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MELISSA

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Edible biomass conservation and preparation.

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1 INTRODUCTION

The general concept of MELISSA includes the use of the biomass generated in the two photosynthetic reactors, *Spirulina* in compartment IV and *Rhodospirillum* in compartment II, as food supply. Previous studies have shown that both microorganisms can be used as supplement in the food diet of rats (Borowitzka 1988, Tranquile and Emeis 1997). On the other hand, *Spirulina* has been used as an important source of proteins in children suffering from malnutrition and different types of food and pills based on *Spirulina* are commercialised widely. Also has been used as human food for centuries, and forms part of the diet of tribes of Lake Chad and was used as food by aztecs in Mexico (Becker 1991).

In order to be used as food supply, the biomass obtained in the photosynthetic bioreactors has to be first harvested. The process of cell harvesting has already been described in TN 37.3. Basically, the system proposed in this study consists of two units: a centrifuge and a membrane module. The centrifuge is envisaged to recover the cells, producing a paste with a high percentage of liquid elimination (75% - 80%). The clear liquid obtained in the centrifuge would be then passed through a membrane module, with the objective to provide a complete clear liquid stream to be pumped to the next compartment. As the continuous centrifuge uses water to discharge the solid paste retained in it, that is the cells, there is a certain final degree of dilution of the cells, that typically are discharged from the centrifuge at a concentration of about 10-20 g/L.

It is clear that if the final use of the cells as food additive requires a lower percentage of water (for example, if they are required completely dried, or freeze-dried), then an additional step will be necessary to be incorporated for the elaboration of the final product.

There are as well other factors that should be considered at this point in order to define more precisely the process of preparation of the biomass as food. These are, at least the washing of the cells, in order to eliminate their excess of salts and the treatment of the food to get it free of microbiological activity and with the required degree of

water content. Clearly, these steps can be combined, as some operations can enable to reach simultaneously two of the objectives. Their selection and application depends also on the definition of the conditions envisaged in the final product.

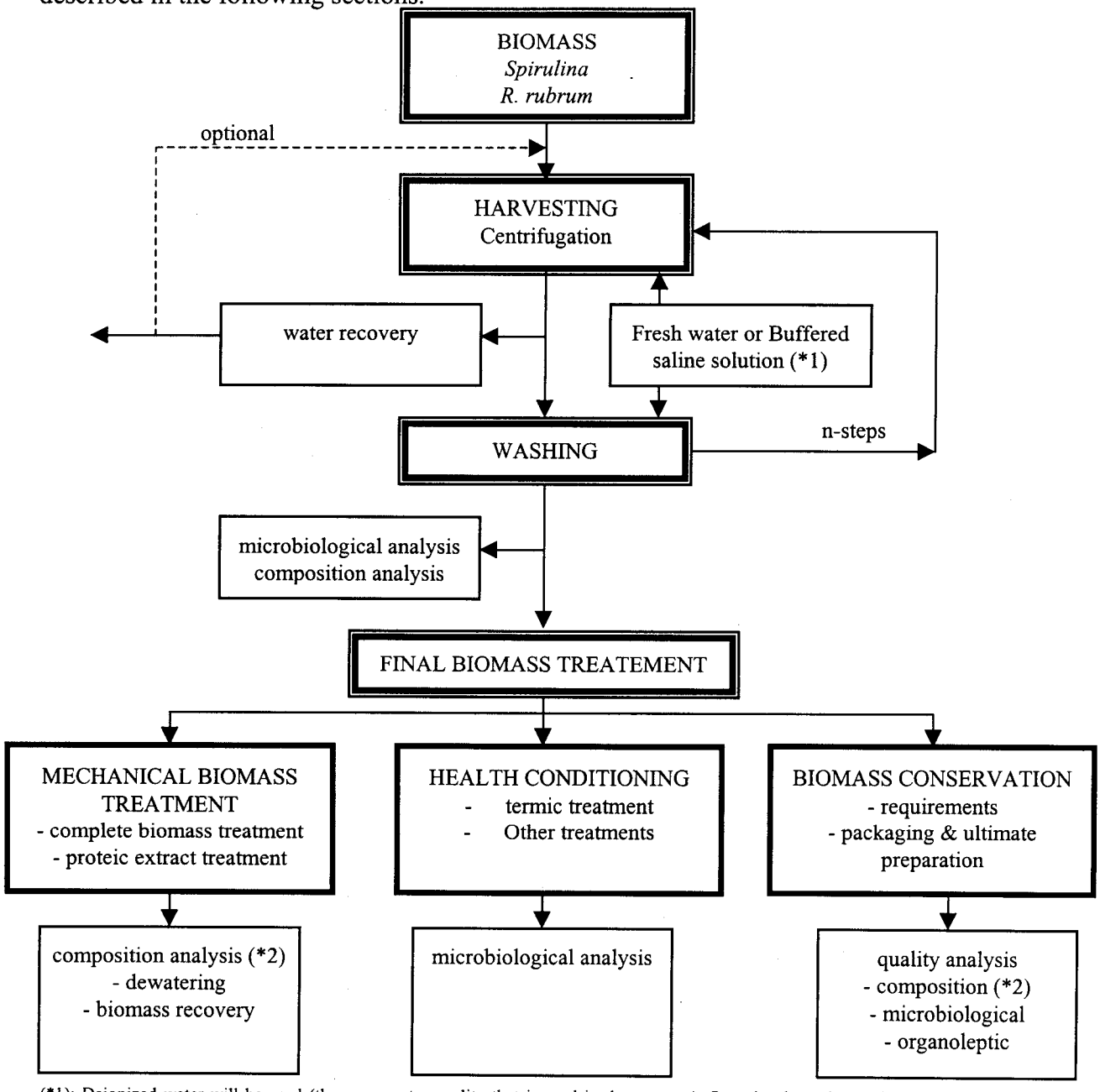
In this Technical Note, the definition of a procedure for the biomass conservation and preparation for the crew compartment is discussed, and two final possibilities are presented for consideration.

This work is presented in three parts. In the first part, harvesting, dewatering and additional specific treatment of biomass (mechanical treatment, health conditioning) and the effect of these treatments on the biomass quality have been included. In the second part, the conservation procedure is considered. In the third part the issue of the definition of the quality of the obtained biomass (composition and microbiological analysis) is addressed, and the corresponding analytical methodologies to characterise it are documented in the annexes at the end of the document.

2 PART I – TREATMENT OF BIOMASS

2.1 GENERAL PROCESS

The general scheme for the process required for the biomass recovery and use as food supply is described in the next figure. The details on each step the process is described in the following sections.



(*1): Deionized water will be used (the same water quality that is used in the reactors). Quantity depends on biomass volume and concentration of this biomass.

(*2): If biomass composition analysis is carried out after preliminary biomass processing, it may not be necessary to perform it again later.

Figure 1: General process out-line for biomass recovery and use as food supply.

2.2 BIOMASS HARVESTER

The function of the cell harvesting system at the outlet of the photosynthetic compartment is to separate the cells, to be further processed for food preparation, and the water, to be used in the next compartment of the loop. The different possibilities to carry out this function were analysed in TN 37.30, and a combination of two techniques, centrifugation for cell recovery, and membrane filtration of the liquid stream before connection to the next compartment were selected as the most appropriate processes for the pursued objective.

Spirulina as well as *Rhodospirillum* can be harvested by centrifugation, and many studies demonstrate that the same method can be carried out for both microorganisms (Becker 1981, Borowitzka 1988, Ripley and Fox 1996, Grizeau et al. 1996).

The centrifugation system proposed in TN 37.30 is a continuous disc stack centrifuge. In it, liquid is eliminated continuously from the centrifuge, while solids are retained, and discharged in a discontinuous mode. Water is used to proceed to such intermittent discharge of the solids from the centrifuge. This operation causes a certain dilution of the solids, but on the other hand it makes possible to combine the solid recovery from the centrifuge with water washing, as this is one of the steps required in the process, as discussed in the next point. Both steps, centrifugation and washing, can be carried out alternatively using centrifugation cycles, recovering cells and suspending them with fresh washing solution (buffered or saline solution if necessary).The use of buffered water in the centrifuge cycles allows to preserve cell integrity and therefore minimises the losses of cell components.

The global configuration of both steps is shown in Figure 2.

2.3 BIOMASS WASHING PROCEDURE

The purpose of biomass washing is to eliminate salts and some undesired compounds such as metals or residues from growth medium than could affect biomass quality. Some studies made with this type of biomass have verified that biomass washing reduces considerably the contents of elements that could produce problems related to human health (Borowitzka 1988, Ripley and Fox 1996).

The washing processes could be carried out before or after the harvesting processes. Both possibilities have advantages and disadvantages, and the decision also depends on the final harvesting system to be used.

To wash the cells before harvesting seems to be a first alternative, to considered. In this cell-washing mode, a membrane filter retains cells, and fresh wash solution is added to cell suspension at the same time filtrate is removed. Due to the occurrence of membrane clogging when a high cell concentration is used, this method of cell washing was discarded (See TN 37.30, part II).

In industrially used methods, biomass (in this case, *Spirulina)* fall onto a filtration screens and fresh water jets which also rinse salts from biomass. Later, slurry is taken to a vacuum-belt filter where it is dewatered and a paste-slurry is recuperated (Ripley and Fox 1996).

The harvesting method selected for food application is the centrifugation. Using a centrifugation system to harvest biomass cells can be washed with centrifugation cycles, recovering cells and suspending them with fresh wash solution. This alternative has the risk of cell disruption and some compound may leak out to the medium. This system may require a large amount of water (although the precise quantity will be optimised during the experimental phase in order to minimise it, and depends on cells concentration, and the operating conditions: feed flow rate and rpm that which the centrifuge works) that has to be later recovered.

Another important point to consider is pH and salinity of the washing solution Washing solution could produce cell disruption if pH it is not adequate. *Spirulina* grows in a basic pH (10.0 ± 0.5), very different from *Rhodospirillum* pH which tends to be

acid (6.9 ± 0.1). Fresh washing solution ought to have similar pH than cell growth medium. All practical references of *Spirulina* production show a washing step with fresh water (Becker 1981, Borowitzka 1988, Ciferri 1983, Lembi, Wharton, Smernoff, Averner 1988). This can be possible due to the cell wall structure of *Spirulina.* Therefore, it seeems that a washing step with water does not produce important osmotic changes in the cell, even though this point will require to be tested.

Rhodospirillum can be more sensible to osmotic changes than *Spirulina*, but on the other hand its culture medium pH is very close to neutrality and should not present any problem from a practical point of view.

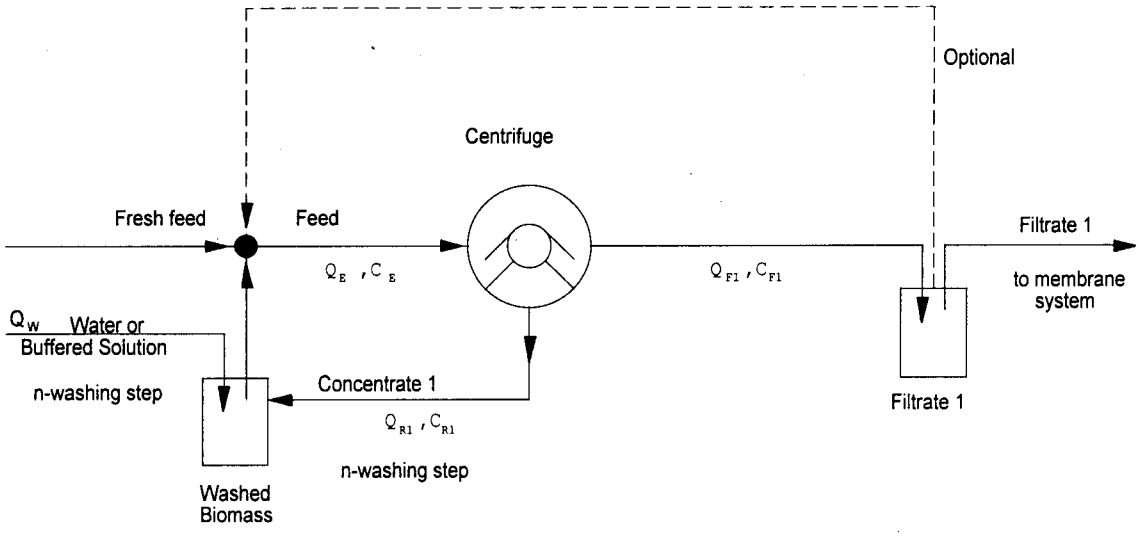


Figure 2: Global configuration of the biomass harvester and washing procedure.

Harvesting and washing proposed procedure is the same for *Spirulina* and for *Rhodospirillum.* Therefore, the same equipment can be used for both microorganisms.

Disc stack centrifuge will work at higher flow-rates that the ones described in TN 37.30 (5-10 l/h instead of 2 l/h). Increasing flow rates provide higher separation efficiency and minimize water consumption because a bigger amount of biomass is treated per each water discharge.

During the washing procedure some amount of filtrate 1 (see Figure 2) may be recycled to the centrifuge inlet in order to increase the inlet flow. This filtrate 1 will reduce additional water consumption and a better concentration factor for the biomass

will be obtained. N-washing steps will be conducted until salts content will be reduced at a specified value.

2.4 BIOMASS HEALTH CONDITIONING TREATEMENT

After the completion of the washing process it is necessary to ensure that biomass does not content any microbial contamination and, therefore can be consumed. There are different methods to reduce microbiological charge: thermal treatments as pasteurisation; sterilisation (Bettison and Rees 1991) and others methods that have been developed recently such as microwave, ohmic heating (Bettison and Rees 1991) and radiation (Chadwick and Ehlerman 1977, Stuart Thorne 1991).

Depending of the final product use and treatment, biomass has to be sterilised. In some techniques, this requirement is intrinsically satisfied by the technique. For example, in a drum-drying process,(Becker 1981) biomass temperature reaches high enough levels to assure biomass sterility. The same occurs if an extrusion-cooking (Fellows 1996, Stuart Thorne 1983) treatment is made. So, a previous and independent step of biomass sterilisation may be not always necessary.

Water percentage in biomass has also to be considered. Usually, it is easier to sterilise a low concentrated liquid rather than a high viscosity one and the hardware equipment required is not the same. There are less possibilities to use different methods when biomass is more concentrated. Another possibility to take into account is the biomass treatment inside the final packaging process.

Table 1 summarises advantages and disadvantages of the different biomass treatments considered.

Table 1: **Main characteristics of different biomasss treatment procedures.**

Technique	Advantages	Disadvantages
Pasteurisation	- Easy method, well known - Used in the *Spirulina* products factories - Destruction of pathogen micro-organisms - More economic than other methods if product has not to be stored for an indefinite time	- Destruction of microbiological charge is not total. Limited shelf life - Can produce losses of some nutrients as vitamins, pigments and protein denaturalisation if treatment is not adequate (time/temp. incorrect) - It needs special equipment if biomass is not liquid. Special heat exchangers are necessaries
UHT (ultra high temperature)	- It is an easy and well known system - Destruction of pathogens and others microorganisms that could damage the final product - Less modifications or losses of nutrients are produced - Product can be stored for a long time period	- It is recommended to use an aseptic packaging line next to UHT system. - It needs especial equipment if biomass is not liquid. Special heat exchangers are necessary
Ohmic heating	- Produces long-life products, does not require storage under refrigerated conditions - Does not degrade the product - Similar to high sterilisation	- It is know what effects can produce .on the cells. System only tested for foods that have big particles into a liquid - It is recommended to use a packaging aseptic line next to ohmic system.
Microwave	- Vegetative cells and spores are inactivated and water content reduced - Does not produce important modifications to product.	- Method in development. It is not known what effects could produce on the cells - Best for packaged food (in plastic)
Radiation	- Automated and clean method - Microbiological contaminants are destroyed - Product conserve nutrients and organoleptical properties	- Very expensive method, only recommended for high productions - In some countries it is not authorised because of problems with human health and safety - Very indicated for packaged food as a final method to preservation

Due to the biomass characteristics the best methods for sterilisation are classic

thermal treatments and radiation, but cost and safety problems of radiation are too high.

2.5 BIOMASS MECHANICAL CONDITIONING TREATMENT

To preserve biomass it is necessary to reduce its water content to less than 10%, otherwise different microbial growth could take place. Two systems allow to achieve a water activity reduction up to a totally safe level: froze and dry. From that point there are different possibilities. Selection of the method is based principally on some parameters like volume of biomass to be treated, production cost and biomass utilisation (to immediately consume or for a long conservation before consumption).

The presentation choosed when the product will be used shortly after its production (immediate consume) is the concentrated drink, like a "tomato juice". This presentation has a number of advantages with respect to others. One of them, and not the less important, is that the mechanical treatment for such presentation is much simple than for other presentations. It only requires a certain dewatering up to the desired concentration for an agreeable ingestion and a pasteurisation to provide the required safety protection.

The most commonly used methods to dry liquid products are drum-drying or spray-drying (Fellows 1996). The drum drying process ruptures the algae cell wall, making the valuable protein digestible, and at the same time sterilises the product (Becker 1981). Those advantages together with the fact this method is used in food industries make of it a good option to treat the biomass.

Modern methods such as extrusion can be a good alternative in the consumption of this kind of food, especially if organoleptic properties are consireded. Cooking extrusion process rises biomass temperatures around 170 °C and so it is sterilised. However, this method is only applicable for products with high added value (proteins, carbohydrates).

