.291	6	25
0.570	2.339	2.339	2.338	2.337	2.337	2.336	2.336	2.335	2.334	2.334	2.333	7	29
0.580	2.381	2.381	2.380	2.379	2.379	2.378	2.378	2.377	2.376	2.376	2.375	8	33
0.590	2.424	2.423	2.422	2.422	2.421	2.420	2.420	2.419	2.418	2.418	2.417	9	38
0.600	2.466	2.465	2.464	2.464	2.463	2.462	2.461	2.461	2.460	2.460	2.459	0	00
0.610	2.508	2.507	2.507	2.506	2.505	2.505	2.504	2.503	2.502	2.502	2.501	1	04
0.620	2.550	2.549	2.549	2.548	2.547	2.547	2.546	2.545	2.545	2.544	2.543	2	08
0.630	2.593	2.592	2.591	2.590	2.590	2.589	2.588	2.587	2.587	2.586	2.585	3	13
0.640	2.635	2.634	2.634	2.633	2.632	2.631	2.630	2.630	2.629	2.628	2.627	4	17
0.650	2.677	2.677	2.676	2.675	2.674	2.673	2.673	2.672	2.671	2.670	2.669	5	21
0.660	2.720	2.719	2.718	2.717	2.716	2.716	2.715	2.714	2.713	2.712	2.712	6	25
0.670	2.762	2.761	2.761	2.760	2.759	2.758	2.757	2.756	2.755	2.755	2.754	7	30
0.680	2.805	2.804	2.803	2.802	2.801	2.801	2.800	2.799	2.798	2.797	2.796	8	34
0.690	2.847	2.846	2.846	2.845	2.844	2.843	2.842	2.841	2.840	2.839	2.838	9	38
0.700	2.890	2.889	2.888	2.887	2.886	2.885	2.884	2.883	2.882	2.881	2.881	0	00
0.710	2.933	2.932	2.931	2.930	2.929	2.928	2.927	2.926	2.925	2.924	2.923	1	04
0.720	2.975	2.974	2.973	2.972	2.971	2.970	2.969	2.968	2.967	2.966	2.965	2	09
0.730	3.018	3.017	3.016	3.015	3.014	3.013	3.012	3.011	3.010	3.009	3.008	3	13
0.740	3.061	3.060	3.059	3.058	3.056	3.055	3.054	3.053	3.052	3.051	3.050	4	17
0.750	3.103	3.102	3.101	3.100	3.099	3.098	3.097	3.096	3.095	3.094	3.093	5	21
0.760	3.146	3.146	3.145	3.144	3.143	3.142	3.141	3.140	3.138	3.136	3.135	6	26
0.770	3.189	3.188	3.187	3.186	3.185	3.184	3.182	3.181	3.180	3.179	3.178	7	30
0.780	3.232	3.231	3.230	3.229	3.227	3.226	3.225	3.224	3.223	3.222	3.220	8	34
0.790	3.275	3.274	3.273	3.271	3.270	3.269	3.268	3.267	3.266	3.264	3.263	9	38

Paper Chromatographic Detection of Synthetic Dyestuffs

The examination is carried out essentially according to the method of Thaler and Sommer. The dye itself is isolated initially by adsorption on wool.

Isolation of the dyes

Reagents:

Ammonia solutions:	5% in 70% alcohol (vol.)
	5% in water
Potassium bisulfate solution:	10% in water.

White lamb's wool of good quality is thoroughly defatted by extraction for one hour in a Soxhlet. The wool is air-dried, contact with the hands being avoided as far as possible. It is then heated on a water-bath with an excess of ammonia (5%) for one hour followed by thorough washing with water. After drying overnight (on glass rods) the wool is ready for use.

10 g or more of the finely ground material to be tested is suspended in 50 ml of a solution of 5% ammonia in 70% (vol.) alcohol. After standing for two hours the insoluble part is separated by centrifuging. The alcohol is evaporated from the solution in a porcelain dish, the volume made up to 30 ml with water, acidified with 5 ml of the potassium bisulfate solution, and a 20 cm long wool thread is immersed. The whole is kept boiling gently until all the dye has been adsorbed as completely as possible by the wool. After thorough washing with distilled water the wool is warmed in a porcelain dish with 25 ml of 5% ammonia in order to remove the dye again. The woolen thread is taken out and the solution concentrated to a sufficiently intense colour. This solution can then be used directly for chromatography. If, however, it is strongly contaminated with by-products, it is advisable to repeat the adsorption on wool.

Chromatography of Dyestuffs:

The ascending chromatographic technique as well as circular chromatography can be applied. In order to identify the dye ascending chromatography is preferable.

Paper:	Schleicher and Schüll 2043b
Mobile phase:	2 g of trisodiumcitrate dissolved in 100 ml 5% ammonia.

Control of the mobile phase:

As the sharpness of separation depends greatly upon the concentration of the ammonia, this should be checked from time to time. The easiest way of doing this is to chromatograph a solution containing 0.25 g Fast Red E and 0.25 g Azorubin S (Carmoisine) in 100 ml water. As long as these two dyes are well separated the mobile phase is satisfactory.

Literature:

Thaler H. and Sommer G: Studien zur Farbstoffanalytik, Z. Lebensmittel-Untersuch. Forsch. *97*, 364 (1953).

Beythien-Diemair: Laboratoriumsbuch für den Lebensmittelchemiker, 7th edition 1957, p. 81.

SENSORY ASSESSMENT

2.5–5 ml of the seasoning is dissolved in 250 ml of boiling drinking water in a white porcelain dish.

The test is performed in regard to

- appearance
- odour
- taste

at a temperature of 60–65 °C.

Details of methodology: see chapter 8.

DETERMINATION OF RELATIVE DENSITY

February 1978 – original text in French

1. SCOPE

This paper describes the method of determining the relative density of homogenous liquids.

2. FIELD OF APPLICATION

Liquid protein hydrolysates (seasonings)
All homogenous liquids

3. DEFINITIONS

3.1. The relative density d 20/4 of the sample is the ratio of its mass per unit volume at 20°C to the mass per unit volume of water at 4°C. It is dimensionless.

3.2. The mass per unit volume ρ (t) at a temperature t is the quotient of the mass divided by the volume; $\rho(t) = \frac{m}{V(t)}$ (see 9.1.).
This is expressed as g/ml.

4. PRINCIPLE

Identical volumes of a sample and water are weighed consectively in the same pycnometer under the same conditions. From the ratio of these values the relative density is calculated.

5. REAGENT

5.1. Distilled water (freshly boiled and cooled).

6. APPARATUS

6.1. 25 ml pycnometer (50 or 20 ml sizes are also satisfactory) with thermometer, fitted with a lateral capillary arm with ground glass stopper. Clean and dry the pycnometer carefully. Avoid any abrupt changes in temperature and mechanical shocks such as those brought about by heating in the oven and/or by vacuum. The resulting changes in volume only recover very slowly.

6.2. Absorbent paper (e.g. filter paper)

6.3. Pleated filters, 15 cm diameter

6.4. Glass funnel

6.5. Watch glass

6.6. Centrifuge

6.7. Stoppered glass tubes for centrifuge

7. SAMPLING AND SAMPLES

Generally speaking a seasoning is a clear solution not requiring any preparation for this test.

However, if a sample contains substances that are insoluble at ambient temperature, filter it (6.3.) or centrifuge it before measuring the density. Cover the funnel or stopper the centrifuge tube (6.7.) to prevent concentration of the sample due to evaporation of water. If the filtration method is used, discard the first 10 ml of the filtrate.

8. PROCEDURE

8.1 Weigh to an accuracy of 0,1 mg a dry, empty and clean pycnometer – mass m_1.

8.2. Fill the pycnometer with distilled water having a temperature of about 15°C.

8.3. Insert the thermometer without trapping any air bubbles and wipe away all traces of moisture on the surface of the pycnometer.

8.4. Place a piece of filter paper (4–5 cm^2) over the open capillary tube to absorb the water escaping as the temperature of the liquid rises.

8.5. Leave to stand at a temperature a little above 20°C (usually ambient temperature) until the pycnometer thermometer is indicating 20°C exactly.

8.6. At this moment, remove the piece of filter paper, close the capillary tube with the ground glass stopper, quickly remove all traces of moisture on the surface of the pycnometer and weigh without delay to the nearest 0,1 mg – mass m_2.

8.7. Empty and dry the pycnometer.

8.8. Repeat the above operation, but this time filling the pycnometer with the test sample – mass m_3.

9. EXPRESSION OF RESULTS

9.1. Method of calculation:
(express the relative density to 4 decimal places)

$$d\,{}^{20}_{4} = \frac{\rho\,(\text{sample};\,20)}{\rho\,(H_2O;\,4)}$$

$$\rho\,(\text{sample};\,20) = \frac{m_3 - m_1}{V_{Pyc}}\ \text{g/ml}$$

$$V_{Pyc} = \frac{m_2 - m_1}{\rho\,(H_2O;\,20)}\ \text{ml}$$

m_1=mass of empty pycnometer in g
m_2=mass of pycnometer with water in g
m_3=mass of pycnometer with test sample in g
$\rho\,(H_2O;\,20)$=0,9982 g/ml
$\rho\,(H_2O;\,4)$ =1,0000 g/ml

9.2. Repeatability: 0,0005

10. NOTES ON PROCEDURE

Generally the volume of each pycnometer is determined once only. Obviously, it will have to be redetermined if the thermometer is changed.

When the volume of a pycnometer is known it is only necessary to carry out steps 8.1. and 8.8.

11. BIBLIOGRAPHICAL REFERENCES

Definitions of relative density: ISO, Recommendation R 31, part III – 1960, (E), Definitions 3–2.1. and 3–3.1.

CAPILLARY GAS CHROMATOGRAPHIC DETERMINATION OF 1,3-DICHLORO-2-PROPANOL (DCP)

December 1992 – Original text in English

1. SCOPE

This method describes a procedure for the gas chromatographic determination of 1,3-dichloro-2-propanol (DCP), a contaminant formed during the manufacture of protein hydrolysate.
(see: Bibliographical References).

2. FIELD OF APPLICATION

The method is restricted to the determination of DCP in protein hydrolysates. Determination in products containing other ingredients besides hydrolysates may give rise to interfering peaks, originating from these ingredients.

3. PRINCIPLE

A sample portion of the product is adsorbed on an Extrelut column. DCP is eluted afterwards with a pentane-diethyl ether mixture.

End determination is carried out by capillary gas chromatography (GC) with an electron capture detector (ECD).

4. REAGENTS

4.1 Eluent:

Pentane: e.g. Merck no 6145
Diethyl ether: e.g. Merck no 3434

Mix well in a suitable container 850 ml pentane with 150 ml diethyl ether.

Before use inject 2.0 µl of the mixture in the gas chromatograph as a test for interfering impurities. If required buy purer reagents or distil.

4.2 Standard solutions:

Always prepare internal standards and DCP solutions simultaneously.

4.2.1 Internal standard solutions

4.2.1.1 Trichlorobenzene solution:

Trichlorobenzene: e.g. Merck no 821 152

Dissolve 50 mg trichlorobenzene in a 50 ml volumetric flask with eluent (4.1) as solvent. Fill up to the mark and mix well.

Pipette 1 ml of the concentrated solution into a 250 ml volumetric flask, fill up to the mark with eluent (4.1) and mix well.

This solution is stable for 2 months, when stored in a refrigerator.

Pipette 10 ml of the diluted internal standard solution into a 100 ml volumetric flask, fill up to the mark with eluent (4.1) and mix well.

This solution is stable for five days, when stored in a refrigerator.

4.2.1.2 Trichlorobenzene-dibromoethane solution:

1,2-Dibromoethane: e.g. Merck no 800 952

Dissolve 50 mg trichlorobenzene and 100 mg 1,2-dibromoethane in a 50 ml volumetric flask with eluent (4.1). Fill up to the mark and mix well.

Dilute this solution 2500 times (see 4.2.1.1).

4.2.2 DCP solutions

4.2.2.1 DCP Stock solutions:

DCP: e.g. Fluka no 36 280 puriss.

Dissolve 50 mg DCP with eluent (4.1) in a 50 ml volumetric flask, and fill up to the mark. Mix well. Dilute this solution 1000 times in three steps with eluent (4.1)

The final solution contains 1 µg DCP/ml.

4.2.2.2 Standard curve:

Pipette 1.0-2.0-3.0-4.0 ml solution 4.2.2.1 into 50 ml volumetric flasks. Add 1.0 ml of the most diluted trichlorobenzene solution (4.2.1.1). Fill up to the mark with eluent (4.1) and mix well.

These standard solutions contain:

A) 1.0 µg DCP and 0.4 µg trichlorobenzene
B) 2.0 µg DCP and 0.4 µg trichlorobenzene
C) 3.0 µg DCP and 0.4 µg trichlorobenzene
D) 4.0 µg DCP and 0.4 µg trichlorobenzene.

These solutions can be kept for one week, when stored in a refrigerator.

4.3 20% Sodium chloride solution:

Dissolve 200 g sodium chloride p. a. into water. Use a 1000 ml volumetric flask to bring the volume of the solution to one liter. Mix well.

Remark:

Water used for making dilutions or otherwise should be demineralised or distilled and not contain any interfering substances.

5. APPARATUS

5.1 Capillary gas chromatograph with split injector, make-up-gas device, 63 Ni electron capture detector (ECD) and the necessary utilities.

Use either Carbowax 20M or OV-101 capillary columns (see 9. gas chromatography).

5.2 Integrator and/or recorder.
Carrier gas: hydrogen or helium.

Warning:

Hydrogen is explosive when mixed with air. If hydrogen is chosen as carrier gas, it should not come into the oven!

Before opening the hydrogen inlet, check to see if the column is properly mounted. To ensure safety, install a hydrogen sensor in the chromatograph.

Rooms in which instruments are placed should be aerated continuously.

5.3 Reaction gas: argon:methane, 95:5.

The gas should be continuously purified with a moisture trap. Regenerate the filter at weekly intervals by heating overnight at 350° C with helium flushing (60 ml/min). The molecular sieve should be replaced after 5–10 regenerations

Alternatively, nitrogen may be used as a reaction gas.

5.4 Injector:

A glass tube in the injector regularises the gas flow. In order to protect the column from solid particles, fill the glass tube with about 0.15 g Chromosorb GAW-DMCS (with 3% SE 30). Use glasswool as a support.

6. MATERIALS

6.1 Volumetric flasks, 50 ml, 100 ml, 250 ml

6.2 Graduated cylinder, 50 ml

6.3 Rocker dispenser, 20 ml, with 1 litre reservoir

6.4 Graduated pipettes, 1 ml

6.5 Volumetric pipettes 1.0-2.0-3.0-4.0 and 10 ml

6.6 Volumetric flask of 1000 ml

6.7 Balances, analytical and top loading with accuracy of 0.1 mg and 10 mg respectively,

6.8 Extrelut 20 column (Merck) with standard and clamp

6.9 Stopwatch

6.10 Folded filters as e.g. S & S 593 1/2 or equivalent, together with fitting funnels and other necessities for filtering samples.

7. PREPARATION OF THE TEST SAMPLES

7.1 Liquid samples

Filter a sufficient amount of liquid product. Samples supposed to contain more than 0.2 mg/kg DCP are diluted with a known quantity (by weight) of the 20% sodium chloride solution (4.3). Mix well.

7.2 Solids/powdery samples

Solid samples may be dissolved into a known quantity (by weight) of warm water, so that a salt concentration of 20% is reached. Cool and mix well. Filter through a folded filter, when strictly needed.

8. PROCEDURE

Remove the lower cap of an Extrelut column, and put the needle in place.

Place the column in a suitable holder on a balance, and weigh about 20 g sample to 0.01 mg accurate directly into the column.

Let the product penetrate for 15 minutes (stopwatch!). For this purpose fasten the column with a clamp to the stand (do not squeeze the column) and place a 50 ml graduated cylinder under the column for collecting the eluate.

After 15 min pour 3 × 20 ml eluent (4.1) on the column by means of a rocker dispenser and collect about 40 ml eluate (this takes 20–25 min).

Bring the eluate with more eluent (4.1) into a 50 ml volumetric flask. Add 1.0 ml of the most diluted trichlorobenzene solution (4.2.1.1) and fill to the mark with more eluent.

Alternatively collect about 40–45 ml eluate directly into a 50 ml volumetric flask, add 1.0 ml of the most diluted trichlorobenzene solution (4.2.1.1) and fill to the mark with more eluent.

Control the procedure for disturbing peaks with a blanc determination. Substitute the sample by the 20% sodium chloride solution (4.3).

Remarks:

The times prescribed for the penetration and elution are critical. Speeding up the process gives lower recoveries. When needed, increase the time needed for the elution of DCP by using a finer needle.

When necessary, samples can be diluted with a known quantity (by weight) of the 20% sodium chloride solution (4.3). Pipette 20 g of the diluted and possibly filtered solution on the extrelut column. Correct the result obtained with the formula under 11. (Calculation and interpretation of the results) with the dilution factor applied when preparing the sample solution.

9. GAS CHROMATOGRAPHY

Before starting the analysis, condition the column at 200° C and the detector at 300° C for 24 hours.

9.1 **Carbowax 20M capillary column**

Column: 50 m × 0.2 mm, fused silica with 0.2 µm Carbowax 20M.

Temperature programme	10 min at 115° C; heating rate: 30° C/min and 12 min at 200° C
Injector temperature	250° C
ECD temperature	300° C
Injection volume	2.0 µl
Solvent retention time	about 2.9 min
Trichlorobenzene retention time	about 5.9 min
DCP retention time	about 8.5 min

Carrier gas	hydrogen, initial pressure = 1.4 bar, split about 1:10
Reaction gas	30 ml/min argon: methane, 95:5 (or use nitrogen)

9.2 **OV-101 capillary column**

Column: 25 m x 0.3 mm, fused silica with 0.2 µm OV-101 (to be used for the checks, see 10).

Temperature program	7 min at 50° C; heating rate: 30° C/min and 12 min at 200° C
Injector temperature	250° C
ECD temperature	300° C
Injection volume	2.0 µl
Solvent retention time	about 0.85 min
Dibromoethane retention time	about 2.7 min
DCP retention time	about 5.6 min
Carrier gas	hydrogen, initial pressure = 0.4 bar, split about 1:8
Reaction gas	as under 9.1.

Inject 2 µl blanc, 2 µl standard solution B (4.2.2.2) and after 4 samples again 2 µl standard solution B (4.2.2.2) in the gas chromatograph.

Calculate the quantity of DCP in the sample, using the internal standard method and peak height, according to the formula (see 11).

10. AUTOCONTROL PROCEDURES

Check once a week (or when introducing new equipment) the linear response of the electron capture detector (ECD) by injecting all standard solutions (4.2.2.2).

About every week (or in case of doubt) analyse a reference sample to check chromatographic conditions and accuracy of the results.

Add 2.5 ml of the final DCP standard solution (4.2.2.1) to 50.0 ml of a liquid sample with as low a DCP content as possible. Analyse the spiked and unspiked sample for DCP, as described.

Perform gas chromatography on the Carbowax 20M column and correct the end result for:

- The quantity of DCP originally present in the sample,
- The volume dilution owing to the 2.5 ml DCP standard added when spiking the sample,

– The estimated recovery rate (should be better than 90%).

REMARK:

The dibromoethane internal standard (4.2.1.2) at a comparable concentration should be used, when the OV-101 column is applied instead of the Carbowax 20M column.

11. CALCULATION AND INTERPRETATION OF THE RESULTS

The same amount of internal standard has been added to the standard and sample solutions. For the calculation with the **internal standard** use the following formula:

$$\frac{A \times C \times E \times 1000}{B \times D \times M} = \mu g \text{ DCP/kg}$$

Where:

A = peak height of DCP in sample solution

B = peak height of DCP in standard solution

C = peak height of internal standard in standard solution

D = peak height of internal standard in sample solution

E = quantity of DCP in µg/50 ml standard solution B

M = Mass in g of the sample portion taken.

REMARKS:

- The mass M of the sample portion should be corrected accordingly when analysing diluted liquid or dissolved dry samples.
- For the routine determinations use the standard solution B (4.2.2.2) as the reference.
- The use of an internal standard may be deleted for control purposes when, based on monitoring, no significant amounts of DCP are expected.
  The "response" of the GC instrument with the external DCP standard should not show deviations. Delete in this case the factors C and D from the formula used for the calculation of the results.
- Use reference standard D (4.2.2.2) when more DCP is present and/or dilute the sample with the 20% sodium chloride solution before analysing.
- The concentration of the internal standard solution should be adapted, when the GC instrument shows an unfavourable relation between the peak heights of the internal standard and the DCP.

- Confirmation procedure should be executed when necessary: Use either the second column mentioned or a derivatisation procedure, as described in the methods for 3-MCPD (see chapt. 3/10a and 3/10b).

  Alternatively, use a "Full scan GC/MS" confirmation procedure. (MS = Mass spectrometric identification)

12. LIMIT OF DETECTION AND RECOVERY

The limit of detection is slightly better than 50 µg DCP/kg. The extraction yield is better than 90%.

The standard deviation for repeatability is about 12 µg DCP and the standard deviation for reproducibility about 15–17µg DCP.

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J. Chromatogr. 555 (1991) 311–314

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Determination of chloropropanols in protein hydrolysates
J. Chromatogr. 589 (1992) 109–119

CAPILLARY GAS CHROMATOGRAPHIC DETERMINATION OF 3-CHLORO-1,2-PROPANEDIOL (3-MCPD)

(PBA METHOD)

December 1992 – Original text in English

1. SCOPE

This method describes a procedure for the gas chromatographic determination of 3-chloro-1,2-propanediol, resp. 3-Monochlorpropanediol (3-MCPD), a contaminant formed during the manufacture of protein hydrolysate.
(see: Bibliographical References).

PBA: abbreviation of phenylboric acid (see reagent 4.1)

2. FIELD OF APPLICATION

The method is restricted to the determination of 3-MCPD in protein hydrolysates. Determination in products containing other ingredients besides hydrolysates may give rise to interfering peaks, originating from these ingredients.

3. PRINCIPLE

3-MCPD in a sample portion of the product is derivatized with phenylboric acid (PBA). The resulting reaction product is extracted with hexane.

End determination is carried out by capillary gas chromatography (GC) with flame ionisation detection (FID).

4. REAGENTS

4.1 Phenylboric acid (PBA, e.g. Aldrich no 10727-1)

4.2 Acetone (e.g. Baker no 8003)

4.2.1 Derivatization solution:

Weigh 5 g phenylboric acid into a 50 ml conical flask with stopper and dissolve in 19 ml acetone and 1 ml water. Mix well.

4.3 n-Heptadecane (e.g. Merck no 9604)

4.4 n-Hexane (e.g. Merck no 4367)

4.4.1 Internal standard solution:

Weigh 10 mg n-heptadecane into a 100 ml volumetric flask, dissolve in n-hexane, fill up to the mark and mix well.

4.4.2 Pipette 10 ml internal standard 4.4.1 into a 100 ml volumetric flask, fill up to the mark with n-hexane and mix well.

The final concentration is 0.01 mg n-heptadecane/ml.

4.5 Standard 3-chloro-1,2-propanediol (3-MCPD, e.g. Merck 802 637)

4.6 Standard solutions 3-MCPD:

4.6.1 Weigh approximately 25 mg 3-MCPD into a 250 ml volumetric flask and dissolve in 20% sodium chloride solution (4.7), fill up to the mark and mix well.

4.6.2 Pipette 10 ml of the standard solution 4.6.1 into a 100 ml volumetric flask, fill up to the mark with the 20% sodium chloride solution (4.7) and mix well.

4.6.3 Pipette 1, 2.5 and 10 ml of the solution 4.6.2 into 25 ml volumetric flasks. Fill up to the mark with the 20% sodium chloride solution (4.7) and mix well.

4.7 20% sodium chloride solution:

Dissolve 200 g sodium chloride p.a. into water. Use a 1000 ml volumetric flask to bring the volume of the solution to one litre. Mix well.

Remark:

Water used for making dilutions or otherwise should be demineralised or distilled and not contain any interfering substances.

5. APPARATUS

5.1 Gas chromatograph: GC-FID system, preferably with automatic injection system, integrator and necessary utilities.

Gases used are:
helium as carrier gas, and nitrogen as make-up gas.

