

APPLICATION OF DIFFERENT METHODS FOR THE PREPARATION OF CHLOROPHYLL PIGMENTS FOR FOOD AND PHARMACEUTICAL INDUSTRIES

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تطبيق طرق مختلفة لتحضير صبغ اليخضور للصناعات الغذائية والصيدلانية

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يهدف هذا البحث لاستنباط طريقة تطبيقية اقتصادية لتحضير صبغ اليخضور وخاصة مشتق النحاس من مصادر نباتية في شكل مضافات لونية . وقد تم تطبيق طريقة معدله لإنتاج صبغ اليخضور صناعياً مع الأخذ في الاعتبار كمية ونوع المذيبات العضوية المستخدمة وكذا طرق الاستخلاص والتحضير . ثم أستتبع ذلك تطبيق طريقتين تقليديتين للمقارنة هما طريقة شيرتز ١٩٦٤ وطريقة اريما ١٩٧٤ .

وقد تم استخدام نبات السبانخ إما في الصورة الجافة أو الطازجة بعد التزهيه كمصدر لإنتاج مترابك النحاس في الطريقة المطورة المتبعة ، وتم تقييم المنتج النهائي طيفياً (للتجارب المطبقة) على هيئة الصورة الذاتية في الماء مع الأخذ في الاعتبار قوة اللون ودرجة نقاء المنتج النهائي وكمية المذيبات العضوية المستخدمة والوقت المستهلك... الخ . وقد أظهرت المقارنة أن الطريقة المطورة المتبعة تعطي نتائج أفضل من الطرق التقليدية وقد تم تصعيد التجربة المطورة على النطاق النصف صناعي .

Key Words: Chlorophyll pigments, Cu-pheophytin complex, Na-Cu chlorophyll, Spectrophotometric evaluation.

ABSTRACT

The main objective of this work is to establish an economic and applied technique for the production of chlorophyll pigments and their derivatives namely Cu-chlorophyll complex in the form of colour additives. These trials include, the introduction of some modifications concerned with the processing of plant materials, type of extracting solvent, procedures of chemical reactions and purification followed by the application of two of the classical methods previously reported by Schertz 1964 and Iryama 1974 for comparison. The achieved modified method with oven dried powder or fresh plant material of Spinach for the preparation of Cu-chlorophyll complex has been adopted. Spectrophotometric evaluation of Na-Cu chlorophyllin *viz.* colour strength and purity and other factors controlled the experimental conditions (bulk of solvent used, time consumed etc.) were carried out. The comparative study of the applied methods revealed that the achieved modified method gave satisfactory results. Preparation of the chlorophyll pigments on experimental scale and semi-pilot scale were carried out by applying the achieved modified method.

INTRODUCTION

Egypt is known to be rich in plant sources required for the production of natural colour additives on industrial scale in economic manner, away from the high costs of importation of such products. One of the important colours is the chlorophyll as a natural green colour, which has been admitted in several countries.

Several attempts have been made to simplify the methods of production of chlorophyll. The commercial

production of copperd chlorophyll products for food colouring has been taking for a long time as long ago[1-5].

Different methods were applied comprising processing of plant materials *viz.* blanching, non blanching followed by drying in air or electrical oven or in solar dehydrating oven. The qualitative and quantitative evaluation of the different plant materials as well as different processing techniques were carried out *viz.* TLC, HPLC and spectrophotometric analysis as mentioned [6]. These techniques revealed that Spinach contains the highest

chlorophyll contents followed by Alfalfa then Parsely and finally Chard. Electrical and solar dehydrating ovens were shown to be more efficient for drying of plant materials. Spinach which has been found to contain the highest chlorophyll content among the selected plant materials was taken as a model for applying the methods of preparation of chlorophyll pigments.

This work deals with the modification and/or improvement of the methodology and processing steps to be applied for the production of chlorophyll pigment derivatives. These developments include stabilization of the chlorophyll pigment, minimizing the time consumed during the steps of preparation and reduction of the bulk of solvents used in extraction.

EXPERIMENTAL

Plant Material

Spinacia oleracea Linn. (Spinach), was used as local source for the preparation of chlorophyll pigments.

Apparatus and Techniques

Pyc-Unicam double beam spectrophotometer SP 8-100 was used.

Preparation and Purification of Chlorophyll Pigments

Several trials for the production of chlorophyll pigments and their derivatives namely Cu-complexes either as the oil soluble form (Cu-pheophytin) and the water soluble form (Na-Cu-Chlorophyllin) have been carried out. These trials included the application of the achieved modified method with oven dried powder or fresh blanched plant material of Spinach in comparison with the classical methods previously reported[1,2].