The possibility to use the microalgae protein as a single cell protein (SCP) (Bourland and Vodovotz 1998, Lembi and Jassby 1988, Nakhost and Karel 1989) has now gained prominence. This method has some advantages and disadvantages (see table 2), but it is an interesting application. The methodology to be used has many steps as shown in Figure 3 (Nakhost and Karel 1989) and only makes good use of a portion of the total biomass.

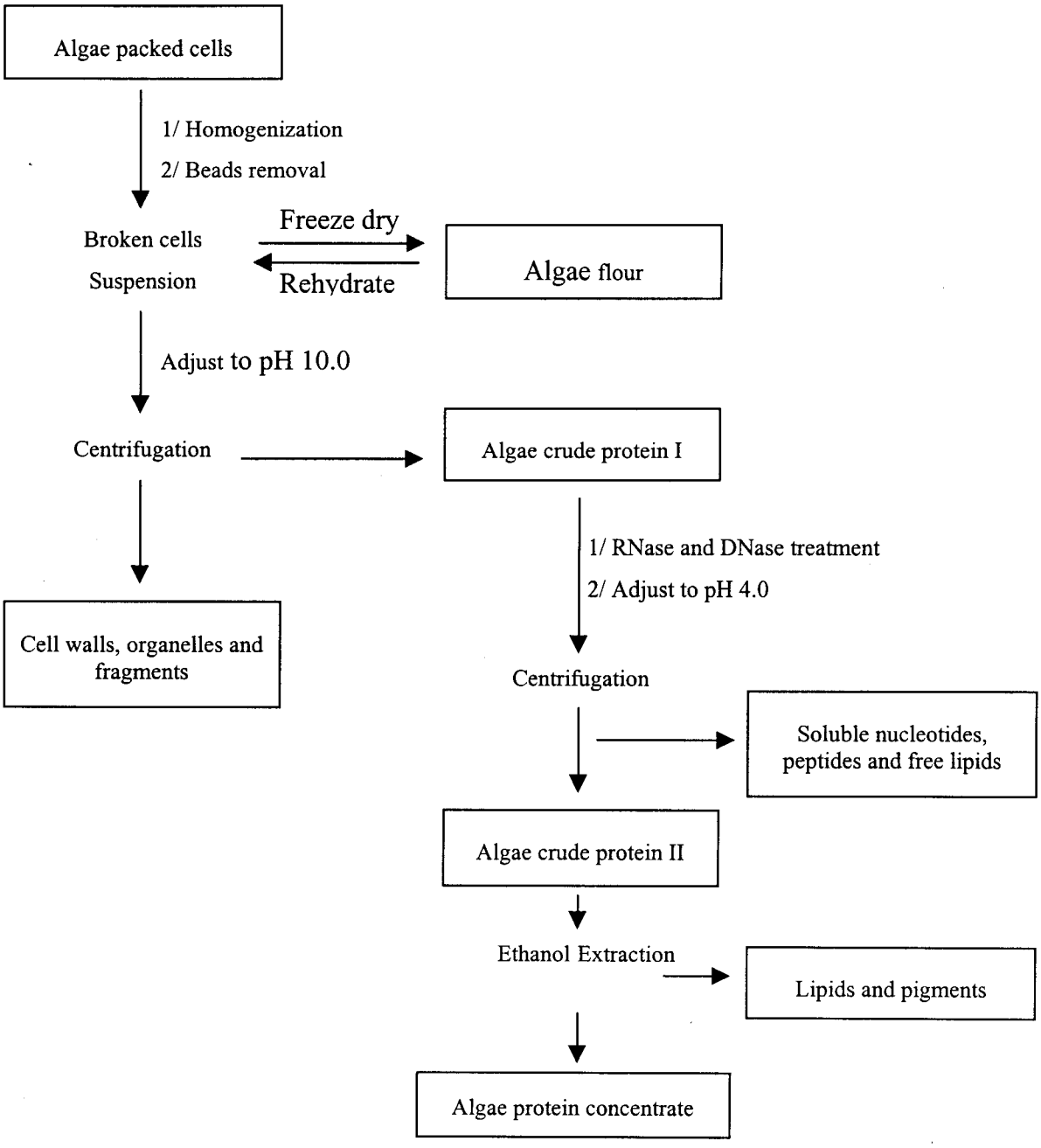


Figure 3: Stepwise procedure for algae protein concentrate preparation.

Another similar possibility (not considered in Table 2) would be to disrupt cell components with a mechanical method, for example, to obtain two main parts: one with the cell wall fiber components, and another with the rest of the cellular material. After disruption, these two fractions can be separated by centrifugation: in one hand, a liquid portion with nutrients and in the other hand one fibre compound. The liquid portion, could be treated with any of the previously exposed methods (pasteurisation, spray-drier, etc.) and so, work in better conditions than concentrated biomass. Fibre portion could be further treated to be digestible and to have a better texture.

Table 2 summarises the main advantages and disadvantages of the most common methods that could be used.

Table 2: **Mechanical biomass treatment summary.**

Technique	Advantages	Disadvantages
Fresh biomass - pasteurised	- Treatment is not necessary - Avoids losses of nourishes. - Aqueous presentation allows easy use of additives.	- Immediate consumption is necessary, otherwise can be preserved for a few days from 0 to 2°C - May produce organoleptic and physiological problems
Frozen	- It is an easy system to handle - Biomass may be preserved for a long time with crioprotectors - Best system from biomass storage	- Cell wall rupture may take place if not frozen properly - There are not standard criteria on which is the best freezing system and what temperature
Freeze drying	- High quality product - Does not degrade product	- Expensive method when production is high
Drum drying	- Cell wall rupture takes place and consequently digestibility of product is better - Increases protein efficiency ratio - Sterilises the product - High quality product	- Very expensive method - Loses of vitamins (B complex)
Spray drying	- Commercially widely used - It is fast and few space is required - Uniform product appearance - Easy packaging - May be mixed with other ingredients - High quality product	- Low solubility in water and juices - Oxidation is easy and needs to be packaged and conserved in darkness - Need a previous step of biomass sterilisation
Protein concentrate (SCP)	- Cell wall, nucleic acids and pigments are removed - Easy to incorporate in other products	- Expensive method - Maximum efficiency in operation is necessary
Extrusion	- Possibility to mix with other products (cereals, scentigs, flavours) - Enhances organoleptic properties (better taste and apearence) - Cooking extrusion sterilise product	- The capability to use this procedure is often unknown

3 PART II - BIOMASS CONSERVATION GUIDELINES

Conservation process depends on biomass treatment. In general, the following points have to be considered:

- Biomass should be conserved in anoxigenic atmosphere, without humidity and heat. Before storage it is necessary to make humidity analysis to assure proper product quality.
- The β-carotene is easily oxidizable, the product can lose colour, and produce organoleptic problems.
- Fatty acids can also be oxidizable. This problem may be avoided if an antioxidant is added and the product is packed in inert or modified atmosphere inside a metallic or plastic package.
- It is recommended to pack the powder in metal–coated plastic film. This kind of packaging protects against oxidation of β-carotene and accumulation of moisture.
- An aseptic packaging line after biomass treatment will minimize contamination risk.
- The type of package to be used depends on product characteristics, shelf life, package-food interactions, storage volume, etc.
- Conservation system depends on treatment and it will be necessary, in some cases, a microbiological and organoleptic analysis to assure product quality before product consumption.

4 PART III - BIOMASS QUALITY

4.1 PRELIMINARY CONSIDERATIONS

The traditional methods used in algae culture take place in lakes with moderate/high alkalinity. The microalgae are collected with a filtering system and then, dried in the sun. This method involves some risk, principally those related to the contamination of the biomass. A more advanced approach is the biomass cultivation in a tank specifically designed to enhance algae production. Harvest technologies have been tested as alternatives to the filtering system, i.e. coagulation, flocculation, sand filtration. Among them, the most commonly used method is centrifugation, due to the small size of the cells (Becker 1981). In most cases, a further concentration step or drying of the harvested microalgae slurry is necessary. In these cases methods such as drum drying or spray drying are normally used.

Microalgae produced in clean cultures will be utilised primarily as food, but one of the major drawbacks is the production cost. In some cases, in large-scale production when *Spirulina* grows naturally in a lake a pasteurisation step to exclude the presence of faecal bacteria is applied (Ciferri 1983).

Taking into account the previous literature concerning to microalgae production and utilisation, various criteria of quality for the utilisation of biomass as human food should be developed, including some aspects of nutritional-quality and toxicological criteria, in particular: approximated chemical composition, biogenic toxic substances (microtoxines, other toxins), non-biogenic toxins (wastes from cultivation and processing), biochemical nutritional studies, safety evaluations (feeding trials in animals) and acceptability.(Becker 1981, Borowitzka 1988, Ciferri 1983, Ripley and Fox 1996, Ortega 1991).

Data on the chemical composition of biomass (*Spirulina* and *Rhodospirillum)*

give key basic information on the nutritive potential of biomass. In addition, this proportion can be modified by specific cultivation conditions such as composition of the culture medium, and light intensity.

Evidence of the nutritional quality is only one of the basic requirements for successful utilisation of biomass in food preparations. Equally important is to guaranty the toxicological safety of the material. Some toxicological investigations have been successfully performed jointly with a satisfactory nutritional quality obtention by several authors (Ortega 1991, Saiz et al. 1993, Tranquille and Emeis 1997).

Critical biomass components have to be within specifications as defined by the corresponding analysis in order to have enough precision and fiability of the data obtained. In some cases the analysis error can make not possible the obtention of ultimate results and conclusions

Some compounds may change depending on treatment and preservation processes, therefore in some cases additional analysis will be required to verify final product quality, in addition to microbiological analysis.

All the pilot plant installation has to be built taking into account sanitary aspects that may produce biomass contamination like residence time in holding tanks, sedimentation, and others. For instance, the installation has a couple of tanks that work as buffer tanks between the biomass production in the reactor and its further processing. The tanks begins empty or almost empty and stores biomass until it can be processed in the following step, then the tank is drained until it becomes empty again. It is necessary to verify if the residence time of the biomass in the tank installed between the reactor and the centrifuge unit develops any contamination. The same problem has to be checked in the tank installed between the washing step and the final treatment step.

4.2 *Spirulina* BIOMASS QUALITY

Spirulina is the microalgae that has received more attention in terms of toxicological characteristics and properties. Relating to human studies there are less published results, but only in few cases intestinal problems were detected.

Chemical composition of *Spirulina* cells will give a basic information about their nutritional potential. The determination of the contents of their main compounds, and also toxic substances is important to determine if it can be used as food for human nutrition.

Firstly it is necessary to make chemical composition analysis of biomass, either freeze dried or fresh (results may change). Also, additional determinations of the quality of product will be necessary, for example, total protein, nucleic acids, chlorophyll, minerals and metals, in general any compound that can produce some toxicity and digestibility problems.

The following chemical analysis are proposed to characterise the quality of *Spirulina* as food:

- Water content
- Ash and ash compounds
- Chemical elemental composition: C, H, N, S , P(optional)
- Total protein
- Carbohydrates
- Fatty acids
- Nucleic acids
-Chlorophyll, phycocyanin and carotene

Frequency of analysis will depend on the duration of each step of the process and the source of the biomass. Analysis will be made in the steps pointed out in the general and final schemes shown in this TN.

4.2.1 Analysis of biomass compounds affecting quality

-Ash compounds: some compounds can produce health problems, and it is necessary to check if they are present within the security range (Na^+ and K^+ for example). Moreover it is possible to have a contamination with heavy metals (residues from cultivation and processing steps)

-Protein: protein ought to represent a 60-70% of total dry weight. Also it is necessary to make aminoacids analysis to check whether the proportion of essential and non-essential aminoacids is appropriate (high quality protein).

-Carbohydrates: carbohydrates percentage is a relevant parameter, as directly influences protein efficiency ratio (PER). Also, the quantity of carbohydrates that are difficult to degrade, because they are part of the cell wall, should be determined. Cell wall of cyanobacteria is composed of lipopolisaccharide and peptiglucan and does not contain cellulosic material.

-Vitamins: The data available on the vitamin cintent of micro-algae are very limited. S*pirulina* have highest vitamine B_{12} (unprocessed): 20g of *Spirulina* provide all the daily body requirements of B_{12} Vitamin B_{12} does not exist in the vegetables only in the meat, so it is important in the vegetarian's diets. It is combined with proteins and increases the value of SCP (single cell protein). It is interesting to know how processing of the algae after harvesting procedure affects vitamin content. HTST system do not affect vitamin B_{12} contens, but others termic treatments does. In the organism prevents against megaloblastic anaemia and contributes to the lipid metabolism and protein synthesis.

-Fatty acids: the ratio between unsaturated and saturated fatty acids (PIN/SAT) is an important coefficient for the definition of the biomass quality. For nutritional aspects (low cholesterol). *Spirulina* contains large amounts of polyunsaturated lipids, what makes it an interesting property as food supply

-Nucleic acids: may produce toxicity problems (uric acid in humans) when the intake is higher than permissive values (2g/day).

- **Chlorophyll and pigments:** toxicity problems caused by their degradation have been described, so high percentage is not permitted.

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4.3 *Rhodospirillum* BIOMASS QUALITY

Rhodospirillum have not been so extensively studied as *Spirulina* in human nutrition and toxicity data are less available. In rats food acceptability studies took place with success (Tranquille and Emeis 1997). The same considerations previously made for *Spirulina* can be extended to biomass analysis of *Rhodospirillum* with some differences

General chemical composition is slightly different and obviously it is very important to consider the complete biomass analysis.

Protein content is less high than for *Spirulina* Fatty acids content is different and polyunsaturated acids are not present in important quantities. Vitamin content of B_{12} is also important. *Rhodospirillum* can produce PHB and glycogen, and so accumulate them in the cell and decrease relative protein content.

Culture medium and pH are also different from *Spirulina* and ash compounds (minerals and heavy metals) may have other percentages.

4.3.1 Quality parameters

Taking into account the above related considerations, the analysis to be made for *Rhodospirillum* biomass quality will be the same as for *Spirulina*, except for special compounds such as:

-**PHB:** poly-B-hidroxibutiric acid is accumulated in the cell and may produce problems in harvesting step and in nutritional biomass value because reduce protein efficiency ratio (PER).

-**Glycogen:** is a reserve polisaccharide and also reduce protein efficiency ratio. Total value of glycogen can be compared with total carbohydrates value.

These two compounds increase their presence in biomass when culture medium is nitrogen limited as described in the literature (Grizeau et al.1996).

4.4 MICROBIOLOGICAL QUALITY

Biomass quality includes also microbiological quality. To assure this quality some characterisation in this aspect is also proposed. An analysis after harvesting procedure will determine eventual contamination degree and will enable to set the intensity of the treatment requirements to avoid the contamination.

Before biomass sterilisation or pasteurisation microbiological analysis must guarantee absence of:

-Total bacteria (4000 c.f.u/g- 20000 c.f.u/g)

-*E.coli* (absence)

-*Salmonella* (absence)

-Enterobacteria (absence)

-*Staphylococccus aureus* (absence)

-*Pseudomonas aeruginosa* (absence)

Bacterial detection test will be made as described in the Annex 1shown in this TN. List of pathogens is coming from literature, FAO/WHO food standards (Codex Alimentarius Comision) and NTS Ingredients S.L Company.

Permissive values change depending on each aliment and the particular standard for pathogens, bacterial absence it is necessary after sterilise or if the product will not be termically treated. Before sterilisation these values are not expected to be high. Depending on the contamination, treatment has to be more or less severe. For example:

E.coli: killed in 1h. at 55ºC and 15'-20' at 60 ºC.

St.aureus: killed at 60 ºC in 1h.

After the microbiological treatment, an additional analysis will determine if treatment has been applied successfully. The final product will be tested and controlled by an additional analysis made on a sample product.

5 PROPOSED PROCESS FLOWSHEET FOR BIOMASS RECOVERY AND UTILISATION AS FOOD SUPPLY

The complete process proposed includes two final possibilities (two product presentations). In the first presentation, concentrated drink like a "green/red (*Spirulina/Rhodospirilum*) juice", the biomass is taken after harvesting and washing process at a concentration that ranges from 10 to 20 gr/l. This concentration may be increased up to the desired value in order to provide a reasonable amount of biomass per litter of drink. Additionally, the biomass has to be pasteurised in order to guarantee the proper elimination of any possible microbiological contamination. After those two treatments the product can be packed and preserved in refrigerator at temperatures not higher than 4ºC, and has to be consumed no later than a few days after.

During the pasteurisation process as well as during the packaging and storage of the product, it may experiment some changes in their composition (lost and/or degradation of some components), and organoleptic changes (colour, flavour and taste) that have to be controlled. To make the product more attractive to the consumer it may be certainly possible to add some additives to correct the colour, flavour and taste or some preservative to prevent its degradation. All those aspects will be tested at the end of the process according the quality analysis results.

The second presentation proposed is as powdered biomass. In this case the biomass is taken after harvesting and washing process and is driven to a drum-drying system. This system has two heated rotary drums. The biomass falls over the rotary drums and the excess water is eliminated by evaporation due to heated drums surface. In this process the biomass temperature rises so high values that it becomes sterilised. This fact together with the remaining advantages exposed in Table 2 lead to the inclusion that this system as another good alternative for the biomass treatment. The product may be vacuum-packed, or modified atmosphere packed and can be stored for long time periods. In order to verify final product quality the required analysis will be carried out.