5.2 Capillary column (e.g. fused silica CP-Sil 5-CB, ID = 0.32 mm, length = 50 m, F = 0.12 µm).

6. MATERIALS

6.1 Mechanical shaker

6.2 Volumetric flasks of 25, 100 and 250 ml

6.3 Volumetric flask of 1000 ml

6.4 Measuring pipettes of 1-2.5-3-5 and 10 ml

6.5 Conical flasks of 50 ml, with glass stopper

6.6 Analytical balance and top loading balance with accuracy of 0.1 mg and 10 mg respectively

6.7 Sample vials of 20 ml with aluminium or plastic caps, both supplied with teflon lined close fitting septa

6.8 Water or glycerol bath that can be kept with sufficient accuracy at 90° C.

6.9 Waterbath at ambient temperature

6.10 Folded filters as e.g. S & S 593 1/2 or equivalent, together with fitting funnels and other necessities for filtering samples.

7. PREPARATION OF THE TEST SAMPLES

7.1 Liquid samples

Weigh around 75 g sample into a 100 ml volumetric flask, fill up to the mark with the 20% sodium chloride solution (4.7) and mix well.

7.2 Solid or powdery samples

Weigh around 30 g sample into a glass beaker of 100 ml. Dissolve with enough hot water, so that the sodium chloride concentration in the resulting dilution is approximately 20%. Transfer quantitatively this sample solution with small portions of the 20% sodium chloride solution into a 100 ml volumetric flask. After cooling, fill up to the mark with the same sodium chloride solution and mix well. Filter through a folded filter when strictly necessary.

Remark:

It is assumed that the solid samples consist of protein hydrolysates with enough sodium chloride to arrive at a concentration of about 20% when completely dissolved. The final salt concentration somewhat influences the equilibrium during the extraction, described under 8.1 (See the original publication, mentioned under 13).

8. PROCEDURE

8.1 Derivatization and Extraction:

Pipette 5 ml of the prepared sample solution (7.) into a sample vial. Pipette 1 ml of the derivatization reagent (4.2.1) into the same vial and seal the vial accurately with septum and vial cap. Place the vial for 20 min into the waterbath at 90° C and cool afterwards to room temperature (standardise with the waterbath at room temperature).

Remove the vial cap and septum, and add by pipette 3 ml of the internal standard solution (4.4.2). Place the septum and cap on the

vial and shake for 30 sec on the mechanical shaker. Allow the organic and water layers to separate.

8.2 Calibration:

For the calibration of the system, pipette 5 ml of solutions 4.6.2 and 4.6.3 into vials. Continue as described under 8.1.

8.3 Blanc:

Execute a blanc by substituting 5 ml sample solution by 5 ml of the 20% sodium chloride solution (4.7) and continuing according to 8.1

8.4 Samples:

Inject 2 µl of the organic layer of each of the standards 8.2 and the blanc 8.3 into the GC system. The calibration should give a straight calibration curve. Inject afterwards the samples, but repeat the injection of the proper standard after every 4th sample.

Remark:

It is not necessary to repeat the calibration of the system for each series of analyses. Use the standard solution giving a reasonable peak height or area in relation to the peak heights or areas of the samples.

Adapt if necessary the concentration of 3-MCPD in the standard solutions in 4.6.2 and 4.6.3 in order to have a suitable calibration curve in the range of the 3-MCPD concentrations in the samples.

9. GAS CHROMATOGRAPHY

The description given below is a practical example, that should be adapted to the equipment present in the individual laboratories, and to the samples.

Capillary column: see 5.2

Column pressure	150 kPa
Make-up gas	nitrogen 30 ml/min
Split flow (Bypass)	10 ml/min
Initial column temperature	40° C
Initial time	1 min
Temperature rate	7.5° C/min
Final temperature	182.5° C
Final time	0 min
Temperature rate	40° C/min

Final temperature	280° C
Final time	13 min
Injected volume	2 µl
Injector temperature	200° C
Detector temperature	280° C
Retention times	around 16 min for 3-MCPD, around 20 min for the internal standard

10. AUTOCONTROL PROCEDURES

Check once a week (or when introducing new equipment) the linear response of the flame ionisation detector (FID) by injecting a properly diluted series of standard solutions with 3-MCPD contents in the range of the 3-MCPD concentrations in the samples.

About every week (or in case of doubt) analyse a reference sample to check chromatographic conditions and accuracy of the results.

Spike a sample, with the lowest concentration 3-MCPD, with enough 3-MCPD standard solution to obtain a level between 1–3 times the concentration, normally found in the samples. Analyse the spiked and unspiked samples for 3-MCPD and calculate the recovery.

Correct the result for:

- The quantity of 3-MCPD originally present in the sample,
- The volume dilution owing to the addition of 3-MCPD standard when spiking the sample.

Perform a blanc with 5 ml of the 20% sodium chloride solution (4.7), as described in 8.3

REMARKS

- The concentration in the internal standard solution should be adapted, when the GC instrument shows an unfavourable relation between the peak areas of the internal standard and the 3-MCPD.
- Confirmation procedure should be executed when necessary. Use either the HFBI method (see Chapt. 3/10b) or a "Full scan GC/MS" confirmation procedure (use of FID detection is not possible here). (MS = Mass spectrometric identification).

11. CALCULATION AND INTERPRETATION OF THE RESULTS

Calculate the results using the following formula:

$$\frac{A \times C \times E \times 1000 \times 100}{B \times D \times M \times 5 \times 1000} = \text{mg 3-MCPD/kg}$$

Where:

A = peak area of 3-MDCP in the sample solution

B = peak area of 3-MDCP in the standard solution

C = peak area of internal standard in the standard solution

D = peak area of internal standard in the sample solution

E = quantity of 3-MDCP in µg in 5 ml standard used for the calculation

M = Mass in g of sample portion taken.

12. LIMIT OF DETECTION AND RECOVERY

The limit of detection is slightly better than 0.5 mg 3-MCPD/kg. The recovery of spiked samples should be better than 90%.

At a level of 10 mg 3-MCPD, the standard deviation for repeatability is about 0.18 mg and the standard deviation for reproducibility about 1.34 mg 3-MCPD.

At a level of 1 mg 3-MCPD/kg the figures are approximately 0.034 mg and 0.096 mg 3-MCPD respectively.

13. LITERATURE

Original publication:

W. J. Plantinga, W. G. van Toorn and G. H. D. van der Stegen, *Journal of Chromatography* 555 (1991) 311-314.

SENSORY ASSESSMENT

The product is prepared according to the recipe.

1 plate = 250 ml
1 cup = 150 ml
1 little cup = 100 ml (for exotic soups)

The test is performed in regard to

- appearance
- odour
- taste

at a temperature of 60–65 °C.

Details of methodology: see chapter 8.

DETERMINATION OF DRY MATTER

For method see 2/2

Notes:

For products containing significant quantity of tomato puree, powder or flake, a vacuum oven should be used preferably.

Recommended drying conditions are:

Temperature	70 ±1°C
Pressure	0,1 bar (absolute)
Time	4 hours

DETERMINATION OF THE ASH

For method see 2/3

With liquid soups, the entire contents of the can are thoroughly homogenised and 100 g of the homogenised sample is put into a 200 ml measuring flask. The sample is made up to volume with distilled water and the flask is shaken well. An aliquot part, which should contain 1–2 g dry matter, is then used for the determination.

DETERMINATION OF THE SAND

March 1975 – Original text in German

1. SCOPE

Determination of the content of inorganic impurities.

2. FIELD OF APPLICATION

Soups

Raw materials of vegetable origin.

3. DEFINITION

The ash component insoluble in hydrochloric acid is designated as sand.

4. PRINCIPLE

The ash is dissolved in hot hydrochloric acid, the insoluble components are filtered off, incinerated together with the filter.

5. REAGENTS

5.1. Hydrochloric acid 10%.

6. APPARATUS

6.1. see ash determination 2/3 (6.1. to 6.7.). However, only platinum bowls and *no* quartz bowls should be used.

6.2. ash-free filters.

7. PROCEDURE

The cool ash (see ash determination 2/3) is mixed with 20 ml of 10% hydrochloric acid, and heated on a water bath, while being stirred, for 15 minutes. The mixture is then strained through an ash-free filter, washed with hot distilled water until no Cl-ions are detectable, after which the filter with the residue is incinerated in the muffle furnace at 550 °C. After cooling in the desiccator the bowl with contents is weighed to an accuracy of 0.1 mg. Having removed the contents (i.e. the sand) from the bowl with a brush, the empty bowl is again weighed to an accuracy of 0.1 mg.

8. EXPRESSION OF RESULTS

8.1. Method of calculation:

(specification in percentage by weight to two decimal places)

$$\text{Sand content} = \frac{\text{mg final weight (sand)} \times 100}{\text{mg test portion}} \%$$

8.2. Repeatability: 0.02 g/100 g test sample.

DETERMINATION OF TOTAL NITROGEN

For method see 2/6

With liquid soups the entire contents of the container should be thoroughly homogenised. An aliquot part is then used for the determination.

Determination of Total Glutamic Acid

Preparation of Solution for Analysis:

10 g of the finely ground soup mass is mixed with about 50 ml of water and left to stand at room temperature for one hour, being shaken at frequent intervals. After centrifuging the supernatant fluid is filtered through glass wool into a 250 ml volumetric flask. The residue is washed 3 times with 25 ml water and centrifuged each time. The combined filtrates are brought to a pH specific for the enzyme to be used. After making the solution up to volume it can be used directly for analysis.

With liquid soups, 40 ml of the suspension as mentioned in 4/2 is adjusted to the required pH value and then made up to 100 ml with water.

Procedure:

See Broth and Meat Broth Products 2/8a and 2/8b.

DETERMINATION OF TOTAL FAT CONTENT BASED ON WEIBULL-STOLDT METHOD

July 1989 – Original text in English

For method see 2/9a

– Dry soups
containing fat difficult to extract (e. g. from milk powder).

Thoroughly homogenise the laboratory sample prior to the acid treatment.

– Liquid soups
containing fat difficult to extract.

Thoroughly homogenise the entire contents of the tin or final sales unit prior to the acid treatment.

DETERMINATION OF FREE FAT CONTENT BY DIRECT SOXHLET EXTRACTION

July 1989 – Original text in English

For method see 2/9b

– Dry soups
containing easily extractable fat.

Thoroughly homogenise the laboratory sample prior to the extraction.

– Liquid soups
containing easily extractable fat.

Prior to the extraction, thoroughly homogenise the entire contents of the tin or final sales unit and dry a test portion on seasand (see Method 2/2: 'Bouillon and Meat Bouillon').

Qualitative Detection of Antioxidants

The detection of antioxidants is carried out in three stages:

1. Extraction of the fats containing the antioxidants.
2. Isolation of the antioxidants from the fat.
3. Identification of the individual antioxidants.

The extraction of the fats from dry materials is performed by simply extracting with a solvent. With fluid samples a fat separation can be effected by dilution with hot water.

In isolating the antioxidants from fats, distinction should be made between the following two groups of substances:

1. Substances which can be extracted from the solution of fat with 75% alcohol. To this group belong the gallates, butylhydroxyanisole (BHA) and nordihydroguaiaretic acid (NDGA).

2. Substances which cannot be extracted with 75% alcohol. This is the case primarily with butylhydroxytoluene (BHT). This antioxidant can be isolated either by distillation with superheated steam (1), or by chromatographic adsorption (2). The latter is more time-consuming but also more accurate and very sensitive. It enables 0.001% of BHT to be identified with certainty.

For the identification of the individual antioxidants, chromatographic methods are preferable. In order to obtain a general idea of the antioxidants present a thin-layer chromatogram is prepared. This gives definite data on BHA, BHT, and often about gallates as well. In some cases, however, the separation of gallates is greatly hampered by the presence of substances such as curcuma and chlorophyll which may occur naturally. In this case they hardly move beyond the starting point. If, therefore, a spot appears here – though it need not necessarily have been produced by one of the last-named antioxidants – the sample must be re-examined by means of two-dimensional chromatography.

Procedure:

1. Extraction of Fats

For dry samples a portion containing at least 10 g of fat is extracted by shaking with 50 ml of cyclohexane. This is followed by filtering or centrifuging.

When dealing with fluid samples, an amount containing at least 10 g of fat is treated with enough hot water to cause the fat to form a supernatant layer. After cooling the fat is removed and dissolved in 50 ml of cyclohexane. Any water adhering can easily be removed with filter paper.

2. Isolation of Antioxidants

a) Substances which can be extracted with 75% ethanol (3).

The fat solution obtained under 1. is extracted in a separatory funnel for two minutes, first with 40 and then with 20 ml of ethanol (75%). The combined fractions are evaporated at a maximum temperature of 40° C in vacuo until most of the alcohol has been removed. The resulting turbid aqueous phase is

kept for several hours at 0° C and then filtered. The filtrate is extracted with 30 ml ethyl acetate. After separation into layers (accelerated by addition of sodium sulphate) the ethyl acetate layer is dried with anhydrous sodium sulfate, evaporated in vacuo to about 0.5 ml, and then used to obtain a chromatogram.

This method of separation is rather complicated and time-wasting. It is, however, indicated when small quantities of antioxidants have to be identified. For routine analyses the following abbreviated procedure is recommended:

A fat solution containing 1 g of fat in 1–2 ml of cyclohexane or petroleum ether (40–60° C) is shaken for 2 minutes with 1 ml ethanol (75%). After separation into two layers the lower alcoholic layer is drawn off and can immediately be used for chromatography. As a rule it is sufficient to apply 0.1–0.2 ml of this extract.

b) Isolation of BHT

Apparatus and reagents:

A column of about 2 cm inner diameter, 25–35 cm long, closed at the lower end with a stopcock and at the upper, wider end with an arrangement permitting the use of pressure.

Silica gel fine powder for partition chromatography Merck 7729.

Preparation of the Adsorption Column

30 g of silica gel is stirred with 80 ml cyclohexane and left to stand for 15 minutes. After repeated vigorous stirring the mixture is poured into the glass tube, the lower end of which has been plugged with glass wool. The beaker is rinsed with 20 ml cyclohexane and the washing is added to the contents of the column. The solvent is allowed to run through under weak pressure (nitrogen, or compressed air) until it has almost reached the surface of the column. Then a cotton plug is placed on top of the silica gel.

Preparation of the Fat Solution

A solution of the fat in cyclohexane, 10 g/50 ml, is used, or the solution from which the gallates and the BHA have already been removed by extracting with alcohol.

Isolation of BHT

10 ml of the fat solution is poured on to the prepared column and the solution lowered, by slightly increased pressure, to the level of the cotton plug. Now 10 ml of pure cyclohexane is added and the level again brought down to the cotton plug by slight pressure. After discontinuing the pressure, one hour waiting time is necessary before resuming the elution. During this period the fat will have been adsorbed. If, however, the elution is continued immediately, fat may be carried along with the elutant, and the detection of BHT is no longer possible.

The elution is further continued with cyclohexane under slightly increased pressure, so that a flow rate of 2–3 ml per minute is attained. The first 50 ml, measured from the beginning of percolation of the fat solution, is practically free from BHT. It is returned to the column. The next 100 ml of elutant contains most of the BHT. It is collected and subsequently concentrated to about $^1/_2$ ml.

3. Chromatographic Identification of the Individual Antioxidants

For general investigations, one-dimensional thin-layer chromatography by the Stahl method (4) is best suited. Seher (5) has suggested chloroform as the mobile phase, with which a good separation of BHA, guaiacol, eugenol, and BHT is achieved. The gallates and NDGA are retained at the starting point. By changing the mobile phase and the stationary phase the following substances can be separated:

1. Nordihydroguaiaretic acid
2. Propyl gallate
3. Butyl gallate
4. Octyl gallate
5. Dodecyl gallate
6. Vanillin
7. Butylhydroxyanisole (BHA)
8. Eugenol
9. Thymol
10. Butylhydroxytoluene (BHT)

The Rf values are very difficult to reproduce when chloroform and hexane-glacial acetic acid are used for development. With each chromatogram, therefore, it is necessary to run a standard solution of the pure antioxidant simultaneously.

Reagents and Apparatus

Phosphomolybdic acid, 5% in ethyl alcohol.

Concentrated ammonia.

Silica gel G Merck, diatomaceous earth (Kieselgur G) Merck.

0.1% solutions of the individual antioxidants in ethyl alcohol. The solutions for comparison are to be prepared separately. When the various substances are combined, their stability is very restricted. It must also be remembered that the gallates in alcoholic solution become slightly esterified after long standing.

Assembly for thin layer chromatography by the Stahl method.

Materials for the stationary phase: silica gel G 25 parts + diatomaceous earth (Kieselgur G) 5 parts.

Preparation of the plates: see ref. (4).

Mobile phase: hexane-glacial acetic acid (2 vol. + 0.5 vol.).

This mobile phase must not be used too often, as its concentration changes very rapidly on use.

The solutions for analysis, prepared according to 2a) and 2b), are applied to the starting points in several small portions. On a plate 20 × 20 cm 6–8 samples can be analyzed simultaneously. For comparison, solutions of the pure antioxidants are also applied – preferably in the centre of the plates. After the spots have dried (drying being accelerated by a current of warm air), they

are developed at once with hexane-glacial acetic acid. This takes about 35 minutes. The plates are taken from the chamber, allowed to dry for a short time, and then chromographed once more. After evaporation of the adhering mobile phase the plates are sprayed heavily with phosphomolybdic acid. The plates should then be uniformly yellow in colour. During spraying, or shortly afterwards, the antioxidants appear as blue (BHA, eugenol), blue-grey (BHT), brownish (guaiacol) or brownish-green (NDGA, gallates) spots. When the plates are treated with ammonia vapour the background changes to a pure white, and the spots can be more readily distinguished. They fade, however, relatively quickly.

Interfering substances

Certain ingredients of condiments and other substances which pass into fat during smoking may give the erroneous impression of the presence of synthetic antioxidants. Particularly disturbing in this connection are thymol, the main ingredient of the essential oils of the Labiatae, and also eugenol. Thymol has practically the same Rf value as Butylhydroxyanisol. The separation of both spots can be achieved by two-dimensional chromatography. In case of doubt, it is always recommended that a mixed chromatogram be run.

Another possible source of interference may result when fats or oils have been in contact with rubber or certain plastic materials. Therefore rubber stoppers should never be used when extracting fats or when isolating antioxidants. Even after prolonged use rubber stoppers and tube connections release stabilisers, which appear in the chromatogram although mostly with a different Rf value than the antioxidants.

In general it can be said that during separation of BHT by steam distillation (2. b) one can expect the presence of other substances which volatilize with steam. By this method marked tailing may occur and also the Rf values of the pure standards will not agree with those of the antioxidants isolated from the fat. In order to prevent tailing the so-called 'technique in steps' is applied. In this case chromatography is first carried out with hexane. Thus the undesirable contaminants causing the tailing are moved to the upper part of the chromatogram, while the antioxidants remain practically on the starting line.

The possibility of interfering substances does not occur when BHT has been separated chromatographically. Also, other colouring substances that may be present are retained as well as the stabilisers from rubber already mentioned and also BHA, NDGA and the gallates.

The fat-soluble dyes and other substances extractable with alcohol sometimes interfere with the chromatographic separation of antioxidants, and particularly of the gallates. In this case two-dimensional chromatography has given good results: chloroform is first applied, and then hexane-glacial acetic acid in the other direction.

Literature:

(1) C. Anglin, J. H. Masson and R. A. Chapman: J. Agr. Food Chem. *4*, 1018 (1956).
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(3) R. ter Heide: Fette, Seifen, Anstrichmittel *60*, 360 (1958).
(4) E. Stahl: Chemiker-Zeitung *82*, 323 (1958).
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Sulphate Ash

June 1973 – Original Text in Dutch

1. Definition

Sulphate ash is the residue after the incineration in the presence of sulphuric acid.

2. Reagents

Sulphuric acid 95–98% (d=1.84)
Solid ammonium carbonate.

3. Procedure

Burn in porcelain or platinum dish, leave to cool in desiccator and weigh accurately to 0.1 mg (W_0).

Weigh the ground sample into the dish (weight of sample+dish=W_1).

Add a few drops of water and mix with 1 ml sulphuric acid. Expel excess sulphuric acid by heating moderately and incinerate.
If the ash still contains carbon particles, repeat the process with some drops of sulphuric acid. Leave to cool. Add some pieces of ammonium carbonate and incinerate again.

Leave to cool in desiccator and weigh (W_2). See also the remark below under 6.

4. Calculation

Calculate the content of inorganic matter as sulphate ash (A) in %.

$$A = \frac{W_2 - W_0}{W_1 - W_0} \times 100$$

in which:

W_0 = weight of empty dish in g
W_1 = weight of dish and sample in g
W_2 = weight of dish and ash in g

5. Report

Mention in the report the result as sulphate ash to an accuracy of 0.01%.

6. Remark

In case the content of crude sand of the same sample has to be determined, use the sulphate ash obtained as described here above.

Crude Sand

June 1973 – Original Text in Dutch

1. Definition

Crude sand is the residue of the sulphate ash after treatment with hydrochloric acid.

2. Reagent

Hydrochloric acid 4 n

3. Analysis Sample

Start from the sulphate ash which is obtained by following the procedure described under 6/1.

4. Procedure

Add 25 ml hydrochloric acid to the sulphate ash. Cover the dish with a watch glass and boil gently for 10 minutes. Then leave to cool.

Filter the residue through an ash-free filter and wash with hot water until the filtrate is free of acid.

Dry the filter and the residue in the same dish.

Burn the filter in incinerator, leave it to cool in a desiccator and weigh. Repeat the incineration and the cooling until the difference between two consecutive weighings is less than 1 mg (W_3).

5. Calculation

Calculate the amount of crude sand (CS) in percentages of the sample of the sulphate ash determination.

$$CS = \frac{W_3 - W_0}{W_1 - W_0} \times 100$$

in which:

W_0 = weight of empty dish in g
W_1 = weight of dish and sample in g
W_3 = weight of dish and crude sand in g

6. Report

In the report, mention the amount of crude sand to an accuracy of 0.01%.

DETERMINATION OF SULPHUR DIOXIDE

July 1975 – Original text in English

1. SCOPE

Determination of sulphur dioxide added to foodstuffs (e.g. in the form of sulphites) for preserving purposes or of residual sulphur dioxide from treatments during manufacture.

2. FIELD OF APPLICATION

Dehydrated fruit and vegetables.

3. DEFINITION

The substances that have been stripped under test conditions and are acid or oxidisable to acid are titrated with sodium hydroxide and calculated as sulphur dioxide.

4. PRINCIPLE

The sulphur dioxide is liberated by hydrochloric acid, stripped by boiling in a nitrogen stream, absorbed in and oxidised to sulphuric acid by hydrogen peroxide (5.1.) and finally titrated (5.2.).

Interference by substances containing organic sulphur is greatly reduced by the use of methanol (see 11), which lowers the boiling point (according to Zonneveld and Meyer).

5. REACTIONS

5.1. $SO_2 + H_2O_2 \rightarrow H_2SO_4$

5.2. $H_2SO_4 + 2\ NaOH \rightarrow Na_2SO_4 + 2\ H_2O$

6. REAGENTS

6.1. Water, distilled or demineralised (freshly boiled and cooled before use).

6.2. Methanol, analytical grade.

6.3. Hydrochloric acid, approximately 4N, analytical grade.

6.4. Hydrogen peroxide, 3% V/V freshly prepared and neutral to bromophenol blue (pH approx. 3.6).

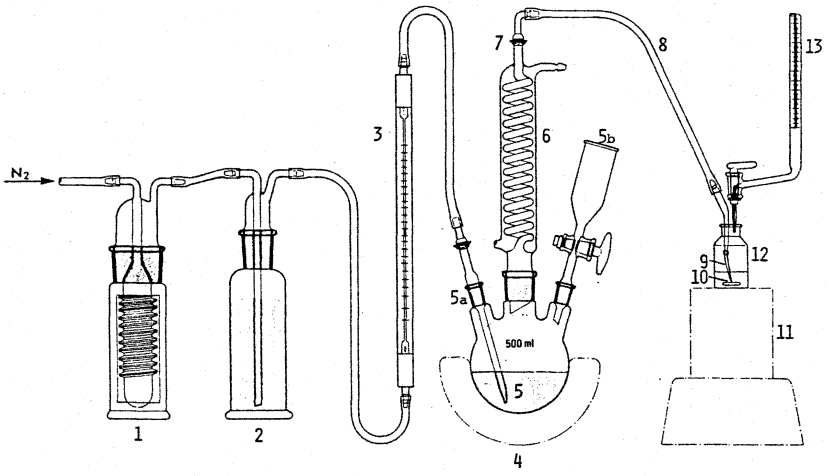
6.5. Bromophenol blue, 0.1 g dissolved in 100 ml 50% V/V ethanol.

