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DECOLORIZATION AND PURIFICATION OF SPIRULINA PROTEIN

PHASE II

An Interim Report Prepared for the
United Nations Industrial Development Organization

by

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ABSTRACT

A laboratory investigation of decolorization techniques has been completed as the second phase in a program to develop a process for purification and decolorization of Spirulina algae. The overall objective of Phase II has been to identify and assess possible means of purifying the algae so as to provide guidance for conducting pilot scale trials leading to a process design.

Previous work on decolorization of algae is reviewed and the results of preliminary experimentation on each of several possible decolorization methods are reported. It is concluded that solvent extraction is the most promising process for achieving decolorzation although other methods may have potential for brightening a final protein product.

Lipids, chlorophyll and carotenoids can be extracted from Spirulina algae by conventional means to produce an intermediate product which contains biliprotein pigment as the major colorant. Biliprotein pigment is chemically bound to the protein molecule. Several methods for detaching pigment from protein have been investigated and transesterification by reaction with alcohol has been found to be the most effective.

Bench scale extraction experiments have been performed to determine the most suitable solvent, extraction mode and raw material form. Methanol and ethanol appear to be the most effective solvents for removing lipids, chlorophyll and carotenoids to yield the intermediate product. Ethanol is preferred as residual solvent in the final product would not be toxic and it appears to give a higher protein yield. A dry algal powder is the preferred raw material form. Good results have been obtained using spray dried

powder although a roller dried powder, dried without scorching the protein, may be equally acceptable. Direct extraction of dewatered algal paste containing of the order 15% solids is not practical because of the high solvent ratios required to accomplish extraction in a reasonable number of stages. Milling of the algae to produce cell fragments in the 20 micron size range is desirable for complete and rapid extraction.

Laboratory experiments indicate a two stage extraction system with intermediate and final filtering and solvent wash is capable of producing a suitably clean intermediate product containing biliprotein pigment as the major remaining colorant. Acceptable intermediate products have been obtained with solvent ratios in the range 4:1 to 8:1. Yields of solids and protein using ethanol as solvent are estimated at 78 and 88% respectively. Miscella solids content is of the order 2%.

A systematic investigation of the detachment of pigment from biliprotein by transesterification with alcohol has been undertaken. Methanol and ethanol have been found to be more effective than isopropanol. It appears the reaction can proceed at solvent ratios as low as 8.5:1. Increasing the temperature serves to increase reaction rate to the point where pigment can be removed in a matter of minutes at 135°C. Temperatures higher than 135°C. are not recommended due to the risk of product scorching.

Preliminary specifications for final protein product and pigment by-product are presented. There are indications that the extraction process may have a slight adverse effect on protein quality in that pepsin digestibility, lysine and methionine content are lower for the purified product. Comparison of the total amino acid contents of original algae and final product indicates that at least part of the "crude protein" apparently lost during extraction is non-protein nitrogen.

1. INTRODUCTION

The Research and Productivity Council was commissioned by the United Nations Industrial Development Organization to develop a suitable process for producing a high quality colorless Spirulina protein product for human consumption from the species of algae occurring naturally in the Caracol Basin of Sosa Texcoco S.A., Mexico City. The process is to include the production of marketable by-products from the algae. This interim report presents our findings and results from Phase II of the project and is submitted in accord with the requirements of UNIDO Contract 73/28.

2. BACKGROUND

The approach that has been adopted for the project comprises five phases - preliminary assessment of economic requirements, laboratory investigation, pilot scale trials, process design and feasibility analysis. During the course of Phase I it was established that markets may exist for two products from the algae - a quality protein product for human consumption having acceptable color, odor and flavor characteristics and a pigment by-product for use in poultry feeds. Cost guidelines for a purification process based on likely value of products were developed. Phase II, the laboratory investigation, is intended to identify and assess possible means of purifying the algae to provide guidance for conducting pilot scale trials leading to a process design. Objectives for Phase II are as follows:

(1) Select the most promising process for achieving decolorization and deodorization of Spirulina algae from literature and patent review and from preliminary experimentation. The objectives

which follow presuppose that solvent extraction is chosen as the best route to pursue.

(2) Investigate the effect of pretreatment of algae to rupture cell walls.

(3) Select the most promising solvent for removing color and odor considering such factors as legality, solubility, effect on protein, cost and availability and physical properties.

(4) Prepare samples (of the order 100 g) of protein product to provide an indication of product yield and composition.

(5) Prepare samples of by-product(s) to obtain estimates of yield and composition.

During the course of Phase II, a literature and patent review has been undertaken relating to previous work on decolorization of algae. Bench scale experiments have been performed using harvested algae (filtered but not dried) obtained from Sosa Texcoco S.A. in December/73. This report presents an account of the major relevant findings from the literature and patent search and presents results obtained from the bench scale experimentation program undertaken to accomplish the above objectives.

3. LITERATURE AND PATENT REVIEW

3-1 Solvent Extraction

Decolorization of algae by solvent extraction has been investigated in several laboratories. Major findings are reviewed below.

Patents have been filed by the Institut Francais du Petrole (e.g. French Patent No. 1,591,570 June 12, 1970) describing a procedure for extracting algae with water and/or an organic solvent. Patent coverage is claimed for all of the common practical organic solvents - those containing "1 to 20 and preferably 1 to 8 carbon atoms per molecule". In the example cited, 55 g of dewatered Spirulina platensis (82% moisture content) is extracted in a Soxhlet apparatus with 1.5 liters of an equal volume mixture of water and ethanol. It is claimed that the algae are progressively discolored to a greyish white mass containing 65% protein (dry basis) and that during the course of the extraction the solvent is colored blue-green after 10 minutes changing to yellow after 15 minutes.

We have been unable to locate any published information or obtain any private comment concerning IFP work on decolorization of algae beyond that contained in the above patent. From our reading of the patent it appears that a preliminary investigation has been made to demonstrate that color components can be removed from algae as from other plant material using the common extraction solvents. No attempt appears to have been made to optimize the extraction procedure specifically for algae or to develop a practical process. In view of the broad coverage claimed and the limited scope of the supporting investigation, the patent may well be void on the grounds of prior art. We note that many of the common analytic procedures for determining the pigment

content of plant material (for example, the chlorophyll content of grass) specify extraction of the material with solvents such as are specified in the above patent. Also, several publications have been located which predate the IFP patent and which describe the extraction of Chlorella algae with common solvents to remove color and flavor components (see following).

Studies at the National Institute of Nutrition in Japan (Refs. 1,2) have indicated that methanol is more effective than acetone and ethanol in removing color from Chlorella algae. Acting on this observation, a later investigation (Ref. 3) carefully studied the extraction of Chlorella with methanol. An aqueous algal paste containing 30% solids was produced by centrifuging at 25,000 rpm. A quantity of paste equivalent to 40 g dry solids was blended in a Waring blender in 200 ml of methanol for 30 seconds. It was noted that the heat evolved by blending in excess of 1 minute appeared to fix pigment and produce a brownish discoloration that could not be extracted. The blended suspension was transferred to a Soxhlet thimble and extracted with 1 liter of methanol. Distillation rate was 2.5 l/hr with a siphon interval of 3 minutes. The mass was stirred during extraction (200 rpm). Complete extraction was accomplished in 2.5 hours.

On filtering, it was found that the methanol extracted algae had a noticeable green color. This was removed by suspending in a 3% solution of hydrogen peroxide for 18 hrs or a 5% solution for 5 hrs. The final product was white with a slight green tinge. Yield and protein values are reproduced below.

Extraction of Chlorella with methanol with subsequent bleaching (Ref. 1)

Sample	Dry Weight (g)	Yield (%)	Crude Protein (% dry basis)
Raw algae	39	-	48.0
Methanol extracted	27	69.2	68.2
Methanol extracted, treated with 3% H_2O_2	25	64.1	69.0

The bitter taste characteristic of the raw algae was found to have been completely removed by the methanol extraction. Various methods of cell rupture were investigated including sonic oscillation at 10 Kc/s, extended treatment in a Waring blender and homogenizer, and grinding in a ball mill. These were all effective but required treatment for extended periods. The methanol extraction described was judged to be as effective as any mechanical treatment in terms of number of resultant fractures of the cell membrane.

Samples of spray dried algae have been extracted in a soxhlet apparatus by the technical staff of Sosa Texcoco S.A. (private communication). Sample charge was 3 g. Boiling vessel capacity was 250 ml. Extraction time averaged 12 hours. Results are summarized as follows:

Solvent	Color of Product
Ethanol/water (variable proportions)	intense green
Ethanol/acetone/water (80:10:10)	yellow
Ethanol/acetone (80:20)	yellow
Isopropanol/acetone (80:20)	intense green

Stagewise extraction experiments have been conducted by Sosa Texcoco S.A. using samples of spray dried algae. The extraction vessel consisted of a ball mill to provide adequate cell destruction and agitation. A contact time of 30 minutes in each stage was required to reach equilibrium - i.e. after 30 minutes the absorbance of the solvent was constant. Fresh solvent (equal volume mixture of ethanol and iso-propanol) was used in each stage.

Trial 1

Solvent ratio, 2.5:1 w/w initial stage
1.5:1 w/w subsequent stages.

No. of extraction stages - 4.

Extraction was not complete after 4 extraction stages.

Absorbance of final extract (dilution 333:1) @ 440 nm - .045
@ 680 nm - .02

The residue was further extracted in a fabricated vessel at 150°C. for 3 hours. After 3 hours extraction was apparently complete and a grey-green residue obtained.

Trial 2

Solvent ratio, 1.19:1 w/w initial stage
0.79:1 w/w subsequent stages.

No. of stages - 6.

Extraction was not complete after 6 stages.

Absorbance of final extract (dilution 333:1)	@ 440 nm - 0.18
	@ 680 nm - 0.07

The light green residue obtained after six extraction stages was further extracted for 15 hours at high temperature. A yellow green residue was obtained.

In summary, the work at Sosa Texcoco S.A. has indicated that much of the color can be removed from samples of dry algae by solvent extraction. Cell disruption and agitation during extraction are both important. Equilibrium conditions are reached in about 30 minutes on contacting algae with solvent at low solvent ratios. A large number of extraction stages is required (greater than six) to obtain complete extraction at low solvent ratios (of the order 1:1). An acceptable product has not been obtained to date by stagewise extraction. A pressure extractor has been used, operating on a soxhlet principle, to produce yellow/green residues. It is noted that severe processing conditions such as are encountered in the fabricated pressure extractor could adversely affect product quality.

3-2 Chemical Bleaching

Treatment of algae with hydrogen peroxide has been described in U.S. patent 3,288,613 (Nov. 29, 1966) assigned to the General Electric Company. To a 1% aqueous suspension of algae there is added 1% of a 30% hydrogen peroxide solution. After reacting for fifteen minutes, it is claimed the algae can be consumed directly as algae soup. It is postulated that the peroxide oxidizes some of the cellular material, making the algae more digestible. Color is not removed by the process.

It has been noted (Ref. 4) that chemicals used for bleaching algae have a tendency to adversely affect the nutritional value of the product. Also bleached algae has been found to be unfit for human consumption due to fat rancidity.

Chemical bleaching may be more appropriate as a means of removing the last traces of color from solvent extracted algae. Residual coloration was removed by treating methanol extracted algae (Chlorella) for 5 hrs in a 5% solution of hydrogen peroxide (Ref. 3). The product was free from rancidity problems as the fat as well as most of the pigments were removed during solvent extraction.

3-3 Intense Light Treatment

A process for permanently bleaching algae without affecting the nutritional qualities by exposing an algae culture to intense white light has been described in U.S. patent 3,197,309 (July 27, 1965) assigned to the Boeing Company. The algae suspension is exposed to artificial white light at an intensity in excess of 5,000 foot candles for 8 to 16 hours. The pH of the culture is maintained at 8.0 to 8.5. Agitation is achieved by introducing gaseous oxygen. It is essential to exclude carbon dioxide from the system before exposure. This is accomplished by aging the suspension for 72 to 96 hours prior to treatment.