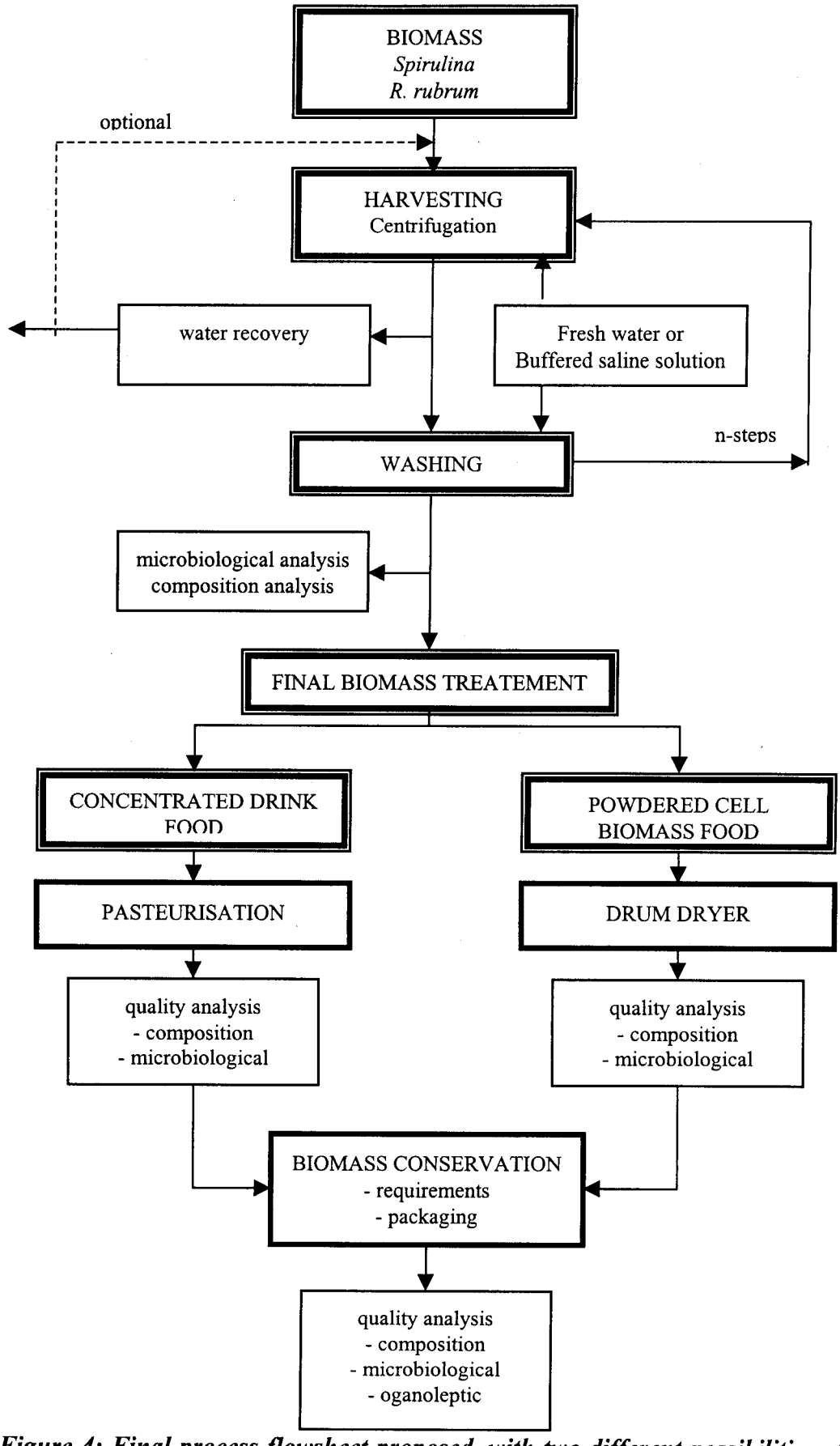

Figure 4: Final process flowsheet proposed, with two different possibilities of final food presentation, concentrated drink or powdered biomass

The experimental runs in order to demonstrate the application of the different techniques and its effect on biomass quality will be performed in the next part of the work, taking profit of the operation of the MELISSA pilot plant reactors, and will be reported in TN 43.222. In these tests several equipment will be required. Continuous centrifugation is available in the Chemical Engineering Department. Pasteurisation will be applied in cooperation with the Food Technology pilot plant in UAB. Drum drying and also atomisation are available through the cooperation with two local companies using the thecniques intensively, which have offered the use of their pilot plants. No attempts will be made to purchase this costly equipment, as the main objective is the trade-off of the methodology and the assessment of its impact on the food quality.

6 REFERENCES

Becker E.W. Algae mass cultivation-Production and utilisation. *Process Biochemistry*, Aug/Sep (1981),p.10-27.

Bettison J, Rees J.A.G. Procesado térmico y envasado de los alimentos. De. Acribia S.A, 1991.

Borowitzka & Borowitzka. Micro-algal biotechnology. Cambrige University press (1988)

Bourland Ch, Vodovotz Y. NASA experiments with food for interplanetary travel. *Food Technology*.. Vol.52, nª6 . (June 1998), p.13-14.

Chadwick K.H, Ehlermann D.A.E. Manual of Food Irradation Dosimetry. International Atomic Energy Agency, Vienna. Technical Reports series. nº 178.1977.

Cheftel. Introduction a la biochmie et a la technologie des aliments. Volume 2.(1977). Technique et documentation. Enterprise modernes d'edition.

Ciferri Orio. Spirulina, the edible micro-organism. *Microbiological reviews.* (Dec.1983), p.551-578.

Ciferri O, Tiboni O. The biochemistry and industrial potential of *Spirulina .Ann.Rev.Microbiol.*(1985).39:503-26.

Fellows P.J. Food processing technology. Principles and practice. Woodhead publishing limited. Cambridge.(1996).

Grizeau D, Dupre C, Guary J.C, Bernard S. Production of freeze-dried biomass of *Rhodospirillum rubrum.* Furniture and analytical results. (T.N. 29.2) Equipe Biotechnologies Marines. Conservatoire National des Arts el Métiers. Institut National des sciences et Techiniques de la mer. Cherbourg. Delivery to ESA_ESTEC 1996.

Lembi Carole. Algae and human affairs **Jassby A** Spirulina a model for micro algae as human food. Cambrige University press. (1988),p.151-179.

Lembi Carole. Algae and human affairs. **R Wharton, D Smernoff, M Averner**. Algae in space.. Cambrige University press. (1988),p.485-509.

Nakhost, Z., Karel M.. Potential utilisation of algal protein concentrate as a food ingredient in space habitats. *Science des aliments* 9 (1989),p. 491-506.

Oguchi Mitsuo, Otsubo Koji. Closed and continuous algae cultivation system for food production and gas exchange in cells. Space reserche group. *Adva.Space Res* vol 9, n8, (1989),p.169-177.

Ortega Calvo J.J. Evaluación nutricional y toxicológica de algas comestibles:composición quómica.Ph. D. IRNAS. Sevilla (1991).

Ripley and Fox. Spirulina, production and potential. Edisud (1996). Aix-en-Provence.

Saiz-Jimenez Ortega Calvo J, Mazuelos C, Hermosín B. Chemical composition of Spirulina and eukaryotic algae food products marketed in Spain. *Journal of Applied Phycology* 5, (1993),p.425-435.

Stuart Thorne. Developments in food preservation. Developments series. Elsevier Applied science publishers, (1983).

Tranquille N, Emeis J.J. *Rhodospirillum rubrum.*Food acceptability study. TNO Prevention and Health. Division of Cardiovascular Research. Leiden, the Netherlands, (1997).

ANNEX 1. METHODS OF ANALYSIS FOR EDIBLE BIOMASS CONSERVATION AND PREPARATION

Table of Contents

1 Mineral composition [1]

Analysis of Ca, P, Fe, Na, CL, Mg, Mn, Zn, K

a) Material and reagents

- Silica capsule
- Oven at 450°
- DW. Distilled water
- $HClO_4$
- HNO_3
- HF
- Atomic absorption spectrophotometry

b) Procedure:

- Dry the sample during 16h at 70-80°C. Weight 1g of sample in a silica capsule. Put into an oven during 2h at 450°C. Mix with 2ml water and add 5ml of $HClO_4$ 1:4, 5ml to H_3NO 1:4 and 5ml HF. Dry with hot. Add HCl ate and few ml of distilled water. Cold slowly and filter and dilute to 30ml with distilled water.
- Use the spectrophotometer to determine every compound. For the calibration procedure use standard solution treated in the same way as the samples.

[1] This analysis will be done by the Chemical Analysis Deparment of UAB (Universitat Autònoma de Barcelona)

2 Total Proteins

Lowry modified method

a) Reagents

- (i) 5% Na_2CO_3
- (ii) 0.5 % $CuSO_45H_2O$ in 1% sodium potassium tartrate
- (iii) To 50 mL of reagent (i) add 2mL of reagent (ii), prepare immediately before use and do not keep.
- (iv) Diluted Folin-Ciocalteau reagent. The total acidity of the concentrated reagent is determined by teatring a sample with 1.0 N NaOH using phenolphthalein as indicator. It is then diluted with distilled water to make the total acidity exactly 1.0 N
- (v) Protein standard: bovine serum albumin, 0.2 mg/ml.

b) Procedure.

Measure 0.5 mL of washed cell suspension (0.1-mg dry weight of cells) into an ordinary test tube, add 0.5 mL of 1.0 N NaOH, place in a boiling water bath for 5 min, and cool in cold water. Add 2.5 mL of reagent (iii), allow to stand 10 min, and rapidly add 0.5 mL of reagent (iv). A reagent blank containing 0.5 mL of distilled water instead of cell suspension, and a set of standard protein solutions (0.05-0.2 mg protein) are treated in the same way, including the heating stage. After standing 30 min to allow full colour development, measure all optical densities against the reagent blank at 750 nm.

. Calculate the protein concentration of the sample by interpolation of its net absorbance value in the standard curve.

Note: The absorbance obtained is pH and time dependent.

3 Aminoacids composition

Aminoacid concentration is determined by high-pressure liquid chromatography HPLC, using an aminoacid derivatization technique, before aminoacid separation.

a) Material and reagents:

- HCl 6 M
- Filter (Millipore 0.45 µm, cellulose acetate filter)
- HPLC equipment:

  Chromatograph: HP-1090 serieII, HP

  Column: Reverse phase column (Aminoquant 200 x 2.1 mm, ref 79916AA-572, HP)

  Guard column cartridge: 20 x 2.1 mm

  Cartridge holder for guard column: 79900CH-010

  Control sample kit: OPA and FMOC (5061-3353, HP)

  Standard aminoacid solution

- Eluent A: sodic acetate (20 mM) / 0.3% THF / 0.018% TRIETHILAMINE(TEA)
- Eluent B: sodic acetate (100 mM) / Acetonitrile/ Methanol (20/40/40)

b) Procedure:

- Hydrolize the sample with HCl 6N at 110ºC, 24 h. Filter the hydrolyse from the cell preparation. Derive with OPA (primary aminoacids) and FMOC (secondary aminoacids) reagents, using Aminoquant method and analyse with the HPLC equipment described. Compare with standards results.
- Analysis method: The detection is done at 338 nm for primary aminoacids and 262 nm for secondary aminoacids. Oven temperature is 40 ºC. This method has a special injection program to derivate the samples before they are injected and a gradient of two eluents to separate all aminoacids in 18 min.

4 Total carbohydrates

Phenol method (Herbert 1971)

a) Reagents:

- (i) 5% (w/v) solution of phenol in water
- (ii) Concentrated sulphuric acid
- Glucose standard

b) Procedure:

Into a test tube, add with a micropipette 1.0 mL of sample containing the equivalent of 0.002-0.1 mg glucose. A reagent blank containing 1 mL of water, and a set of glucose standards (0.025, 0.05, 0.075 mg glucose, in a volume of 1 mL) are prepared at the same part. To all tubes add 1 mL of 5% phenol and mix, then from a dispensing micropipette add 5 mL of concentrated sulphuric acid, directing the stream of acid on the surface of the liquid and shaking the tube simultaneously, to produce fast and complete mixing. The tubes are allowed to stand 10 min, shaken, and placed in a water bath at 25 to 30 °C for 10 to 20 min before measurements are taken. The absorbance of the characteristic yellow colour is measured at 488 nm.

5 Determination of lipid content.

a) Reagents.

- Palmitic acid.
- Lipid standard solution. 1 mg/iml of palimitic acid in chloroform.
- Dichromate solution. 2.5 g/1 of $K_2Cr_2O_7$ in H_2SO_4.

b) Procedure

Add 0.2 volumes of water to the combined chloroform-methanol extracts of the cells. Shake the solution for 5 min to mix well and centrifuge to separate the phases. Collect the organic (lower) phase, leaving behind a precipitate that forms at the interphase. Discard the aqueous (upper) phase. Evaporate the chloroform-methanol solution under a stream of N_2 to a final volume of 2 ml. Transfer 0.05, 0.1, 0.15, 0.20, 0.25 and 0.30 ml of the lipid standard solution to marked 5- or –10 ml screw-capped tubes. Transfer 0.1, 0.2 and 0.5 ml of the unknown lipid sample to marked tubes. Evaporate all tubes to dryness under vacuum or a stream of N_2. Add 2 ml. of dichromate solution to all tubes and cap with Teflon-lined caps. Place all tubes in a boiling-water bath for 45 min. Shake the tubes two or three times during the heating. Cool the tubes, remove 1.0 ml of each, and dilute to 10.0 ml with water. Read the absorbance of each tube at 350 nm against at H_20 blank. Plot a standard curve with the known lipid samples and determine the unknowns graphically or by Beer's law. Note that the assay is based on the disappearance of absorbance at 350 nm as the dichromate is reduced by increasing. amounts of lipids. It is thus convenient to plot the standards as the reciprocal of absorbance against lipid concentration.

6 Fatty acids composition

Fatty acids are analyzed as the methyl esters by acid hydrolysis, esterification and transmethylation , using a H_2SO_4/ CH_3OH

a) Material and reagents:

- Sulfuric acid concentrated
- Chloroform
- Methanol
- Distilled water
- Hexane
- Sulfuric acid/methanol: place 96 mL of methanol in an ambar glass botlle and very carefully add 4 mL of concentrated sulfuric acid.
- Gas chromatography equipment with a FID (260 °C) and automated injection system
- Capillary chromatography column: HP-INNOWax (30m x 0.25 mm x 0.25 μ m)

b) Procedure

- After extraction with chloroform/methanol (2:1) evaporate the solution under a stream of N to a final volume of 2 ml. Add 2 mL of sulfuric acid in methanol Place in a thermosthatic bath 1h at 100 °C. Add 2 mL of distilled water. Extract with hexane (1ml). Filter (0.45 μm, Millipore organics)
- Inject directly into a gas chromatograph/FID. Fatty acid methyl esters are resolved by capillary chromatography using splitless injection. The analysis uses an external standard technique.
- GLC Analysis conditions:

  Inlet: 1.5 μl, 260°C

  Oven: 150 °C (1min), 2,9°C/min, 230 °C (1 min)

  Detector: FID 260 °C.

7 Determination of DNA

a) Reagents

- DNA standard solution.
- Aqueous acetaldehyde.
- Diphenylamine solution.

b) Procedure

Calf thymus DNA is dissolved in 5 mM NAOH at a concentration. of 0.4 mg/ml. A working solution is made every 3 weeks by mixing an aliquot with an equal volume of N $HClO_4$ and heating at 70 °C for 45 min.

Acetaldehyde is cooled and 1 ml is transferred with a cooled pipette into 50 ml of H_2O. This solution is stable at 4 'C for several months (acetaldehyde is flammable and should be stored in the cold and thoroughly chilled before opening,).

Dissolve 1.5 g of diphenylamine in 100 ml of glacial acetic acid and add 1.5 ml of conc. H_2SO_4. Stable at 4 °C for up to 3 months. Just before use add 0.1 ml of aqueous acetaldehyde per 20 ml of reagent.

Add 5 ml of 0.5 N $HClO_4$ to another of the acid-extracted, lipid-free pellets and resuspend the sample by vortexing or inversion. Incubate the sample at 70 °C for 45 min. Centrifuge and remove the supernatant. Transfer 0.1, 0.2, 0.3, 0.4 and 0.5 ml of the calf thymus DNA stock solution to marked screw-cap test tubes and adjust the volume to 2 ml with 0.5 N $HClO_4$ to serve as a reagent blank. Transfer 0.5, 1.0, and 2.0 ml of the unknown DNA sample to marked screw-crap test tubes and adjust all volumes to 2 ml with 0.5 N $HClO_4$. Add 4 ml of the diphenylamine reagent to all tubes and mix well. Incubate all tubes at 30 °C for 16-20 h and read absorbance at 600 nm. Plot a standard curve with the standards and determine unknown DNA concentrations by interpolation if necessary.

8 Determination of RNA

a) Reagents.

- RNA standard solution.
- Orcinol reagent.
- Orcinol stock solution.
- Cupricion solution.

b) Procedure

Dissolve yeast RNA in water to a concentration of 10 μg/ml. Store frozen. Dissolve 0.15 g of $CuCl_2.2H_20$ in 100 ml of conc. HCl. Stable at room temperature. Dissolve 12.5 g of Orcinol to a final volume of 25.0 ml in 95% ethanol to obtain the Orcinol stock solution. For the Orcinol reagent mix 2 ml of Orcinol stock solution with 100 ml of cupric ion solution. This solution should be freshly prepared for each determination.