6.6. Sodium hydroxide, 0.1 N standard volumetric solution.

6.7. Nitrogen.

6.8. Alkaline pyrogallol solution, dissolve 2 g of pyrogallol in 100 ml 30% m/m sodium hydroxide solution.

7. APPARATUS



7.1. Friedrichs wash bottle (1) with pyrogallol solution.

7.2. Wash bottle (2) with glass wool.

7.3. Flow meter graduated from 50 to 500 ml/min (3).

7.4. Heating jacket (4).

7.5. Three neck flask 500 ml (5).
7.5.1. Inlet tube (5a).
7.5.2. Funnel, minimum capacity 50 ml (5b).

7.6. Spiral condenser, 200 mm; spiral internal diameter minimum 6 mm (6).

7.7. Spherical ground joint 13/5 (7).

7.8. Plastic tube: internal diameter approx. 4 mm (8).

7.9. Plastic tube: internal diameter approx. 2 mm (9).

7.10. Magnetic rod (10).

7.11. Magnetic stirrer (11).

7.12. Reagent bottle with conical shoulder, 50 ml (12).

7.13. Burette, 10 ml (13).

Notes:

1. One should not deviate from the above described apparatus and its components to assure a correct determination.

2. The distance between parts 7.9. and 7.10. should be 2–3 mm to allow the gas to be dispersed in the absorption liquid in the form of very small bubbles.

8. SAMPLING AND SAMPLES

The laboratory sample is carefully mixed. For coarse or very heterogeneous materials previous milling is recommended. The samples should be stored in air-tight containers.

The weight of the test portion varies between 10 g and 50 g, depending on the sulphur dioxide content.

It should be weighed to within 0.1 g.

9. PROCEDURE

9.1. Add 25 ml hydrogen peroxide and three drops of bromophenol blue into the reagent bottle (7.12.).

9.2. Introduce the test portion into the three-neck flask (7.5.).

9.3. Add 275 ml water (6.1.) or distilled water/methanol (see 11) and swirl to disperse the test portion in the water.

9.4. Make all connections according to the diagram and start water cooling. It may be advisable to start nitrogen flow before mounting the inlet tube on the three-neck flask in order to avoid plugging of the inlet tube.

9.5. Purge the apparatus for 3 minutes with a stream of nitrogen (400 ml/min) which is made oxygen-free by passing through the Friedrichs wash bottle (7.1.) with alkaline pyrogallol solution (6.8.).

9.6. Start heating.

9.7. Reduce nitrogen flow to 150–200 ml/min.

9.8. Introduce 50 ml of 4 N hydrochloric acid in 7.5. and start magnetic stirrer. The magnetic stirrer must rotate at maximum speed, without jumping.

9.9. After the solution begins to boil (1 drop reflux per second), gentle boiling is continued for one hour.

9.10. Titrate the contents of the receiver with 0.1 N sodium hydroxide to the same colour which the solution had before the sulphur dioxide absorption.

10. EXPRESSION OF RESULTS

10.1. Method of calculation:

$$SO_2 \text{ content} = \frac{3200 \times a}{p} \text{ mg/kg (ppm)}$$

Where a = ml 0.1 N NaOH used
p = weight of the test portion in g.

10.2. Lower limit of sensitivity: about 10 ppm.

10.3. Repeatability: 5 ppm per 100 ppm SO_2.

11. SPECIAL CASES

For the determination of sulphur dioxide in certain vegetable varieties containing organic sulphur (such as cabbage, onion, leek, etc.) the 275 ml water must be replaced by 225 ml methanol and 50 ml water. The lowering of the boiling point reduces the destruction of the organic sulphur-containing compounds and consequently the interference by the resulting volatile S-compounds which react in the absorption liquid like the SO_2.

12. BIBLIOGRAPHICAL REFERENCE

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DETERMINATION OF WATER BY THE KARL FISCHER METHOD

May 1980 – Original text in Englisch

1. SCOPE

Determination of water.

2. FIELD OF APPLICATION

Ingredients, with a water content up to 15%, in which accurate moisture determination by loss of mass is difficult (e.g. dehydrated vegetables, spices, etc.).

3. DEFINITION

The amount of water (including the water of hydrates, see 8.2.) is proportional to the quantity of iodine reduced (5.) by titration with the Karl Fischer reagent, under the conditions of the test.

4. PRINCIPLE

The water extracted from the sample by hot methanol is titrated with Karl Fischer (KF) reagent. The end point of the titration is detected amperometrically ('Dead-stop' titration). The water content is calculated from the KF reagent consumed and the water equivalent of the reagent, previously determined either by using disodium tartrate dihydrate or weighed water (8.2. or 8.3.).

5. REACTIONS

$$SO_2 + I_2 + H_2O + 3C_5H_5N + CH_3OH \rightarrow 2C_5H_5NHI + C_5H_5N \cdot HSO_4CH_3$$

6. REAGENTS

6.1. Methanol, analytical grade, containing less than 0,01% water

6.2. Disodium tartrate dihydrate ($Na_2C_4H_4O_6 \cdot 2H_2O$) with certified water content of $15{,}66 \pm 0{,}05\%$. If kept in a closed container it is stable for several years.

6.3. Karl Fischer reagent (KF), water equivalent approximately 5 mg per ml.
Store in a well-stoppered bottle (away from light). Replace when water equivalent is less than 4 mg/ml. Determine the water equivalent of the KF reagent as described under paragraph 8.2. or 8.3.

6.4. Distilled water.

7. APPARATUS

7.1. Karl Fischer titrator with 'Dead-stop' indicator.
Commercially available manual or automatic KF titrators are suitable. A titrating vessel which allows easy introduction of both solid and liquid substances, is preferred.
The apparatus should be set up according to manufacturer's instructions. The tightness of the assembled titration vessel

should be checked periodically by leaving the apparatus switched on at the end of a titration; the additional reagent consumption should then be less than 0,05 ml during the usual time taken for one titration. Before use, the KF titrator should be prepared as described under 8.1.

7.2. Weighing boat, for direct introduction of solid standard.

7.3. Air oven, capable of maintaining a specified temperature to ±1 °C.

7.4. One-mark pipettes, 10 and 40 ml.
Note: dry at 100 °C before use; store in a pipette cylinder.

7.5. Pipette filler.

7.6. Screw-capped bottles, 100 ml, heat resistant.
Note: dry at 100 °C before use; cool and store in a desiccator

7.7. Desiccators, containing an efficient desiccant (e.g. magnesium perchlorate anhydrous)

7.8. Flasks, 50 ml, with stoppers.
Note: dry at 100 °C before use; cool and store in desiccator.

8. STANDARDISATION

8.1. Preparation

8.1.1. In the prepared apparatus (titration vessel, 7.1.) pipette about 20 ml methanol (6.1.).

8.1.2. Titrate the methanol with KF reagent (6.3.) to the endpoint.
The setting of the apparatus controls (according to manufacturer's instructions) should be retained during the standardisation procedure and the sample determination.

8.2. Standardisation with disodium tartrate

8.2.1. Put into the weighing boat (7.2.) about 300–400 mg of disodium tartrate (6.2.) and weigh to the nearest 0,1 mg (a).

8.2.2. Empty the contents of the weighing boat into the titration vessel (7.1.).

8.2.3. Reweigh the weighing boat to the nearest 0,1 mg (b).
Note: the operation should be carried out in such a way as to minimise the ingress of moisture.

8.2.4. Titrate with KF reagent (6.3.) as soon as the sodium tartrate is dissolved (v).

8.2.5. Calculate the water equivalent F of the KF reagent by means of the equation:

$$F = \frac{(a - b) \times 0{,}1566}{v} \text{ mg } H_2O/\text{ml}$$

where

a is the mass, in mg, of the weighing boat and the disodium tartrate (8.2.1.)
b is the mass, in mg, of the empty weighing boat (8.2.3.)
v is the volume, in ml, of KF reagent used (8.2.4.)

8.2.6. Repeat the standardisation procedure with a second weighing of disodium tartrate.

8.2.7. Calculate mean water equivalent from at least two subsequent determinations which should not differ more than 0,01 mg/ml.

8.3. Standardisation with distilled water

8.3.1. Prepare the equipment before use as described under 8.1.

8.3.2. Weigh a pre-dried 50 ml stoppered flask (7.8.) to the nearest 0,1 mg.

8.3.3. Add about 200 mg distilled water (6.4.) to the flask.

8.3.4. Insert stopper immediately and weigh to the nearest 0,1 mg.

8.3.5. Calculate the weight of added water (m_0).

8.3.6. Pipette 40 ml methanol (6.1.) into the flask. Again, insert stopper immediately. Agitate the contents to ensure complete mixing.

8.3.7. Titrate with KF reagent two 10 ml aliquots from the flask. Calculate mean volume, in ml, of KF reagent used for the titration (V).

8.3.8. Determine the blank value of 10 ml methanol (6.1.) (V_0).

8.3.9. Calculate the water equivalent F of the KF reagent by means of the equation:

$$F = \frac{m_0 \times 10}{40 \times (V - V_0)} \text{ mg } H_2O/\text{ml}$$

where

m_0 is the mass, in mg, of water added (8.3.5.)
V is the volume, in ml, of KF reagent used for titration (8.3.7.)
V_0 is the volume, in ml, of KF reagent used for the blank (8.3.8.)

9. SAMPLING AND SAMPLE

Test samples for analysis should be a powder (preferred particle size $< 500\,\mu$).
Grind samples, if necessary, as quickly as possible to prevent water uptake.
The samples should be stored in air-tight containers in such way that deterioration and change in composition are prevented.

10. PROCEDURE

10.1. Extraction of water from the samples.

10.1.1. Weigh duplicate test portions to the nearest 0,1 mg (m) into pre-dried 100 ml screw-capped bottles (7.6.).
Note: the test portions should preferably contain 100 to 200 mg water.

10.1.2. Pipette 40 ml methanol (6.1.) into the bottles containing the test portions and into an empty bottle (blank sample); securely replace the screw caps and shake vigorously to disperse the samples.

10.1.3. Heat the bottles for 1 hour at 60 °C in the air oven, preferably in a horizontal position to ensure maximum contact between the sample and the methanol.

10.1.4. Remove the bottles from the oven, shake again to ensure complete mixing and allow them to cool down to room temperature and for the solids to settle.

10.2. Titration

10.2.1. Remove the cap from one of the bottles and immediately pipette a 10 ml aliquot from this bottle (10.1.4.) into the titration vessel (7.1.). Replace cap without delay.

10.2.2. Titrate with KF reagent (6.3.).

10.2.3. Repeat procedures 10.2.1. and 10.2.2. for a second aliquot and calculate mean volume, in ml, of the KF titrant required (V_1).

10.2.4. Similarly titrate aliquots from the duplicate test portion

10.2.5. Titrate in duplicate 10 ml aliquots of the blank sample (10.1.2.)

10.2.6. Calculate the mean volume, in ml, of KF titrant required for the blank sample (V_0).

11. EXPRESSION OF RESULTS

11.1 Method of calculation: (results in percentage m/m to one decimal place).

$$\text{Water content} = \frac{(V_1 - V_0) \times F \times 40 \times 100}{10 \times m} \%$$

where

m is the mass, in mg, of test portion (10.1.1.)
V_1, V_0 are the mean volumes, in ml, of KF reagent used for aliquots of sample (10.2.3.) and blank (10.2.6.) respectively
F is the water equivalent of the KF reagent (in mg H_2O/ml) (8.2.5.) or (8.3.9.)

Likewise calculate the water content of the duplicate test portion (10.2.4.).

11.2. Repeatability: 0,2 g/100 g test sample.

12. NOTES

12.1. The KF method is particularly recommended for the determination of moisture in dehydrated vegetables and in other ingredients, such as spices, herbs, flour, starches etc. with water content up to 15 g/100 g.

12.2 For certain raw materials e.g. potato powder, which retain their moisture very strongly, formamide at 100 °C has proved to be an efficient extractant.

12.3. The method can also be used for fats and oils. In that case the moisture should be extracted by using methanol/chloroform (1 : 1) instead of methanol.

12.4. The water of crystallisation of certain substances (e.g. monosodiumglutamate, lactose) reacts with the KF reagent.

12.5. The validity of the method for new applications should be checked by recovery tests using an admixture of sample and standard hydrate, e.g. disodium tartrate (6.2.) at two levels of addition.

13. BIBLIOGRAPHICAL REFERENCES

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DETERMINATION OF DRY MATTER

For method see 2/2

Notes:

For materials such as tomato puree, powder or flake a vacuum oven should be used preferably. Recommended conditions are:

Temperature	70 ±1°C
Pressure	0,1 bar (absolute)
Time	4 hours

DETERMINATION OF THE CHOLESTEROL CONTENT IN (EGG) PASTA PRODUCTS
GAS CHROMATOGRAPHIC METHOD

PREFACE

The AIIBP Technical Commission has made some theoretical and practical comparisons of the methods for determination of cholesterol in (egg) pasta. The aims were:

a) to determine the level of cholesterol in the pasta used in our products,

b) to determine the quantity of eggs used per kg of durum wheat semolina, respectively per kg of dry pasta. To check the declaration of these quantities.

The Technical Commission finally has adopted the German method published in the "Official Collection of Analytical Methods according to § 35[1)]". Before its publication, this official method was subjected to an inter-laboratory test under the direction of the Max-von-Pettenhofer Institute of the Federal Department of Health – Section Chemistry of Food and Consumer Goods.

For this reason, this approved method (which is also already in use) has not been subjected to additional systematic tests by AIIBP. It is therefore published here for information only.

For technical reasons, the original numbering and titles of the paragraphs have been adapted as far as possible to the system used in the AIIBP Manual.

Note

Any amendment to this method – or use of a different method – should be mentioned in the reports of analysis.

1) Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG, Bd I/3, Methode L 22.02/04-1, Mai 1982.

(English: Official Collection of Analytical Methods according to § 35, Food Products and Consumer Goods, Vol. I/3, Method L 22.02/04-1, May 1982).

DETERMINATION OF THE CHOLESTEROL CONTENT IN (EGG) PASTA PRODUCTS GAS CHROMATOGRAPHIC METHOD

May 1994 – Original Text in German, according to:

Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG, Vol. I/3, Methode L 22.02/04-1, Mai 1982

1. SCOPE AND FIELD OF APPLICATION

This official method describes a procedure for the determination of the cholesterol content of (egg) pasta products. It is also applicable for precooked small noodles, like "Spätzle" (German speciality).

2. DEFINITION

The cholesterol content of the products mentioned in section 1. is understood to be that value obtained by the method described here. The results are given as mg cholesterol per 100 gram dry matter.

From the content of cholesterol in the dry matter it is possible to calculate the quantity of egg which was added in the manufacture of the pasta products.

3. PRINCIPLE OF THE METHOD

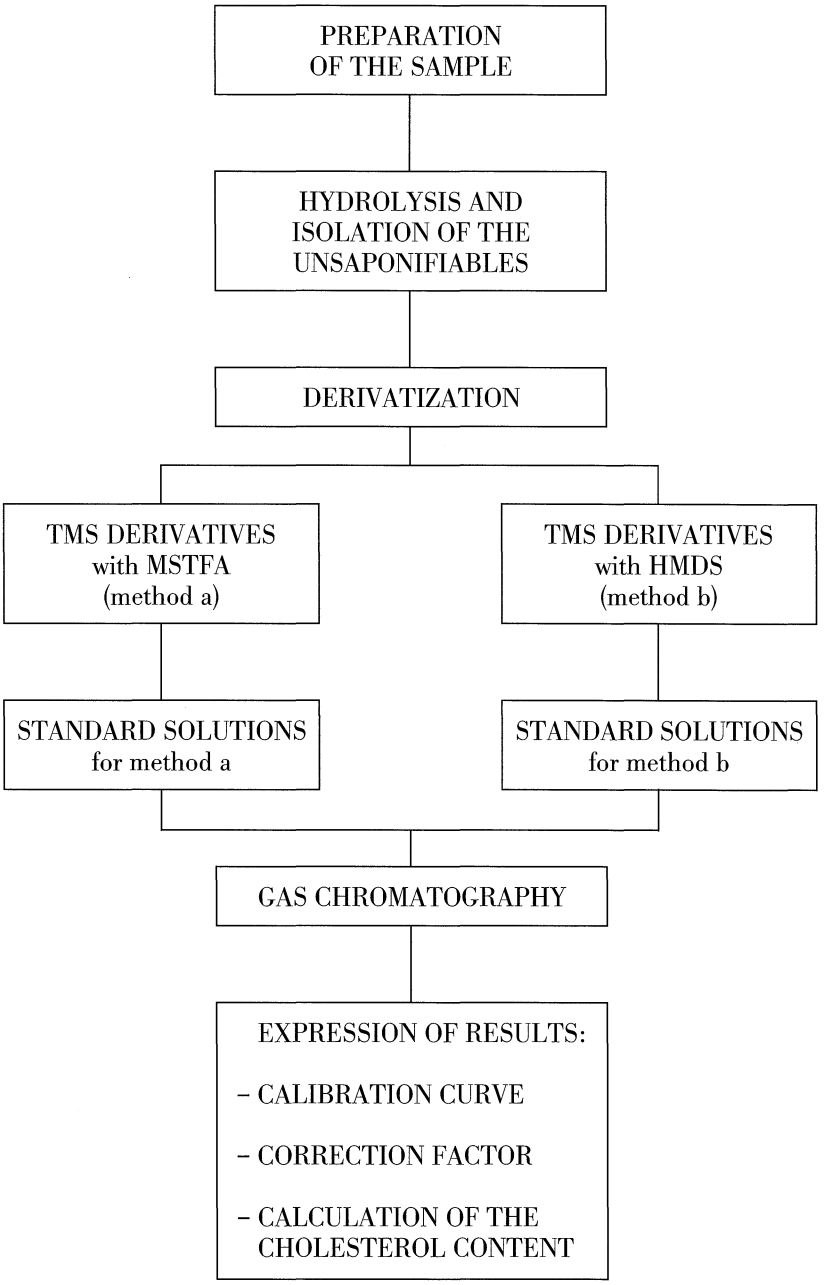
After an acid followed by an alkaline hydrolysis of the finely ground pasta product, the unsaponifiable material is isolated.

The cholesterol content of the unsaponifiables is determined by gas chromatography after conversion of the cholesterol to the Trimethylsilyl (TMS) derivative.
5-α-Cholestane is used as an internal standard.

Two methods of derivatization have been selected from the many possible methods:

- with MSTFA (s. reagent 4.2.3): method a,
- with HMDS (s. reagent 4.3.3): method b.

3.1 SCHEME OF THE METHOD



4. REAGENTS

Unless stated otherwise, all reagents are of analytical quality.
Water must either be distilled or of an equivalent purity.
Where "solution" is mentioned, this means a solution in water.

4.1 Hydrolysis and isolation of unsaponifiables

4.1.1 Potassium hydroxide pellets

4.1.2 Sodium sulphate, anhydrous, heated at 600° C

4.1.3 Hydrochloric acid, diluted:

50 ml water are mixed with 50 ml fuming hydrochloric acid ($\omega = 37/38\%$[1]; specific gravity 1,19 g/ml)

4.1.4 Potassium hydroxide solution $\rho = 1$ g/100 ml[1]

4.1.5 Ethanol $\sigma = 96\%$[1]

4.1.6 Diethyl ether, peroxide free

4.1.7 Acetone

4.2 Determination of sterols as TMS derivative with MSTFA (method a)

4.2.1 Acetone, distilled

4.2.2 Tetrahydrofuran, anhydrous

4.2.3 N-methyl-N-trimethylsilyl-trifluoroacetamide **(MSTFA)**

4.2.4 5-α-Cholestane stock solution, $\rho = 5$ mg/ml:

250 mg 5-α-Cholestane, pure ($\omega > 99\%$) are completely dissolved in about 45 ml acetone in a 50 ml volumetric flask; if necessary by allowing to stand overnight. Fill up to the mark with acetone.

4.2.5 5-α-Cholestane standard solution, $\rho = 0{,}5$ mg/ml:

Pipette 10 ml of the stock solution (4.2.4) into a 100 ml volumetric flask and fill up to the mark with acetone.

4.2.6 Cholesterol standard solution, $\rho = 2$ mg/ml:

In a 100 ml volumetric flask dissolve 200 mg cholesterol pure ($\omega > 98.5\%$) in acetone. Fill up to the mark with acetone.

4.2.7 Nitrogen, purified

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[1] ω = parts by weight
σ = concentration by volume
ρ = concentration by weight

4.3 **Determination of sterols as TMS-derivative with HMDS (method b)**

4.3.1 Acetone, distilled

4.3.2 Tetrahydrofuran, anhydrous

4.3.3 Hexamethyldisilazane **(HMDS)** (1,1,1,3,3,3-hexamethyldisilazane)

4.3.4 Trimethylchlorosilane (TMCS) (chlortrimethylsilane)

4.3.5 5-α-Cholestane stock solution, $\rho = 5$ mg/ml (see section 4.2.4)

4.3.6 5-α-Cholestane standard solution, $\rho = 0.5$ mg/ml (see section 4.2.5)

4.3.7 Cholesterol standard solution, $\rho = 1$ mg/ml:

In a 100 ml volumetric flask dissolve 100 mg cholesterol pure ($\omega > 98.5\%$) in acetone. Fill up to the mark with acetone.

4.3.8 Nitrogen, purified

5. MATERIALS AND APPARATUS

A. MATERIALS

5.1 **Hydrolysis and isolation of the unsaponifiables**

5.1.1 Laboratory mill

5.1.2 Rotary film evaporator or another apparatus suitable for ether distillation

5.1.3 Water bath

5.1.4 Test tube with screw cap

5.1.5 Erlenmeyer flasks, 300 ml, with NS 29 joint

5.1.6 Reflux condensor with NS 29 joint

5.1.7 Separating funnels, 500 ml and 250 ml

5.1.8 Funnels diameter 2 to 3 cm

5.1.9 Round bottomed flasks 250 ml with NS 29 joint

5.1.10 Columm, length 26 cm (see annex)

5.1.11 Folded filter

5.2 **Determination of sterols as TMS derivative with MSTFA (method a)**

5.2.1 Rotary film evaporator

5.2.2 Pressure reducing valve for nitrogen steel-cylinder and feeding system

5.2.3 Drying oven

5.2.4 Pear-shaped flasks, 10 ml, with NS 14.5 joint

5.2.5 Test tubes, 2 ml, with teflon seal and screw cap

5.3 **Determination of sterols as TMS derivative with HMDS (method b)**

5.3.1 Pressure reducing valve for nitrogen steel-cylinder and feeding system

5.3.2 Water bath

5.3.3 Centrifuge

5.3.4 Pipettes 0.5 ml, with 0.05 ml divisions

5.3.5 Centrifuge tubes with joint, approximate volume 15 ml

B. APPARATUS

5.4 **Gas chromatography**

5.4.1 Gas chromatograph, for chromatography with glass columns to an oven temperature of 300° C, equipped with flame ionization detector (FID), writer, possibly with integrator and auto sampler.

5.4.2 Columns: glass columns, length 1 to 2 meters, internal diameter 2 to 3 mm.
Packing: separation phase silicone basis (e.g. 1 to 3% SE-30, UCCW-982, OV-17 or OV-101).
Inert phases (e.g. Chromosorb W, AW, DMCS, 80 to 100 mesh or Gaschrom Q 100 to 120 mesh).

5.4.3 Gases: nitrogen, purified (carrier gas)
hydrogen
synthetic air

5.4.4 Microliter syringe 5 µl for gas chromatography

6. SAMPLING

6.1 Sampling test plan

This has not yet been determined in the framework of the official text (1982!).

6.2 Sampling technique

As protection against changes the sample is kept in a closed container in cool and dark conditions.

7. PROCEDURE

7.1 Preparation of the sample

100 g of the sample are finely ground in the laboratory mill and stored in a closed container, in a cool and dark place.

7.2 Determination

7.2.1 **Hydrolysis and isolation of the unsaponifiables**

5.0 g of the ground pasta product are weighed to an accuracy of 1 mg into a 300 ml Erlenmeyer flask with ground glass joint, and 15 ml diluted hydrochloric acid (4.1.3) are added. The flask is fitted with the reflux condensor and heated for 30 minutes with repeated shaking in order to disperse any lumps and to ensure a complete hydrolysis.

While subsequently cooling the contents of the flask under running water, 15 g potassium hydroxide (pellets) are added carefully while swirling the flask. The contents of the flask will then begin to boil, however vigorous boiling should be avoided.

After cooling again, 20 ml ethanol are added so that this is allowed to flow over the walls of the flask. The flask, fitted with reflux condensor, is heated with repeated shaking in the water bath for 45 minutes.

The flask is again cooled under running water, and its contents are, by repeated washing with in total 75 ml water, quantitatively transferred to a 500 ml separating funnel: the flask is finally washed with 25 ml diethyl ether and this is slowly poured into the separating funnel.