During the course of light treatment, the culture shows a slowly increasing rate of oxygen uptake. As endogenous oxidation progresses, the rate of uptake continues to increase rapidly and may reach a level several times higher than that due to normal respiration. During this period, destruction of chlorophyll begins. It is noted that some easily oxidized cell components are destroyed before chlorophyll breakdown begins. It is claimed that the process yields a fluffy white or light tan

powder of bland flavor which may be used directly as a food supplement.

A decolorization experiment is described in Ref. 3 in which the algae are forced into a state of rapid respiration by intense light treatment accompanied by the cessation of photosynthesis. Decoloration was achieved comparable to that obtained by methanol extraction. However, substantial oxidation of cell components occurred and the final protein content of the product was only 6% compared with 69% for the solvent extracted material.

3-4 Enzyme Treatment

Removal of chlorophyll from algae by the action of the enzyme chlorophyllase has been investigated (Ref. 5). A thirty day reaction time was required to completely remove the chlorophyll from a sample of Scenedesmus algae. Autolysis of algae was found to retard enzyme action. Methanol extraction of algae was improved when chlorophyllase was added to the solution.

4. PRELIMINARY TRIALS

Four methods which might have potential for removing color from Spirulina algae have been considered. Evaluation has been based on literature and patent review and on the results of preliminary experimentation. Findings are summarized below.

4-1 Intense Light Treatment

Illumination of algae (5,000 foot candles) in the absence of carbon dioxide for periods up to sixteen hours is reported to remove color (see literature review). The results of preliminary experiments with Spirulina algae indicate that it is necessary to work in very dilute solutions to achieve decoloration. A 0.3% aqueous algae solution was decolored in 24 hours under 5,000 foot candle illumination. No change in color was apparent on illuminating a 4% solution at 100,000 foot candles for 24 hours.

Although color can be removed by light treatment, there are a number of disadvantages to the concept:

- the process is incompatible with recovery of a pigment by-product.
- fat is not removed during treatment with the result that the final product is likely to be rancid.
- the requirements of low concentration (less than 0.5%) and relatively long illumination periods would necessitate large holding capacities and an impractical number of light sources.

The process may be more effective as a means of brightening a product from which most of the color has been removed by other means. Lower color intensities would permit higher working concentrations and/or shorter illumination periods.

4-2 Chemical Bleaching

Chemical bleaching using hydrogen peroxide has been investigated as a means of removing color from algae in aqueous suspensions. A suspension containing 5% peroxide - 1% Spirulina algae was stirred at 40°C. After 23 hours, the color of the suspension had changed from the original deep green to yellow-brown. Odor was not removed by peroxide treatment.

Chemical bleaching of whole algae in aqueous suspension was not pursued beyond the above preliminary experiments due to the large bleach requirement and other predictable disadvantages including rancidity and odor in the final product, incompatibility with by-product recovery and possible toxic effects. Reaction of peroxide with unsaturated fatty acid substrates could lead to the formation of polymerized products, certain of which are currently suspected of having carcinogenic properties. Further, hydroperoxide derivatives of fatty acids have been shown to have a deleterious effect on the synthesis of lipids in the livers of rats (Ref. 6). Thus peroxide treatment of whole algae containing unsaturated fats would probably be suspect from a toxicity viewpoint. However, it is noted that a final peroxide treatment to brighten a protein product after fats have been removed by some other method might be both safe and effective.

4-3 Enzyme Treatment

Investigation of enzyme treatment has indicated that the concept does not have sufficient potential as a means

of removing color from algae. Difficulties include the following:

- Availability - At least three specific enzymes would be required to achieve complete decoloration by removing biliprotein pigments, chlorophyll and carotenoids. No commercial source for the specific enzymes required has been found. In fact, the scientific literature does not make mention of specific enzymes for carotenoids and biliproteins.

- Selectivity - Enzymes sufficiently cheap to be considered would be severely contaminated with other enzymic activities. For example, an enzyme which would degrade the biliprotein phycocyanin would have a proteolytic activity serving to increase the solubility of protein and reduce protein yield.

- By-product Recovery - The enzymically degraded xanthophylls would not have the desired pigmenting value for poultry, hence the process would not be compatible with by-product recovery.

It is noted that the autolytic enzymes present in the algae itself have color removal potential. There is evidence that these assist in the removal of biliprotein pigment but unfortunately autolysis serves to reduce the yield of protein as well.

4-4 Solvent Extraction

Solvent extraction of algae has been selected as the most promising method for removing color from algae. Literature reports and private communications indicate that

chlorophyll and carotenoid pigments can be removed by organic solvents, particularly the low boiling alcohols. An extraction process is also well suited to recovery of a pigment by-product, shown in Phase I to have commercial potential. There are a number of other inherent advantages to extraction when compared with other possible processes reviewed above. The raw material can be either in dry powder or dewatered paste form - i.e. there is no need to work with dilute suspensions as is the case for intense light treatment. Extraction conditions can be controlled to minimize loss of protein. As solvent can be recovered for re-use, there is no direct chemical consumption (apart from solvent loss) such as is incurred in chemical bleach or enzymic processes.

As a first step in developing an extraction process for algal decolorization, preliminary trials were conducted to investigate in a general way the degree of decolorization attainable, likely yields and requirements with regard to extraction conditions.

4-4-1 Ethanol Extraction by Blending/Centrifuging

A 100 g sample of algae paste, obtained from Sosa Texcoco S.A. in December/73 and maintained frozen at -35°C. for one month, was thawed and mixed with 500 ml absolute ethanol. The paste contained 15.6% solids. The solvent to solids ratio was 25.6 w/w.

The suspension was blended with a high speed Polytron blender at 50°C. for 5 minutes. Blending served to fracture algal cells and reduce particle size. The suspension was centrifuged at 5,000 rpm for 10 minutes.

The above procedure was repeated twice for a total of three extractions using fresh solvent in each stage.

Extraction No.	1	2	3
Supernatant	dark green	dark yellow	yellow
Residue	light green	blue green	blue

Final recovery of blue residue after three extractions was 9.79 g for a solids yield of 63%.

Subsequent extractions with ethanol failed to remove the blue color from the residue obtained above. A confirmatory experiment with ethanol gave a solids yield of 62%.

The procedure was repeated using acetone as solvent (solvent ratio 37:1). After three extractions, a blue green residue was obtained. A fourth extraction with ethanol resulted in a blue color similar to that obtained above.

4-4-2 Characterization of Blue Residue

The blue residue obtained by successive low temperature extractions with ethanol contains phycocyanin, the major blue pigment of blue-green algae. Phycocyanin is a biliprotein - a protein which has a bile pigment (a linear tetrapyrrole)chemically bonded to the protein chain. The pigment or chromophore attached to the protein and imparting the characteristic blue color to phycocyanin is phycocyanobilin.

Cleavage of chromophores from biliproteins, and particularly cleavage of phycocyanobilin from phycocyanin, has been studied and the results reported in the scientific literature. Because the chromophore is chemically bonded to the protein molecule, it cannot be removed by simple contact with

organic solvents as is the case for the other algal colorants - chlorophyll and the carotenoids. Four methods for chromophore detachment have been reported:

- Methanol reflux - Denatured phycocyanin was refluxed for 16 hours (Ref. 7) in methanol with constant stirring.

- Acid cleavage - Phycocyanin was dispersed in concentrated HCl at 25°C. for 30 minutes, diluted to 3 M HCl and filtered (Ref. 7).

- Enzymatic cleavage - Various proteolytic enzymes were investigated and Nagarase, obtained from Enzyme Development Corporation of New York, was the only one found to be effective (Ref. 8). Samples of 10 g of denatured phycocyanin were suspended in 50 ml of 0.25 M potassium phosphate buffer, pH 7.0, and incubated with 100 mg Nagarase at 37°C. for 16 hours.

- Alkaline cleavage - Refluxing phycocyanin in 10% KOH in methanol for 15 minutes has been shown to convert phycocyanobilin to mesobiliverdin, with detachment of the green pigment from the protein molecule (Ref. 7).

Methanol reflux is claimed to be the most effective method for removing phycocyanobilin from phycocyanin. Phycocyanobilin yields, assuming the phycocyanin contained 5% chromophore, were:

methanol reflux	32 %
enzyme cleavage	16 %
HCl cleavage	8 %
alkaline cleavage	not reported

The mode of attachment of phycocyanobilin to the protein has been investigated (Ref. 9). While there is still some uncertainty as to the location of the linkage sites on the phycocyanobilin molecule, it appears that the chromophore is covalently linked to the protein through an ester bond. There is also evidence to suggest that the chromophore is well buried in the protein moiety and perhaps hydrogen bonded as well through the pyrrolenine nitrogen. It has been postulated that detachment of the chromophore by hot neutral methanol, ethanol and other alcohols could proceed by a transesterification reaction.

4-4-3 Detachment of Blue Pigment from Ethanol Extracted Algae

Literature reports indicate that refluxing in methanol is the most effective method for removing phycocyanobilin (blue pigment) from protein. Four grams of blue residue obtained by ethanol extraction of Spirulina algae were refluxed in 1 liter of methanol. After 16 hours the hot suspension was filtered. The filtrate was blue indicating that the pigment had been successfully detached from the protein. The residue was air dried and milled to produce a near white powder. The yield of solids, based on the original blue residue charge, was 88%.

Refluxing for longer periods up to 40 hours did not significantly improve product color. A slight tan or fawn tinge became apparent after prolonged reflux, possibly indicative of protein degradation and/or scorching.

The effectiveness of various solvents for detaching blue pigment was investigated by refluxing samples of blue residue for 16 hours and noting product color and yield (Table 1).

Table 1. Effectiveness of Various Solvents for Detaching Blue Pigment

Solvent	Product Color	Yield (%)
Methanol	near white	88
Ethanol	pale green	90
Iso-propanol	gray	90

The results of the alcohol reflux experiments are consistent with the view that the blue pigment is chemically bonded to the algal protein but can be detached by means of a transesterification reaction involving alcohol. Methanol appears to give the best product, followed by ethanol and iso-propanol. It is possible that the effectiveness of the solvent may be related to steric factors. If the pigment prosthetic group on the protein is shielded by the large protein molecule, as literature reports indicate, then the size of the alcohol molecule might be significant - i.e. in terms of effectiveness methanol >ethanol >iso-propanol.

Alkaline cleavage of blue pigment was investigated by suspending 2 g blue residue in 1 l. of 1 M KOH in methanol. The residue turned green immediately on contacting the alkaline solution. The green color was removed from the residue by refluxing the solution for 1/2 hour. On filtration and drying, a white residue was obtained.

The yield of product obtained from alkaline reaction was 45%, only one-half that obtained by methanolysis. It appears that hydrolysis of the protein occurs under the alkaline conditions employed. Alkaline cleavage was not pursued further because of the low yield evident in preliminary experimentation.

Acid hydrolysis of blue residue was investigated using acid strengths up to 5% v/v in alcohol and in acetone. No color was extracted, in fact the blue color of the solids became more intense. Hydrolysis in concentrated HCl is reported to have removed pigment (see above) however this was not considered a practical course to pursue due to the obvious risk of protein loss in strong acid.

Enzyme cleavage was not pursued as literature reports indicate that the yield of detached pigment is below that for methanolysis. Further, a proteolytic enzyme is required which would have a deleterious effect on the yield of protein product.

4-4-4 Effect of Pretreatment on Extraction and Product Quality

Tests were undertaken to establish the effect of algae drying mode on product quality. Fat, chlorophyll and carotenoids were removed by successive extractions with alcohol at high solvent ratio*. The intermediate residue was refluxed in methanol for 16 hours. The extracted material was dried in an air oven at 105°C. and yield and color noted (Table 2).

* During each extraction stage the algae sample was dispersed in the appropriate amount of alcohol, heated to 50°C. and blended for 5 minutes with a Polytron blender. Separation was achieved by centrifugation at 5,000 rpm for 10 minutes.