Add 2 ml of 0.3 ml N KOH to the remaining acid-extracted, lipid-free pellet and incubate for 18-24 h at 30 °C. After the hydrolysis period, cool the sample on ice and acidify the solution to pH 1.0 (pH paper) with conc. $HClO_4$. Centrifuge and carefully remove the supernatant. Wash the pellet with 1 ml of cold 0.2 N $HClO_4$. Combine the supernatants. Transfer 0.2, 0.4, 0.8, 1.0, 1.5 and 2.0 ml of the RNA standard solution to marked screw-cap tubes. Transfer 0. 1, 0.2, and 0.5 ml of the unknown RNA solution to marked tubes and adjust the volume of each to 2.0 ml with H_2O. Include a blank containing 2.0 ml of H_2O. Add 2 ml of the Orcinol reagent to each tube, seal with teflon-lined caps, and place the tubes in running water and read the absorbance of each tube at 665 nm. Plot the absorbance of the RNA standards against the RNA concentration to obtain a standard curve. Determine the concentration of he unknowns against the standard curve.

9 Chlorophyll and phycocyanin measurement.

Chlorophyll Measurement.

a) Material.and reagents

- Acetone 80%.
- 0.45 μm Millipore filters (acetone resistant).
- Ultrasonic homogeneizer.
- Dual cell spectrophotometer.

b) Simple treatment and procedure.

- Filter 10 ml of a culture suspension to separate the culture media.
- Add the filtrate to a 5 ml 80% acetone in a test tube.
- Submit sample to sonication 30 min to disperse the sample.
- Wait 2 min.
- Filter the sample with the Millipore filter.
- Measure absorbance in the spectrophotometer at 663 nm against an acetone blank.
- Calculate concentration using 8200 m^{-2}/Kg at 663 nm as extinction coefficient. Or alternatively as (Sestak 1971):
- A_1= Absorbance at 664 nm.
- A_2= Absorbance at 647 nm.

Chlorophyll a ($x10^{-3}$ Kg/m^3)=11.78 A_1-2.29 A_2

Phycocyanin Measurement

a) Material and Reagents

- 0.45 μm *Millipore* filters.
- Ultrasonic homogeneizer (Vibra Cell , Sonics materials inc.Danbury.USA).
- 0.05M K-Phosphate buffered solution pH 7.25 mM EDTA.
- Liquid nitrogen.
- Dual cell spectrophotometer.

b) Simple treatment and procedure.

- Filter 10 ml of a culture suspension to separate tile culture media.
- Add 5 ml of phosphate buffered solution previously used.

- Submit sample to sonication during 30 min to disperse the sample and break tricomes.
- Freeze the sample in liquid nitrogen.
- Let the sample equilibrate overnight at 20 °C.
- Measure the absorbance of the sample at 652 and 615 nm against a phosphate blank
- Calculate phycocyanin concentration as (Siegelman 1980)
- A_1= Absorbance at 615 nm.
- A_2= Absorbance at 652 nm.

Phycocyanin (PC)(Kg/m^3)=(A_1-0.474 A_2)/5.34
Allophycocyanin (APC) (Kg/m^3)=(A_2-0.208 A_1)/5.09

10 Carotenoid Determination

Spectrophotometric method

Liaaen-jensen (1971) (Carotenoid)).

a) Material and reagents:

- Spectrophotometer: fixed wavelength 475 nm.
- Acetone:methanol solution 7:2 (v:v).

b) Sample treatment:

- - Add 10 ml of acetone/methanol solution to a freeze dried sample (v.g. 5 mg) and dissolve it. Alternatively centrifuge (10 min., 15,000 *g)* 10 ml of culture solution (or the equivalent to have about 5 mg of distilled water). Discard the supernatant. Wash the pellet with double distilled water. Centrifuge again in the same conditions. Discard the supernatant. Add 10 ml of the acetone/ methanol solution. Redissolve the pellet.
- Centrifuge again 10 min. 15,000 g.
- Retain the supernatant.

c) Analysis:

- Transfer the supernatant to a quartz cuvette.
- Read the absorbance at 475 for carotenoids. Read the absorbance of a acetone/methanol solution blank at the same wavelength.

d) Results.

- Substract absorbance of the sample from the blank.
- Calculate pigment concentration using the following absortion coefficients:

Carotenoids: E= 250 (ml mg^{-1} cm^{-1})

11 PHB content determination.

Law & Slepecky (1960).

a) Material and reagents.

- Spectrophotometer: fixed wavelength 235 nm.
- Reactants:
- Sodium hypochlorate solution (10-40% av. chlorine),
- Acetone.
- Absolute ethanol.
- Chloroform.
- Concentrated sulphuric acid.

b) Sample treatment.

- Add 1 ml of sodium hypochlorate to a freeze dried sample (10-20 mg).
- Incubate 1 h at 37 °C.
- Add 4 ml of double distilled water.
- Mix and centrifuge 10 min. 12000 g. Discard the supernatant.
- Wash the pellet with 5 ml of acetone.
- Centrifuge in the same conditions. Discard the supernatant.
- Wash the pellet with 5 ml of absolute ethanol.
- Centrifuge in the same conditions. Discard the supernatant.
- Add 3 ml of hot chloroform. Wait 1 or 2 min. Mix it
- Centrifuge in the same conditions. Keep the supernatant in a different tube.
- Repeat the chloroform extraction two more times and mix the extracts.
- Complete the extracts to a known volume (vg. 10 ml).
- Take a sample of the chloroform extract containing *5-50 μg.*
- Evaporate the chloroform in a boiling bath.

- Add 5 ml of concentrated sulphuric acid, and cap the tube
- Incubate 10 min. in a boiling bath.
- Cool and mix it.

c) Analysis.

- Transfer the sample to a quartz cuvette.
- Read absorbance at 235 nm against concentrated sulphuric acid (95%-98%).
- Read absorbance of a heated sulphuric acid blank.
- Substract the absorbance of the sample from the blank.

d) Results.

Calculate the concentration of the PHB using a molar extinction coefficient of 1.56×10^4 ($l.mol^{-1}.cm^{-1}$) (crotonic acid)) and assume as 86 the average residue weight of the polymer. Hence 0.551 μg /ml of PHB give an absorbance of 0.1.

PHB concentration = net absorbance * 137.5 / dry weight.

Note: It is advisable to run spectra on pure samples of treated PHB or crotonic acid to check stray light on the spectrophotometer. Use high quality sulphuric acid.

12 Glycogen measurement

Glycogen measurement: Palmsternia modified method.

a) Reagents:

1.- Absolute ethanol

2.- 60% Ethanol

3.- Potassium hydroxide 30%.

4.- Ether.

b) Sample treatment:

- Add 1 ml KOH 30% to the biomass sample (about 5 mg)
- Incubate 30 min. in a boiling water bath.
- Add 3 volumes of water and 8 volumes of ethanol.
- Centrifuge 10 min. 12000 g. Discard the supernatant.
- Wash the pellet twice with 60% ethanol.
- Centrifuge each time in the same conditions.
- Dry the pellet with absolute ethanol.
- Wash the pellet with ether.

(Further purification is possible by dissolution in water (100 mg/ml) and precipitation at 0 °C in acetic acid 80%).

(In case of high lipid content, wash the sample with ether prior to KOH treatment (Herbert 1971)).

To determine the glycogen concentration, follow the analysis for the total carbohydrate method. Express the results as glucose equivalents per unit of sample dry weight sample.

13 Microbiological analysis

13.1 TOTAL BACTERIA

Microbiological analysis will be done with a sample suspension in a sterile solution from which it can be made successive dilutions. Before this, it is necessary to grin and homogenise the sample

a) Material and reactants

- Sterile flask of 200ml.
- Sterile pipette of 1ml
- Agitator
- Sterile Petri dishes
- Oven at 30 °C
- Thermostatic bath
- Tryptone water[2]
- Plate count agar (PCA)

b) Procedure

- Weight in aseptic conditions 10g of sample approximately. Add so many ml. of tryptone water than weighted sample multiplied by 9, for obtain a dilution of 1/10
- Transfer 1ml of this suspension to a tube with 9 ml of tryptone water. Repeat this operation until obtain a dilution of 10^{-4}.
- Mark two Petri dishes for every dilution, including the 1/10 dilution
- With a sterile pipette add 1 ml of every dilution in agar plates, in order from

[2] Composition of tryptone water: Tryptone (10g), Sodium chloride (5g), distilled water (1000 mL). Dissolve all ingredients in distilled water and ajust the pH to 7.5. Sterilize at 120 °C for 20 min.

more diluted to less diluted

- Add 15 ml of culture medium, cooled at 45 °C, every plate.
- Swirl plates to mix thoroughly and allow to cool.
- Solidify the agar, reverse the plates and incubate them 72 h at 30 °C.
- Count all colonies on selected plates containing 25 - 250 colonies promptly after the incubation period. If plates from two consecutives decimal dilutions yield 25 to 250 colonies each, compute the count per gr or ml for each dilution and report the arithmetic average as the CFU per gr or ml, unless the higher computed count is more than twice the lower one.

13.2 –*E.coli*

For *E. coli* investigation it is necessary to start from the results obtained in the coliform counting.

It is used the more probably number (MPN) technique for a recount of coliforms and *E. coli*

a) Material and reagents

- Sterile Erlenmeyer flask of 200 ml.
- Sterile pipettes of 10 ml. and 1ml.
- Agitator
- Standard sterile loop or needle
- Oven culture at 35 °C
- Tryptone water
- Lactose broth, brilliant-green bile (BGBL)
- Mac Conkey agar
- Saline solution
- Peptone water (test for indol probe)
- Clark and Lubs broth
- Simmons citrate agar
- VP reagent (Voger-Proskauer)
- Red methyl indicator
- Indol indicator

b) Procedure

- Obtain a dilution of 1/10 with 10 g of sample.
- Transfer 1 ml of this suspension to a tube with 9 ml to tryptone water. Repeat this operation until obtain a dilution of 10^{-2}.
- Prepare three series with three tubes with 10 ml of BGBL medium. Mark all

tubes of each serie with its corresponding dilution.

- In each tube of one first serie add 1 ml of initial solution. In each tube of the second serie add 1 ml to 10^{-1} dilution. In each tube of the thirst serie add 1 ml to 10^{-2} dilution.
- Incubate the series at 35 °C during 24-48 h.
- Calculate the MPN of coliforms from the positives tubes of each serie (reaction is positive when gas is produced into a Durham bell) and from correlation in a MPN table.
- Transfer one sterile loop from each tube of BGBL positives (with gas), to another that contains 10 ml of BGBL broth
- Incubate 24 h at 45 °C
- From positives tubes, transfer a loopful to Mac Conkey agar plates using a streak technique.
- Incubate 24 h at 35 °C. In Mac Conkey agar *E. coli* colonies have a red violet colour.
- To confirm *E. coli* presence make the following biochemistry tests (test IMViC):
  - Indol production
  - Methyl red
  - Voger-Proskauer (VP)
  - Citrate utilisation
- Select two or three colonies and resuspend it with physiologic serum
- Transfer two or three drops of suspension to every biochemistry probe. Incubate them at 35 °C during 24 h.
- All lactose fermentative cultures, with a gas production and which IMVIC result are ++-- or -+-- are probably *E. coli.* MPN is determined from all tubes that show contain *E. coli* reading at the MPN tables.

13.3 *Salmonella*

a)Material and reagents

- Sterile flask of 250 ml.
- Sterile pipettes of 10 and 1 ml.
- Agitator.
- Sterile loop or needle
- Oven culture (35and 42 °C)
- Microscope slides
- Peptone water[3]
- Selective broth Muller-Kaufman (tetrathionate $S_40_6^=$)
- Selective selenite broth cystine
- Xylose-lysine desoxycholate citrate(XLD)
- Iron triple sugar agar (Kliger)
- Lysine- iron-cystine agar (LIA)
- Identification serie with a comercial biochemical multitest systems (API 20E biomerieux)
- Serological test: polyvalent agglutination serum anti 0

b) Procedure

For *Salmonella* isolation few steps are used

- 1-To preenriched
- 2-Enriched
- 3-Isolation

[3] Composition peptone water: :peptone (10g), Sodic chloride (5g), Sodium Hidrogenphosphate (9g), Potassium dihydrogenphosphate (1.5g), Distilled water (1000 ml). Dissolve all ingredients in distilled water, and ajust the pH to 7.0. Sterilize at 120 °C for 20 min.

- 4-Biochemistry confirmation
- 5-Serologic confirmation

- Weight in aseptic conditions 5g of sample approximately. Add so many peptone water ml than sample quantity multiplied by 9, to obtain a 1/10 dilution
- Incubate at 37 ºC during 18-24 h.
- Transfer 1 ml of 1/10 dilution to 10 ml tetrationate broth and 1 ml of selenite cystine broth.
- Incubate selenite broth at 35 ºC during 24 h and tetrationate broth at 42 ºC during 24 h.
- From every medium, spread with a needle one XLD plate. Incubate at 35ºC during 24h.
- Spread possible colonies of *Salmonella* (red colognes with a black centre) in a Kliger and LIA. Incubate 24 h at 37 ºC.
- Confirm their identification looking at identification tables.
- Biochemistry and serologic identification with a commercial multitest kit

13.4 -Enterobacteria

Colony count method

a) Material and reagents

- Sterile Erlenmeyer flask of 200 ml.
- Sterile pipettes of 10 and 1 ml.
- Sterile Petri dishes
- Oven culture (35 °C)
- Thermostathic bath
- Tryptone water
- Violet glass agar, neuter red, bilis, glucose (VRBG)

b) Procedure

- Weight in aseptic conditions 10g of sample approximately. Add so many tryptone water ml than weight sample quantity multiplied by 9, to obtain a 1/10 dilution
- Transfer 1ml to this suspension to a tube with 9 ml of tryptone water. Repeat this operation until obtain a 10^{-4} dilution.
- Mark two Petri dishes for every dilution, including the 1/10 dilution.
- Add 15 ml of culture media, and cooled at 45 °C, every plate.
- Swirl plates to mix thoroughly and allow to cool.
- Solidify the agar, reverse the plates and incubate 24 h at 35 °C.
- After incubation count the purplish red colonies which are surrounded by a reddish zone of precipitated bile which have between 25 and 250 colonies. If plates from two consecutives decimal dilutions yield 25 to 250 colonies each, compute the count per gr for each dilution and report the arithmetic mean as the CFU per gr, unless the higher computed count is more than twice the lower one.

13.5 -*Staphylococccus aureus*

S.aureus presence can be used as indicator of good cleanliness and disinfecting practices and as well as good temperature control .

a) Material and reagents

- Sterile flask of 200 ml
- Sterile pipettes of 10, 1 and 0.1 ml
- Agitator
- Sterile loop or needle
- Oven culture (35 °C)
- Tryptone water
- Baird-Parker agar
- Blood agar
- Coagulase

b) Procedure

- Weight in aseptic conditions 10g of sample approximately. Add so many tryptone water ml than weight sample quantity multiplied by 9, to obtain a 1/10 dilution.
- Transfer 1ml of this suspension to a tube with 9 ml. of tryptone water. Repeat this operation until obtain a 10^{-2} dilution.
- Transfer 0.1 ml of every dilution in a Braid-Parker agar plate. Distribute over all plate with a sterile Pasteur pipette.
- Incubate 24-48 h at 35 °C.
- After incubation count black colonies with a white marge and surround with a transparent "halo" to plates that have between 25 and 250 colonies. If plates from two consecutives decimal dilutions yield 25 to 250 colonies each, compute the count per gr for each dilution and report the arithmetic mean as the CFU per gr, unless the higher computed count is more than twice the lower one.

- Confirm possible *S. aureus* colonies with coagulase commercial test
- If *S. aureus* is present, it can be detected and typified its enterotoxine whit a standard agglutination assay (commercial identification kit).

13.6 -*Pseudomonas aeruginosa*

a) Material and reagents

- Sterile pipettes of 1 and 0.1 ml
- Sterile loop or needle
- Culture oven
- Sterile Erlenmeyer flask
- Concentrate asparragine broth
- Simple asparragine broth
- Confirmative acetamide broth

b) Procedure

- Weight in aseptic conditions 10g of sample approximately. Add so many peptone water ml than weighted sample quantity multiplied by 9, to obtain a 1/10 dilution. Incubate 18-24h at 37 °C.
- Transfer 1ml of 1/10 dilution in a series of 5 tubes with 10ml-asparragina-concentrate broth
- Transfer 1 ml of 1/10 dilution in a series of 5 tubes with 10ml-asparragina simple broth
- Transfer 0,1 ml of 1/10 dilution in a series of 5 tubes with 10ml asparragina simple broth
- Incubate all three series 48h at 37 °C.
- Turbidence indicates that *P. aeruginosa* his grow. Read the results in MPN tables
- Transfer 1 ml of positives tubes in tubes with acetamidebroth.
- Incubate 48h at 37 °C
- If in this tubes with acetamina appear a purple red coloration, result is positive

14 BIBLIOGRAPHY

- **Herbert D.** Methods in microbiology. Vol 5b Acad. Press (1971).
- **ICMSF.** Microorganismos de los alimentos. Tomo 1 y 2. (2ª edicion). Editorial Acribia. Zaragoza.
- **Law and Slepecky.** Assay of poly-b-hydroxybutyric acid. *Anal. Chem.* 82, 33-36.
- **Liaaen-Jensen.** Quantitative Determination of carotenoids in Photosynthetic Tissues. Methods Enzymol, Academic Presss Inc. N.Y, 23, p 586-602 (1971).
- **Janet R Stein**. Handbook of Phycologycal methods. Cambridge University press. (1979).
- **Palmsternia.** Glycogen measurement. *Acta Chem. Scand.* 10, 567-577.
- **Sestak Z, Castsky J, Jarvis P** Determination of Chlorophylls A and B in Plant Photosynthetic Production. Manual of Methods. 672-701(1971).
- **Siegelman HW, Kycia JH.** Algal biliproteins in Handbook of Phycological Methods ED. Hellebnat JA and Sraigie JS . Cambridge Univ. Press 71-80(1980).
- Técnicas para el analisis microbiologico de alimentos y bebidas del centro nacional de nutrición y alimentación. **Ministerio de sanidad y consumo**(1982).