After the addition of an extra 50 ml diethyl ether, the funnel is shaken vigorously for 1 minute and then allowed to stand for so long as is necessary for the phases to separate (about 30 minutes).

The saponified solution is slowly drawn off into a 250 ml separating funnel but emulsion and insoluble matter are withheld at the phase boundary . The walls of the 500 ml separating funnel are rinsed with 10 ml potassium hydroxide solution (4.1.4) and this solution is also added to the 250 ml separating funnel.

Then 50 ml diethyl ether are added to the 250 ml separating funnel and this is shaken vigorously for 1 minute. After separation of the phases, the saponified solution is drawn off into a second 250 ml separating funnel and this is shaken with a further 50 ml diethyl ether.

The diethyl ether layers are transferred to a 500 ml separating funnel and the 250 ml separating funnels are each rinsed with 10 ml diethyl ether. The combined diethylether solutions are washed once with 50 ml potassium hydroxide solution and at least three times with 50 ml water until the water washings give a neutral reaction. During the washing the insolubles or emulsions are retained in the separating funnel. During the washing process overvigorous shaking should be avoided.

Finally the aqueous layer is drawn off. In order to remove the last traces of water, the ether phase is transferred with slight overpressure above a column (see annex) in which an approximately 4 cm thick layer of sodium sulphate is placed above a cotton wool plug.
A 250 ml round bottomed flask is used as collection vessel. (It is also possible to carry out the usual drying over sodium sulphate and the subsequent filtration in a 250 ml round bottomed flask).
The separating funnel and column (or filter) are rinsed 2 or 3 times with about 20 ml diethyl ether.

The ether solution of unsaponifiables is evaporated in the rotary film evaporator to a volume of a few milliliters.

The isolation of the unsaponifiables is carried out without interruption.

7.2.2 Preparation of the unsaponifiables for the gas chromatography

The cholesterol and the phytosterols present in the unsaponifiables can be determined gas chromatographically without derivatization after addition of an internal standard (5-α-cholestane) so long as the necessary apparatus (glass column, direct injection system, completely deactivated carrier material/gas, etc.) are available and as short a time as possible is realized between the isolation of the unsaponifiables and their gas chromatography.

Because of better repeatability, it is however recommended that the sterols are derivatized followed by gas chromatography of their derivatives.

7.2.2.1 **Conversion of the sterols to TMS derivatives with MSTFA (method a)**

The solution of unsaponifiables is quantitatively transferred with a few ml diethyl ether into a 10 ml pear shaped flask and evaporated almost to dryness in the rotary film evaporator. The last traces of solvent are removed with a stream of nitrogen. Then the residue is dissolved in 2.0 ml 5-α-cholestane standard solution (section 4.2.5) for normal egg content and in 4.0 ml for high egg content.

1.0 ml of this acetone solution is pipetted into a small test tube (section 5.2.5) and evaporated to dryness in a stream of nitrogen. The residue is dissolved in 0.5 ml of tetrahydrofuran and treated with 0.5 ml MSTFA. The test tube is immediately closed with a teflon screw cap and shaken. After 90 minutes heating in a drying oven at 70° C the sample solution can be gas chromatographed.

7.2.2.1.1 Preparation of the standard solutions

The following solutions are pipetted into seven 10 ml volumetric flasks:
In each, 1 ml 5-α-cholestane stock solution (section 4.2.4) and successively in each 2, 3, 4, 5, 6, 7 and 8 ml cholesterol standard solution (section 4.2.6) and filled up to the 10 ml mark with acetone.

1.0 ml from each solution with contents of 0.4-0.6-0.8-1.0-1.2-1.4 and 1.6 mg cholesterol per ml and 0.5 mg 5-α-cholestane per ml as internal standard are pipetted into test tubes and treated as described under 7.2.2.1.

7.2.2.2 **Conversion of the sterols to TMS-derivatives with HMDS (method b)**

The solution of unsaponifiables is quantitatively transferred with the help of a few milliliters of diethyl ether to a centrifuge tube with a ground glass joint.
After evaporation of the solvent in a stream of nitrogen, the residue is dissolved in 0.15 ml of tetrahydrofuran and 0.4 ml hexamethyldisilazane and 0.2 ml trimethylchlorosilane are added.
The mixture is heated for 30 minutes at 60–70° C and the excess reagent is removed in a stream of nitrogen.

According to the egg content of the sample, this is digested with corresponding quantities of 5-α-cholestane standard solution (sections 4.2.5 and 4.3.6)

in order to achieve a cholesterol concentration of 0.8 to 1.1 mg cholesterol per ml (2.0 ml for pastas with 2 eggs per kg semolina, 5.0 ml for pastas with 4 eggs)*. The solution is vigorously shaken and centrifuged (test solution).

* *(Interpolate in the case of pastas with 3, resp. 6 eggs per kg semolina/Red.)*

7.2.2.2.1 Preparation of the standard solutions

Pipette into seven centrifuge tubes 2, 3, 4, 5, 6, 8 and 10 ml of the cholesterol standard solution (section 4.3.7). After removal of the solvent, these are silylated as described above (see 7.2.2.2).

The residue is dissolved in 5.0 ml 5-α-cholestane standard solution (sections 4.2.5 and 4.3.6) in order to obtain standard solutions with cholesterol contents of 0.4-0.6-0.8-1.0-1.2-1.6 und 2.0 mg/ml.

8. GAS CHROMATOGRAPHY

1 to 2 µl portions of the standard solutions (sections 7.2.2.1.1 and 7.2.2.2.1) and of the test solutions (sections 7.2.2.1 and 7.2.2.2) are gas chromatographed in triplicate for each solution.

The following conditions are used:

Temperatures:	Injection block	250–275° C
	Column oven	220–270° C (according to column length and carrier gas flow rate)
	FID	275–300° C
Attenuation:	By injection of 1 to 2 µl 5-α-cholestane standard solution (sections 4.2.5. and 4.3.6), the peak heights should cover 50 to 70% of the graph.	

The carrier gas flow rate can be optimalized under the chosen test conditions, the flow rate of hydrogen and air is determined from the equipment manufacturers' information.

9. EXPRESSION OF RESULTS

9.1 Making the calibration curve

After calculating the peak area (height × average breadth) or determination of the peak area integral of 5-α-cholestane and cholesterol peaks, from the three values for each concentration, the average peak area is determined.

The cholesterol content ρ in mg/ml of the silylated standard solution is calculated from the following formula:

$$\rho = \frac{\rho_{\text{cholestane}} \times S_{\text{cholesterol}}}{S_{\text{cholestane}}} \qquad (1)$$

where:

$\rho_{\text{cholestane}}$ = concentration of 5-α-cholestane ($\rho = 0.5$ mg/ml)
$S_{\text{cholesterol}}$ = average peak area of cholesterol in mm^2
$S_{\text{cholestane}}$ = average peak area of cholestane in mm^2

The observed cholesterol contents ($\rho_{\text{cholesterol obs.}}$) are plotted on the abcissa and the theoretical cholesterol contents ($\rho_{\text{cholesterol theor.}}$) on the ordinate of a diagram, in mg/ml, and the calibration line is drawn through the cross points. Because it can be expected by the above conditions that the majority of the cholesterol concentrations will be between 0.8 and 1.1 mg/ml, it is sufficient to check the corresponding points on the calibration curve each day.

9.2 Calculation of the correction factor

From the theoretical and observed cholesterol content a correction factor ($F_{\text{corr.}}$) can be calculated.

$$F_{\text{corr.}} = \frac{\rho_{\text{cholesterol theor.}} \times S_{\text{cholestane}}}{\rho_{\text{cholestane}} \times S_{\text{cholesterol}}} \qquad (2)$$

where:

$\rho_{\text{cholesterol theor.}}$ = cholesterol content of the injected solution (theor. value) in mg/ml
$\rho_{\text{cholestane}}$ = cholestane content of the injected solution (0.5 mg/ml)
$S_{\text{cholesterol}}$ = average peak area for cholesterol in mm^2
$S_{\text{cholestane}}$ = average peak area for cholestane in mm^2

9.3 Calculation of the cholesterol content of the sample

From the triplicate chromatograms for one sample, the average peak area for 5-α-cholestane and cholesterol are calculated.

The cholesterol content ρ in mg/ml of the silylated sample solution is calculated according to formula (1) (see section 9.1). The theoretical cholesterol value corresponding to the determined value may be read from the calibration curve, or the average value may be corrected with the valid correction factor ($F_{corr.}$) for the concentration range in question.

The cholesterol content ω in mg/100 g dry matter of the sample is calculated according to the following equation:

$$\omega = \frac{\rho_{\text{cholesterol theor.}} \times V \times 100 \times 100}{m \times TS}$$

where:

$\rho_{\text{cholesterol theor.}}$ = cholesterol content of the silylated sample solution in mg/ml (theoretical value, that is already including the correction factor $F_{corr.}$, or from the value taken from the calibration curve)
V = Volume of the 5-α-cholestane standard solution in ml
m = weight of sample in g
TS = dry matter content of the sample in g/100 g

9.4 Reliability of the method

9.4.1 Repeatability (r)
$r = 4.2$ mg/100 g $\quad s_{(r)} = \pm\ 1.484$ mg/100 g

9.4.2 Reproducibility (R)
$R = 0.2\ \bar{y}$ mg/100 g $\quad s_{(R)} = \pm\ 0.071\ \bar{y}$ mg/100 g
$\bar{y}$ = mean of two or more values

10. TEST REPORT

In the test report it is necessary besides the mention of this official method to give at least the following information:

- Type, origin and coding of the sample
- Type and date of the sampling
- Dates of receipt and test in the laboratory
- Result of the test
- The motivation for possible deviations from this official method

11. REMARK

This official method was subjected to a ring test under the direction of the Max-von-Pettenkofer Institute of the Federal Department of Health, Section Chemistry of Food and Consumer Goods.

12. BIBLIOGRAPHICAL REFERENCES

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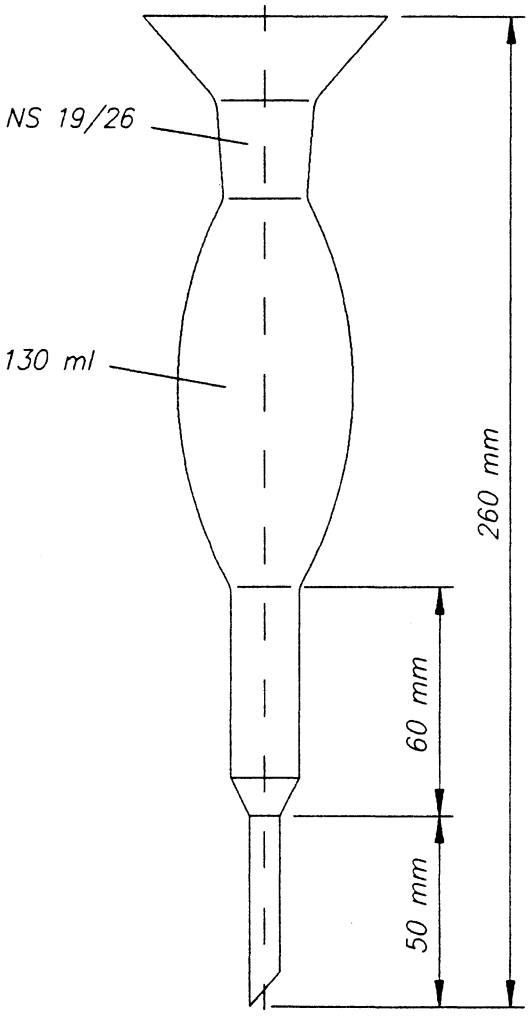
ANNEX



Column for drying the ether phase (see 5.1.10 and 7.2.1).

Introduction

June 1973 – Original Text in English

A. Scope

This chapter contains methods for the determination of the number of viable aerobic mesophilic microorganisms ('total count'), *Escherichia coli*, *Staphylococcus aureus* and spores of *Clostridium perfringens* as well as for the detection of *Salmonella* in dried soups and the raw materials used in their preparation. The object of such determinations is to produce soups with a high standard of bacteriological quality.

B. Field of application

It must be emphasised that soup products have a variety of compositions, and may be manufactured as dried, instant, deepfrozen, or canned soups. The microbiological composition of the finished products may be influenced by the manufacturing procedures employed, and by the microbial content of the raw materials used.

I. Dried soups

Dried soups may contain a number of ingredients such as spices and dried vegetables, and as is well known these ingredients may occasionally contain quite large numbers of harmless microorganisms. These varied and complex influences must be borne in mind when evaluating the results of a bacteriological examination of soups.

In order to obtain a realistic analysis of the microbiological content of dried soups, the following tests are recommended:

1. Count of viable aerobic mesophilic microorganisms
2. Count of *Escherichia coli*
3. Examination for *Salmonella*
4. Count of *Staphylococcus aureus*
5. Count of spores of *Clostridium perfringens*

These procedures will permit satisfactory evaluation of the microbiological condition of dried soups or their ingredients. It is not recommended to examine for *Bacillus cereus* and group D-streptococci (or the enterococcus group), as these types are of interest only when many millions are present in a food. Their control in soups is adequately guaranteed by the enumeration of viable aerobic mesophilic microorganisms.

The following specific points should be noted in respect of the tests recommended in this report.

1. Count of viable aerobic mesophilic microorganisms ('total count')

Although in these products the determination of viable aerobic mesophilic microorganisms itself does not furnish indications concerning a possible health hazard or decomposition, the count should not be too high for reasons of general hygiene. In evaluating the viable count, however, the specific bacterial flora of the soup components has to be taken into consideration. For example, it is necessary to accept a higher count in dried soups containing vegetables than in other soups.

2. Count of Escherichia coli

The main purpose of this examination is to determine the general hygiene of the manufacturing process. Positive findings have always to be followed by checking each individual operation in order to find the reasons for such results. Also in this case the composition of the soup product (for example its possible content of components of animal origin) must be considered in the evaluation.

3. Examination for Salmonella

Salmonella can occur in a wide range of raw materials and food products, and in order to protect the consumer, *Salmonella* testing is recommended for soup products. This examination is proposed even though many soup products are given a heat treatment during production and/or are cooked by the consumer. Tests for *Salmonella* are particularly important for instant and deepfrozen soups, which may be given only a moderate heating or no heating prior to eating.

4. Count of Staphylococcus aureus

In dehydrated soups, as in other foods, possible health hazards due to bacteria might be caused by the presence of toxins formed by the growth of *Staphylococcus aureus*. A method must therefore be available for the determination of Staphylococcus aureus.

5. Count of spores of Clostridium perfringens

This species might survive the heat treatments given to soups during production and preparation, and be able to germinate in the prepared soup. It is therefore necessary to make certain that, if these spores are present in the product, there is only a small number of them.

II. Canned soups

Bacteriological counting is not necessary for canned soups; pH determination and organoleptic examination are more important. After incubation of cans the contents should be of satisfactory appearance and odour and should not have changed significantly in pH value.

If for any reason canned soups require bacteriological analysis, then this may be done with the same methods used for other canned foods.

General remark

The methods described in this report should only be applied by competent bacteriologists, and it is emphasised that the results obtained will require expert evaluation, based on a full understanding of the microbiological influences underlying the microbial content of soup products.

C. Diluant, media and reagents

1. Diluant

Solution of tryptone-salt (T.S.)

Tryptone	1 g
NaCl	8 g
Distilled water	1000 ml
pH	7.0–7.2

Dissolve the tryptone and the salt in the distilled water by gentle warming. Adjust the pH. Dispense into screw capped bottles as follows:

(i) Exactly 50 ml into 200 ml bottles for the 1:10 dilution.
(ii) Exactly 90 ml into 200 ml bottles for the 1:100 and 1:1000 dilutions.
(iii) Approximately 300 ml into 500 ml flasks for the preparation of the mother suspension (1:5).

Sterilize in the autoclave at 121°C for 20 to 30 min according to volumes in the bottles (20 min for 50 and 90 ml; 30 min for 300 ml). Check the sterility of the medium before use by incubation for 48 h at 30°C.

2. Media and reagents

The described methods were checked using in general Difco media or media composed from Difco ingredients. If media or ingredients from other sources—e.g. Oxoid, BBL, Merck, etc.—are used their performance in principle should be checked. The composition of the media and the reagents needed is given in the procedures for the different bacteria. The amount of agar may have to be adapted to the kind used.

D. Glassware, etc.

1000 ml flat bottomed flask (with conical shoulder)
Measuring cylinder graduated 200, 100 and 50 ml
10 ml pipettes, graduated in 0.1 ml
1 ml pipettes graduated at 1 ml and 0.1 ml
200 and 500 ml bottles closed by metal screw caps
Glass Petri dishes, 85 or 100 mm in diameter
Test tubes 160 × 16 mm closed by non-absorbent cotton wool plugs or metal caps, containing Durham tubes
Mixer: e.g. Polytron P.T. 20, Ultra Turrax, Atomix, etc.

Cleaning and sterilization

After careful washing, the glassware is rinsed with water of drinkable quality, free from detergents. It is dried and then prepared for sterilisation, the use of cotton wool plugs to close the tubes and bottles should be avoided as far as possible; those which are covered by metal caps or lids are preferred. Cotton wool would only be used for pipettes and flasks. After suitable wrapping (metal containers are preferable for pipettes and Petri dishes), the prepared glassware is sterilized in the following way:

- By dry heat at 160°C for at least 2 hours or preferably at *175°C for 35 min* or at 180°C for 25 min. In each case, allow to cool slowly in the oven.

N.B. Glass Petri dishes can be replaced by suitable plastic dishes. These are usually supplied after sterilization by the manufacturer; they must not be submitted to another treatment.

E. Sampling

1. Laboratory sample

Recommendations concerning the level of sampling are not included here. For general guidance on the sampling of soups see Chapter 0.

2. Test sample

Preparation of the mother suspension and homogenization

The contents of a packet of dried soup, at least 50 g, at most 100 g, is weighed using proper aseptic precautions. It is placed in a 1000 ml flask. Sufficient quantity of tryptone salt (T.S.) solution is added to make a 1: 5 dilution, e.g. 400 ml diluent for 100 g soup. Graduated flasks and sterile 10 ml pipettes graduated at 0.1 ml are used for this purpose. Mix and leave at laboratory temperature (18–25°C) for 50 min, repeating the shaking every 10 to 15 minutes.

After making a uniform suspension of the insoluble particles by manual agitation, approximately 200 ml are put into the sterile bowl of a mixer. The mixture is macerated for 3 min at 10,000–15,000 r.p.m. (or equivalent). One ml of this mother suspension corresponds to 0.2 g of dried soup.

The mixture obtained is used to make the dilutions. These dilutions and inoculations must be made during the following 15 min, the mixture being left at the temperature of the laboratory (18–25°C).

Certain soups diluted to $^1/_5$ (mother suspension) give very viscous liquids of which the homogenisation is difficult. In this case it is preferable to make an original dilution of $^1/_{10}$. One should take account of this in the subsequent calculation of the number of colonies of aerobic bacteria and of *E. coli* and of *S. aureus*.

Dilutions

Each dilution is made with a fresh sterile 10 ml pipette; to a bottle containing 50 ml T.S. is added 50 ml of the mother suspension. The mixture is carefully shaken 20 times. A 1:10 dilution of the dried product is thus obtained (the mother suspension being a $^1/_5$ dilution).

Another sterile bottle containing 90 ml of T.S. receives 10 ml of the preceding 1:10 dilution. It is carefully shaken 20 times. A 1:100 dilution of the dried product is then obtained.

A third bottle like the previous one receives 10 ml of the 1:100 suspension; it is mixed as before and provides a 1:1000 dilution.

'Total count' (Count of viable aerobic mesophilic microorganisms)

June 1973 – Original Text in English

A. Media

Tryptone yeast extract agar (T.L.)

Tryptone	6 g
Yeast extract	3 g
Agar	15 g
Distilled water	1000 ml
pH	7.0–7.2

Dissolve the 3 ingredients in distilled water by gentle heating. Adjust the pH. Dispense in 50 ml volumes into 100 ml screw capped bottles or 100 ml volumes into 200 ml bottles.

Sterilize in the autoclave at 121°C for 20 min. Store the medium in a refrigerator at +4°C; its maximum storage time is 1 month at most.

Non-nutritive agar:

Agar	15 g
Distilled water	1000 ml
pH	7.0–7.2

Dissolve the agar in the distilled water by heating. Adjust the pH. Dispense in 100 ml volumes into 200 ml screw capped bottles. Sterilize in the autoclave at 121°C for 20 min.

B. Procedure

1 ml of the 10^{-2}, 10^{-3} dilutions and 0.1 ml of the 10^{-3} dilution are placed into glass or plastic Petri dishes (85–100 mm diameter), using different pipettes for each dilution.

Within 5 min, 15–20 ml quantities (according to the size of the dish) of T.L. agar are poured into each dish, the agar having been melted in a boiling water bath and then cooled to 48–50°C. To improve to some degree the accuracy of this counting procedure it is essential to inoculate two Petri dishes with each of the dilutions. Two dishes receive 1 ml of the dilution 10^{-2}, 2 others 1 ml of the dilution 10^{-3}, etc.

The dilution of soup and the agar are carefully mixed. The plates are allowed to cool on a perfectly flat cool surface. When completely set, a layer of 2–3 mm thickness of non-nutritive agar is poured onto the surface of each dish. To do this, the non-nutritive agar is melted in a boiling water bath and then cooled to 48–50°C.

The dishes are once again left on a flat horizontal surface until the upper layer of agar has set. They are then inverted and incubated at 30 ±1°C for 72 hours.

The colonies are counted after these 72 hours (±2 hr) in each of the dishes containing at least 30 and at the most 300 colonies. If necessary, a magnifying glass (magnification ×6 to ×8) is used. The dilution at which the selected dishes were made is recorded; to obtain the number of viable aerobic mesophilic microorganisms in 1 g dried soup, the number found is multiplied by this dilution. It must not be forgotten that the last dish of the series

contains 0.1 ml of the 10^{-3} dilution, corresponding finally to a dilution of 10^{-4} per ml.

Example:

1 dish containing 1 ml of the 1:100 dilution supports 500 colonies. That containing 1 ml of the 1:1000 dilution has 70 colonies. Only the latter count is used, i.e. it is recorded that 1 g of the dried soup contains $70 \times 1000 =$ 70,000 viable aerobic mesophilic microorganismus.

COUNT OF ESCHERICHIA COLI

October 1975 – Original Text in English

A. MEDIA

Lactose glutamic acid broth (L.G.A.)

Sodium glutamate	12.7 g
Lactose	20.0 g
Sodium formate	0.5 g
L-cystine	0.04 g
L(−)Aspartic acid	0.048 g
L(+)Arginine	0.04 g
Thiamine	0.002 g
Nicotinic acid	0.002 g
Pantothenic acid	0.002 g
Magnesium sulphate ($MgSO_4 \cdot 7H_2O$)	0.200 g
Ferric ammonium citrate	0.020 g
Calcium chloride	0.020 g
Dipotassium hydrogen phosphate	1.80 g
Bromcresol purple	0.020 g

Dissolve 17.7 g in 1 litre of distilled water containing 2.5 g ammonium chloride. Adjust the pH to 6.7. Dispense in 10 ml volumes into 160 × 16 mm tubes containing Durham tubes.

Sterilize with autoclave at 116 °C for 10 minutes.

Brilliant green lactose bile broth (B.L.)

Peptone	10 g
Lactose	10 g
Fresh beef bile or 10% solution of dehydrated bile,	200 ml
Distilled water	790 ml

Adjust the pH to 7.0–7.2 after dissolving the ingredients in water by gentle warming.

After cooling, add 13.3 ml of a 0.1% aqueous solution of brilliant green (e.g. Bacto brilliant green, Difco).

Dispense in 10 ml volumes into 160 × 16 mm tubes or 20 ml screw capped bottles containing Durham tubes.

Sterilize in the autoclave at 121°C for 20 min.

To standardise the preparation, a commercial dried preparation of Brilliant Green Lactose Bile Broth (2% broth) (e.g. Difco B7 or Oxoid CM 31) may be used.

Peptone water (E.P.):

Tryptone	10 g
NaCl	5 g
Distilled water	1000 ml
pH	7.0–7.2

Dissolve the peptone and the salt in the distilled water by gentle warming. Adjust the pH. Dispense in approximately 10 ml volumes into 160×16 mm tubes or 20 ml screw capped bottles.

Sterilize in the autoclave for 20 min at 121°C

B. PROCEDURE

1 ml of the 1:10 dilution is inoculated into each of 3 tubes containing Durham tubes and 10 ml of lactose glutamic acid broth (L.G.A.). The pipette is rinsed by drawing up the liquid from the tube or bottle a few times. During the manipulations the introduction of air bubbles into the Durham tubes is to be avoided.