Table 2. Variation of Yield and Product Quality with Drying Mode

Sample	Solvent Ratio	No. of Extraction Stages	Intermediate Color	Intermediate Yield	Product Color	Product Yield
Dewatered algae (15.6% solids)	25:1	3 (ethanol)	blue	63	white	56
Roller dried[1]	25:1	5 (methanol)	gray-green	68	brown	64
Freeze dried[2]	25:1	3 (methanol)	blue	68	light tan	61
Spray dried[3]	25:1	3 (methanol)	blue	61	pale green	56

Notes

[1] Roller dried product obtained from Sosa Texcoco, December/73.

[2] Dewatered algae freeze dried at R.P.C.

[3] Dewatered algae spray dried at R.P.C., drier outlet temp. 80°C.

The final products obtained from the dewatered, freeze dried and spray dried samples were judged to be acceptable in terms of color and odor. The product obtained from roller dried algae was brown indicating that the protein had been scorched during the drying process. Extractable components were more difficult to remove from the roller dried product, possibly because of bonding with the protein at the high temperatures encountered during roller drying.

Variation in final product yield appears to be due to autolysis of the algae during storage. The material which was subsequently freeze dried was held frozen in dewatered form for three weeks vs. six weeks for the material which was subsequently spray dried. The sample of dewatered algae used in the above experiment had been held frozen for six weeks.

The degree of cellular damage sustained by the algae prior to or during extraction has been found to affect the ease with which extractable components are removed. Polytron blending, to provide maximum particle attrition during extraction, was found to be more effective and result in fewer extraction stages, than high speed mixing. On a production scale, the required degree of cellular damage could be attained by comminuting an algal paste in a colloid mill or by grinding an algal powder in a ball mill prior to extraction.

Tests were conducted on methanol extraction of freshly harvested algal paste in both milled and unmilled form. The results showed that the freshly harvested algae was somewhat more difficult to extract - i.e. one or two further cycles were required than for frozen material previously investigated; the extracts were much greener indicating that the chlorophyll was not degraded; the intermediate blue residue was obtained in higher yield (72-78% vs. 63-65%) and had a deeper color indicating less autolytic action; the unmilled material was very easily filtered compared with the milled fresh and the frozen material. The results indicate that the frozen material has suffered cellular disruption aiding extraction and some degree of autolysis causing decreased yield and marginally improved extraction of biliprotein pigment.

5. INVESTIGATION OF EXTRACTION PARAMETERS

Preliminary trials have indicated that solvent extraction is the most suitable process to pursue for decolorization of algae although there may be difficulty in removing biliprotein pigment. To further the laboratory investigation of decolorization, a two step process is considered in which fat, chlorophyll and carotenoid components are removed by conventional extraction followed by a reaction step to detach the biliprotein pigment. In this section, extraction of algae to produce an intermediate product is investigated to acquire information as to the most suitable extraction mode, solvent and raw material form.

5-1 Laboratory Soxhlet Extraction Experiments

Soxhlet extraction, in which the material to be extracted is retained in a cellulose thimble and contacted with a continuous stream of fresh solvent, is a convenient means of comparing the effectiveness of various solvents for removing color from algae. In the experiments which follow, the progress of the extraction was followed by sampling periodically the colored solvent in the reboiler and measuring absorbance at 436 nm (carotenoids) and at 660 nm (chlorophyll). The contents of the cellulose thimble were stirred briefly at each sampling to prevent solids adhering to the thimble walls.

Extraction experiments were performed at the outset with methanol as solvent, using algae paste and spray dried powder as raw material. Raw material charge was 9 g (dry basis). Flow of fresh solvent was 15 ml/min. Absorbance of the solvent in the reboiler flask is plotted in Figure 1. The curves obtained for absorbance at 660 nm are similar to those plotted for 436 nm in Figure 1. A blue residue was visible after 17 hours

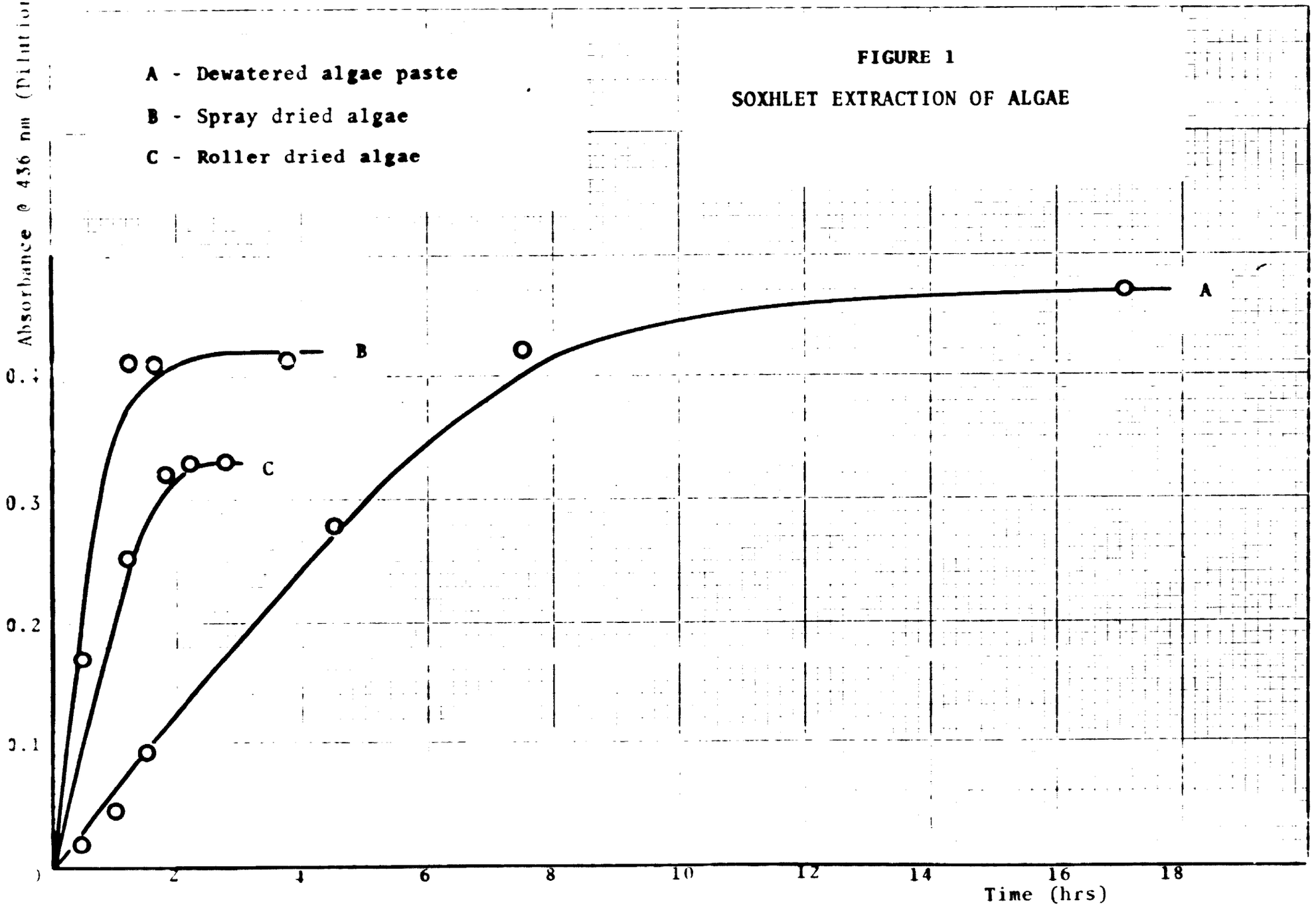


FIGURE 1

SOXHLET EXTRACTION OF ALGAE

in the case of algae paste and after 1 hour with spray dried powder. Solids yield was 47% for paste, 60% for powder. The results indicate that static extraction of algae should be considered only for algal powder. The presence of water tends to retard the progress of the extraction unless violent mixing is employed to thoroughly mix water and solvent so that the water is removed rapidly from the paste. The solvent ratio for the extraction of spray dried powder, calculated as the fresh solvent required to reach the blue residue stage, was 79:1.

A similar extraction performed with roller dried algae is plotted in Figure 1. The extraction was complete after 3 hours vs. 1 hour for spray dried material. A gray-green residue was obtained. Some of the color components are apparently bonded to the protein by roller drying and are difficult to extract. The lower final absorbance value for the experiment is consistent with analytic findings of lower carotenoid in the roller dried product.

Soxhlet extractions of spray dried algae were performed using various solvents as shown in Figure 2. The solvents selected were those which have application in food processing and with the possible exception of methanol would likely be readily approved for use in decolorizing algae for human consumption. Product yield and appearance were noted (Table 3).

On the basis of the data in Table 3, methanol and ethanol are the preferred solvents for decolorization. These gave the highest absorbance values in the series of controlled experiments indicating best color removal performance. The products obtained using ethanol and methanol were the most acceptable of those obtained in terms of residual color.

FIGURE 2

SOXHLET EXTRACTION OF SPRAY DRIED ALGAE

EFFECTIVENESS OF VARIOUS SOLVENTS

A - Ethanol
B - Methanol
C - Iso-propanol
D - Acetone
E - Ethylene Dichloride
F - Hexane

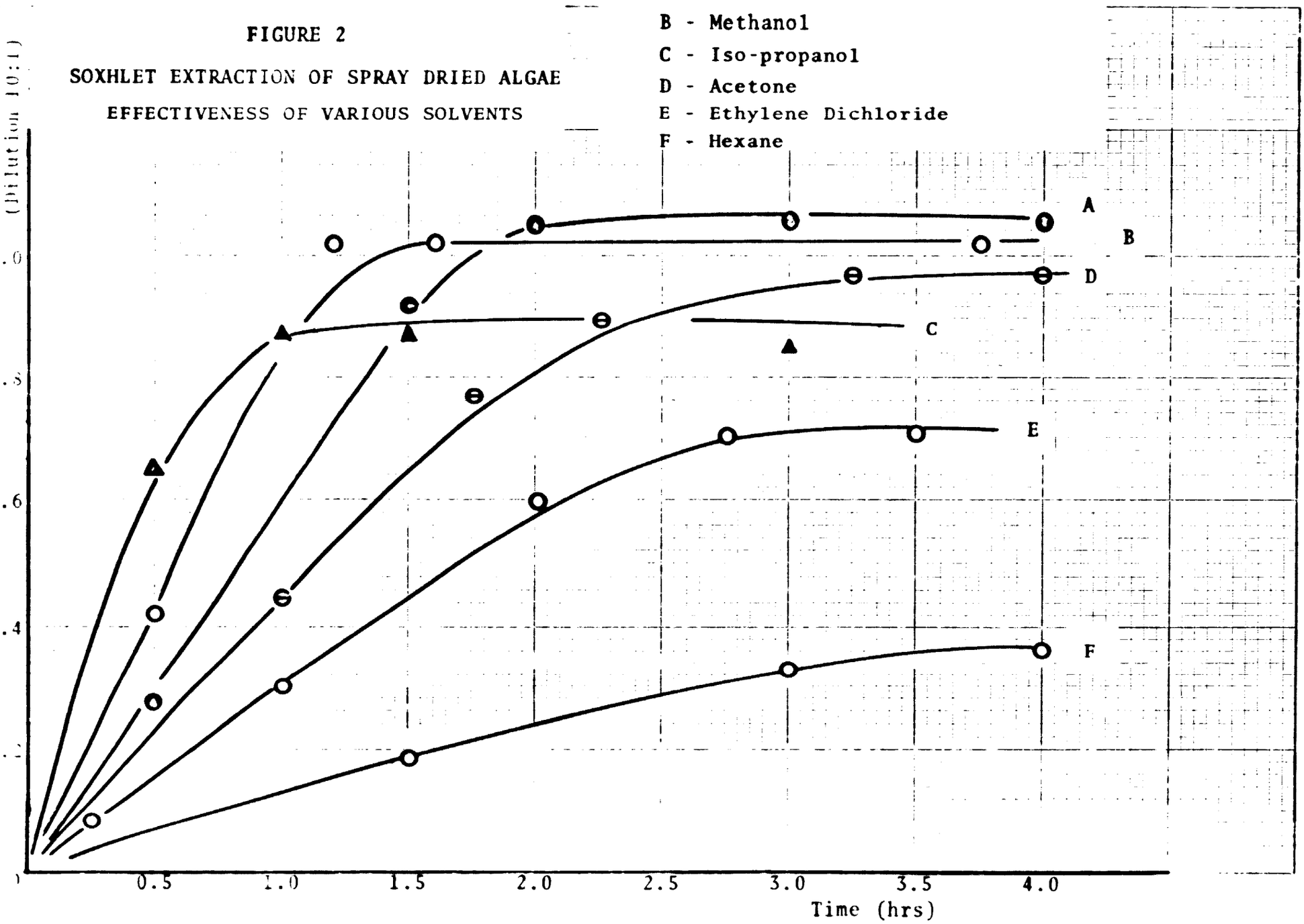


Table 3. Soxhlet Extraction of Spray Dried Algae

Solvent	Extraction Time (hrs)	Final Absorbance @ 436 (10:1)	Product Yield (%)	Product Color
Methanol	1.5	1.02	60	blue
Ethanol	2.5	1.05	72	pale green
Iso-propanol	1.5	.88	82	green
Acetone	4.0	.95	81	deep green
Ethylene Dichloride	3.0	.71	74	deep green
Hexane	4+	.36	79	deep green