ANNEX 2. TECHNICAL DATA

PRINCIPLES FOR THE ESTABLISHMENT AND APPLICATION OF MICROBIOLOGICAL CRITERIA FOR FOODS

CAC/GL 21 - 1997

INTRODUCTION

These Principles are intended to give guidance on the establishment and application of microbiological criteria for foods at any point in the food chain from primary production to final consumption.

The safety of foods is principally assured by control at the source, product design and process control, and the application of Good Hygienic Practices during production, processing (including labelling), handling, distribution, storage, sale, preparation and use, in conjunction with the application of the HACCP system. This preventive approach offers more control than microbiological testing because the effectiveness of microbiological examination to assess the safety of foods is limited. Guidance for the establishment of HACCP based systems is detailed in Hazard Analysis and Critical Control Point

System and Guidelines for its Application (Annex to CAC/RCP 1-1969, Rev. 3-1997).

Microbiological criteria should be established according to these principles and be based on scientific analysis and advice, and, where sufficient data are available, a risk analysis appropriate to the foodstuff and its use. Microbiological criteria should be developed in a transparent fashion and meet the requirements of fair trade. They should be reviewed periodically for relevance with respect to emerging pathogens, changing technologies, and new understandings of science.

1. DEFINITION OF MICROBIOLOGICAL CRITERION

A microbiological criterion for food defines the acceptability of a product or a food lot, based on the absence or presence, or number of microorganisms including parasites, and/or quantity of their toxins/metabolites, per unit(s) of mass, volume, area or lot.

2. COMPONENTS OF MICROBIOLOGICAL CRITERIA FOR FOODS

2.1 A microbiological criterion consists of:

- a statement of the microorganisms of concern and/or their toxins/metabolites and the reason for that concern (see § 5.1);
- the analytical methods for their detection and/or quantification (see § 5.2);
- a plan defining the number of field samples to be taken and the size of the analytical unit (see § 6);
- microbiological limits considered appropriate to the food at the specified point(s) of the food chain (see § 5.3);
- the number of analytical units that should conform to these limits.

2.2 A microbiological criterion should also state:

- the food to which the criterion applies;
- the point(s) in the food chain where the criterion applies; and
- any actions to be taken when the criterion is not met.

2.3 When applying a microbiological criterion for assessing products, it is essential, in order to make the best use of money and manpower, that only appropriate tests be applied (see § 5) to those foods and at those points in the food chain that offer maximum benefit in providing the consumer with a food that is safe and suitable for consumption.

3. PURPOSES AND APPLICATION OF MICROBIOLOGICAL CRITERIA FOR FOODS

3.1 Microbiological criteria may be used to formulate design requirements and to indicate the required microbiological status of raw materials, ingredients and end-products at any stage of the food chain as appropriate. They may be relevant to the examination of foods, including raw materials and ingredients, of unknown or uncertain origin or when other means of verifying the efficacy of HACCP-based systems and Good Hygienic Practices are not available. Generally, microbiological criteria may be applied to define the distinction between acceptable and unacceptable raw materials, ingredients, products, lots, by regulatory authorities and/or food business operators. Microbiological criteria may also be used to determine that processes are consistent with *the General Principles of Food Hygiene* (CAC/RCP 1-1969).

3.1.1 APPLICATION BY REGULATORY AUTHORITIES

Microbiological criteria can be used to define and check compliance with the microbiological requirements.

Mandatory microbiological criteria shall apply to those products and/or points of the food chain where no other more effective tools are available, and where they are expected to improve the degree of protection offered to the consumer. Where these are appropriate they shall be product-type specific and only applied at the point of the food chain as specified in the regulation.

In situations of non-compliance with microbiological criteria, depending on the assessment of the risk to the consumer, the point in the food chain and the product-type specified, the regulatory control actions may be sorting, reprocessing, rejection or destruction of product, and/or further investigation to determine appropriate actions to be taken.

3.1.2 APPLICATION BY A FOOD BUSINESS OPERATOR

In addition to checking compliance with regulatory provisions (see § 3.1.1) microbiological criteria may be applied by food business operators to formulate design requirements and to examine end-products as one of the measures to verify and/or validate the efficacy of the HACCP plan.

Such criteria will be specific for the product and the stage in the food chain at which they will apply. They may be stricter than the criteria used for regulatory purposes and should, as such, not be used for legal action.

3.2 Microbiological criteria are not normally suitable for monitoring Critical Limits as defined in *Hazard Analysis and Critical Control Point System and Guidelines for its Application* (Annex to CAC/RCP 1-1969, Rev. 3-1997). Monitoring procedures must be able to detect loss of control at a Critical Control Point (CCP). Monitoring should provide this information in time for corrective actions to be taken to regain control before there is a need to reject the product. Consequently, on-line measurements of physical and chemical parameters are often preferred to microbiological testing because results are often available more rapidly and at the production site. Moreover, the establishment of Critical Limits may need other considerations than those described in this document.

4. GENERAL CONSIDERATIONS CONCERNING PRINCIPLES FOR ESTABLISHING AND APPLYING MICROBIOLOGICAL CRITERIA

4.1 A microbiological criterion should be established and applied only where there is a definite need and where its application is practical. Such need is demonstrated, for example, by epidemiological evidence that the food under consideration may represent a public health risk and that a criterion is meaningful for consumer protection, or as the result of a risk assessment. The criterion should be technically attainable by applying Good Manufacturing Practices (Codes of Practice).

4.2 To fulfil the purposes of a microbiological criterion, consideration should be given to:

- the evidence of actual or potential hazards to health;
- the microbiological status of the raw material(s);

- the effect of processing on the microbiological status of the food;
- the likelihood and consequences of microbial contamination and/or growth during subsequent handling, storage and use;
- the category(s) of consumers concerned;
- the cost/benefit ratio associated with the application of the criterion; and
- the intended use of the food.

4.3 The number and size of analytical units per lot tested should be as stated in the sampling plan and should not be modified. However, a lot should not be subjected to repeated testing in order to bring the lot into compliance.

5. *MICROBIOLOGICAL ASPECTS OF CRITERIA*

5.1 MICROORGANISMS, PARASITES AND THEIR TOXINS/METABOLITES OF IMPORTANCE IN A PARTICULAR FOOD

5.1.1 For the purpose of this document these include:

- bacteria, viruses, yeasts, moulds, and algae;
- parasitic protozoa and helminths;
- their toxins/metabolites.

5.1.2 The microorganisms included in a criterion should be widely accepted as relevant - as pathogens, as indicator organisms or as spoilage organisms - to the particular food and technology. Organisms whose significance in the specified food is doubtful should not be included in a criterion.

5.1.3 The mere finding, with a presence-absence test, of certain organisms known to cause foodborne illness (e.g. *Clostridium perfringens, Staphylococcus aureus* and *Vibrio parahaemolyticus*) does not necessarily indicate a threat to public health.

5.1.4 Where pathogens can be detected directly and reliably, consideration should be given to testing for them in preference to testing for indicator organisms. If a test for an indicator organism is applied, there should be a clear statement whether the test is used to indicate unsatisfactory hygienic practices or a health hazard.

5.2 MICROBIOLOGICAL METHODS

5.2.1 Whenever possible, only methods for which the reliability (accuracy, reproducibility, inter- and intra-laboratory variation) has been statistically established in comparative or collaborative studies in several laboratories should be used. Moreover, preference should be given to methods which have been validated for the commodity concerned preferably in relation to reference methods elaborated by international organizations. While methods should be the most sensitive and reproducible for the purpose, methods to be used for in-plant testing might often sacrifice to some degree sensitivity and reproducibility in the interest of speed and simplicity. They should, however, have been proved to give a sufficiently reliable estimate of the information needed.

Methods used to determine the suitability for consumption of highly perishable foods, or foods with a short shelf-life, should be chosen wherever possible so that the results of microbiological examinations are available before the foods are consumed or exceed their shelf-life.

5.2.2 The microbiological methods specified should be reasonable with regard to complexity, availability of media, equipment etc., ease of interpretation, time required and costs.

5.3 MICROBIOLOGICAL LIMITS

5.3.1 Limits used in criteria should be based on microbiological data appropriate to the food and should be applicable to a variety of similar products. They should therefore be based on data gathered at various production establishments operating under Good Hygienic Practices and applying the HACCP system.

In the establishment of microbiological limits, any changes in the microflora likely to occur during storage and distribution (e.g. decrease or increase in numbers) should be taken into account.

5.3.2 Microbiological limits should take into consideration the risk associated with the microorganisms, and the conditions under which the food is expected to be handled and consumed. Microbiological limits should also take account of the likelihood of uneven distribution of microorganisms in the food and the inherent variability of the analytical procedure.

5.3.3 If a criterion requires the absence of a particular microorganism, the size and number of the analytical unit (as well as the number of analytical sample units) should be indicated.

6. *SAMPLING PLANS, METHODS AND HANDLING*

6.1 A sampling plan includes the sampling procedure and the decision criteria to be applied to a lot, based on examination of a prescribed number of sample units and subsequent analytical units of a stated size by defined methods. A well-designed sampling plan defines the probability of detecting microorganisms in a lot, but it should be borne in mind that no sampling plan can ensure the absence of a particular organism. Sampling plans should be administratively and economically feasible.

In particular, the choice of sampling plans should take into account:

- risks to public health associated with the hazard;
- the susceptibility of the target group of consumers;
- the heterogeneity of distribution of microorganisms where variables sampling plans are employed; and
- the Acceptable Quality Level[3] and the desired statistical probability of accepting a non-conforming lot.

For many applications, 2-or 3-class attribute plans may prove useful. [4]

6.2 The statistical performance characteristics or operating characteristics curve should be provided in the sampling plan. Performance characteristics provide specific information to estimate the probability of accepting a non-conforming lot. The sampling method should be defined in the sampling plan. The time between taking the field samples and analysis should be as short as reasonably possible, and during transport to the laboratory the conditions (e.g.

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[3] The Acceptable Quality Level (AQL) is the percentage of non-conforming sample units in the entire lot for which the sampling plan will indicate lot acceptance for a prescribed probability (usually 95 per cent).

[4] See ICMSF: Microorganisms in Foods, 2. Sampling for Microbiological Analysis. Principles and Specific Applications, 2nd Edition, Blackwell Scientific Publications, 1986 (ISBN-0632-015-675).

temperature) should not allow increase or decrease of the numbers of the target organism, so that the results reflect - within the limitations given by the sampling plan - the microbiological conditions of the lot.

7. *REPORTING*

7.1 The test report shall give the information needed for complete identification of the sample, the sampling plan, the test method, the results and, if appropriate, their interpretation.

PUBLICATION HISTORY

This booklet is an extract of Volume 1B - **General Requirements (Food Hygiene)** of the *Codex Alimentarius*. The following table indicates previous versions of these texts and the reference to the draft texts prepared by the Codex Committee on Food Hygiene.

DOCUMENT	REFERENCES
Recommended International Code of Practice - General Principles of Food Hygiene:	CAC/RCP-1 (1969)
Revision 1	CAC/RCP-1 (1969), Rev.1 (1979)
Revision 2	CAC/RCP-1 (1969), Rev.2 (1985)
Revision 3 (Current)	CAC/RCP-1 (1969), Rev.3 (1997)
Draft adopted by the 22nd Session of the Commission	ALINORM 97/13, Appendix II
Hazard Analysis and Critical Control Point (HACCP) System and Guidelines for its Application	CAC/GL 18-1993
Revision 1 (Current)	Annex to CAC/RCP-1 (1969), Rev.3 (1997)
Prior draft	ALINORM 93/13A, Appendix II
Draft adopted by the 22nd Session of the Commission	ALINORM 97/13A, Appendix II

Principles for the Establishment and Application of Microbiological Criteria for Foods	Published in the Procedural Manual of the Codex Alimentarius Commission, Sixth to Ninth Editions (1986-1995)
Revision 1 (Current)	CAC/RCP-22 (1997)
Draft adopted by the 22nd Session of the Commission	ALINORM 97/13A, Appendix III

INDEX

NTS *Ingredients s.l.*

DAGUEL

C/ LLULL, 252 Bajos, Local 6, 08005 Barcelona
Tel. 93 - 266 39 42
Fax 93 - 266 40 68
E-mail: nts@mx3.redestb.es

PRODUCTO: **Spirulina en polvo**

DESCRIPCIÓN: Polvo fino de color verde oscuro con olor y sabor similar a los vegetales marinos.

ANALÍTICA:	Típica	Lote nº 1268
Proteina (Kjeldahl)	60 - 70 %	61.3 %
Ceniza	6.4 - 9 %	8.1 %
Humedad (T:105° C - 3h.)	4 - 7 %	6.9 %
Carotenoides	0.2 - 0.4 %	0.2048 %

MICROBIOLOGIA:	Típica	Lote nº 1268
Bacterias totales	4000 / g - 20000 / g	17000 / g
E. Coli	------	Neg.
Salmonella	------	Neg.
Enterobacterias	------	Neg.
Staphylococcus aureus	------	Neg.
Pseudomonas aeruginosa	------	Neg.

Lote nº: 1268
Envases: 25 Kg.
Caducidad: Consumir antes de fin de 1.999
Origen: China

REGISTRO MERCANTIL DE BARCELONA, FOLIO 162, TOMO 27400, HOJA Nº B-117616, INSCRIPCION 1ª - NIF B60841131

NTS *Ingredients s.l.*

DAGUEL

C/ LLULL, 252 Bajos, Local 6 08005 Barcelona
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Fax 93 - 266 40 68
E-mail: nts@mx3.redestb.es

SPIRULINA

PROTEÍNAS (60 % - 71 %)

* Aminoácidos esenciales		% mín.	% máx.
Isoleucina	:	3.69	4.13
Leucina	:	5.56	5.80
Lisina	:	2.96	4.00
Metionina	:	1.59	2.17
Fenilalanina	:	2.77	3.95
Treonina	:	3.18	4.17
Triptófano	:	0.82	1.13
Valina	:	4.70	6.00

* Aminoácidos no esenciales			
Alanina	:	4.97	5.82
Arginina	:	4.46	5.98
Asparragina	:	5.97	6.43
→Cisteína	:	0.56	0.67
Glutamina	:	8.29	8.94
Glicina	:	3.17	3.46
Histidina	:	0.89	1.08
Prolina	:	2.68	2.97
Serina	:	3.18	4.00
Tirosina	:	1.98	2.99

CARBOHIDRATOS (13 % - 16.5 %)

Ramnosa	:	9 %
Glucano	:	1.5 %
Ciclitoles	:	2.5 %
Glucosamina y		

Glucosamina y ácido murámico	:	2.0 %
Glicógeno	:	0.5 %

REGISTRO MERCANTIL DE BARCELONA, FOLIO 162, TOMO 27400, HOJA Nº B-117816, INSCRIPCION 1ª - NIF B60641131

[redacted]

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VITAMINAS

Biotina (H)	:	0.4	mg / kg
Cianocobalamina (B 12)	:	2.0	mg / kg
Ca - Panlotenato	:	11	mg / kg
Inositol	:	350	mg / kg
Nicotinamida	:	118	mg / kg
Piridoxina (B6)	:	3.0	mg / kg
Riboflavina (B2)	:	40.0	mg / kg
Tiamina (B1)	:	55.0	mg / kg
Tocoferol (E)	:	190.0	mg / kg

[redacted]

MINERALES

		mg / kg mín.	mg / kg.máx.
Calcio (Ca)	:	1045	3115
Fósforo (P)	:	7617	8942
Hierro (Fe)	:	475	580
Sodio (Na)	:	275	412
Cloro (Cl)	:	4000	4400
Magnesio (Mg)	:	1410	1915
Manganeso (Mn)	:	18	25
Zinc (Zn)	:	27	39
Potasio (K)	:	13305	15400

SET-RPLA

STAPHYLOCOCCAL ENTEROTOXIN TEST KIT

INTRODUCTION

Staphylococcal food poisoning is caused by eating foods contaminated with enterotoxins produced during the growth of certain strains of *Staphylococcus aureus*. Reports on the assay of these toxins by reversed passive latex agglutination (RPLA) have been published.[1,2,3] The technique of reversed passive latex agglutination (RPLA) enables soluble antigen such as bacterial toxins to be detected in an agglutination assay.

In a standard agglutination assay, soluble antibody reacts with particulate antigen such as bacterial cells. However, in a REVERSED agglutination assay the antibody, which is attached to particles, reacts with the soluble antigen. The particles (in this case, latex) do not themselves play a part in the reaction and they are therefore PASSIVE. The cross-linking of the latex particles by the specific antigen/antibody reaction results in the visible LATEX AGGLUTINATION reaction.

The SET-RPLA test kit is based upon the reports by Shinagaki *et al.*[1] and Oda *et al.*[4] It was developed under the guidance of the Tokyo Metropolitan Research Laboratory of Public Health.