Using different 1 ml pipettes, 1 ml of each of the 10^{-2} and 10^{-3} dilutions is inoculated into each of 3 tubes containing 10 ml L.G.A. broth. The pipette is rinsed as previously during the course of the inoculation.

All the tubes thus inoculated are incubated at 30±1°C for 48 h (±1 h).

Tubes which show gas in the Durham tube (approx. $^1/_{10}$ of its total volume) are examined for *E. coli* which ferments lactose with production of gas in B.L. incubated at 44.0°C for 48 h, and produces at the same time indole from peptone water incubated in the same way.

Each tube which contains gas in the Durham tube is carefully shaken.

A loopful of its contents (platinum or nichrome wire formed into a 5 mm loop) is then transferred to a tube containing 10 ml B.L. An identical loopful is transferred immediately afterwards to a tube containing 10 ml peptone water.

The tubes thus inoculated are immediately placed in a water bath with an accurate thermostat at a temperature of 44.0±0.1°C for 48 h. The presence or absence of gas in the Durham tubes of the B.L. is then noted, and the peptone water tubes examined for indole.

Where subculture from a gas producing tube from the 30°C incubation gives rise to both indole production from peptone water and gas from B.L. at 44°C, it is recorded as positive for *E. coli* and the number of *E. coli* present in the original sample is determined from Table 1.

Test for indole

Add to the peptone water culture 3–4 drops of nitrous-nitric acid (nitric acid with sufficient crystals of sodium nitrite added to obtain nitrous fumes) then 2–3 ml of iso-amyl alcohol. Mix and let it stand. The presence of indole is shown by the immediate appearance of a pink or red colour in the upper amyl alcohol layer.

Table 1

Most Probable Number (MPN) of organisms per gram sample using three tubes each inoculated with 1 ml of 10^{-1}, 10^{-2} and 10^{-3} dilutions of the sample.

MPN/g (ml)
3×0.1; 3×0.01; 3×0.001 g (ml)

Result			MPN	Category 1	Category 2	Confidence limits 99%		Confidence limits 95%	
0	0	0	< 3						
0	1	0	3		*	< 1	23	< 1	17
1	0	0	4	*		< 1	28	1	21
1	0	1	7		*	1	35	2	27
1	1	0	7	*		1	36	2	28
1	2	0	11		*	2	44	4	35
2	0	0	09	*		1	50	2	38
2	0	1	14		*	3	62	5	48
2	1	0	15	*		3	65	5	50
2	1	1	20		*	5	77	8	61
2	2	0	21	*		5	80	8	63
3	0	0	23	*		4	177	7	129
3	0	1	40	*		10	230	10	180
3	1	0	40	*		10	290	20	210
3	1	1	70	*		20	370	20	280
3	2	0	90	*		20	520	30	390
3	2	1	150	*		30	660	50	510
3	2	2	210		*	50	820	80	640
3	3	0	200	*		<100	1900	100	1400
3	3	1	500	*		100	3200	200	2400
3	3	2	1100	*		200	6400	300	4800
3	3	3	>1100						

Category 1: Normal results, obtained in 95% of cases.
Category 2: Less probable results, obtained only in 4% of cases, that should not be employed in case of important decisions.

Results not mentioned in the table are always unacceptable, because they are even less probable than those of category 2.

This table has been calculated according to J.C.de Man 'The probability of most probable numbers' European J. Appl. Microbiol. 1, 67–78 (1975).

Examination for Salmonella

June 1973 – Original Text in English

The simplest procedure which gives satisfactory results is to use pre-enrichment in mannitol broth, enrichment in selenite broth, plating on brilliant green agar, and subsequent confirmation of suspect *Salmonella* colonies (Kliglers iron agar, serology, etc.). However, alternative methods after pre-enrichment, such as enrichment in tetrathionate broth with novobiocin followed by plating on desoxycholate citrate agar also give satisfactory results, and the combined use of several methods may result in the detection of more *Salmonella*–positive samples. These methods are also included here in case a more elaborate system is required.

A. Proposed procedure

a) Media

Double strength mannitol broth (M.B.)

Mannitol	5 g
Meat extract	1 g
Proteose peptone No. 3	10 g
Sodium chloride	5 g
Bromocresol purple (1.6 % alcoholic solution)	1 ml
Distilled water	500 ml

Dissolve the ingredients (except the bromocresol purple) in water by gentle heating add the bromocresol purple: adjust the pH to 7.0–7.2. Fill 50 ml quantities into 100 ml screw capped bottles. Autoclave for 20 min at 121°C

Selenite broth

Tryptone	5 g
Lactose	4 g
Anhydrous disodium hydrogen phosphate	10 g
Acid sodium selenite	4 g
Distilled water	1000 ml
pH	7.0

Dispense in 10 ml volumes into 16 × 160 mm tubes (or into screw capped 1 oz bottles). Do not autoclave. Heat in flowing steam for 30 min.

Brilliant Green agar (B.G.)

Yeast extract	3 g
Proteose Peptone No. 3	10 g
Sodium chloride	5 g
Lactose	10 g
Saccharose	10 g
Phenol Red	0.08 g
Brilliant Green	0.0125 g
Agar	20 g

Suspend 58 g in 1000 ml distilled water and heat to boiling to dissolve the medium completely. Distribute in bottles and sterilise for 15 min at 121° C. Do not overheat. Store in a dark place.

Kliglers Iron Agar (K.I.)

Beef extract	3 g
Yeast extract	3 g
Peptone	15 g
Proteose peptone	5 g
Lactose	10 g
Dextrose	1 g
Ferrous sulphate	0.2 g
Sodium chloride	5 g
Sodium thiosulphate	0.3 g
Agar	12 g
Phenol red	0.024 g

Suspend 55 g of the dehydrated medium in 1000 ml distilled water and heat to boiling to dissolve the ingredients completely. Distribute in bottles or tubes, sterilise at 121°C for 15 min, and allow to set on the slant so that there is a butt of about 2.5 cm and a slope of about 2.5 cm also.

B. Procedure

Pre-enrichment: 100 ml of the mother suspension (=25 g sample) is added to 100 ml double strength mannitol broth in a 500 ml screw capped bottle. The mixture is incubated for 18–24 h at 37°C. If the indicator retains its blue colour, the test is probably negative; if it becomes yellow, there is a culture of mannitol positive bacteria. In either case, subculture 0.5 ml quantities into selenite broth. Incubate for 18–24 hours at 37°C.

From each of the enrichment broths, make a subculture onto Brilliant Green Agar. Use a platinum loop and streak to give isolated colonies. The petri dishes containing the isolation media are incubated at 37°C for 18–24 h (not longer). The enrichment cultures are re-incubated at the same time for a further 18–24 h and subcultured again onto Brilliant Green Agar if there are no *Salmonella* colonies on the first streak plates.

Suspect *Salmonella* colonies are pinkish and surrounded by reddening of the media. These should be picked into Kliglers medium, on which *Salmonella* have the following appearance (like some other *Enterobacteriaceae*):

The culture does not spread on the slope
Red slope—lactose negative
Yellow butt—glucose positive—generally blackened by production of H_2S
Gas production.

Such culture is suspected of being a *Salmonella*. After purification, examine serologically with polyvalent *Salmonella* O and H antisera. If positive, send to *Salmonella* typing laboratory for identification of serotype.

Results

If an isolation of *Salmonella* is made, record as *Salmonella* present in 25 g sample, otherwise *Salmonella* absent in 25 g.

B. Alternative procedures

After pre-enrichment has been done as described under A tetrathionate broth can be used for enrichment, under the same conditions as selenite broth.

Cultures from either of these media may be plated onto desoxycholate-citrate-lactose agar.

a) Media

Tetrathionate broth (M.K.) with novobiocin

A) Meat extract 1 g
Yeast extract 2 g
Peptone 5 g
Sodium chloride 5 g
Sodium thiosulphate 40.7 g
Distilled water 1000 ml

Dissolve the ingredients in water by gentle heating. Autoclave at 115°C for 20 min.

B) Calcium carbonate 25 g
Autoclave at 121°C for 20 min

C) Iodine 6 g
Potassium iodide 5 g
Distilled water 20 ml

Grind in a mortar. The solution is not sterilised.

A), B) and C) are prepared separately. Onto powder B) in a 1500 ml or 2000 ml flask, pour first solution A) and then solution C). After mixing manually, dispense in 10 ml volumes into 16 × 160 mm tubes. The base thus obtained may by stored at 4°C for not more than 15 days.

D) A solution of novobiocin in distilled water is added aseptically when the medium is used, to give a final concentration of 40 μg/ml novobiocin in the base. (Novobiocin is obtainable from Théraplix, 98 Rue de Sèvres, Paris 7).

Desoxycholate-citrate-lactose (D.C.L.) agar

1. Dissolve 20 g of meat extract in 200 ml of distilled water. Boil, adjust to pH 7.3. Filter through paper, and collect the filtrate in a flask containing 20 g proteose peptone. Dissolve the peptone and sterilise at 121°C for 20 min.

2. Dissolve 90 g agar in 4000 ml distilled water. Filter hot (in flowing steam in the autoclave) through filter paper. Add 1) to 2), then add 5 ml of a 2% solution of neutral red, and 40 g lactose. Fill in accurate 100 ml quantities in screw capped bottles. Sterilise by heating first in flowing steam for 1 hour and then at 115°C for 10 min.

When using the medium, melt the agar base in a boiling water bath. Add then to each bottle 5 ml of each of solutions A and B below, using different pipettes for the two solutions. Mix between each addition, and pour into Petri dishes.

Solution A:	Sodium citrate	17 g
	Sodium thiosulphate	17 g
	Iron ammonium citrate	2 g
	Sterile distilled water	100 ml

Do not sterilise. Dissolve by gentle heating, and leave standing at room temperature for 24 hours.

Solution B:	Sodium desoxycholate	10 g
	Sterile distilled water	100 ml

Do not sterilise. Dissolve by gentle heating.

The mixing of solutions A and B with the agar base must be made quickly. When the medium is poured into petri dishes, it must be cooled rapidly.

Disregard of the precaution gives a soft agar which it is difficult to inoculate correctly.

Procedure using desoxycholate-citrate-lactose agar

If there is growth on the desoxycholate-citrate-lactose agar, mark carefully (with the help of a magnifying glass, $\times 8$) the different sorts of colourless colonies (lactose negative), with or without a black centre. Where they are well isolated, pick at least two of each kind of colony with the aid of a straight wire into Kliglers medium, making a central streak on the slope and stabbing into the butt. If the colonies are not well isolated, a re-streaking is necessary. Desoxycholate-citrate-lactose agar plates may be re-examined after a second period of incubation of 18–24 h at 37°C, and any further suspect colonies treated as previously.

Count of Staphylococcus aureus

June 1973 – Original Text in English

a) Medium

Baird-Parker's agar (E.T.G.P.A.)

Prepare the following basal medium:

Tryptone	1.0 g
Meat extract	0.5 g
Yeast extract	0.1 g
Lithium chloride (hydrated)	0.5 g
Agar	2.0 g
Distilled water	100 ml

The above ingredients are dissolved in the distilled water by steaming, and the pH of the medium adjusted to 6.8. A 0.2% w/v stock solution of the sodium salt of sulphamezathine is prepared by dissolving 0.5 g of pure sulphamezathine (Imperial Chemical Industries) in 25 ml of *N*/10 NaOH and making up to 250 ml with distilled water. 27.5 ml of this solution are added to each 1 litre of base. The medium is dispensed, unfiltered, in 90 ml amounts and sterilised by autoclaving at 121°C for 15 min; the pH after autoclaving should be 6.8–7.0.

For use of the medium the following 4 pre-warmed Seitz filtered solutions are added to 90 ml of the molten base held at 45–50°C.

1)	20% (w/v) solution of glycine (Hopkins and Williams Ltd)	6.3 ml
2)	1.0% (w/v) solution of potassium tellurite (British Drug Houses B.D.H.; not less than 97% pure when assayed oxidometrically)	1.0 ml
3)	20% (w/v) solution of sodium pyruvate (L. Light & Co., Colbrook, Bucks.)	5.0 ml
4)	Egg Yolk Emulsion (e.g. Oxoid New Improved)	5.0 ml

After the above additions, contents of bottles are rapidly mixed and poured immediately into Petri dishes; 10–15 ml are poured into each Petri dish.

Storage of Medium

The basal medium can be stored for several months at room temperature in screw capped bottles without loss of selectivity or increased toxicity to *Staph. aureus*. Stock solutions of 20% glycine and 1% tellurite can be stored for a similar period at room temperature, but the sodium pyruvate is best stored at 5°C and replaced every month.

Poured plates of the complete medium cannot be stored satisfactorily either at room temperature or 5°C and should be, therefore, freshly prepared and used, preferably within 24 hours of pouring and not after longer than 48 hours storage.

Alternatively, a commercial version of the medium, e.g. as supplied by Oxoid or B.B.L. may be used.

Use of Medium

Plates should be well-dried by incubation at 37°C for 1–2 hours, or by use of some similar procedure.

b) Procedure

Replicate 0.1 ml amounts of the mother suspension are spread on the surface of 2 Petri dishes containing Baird-Parker agar with sulphamezathine, and incubated for 24–48 h at 37°C. This represents a dilution on the plate of 1 in 50.

Small, convex, shiny, intensely black colonies with a narrow white margin surrounded by a zone of clearing of the medium are presumptive pathogenic staphylococci. (The zone of clearing may be difficult to observe due to the particulate matter from some soups).

Typical colonies are confirmed as follows:

(i) Gram stain : Gram positive cocci
(ii) Coagulase test : positive

Remove a whole colony with an inoculating wire and suspend it in 0.1 ml of heart infusion broth. This is then incubated for one hour at 37°C and then 0.3 ml of rabbit plasma added and re-incubated for a further 2–4 hours; any degree of coagulation of the plasma is recorded as positive coagulase activity.

Catalase negative faecal streptococci which grow in the medium as black colonies *not* surrounded by clear zones can cause false clotting of citrated rabbit plasma unless this contains 0.1% E.D.T.A. (ethylene diamine tetracetic acid). Therefore such colonies should be tested for catalase by spotting with 3% hydrogen peroxide from a Pasteur pipette before proceedsing with the coagulase test; *S. aureus* produces catalase.

Results

If there are no colonies of *S. aureus* on the plates the sample contains < 25/g. Where present, $50 \times$ the average number of colonies present on the two plates gives the estimated number of *S. aureus* per gram.

Count of spores of Clostridium perfringens

June 1973 – Original Text in English

a) Media

Brain heart infusion agar with sulphite and iron

Dissolve 52 g of dehydrated Brain Heart Infusion Agar (e.g. Difco B 418) in 1000 ml distilled water, fill in 20 ml quantities into 200 × 20 mm tubes, and autoclave for 15 min at 121°C.

When required for use, melt the medium in a boiling water bath, cool to 55°C and add 1 ml sodium sulphite solution (6.25 g $Na_2SO_3 . 7H_2O$ in 100 ml distilled water) and 4 drops of 5% ferric citrate solution to each 20 ml tube of medium. It is not normally necessary to sterilise these two solutions, but sterile water should be used for their preparation.

Modified Willis and Hobbs' medium

Stir 33 g of dehydrated azide blood agar base (e.g. Difco B 409 or Oxoid CM 259), 10 g of lactose and 0.03 g of neutral red into 1000 ml cold distilled water, heat to boiling to dissolve and distribute in 20 ml quantities in 200 × 20 mm tubes and autoclave for 15 min at 121°C. When required for use; melt, cool to 45–50°C, add 10% egg yolk suspension (equal parts egg yolk and tryptone salt solution) and pour into Petri dishes. After cooling, spread 3 drops *Cl. perfringens* Type A antiserum on half of each plate.

b) Procedure

Heat treatment to kill vegetative cells

Aliquots of a 1:10 dilution, of approx 7 ml in each of two sterile tubes, are held in a water bath at 80°C for 10 min and then cooled immediately under the tap. Care must be taken that the sides of the tube are not touched when pipetting the sample in, and that none of the sample is above water level in the bath. An estimation should be made of the time it takes for the contents of the tubes to reach 80°C and this should be added to the 10 minute holding time at 80°C.

Inoculation of culture medium

4 tubes of melted brain heart infusion agar (containing iron and sodium sulphite) at 45° C are inoculated as follows:

2 tubes each receive 5 ml heated suspension
1 tube receives 1.0 ml heated suspension + 4 ml tryptone salt
1 tube receives 0.1 ml heated suspension + 5 ml tryptone salt

The tubes are mixed by rolling in the hands without aeration, and are cooled under running tap water.

Incubation

At 44°C, preferably in a water bath.

Counting

Count after 18, 24 or 48 h incubation as appropriate (if left too long, the whole tube may blacken). The *Cl. perfringens* count can be estimated from the number of confirmed colonies.

Confirmation of colonies as Cl. perfringens

Cut the tube near the black colonies to be examined and streak all, or a significant proportion of the colonies onto plates of the modified Willis and Hobbs' medium one half of which has been spread with *Cl. perfringens* Type A antiserum. Streak the organisms from the plain to the antiserum treated side of the plate; several colonies can be streaked on the same plate. Incubate anaerobically at 37°C for 18–24 hours. All strains forming acid from lactose and a lecithinase which is inhibited by the Type A antiserum are identified as *Cl. perfringens*.

SENSORY ASSESSMENT OF PRODUCTS OF THE SOUP INDUSTRY

8

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SENSORY ASSESSMENT OF PRODUCTS OF THE SOUP INDUSTRY

8

October 1983 – Original text in English
Revised 1989

1. SCOPE

Procedures are described for the evaluation of appearance, consistency, odour and flavour for discriminative, preference and acceptability tests.

N. B. The paper does not contain an account of statistical evaluation. References are given to the most appropriate procedures and relevant literature in Section 11.

2. FIELD OF APPLICATIONS

Meat extract
Bouillons and meat bouillons
Seasonings (protein hydrolysates)
Soups and related products
Raw materials

3. DEFINITIONS

3.1 The quality attributes that are contributing to the overall character of the product are described and, if necessary, rated according to the intensity rating scale (3.5.1)

3.2 In the quality rating test, the product is rated according to the product quality scale (3.5.2).

3.3 In the preference test (8.2.5) the product acceptance is rated by means of the hedonic scale (3.5.3).

3.4 Quality attributes

3.4.1 Appearance

3.4.1.1 Overall

3.4.1.2 Detailed

- surface
- liquid phase
- solid phase (garnish)

3.4.2 Rheological properties – texture

3.4.2.1 Consistency of liquid phase

3.4.2.2 Consistency of solid phase (garnish)

3.4.3 Odour

3.4.4 Taste

3.4.5 Mouthfeel

3.4.6 After-taste

3.5 Rating scales

3.5.1 Intensity scale

0 none
1 weak
2 moderate
3 strong
4 very strong

3.5.2 Quality rating scale

7 excellent
6 very good
5 good
4 satisfactory
3 poor
2 very poor
1 extremely poor

3.5.3 Hedonic scale

9 like extremely
8 like very much
7 like
6 like slightly
5 neither like nor dislike
4 dislike slightly
3 dislike
2 dislike very much
1 dislike intensely

4. PRINCIPLES OF THE SENSORY ASSESSMENT

4.1. Planning

Before planning experiments using sensory testing methods, the problem under investigation should be considered carefully in order to define the information sought. An appropriate method of testing should then be selected, having regard to the degree of precision deemed necessary. Where many samples are concerned, it may be desirable to assess them at more than one session because of limitations such as fatigue.

Consideration should be given to appropriate statistical experimental design, for disregard of design may lead to bias and certainly to inefficiency. The special measures which are used in sensory testing, such as the controlled environment and conduct of testing, must be carefully observed to avoid biased results and faulty decisions.

4.2 Preparation

The sample is to be prepared as described in paragraph 9, and its quality attributes (3.4) are to be rated by means of the rating scales (3.5), using one of the methods described in paragraph 8.

5. EQUIPMENT

5.1 Facilities for sample preparation (electric heating plates, water baths, etc.)

5.2 Measuring vessels, cooking pans, covered pots, whisks, ladles and spoons made of corrosion-proof metal for preparing samples (9).

5.3 Glass and/or porcelain containers with lid for intermediate storage of fully prepared samples.

5.4 Plates and cups of similar material and spoons made of glass, porcelain, plastic or stainless steel as well as disposable equipment. Utensils must not impart any flavour to the food.

5.5 Forms for recording results: see Appendices 1 to 5.

6. TASTING ROOM

An environment conducive to concentration and the prevention of distractions, including sample preparation, is most important.

The room should

- offer a pleasant atmosphere,
- be well lit by daylight lamps,
- be equipped with coloured lighting to mask the influence of product (colours), as necessary
- have walls in neutral colours,
- be easy to clean,
- be free from foreign odours, noise and other distracting factors.

If possible, the room should be divided into

- an area for sample preparation

  equipped with hot plates and water baths (5.1) and facilities for washing dishes, etc. and disposing of leftovers,

- an area for sensory evaluation

  with separate tasting booths or tables equipped with separating walls (panel members should not sit opposite each other) and equipped with facilities for rejection of test material.

7. ASSESSORS

7.1 Trained assessors

Discriminatory assessments should only be made by individuals who have been selected and trained by means of appropriate methods (see references: 11).

Factors which should be considered in selecting individuals to serve on sensory evaluation panels include availability, good health and motivation. The method of selection should include a preliminary training period designed to acquaint assessors with the quality factors involved in the product. They should then be selected for their sensitivity and consistency in evaluating a product using a replica of actual test conditions. More assessors should be selected for further training than are needed for a given panel to allow for normal absence.

A procedure which may be used to select assessors with special sensitivity, e.g. for a specific off-flavour, involves the presentation to the assessor in a random order of four identical samples of product and four having the difference it is required to be distinguished. The probability of identifying the two sets of four samples correctly by chance alone is 2.9%.

Subsequent participation should be on the basis of random selection. However, the composition of a panel should preferably be constant throughout the same series of tests.

It is recommended that the performance of individuals should be frequently inspected to monitor changes in ability.

7.2 Non-trained assessors

Training is contrary to the purpose of consumer tests. Ability to discriminate is not a valid criterion for this type of assessment and selection should be in respect of representativeness of some consumer population. Consideration should be given to factors such as age, sex, membership of social groups and eating habits.

8. METHODS

Testing and evaluation are to be performed according to the principles outlined in paragraph 4.

8.1 Evaluation of an individual sample

Evaluation of an individual sample can be carried out using any scales given in paragraph 3.

8.1.1 Product description

A qualitative description of the individual attributes which contribute to the overall character of the product.

Trained assessors make an independent appraisal of the product by selecting from a systematically arranged list of potential quality attributes and descriptions which are apposite to the product. The following scale of intensity is used:

0 none
1 weak
2 moderate
3 strong
4 very strong

A group discussion of the preliminary observations follows and adjustments made in order to obtain a consensus.

Quality attributes

1. Appearance

   1.1 Overall

   1.2 Detailed

   - surface
   - liquid phase
   - solid phase (garnish)

2. Rheological properties – texture

   2.1 Consistency of liquid phase

   2.2 Consistency of solid phase (garnish)

3. Odour

4. Taste

5. Mouthfeel

6. After-taste

A table of Descriptive Terms according to the above classification is given in Appendix 6.

8.1.2 Product quality score

The product is classified according to the following quality rating scale:

7 excellent
6 very good
5 good
4 satisfactory
3 poor
2 very poor
1 extremely poor

Trained assessors appraise the product in relation to those characteristics which are agreed to be important to consumers.

Expression of results: see 10.2.

8.1.3 Acceptability rating

The product is classified according to the following hedonic scale:

9 like extremely
8 like very much
7 like
6 like slightly
5 neither like nor dislike
4 dislike slightly
3 dislike
2 dislike very much
1 dislike intensely

A panel of 60 or more untrained assessors, suitably selected to be representative of the consumer population is used.

This examination may be made under domestic conditions as an alternative to the use of a controlled environment, provided that the full test instructions are suitably explicit.

Expression of results: see 10.2.

8.2 Comparison of two samples

8.2.1 Establishment of a difference

A 'forced choice' method is applied to determine sensory differences:

- Comparison of pairs (see Appendix 3)
- Triangle test (see Appendix 4)
- Duo-Trio test (see Appendix 5)

There are two distinct risks involved in using these tests:

Type I (known also as 'error of the first kind') when the samples are stated to be distinguishable when in fact they are not, and

Type II (known also as 'error of the second kind') when the samples are stated to be not distinguishable when in fact they are.