Iso-propanol is less effective, removing less color than methanol and ethanol, yielding a product having a distinct green color. Acetone appeared to remove a comparable amount of color (as iso-propanol) judging from absorbance measurements on the solvent, but the final product was a deeper shade of green relative to the iso-propanol product.

Ethylene dichloride was inferior to acetone and iso-propanol. Hexane was clearly not acceptable as the final product retained the characteristic algal odor.

5-2 Stagewise Extraction of Algae

In view of the Soxhlet extraction results obtained using algal paste and the very fine particle size of algal powders, it is more appropriate to consider stagewise extraction with alternate mixing and separation rather than fixed bed extraction with percolation.

Experiments were undertaken to determine the number of extraction stages required to remove color (to reach the blue residue form or equivalent) as a function of solvent ratio (wt solvent:wt solids). An extraction stage consisted of blending algal solids with the appropriate amount of fresh solvent at 50°C. for 5 minutes using a Polytron blender, followed by centrifugation at 5,000 rpm for 10 minutes. Absorbance of the supernatant was recorded. Extraction was assumed to be complete when the absorbance of the undiluted supernatant was less than 0.1.

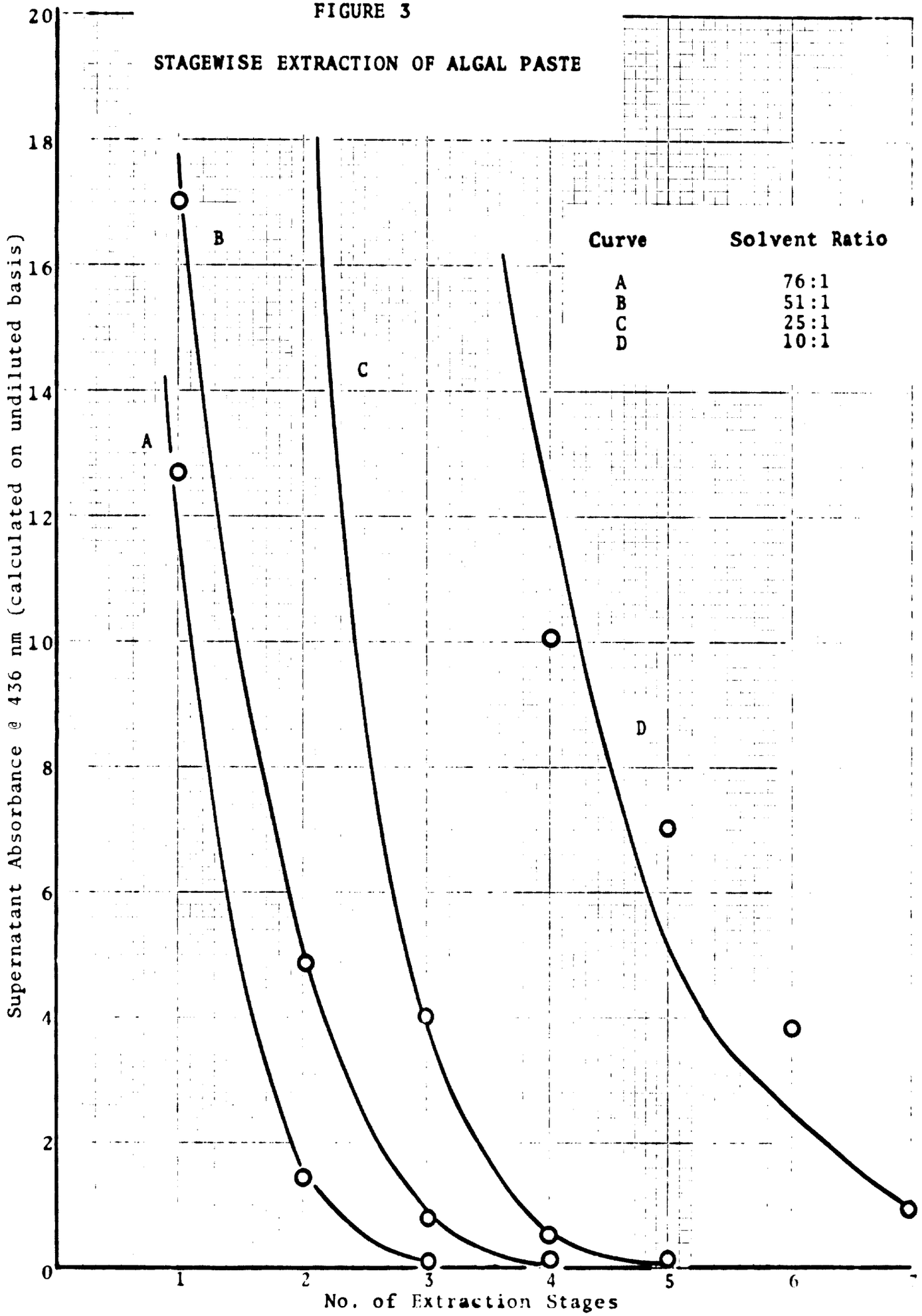
The results of the stagewise extraction experiments using algal paste and spray dried algae as raw material are presented in Figures 3 and 4 respectively. Average yield of solids for the four experiments using algal paste was 60%, for spray dried algae - 57%.

As a rough guide, the solvent ratio should be below 10:1 and preferably as low as 7:1 for economic extraction of fine powders. The lower the ratio, the lower the energy requirement for solvent recovery and the lower the solvent loss. The number of stages considered to be practical will vary with the separation equipment used. If belt filters with countercurrent wash are used between stages, two stages would probably be the economic limit. If hydrocyclones can be used, three to five stages might be acceptable.

It is apparent from Figure 3 that stagewise extraction of algal paste is not practical as the number of stages required at low solvent ratios is unacceptably high. Using spray dried algae (and possibly roller dried algae if dried under conditions which do not result in product scorching) stagewise extraction might be feasible particularly if liquid cyclones are proven effective for separation between stages (Figure 4).

FIGURE 3

STAGEWISE EXTRACTION OF ALGAL PASTE



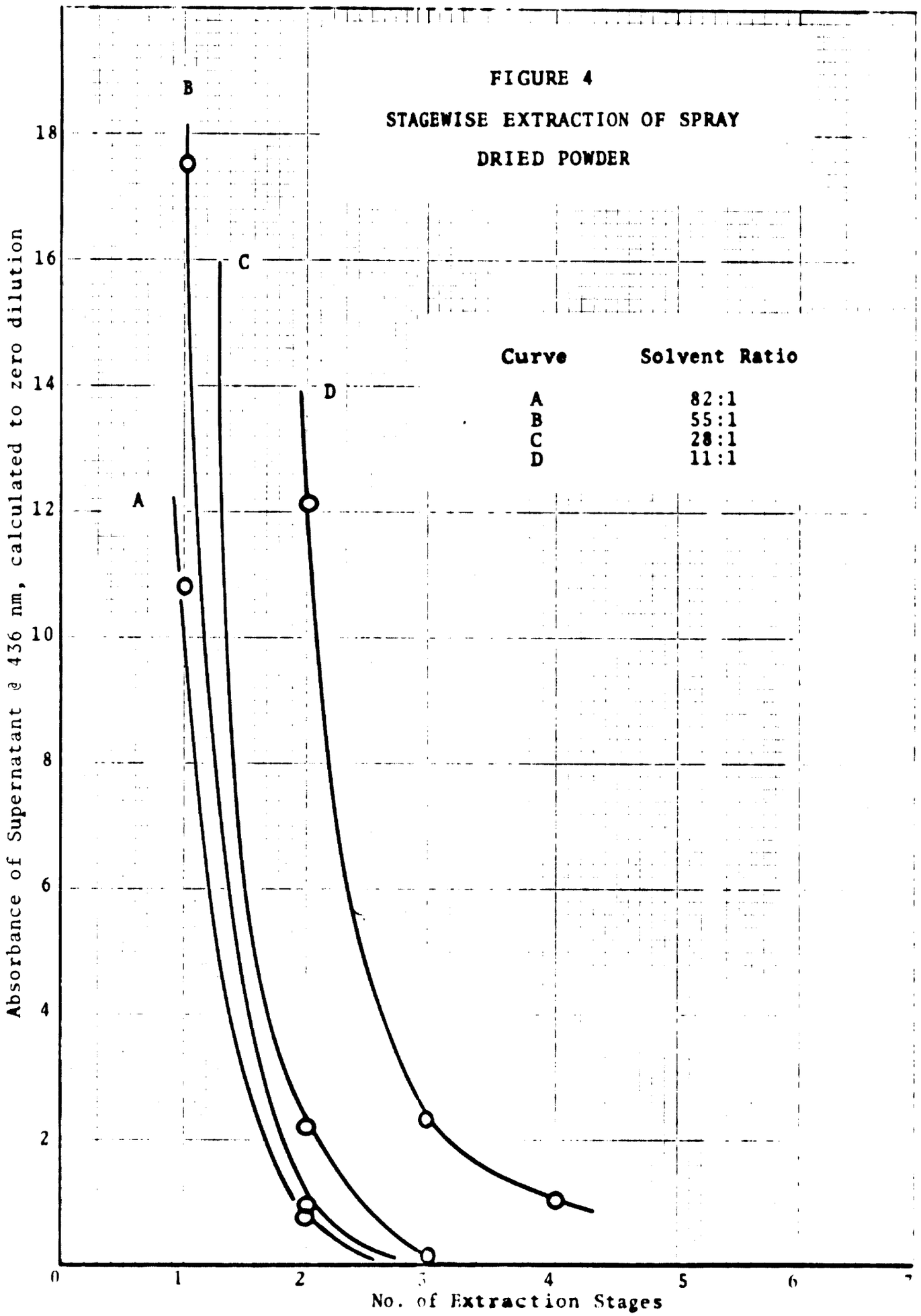


FIGURE 4
STAGEWISE EXTRACTION OF SPRAY DRIED POWDER
Curve
Solvent Ratio
A
82:1
B
55:1
C
28:1
D
11:1
Absorbance of Supernatant @ 436 nm, calculated to zero dilution
No. of Extraction Stages
0
1
2
3
4
5
6
2
4
6
8
10
12
14
16
18

The composition of product (blue intermediate) and extracts obtained from a stagewise extraction experiment are presented in Table 4. An approximate material balance for the extraction is contained in Table 5.