The SET-RPLA test may be used to detect staphylococcal enterotoxins in a wide variety of foods and to give a semi-quantitative result. The test may also be used to demonstrate enterotoxin production in isolates of *S. aureus* grown in culture. It should be noted that coagulase-negative staphylococci have been isolated which also produce enterotoxin in staphylococcal food poisoning.[5]

PRINCIPLE OF ASSAY

Polystyrene latex particles are sensitised with purified antiserum taken from rabbits, immunised individually with purified staphylococcal enterotoxins A, B, C and D. These latex particles will agglutinate in the presence of the corresponding enterotoxin. A control reagent is provided which consists of latex particles sensitised with non-immune rabbit globulins. The test is performed in V-well microtitre plates. Dilutions of the food extract or culture filtrate are made in five rows of wells, a volume of the appropriate latex suspension is added to each well and the contents mixed. If staphylococcal enterotoxins A, B, C or D are present, agglutination occurs, which results in the formation of a lattice structure. Upon settling, this forms a diffuse layer on the base of the well. If staphylococcal enterotoxins are absent or at a concentration below the assay detection level, no such lattice structure can be formed and, therefore, a tight button will be observed.

The diluent provided contains sodium hexametaphosphate, which has been shown to reduce the incidence of non-specific reactions with components of food matrices.[6]

PRECAUTIONS

This product is for *in vitro* diagnostic use only.
Do not freeze.
Reagents with different lot numbers should not be interchanged.
Reagents and diluent contain 0.1% sodium azide as a preservative. Sodium azide may react with lead or copper plumbing to produce metal azides which are explosive by contact detonation. To prevent azide accumulation in plumbing, flush with copious amounts of water immediately after waste disposal.

STORAGE

The SET-RPLA Kit must be stored at 2°C to 8°C. Under these conditions the reagents will retain their reactivity until the date shown on the kit box. After reconstitution, the enterotoxin controls should be stored at 2°C to 8°C. Under these conditions, the reconstituted enterotoxin controls will retain their reactivity for 3 months, or until the date shown on the kit box, whichever is the sooner.

SAMPLE PREPARATION

1. **Food Matrices**

1.1 A wide range of foods may be tested for staphylococcal enterotoxins; the extraction procedure may, however, require modification for particular foods. The main requirement is to achieve a non-turbid, fat-free extract. A low dilution factor is desirable for optimum sensitivity, but if the nature of the food dictates a greater dilution during extraction, a reduced sensitivity will result.

1.2 To gain a representative sample of a batch, a series of 10g portions are collected from different locations within the batch (see T.P.I., U.S.D.A. sampling plans or equivalent).

2. **Culture Filtrates**

Staphylococci from either clinical sources or food matrices may be recovered and identified using suitable techniques described in standard textbooks.

METHOD OF USE

1. **Materials required but not provided.**

Blender or homogeniser
Microtitre plates (V-well) and lids
Fixed or variable pipette and tips (25µl)
Centrifuge capable of generating 900g (typically 3000rpm in a small bench top centrifuge)
Membrane filtration unit using low protein-binding disposable filters with a porosity of 0.2µm–0.45µm (such as Millipore SLGV)
Tryptone Soya Broth (Oxoid CM129)
Sodium chloride solution (0.85%)
Sodium hypochlorite solution (>1.3% w/w)
25µl dropper (optional)
25µl diluter (optional)
Micromixer (optional)
Moisture box (optional)

2. **Components of the Kit**

TD901 Latex sensitised with anti-enterotoxin A. Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin A.

TD902 Latex sensitised with anti-enterotoxin B. Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin B.

TD903 Latex sensitised with anti-enterotoxin C. Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin C.

TD904 Latex sensitised with anti-enterotoxin D. Latex suspension sensitised with specific antibodies (rabbit IgG) against staphylococcal enterotoxin D.

TD905 Latex control. Latex suspension sensitised with non-immune rabbit globulins.

TD906 Staphylococcal enterotoxin A control.

907 Staph ...l enterot... ...
contr...
908 Staph ...l entero... ...C
contr...
909 Staph ...l entero... ...D
contr...
910 Dilue... ...phate buff...ed saline
conta... ...ine serum ...l, ...min and
...odiu... ...exa... ...eta...phosp... ...at...
Instru... ...ction leaflet

3. Toxin ...xtraction or Pr... ...tion
Extra... ...on from Food M... ...uces

3.1 ...llenc... 10g of sample wi... 10ml of
...odi... ...chloride solutio... (0.85%) in a
...llenc... ...r or homogeniser.

3.2 ...ent... ...uge the blended samp... at 900g
...t 4°... for 30 minutes.

NOTE: If a r... ...igerated centrifuge is not
...avail... ...le, cool the sam...le to 4°C before
...cent... ...ugation..

3 ...filte... ...he supernatant through a 0.2μm–
0.45 ...m low protein-binding membrane
...filte... **Retain the filtrate for assay of**
toxi... content.

Prod...iction of Enterotoxins in
Cultu...re Fluids

3.4 Inoc...late the isolated organis... into
Tryp...tone Soya Broth (CM129) and
incu...bate at 37°C for 18–24 hours,
pref...erably with shaking.

3.5 Afte... growth, either centrifuge at 900g
for 2... minutes at 4°C or membrane filter
usin... a 0.2μm–0.45μm low pr...tein-
bind...ing filter. **Retain the filtrate for**
assa...y of toxin content.

4. Con...rol

Each reconstituted toxin control will cause agglutination with its respective sensitised latex. The use of the toxin controls will provide references for the positive patterns illustrated below (see Interpretation of Test Results). The controls should be used from time to time only to confirm the correct working of the test latex. The toxin controls are not provided at a specified level and therefore must not be used as a means of quantifying the level of toxin detected in the test sample

5. Assay Method

5.1 Working Reagents

The latex reagents and diluent are ready for use. The latex reagents should be thoroughly shaken before use to ensure a homogeneous suspension. To reconstitute the control reagents, add 0.5ml of diluent (TD910) to each vial. Shake gently until the contents are dissolved.

5.2 Arrange the plate so that each row consists of 8 wells. Each sample needs the use of 5 such rows.

5.3 Using a pipette or dropper, dispense 25μl of diluent in each well of the 5 rows.

5.4 Add 25μl of test sample to the first well of each of the 5 rows.

5.5 Using a pipette or diluter and starting at the **first well** of each row, pick up 25μl and perform doubling dilutions along each of the 5 rows. **Stop at the 7th well** to leave the last well containing diluent only.

5.6 To each well in the first row, add 25μl of latex sensitised with anti-enterotoxin A.

5.7 To each well in the second row, add 25μl of latex sensitised with anti-enterotoxin B.

5.8 To each well in the third row, add 25μl of latex sensitised with anti-enterotoxin C.

5.9 To each well in the fourth row, add 25μl of latex sensitised with anti-enterotoxin D.

5.10 To each well in the fifth row, add 25μl of latex control.

5.11 To mix the contents of each well, rotate the plate by micromixer or agitate by hand. Take care that no spillage occurs from the wells.

5.12 To avoid evaporation, cover the plate with a lid. Placing the plate in a moisture box is an acceptable alternative. **Leave the plate undisturbed** on a vibration-free surface at room temperature for 20 to 24 hours. It will help subsequent reading of the test if the plate is placed on black paper for the duration of this incubation.

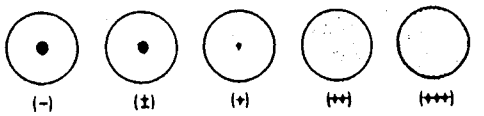
5.13 Examine each well in each row for agglutination, against a black background.

5.14 Centrifuge tubes, membrane filters, microtitre plates, lids and pipette tips should be sterilised by autoclaving at 121°C or disinfected before disposal in hypochlorite solutions (>1.3% w/w).

5.15 Dispose of culture extracts, food extracts, samples and toxin controls in hypochlorite solutions (>1.3% w/w).

INTERPRETATION OF TEST RESULTS

The agglutination pattern should be judged by comparison with the following illustration:



Results classified as (+), (++), and (+++) are considered to be positive.

Results in the row of wells containing latex control should be negative. In some cases, non-specific agglutination may be observed. In such cases the results should be interpreted as positive, provided that the reaction with sensitised latex is positive to a higher dilution of test sample than that seen with the latex control. The last well in all rows should be negative. If positive patterns are observed in some of these wells, the reaction should be regarded as invalid.

NOTE: Certain staphylococcal strains are known to produce more than one enterotoxin.

LIMITATIONS OF THE TEST

The sensitivity of this test in detecting the enterotoxins has been reported to be 0.5ng/ml in the test extract. When a food extract is made with a dilution ratio of 1:1 with diluent, the sensitivity is, therefore, 1ng/g of food matrix. The detection limit will vary according to any extra dilution conditions dictated by the type of food matrix. Concentration of the enterotoxin in the food extract can be effected by a variety of methods, such as ultrafiltration. Production in culture of SETs depends on the growth conditions. A positive result obtained by the culture demonstrates the production of one or more SET under those circumstances; it does not imply the *in vivo* production of toxins to those levels.

REFERENCES

1. Shingaki, M., *et al.* (1981). *Ann. Rep. Tokyo Metro. Lab. Public Health* **32:** 128.
2. Oda, T., *et al.* (1979) *Ann. Rep. Fukuoka City Lab Hyg.* **4:** 33.
3. Park, C. and Szabo, R. (1986). *Can. J. Microbiol.* **32:** 723.
4. Oda, T. (1978). *Jap. J. Bacteriol.* **33:** 743.
5. Crass, B. and Bergdoll, M. (1986). *J. Clin. Microbiol.* **23:** 43.
6. Rose, S., Bankes, P. and Stringer, M. (1989). *Int. J. Food Microbiol.* **8:** 65-72.

Table 4—Typical Biochemical and Serological Reactions of *Salmonella*

Test or Substrate	Positive	Negative	*Salmonella* species reaction[a]
1. Glucose (TSI)	yellow butt	red butt	+
2. Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3. H_2S (TSI and LIA)	blackening	no blackening	+
4. Urease	purple-red color	no color change	−
5. Lysine decarboxylase broth	purple color	yellow color	+
6. Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	+[b]
7. KCN broth	growth	no growth	−
8. Malonate broth	blue color	no color change	−[c]
9. Indole test	red color at surface	yellow color at surface	−
10. Polyvalent flagellar test	agglutination	no agglutination	+
11. Polyvalent somatic test	agglutination	no agglutination	+
12. Phenol red lactose broth	yellow color and/or gas	no gas; no color change	−[d]
13. Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	−
14. Voges-Proskauer test	pink-to-red color	no color change	−
15. Methyl red test	diffuse red color	diffuse yellow color	+
16. Simmons citrate	growth; blue color	no growth; no color change	V

[a] +, 90% or more positive in 1 or 2 days; −, 90% or more negative in 1 or 2 days; V, variable.
[b] Majority of *Salmonella* subspecies 3a, 3b, 4, and 6 are negative (see Table 1).
[c] Majority of *Salmonella* subspecies 2, 3a, and 3b are positive (see Table 1).
[d] Majority of *Salmonella* subspecies 3b are positive (see Table 1).

Table 1—Selected MPN Estimates and 95% Confidence Limits[a] of Estimates for Fermentation Tube Tests When Three Tubes with 0.1-g, 0.01-g, and 0.001-g Volumes Are Used[a]

No. of positive tubes/3 tubes					
0.1 g	0.01 g	0.001 g	MPN/g[b]	95% confidence limits	
				Lower	Upper
0	0	0	<3	–	–
0	1	0	3+	<1	17
1	0	0	4	<1	21
1	0	1	7+	2	27
1	1	0	7	2	28
1	2	0	11+	4	35
2	0	0	9	2	38
2	0	1	14+	5	48
2	1	0	15	5	50
2	1	1	20+	7	60
3	2	0	21	8	62
3	0	0	23	9	130
3	0	1	39	10	180
3	1	0	43	10	210
3	1	1	75	20	280
3	2	0	93	30	380
3	2	1	150	50	500
3	2	2	210+	80	640
3	3	0	240	90	1400
3	3	1	460	100	2400
3	3	2	1100	300	4800
3	3	3	>1100	–	–

[a]Normal results, obtained in 95% of tests, are *not* followed by a plus. Less likely results, obtained in only 4% of tests, are followed by a plus. Combinations of positive tubes not shown occur in less than 1% of tests, and their frequent occurrence indicates that technique is faulty or that assumptions underlying the MPN estimate are not being fulfilled. MPN estimates for combinations that are not shown may be obtained by extrapolation (or by Thomas's formulae, Section 6.6) to the next highest combination that is shown in the table. For example, a result of 2, 0, 2 would have an MPN of approximately 20, which is the MPN for a more likely result of 2, 1, 1.

[b]All figures under "MPN/g" in this table may be multiplied by 100 for reporting MPN/100 g.

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Table 1—Examples for Computing Colony Count per Gram or Milliliter

Sample no.	Colonies counted, Dilution 1:100	Colonies counted, Dilution 1:1000	Count ratio[a]	Colony count[b] (CFU/g or ml)	Rule
Common application, one plate from each of two dilutions					
1001	234[c]	23	—	23,000	*a*
1002	243	34	1.4	29,000	*c*
1003	140	32	2.3	14,000	*c*
1004	Spr[d]	31	—	31,000	*a, h*
1005	0	0	—	<100 Est	*f*
1006	TNTC	7150	—	>5,600,000 Est	*g*
1007	18	2	—	1800 Est	*e*
1008	Spr	Spr	—	Spr	*h*
1009	325	20	—	33,000 Est	*d, g*
1010	27	215	—	LA[e]	*h*
1011	305	42	—	42,000	*a*
1012	243	LA	—	24,000	*a, h*
1013	TNTC	840	—	840,000 Est	*g*
Procedure where two plates per dilution are poured					
1111	228 240	28 26	1.2	25,000	*b, c*
1112	175 208	16 17	—	19,000	*b*
1113	239 328	16 19	—	28,000	*b*
1114	275 280	24 35	—	30,000	*b*
1115	138 162	42 30	2.4	15,000	*b, c*
1116	228 240	28 23	1.1	24,000	*b, c*
1117	224 180	28 Spr	1.4	24,000	*b, c*
1118	287 263	23 19	—	28,000 Est	*d, g*
1119	18 16	2 0	—	1700 Est	*e*
1120	0 0	0 0	—	<100 Est	*f*

[a]Count ratio is the ratio of the greater to the lesser plate count, as applied to plates from consecutive dilutions having between 25 and 250 colonies.

[b]All counts should be made in accordance with instructions in Section 4.51, No. 8, as well as any other rules listed or given in the text.

[c]Underlined figures used to calculate count.

[d]Spreader and adjoining area of repressed growth covering more than one-half of the plate.

[e]LA = Laboratory accident.

TABLAS.

Tabla I. Tabla de números al azar.

Filas	Columnas																												
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
1	0	7	4	0	3	4	1	7	2	1	9	9	1	2	2	1	3	6	0	5	2	1	2	3	1	4	4	0	9	4
2	0	1	3	0	2	0	1	8	5	1	6	8	0	0	7	1	6	1	1	1	4	0	4	3	1	8	2	0	5	5
3	0	2	4	1	9	5	0	4	4	0	2	6	0	5	7	0	3	3	0	7	5	0	0	2	1	9	6	0	5	3
4	0	4	8	1	1	0	1	2	7	1	6	9	0	9	6	0	6	9	0	7	7	1	3	2	1	2	8	0	4	5
5	1	1	1	0	5	1	0	7	3	1	3	4	0	8	4	0	4	0	0	7	9	1	0	9	0	4	7	0	1	4
6	1	7	1	1	0	5	1	9	1	0	4	2	0	6	6	0	0	6	1	5	9	1	0	4	1	7	9	0	1	9
7	0	2	2	1	1	8	1	5	1	0	1	5	0	9	2	0	1	1	0	5	4	0	0	1	1	5	8	0	3	5
8	0	0	5	1	2	4	1	4	7	1	9	8	1	5	0	1	3	9	0	6	2	1	1	5	1	4	3	0	8	9
9	0	5	9	1	0	7	1	3	3	0	9	8	0	2	7	1	3	1	1	4	9	0	0	4	0	9	0	1	1	7
10	0	4	1	0	8	3	0	0	9	1	0	3	0	1	8	0	1	6	1	5	0	1	8	8	1	7	0	0	0	3
11	1	8	0	1	3	8	1	6	0	1	8	9	0	6	0	0	3	8	1	6	3	0	0	8	1	0	0	1	1	2
12	0	6	5	0	9	3	1	2	0	0	5	0	0	6	1	1	0	6	0	5	6	0	3	0	1	6	4	1	2	5
13	0	9	5	1	5	5	0	8	5	1	5	3	0	4	6	0	5	8	0	3	2	1	9	7	0	6	8	1	9	0
14	1	6	5	1	4	5	2	0	0	0	9	7	0	2	5	1	8	7	0	2	1	1	7	5	0	7	2	0	1	7
15	0	7	1	1	5	7	1	7	4	1	4	6	1	8	1	0	9	9	0	3	9	0	8	6	1	9	3	0	2	8
16	1	0	1	0	1	0	1	4	1	0	7	8	0	2	3	0	8	1	1	7	7	1	7	6	1	9	2	0	3	2
17	0	8	7	0	7	0	0	8	8	1	5	2	1	2	6	0	3	1	0	1	2	1	3	7	1	8	4	1	1	6
18	1	3	0	0	2	9	1	6	7	0	6	7	1	1	3	1	3	5	0	7	6	0	3	6	1	2	1	1	8	6
19	0	6	4	1	8	3	1	9	4	1	1	6	1	4	5	1	4	0	0	4	9	1	5	4	1	6	2	1	2	9
20	0	9	1	1	4	2	0	8	0	1	0	2	1	7	3	0	6	3	1	7	8	0	3	7	1	0	8	1	6	6

MEDIOS DE CULTIVO.