Only the 'first mentioned risk of error' can easily be taken into account in practise; it is the significance level chosen and is usually indicated in statistical tables.

It is necessary to select the conditions of the test with care, having regard to its objective. Frequently there must be a compromise between that which is desired and that which can be achieved in practice.

For example, in a triangle test where it is necessary to know whether two products can be differentiated by 10% of the population, it may be calculated that with 'errors of the first and second kind', each set at 5%, at least 550 assessments are required.

Smaller panels may be used if selected and trained assessors of better acuity are used. In the case above, a panel so selected that one half is able to discriminate between the products need be no more than 22 with the same risk of the two kinds of error as before. Smaller panels may also be used in situations where it is possible to accept higher risks of error.

Examples which illustrate the selection of test conditions are as follows:

a) In considering whether a replacement of an expensive ingredient by a cheaper one will lead to a significantly different product, it is necessary to minimize 'the error of the second kind', but unless 'the error of the first kind' is limited, no decisions would ever be taken involving a change in the product.

   A 90% or 95% significance level would be appropriate.

b) In investigating whether a high quality but expensive ingredient is capable of affecting a product, it is necessary to limit severely an error of the first kind and consequently accept a high risk of finding no difference when in fact there is one.

   A 99% or even 99,9% significance level would be selected and it would also be usual to establish whether any difference is detected using procedure 8.2.5.

8.2.2 Establishment of a directional difference of a defined criterion ('one-sided' or 'two-sided' test)

When it is necessary to determine whether or not there is a difference in sensory properties between two products in respect of some specific quality which can be clearly defined, a 'paired comparison test' employing trained assessors is used.

Before conducting the test, it should be carefully determined whether the test is going to be a 'one-sided' (one-tailed) or 'two-sided' (two-tailed) one. The corresponding statistical table for the interpretation of the result will have to be used.

As regards the sidedness of the test, the following may serve as explanation:

a) When it is necessary to know whether a soup with a salt level of 10 g/l, the test is 'one-sided' because it is not expected that the latter soup can be the saltier one.

b) When it is necessary to know which of two different meat extracts, tested at an equal cost-price level, gives a meatier soup, the test will be 'two-sided'.

   When, on the other hand, it is necessary to know whether a more expensive meat extract gives a meatier soup than a less expensive one (both tested at equal concentration), the test is 'one-sided' as the more expensive meat extract is only accepted when it gives a significantly meatier product.

In cases of doubt whether the test is 'one-sided' or 'two-sided' it is advised that a statistician be consulted. Where such advice cannot be sought, then the 'two-sided' test should be selected.

8.2.3 Characterisation of a difference between two samples

The technique of product description 8.1.1 is applied to each sample separately, followed by directional difference tests of selected criteria referred to in 8.2.2 ('one-sided' or 'two-sided' test).

8.2.4 Establishment of the degree of difference between two samples

The difference may be a defined attribute or general quality. Each sample is evaluated using the product quality score described under 8.1.2, and the results examined statistically for significance.

8.2.5 Establishment of a preference between two samples

A panel of untrained assessors (7.2) rates the samples according to the hedonic scale (8.1.3) using the design form in Appendix 1. The significance of the results is obtained statistically.

The samples can also be directly compared by means of 'comparison of pairs' (8.2.1) whereby questions like,

- is there any difference?
- if so, which sample do you prefer?

can be answered.

Before conducting the test it is to be determined whether a 'one-' or 'two-sided' test is concerned.

Examples:

- Preference between two products in a consumer test = 'two-sided test'.
- Preference between two different recipes of identical cost = 'two-sided test'.

But:

- If new ingredients or new recipes leading to higher cost of the product are to be tested, a 'one-sided' test needs to be performed, because the new (more expensive) product will be accepted only if the preference is significant.
- If a recipe is to be tested which will result in a cheaper product, a 'one-sided' test is also required because the cheaper product will not be accepted, if the preference of the standard product is significant.

In the last example, the 'errors of the second type' need to be kept as small as possible since a wrong decision in favour of the cheaper product could lead to serious damage to the product.

Therefore, the panel should be as large as possible. (If this cannot be realized, a bigger 'error of the first type' should be accepted.)

In addition to determining a preference, each untrained assessor (7.2) should be asked to indicate which product description definitely fits the preference expressed by him.

In doubtful cases a statistician should always be consulted.

8.3 Comparison of more than two samples

8.3.1 Establishment of a variation between several samples

Each sample is given a quality rating in accordance with 8.1.2. The variation between samples is tested statistically for significance for example by the chi-squared test (χ^2).

8.3.2 Establishment of a rank order

Three or more samples submitted at the same time are arranged in order of some specified attribute by trained assessors (Appendix 2), and the ranking orders analyzed statistically.

8.3.3 Establishment of an order of preference

As with 8.3.2 but arrangement is made in order of preference. Untrained assessors may be used.

9. SAMPLES AND SAMPLE PREPARATION

9.1 Sample preparation

The sample is made according to the directions of use, taking the effective weight of the product in the package.

Commonly available drinking water without foreign odour, colour, etc., can be used generally. It should be noted that the hardness of the drinking water may influence the sensory properties of the product to be tested.

Products to be tasted hot are transferred from the cooking pans into covered containers for intermediate storage. They are kept at about 70 °C during testing. Ensure that the intermediate storage time is kept to a minimum having regard to the circumstances and type of product under examination.

The samples should be coded in such a way as to avoid giving information to the assessors. Assignment of code markings by some method which ensures randomness is essential. One recommended procedure is to use three digits selected from a table of random numbers. Another method is to use signs with no suggested sequence.

9.2 Test portions

The intermediate storage containers should not be used for communal samples. Each assessor must receive his own set of sample which

can be taste tested undisturbed at his own place. The prepared sample must be stirred before serving to ensure proper distribution of any garnish.

Samples are served preferably in pre-heated plates or cups and tasted at a temperature between 60 and 65 °C for products which are normally consumed warm.

The amount of each sample should be the same since the quantity affects the rate of cooling.

10. PROCEDURE

10.1 Tasting technique

It is recommended that the period immediately before or after a meal should be avoided. Assessors should be asked to refrain from smoking for at least 30 minutes before a test. In the testing room, smoking is on principle not allowed. Similarly the excessive use of odourous cosmetics is undesirable.

Before tasting, the contents of the plate or cup should be mixed properly. The samples are assessed for the properties under investigation according to the instructions given under 8: Methods.

Where necessary, the liquid or the garnish may be examined separately. It is preferable that test material is rejected after tasting.

Assessors should be provided with neutralizing and rinsing agents to be used before and between each tasting test. White bread, cream crackers, milk, unsweetened black tea or still water, are most suitable.

In discriminatory assessment it is often desirable to test the samples for other characteristics when they differ in appearance.

Differences can be eliminated in various ways, such as the use of coloured lights or by the addition of colouring to the samples, providing the taste is not affected.

Differences in other irrelevant factors may be masked by appropriate means. For example, differences in texture or consistency can be eliminated by homogenisation.

10.2 Results

The results are entered on the forms (Appendices 1 to 5), using rating scales appropriate to the criteria.

Results should only be summarized as the frequency of classification in each category and not expressed mathematically.

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Appendix 1

QUESTIONNAIRE
ACCEPTABILITY TEST

Date: _____ Assessor: _____

Product: _____

Please indicate how much you like or dislike the sample. Use the scale below to indicate your attitude by checking at the point which best describes your feeling about it.

Code	No.	No.	No.	No.
like extremely	☐	☐	☐	☐
like very much	☐	☐	☐	☐
like	☐	☐	☐	☐
like slightly	☐	☐	☐	☐
neither like nor dislike	☐	☐	☐	☐
dislike slightly	☐	☐	☐	☐
dislike	☐	☐	☐	☐
dislike very much	☐	☐	☐	☐
dislike intensely	☐	☐	☐	☐

Comments: _____

_____

_____

_____

Appendix 2

QUESTIONNAIRE
ESTABLISHMENT OF RANKING ORDER

Date: _____ Assessor: _____

Product: _____

Please rank the samples in order of the intensity of _____

in increasing order.

☐	☐	☐	☐	☐	☐
slight					very strong

Comments: _____

_____

_____

_____

Appendix 3

QUESTIONNAIRE
PAIRED-COMPARISON TEST

Date: _____ Assessor: _____

Product: _____

Please indicate which sample you prefer for overall quality.

Please check one.

No.	

No.	

Comments: _____

_____

_____

_____

Appendix 4

QUESTIONNAIRE
TRIANGLE TEST

Date: _____ Assessor: _____

Product: _____

You are receiving three samples; two of these samples are identical and one is different.
Please indicate the odd sample.

No.	

No.	

No.	

Please indicate why the odd sample is different from the others:

_____

_____

_____

_____

Appendix 5

QUESTIONNAIRE
DUO-TRIO-TEST

Date: _____ Assessor: _____

Product: _____

You are receiving three samples, from which one is the reference standard and one of the other samples is a duplicate of the reference.

Please indicate which sample is the same as the reference.

× = the identical sample

reference standard No.		sample No.		sample No.	

Please indicate why the other sample is different:

_____

_____

_____

_____

English	German	French	1. Colour Appearance: Overall	1. Colour Appearance: Surface	1. Colour Appearance: Liquid ph.	1. Colour Appearance: Solid ph.	2. Consistency Texture (rheol. properties): Liquid ph.	2. Consistency Texture (rheol. properties): Solid ph.	3. Odour	4. Taste	5. Mouthfeel/Texture	6. After-taste
acid	*sauer*	*acide, aigre*							x	x		x
acidulous	*säuerlich*	*acidulé*							x	x		x
acrid	*beissend*	*âcre*							x		x	
alkaline	*alkalisch*	*alcalin*								x	x	x
aromatic	*aromatisch*	*aromatique*							x	x		x
artificial	*künstlich*	*artificiel*	x						x	x		x
astringent	*adstringierend*	*astringent*									x	
balanced	*ausgewogen*	*équilibré*	x						x	x		
biscuit-like	*biskuitartig*	*biscuité*						x		x		
biting	*beissend*	*mordant, piquant*							x		x	
bitter	*bitter, herb*	*amer*								x		x
blended	*harmonisch*	*harmonieux*							x	x		
bouillon flavour	*Bouillon-geschmack*	*goût de bouillon*								x		
bound	*gebunden*	*lié*					x					
burning	*brennend, heiss*	*brûlant*								x		x
burnt	*verbrannt, brandig*	*brûlé (roussi)*							x	x		x
caramelized	*karamelisiert*	*caramélisé*	x		x				x	x		x
characteristic of	*charakteristisch*	*caractéristique de*	x			x	x		x	x	x	
chemical	*chemisch*	*goût/odeur chimique*							x	x		x
clear	*klar*	*clair, limpide*	x	x	x							
cloudy	*trübe*	*trouble*	x	x	x							
coloured	*farbig, gefärbt*	*coloré*	x		x	x						
colourless	*farblos*	*incolore*	x	x	x	x						

English	German	French	1. Colour Appearance: Overall	1. Colour Appearance: Surface	1. Colour Appearance: Liquid ph.	1. Colour Appearance: Solid ph.	2. Consistency Texture (rheol. properties): Liquid ph.	2. Consistency Texture (rheol. properties): Solid ph.	3. Odour	4. Taste	5. Mouthfeel/Texture	6. After-taste
creamy	*cremig, sahnig*	*crémeux, goût de crème*	x		x		x			x x	x	
creamy with fine particles	*sämig mit feinen Partikeln*	*grémillé*	x		x		x				x	
curdled	*geronnen*	*coagulé*	x		x		x				x	
diluted	*verdünnt*	*dilué*	x		x		x					
dull	*blind, matt, glanzlos*	*terne*		x								
earthy	*erdig*	*terreux*							x	x		x
faded	*entfärbt*	*décoloré*	x		x	x						
fatty	*fettig*	*gras*	x	x						x	x	
fermented	*fermentiert, gegoren*	*fermenté*							x	x		x
fermenting	*gärig*	*goût de (en) fermentation*							x	x		x
fibrous	*faserig, strohig*	*fibreux*				x		x			x	
fine	*fein*	*fin*							x	x	x	
firm	*fest*	*ferme*						x				
fish-oil taste	*tranig*	*goût d'huile de poisson*							x	x		x
fishy	*fischig*	*goût/odeur de poisson*							x	x		x
flat	*flach*	*plat*							x	x		
flocculated	*ausgeflockt*	*floculé*	x		x		x				x	
floury	*mehlig*	*farineux*								x	x	
fresh	*frisch*	*frais*	x			x			x	x		x
fruity	*fruchtig*	*fruité*							x	x		
full-bodied	*vollmundig*	*riche*								x	x	
gelatinous	*gallertartig*	*gélatineux*	x		x		x				x	

English	German	French	1. Colour Appearance: Overall	1. Colour Appearance: Surface	1. Colour Appearance: Liquid ph.	1. Colour Appearance: Solid ph.	2. Consistency Texture (rheol. properties): Liquid ph.	2. Consistency Texture (rheol. properties): Solid ph.	3. Odour	4. Taste	5. Mouthfeel/Texture	6. After-taste
glossy	*glänzend*	*brillant*		x								
gluey	*leimig*	*gluant*					x				x	
glutamate taste	*Glutamat-geschmack*	*goût de glutamate*								x		x
granulous, gritty	*körnig, griessig*	*granuleux*	x			x	x	x			x	
greasy	*schmierig*	*graisseux*	x	x						x	x	
harmonious	*harmonisch*	*harmonieux*							x	x		
harsh	*harsch, rauh*	*âpre, rêche*								x	x	
hay-like	*heuig*	*goût/odeur de foin*							x	x		
herby	*kräuterig*	*goût/odeur de fines herbes*							x	x		
hot	*brennend, heiss, scharf*	*brûlant, piquant*								x		x
hydrolysed protein flavour	*Würze, «nach Würze» (Speisewürze, Hydrolysat)*	*goût/odeur d'hydrolysat*							x	x		x
insipid	*fade, schal*	*insipide*							x	x		
irritant	*kratzig*	*irritant*									x	
juicy	*saftig*	*juteux*						x				
ligneous	*holzig*	*ligneux*				x		x			x	
liquid	*flüssig, dünn*	*liquide*	x		x		x				x	
long	*lang*	*long*					x				x	
lumpy	*klumpig*	*grumeleux*	x		x		x				x	
matt	*matt*	*mat, terne*		x								
meaty	*fleischig*	*goût de viande*								x		x
mellow	*weich*	*moëlleux, mou, tendre*						x			x	

English	German	French	1. Colour Appearance: Overall	1. Colour Appearance: Surface	1. Colour Appearance: Liquid ph.	1. Colour Appearance: Solid ph.	2. Consistency Texture (rheol. properties): Liquid ph.	2. Consistency Texture (rheol. properties): Solid ph.	3. Odour	4. Taste	5. Mouthfeel/Texture	6. After-taste
metallic	*metallisch*	*métallique*								×		×
mild	*mild*	*doux*								×		
mouldy	*schimmelig*	*moisi*	×	×					×	×		
musty	*muffig, dumpf*	*goût/odeur de renfermé*							×	×		×
oily	*ölig*	*huileux*	×	×						×	×	
pale	*blass*	*pâle*	×	×	×	×						
particular	*eigenartig*	*particulier*	×						×	×		
pasty	*teigig*	*pâteux*	×			×		×			×	
peculiar	*eigenartig*	*particulier*	×						×	×		
persistent	*anhaltend*	*persistant*							×	×	×	
pharmaceutical	*pharmazeutisch, Apotheke-*	*pharmaceutique*							×	×		×
pungent	*stechend, scharf*	*piquant*							×		×	
putrid	*faul, faulig*	*pourri*							×	×		×
rancid	*ranzig*	*rance*							×	×		×
raw	*roh*	*crû*								×		
rich	*kräftig*	*corsé, vigoureux*							×	×		
rotten	*faulig*	*pourri*							×	×		×
rounded-off	*abgerundet*	*arrondi*	×						×	×		
rubber taste/ ... of flavour	*Gummigeruch, -geschmack*	*goût/odeur de caoutchouc*							×	×		×
rubbery	*gummi-artig*	*caoutchouteux*						×				
salty	*salzig*	*salé*								×		×
savoury	*schmackhaft*	*savoureux*								×		

English	German	French	1. Colour Appearance: Overall	Surface	Liquid ph.	Solid ph.	2. Consistency Texture (rheol. properties): Liquid ph.	Solid ph.	3. Odour	4. Taste	5. Mouthfeel/Texture	6. After-taste
seasoned	*gewürzt*	*assaisonné, relevé*							×	×		×
short	*kurz*	*court (furtif)*					×				×	
slimy	*schleimig, dick*	*épais*			×		×				×	
smoke flavour	*Rauch- (Räucher-) Aroma/ Gechmack*	*goût/odeur de fumé*							×	×		×
smoked	*geräuchert*	*fumé*							×	×		×
smoky	*rauchig*	*fumé*							×	×		×
smooth	*glatt, sämig*	*lisse, velouté, onctueux*	×		×		×			×	×	
soapy	*seifig*	*savonneux*							×	×		×
soft	*weich*	*mou (molle), tendre, moëlleux*						×			×	
sour	*sauer*	*acide, aigre*							×	×		×
sourish	*säuerlich*	*aigrelet*							×	×		×
spicy	*würzig*	*épicé*							×	×		×
stale	*abgestanden, alt*	*pas frais, vieux (goût de)*							×	×		×
'starch paste'	*kleisterig*	*«colle d'amidon»*					×	×			×	
sticky	*klebrig*	*collant*					×	×			×	
stringy	*faserig*	*filandreux*				×		×			×	
strong	*kräftig, streng*	*vigoureux, corsé*							×	×		×
sweet	*süss*	*doux, sucré*								×		×
sweetish	*süsslich*	*douceâtre*							×	×		×
tallowy	*talgig*	*suiffé*								×	×	×
tender	*zart*	*tendre*						×			×	

English	German	French	1. Colour Appearance: Overall	1. Colour Appearance: Surface	1. Colour Appearance: Liquid ph.	1. Colour Appearance: Solid ph.	2. Consistency Texture (rheol. properties): Liquid ph.	2. Consistency Texture (rheol. properties): Solid ph.	3. Odour	4. Taste	5. Mouthfeel/Texture	6. After-taste
thick	*dick, schleimig*	*épais*				x		x			x	
thin	*dünn*	*mince, liquide*	x		x		x x	x			x x	
tough	*zäh*	*coriace*						x			x	
transient	*flüchtig*	*fugace, éphémère*							x			
turbid	*trübe*	*trouble*	x	x	x							
typical of...	*typisch von...*	*typique de ...*	x		x	x	x	x	x	x	x	
unbalanced	*nicht ausgewogen*	*manque d'équilibre*	x						x	x		
violet flavour	*Veilchengeschmack*	*goût de violette*							x	x		
viscous	*viskos, zähflüssig*	*visqueux*					x					
volatile	*flüchtig*	*volatil*							x			
watery	*wässerig*	*aqueux*	x		x		x			x	x	
weak	*schwach*	*faible*							x	x		
wine-like	*weinig*	*goût/odeur de vin (vineux)*							x	x		x
yeasty	*hefig, Hefe-Note*	*goût/odeur de levure*							x	x		x

ASSURANCE OF THE MICROBIOLOGICAL SAFETY OF DRY SOUPS AND BOUILLONS

September 1993 – Original text in English

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ASSURANCE OF THE MICROBIOLOGICAL SAFETY OF DRY SOUPS AND BOUILLONS

1. SCOPE

This document is intended to give guidance to dry soup manufacturers on the safe production of dry soups, dry bouillons, bouillon paste and semi-finished soup base. The term soups and bouillons used throughout this text is meant to represent all these product types. For bouillons and consommés a further product definition can be found in the AIIBP Code of Practice (1).

Dry soups and bouillons are manufactured by mixing a variety of dry ingredients which together will form the dry soup or bouillon mix.

The ingredients themselves are mostly dry like dry vegetables, spices, flour and starches, salt and seasonings but some may be added in the form of a paste like meat extract or as a liquid like fats and oil. They have in common that they are preserved by a low water activity due to a low moisture content. Thus they can be stored without loss of microbiological quality.

Dry soups and bouillons are neither pasteurized nor sterilized during the manufacturing process and they carry the unavoidable microflora of the ingredients used to compose the dry mix. Due to the low water activity of the finished products the microorganisms present will be unable to multiply and thus dry soups and bouillons commonly have a long shelf life of 1–2 years (or more), which shelf life is not restricted by microbiological factors.

The dry soups, however, should be properly packed and stored to be protected against ingress of moisture and chemical deterioration due to light, oxygen and elevated temperatures.

Proper selection and hygienic handling of ingredients can assure that no microorganisms in the final product are present in levels that are hazardous to the health of the consumer. The AIIBP has in 1992 updated its guideline values for those microorganisms that could lead to food poisoning or disease (2).

After cooking in water (regular soups) or reconstitution with boiling water (instant soups) the soups and bouillons will give the consumer an attractive convenience product, which commonly is to be consumed hot. This way of preparation represents a factor which contributes to the excellent safety record of industrial dry soup mixes.

2. HACCP AND GMP

Dry soups and bouillons should be manufactured under hygienic conditions applying **Good Manufacturing Practices (GMP).** The basic GMP rules are given in the General Principles of Food Hygiene recommended by the Joint FAO/WHO Codex Alimentarius Commission (3). Further hygiene requirements are given in the EEC Council Directive on the Hygiene of Foodstuffs (4).

The common GMP rules which are applicable to all food operations are not mentioned again in this document. Each dry mix plant should prepare a written document in which the general hygiene rules to be followed are recorded for its own specific operation. This should include a.o. personal hygiene, cleaning of plant and equipment, environmental hygiene, pest control and handling of waste.

The safety of foods is best assured by applying the principles of the HACCP **(Hazard Analysis Critical Control Point)** system (5), which aims to identify Critical Control Points and their control and monitoring requirements. This document is restricted to microbiological hazards.

Application of HACCP during the manufacture of dry soups and bouillons enables a producer to control known microbiological hazards and to manufacture products which meet the safety demands set by law or formulated by a professional body like AIIBP. A survey of official microbiological limits and AIIBP recommended specifications for dry soups and bouillons is given in the AIIBP article published in 1992 (2).

True application of HACCP is the starting point for the product safety of dry soups and bouillons and only a sporadic or even routine verification of the microbiological condition of finished goods can not replace the implementation of the concepts of HACCP.

This is equally valid for the traditional inspection of a plant operation to check compliance with **Good Manufacturing Practice (GMP).** Also this approach has many shortcomings that can be overcome only by an inspection based on the principles of HACCP.

The outcome of such a specific HACCP study for a given production facility should be recorded and implemented in the quality assurance procedures and GMP requirements for that plant.

This AIIBP document addresses the key elements of Quality Assurance (QA) which most dry mix operations are likely to have in common but it can not provide the detail and specificity of an actual Hazard Analysis study performed for an individual plant. It is recommended that such study is done for all dry soup and bouillon operations.

3. HACCP FOR DRY MIXED PRODUCTS

In the eighties and nineties a number of excellent documents were published on the application of HACCP. The 1993 Codex document (6) gives the agreed principles to be followed. These principles are further discussed in the 1992 publication of the NACMCF (7).

The Campden HACCP Manual of the same year (8) has transformed this into a practical guide. Readers are referred to these reports for a complete introduction into HACCP as this report will give only general guidance, which can not be a substitute for an actual HACCP study.

The HACCP study should follow the 7 principles detailed by Codex i.e. hazard assessment, CCP identification, establishing critical limits, monitoring procedures, corrective actions, verification and documentation procedures.

Here we will give only a summary of the requirements of such study, to get an understanding of the procedures to be followed.

When the objective of the study has been agreed and a brief for the HACCP team has been prepared by the management, the general procedure for an HACCP study can be outlined as follows:

- Assemble the HACCP team
- Describe the product and its intended use
- Draw a diagram of the process flow
- Analyze the microbiological hazards
- Identify the Critical Control Points (CCPs)
- Evaluate and select effective controls
- Establish an effective monitoring system for CCPs
- Establish corrective action for deviations
- Verify that the HACCP programme is working correctly
- Keep records on CCPs, monitoring and corrective action

The flow diagram of Fig. 1 gives the successive steps in the manufacturing process to consider for dry soups and bouillons. It shows that the microbiological status of the raw materials represents the main factor which determines the microbiological quality of the final mix and thus is of prime importance.

4. RAW MATERIALS

4.1 **Raw materials: Approved Suppliers**

A key element in the manufacture of dry mixes is that no ingredients are used which present a risk to the safety of the dry mix. This means that ideally any concern about the microbiological condition of an ingredient should be eliminated before its use in the dry mix is considered. The use of approved suppliers is the first step to be taken. For selection of qualified suppliers guidance is given in ref. 9 (ISO 9004).