Table 4. Composition of Algae, Blue Intermediate Product and Successive Extracts

Solvent - methanol
Raw material - algal paste
Solvent ratio - 50:1 w/w

	Algae (% dry basis)	Blue Intermediate (% dry basis)	Extracts (% w/v)			
			1	2	3	4
Protein	53.3	61.7	0.101	0.019	0.006	0.004
Fat	2.2	0.3	-	-	-	-
Ash	5.3	2.7	0.049	0.012	0.004	ND
Carbohydrate	37.8	35.3	-	-	-	-
Water			8.51	1.03	0.45	0.18
Total Solids			0.375	0.049	0.017	0.011

ND - Not detected.

Table 5. Material Balance - Stagewise Extraction to Blue Intermediate

Basis - 100 g algal paste
- 4 extractions with 1 l. each of methanol

Component	Extracts					Blue Intermediate	Total Output	Input (Algae)
	1	2	3	4	Total			
Water (g)	70.60	8.60	3.70	1.50	84.40	-	84.4	84.4
Solids (g)	3.10	0.40	0.10	0.10	3.70	10.50	14.2	15.6
Protein (g)	0.80	0.20	0.05	0.03	1.10	6.50	7.6	8.3
Ash (g)	0.40	0.10	0.03	-	0.53	0.28	0.8	0.8

The yield of recovered solids (blue intermediate) was 67%. Protein yield was 78%. All of the solid material apparently lost during extraction could not be accounted for in the extract solution. It appears that some metabolism of organic material may have occurred during refrigerated storage of the extract samples prior to analysis. No loss of the ash component by degradation on storage would be expected. All of the ash present in the original algae can be accounted for by material balance.

5-3 Two Stage Countercurrent Extraction

A countercurrent extraction system consisting of two extraction stages with intermediate and final filtering and solvent wash was devised (Schematic 1) for bench scale experimentation. An attempt was made to approximate the results which might be obtained in a continuous countercurrent unit by running several batch experiments and storing intermediate solvent streams between runs.

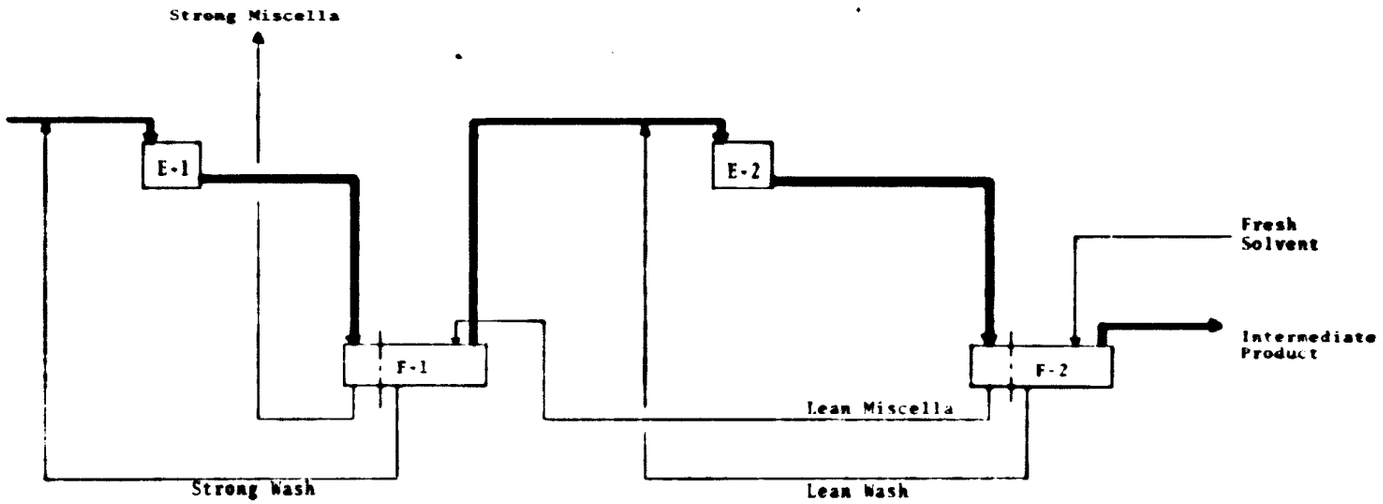
REVISIONS

E-1, E-2 - Extraction Vessels

F-1, F-2 - Horizontal Belt Filters

SCHEMATIC 1 - CONCEPTUAL DIAGRAM

2-STAGE COUNTERCURRENT EXTRACTION



Product Flow

Solvent Flow

DWG NO.

RELATED DWGS

JOB No. 3402	PROJECT ALGAE DECOLORIZATION
DESCRIPTION Conceptual Diagram	PATTERN No / MATERIAL
	DR PAL / SCALE
	CH / DWG No

NEW BRUNSWICK RESEARCH & PRODUCTIVITY COUNCIL, FREDERICTON

The extraction vessel consisted of a 300 ml flat bottomed boiling flask fitted with a condenser and heated on a magnetic stirrer hot plate. Filtration was accomplished using a Buchner funnel with Watman qualitative paper. Appropriate quantities of spray dried algae were blended in solvent at 50°C. using a Polytron blender. The algal suspensions were refluxed for one hour in each stage. Results are summarized in Table 6.

Table 6. Two Stage Countercurrent Extraction

Solvent	Solvent Ratio	Filter Cake after Intermediate Wash	Filter Cake after Final Wash	Absorbance of strong Miscella at 436 nm (Dil. 500:1)	Yield of Solids
Methanol	8:1	blue-green	blue	0.22	61
Methanol	6:1	blue-green	blue	-	64
Methanol	4:1	blue-green	blue	0.44	64
Ethanol	6:1	green	pale green	-	74
Ethanol	4:1	green	pale green	0.42	78

Extraction of spray dried algae with methanol in two stages produced a blue intermediate product at solvent ratios as low as 4:1. A similar extraction with ethanol produced a pale green intermediate in significantly higher yield (74% vs. 64%).

Product and miscella composition and approximate material balance for countercurrent extraction using methanol and ethanol at a solvent ratio of 4:1 are presented in Tables 7 and 8.

Table 7. Countercurrent Extraction - Composition of Products and Miscella

	Raw Algae	Methanol Extraction Product	Methanol Extraction Miscella	Ethanol Extraction Product	Ethanol Extraction Miscella
Protein (% dry basis)	53.3	61.8	30.6	59.7	25.2
Fat (% dry basis)	2.3	0.6	5.5	ND	10.7
Ash (% dry basis)	5.3	4.6	9.0	5.1	6.1
Carbohydrate	37.7	33.0	46.0	35.2	46.6
Chlorophyll	0.8	ND	2.2	ND	3.7
Carotenoids	0.6	ND	1.6	ND	2.7
	100.0	100.0	94.9	100.0	95.0

ND - Not detected.

Note: Chlorophyll and carotenoid composition of miscella are estimated values obtained by difference (see material balance Table 8).

Table 8. Material Balance - Countercurrent Extraction

Basis - 15 g spray dried algae. Solvent ratio 4:1.

	Methanol Extraction				Ethanol Extraction			
Component	Product	Miscella	Total Output	Input (Algae)	Product	Miscella	Total Output	Input (Algae)
Solids (g)	8.84	4.91	13.75	13.75	10.77	2.98	13.75	13.75
Protein (g)	5.46	1.50	6.96	7.33	6.43	0.75	7.18	7.33
Fat (g)	0.05	0.27	0.32	0.32	ND	0.32	0.32	0.32
Ash (g)	0.41	0.44	0.85	0.73	0.55	0.18	0.73	0.73
Carbohydrate	2.92	2.26	5.18	5.18	3.79	1.39	5.18	5.18
Chlorophyll	-	0.11	0.11	0.11	-	0.11	0.11	0.11
Carotenoids	-	0.08	0.08	0.08	-	0.08	0.08	0.08

The results indicate there is a significant difference in extraction performance between methanol and ethanol in terms of yield and product color. Being more polar, it is to be expected that methanol will extract more ash. The material balance (Table 8) however, indicates that methanol extracts more protein and organic solids as well. Half the difference in yield is due to higher loss of protein values during methanol extraction. Protein yield for ethanol extraction was 88% vs. 74% for methanol.

There is little evidence to suggest that extraction of color is less complete with ethanol. The blue biliprotein pigment is thermally unstable, tending to turn green at high temperature or on prolonged heating at lower temperature. The green coloration of the ethanol extracted product appears to have resulted from degradation of the biliprotein pigment at the ethanol reflux temperature (78°C. vs. 65°C. for methanol) rather than from incomplete removal of fat soluble color components.

To test the hypothesis, a low temperature stagewise extraction of algal paste was performed using ethanol as solvent rather than methanol as used previously (section 5-2). Solvent ratio was 25:1. A blue residue comparable to that obtained previously was produced after 4 extraction stages. Solids yield was 73% using ethanol vs. 60% for methanol. Ethanol would appear to be preferable to methanol from a protein yield viewpoint.

6. REMOVAL OF BILIPROTEIN PIGMENT

Preliminary trials have indicated that the blue residue obtained by exhaustive solvent extraction of algae can be decolored to produce a near white powder by prolonged refluxing in methanol. Literature reports indicate that the blue pigment is biliprotein in which the chromophore is chemically bound to the protein molecule. The chromophore appears to be best detached by a transesterification reaction with alcohol. In this section, detachment of pigment from blue residue is investigated to establish the most suitable reaction conditions consistent with product yield and quality.

6-1 Effectiveness of Various Solvents

Blue residue prepared by methanol extraction of spray dried algae (yield - 58%) was refluxed with various solvents at a solvent ratio of 300:1. Progress was monitored by sampling the solution periodically, filtering and measuring the absorbance of the clear filtrate. It is noted that the extracted blue pigment is unstable and degrades on standing. Spectrophotometric results for blue solutions can therefore only be used as a guide to the efficacy of similar tests. Final product yield and color were noted. Results are presented in Figure 5 and Table 9.

Table 9. Decoloration of Blue Residue with Various Solvents

Solvent ratio 250:1. Reflux time - 18 hours.

Solvent	Product Color	Yield (%)
Methanol	cream with green tinge	88
Ethanol	light tan	93
Iso-propanol	pale blue	95
Acetone	blue-green	96
Chloroform	blue	87