Agua de triptona.

- Triptona	10 g
- Cloruro sódico	5 g
- Agua destilada	1000 ml

Calentar hasta disolver. Ajustar el pH a 7.5. Esterilizar durante 20 min a 120°C.

Agua de peptona tamponada.

- Peptona	10 g
- Cloruro sódico	5 g
- Fosfato disódico ($Na_2HPO_4.12H_2O$)	9 g
- Fosfato monopotásico (KH_2PO_4)	1.5 g
- Agua destilada	1000 ml

Calentar hasta su disolución. Ajustar el pH a 7.0. Esterilizar 20 min. a 120°C.

Agar para recuento en placa P.C.A.

- Triptona	5 g
- Extracto de levadura	2.5 g
- Glucosa	1 g
- Agar	12 g
- Agua destilada	1000 ml

Calentar hasta la disolucion. Ajustar el pH 7.0. Esterilizar durante 20 min a 120°C.

Agar VRBD (Agar-Violeta cristal-Rojo neutro-bilis-glucosa seg. MOSSEL)

VRBD Agar (Violet-red Bile Dextrose Agar acc. to MOSSEL)

Agar selectivo para el aislamiento y numeración de gérmenes totales de tipo Enterobacteriáceas en alimentos, según MOSSEL y col. (1962, 1963).

Por su formulación, este medio de cultivo corresponde a las recomendaciones de la International Organization for Standardization (ISO) (1977) y a las del Ministerio Federal Alemán de Sanidad (1967) y en gran medida a la European Pharmacopeia II. Corresponde además a las Prescripciones analíticas apartado 35 de la LMBG para el análisis de alimentos.
HECHELMANN y col. (1973) han informado sobre los buenos resultados experimentales obtenidos con este medio de cultivo.

Forma de actuación

El Violeta cristal y las sales biliares inhiben considerablemente la flora acompañante. La degradación de la glucosa con la consiguiente formación de ácido se pone de manifiesto por el viraje a rojo, y eventualmente por la precipitación de ácidos biliares alrededor de las colonias correspondientes. Puesto que todas las Enterobacteriáceas degradan la glucosa, con formación de ácido, son fácilmente reconocibles por este motivo. No obstante, este medio de cultivo no es completamente específico para dichos microorganismos, pues algunos gérmenes de acompañamiento (por ejem. Aeromonas) que crecen al mismo tiempo, pueden dar también la misma reacción.

Composición (g/litro)

Peptona de carne 7,0; extracto de levadura 3,0; cloruro sódico 5,0; D(+)-glucosa 10,0; mezcla de sales biliares 1,5; Rojo neutro 0,03; Violeta cristal 0,002; Agar-agar 13,0.

Preparación

Disolver 39,5 g/litro y esterilizar con cuidado (30 min. a vapor fluente).
■ ¡No esterilizar en autoclave!
pH: 7,3 ± 0,1.

El medio de cultivo preparado es claro y ligeramente rojizo.

Empleo e interpretación

Sembrar el medio de cultivo a partir de un cultivo de enriquecimiento o directamente con el material objeto de ensayo, utilizando el procedimiento de vertido en placa o sembrando las placas en superficie, por estría.
Incubación: 16-18 horas a 37 °C.

Colonias	Microorganismos
Rojas, con halo de precipitación rojizo	Enterobacteriáceas y otros
Incoloras	Microorganismos no clasificables como Enterobacteriáceas

Las colonias de Enterobacteriáceas sospechosas, deberán ser reinvestigadas para su confirmación.

Control de calidad del medio de cultivo

Cepas de ensayo	cuotas de recuperación método espiral	colonias rojas	precipitado
Escherichia coli ATCC 11775	> 30 %	+	+
Salmonella gallinarum NCTC 9240	> 30 %	+	+
Shigella flexneri ATCC 29903	> 30 %	+	+
Yersinia enterocolítica	> 30 %	+	+
Staphylococcus aureus ATCC 6538	< 0,01 %	–	–
Micr... luteus ATCC 9341	< 0,01 %	–	
Streptococcus lactis ATCC 19435	< 0,01 %	–	–
Bacillus cereus ATCC 11778	< 0,01 %	–	–
Lactob... plantarum ATCC 1...	< 0,01 %	–	–

Caldo BRILA (Caldo-Verde brillante-bilis-lactosa)

Brilliant-green Bile Broth

Para el enriquecimiento selectivo y numeración de Escherichia coli en aguas, leche, alimentos y otros materiales mediante la determinación del título, o según la técnica NMP.

Este medio de cultivo corresponde a las recomendaciones de la Federación Internacional de productos Lácteos (FIL-IDF) (1971, 1974), a las de los Standard Methods for Examination of Water and Wastewater (1981), a las de la Internacional Organization for Standardization (ISO) (1975) a la Norma DIN 10172 y a las Prescripciones analíticas apartado 35 de la LMBG.

Forma de actuación

La bilis y el Verde brillante inhiben notablemente el crecimiento de la flora indeseable acompañante, incluso Clostridios degradadores de la lactosa (por ejem. Cl. perfringens) (MACKENZIE y col. 1948). La fermentación de la lactosa con formación de gas, que es un indicativo de la presencia de E. coli, se demuestra mediante campanas de DURHAM. Los restantes Coliformes no fecales también crecen en este medio, pero casi siempre sin formación de gas.

Composición (g/litro)

Peptona 10,0; lactosa 10,0; bilis de buey, desecada 20,0; Verde brillante 0,0133.

Preparación

Disolver 40 g/litro, distribuir en tubos de ensayo provistos de campana de DURHAM y esterilizar en autoclave (15 min. a 121 °C).
pH: 7,2 ± 0,1.

El Caldo preparado es claro y de color verdoso.

Empleo e interpretación

Sembrar los tubos.
Incubación: 24-48 horas a 37 °C o a la temperatura descrita en cada caso.

El título de E. coli corresponde al volumen más pequeño de material a investigar que todavía produce gas. Como garantía de la investigación, hay que complementar el estudio con la diferenciación del cultivo desarrollado.

Aditivos y productos auxiliares

Campanas de DURHAM

Control de calidad del medio de cultivo

Cepas de ensayo	crecimiento	gas
Escherichia coli ATCC 25922	bueno	+
Escherichia coli ATCC 11775	bueno	+
Citrobacter freundii ATCC 8090	bueno	+
Staphylococcus aureus ATCC 6538	inhibido	—
Micrococcus luteus ATCC 10240	inhibido	—
Bacillus cereus ATCC 11778	inhibido	—
Lactobacillus plantarum ATCC 8014	inhibido	

Bibliografía

American Public Health Association, American Water Works Association and Water Pollution Control Federation: Standard Methods for the Examination of Water and Wastewater, 15th Ed. Washington, 1981.

Gesundheitsamt: Amtliche Sammlung von Untersuchungsverfahren nach §G. Beuth Verlag Berlin, Köln.

DIN Deutsches Institut für Normung e.V.: Mikrobiologische Milchuntersuchung. Bestimmung der coliformen Keime. Referenzverfahren. — DIN 10172.

Internationaler Milchwirtschaftsverband: Coliformenzählung in Sauermilcherzeugnissen. Internationaler Standard FIL/IDF 65: 1971. — Milchwiss.; 28; 345-347 (1973).

Internationaler Milchwirtschaftsverband: Coliformenzählung in Milchpulver und Rahmpulver. Internationaler Standard FIL/IDF 64: 1971 — Milchwiss.; 29; 527-529 (1974).

Internationaler Milchwirtschaftsverband: Zählung coliformer Bakterien in Milch und Milchprodukten. Internationaler Standard FIL/IDF 73: 1974. — Milchwiss.; 31; 606-611 (1976).

International Organization for Standardization: Meat and meat products — Detection and enumeration of presumptive coliform bacteria and presumptive Escherichia coli (Reference method). — Draft International Standard ISO/DIS 3811 (1975).

MACKENZIE, E.F.W., TAYLOR, W.E. a. GILBERT, W.E.: Present experiments in the rapid identification of Bacterium coli type I. — J. Gen. Microbiol., 2; 177-204 (1948).

Agar MacCONKEY

MacCONKEY Agar

Agar selectivo para el aislamiento de Salmonellas, Shigellas y bacterias coliformes, a partir de heces, orina, alimentos, aguas residuales, etc. según MacCONKEY (1905).

La formulación de este medio de cultivo corresponde considerablemente con las recomendadas por la United States Pharmacopeia XXI (1985), European Pharmacopoeia I y Deutschen Artzneibuch. Corresponde además con las normas de ensayo, apartado 35 de la LMBG para el análisis de alimentos.

Forma de actuación

Las sales biliares y el Violeta cristal inhiben considerablemente la flora gram—positiva. La lactosa, junto con el indicador de pH Rojo neutro, sirven para la comprobación de la degradación de dicho azucar.

Composición (g/litro)

Peptona de caseína 17,0; peptona de carne 3,0; cloruro sódico 5,0; lactosa 10,0; mezcla de sales biliares 1,5; Rojo neutro 0,03; Violeta cristal 0,001; Agar-agar 13,5.

Preparación

Disolver 50 g/litro, esterilizar en autoclave (15 min. a 121 °C) y verter en placas.
pH: 7,1 ± 0,1.

Empleo e interpretación

Sembrar las placas por el procedimiento de estría.
Incubación: 18-24 horas a 37 °C.

Las colonias lactosa-negativas son incoloras y las lactosa-positivas son rojas con un halo turbio debido al descenso de pH provocado por los ácidos biliares.

Colonias	Microorganismos
Incoloras, transparentes	Salmonella, Shigella y otros
Grandes, rojas, halo turbio	Escherichia coli
Grandes, rosadas, mucosas	Enterobacter, Klebsiella
Diminutas, de crecimiento aislado, opacas	Enterococos, Estafilococos y otros

Control de calidad del medio de cultivo

Cepas de ensayo	cuotas de recuperación	color de las colonias	color del medio de cultivo	precipitado
Escherichia coli ATCC 11774	**> 30 %**	**rojo**	**rojo**	**+**
Salmonella typhimurium ATCC 13311	**> 30 %**	**incoloras**	**amarillo**	**—**
Salmonella dublin	**> 30 %**	**incoloras**	**amarillo**	**—**
Shigella sonnei ATCC 11060	**> 30 %**	**incoloras**	**amarillo**	**—**
Proteus mirabilis ATCC 29906	**> 30 %**	**incoloras**	**amarillo**	**—**
Bacillus cereus ATCC 11778	**< 0,01 %**			
Staphylococcus aureus ATCC 6538	**< 0,01 %**			
Streptococcus faecium ATCC 8043	**< 0,01 %**			

Caldo de Clark el Lubs (Rojo de Metilo y Voges Proskauer).

- Peptona	5 g
- Fosfato bipotasico	5 g
- Glucosa	5 g
- Agua destilada	1000 ml

Calentar hasta disolver. Ajustar a pH 7.0 y esterilizar 15 min a 120°C.

Reactivo de rojo de metilo.

- Rojo de metilo	0.1 g
- Alcohol	300 ml
- Agua destilada	200 ml

Disolver el colorante en el acohol y añadir el agua destilada.

Reactivos Voges Proskauer.

Sol. 1

- Alfa - naftol	6 g
- Alcohol	100 ml

Sol. 2

- KOH	40 g
- Agua destilada	1000 ml

Citrato de Simmons.

- Citrato sódico	2 g
- Cloruro sódico	5 g
- Fosfato dipotásico	1 g
- Fosfato amónico	1 g
- Sulfato magnésico	0.2 g
- Azul de bromotimol	0.08 g
- Agar	15 g
- Agua destilada	1000 ml

Calentar hasta su disolución. Ajustar a ph 6.9. Alicuotar en tubos y estarilizar durante 20 min a 120ºC. Solidificar en posicion inclinada.

Agua de peptona (indol)

Ver agua de peptona pag. 20 .

Reactivo de Kovacs (indol).

- Para-dimetilaminobenzaldehido	5 g
- Alcohol amílico	75 ml
- ClH	25 ml

Disolver el para-dimetilaminobenzoaldehido en el acohol y añadir gota a gpta el ClH.

Caldo de enriquecimiento tetrationato seg. MULLER-KAUFFMANN (base)

Tetrathionate Broth Base acc. to MULLER-KAUFFMANN

Para el enriquecimiento selectivo de Salmonellas a partir de diversos materiales, especialmente carnes, productos cárnicos y otros alimentos.

El medio de cultivo corresponde a las recomendaciones de la ISO (1975), de la Norma DIN 10160 para el análisis de carne y de la Norma DIN 10181 para el análisis de leche. Corresponde además a las Prescripciones analíticas apartado 35 de la LMBG.

■ El Caldo basal preparado, puede almacenarse durante largo tiempo, pues el tetrationato sólo es sintetizado cuando se añade el yodo antes del uso.

KAUFFMANN (1930, 1935) desarrolló el medio de cultivo combinando el Caldo-tetrationato según MULLER (1923) con el Caldo-verde brillante-bilis.

Forma de actuación

A partir del tiosulfato, en el medio de cultivo se forma tetrationato al añadir yodo, que inhibe el crecimiento de Coliformes y otras bacterias intestinales. Las Salmonellas y también Proteus y algunos otros gérmenes reducen al tetrationato y por tanto no son inhibidas. El carbonato cálcico tampona al ácido sulfúrico liberado en la reducción del tetrationato. La bilis actúa estimulando el crecimiento de Salmonellas, pero inhibe más o menos a los gérmenes acompañantes. El Verde brillante inhibe sobre todo a los gérmenes gram-positivos.

Composición (g/litro)

Extracto de carne 0,9; peptona de carne 4,5; extracto de levadura 1,8; cloruro sódico 4,5; carbonato de calcio 25,0; tiosulfato sódico 40,7; bilis de buey, desecada 4,75.
Aditivos: yoduro potásico 5,0; yodo 4,0; Verde brillante 0,01.

Preparación

Suspender 82 g/litro y en caso necesario calentar brevemente y enfriar rápidamente. Queda un sedimento de carbonato cálcico.

■ ¡No esterilizar en autoclave!.

Antes del uso, añadir 20 ml/litro de solución de yodo y yoduro potásico y 10 ml/litro de solución al 0,1% de Verde brillante, distribuir en tubos repartiendo previamente de forma homogénea el precipitado que eventualmente hubiera podido formarse. Una vez añadidas estas sustancias, no volver a calentar.
pH: 7,6 ± 0,2.

Preparación de la solución de yodo y yoduro potásico: 5 g de yoduro potásico, 4 g de yodo, 20 ml de agua destilada.

■ El Caldo listo para el uso, debe ser utilizado, a ser posible, el mismo día de su preparación.

Empleo e interpretación

Suspender directamente en 100 ml de Caldo-tetrationato aprox. 10 g del material objeto de investigación.
Incubación: 18-24 o 48 horas a 37 °C o bien a 43 °C (BÄNFFER 1971, EDEL y KAMPELMACHER 1969).

Los cultivos desarrollados deben ser reinvestigados. Si se sospecha la existencia de Proteus, se recomienda hacer subcultivos en Agar-Pril®-manitol.

Aditivos y productos auxiliares

Merck art. núm.	producto
1310	Verde brillante
4761	Yodo resublimado p.a.
5043	Yoduro potásico p.a.
10727	Agar-Pril® manitol

Control de calidad del medio de cultivo

Cepas de ensayo	concentración del inóculo	crecimiento a	
		6 h.	24 h.
Escherichia coli ATCC 25922	99 %	< 30 %	< 5 %
Salmonella typhimurium ATCC 14028	1 %	> 70 %	> 95 %

Caldo de enriquecimiento selenito, seg. LEIFSON

Selenite Enrichment Broth acc. to LEIFSON

Para en enriquecimiento selectivo de Salmonella, a partir de heces, orina, agua, alimentos, etc. según LEIFSON (1936).

El medio de cultivo corresponde a las recomendaciones de la APHA (1984) para el análisis de alimentos.

Forma de actuación

El selenito inhibe el crecimiento de bacterias intestinales Coliformes y Enterococos, principalmente en las primeras 6 a 12 horas de incubación. Salmonella, Proteus y Pseudomonas no son inhibidos.

Composición (g/litro)

Peptona de carne 5,0; lactosa 4,0; selenito sódico 4,0; hidrogenofosfato dipotásico 3,5; dihidrogenofosfato potásico 6,5.

Preparación

Disolver 23 g/litro a temperatura ambiente. En caso necesario calentar brevemente como máximo a 60 °C, esterilizar por filtración si se prevé un almacenamiento prolongado y distribuir en tubos.
■ ¡No esterilizar en autoclave!
pH: 7,0 ± 0,1.

El Caldo preparado es claro y ligeramente amarillento a rojizo.

■ Si se presenta un sedimento de color rojo ladrillo, de selenito precipitado, el medio de cultivo no es utilizable.

Empleo e interpretación

El material sólido sometido a ensayo se introduce en el Caldo preparado (concentración sencilla). Si el material a ensayar fuere un líquido, mezclar en la proporción de 1:1 con un Caldo preparado a concentración doble de la anteriormente indicada.
Incubación: hasta 24 horas a 37 °C, o mejor —según BÄNFFER (1971)— a 43 °C. Al cabo de 6-12 horas (y eventualmente, al cabo de 18-24 horas) se resiembra en medios de cultivo selectivos.