It is thus recommended that all sensitive raw materials are purchased from inspected and approved suppliers. During supplier visits a manufacturing flow chart should be constructed on which sources and sites of contamination are indicated together with the ways to control such contamination. Such supplier audits are preferably done by a team of specialists with a good understanding

of microbiology, processing, QA and HACCP. The audit findings also will help to specify the risk category for a given ingredient.

A verification that the supplier ensures effective control of Critical Control Points (CCPs) by a thorough monitoring of critical raw materials and process parameters should be made from time to time.

4.2 Raw materials: Risk Categories

In an HACCP analysis of raw materials it proves useful to group all ingredients into **Risk Categories,** based on the microbiological hazards which might be associated with each of the ingredients. Such grouping into **Risk Categories** helps to manage and control the microbiological risks as it targets analytical resources and attention to those materials which deserve this most. Furthermore it simplifies communication within the company about microbiological safety and quality. The **risk categorization** can only be done by a competent dry soup manufacturer, who has developed a good understanding of the hazards involved.

Risk Category 1: no pathogen hazard

The first group with the lowest microbiological risk is presented by materials which, when bought from an approved supplier, rarely require analysis of their microbiological condition as their microbial load will be limited and presence of pathogens is not to be expected.

Examples are salt, sugar, glutamate, lactose, modified starches and many flavours.

Risk Category 2: pathogen hazard known and in control

The second group is composed of ingredients which might present a microbiological hazard like contamination with staphylococci or salmonella, but these ingredients are bought from qualified and inspected suppliers with documented procedures and QA programmes to control these hazards.

The actual risk for contamination thus is in control. The general microbiological condition of these materials is regularly checked and when deviations are observed pathogen testing is included as a default test and a new inspection of the production facilities of the supplier is carried out.

Examples are milk powder, meat extract, dried meat, cream, noodles, decontaminated spices.

Risk Category 3: possible pathogen hazard

The third group is formed by raw materials known from previous history to be susceptible to microbiological contamination with ha-

zardous microorganisms. These could be very sensitive ingredients like spices and some sources of vegetables even when supplied by a well known supplier. Such raw materials have to be inspected carefully for all relevant microorganisms, including pathogens, using an appropriate sampling plan.

Examples are formed by most natural herbs and spices and unblanched dry vegetables like asparagus, mushrooms, onions and red pepper from non-industrialized countries. **Risk Category 2** materials from a new supplier or new origin initially could be placed also in this **Category 3** to collect evidence that they do belong in **Category 2.**

Over the years sufficient information has been provided by the microbiological analysis of raw materials and by publications on the ecology of dry mix ingredients that a ranking into risk groups as mentioned before will be possible.

Reference is made to the standard work of the ICMSF on the Microbial Ecology of Foods (10), which 1980 edition is in revision and to a publication by the National Research Council in the US (11).

4.3 Raw materials: Critical Control Points (CCPs)

The division of raw materials into risk categories helps in identifying which materials could present a microbiological hazard. Normally only materials in **Categories 2** and **3** are considered a CCP. As a priority these materials should get adequate attention. It is important for these to select a good source and to give strict preference to buying from a raw material manufacturer over buying from a trader, as the latter will not be in control of the process and might be unaware of the microbiological hazards associated with the various processing steps. Use of **decontaminated** materials is acceptable when the decontamination process is shown to be effective and well controlled.

4.4 Raw materials: Sampling and Analysis

It is recommended that a positive release system is followed for all ingredients arriving in a dry mix plant. Such acceptance/rejection system could be based on the division of ingredients in **Risk Categories** as mentioned before.

It is recommended that each company applies a written **sampling** and **monitoring scheme** using specifications and sampling plans which are agreed with the suppliers. These schemes should take into consideration the **Risk Category** and the test relevant for a particular ingredient.

Risk Category 1

For materials with no pathogen hazard the release could be directly, once the identity and origin (approved supplier) is confirmed.

Microbiological testing is not required except for a limited number of checks to verify that products meet the agreed specification ('due diligence', see ref. 12). A few tests per year would be adequate.

Materials of **Categories 2** and **3** could present a pathogen hazard and thus their microbiological condition is considered a **Critical Control Point*.**

Risk Category 2

For **Category 2** ingredients bought from an approved supplier, who is in control of his process, one can restrict the microbiological analysis to monitoring. This means collection of one or more samples per delivery and analysis of these with tests which can indicate whether the supplier is still performing well. Such general hygiene or GMP tests could be:
Aerobic Plate Count, Enterobacteriaceae or coliforms and a yeast/mould count.

The test for Enterobacteriaceae or coliforms is particularly relevant for ingredients which during their manufacture have undergone a treatment which completely eliminates infective pathogens i.e. which have been processed for safety. One might extend this testing programme with occasional tests on pathogens like:
Salmonella, S. aureus, B. cereus and C. perfringens (as appropriate) but this has mainly due diligence value.

One should extend the routine monitoring when deviations are found. This default testing programme could include tests for pathogens and a new inspection of the processing conditions of the raw material supplier.

Risk Category 3

A full **microbiological analysis including tests for the relevant pathogens** has to be done for each delivery when ingredients of **Category 3,** with a risk for pathogen contamination, are to be used. The tests can be performed by the company, the supplier or a third party laboratory. For the sampling schemes reference is made to buying specifications agreed with the supplier and the ICMSF book on sampling (13). Such testing will, however, be unable to detect low levels of contamination, but it will help to minimize the hazards. The best way to control pathogen hazards is to eliminate the use of **Category 3** ingredients, but this is not always possible.

Data handling

It is recommended that **trend analysis** is applied on the raw material data collected to evaluate the performance of the supplier of a

* The Critical Control Points (CCPs) referred to in this document are in fact Design Control Points (DCPs) as they are based on a logical analysis of the dry soup process, and not the result of an actual HACCP study of a real operation. Only the latter are – strictly speaking – genuine CCPs.

particular ingredient. Based on the findings of such statistical techniques the level of acceptance/rejection sampling can be decreased or increased. Continued good performance of a supplier of a **Risk Category 2** ingredient could thus result in e.g. skip lot sampling where only 1 out of a given number of deliveries is analyzed.

Methods

For the microbiological methods to be used for the analysis of ingredients for dry soups and bouillons the use of ISO methods is recommended. Reference is made to the AIIBP publication of 1992 (2), which lists the available methods.

5. CONDITIONING

Some raw materials have to be made suitable for use in a dry soup mix. This could be done by agglomerating, drying, fat coating, milling, etc. It is beyond the scope of this document to discuss the CCPs of these operations. The manufacturer should be aware whether or not such operations can affect the microbiological condition of a particular ingredient. Where moisture is used he should consider the possibility of evaporation and condensation and the potential formation of wet spots. It is the responsibility of the manufacturer to determine whether in his operation conditioning of raw materials will be a CCP or not. When it is a CCP he has to apply measures which control the hazard.

6. MIXING

The key operation in a dry soup and bouillon plant is the mixing of ingredients in a mixer. By dumping known weights of the various dry components from silos, bags, boxes, etc. into a mixer a batch of dry soup or bouillon is made, which ingredients are evenly distributed in the batch by mixing. Sometimes liquid oil or fat is sprayed onto this mix during the mixing operation.

Ready made mixes are mostly filled into large containers or big bags and are then ready for the packing operation.

The mixing itself is not a critical control point (CCP) but the hygienic condition of the mixer could be a CCP. No microbial multiplication will take place as long as the mixer is operated under dry conditions, therefore **dry cleaning** should be applied whenever possible. Sometimes, however, the mixer has to be cleaned to eliminate product sticking to the mixer or to prevent contamination between batches by flavour or dry soup ingredients. When **wet cleaning** is applied this will be a CCP.

Preferred for wet cleaning is a mixer of a hygienic design in which no difficult to clean spots are present nor places where water could sit after the cleaning. As most mixers are not designed for wet cleaning measures should be taken to prevent that after wet cleaning moisture remains in the mixer, as otherwise microbial multiplication could take place. Quick drying of the mixer can be achieved by e.g. washing with hot water and/or forced ventilation with hot air.

Disinfection of the mixer is normally not required as the microbial load of a cleaned mixer will not significantly affect the condition of the dry soup or bouillon mix.

Visual inspection shortly after the wet cleaning is essential to detect any moisture trapped in the mixer. Microbiological checks of the interior of the mixer and of the first batch produced after wet cleaning are optional depending on the reliability of the cleaning operation.

A written cleaning procedure should be available together with the agreed control and monitoring procedures.

7. FILLING/PACKING

The filling and packing of the dry mix in pouches or cartons are dry operations, with normally no concern for microbial contamination when these are done on a visually clean and tidy packing machine free of product rests. Vacuum cleaning of the filling machine will be adequate to eliminate dust and product rests and wet cleaning is normally not required nor recommended. A fully dry filling/packing operation is thus not a CCP.

The packaging material should be able to prevent ingress of moisture into the product as otherwise quality loss and – in exceptional cases – yeast or mould growth could be possible, when the content is hygroscopic.

The packaging material should be stored and kept clean to prevent contamination of the product with dust or foreign material.

8. STORAGE

The packed dry soups and bouillons are stored in a warehouse before they are delivered to distribution centres or shops. The storage in the warehouse is not a CCP with respect to microbial contamination.

End product analysis

Samples of product ready for distribution will be regularly analyzed to be able to document the good hygienic status of the product. The microbiological limits to be met are the relevant legal limits or the AIIBP guidelines of 1992 (2), which could be extended by company limits for those microbial groups not mentioned in legislation or by the AIIBP.

This end product analysis is mainly for due diligence reasons as the hygienic quality of the soups and bouillons should be assured in a preventative way by the HACCP procedures mentioned in this document. This analysis therefore should be a relatively minor activity compared to the attention given to **the raw materials** and **the dry mix operation** itself.

9. DISTRIBUTION

Distribution is not a CCP for dry soups or bouillons, when distribution is performed properly and packs are not damaged during transport.

10. CONSUMER USE

All consumer packages as well as products for professional use should have clear instructions about the preparation of the soups or bouillons. The way of preparation for instant soups (like 'prepare with boiling water') or regular soups (like 'bring to boil' or 'cook for x minutes') should be unambiguous. When soups are to be prepared with cold water only this should be indicated. Similarly clear preparation instructions should be present on soup or bouillon pastes and semi-finished soup bases for consumer use or for professional use.

Proper instructions for the preparation of dry soups and bouillons form in principle not a critical control point when the products meet the microbiological specifications recommended by the AIIBP (2).

Preparation with boiling water or cooking will give, however, additional security with respect to infective pathogens. The instructions furthermore should also cover the use of any left overs. When these are cooled slowly or kept overnight at ambient temperatures microbiological hazards will occur. Thus consumer instructions are a CCP.

REMARKS

a) **Survey of typical CCPs**

In table 1 a summary is made of the Critical Control Points typical for the production of dry soups and bouillons, together with the control options and the monitoring options. This survey could be used as a rapid check on the most common CCPs of a dry mix operation.

b) **Example**

A worked out example for a meat bouillon mix is given in Annex 1.

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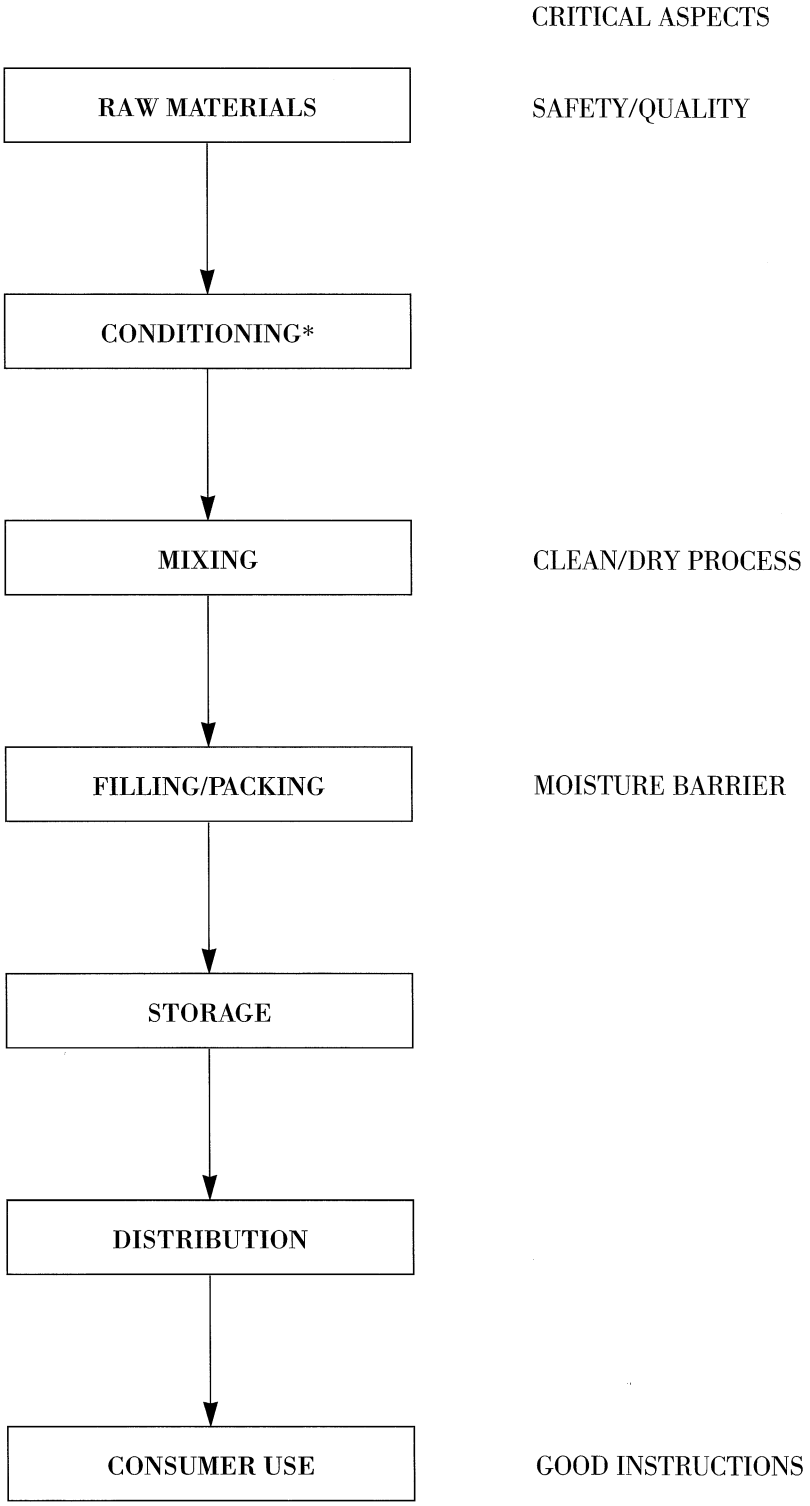
* The 1987 version of this manual (Technical Manual 19) includes an HACCP analysis of instant dry soups, which is not supported by the AIIBP, as only heat resistant sporeforming bacteria and toxin were considered a hazard in this study.

FIG. 1

FLOW DIAGRAM FOR DRY SOUP AND BOUILLON MIXES



* Whether conditioning is critical depends on outcome of specific HACCP study.

TABLE 1: SUMMARY OF CCPs, CONTROL & MONITORING OPTIONS FOR DRY SOUPS AND BOUILLONS

PRODUCTION PHASE	CRITICAL CONTROL POINT	CONTROL OPTION	MONITORING OPTION
RAW MATERIAL	PATHOGEN CONTAMINATION		
RISK CAT. 1	NO CCP	APPROVED SUPPLIER	
RISK CAT. 2	CCP	SPECIFICATION; APPROVED SUPPLIER	INSPECTION; GMP-TESTS
RISK CAT. 3	CCP	FULL SAMPLING AND PATHOGEN ANALYSIS	CHECK DATA: GMP-TESTS & PATHOGENS
CONDITIONING*			
MIXING DRY CLEANING	NO CCP	KEEP DRY	INSPECTION
WET CLEANING	MOISTURE	RAPID DRYING	INSPECTION
FILLING/ PACKING	NO CCP	KEEP CLEAN, TIDY & DRY	VISUAL INSPECTION
STORAGE	NO CCP		
DISTRIBUTION	NO CCP		
CONSUMER USE	ABUSE PREPARATION	CONSUMER INSTRUCTION	CHECK LABEL

* Conditioning could be a CCP; depends on result of HACCP study

ANNEX 1

EXAMPLE MEAT BOUILLON

Meat bouillon is made by blending the following ingredients:

Salt, monosodium glutamate, hydrogenated vegetable oil, beef extract, hydrolyzed vegetable protein (HVP), yeast extract, onion powder, white pepper, paprika, laurel leaves, parsley, coriander and fenugreek.

According to the principles discussed in section 4 the ingredients used should be of good quality (no pathogens in levels which could present a health hazard) and contamination during manufacture should be prevented.

RAW MATERIALS

The raw materials can be divided in Risk Categories (RC) as explained in 4.2.

Salts and oils

Salt, monosodium glutamate and hydrogenated vegetable oil are **Risk Category 1** materials (no CCP). They are bought from an approved supplier and not routinely tested for their microbiological condition.

Extracts, HVP

Beef extract, hydrolyzed vegetable protein (HVP) and yeast extract are **Risk Category 2** ingredients due to the possible presence of Salmonella, Staph. aureus and C. perfringens. They are bought on a microbiological specification from approved suppliers, who are known to control the microbiological hazards which are associated with these products.

The following specification could be applied, but the values quoted should be seen as examples only:

	Limit per g			
	n	c	m	M
Bacillus cereus	5	3	10^2	10^4
Clostridium perfringens	5	3	10	10^2
Staphylococcus aureus	5	2	10^2	10^3
Salmonella	5	0	absent in 25 g	

ANNEX 1 (continued)

Herbs and spices

Ground coriander, fenugreek, white pepper, paprika, laurel leaves and parsley are herbs and spices for which contamination with Salmonella is known to occur or suspected i.e. **Risk Category 3** materials. Decontaminated ingredients are preferred but these are not always available or suitable. In this example it is assumed that pepper and paprika are heat decontaminated with a validated process by an approved supplier, and they are now considered to be **Risk Category 2** materials. A decontamination which equals pasteurization is adequate, which means that the spore load will not be affected by such process and the same specification can be applied for all these herbs and spices, whether RC 3 or 2.

The following specification could be applied (but the values given are examples only):

	Limit per g			
	n	c	m	M
Bacillus cereus	5	3	10^4	10^5
Clostridium perfringens	5	3	10^4	10^5
Salmonella	5	0	absent in 25 g	

The **Risk Category 3** materials are fully analyzed on Salmonella (5 samples of 25 g per lot) before they are accepted for use in dry soups and bouillons and similarly tests on B. cereus and C. perfringens could be applied.

The incoming lots of decontaminated pepper and paprika **(Risk Category 2),** are accepted when the GMP tests (Enterobacteriaceae or coliforms and optionally yeasts & moulds and Aerobic Plate Count) indicate that the decontamination was properly carried out. A routine testing on heat sensitive pathogens like Salmonella is not required.

Dry vegetable

Onion powder is a dry vegetable, which can be considered **Risk Category 2** when bought from an approved supplier, who has good control over hygiene during harvest and manufacture. As the onions are normally not blanched, in particular contamination with pathogens should be prevented. GMP tests (see above) could be used to monitor the general hygienic status of incoming lots and tests for pathogens are required only as a default test when deviations from the normal counts are observed.

However, such onion powder should be considered **Risk Category 3,** when bought from a trader or from a supplier in an area where general hygiene is known not be optimal. Under such conditions Salmonella contamination and elevated levels of B. cereus have been observed. A complete test on the

ANNEX 1 (continued)

relevant pathogens, in particular on Salmonella, should be carried out for each lot.
The buying specification for unblanched onion powder could be set as follows (values given are examples only):

	Limit per g			
	n	c	m	M
Bacillus cereus	5	3	10^3	10^4
Clostridium perfringens	5	3	10^2	10^3
Salmonella	5	0	absent in 25 g	

MIXING

Mixing is not a CCP when no wet cleaning of the mixer is applied and general hygiene rules (keep mix department clean, dry and tidy) are followed.

Wet cleaning of mixers or of pumps to dose vegetable oil will be a CCP and thorough drying and visual inspection is essential. Microbiological checks of equipment can be performed to verify that no microbial load builds up in the mixing department. Records should be kept of cleaning and inspection.

FILLING/PACKING

This is normally not a CCP. Area should be kept clean and dry. Visual checks on this will be adequate.

DISTRIBUTION

No CCP, but occasionally microbiological tests are carried out on final packed product to check compliance with the national legal limits, the AIIBP specifications or internal company limits.

CONSUMER USE

Presence of clear consumer instructions on all dry soups and bouillons is a CCP and should be checked when new packs are printed.

ASSURANCE OF THE MICROBIOLOGICAL SAFEETY OF ASEPTICALLY PACKED SOUPS

September 1997

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1. INTRODUCTION

This is an AIIBP document to give general guidance on assurance of the microbiological safety of aseptically processed and packed acid and low acid soups in cartons. The AIIBP is aware that the Critical Control Points (CCPs) from a real production operation can not be addressed adequately and completely in a simple guide. The intention therefore is restricted to provide helpful guidance to members, who have a legal obligation to base the safety of their operation on a properly performed HACCP (Hazard Analysis Critical Control Point) study or equivalent system to control product safety. The section on quality assurance in particular can be used as a complement to local expertise in the execution of such HACCP study.

The document can be of help also in discussing the principles of aseptic packing of soups with local authorities like Food Inspectors. For the relevant general hygiene requirements the document refers to the General Principles of Food Hygiene of FAO/WHO (1) and the EC Council Directive 93/43/EEC on the Hygiene of Foodstuffs (2). Additional guidance on aseptic processing can be found in the Codex Code of Hygienic Practice for Aseptically Processed and Packaged Low-Acid Foods (3).

2. SCOPE

The document is restricted to soups sauces and gravies in cartons as these represent the main volume of aseptically filled soups and sauces. The aseptic packing is discussed for soups, with or without particles, packed in cartons made from the reel (like Tetra Pak) as well as for soups packed in preformed blanks (like Combi Block) or other aseptic packs. This approach will help to clarify the CCPs for the aseptic packing of these two types of packaging.

This document covers aseptically packed low acid soups (pH > 4.6), acid or acidified soups (pH 4.2 to 4.6) and high acid soups (pH ≤ 4.2). *All QA measures and CCPs, which are specific for high acid and/or acid(ified) soups will be represented in the text in italics.*

3. REQUIREMENTS FOR ASEPTIC PROCESSING

3.1 General.

Plant Facilities

The plant facilities for the production of aseptically packed soup should be adequate for the function, which they have to perform. Plant lay-out should meet the requirements formulated in the Codex General Principles (1) and of the relevant Code of Practice for Aseptically Packaged Foods (3). Equipment should be of hygienic design in line with the EC Machinery Directive (5). Additional recommendations on hygienic and aseptic equipment were published by the European Hygienic Design Group (EHEDG, 4) and in the Technical Manual No 11 of CCFRA (Campden Food & Drink Research Association, 6)

Suitable rooms for raw material storage, pre-processing, mixing and the aseptic heat processing and cooling should be available. Additionally the plant lay-out should allow the **aseptic filling operation** to be performed in a room separated from the mixing room (and heat processing) and the secondary packaging room. This aseptic filling room should meet particular high standards of environmental hygiene to prevent (cross) contamination. The room should have a slight overpressure with filtered air to prevent contamination with airborne microorganisms especially during the aseptic filling operation of low acid soups.

Suitable, hygienic drains accessible for cleaning, should be available in the filling hall to prevent the build up of microbial contaminants, which could present a safety or spoilage hazard for the aseptically packed products.

There should be an adequate supply of steam for the heat processing operation. The formation of condensate due to excessive steam loss from steam barriers to the environment should be prevented.

Access of personnel to the heat processing and filling area should be limited to dedicated personnel only, who are directly concerned with processing and associated activities. For pack inspection (tear down) suitable facilities should be available in the filling room as part of the routine QA procedure.

3.2 Personnel

Training of staff

The staff responsible for the operation of an aseptic processing and packing plant should have a good understanding of the factors which are important for product safety. A qualified technologist and microbiologist, with professional training in food technology and microbiology should be part of the staff. Education and training can be provided by universities, specialized institutes and by the major companies manufacturing aseptic processing facilities.