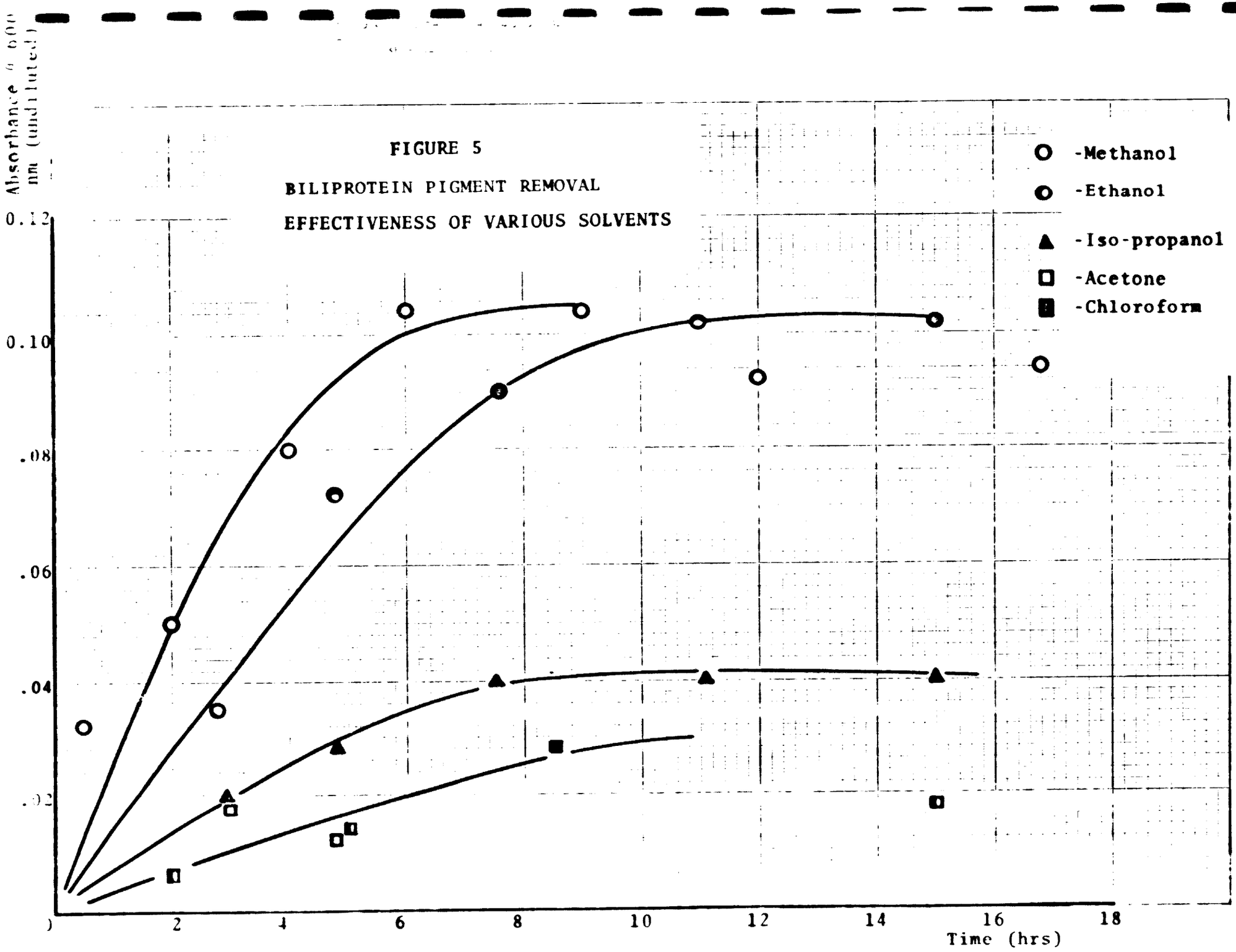


FIGURE 5
BILIPROTEIN PIGMENT REMOVAL
EFFECTIVENESS OF VARIOUS SOLVENTS
-Methanol
-Ethanol
-Iso-propanol
-Acetone
-Chloroform
0.12
0.10
.08
.06
.04
2
4
6
8
10
12
14
16
18
Time (hrs)

The results indicate that methanol and ethanol are the most effective solvents for removing biliprotein pigment. Iso-propanol is markedly less effective, followed by acetone and chloroform. The products obtained from ethanol and methanol treatment were both light in color with acceptable odor and flavor characteristics. As the final absorbance values were similar for methanol and ethanol, the lower yield obtained with methanol reflux appears to be due to loss of crude protein rather than to more complete removal of color. Color removal was apparently complete in 8 hours with methanol, 11 hours for ethanol.

6-2 Solvent Ratio

Three experiments were performed to remove biliprotein pigment from blue residue by methanol reflux at various ratios of solvent to solids. The progress of each reaction is plotted in Figure 6. In each case, the product obtained on drying and milling was light in color with a green tinge. Absorbance values indicated that the pigment detachment reaction was essentially complete after 8 to 10 hours. Product quality and reaction time appeared to be insensitive to solvent ratio over the range 208:1 to 8.5:1.

6-3 Effect of Catalyst

The effect of alkaline and acid catalysts on biliprotein pigment detachment was investigated using sodium methoxide (an alkaline catalyst widely used in fat transesterification reactions) and sulfuric acid.

Sodium methoxide - Two trials were conducted using 5% w/w and 50% w/w catalyst (pH 9.1 and 10.7 respectively) at a solvent ratio of 300:1. Absorbance of the supernatant is plotted as a function of time in Figure 7. Absorbance values cannot be

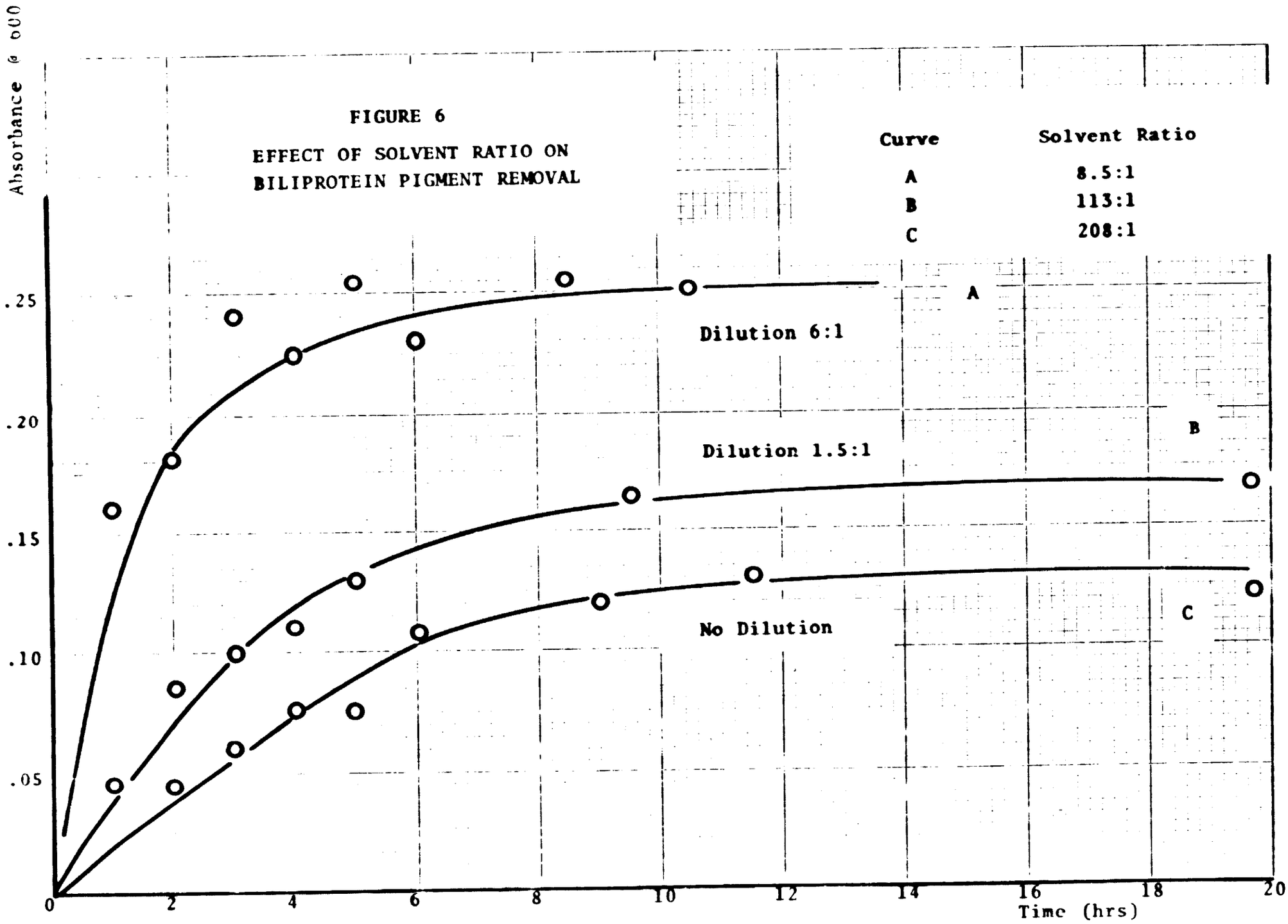
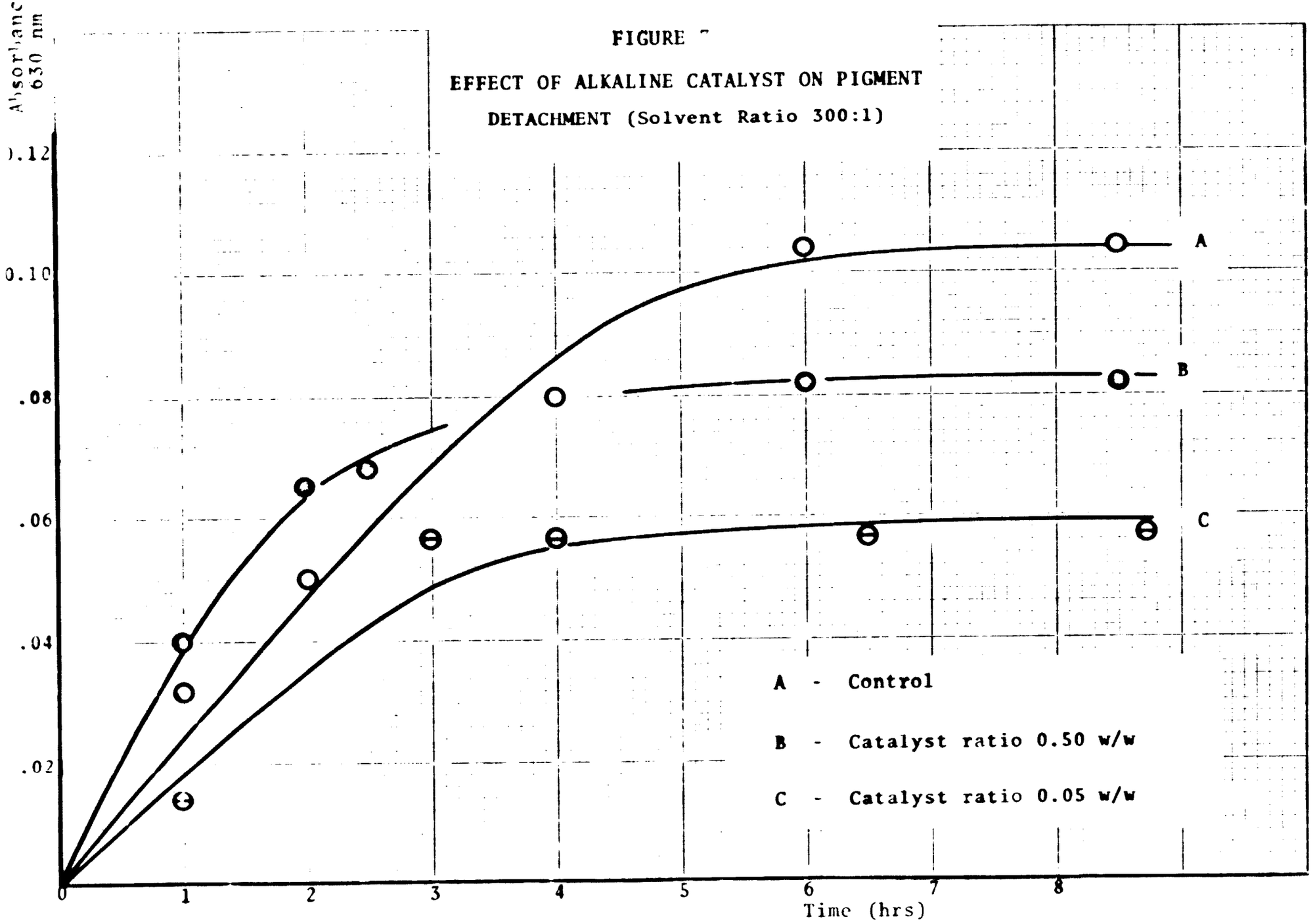


FIGURE 6
EFFECT OF SOLVENT RATIO ON
BILIPROTEIN PIGMENT REMOVAL
Curve
Solvent Ratio
A
8.5:1
B
113:1
C
208:1
Absorbance @ 600
.25
.20
.15
.10
.05
0
2
4
6
8
10
12
14
16
18
20
Time (hrs)
Dilution 6:1
Dilution 1.5:1
No Dilution
A
B
C

FIGURE

EFFECT OF ALKALINE CATALYST ON PIGMENT DETACHMENT (Solvent Ratio 300:1)



directly compared to those obtained from a control experiment without catalyst as the blue color is observed to change to green under alkaline conditions, possibly because of the conversion of phycocyanobilin to mesobiliverdin. The presence of catalyst appeared to have speeded up the reaction somewhat; absorbance values levelled off after 4 to 6 hours vs. 6 to 8 hours for the control. The final product obtained from the trials with catalyst was similar in color to the control (light color with green tinge). However, solids yield fell off with increasing catalyst concentration:

Catalyst ratio	Yield
0	88%
5%	82%
50%	75%

The use of alkaline catalyst was not pursued as only a slight effect on reaction rate was noted even at high catalyst concentrations and also because of the detrimental effect on product yield.