Control de calidad del medio de cultivo

Cepas de ensayo	concentración del inóculo	crecimiento a	
		6 h.	24 h.
Escherichia coli ATCC 25922	99 %	< 30 %	< 5 %
Salmonella typhimurium ATCC 14028	1 %	> 70 %	> 95 %

Agar XLD (Agar-xilosa-lisina-desoxicolato)

XLD Agar (Xylose Lysine Deoxycholate Agar)

Para el aislamiento y diferenciación de Enterobacteriáceas patógenas, especialmente de especies de Shigella y Salmonella, según TAYLOR (1965), TAYLOR y HARRIS (1965, 1967) y TAYLOR y SCHELHART (1967).

Por su formulación, este medio de cultivo corresponde a las recomendaciones de la United States Pharmacopoeia XXI (1980), de la European Pharmacopoeia II y de la APHA (1984).

■ En combinación con un enriquecimiento adecuado, con el Agar XLD se puede detectar un número notablemente mayor de Salmonellas y Shigellas que con otros medios de cultivo selectivos (TAYLOR y SCHELHART 1967, 1968; DUNN y MARTIN 1971). En el procedimiento de siembra directa por estría, el Agar XLD aventaja también en este sentido a otros medios de cultivo (ROLLENDER y col. 1969; BHAT y RAJAN 1975).

Forma de actuación

La degradación a ácido de la xilosa, lactosa y sacarosa produce un viraje a amarillo del Rojo de fenol. El tiosulfato y la sal de hierro (III) revelan la formación de ácido sulfhídrico por la precipitación de sulfuro de hierro negro en las colonias. Las bacterias que descarboxilan la lisina, produciendo cadaverina, se reconocen por la presencia de un color rojo-purpúreo, debido al aumento del pH, alrededor de sus colonias.

Varias de estas reacciones pueden presentarse simultáneamente o sucesivamente, lo que puede dar lugar a diversos matices de color del indicador de pH, o a un viraje de amarillo a rojo en el transcurso de una incubación más prolongada. El efecto inhibidor de este medio de cultivo es débil.

Composición (g/litro)

Extracto de levadura 3,0; cloruro sódico 5,0; D(+)-xilosa 3,5; lactosa 7,5; sacarosa 7,5; L(+)-lisina 5,0; desoxicolato sódico 2,5; tiosulfato de sodio 6,8; citrato de amonio e hierro (III) 0,8; Rojo de fenol 0,08; Agar-agar 13,5.

Preparación

Disolver 55 g/litro rápida y totalmente y verter en placas.
■ ¡No esterilizar en autoclave!
pH: 7,4 ± 0,2.

Las placas con medio de cultivo son de color rojo y casi siempre claras.

Los precipitados que se presentan ocasionalmente no perjudican la capacidad de rendimiento de este medio de cultivo. Pueden eliminarse por filtración (filtro de pliegues) del medio de cultivo todavía líquido.

Empleo e interpretación

Sembrar el medio de cultivo en superficie, por estría en capa fina.
Incubación: 48 horas a 37 °C.

Colonias	Microorganismos
Amarillas, con zona amarilla alrededor, opacas, halo de precipitación.	Escherichia coli, Enterobacter, Aeromonas
Amarillas, con zona amarilla alrededor, opacas, mucosas, halo de precipitación.	Klebsiella
Amarillas, con zona amarilla alrededor, opacas, a veces con centro negro.	Citrobacter (cepas lactosa-positivas)
Amarillas, con zona amarilla alrededor, opacas.	Serratia, Hafnia
Amarillas, con zona amarilla alrededor, transparentes; centro negro.	Proteus vulgaris; la mayoría de Proteus mirabilis.
Del mismo color que el medio de cultivo, transparente; a veces, con centro negro.	Salmonella.
Del mismo color que el medio de cultivo, transparentes.	Shigella, Providencia, Pseudomonas
Anaranjadas, ligeramente opacas.	Salmonella typhi (cepas xilosa-positivas).

Aditivos y productos auxiliares

Fabricante	producto
Scheleider y Schüll	**Filtro de pliegues, por ejemplo SELECTA-filtro de pliegues Núm. 520 b II.**

Agar lisina-hierro

Lysin Iron Agar

Agar de ensaya para la demostración simultánea de lisinadecarboxilasa (LD) y de la formación de ácido sulfhídrico (H_2S) para la identificación de Enterobacteriáceas, sobre todo de Salmonellas y Arizona, según EDWARDS y FIFE (1961)

JOHNSON y col. (1966), así como TIMMS (1971) obtuvieron buenos resultados con este medio de cultivo. Una combinación con el Agar-hierro-tres azúcares permite una identificación todavía mejor (THATCHER y CLARK 1968). Según HENNER y col. (1982), el Agar lisina-hierro es ventajoso, ante medios de cultivo comparables, en la diferenciación de Proteus frente a Salmonella. Suplementando con sales biliares, Novobiocina, lactosa y sacarosa, RAPPOLD y BOLDERDIJK (1979) desarrollaron un Agar selectivo para el aislamiento, sobre todo también de gérmenes H_2S-negativos.

Forma de actuación

La lisina puede ser descarboxilada por microorganismos LD-positivos, que la transforman en la amina Cadaverina. Esto produce un viraje al violeta del indicador de pH Púrpura de bromocresol. Puesto que la descarboxilación sólo tiene lugar en medio ácido (pH inferior 6,0), es necesario que se produzca previamente la acidificación del medio de cultivo, por fermentación de la glucosa. Por este motivo, este medio de cultivo sólo puede utilizarse para la diferenciación de cultivos que fermentan la glucosa.

Los microorganismos LD-negativos, pero fermentadores de la glucosa, producen un viraje al amarillo de la totalidad del medio de cultivo. La incubación prolongada puede ocasionar una alcalinización en la zona de la superficie del medio de cultivo y en consecuencia, se produce un viraje al violeta. La formación de H_2S produce una coloración negra debida al sulfuro de hierro producido.

Las cepas del grupo Proteus-Providencia, con excepción de algunas cepas de Proteus morganii, desaniman a la lisina a ácido α-cetocarbónico. Este último forma compuestos pardorojizos en la región superficial del medio de cultivo con la sal de hierro y bajo la influencia del oxígeno.

Composición (g/litro)

Peptona de carne 5,0; extracto de levadura 3,0; D(+)-glucosa 1,0; L-lisina monoclorhidrato 10,0; tiosulfato sódico 0,04; citrato de amonio e hierro (III) 0,5; Púrpura de bromocresol 0,02; Agar-agar 12,5.

Preparación

Disolver 32 g/litro, distribuir en tubos y esterilizar en autoclave (15 min. a 121 °C). Dejar enfriar en posición inclinada de forma que, al solidificarse, se produzca una columna de unos 3 cm de altura, y sobre ella, una superficie inclinada de, por lo menos, la misma longitud.
pH: 6,7 ± 0,1.

El medio de cultivo preparado es claro y de color gris violeta.

Empleo e interpretación

Este medio nutritivo se siembra con el cultivo puro sometido a ensayo, tanto por estría sobre la superficie inclinada como por picadura central en la columna vertical subyacente.
Incubación: 16-18 horas a 37 °C.

Microorganismos	color del med. de cultivo: columna vertical	superficie inclinada	formación de H_2S
Arizona	violeta	violeta	+
Salmonella	violeta	violeta	+
Proteus miravilis	amarillo	pardo-rojizo	+
Proteus vulgaris	amarillo	pardo-rojizo	+
Proteus morganaii	amarillo	pardo-rojizo	-
Proteus rettgeri	amarillo	pardo-rojizo	+
Providencia	amarillo	pardo-rojizo	-
Citrobacter	amarillo	violeta	+
Escherichia	amarillo o	violeta	-
Shigella	amarillo	violeta	-
Klebsiella	violeta	violeta	-

* Excepción: Salm. paratyphi A (sin formación de lisina-descarboxilasa, columna vertical = amarillo. Superficie inclinada = violeta)

Control de calidad del medio de cultivo

Cepas de ensayo	crecimiento	columna vertical	superficie inclinada
Shigella flexneri ATCC 12022	**bueno**	**amarillo**	**violeta**
Escherichia coli ATCC 25922	bueno	**amarillo**	violeta
Salmonella typhimurium ATCC 14028	**bueno**	**violeta y negro**	violeta
Salmonella enteritidis NCTC 5188	**bueno**	**violeta y negro**	**violeta**
Citrobacter freundii ATCC 8090	**bueno**	**amarillo y negro**	**violeta**
Proteus mirabilis ATCC 29906	**bueno**	**amarillo y negro**	rojo parduzco
Moraanella morganii ATCC 25830	bueno	amarillo	rojo parduzco

Agar KLIGLER (Agar-hierro-dos azúcares según KLIGLER)

KLIGLER Agar (Double Sugar Iron Agar acc. to KLIGLER)

Medio nutritivo de ensayo para la identificación de bacterias intestinales gram-negativas según KLIGLER (1917-1918)

Mediante la adición de 0,2 % de urea, puede realizarse una modificación del Agar KLIGLER, lo que constituye el Agar-hierro-urea según BADER y HOTZ (1951).

Forma de actuación

Véase: «Agar-hierro-tres-azúcares».

Composición (g/litro)

Peptona de caseína 15,0; peptona de carne 5,0; extracto de carne 3,0; extracto de levadura 3,0; cloruro sódico 5,0; lactosa 10,0; D(+)-glucosa 1,0; citrato de amonio e hierro (III) 0,5; tiosulfato sódico 0,5; Rojo de fenol 0,024; Agar-agar 12,0.

Preparación

Disolver 55,5 g/litro, distribuir en tubos y esterilizar en autoclave (15 min. a 121 °C). A continuación, dejar solidificar como Agar inclinado.
pH: 7,4 ± 0,1.
El medio de cultivo preparado es claro y de color rojizo-parduzco.

Empleo e interpretación

Véase: «Agar-hierro-tres-azúcares»

Aditivos y productos auxiliares

Merck art. núm.	Producto
8487	Urea p.a.

Control de calidad del medio de cultivo

Cepas de ensayo	crecimiento	columna vertical	superficie inclinada
Escherichia coli ATCC 25922	bueno	amarillo	amarillo
Citrobacter freundii ATCC 8090	bueno	amarillo y negro	amarillo
Enterobacter cloacae ATCC 13047	bueno	amarillo	amarillo
Shigella flexneri ATCC 12022	bueno	amarillo	rojo
Salmonella typhimurium ATCC 14028	bueno	amarillo y negro	rojo
Salmonella enteriditis ATCC 13076	bueno	amarillo y negro	rojo
Proteus mirabilis ATCC 14153	bueno	amarillo y negro	rojo
Proteus vulgaris ATCC 13315	bueno	amarillo y negro	rojo

Agar BAIRD-PARKER (Agar selectivo para Estafilococos seg. BAIRD-PARKER [base])

BAIRD-PARKER Agar (Staphylococcus Selective Agar Base acc. to BAIRD-PARKER)

Art. Núm. 5406 (100 g, 500 g)

Para el aislamiento y la diferenciación de Estafilococos en alimentos y materiales farmacéuticos, según BAIRD-PARKER (1962)

Este medio de cultivo corresponde a las recomendaciones de la United States Pharmacopeia XXI (1985), a las de la European Pharmacopoeia II, a las de la Farmacopea Alemana, a las de la International Organization for Standardization (ISO) (1977, 1978), a las de la Federacion Internacional de Lechería (1978) y a las Normas DIN 10163 y 10178.
Es apropiado para la investigación según el apartado 35 de la LMBG.

En la investigación de carnes con diversos medios de cultivo, NISKANEN Y AALTO (1978) obtuvieron los mejores resultados con el Agar BAIRD-PARKER.

Forma de actuación

Este medio de cultivo contiene cloruro de litio y telurito para la inhibición de la flora acompañante, en tanto que el piruvato y la glicocola actúan favoreciendo selectivamente el crecimiento de Estafilococos.

Sobre el medio de cultivo, opaco por su contenido en yema de huevo, las colonias de Estafilococos muestran dos características diagnósticas: por lipolisis y proteolisis, se producen halos y anillos característicos y, debido a la reducción del telurito a teluro, se desarrolla una colonia negra. la reacción con la yema de huevo y la reducción del telurito se presentan con notable paralelismo con la coagulasapositividad, y por tanto, pueden utilizarse como índice de esta última.
Para una demostración directa de Estafilococos coagulasapositivos, ha sido recomendado por STADHOUDERS y col. (1976) el incorporar al medio de cultivo plasma sanguíneo en lugar de yema de huevo.

SMITH y BAIRD-PARKER (1964) recomiendan añadir Sulfametacina para inhibir el crecimiento de Proteus.

Composición (g/litro)

Peptona de caseína 10,0: extracto de carne 5,0; extracto de levadura 1,0; piruvato sódico 10,0; glicina 12,0; cloruro de litio 5,0; Agar-agar 15,0.
Aditivos: emulsión de yema de huevo telurito 50 ml; eventualmente, Sulfametacina 0.05 g.

Preparación

Disolver 58 g en 0,95 litros, esterilizar en autoclave (15 min. a 121 °C), enfriar a 45-50 °C, añadir mezclando 50 ml de emulsión de yema de huevo telurito y, eventualmente, 50 mg/litro de Sulfametacina.
Verter en placas.
pH: 6,8 ± 0,2.

En tanto que el medio de cultivo basal puede guardarse de 1 a 2 meses a 4 °C, el medio de cultivo completo, vertido en placas, ha de ser utilizado dentro de las 24 horas siguientes a su preparación.

Empleo e interpretación

Diluir convenientemente el material a investigar y extenderlo finamente sobre la superficie del medio de cultivo.
Incubación: desde 24 hasta 48 horas a 37 °C.

Colonias	Microorganismos
Negras, lustrosas, convexas, 1 a 5 mm de diámetro, con borde estrecho blanquecino, rodeado por un halo claro de 2 a 5 mm de anchura. Dentro del halo claro presencia de anillos opacos no visibles antes de las 48 horas de incubación.	Staphylococcus aureus
Negras, lustrosas, pero de forma irregular. Al cabo de 24 horas, presencia de zonas opacas alrededor de las colonias.	Staphylococcus epidermidis
Crecimiento ocasional. Muy pequeñas, pardas hasta negras, ausencia de halos de clarificación.	Micrococcus
Pardo-oscuras, mates, presencia a veces de halos de clarificación al cabo de 48 horas.	Bacillus
Blancas, sin halos de clarificación.	Levaduras

Aditivos y productos auxiliares

Merck art. núm.	Producto
3785	Emulsión de yema de huevo telurito

Fabricante	Producto
International Chemical Industries Ltd., Inglaterra	Sulfametacina

Control de calidad del medio de cultivo

Cepas de ensayo	cuotas de recuperación	colonias negras	halos claros alrededor de las colonias
Staphylococcus aureus ATCC 25923	> 70 %	+	+
Staphylococcus aureus ATCC 6538	> 70 %	+	+
Staphylococcus epidermidis NCTC 11047	–	±	–
Streptococcus faecium ATCC 8043	–	±	–
Bacillus subtilis ATCC 6051	< 0,01	–	–
Escherichia coli ATCC 11775	< 0,01	–	–
Proteus mirabilis ATCC 29906	–	pardo-verdoso	–

Bibliografía

BAIRD-PARKER, A.C.: An improved diagnostic and selective medium for isolating coagulase positive Staphylococci. – J. Appl. Bact., 25; 12–19 (1962).

Bundesgesundheitsamt: Amtliche Sammlung von Untersuchungsverfahren nach § 35 LMBG. Beuth Verlag Berlin, Köln.

Deutsches Arzneibuch, 9. Auflage, Kapitel VIII, 10.

DIN Deutsches Institut für Normung e.V.: Nachweis Koagulase-positiver Staphylokokken. Referenzverfahren für Milchpulver. – DIN 10178.

DIN Deutsches Institut für Normung e.V.: Bestimmung Koagulase-positiver Staphylokokken. Referenzverfahren. – DIN 10163.

European Pharmacopoeia II, Kapitel VIII, 10.

Internationaler Milchwirtschaftsverband: Nachweis Koagulase-positiver Staphylokokken in Milchpulver (Referenzmethode). – Internationaler Standard 60 A (1978).

ISO International Organization for Standardization: Dried milk. Enumeration of Staphylococcus aureus. Colony count technique. – Draft Proposal ISO/PP 8869 (1984).

ISO International Organization for Standardization: Meat and meat products – Detection and enumeration of Staphylococcus aureus (Reference methods). – Draft International Standard ISO/DIS 5551 (1977).

NISKANEN, A. a. AALTO, M.: Comparison of selective media for coagulase-positive enterotoxigenic Staphylococcus aureus. – Appl. Envir. Microbiol., 35; 1233–1236 (1978).

SMITH, B.A., a. BAIRD-PARKER, A.C.: The use of sulphamethazine for inhibiting Proteus spp. on Baird-Parker's isolation medium for Staphylococcus aureus. – J. Appl. Bact., 27; 78–82 (1964).

STADHOUDERS, J., HASSINGS, F. a. VAN AALSTEN-VAN MAREN, N.O.: A pour-plate method for the detection and enumeration of coagulase-positive Staphylococcus aureus in the BAIRD-PARKER Medium without egg yolk. – Neth. Milk Dairy J., 30; 222–229 (1976).

United States Pharmacopeia XXI, Kapitel "Microbial Limit Tests", 1985.

Tabla III. Diferenciación de coliformes mediante las pruebas IMViC.

	Indol	R-M*	V-P*	Citrato
Escherichia	+/-	+	-	-
Citrobacter	-	+	-	+
Klebsiella	+/-	-	+	+
Enterobacter	-	-	+	+
Serratia	-	-	+	+
Hafnia	-	-	+	+

*R-M, Rojo de Metilo. V-P, Voges Proskauer