Training of operators

The operators in an aseptic processing plant are the most important people for ensuring the safety of the operation. All key personnel should be properly trained before they start in their job. This initial training should be complemented by regular refresher courses. Most companies marketing aseptic processing and packing equipment can provide various levels of operator training.

3.3 Raw materials

Soup raw materials

The raw materials used for the preparation of soups should be sound and of good quality.

They may be fresh, dry or frozen and should be bought from approved suppliers against suitable specifications covering relevant microbiological requirements.

The mesophilic (and were appropriate thermophilic[1)] spore load of sensitive ingredients like vegetables, dairy products, spices and herbs may be limited (GMP value < 100/g, max 1000/g) in relation to the targeted heat process microorganisms selected and to the pH of the finished product.

Water used in the formulation should be of potable quality and meet the EC limits for drinking water.

Packaging materials

The packaging materials should be bought from approved suppliers against a suitable specification (like no pinholes in inner polymer of pack). Microbiological specifications normally are not used, but obviously the material should be of excellent hygienic quality. The materials should be produced and handled under hygienic conditions which prevent contamination. Whether the material is provided as reels or blanks, this should be clean and fully wrapped in a protective foil to prevent environmental contamination and stored in dedicated rooms. Left-overs of packaging material should be removed from the filling room, rewrapped completely and hygienically stored for later use.

[1)] See chapter 5

3.4 Soup preparation

Pre-treatment of raw materials

This includes general activities like washing, peeling, blanching, size cutting, thawing etc of raw materials to make them suitable for use in the soup.

Reducing or prevention of build-up of contamination with bacterial spores is important, in particular:

- General good hygienic conditions in plant.
- Suitable time/temperature conditions for thawing (with hygienic removal of drip to drain) or hot holding.
- Cleaning/sanitation of plant to prevent build up of fouling to act as source for spores on plant.
- Removal of soil from vegetables, herbs and other raw materials to reduce spore count.
- Proper operation of blanchers (time/temperatures) to prevent formation of thermophilic spores.
- Size cutting of materials to remain below specified maximum size, as heat penetration depends on size of particles.

Soup mixing/cooking

The mixing of the soup ingredients into a soup base and the cooking thereof should be done according to written formulations and specific processing instructions for each soup variety.

Key elements, with respect to getting a soup with the required characteristics and a low microbial load are the following parameters:

- Proper time/temperature of cooking to prevent problems due to thermophilic spore forming bacteria.
- Suitable time/temperature of cooking to prevent inadequate setting of starch or degradation of starch resulting in soup with lower viscosity than designed.
- *Prevention of deviations in composition resulting in out of specification conditions for the pH of acid(ified) soups.*
- Prevention of hygienic shortcomings in equipment, pumps or piping allowing development of (thermophilic) spores
- Prevention of lump formation resulting in spores inside dry lumps, which are not inactivated by heat during processing.
- Assurance of homogenous distribution of particles through the soup mixture.
- Proper defrosting of frozen ingredients at safe time/temperature conditions or during processing.
- Proper rehydration of dry ingredients.
- A double check on particle size of soups with particulate to assure good heat penetration during the designed heat process.
- Elimination or reduction of unnecessary lead times during mixing/cooking.

Soup holding tank before sterilization

The main hazards during holding of the soup before sterilization will be development of thermophilic spores, changes in viscosity or the settling/inhomogeneous distribution of particles .

Attention should thus be given to the following points:

- Controlled time and temperature for holding the soup (> 75°C or max 4 h < 75°C). *This may not be relevant for high/acid soups*.
- Temperature equilibration throughout the whole buffer tank, achieved by jacketing or insulation (prevention of "cold" spots in tanks without stirrer).

- Hygienic design of buffer tank, valves, piping and pumps to permit effective cleaning.
- Viscosity changes.
- *Increase of pH due to buffering.*
- Homogeneous distribution of particles over the batch.
- Hygienic transfer of soup from one batch to another minimizing left overs.

3.5 Sterilization and aseptic packaging

Sterilization/cooling.

Sterilization and cooling of aseptically packed soups is frequently done either by scraped surface heat exchanger, by tubular heat exchanger or steam injection (e.g. for sauces). The hygienic requirements for all types of heat exchangers are given in Annex 2, taken from EHEDG, 1993 (7).

Key elements to be addressed for most sterilization/cooling operations are given below:

- Temperature of product at the exit of the holding tube, to ensure scheduled heating temperature.
- Homogeneous temperature at the entrance of the tubular heat exchanger.
- Constant product flow (in case flow rate is not fixed) below maximum rate set, to ensure scheduled holding time.
- Product viscosity within designed range, particularly for tubular heat exchangers.
- Changes in steam pressure (= temperature) as indicator for fouling of heat exchanger.
- Prevention of leakage in heat recuperation section of heat exchanger.
- Prevention of leakage in piping and couplings downstream the sterilization section.
- Utilization of steam barriers on aseptic valves and couplings to prevent contamination.
- Cooling of soup to a temperature suitable to prevent development of thermophilic spores.
- Pipework and values for diverting or stopping the heating process must have preset temperatures in order to maintain the scheduled process parameters.
- Sampling devices, when present, should be of aseptic design and well cleanable/sterilizable (cleaning/sterilization not during processing).

Sterile holding tank before packaging

The tank should be able to hold the sterilized soup under aseptic conditions at a temperature which does not promote development of thermophilic spores, which may have survived the sterilization (not relevant for high acid soups).

Important parameters:

- Sterile tank should be designed for perfect cleaning and sterilization of tank and all its connections.
- Sterile tank should be properly designed for aseptic holding of soup.
- Tank should be equipped with sterile filters allowing overpressure with sterile air.
- Time/temperature of holding should limit spoilage risk (max 35°C, for max 24 h).

Pack sterilization and aseptic filling

The filling of the sterilized soup in sterilized packs is a critical operation, which should be done in a dedicated part of the plant, where environmental contamination is minimized and controlled. The peroxide commonly used for pack sterilization has a limited sterilization capability and most filling machines are not hermetically sealed from the environment.

For packing in blanks and packing in packs made from the reel different points will be critical. The pack sterilization is integrated in most aseptic filling systems, so the requirements for pack sterilization and aseptic filling are given together.

General requirements.

- Remove left overs of peroxide before longer production stops (over one week).
- Aseptic filling to be done in a "clean room" with a high standard of hygiene, preferably with controlled overpressure of filtered and/or sterile air.
- The air pressure in the sterile zone of the equipment used for packing should be higher than the overpressure in the clean room, where the filling machine is located.
- Contamination of packaging material should be prevented.
- Use of a clean container with peroxide (contamination with organic components can catalyses decomposition of H_2O_2).
- Monitoring of peroxide at the required strength and no overdosing of peroxide to prevent residues in the soup packed.
- Cleaning and sterilization of the filling machine should be ensured.

Filling preformed packs [2)] :

- Hygienic handling and loading of preformed packs onto machine.
- Tightness of the Supplier's seaming should be monitored as incoming goods control procedure.
- Correct formation of preformed packs into pack (correct overlapping of sealing surfaces).
- Proper peroxide spraying of packs (required H_2O_2 dosage and concentration, even distribution) and sterilization of packs (air to be sterile, delivered for required time at specified flow rate and temperature).
- Aseptic filling of packs (prevention of product contamination of seal area; no splashes and no long vegetable fibres in the product).
- Hermetic sealing of filled pack (time, temperature, pressure of sealing jaws, adjust- ment of jaws, correct overlapping of sealing surfaces).
- Performance and hygienic status of final folder (no creation of sharp edges, resulting in leakers).
- Hygienic status of seal cleaning water.

Packs made from reel [3)]

- Prevention of contamination of reels (fully wrapped, minimal manual handling).
- Hygienic change of reels (prevention of contamination).
- Concentration and even distribution of peroxide for sterilization of tubes formed from reel (use of surfactant, inspection of condition and performance of peroxide application roller, measurement of peroxide consumption).
- Time/temperature for peroxide sterilization of tubes.
- Quality of longitudinal seal (time, temperature, pressure, adjustment of sealing jaws).
- Aseptic filling into sealed tubes.

[2)] These systems normally allow the filling of pieces into the pack

[3)] These systems normally do not allow the filling of pieces into the pack

- Quality of lower and upper pack seals (time, temperature, pressure, adjustment of sealing jaws).
- Hygienic quality of seal cooling water.
- Performance and hygienic status of final folder (no creation of sharp edges resulting in leakers).

3.6 Storage and distribution

The processing and handling after the aseptic filling operation should take place outside the hygienic filling room. During secondary packing, palletization, in-plant storage and distribution the cartons with soups should be protected against damage due to mechanical impact or high pressure and against excessive temperature or humidity.

Palletization & storage

Key points requiring attention are:

- Smooth, obstruction free conveyer belt, without sharp points, leading to case packer (otherwise potential damage to packs due to abrasion).
- Lubrication of conveyer belt should not allow microbial growth during operation nor should lubricants, transferred to packs, pick up moisture and allow growth later on.
- Secondary packs should be well designed and strong to give protection of packs.
- Pallets should be smooth and suitable (no nails, no damaged pallets) or be protected by sheets of carton.
- Palletization to be done according to agreed load pattern.
- No excessive temperatures: < 0°C (freezing!!) or > 35°C (thermophile)
- No excessive humidity (> 90%) for extended periods of time, allowing mould growth on pack surface or resulting in collapse of pack due to moisture uptake.
- No intermediate chilling (condensation on packs after return to ambient temperature).
- Pallet stacking should be allowed only, when performed according to an agreed stacking plan.
- Protection of packs against rodents and pests.

Distribution

During distribution the integrity of the soup packs should not be unduly challenged.

The following point should get due attention:

- Temperature of distribution should prevent freezing and temperatures >35°C.
- Pallet handling should prevent pack damage.
- Transport should not create excessive vibration or shocks (eg from brakes) leading to loss of pack integrity or pack damage.
- Pallet stacking not to be allowed, unless an agreed and validated stacking plan is followed.

Sale

Packs should be protected against dirt, mechanical damage, rodent, pest and excessive temperature or humidity during retail sale and careless opening of distribution units by sharp instruments resulting in pack damages.

3.7 Consumer use

It is important that the consumer of the soup gets good instructions about storage and handling of the aseptically packed soups, i.e. :

- Instruction on how to store soup before use.
- Instruction on how to prepare the soup for consumption.
- Instruction on how to store and handle any left over soup.
- Instruction related to shelf-life and conditions of storage after opening the packed soup.

4. CLEANING & STERILIZATION OF PROCESSING PLANT

4.1 Cleaning

Plant and premises should be kept clean and tidy in line with requirements of Codex General Principles (1) and EC Directive on Hygiene of Foodstuffs (2).

Plant and equipment up-stream the sterilization part of the process should be of hygienic design suitable for effective cleaning. Validated cleaning schemes and operating instructions should be available for all open plant. For the closed heat processing and filling operation normally a Cleaning in Place (CIP) system is applied, which should be properly specified and validated.

A good (preventative) maintenance scheme should be in operation to permit adequate cleaning performance for all components of the line.

4.2 Sterilization

Sterilization and aseptic packaging requires the sterilization of the equipment for heat processing, cooling, holding of the soup and the sterilization of the packaging equipment to fill soups in pre-sterilized packs and the sterilization of gases used to maintain a sterile environment within the packaging machines.

The sterilization of all systems should be according to the principles referred to in the Codex Code on Aseptically Packaged Low-acid foods (3). This requires the use of equipment of suitable aseptic design to keep the systems sterile after the sterilization. The sterilization schemes should be properly validated to ensure the correct sterilization of all systems used in the aseptic processing of soups.

The processing line and equipment should be sterilized according to scheduled parameters also in case of processing high acid and in some extend acid(ified) soups.

5. SETTING OF SCHEDULED PROCESS

A scheduled process should be formulated by an expert for each soup, which is aseptically filled into cartons, considering all aspects which are important for the formulation (like microbial load, equilibrium pH, water activity, composition, particulate size and intended storage temperature) and delivery of this process (like heating process, flow rate, consistency, dimensions of holding section) as discussed by Codex (3).

For low acid foods *Clostridium botulinum* [4)] should be taken as the target organism in line with requirements of Codex (3), whereas *B. stearothermophilus* may be the target for soups for areas with ambient temperatures above 35°C.

For acid(ified) soups, with a pH between 4.3 and 4.6, the likely target organism will be Bacillus coagulans. Special attention should be given to neutral ingredients in acid(ified) soups to prevent safety hazards due to rises in pH.

For high acid soups with a pH ≤ 4.2 the likely target organism will be Clostridium pestorianum.

For any soup the scheduled heat process should be carefully formulated and a process should be selected that is demonstrated to deliver this process. For liquid

[4)] Minimum F_o is 3 minutes

soups the time/temperature determination for the process conditions can be based on measurement, at the selected sterilization temperature, of maximum flow rate in a holding tube, considering all aspects which permit the fastest parts to travel through such tube with minimum residence time.

For soups with particulate commonly a combination of measurement of minimal residence time in combination with calculation of heat transfer within the particles is used to set the scheduled process. This is clearly an expert activity, which should not be started without adequate understanding of all aspects which affect the proper sterilization of particulate.

Guidance is given in many publications like those of EHEDG (8), Reuter (9) and McKenna & Tucker (10).

6. QUALITY ASSURANCE OF ASEPTICALLY PROCESSED SOUP

6.1 Raw materials

Soup raw materials

- Visual/organoleptic checks on quality of fresh ingredients.
- Use of sampling and analysis schemes based on relevant criteria for dried, frozen and preserved raw materials.
- Verification, where appropriate, of bacterial spore load (and of thermophilic spore load), as supporting evidence about the performance of suppliers for retrospective trend analysis of data.
- Checks on identity and lot code of ingredients to allow traceability of raw materials used.
- Visual check of integrity of packaging material (cans, bags....).

Packaging materials

- Check whether materials received are fully wrapped: visual inspection.
- Microbiological status of packaging material is optional.
- Prevention of contamination/damage of packaging material during handling: operator training & visual inspection.
- No use of damaged reels: visual inspection.
- Check & record packaging material identity and lot code to allow traceability.
- Check pack integrity for blanks: compression test after filling with water (destructive test) and rhodamine test (leaker test) on representative sample of lot.
- Apply positive release system for blanks.

6.2 Soup preparation

Hygiene status of equipment

- Hygiene checks on equipment before processing: visual inspection, or trend analysis like swabs or agar-contact plates/dip slides and also ATP test.

Pre-treatment of raw materials

- Control size of particles: sieve analysis.
- Check residual soil on washed vegetables, herbs and the like: visual inspection.
- Good instructions for operators to prepare soup: follow ISO 9000 guidelines for formulation of soups and the QA thereof.

Soup mixing/cooking

- *Check pH (acid varieties)*.
- Check whether soup is made according to recipe: sensory evaluation.
- Check viscosity of soup: sensory (routine) and instrumental (as default procedure, when deviations are observed).

- Check time/temperature of cooking: recorder and written forms.
- Monitor hygiene of mixing/cooking operation: visual, ATP or swabs/agar contact.

Soup holding tank

- Record temperature of soup in holding tank: *target > 75°C, > 60°C for acid(ified) soups.*
- *Double check pH (optional).*
- *Double check viscosity (optional, depending on product/process).*
- Check thermophilic spore load (optional): to allow trend analysis of data, where appropriate.
- Check level (when tank runs dry this will upset heat processing): level controller.
- Assure that no left overs remain in holding tank.
- Maintain homogeneity: monitor stirrer speed.

6.3 Sterilization/cooling

- ontrol and record the temperature of product at end of holding section: registration.
- Control and record the temperature of steam (or pressure): registration.
- Control and record the product flow rate: depends on local system and record.
- Calibration of instrumentation for temperature, pressure, flow rate: according to ISO requirements.
- Validate and record functioning of flow diversion valve or shut down procedure and alarms.
- Check operation of steam barriers on couplings etc: visual check or alarm.
- Monitor temperature of cooled product: record temperature and alarm.
- Control of equipment leaks: preventive maintenance programme.

6.4 Aseptic holding tank

- Maintain overpressure: alarmed and/or recorded.
- Sterility of air for overpressure in tank: replace filter regularly.
- Prevention of overflow of tank: high level alarm.
- Control temperature and holding time of soup: registration or record by operator.
- Maintain homogeneity of the product.

6.5 Aseptic filling

Soups packed in blanks [5]:

- Sterile air overpressure: regularly replace filter.
- Overpressure system: record or alarm overpressure.
- Peroxide: level in tank; concentration, time/temperature of application (alarm on temperature) and consumption.
- Control of seal quality: tear down test, basic tests (see 3,5 pack sterilization and aseptic filling) according to agreed schedule.
- Water quality (in contact with pack): monitor hygienic status or disinfect and monitor performance.
- Hygienic status of final folder.

[5] The specific QA measures depend on type of machine, sterilization system, packaging material, product etc..: supplier instructions should be followed carefully.

Soup in packs made from reels [6] :

- Sterile air overpressure: regularly replace filter.
- Peroxide: measure concentration, peroxide temperature, monitor air sterilization temperature, check maintenance of peroxide roller, **check peroxide consumption**. Remove peroxide before long processing stops (> 1 week).
- Water quality (in contact with pack): monitor hygienic status or disinfect and monitor performance.
- Hygienic handling of reels: monitor visually, suitable procedure to ensure a safe change of reels on the machine.
- Control seal quality: temperature, adjustment of sealing jaws; frequent tear down test according to agreed schedule.
- Hygienic status of final folder.

6.6 Storage and sale distribution

- Conveyer belt: check belt for damage and absence of residual lubricant on packs.
- Pallets: visual inspection for damage, dirt, nails, wood splitter.
- Palletization: check whether agreed palletization patterns are followed.
- Storage temperature: record storage temperature (target < 35°C).
- Transport: check whether non-permitted pallet stacking is applied.
- Opening of distribution units: warning on label or easy to open boxes.
- Prevention of unintended chilling of packs.

6.7 Consumer use

- Consumer instructions: check label on pack for preparation instruction.
- Shelf life: check 'best before date' on pack.
- Production code: check presence and readability of correct code (traceability).

7. INCUBATION/TESTING OF PACKED PRODUCT

A low percentage of aseptically packed soup will be incubated and tested for commercial sterility. This end-product testing programme can only detect major problems and its main function is to collect data for trend analysis about the long term performance of the aseptic filling operation. This testing procedure alone is thus unable to ensure safety of batches of packed soup. Safety and quality of soups produced can only be demonstrated from documented adherence to the total QA programme, which is set up for each aseptic packaging operation.

Normally during the testing program, all volume produced remains in quarantine until analysis results on commercial sterility are available. The extent of this procedure depends on confidence in the operation and possible legal requirements.

The sampling and testing regime should be higher for a new operation to collect data to show that the target of commercial sterility is met.

Incubation conditions are not standard, but the following can be taken as a guideline:

- For soups for non-tropical destinations: a minimum of 5 days at 30° or 35°C.
- Alternatively requirements formulated by Codex (3): 10 days at 35°C, EC or national authorities should be followed in line with the characteristics of the product.
- For soups for tropical destinations: 7 days 55°C or another suitable condition recommended by Codex, EC or national authority.
- *High acid soups: a minimum of 7 days at 25°C (yeasts and moulds) Acid(ified) soups: a minimum of conventional 7 days at 30-35° C (Lactobacilli).*

[6] These systems normally do not allow the filling of pieces into the pack.

Incubated packs can be tested with conventional microbiological tests to show presence of microbiological contaminants. Where alternative methods like tests for Adenosine Triphosphate (ATP), dye reduction or residual oxygen are used, these should be properly validated for the type of soup packed.

Measurement of pH can be helpful in addition to microbiological test procedures, but pH measurement alone will not provide a reliable indicator for microbial contamination as many organisms do not change the pH and thus some types of contamination will not be picked up in this way.

When incubated packs are shown to be contaminated, it is recommended that operators check the integrity of the pack, and identify the organisms that have grown in the product. The type of organism can give helpful guidance about the nature of the defect leading to a non-sterile pack (like sporeforming bacteria in case of under processing, cocci after inadequate pack sterilization, *Enterobacteriaceae* with leaker spoilage).

8. VALIDATION OF NEW PLANT

Before a new aseptic plant comes into operation it is recommended to check that the plant has been built and installed according to the agreed plans. When this commissioning is satisfactory, the next step is to document that the plant can produce commercial sterile packs of soup, free from organisms which can lead to spoilage under the intended storage conditions.

One procedure of validation is to produce soup on this plant in three different production runs. From each run 1000 packs are incubated and all of these are tested with reliable traditional microbiological or alternative tests. A maximum acceptable number of defects (e.g. max 1) should be agreed in a validation protocol for these 3x1000 packs to be analyzed. When more defects are detected, the nature of the defect has to be studied, the origin of the defect corrected and a new validation trial carried out before regular production can start.

When the validation trial has shown that no unexpected defective products were observed in the trial phase, actual production is started. During the first months of production an intensified sampling and testing scheme should be applied to collect data about the performance of the plant under normal operation conditions.

It is during this period of intensified sampling that sufficient data should be collected to demonstrate that the plant does perform to the standard set by the Company (typically not more than one or two defects in 10.000 samples). Once evidence is provided that the Company target is met, a reduced level of sampling/testing can be applied as long as performance remains good.

It might be possible to separate part of the produced lot and perform a final visual check on the packs after storage of about 2 weeks at > 15°C, as an additional tool to pick up small defect levels which leads to blowing or leakages. Once it is shown that an agreed low defect level is met, this testing can be reduced to a lower level.

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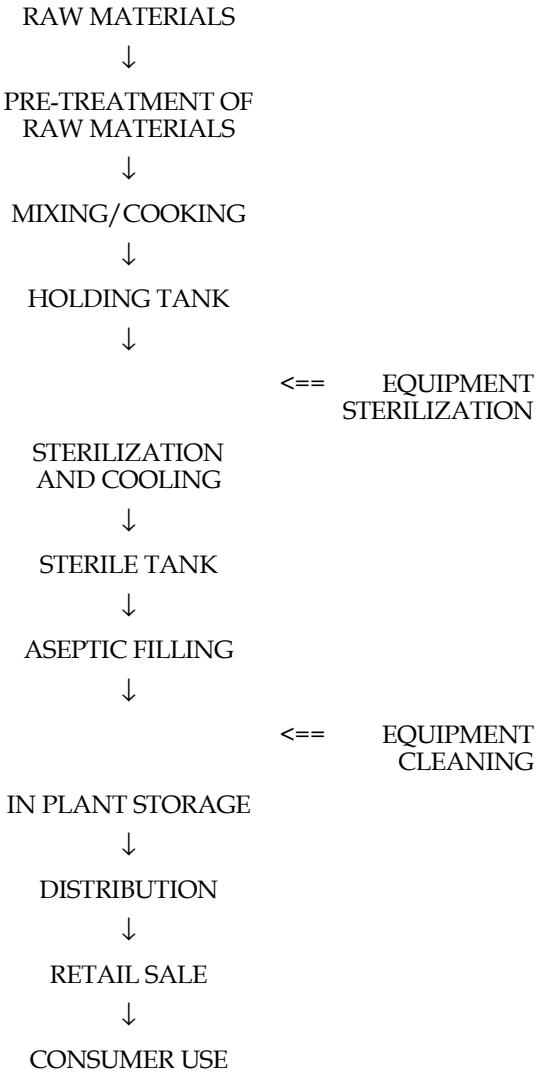
Figure 1 FLOWSHEET FOR ASEPTICALLY PACKED SOUPS



ANNEX 2 HEAT EXCHANGERS

Requirements common to all types of heat exchangers

- They should be fully cleanable and preferably drainable and accessible for inspection.
- Any fluids used for cleaning in place must be compatible with the construction materials under in-use conditions.
- Service fluids should not be corrosive.
- If the service side of the heat exchanger is to be drained, complete removal of all service liquid must be assured.
- Pockets (dead legs) or crevices should not be present at the product side, as they are difficult to clean, and product will reside in them for much longer than the mean residence time.
- All connections in the sterile area of the plant must be aseptic.
- There must always be two gaskets between the product flow and the heating or cooling medium, and it must be impossible to build up pressure between these two gaskets. Hence, the space between these two gaskets, the space between them must have vent-and-drain grooves and be large enough to ensure that they cannot be blocked by leaking product, heating medium or coolant.
- Replacement gaskets must fulfil the requirements specified by the manufacturer of the heat exchanger.
- In the case of high products, the flow passage should not be too narrow, to prevent blockage. For some products, increased velocity helps control fouling and product blockage.
- To prevent stress corrosion, the design should prevent differences in expansion and contraction leading to unacceptable stress at times of maximum differences in temperature, such as start-up, shutdown and cleaning.
- Product flows should be designed to avoid air entrapment within the system.