Sulfuric acid - Blue residue was refluxed in 5% sulfuric acid in methanol to investigate the possible catalytic effect of acid conditions. It was found that the blue color was intensified and could not be removed by refluxing. After 10 hours the algal residue was dark blue and turned black on subsequent air drying.

6-4 Effect of Temperature

Experiments were undertaken to determine the effect of high temperature on the removal of pigment from blue residue. A methanol slurry (solvent ratio 300:1 w/w) was heated to 150°C. with

stirring in a Parr pressure reaction vessel (time-temperature-pressure data in Table 10). After cooling and inspection, the heating cycle was repeated. A pale brown product was obtained which tasted scorched. The supernatant was pale blue after the first heating, pale green after the second. Supernatant absorbance at 600 nm was 0.095, comparable to the absorbance obtained after six hours of refluxing at 65°C (Figure 5). Prolonged heating at high temperature evidently degrades the blue pigment producing a green color.

Table 10. Time-Temperature-Pressure Data for High Temperature Trial

Time (min.)	Temperature (°C.)	Pressure (kg/cm^2)
0	50	-
9	70	0.4
13	90	1.1
16	112	1.1
19	124	4.9
22	134	6.7
24	138	7.0
26	145	8.4
27	146	8.8
28	148	8.9
29	150	9.1
35	70	0.5
38	45	-
	Reheat	
0	34	-
6	65	-
13	70	0.5
19	82	1.1
23	100	4.6
29	130	10.2
32	136	11.2
34	140	12.4
36	142	12.6
38	143	13.1
51	142	12.3
	Cooled	

It is apparent that the rate of removal of blue pigment can be increased by increasing the reaction temperature. Decolorization was accomplished in less than 25 minutes at 125-150°C. compared with a required 6 to 8 hours at 65°C. This is consistent with a doubling of reaction rate for every ten degree rise in temperature.

The scorched odor/flavor apparent in the product obtained above would indicate that the reaction temperature used was too high. An attempt was made to determine the scorch temperature, or temperature at which product scorching is apparent after a brief reaction time sufficient to remove pigment. A small sample of suspension (blue residue in methanol, solvent ratio, 10:1 w/w) was sealed in a tube and immersed in a hot oil bath. After five minutes the tube was quench cooled. Results for various temperatures were as follows:

Temperature (°C.)	Product Color	Quality
135	cream, green tinge	not scorched
145	tan	no scorching but burnt taste
152	light brown	scorched

The results indicate that scorching begins at about 150°C. although a burnt flavor can be detected at 145°C. To ensure good quality, suspensions probably should not be heated above 135°C. Work is in progress to determine the effect of reaction temperature on available lysine in the final product. This will serve to define more precisely the maximum working temperature that is consistent with product quality.

Spray dried algae was extracted at high temperature in the Parr pressure reaction vessel using ethanol at a solvent

ratio of 8.5:1. Samples were heated with stirring to the desired extraction temperature (30 minute heating time required), maintained at that temperature for three minutes and rapidly cooled.

Temperature	Product Color	Solids Yield(%)	Product Quality
105 °C.	light green	64	acceptable taste
112 °C.	tan	61	acceptable taste
125 °C.	tan	67	slight burnt taste

Compared with samples prepared from the blue residue, the samples prepared by high temperature single-stage extraction of whole algae appeared to be darker in color and developed a burnt taste at a lower reaction temperature.

6-5 Material Balance - Biliprotein Removal by Methanolysis

The blue intermediate obtained by exhaustive stagewise extraction of algal paste (material balance Table 5) was refluxed in methanol for 16 hours. The light colored product obtained was dried and analyzed (Table 11). A material balance for the alcohol reflux step is presented in Table 12.

Table 11. Composition of Raw Material and Extraction Products (dry basis)

Sample	Algae	Blue Intermediate	Final Product
Protein	53.3	61.9	62.1
Ash	5.3	2.6	3.2
Fat	2.3	0.3	0.8
Carbohydrate	37.7	35.2	33.9
Carotenoid	0.6	-	-
Chlorophyll	0.8	-	-
	100.0	100.0	100.0

Table 12. Material Balance - Alcohol Reflux to Remove Biliprotein Pigment

Component	Extract	Product	Total Output	Input
Solids (g)	0.45	4.55	5.00	5.00
Protein (g)	0.27	2.83	3.10	3.10
Ash (g)	-	0.15	0.15	0.13
Fat (g)	-	0.04	0.04	0.02
Carbohydrate(g)	0.22	1.54	1.76	1.76

The yield of solids and protein for the alcohol reflux step was 91%.

6-6 Final Treatment to Brighten Product

The product obtained by extraction of algae to remove fat, chlorophyll and carotenoid pigments followed by reflux with alcohol to remove biliprotein pigment is characteristically light in color with a residual green tinge. The removal of the green tinge by chemical bleaching and intense light treatment has been investigated.

Light colored algal powder with a residual green tinge prepared by ethanol extraction of blue residue was resuspended in ethanol (solvent ratio 20:1). The suspension was dosed with hydrogen peroxide (0.65 w/w peroxide:algal solids) and stirred at 30°C. After 4 hours the green coloration had disappeared and a bright yellow-white powder was recovered. The yield of solids was 93%.

A 1% suspension of light algal powder with residual green tinge was stirred at 35°C. under a white light source providing 5,000 foot candle illumination. After 4 hours, the green coloration had disappeared and a bright powder was recovered in

94% yield. The product had less yellow character than the product obtained from peroxide bleach.

Final treatment by illumination appears to be more attractive than bleaching due to the high chemical requirement for the latter process.

7. PRELIMINARY PRODUCT SPECIFICATION

From the results available to date, a preliminary estimate can be made of product composition and yield. Exhaustive stagewise extraction of algae with ethanol or methanol at temperatures up to the solvent reflux temperature produces an intermediate product containing biliprotein pigment as the major colorant. Subsequent reaction with solvent for prolonged periods or at high temperatures serves to detach the biliprotein pigment producing an off-white product having a pale green tinge. Composition and yield data are presented in Table 13.

Table 13. Composition of Algae and Protein Product (Preliminary)
(% on dry basis)

	Algae	Intermediate	Final Product
Protein	53.3	61.9	62.1
Ash	5.3	2.6	3.2
Fat	2.2	0.3	0.8
Carbohydrate	37.8	35.2	33.9
Chlorophyll	0.8	-	-
Carotenoids	0.6	-	-
	100.0	100.0	100.0
Digestible Protein	41.5	43.9	44.7
Digestibility	78	71	72
Yield:			
Solids	-	63-78	57-71
Protein	-	73-88	66-80

The yield of solids and protein varies with the solvent used, as previously noted. The lower values cited above refer to methanol, the higher values to ethanol. Protein digestibility appears to be reduced by about 10% by extraction.

To further investigate the effect of extraction on protein quality, the amino acid content and distribution of samples of blue intermediate and final product were obtained (Table 14). The essential amino acid content and distribution of the purified product is compared with those of soy and algal protein in Table 15.

It is significant that the total and essential amino acid contents, calculated as a percentage of crude protein, are higher for the purified products relative to the original algae. This is apparently due to loss of non-protein nitrogen during extraction. The protein yields cited in Table 13 were calculated on a crude protein basis and thus may well overstate the actual protein loss.

Table 14. Amino Acid Content and Distribution

	g/100 g sample			g/100 g sample		
	Spray Dried Algae	Blue Inter-mediate	Final Pro-duct	Spray Dried Algae	Blue Inter-mediate	Final Pro-duct
iso-leucine	2.27	2.95	3.17	4.67	4.83	5.11
leucine	3.61	4.76	4.97	7.43	7.79	8.01
lysine	2.13	2.63	2.52	4.38	4.30	4.06
methionine	0.99	1.33	1.22	2.04	2.18	1.97
cystine	0.15	0.23	0.21	0.31	0.38	0.34
phenylalanine	1.77	1.95	2.09	3.64	3.19	3.37
tyrosine	1.50	1.88	1.76	3.09	3.08	2.84
threonine	2.26	3.49	3.17	4.65	5.71	5.11
tryptophan	0.53	0.63	0.64	1.09	1.03	1.03
valine	3.50	4.74	5.27	7.20	7.76	8.50
arginine	2.90	4.08	4.22	5.97	6.68	6.81
histidine	0.80	0.88	0.93	1.65	1.44	1.50
alanine	2.94	3.87	4.11	6.05	6.33	6.63
aspartic acid	4.25	5.83	6.21	8.74	9.54	10.02
glutamic acid	6.84	7.81	8.56	14.07	12.78	13.81
glycine	2.04	2.56	2.70	4.20	4.19	4.35
proline	1.64	1.77	1.79	3.37	2.90	2.89
serine	1.98	2.56	2.60	4.07	4.19	4.19
Total	43.08	55.17	57.35	86.62	88.30	90.54

Table 15. Comparison of Essential Amino Acid Content and Distribution

Sample	Soy	Algae	Purified Product
Essential Amino Acid	g/100 g protein		
Histidine	2.9	1.65	1.50
Arginine	7.3	5.97	6.80
Phenylalanine	5.3	3.64	3.37
Methionine	1.7	2.04	1.96
Leucine	8.0	7.43	8.00
Valine	5.3	7.20	8.49
Lysine	6.8	4.38	4.06
Isoleucine	6.0	4.67	5.10
Threonine	3.9	4.65	5.10
Tryptophan	1.4	1.09	1.03
Total	48.6	42.72	45.41

On the basis of total essential amino acid content, the purified product compares more favorably with soy protein than does the original algae. It is significant however that there is an apparent 10% loss of lysine and a somewhat smaller loss of methionine during extraction.

By-Product Specification

The composition of the pigment by-product, obtained by evaporating solvent from the miscella product during extraction, will vary with the solvent used as ethanol appears to extract less protein than methanol.

Estimates of by-product composition and yield are presented in Table 16.

Table 16. By-Product Composition (dry basis)

	Methanol Extraction	Ethanol Extraction
Protein, wt %	30.5	25.2
Fat, wt %	5.5	10.7
Ash, wt %	9.0	6.1
Carbohydrate, wt %	46.1	46.6
Chlorophyll, wt %	2.2	3.7
Carotenoid, wt %	1.6	2.7
	94.9	95.0
Yield (based on original algal solids)	35.7%	21.7%

Estimates have been derived from analysis of miscella samples obtained from countercurrent extraction of spray dried algae and by material balance (Table 8). There is a risk of loss of carotenoid values (xanthophylls) during further processing to produce a stable by-product and to recover solvent. This will be investigated during Phase III when larger quantities of product will be available for experimentation.

The by-product recovery process envisaged at present involves evaporation of solvent to increase solids content of the miscella from 2% to about 50%. If more solvent is removed, the miscella becomes tarry and difficult to handle. The 50% concentrate would then be either absorbed on an inert porous substrate such as diatomaceous earth or mixed with a stabilized oil prior to further solvent recovery by drying in a tumble dryer or by rectification of the oil stabilized product.

8. CONCLUSIONS

(1) Solvent extraction is judged to be the most promising purification method for Spirulina algae. Extraction is compatible with by-product recovery and holds promise for producing a product with acceptable color, flavor and odor characteristics. Chemical bleaching of algae has several disadvantages including high chemical cost and possible toxic effects. Intense light treatment of whole algae is judged to be impractical because of the long illumination periods and dilute working suspensions required. The lack of suitable specific enzymes for removing the various color components precludes enzymic methods.

(2) Lipids, chlorophyll and carotenoid pigments are readily removed from algae by extraction. Biliprotein pigment, responsible for the blue color component of blue-green algae is chemically bound to the protein and cannot be removed by low temperature extraction. The biliprotein pigment can best be detached by a transesterification reaction with alcohol.

(3) Methanol and ethanol have been found to be the best solvents for removing lipids, chlorophyll and carotenoid pigments to produce a blue intermediate product. Isopropanol has been found to be less effective than ethanol and methanol for extracting color but better than acetone, ethylene dichloride and hexane. Carbohydrate, ash and "crude protein" are also extracted with the color components. The yield of protein in the purified product is higher for ethanol extraction than for methanol. This fact, combined with the possibility of toxic effects due to the presence of residual methanol, serves to make

ethanol the solvent of choice for extraction of algae.

(4) A dried powder is the preferred raw material form to minimize solvent requirement. The presence of water serves to dilute the solvent so that a longer contact time or more blending/separation stages are required at a given solvent ratio. It is essential that the algae be dried in a manner that will minimize heat damage to the protein and carotenoids. Spray drying has been found to be much preferred to the roller drying currently performed at Sosa Texcoco S.A. in terms of final product quality. There may be a possibility of roller drying without causing heat damage if the dryer is properly loaded and lower steam pressures or faster roller speeds are employed. This possibility will be pursued during Phase III of the process development program. Milling of the powder prior to or during extraction to reduce cell fragments to the 20 micron size range or below increases the pigment extraction rate.

(5) A two stage extraction system with countercurrent flow of solvent as shown in Schematic 1 appears to be feasible as a means of producing an intermediate product containing biliprotein pigment as the major remaining colorant. Preliminary recommendations as to extraction conditions are as follows:

Raw material - Dry algal powder (spray dried or roller dried under mild conditions).
Solvent - Ethanol.
Solvent ratio - 4:1 to 8:1.
Temperature in extraction vessels - 60°C. to normal boiling point of solvent.

Contact period - up to 1 hour in each stage.
Expected yields - Solids 78%
Protein 88%
Concentration of strong miscella - of the order 2% total solids.

(6) The biliprotein pigment remaining after the initial extraction of fat, chlorophyll and carotenoids can be removed by refluxing in ethanol (or methanol) for 8 to 10 hours at normal atmospheric pressure. Other solvents (iso-propyl alcohol, acetone, chloroform) have been tested but were found to be ineffective in removing the residual color. The reaction can be performed at solvent ratios as low as 8.5:1. Pigment removal can be accomplished in a matter of minutes at high temperature. It appears that reaction temperatures as high as 135°C. can be used without any adverse effect on product quality. A solids yield of the order 90 to 93% is obtained.

(7) The product obtained from the two step extraction-reaction sequence is light in color (off-white) with a pale green tinge. It has acceptable odor, color and flavor characteristics. The residual greenish coloration can be removed by a final treatment with hydrogen peroxide or with intense light. Peroxide treatment does not appear to be feasible because of the high chemical requirement (0.5 w/w). It is doubtful whether a final illumination step to completely remove all traces of color is necessary to satisfy the market for decolored algal protein.

(8) Preliminary experiments indicate that extraction of color can be accomplished in a single stage at high temperature (110°C.). However, the protein product obtained is brown in color, and darker than the product obtained from the two stage extraction-reaction sequence cited above. Also,

it is known from earlier work on roller dried product, that high temperature tends to degrade the xanthophyll components resulting in loss of marketable pigment values. Extraction of lipid, carotenoids and chlorophyll at temperatures up to the normal solvent boiling temperature is therefore preferred to a high temperature single stage process.

(9) The purified algal protein product has a higher protein content (62% vs. 53%) and a significantly lower ash content than the original raw material. Protein digestibility is reduced by the extraction process by about 10%. The reduction in digestibility could result from loss of amino acids (100% digestible by the analytic method employed) and does not necessarily indicate that extraction alters the protein chemically. Approximately 10% of the total lysine appears to be lost during extraction with a somewhat smaller loss of methionine. The total and essential amino acid content (on a g/100 g crude protein basis) is nevertheless higher for the purified product, indicating that extraction serves to improve the overall protein quality by removing some non-protein nitrogen. Crude protein yields cited elsewhere in this report may thus overstate the actual protein loss on extraction.

(10) On the basis of material balance calculation, the pigment by-product from ethanol extraction of algae is estimated to contain of the order 2.7% carotenoid of which up to 42% is xanthophyll. These values have yet to be confirmed by chemical analysis of by-product. Larger quantities of stabilized by-product will be prepared in Phase III of the program and analyzed to determine the effect of processing conditions on xanthophyll recovery.

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APPENDIX

Analytical Methods

The methods used were those published in the Methods of Analysis - A.O.A.C. 11th Edition, 1970, W. Horwitz, Editor, or were modifications of those methods.

Moisture

A known weight of material was dried at 105°C. in an air-circulating oven for 18 hours.

Ash

The dried material from the moisture determination was charred on an electric hot plate and then placed in an electric furnace at 550°C. for 1 hour. The residue was cooled, moistened with distilled water, dried in an air-circulating oven at 105°C. and then placed in the furnace for another hour. The process was repeated until an ash of constant weight was obtained.

Fat

A known weight of material was extracted for 18 hours in a Soxhlet apparatus using diethyl ether and pre-extracted cellulose thimbles. The extract was carefully evaporated to dryness in a stream of low pressure air, and finally dried for 30 minutes in an air-circulating oven at 105°C.

An attempt to use $CHCl_3$:CH_3OH (2:1) resulted in co-extraction of CH_3OH soluble compounds

Crude Protein

A known weight of material was digested with 30 ml concentrated H_2SO_4 and 15.7 g of K_2SO_4 + HgO digestion mixture (Pope Kjeldahl Mixture, Dallas, Texas), cooled and diluted with 250 ml distilled water. Twenty-five mls of 8% $Na_2S_2O_3$ solution were added to the cooled diluted digest, thoroughly mixed and 100 ml of 45% NaOH solution added. The flask was immediately connected to a distillation unit of R.P.C. design and the liberated NH_3 distilled into 50 ml of 4% HBO_3 containing 5 to 10 drops of mixed methylene blue - methyl red indicator. After 30 minutes the receiving flask was removed from the unit, the connecting tube rinsed into the flask and the liberated NH_3 titrated with 0.100 N H_2SO_4. A blank correction was made for the reagents used. The protein concentration was calculated from the formula:

$$\% \text{ protein} = \frac{T_s - T_{bl} \times 0.875}{W}$$

T_s = titration for sample

T_{bl} = titration for blank

W = weight of sample

Pepsin Digestibility of Protein

A known weight of material was digested for 16 hours at 45°C. in 150 ml of 0.2% pepsin in 0.075 N HCl using a rotary agitator to ensure intimate contact between solid and liquid.

At the end of the digestion, 1 g of Hyflo Super-Cel was added and the well mixed solution quantitatively transferred to a Hartley funnel containing a Whatman #2 filter paper. The residue was washed three times with hot water and then the paper and residue were transferred to a Kjeldahl flask and protein (indigestible) determined as described previously.

Calculation:

Digestible Protein, = Total Protein - Indigestible Protein.

Carbohydrate

The carbohydrate content was obtained by difference using the following formula:

Carbohydrate, = 100 - (moisture + ash + fat + total protein + chlorophyll + carotenoids).

Solvent Extracts

Replicate aliquots of the various solvent extracts were carefully evaporated to dryness in 300 ml flasks using a current of low pressure air, and finally dried for 30 minutes in an air-circulating oven at 105°C.

Protein and ash determinations were performed directly on the resultant residues as previously described.

Carotenes, Chlorophyll and Xanthophylls

A known weight of algae was extracted according to A.O.A.C. method 39.020, etc. In the case of the solvent extracts, a known volume of solution was carefully evaporated to dryness and then treated as in 39.020, etc.

Individual pigment components in the algae were separated using suitable column packings and/or TLC plate coating.

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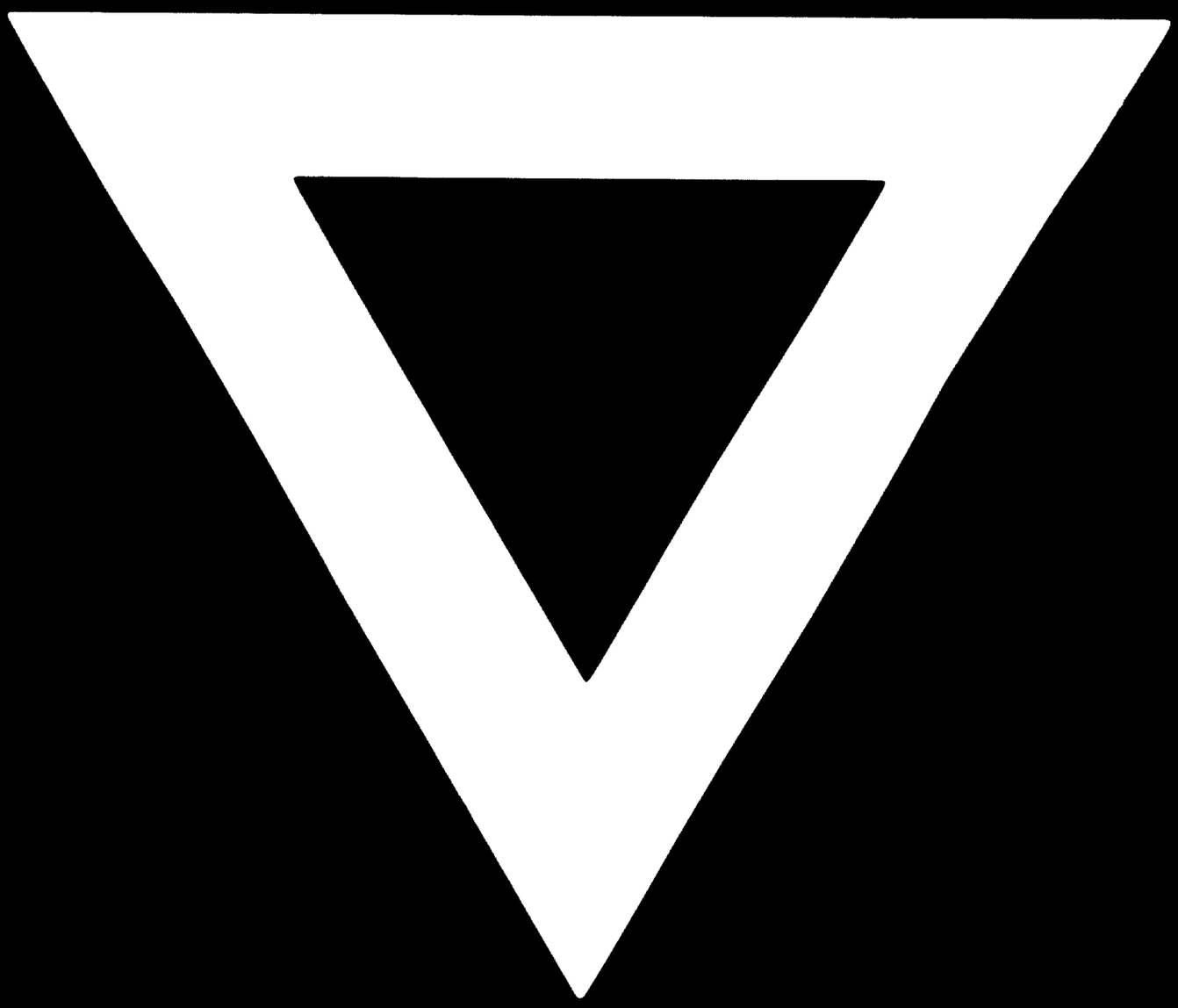

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