A) DEVELOPED METHODS

Method (Ia)

About 2 Kg of dried powdered plant material of Spinach were extracted with 80% acetone (3 x 6 L) in a percolator the addition of magnesium carbonate (20 g). The combined acetone extracts were acidified with 10% HCl till pH 3-4, followed by the addition of 30% CuSO₄ solution (100 ml) and 80 gm Celite 545, then evaporated till acetone-free. The aqueous mixture containing a dark bluish precipitate was filtered and washed with water. The obtained precipitate was extracted by acetone (5L) then filtered. The dark green filtrate was evaporated *in vacuo*, a dark green oily residue was obtained (44.2 g) which represents the oil-soluble form of natural green colour (Cu-chlorophyll complex).

The water soluble form Na-Cu-chlorophyllin was prepared as follows: The oil residue (Cu-chlorophyll complex, 44.2g) was saponified by dissolving it in one litre of 85% ethanol containing 10% NaOH. The ethanol was removed by evaporation during which aliquots of water were added. The clear supernatant was collected and acidified with 10% HCl while the dark green precipitate was separated by centrifugation, then kept in air till complete drying (5.5 g). It was further purified by washing with small quantities of petroleum ether, then suspended in water (450 ml), and neutralized with aqueous NaOH (5%) till pH 8-9.

Method (I b)

Fresh plant material of Spinach was screened from contaminants and the damaged leaves and the roots were removed, spreaded on a perforated stainless steel tray till complete drainage of excess water. The fresh leaves after cleaning and washing (2 Kg) were blanched by packing into a muslin bag then dipped into 2% sodium carbonate aqueous solution at 80° C for 5 minutes, cooled under drain water, and squeezed until most of the water have been removed. The blanched plant material (0.60 Kg) was extracted in dim light at room temperature with acetone while stirring (3 x 1 L), in the presence of magnesium carbonate (10 g), then filtered. The experiment was completed as described before in method (Ia) until a dark green oily residue was obtained (9.5 g) which represents the oil soluble form.

B) CLASSICAL METHODS

Method II [1]

About 2 Kg of the oven dried powder of Spinach were defatted by extraction with petroleum ether (b. r. 40-60 °C) in a Soxhlet apparatus till exhaustion (4 L). The defatted plant material was dried in air (1.980 Kg), then extracted with 80% acetone (3 x 6 L) in a percolator till exhaustion, after the addition of magnesium carbonate (20 g), in the dark at room temperature. The combined acetone extracts were partitioned with petroleum ether (2 x 4 L) after the addition of water saturated with sodium sulphate until the separation of the two phases. The chlorophyll pigment which was transferred to the petroleum ether layer was washed with water, then partitioned several times with 85% methanol (6 L) till the yellow colour was completely removed. The petroleum ether layer was then washed with distilled water to remove any residue of methanol, while the chlorophyll starts to aggregates. The solution was then kept in a deep freeze for complete precipitation of chlorophyll which was separated by centrifugation. The obtained precipitate (30 g) partially purified chlorophyll pigment was washed with cold petroleum ether, then dissolved in acetone. The acetone solution containing the partially purified chlorophyll pigment prepared was acidified with 10% HCl till pH 1. Copper sulphate solution (30%, 100ml), was added, kept in dark at room temperature for 10 hours and celite 545 (80 gm, Fluka) was added. Acetone was evaporated *in vacuo* using rotatory evaporator at 40° C with the addition of aliquots of water to replace the removed acetone during evaporation process. An aqueous suspension (1.5 L) was obtained containing a dark blue precipitate (celite + Cu-chlorophyll complex). The precipitate was collected under suction through Whatman No. 1 filter paper and washed thoroughly with distilled water. The obtained precipitates was kept in air. Extraction of Cu-chlorophyll complex was affected by extraction with acetone (4 L). Upon evaporation of acetone till dryness, an oil residue of Cu-chlorophyll complex (25 g) was obtained. Preparation of the water soluble form was completed as mentioned before in method (Ia).

Method III (Iriyama 1974)

About 2 Kg of fresh plant material of Spinach were screened, washed under stream of water, then suspended in acetone (4 L) and homogenised by using electrical

homogenizer (3 minutes, full speed) after addition of magnesium carbonate (10 g). The acetone extract was filtered. Dioxane (400 ml) followed by addition of water dropwise until a green precipitate was appeared. The solution was allowed to stand for one hour in deep freeze until complete precipitation. The precipitate was separated by centrifugation (1.2 g) as the partially purified chlorophyll pigment, and completed as mentioned before (Ia) yielding 1.20 gm and 0.81 gm of the oil soluble and water soluble forms, respectively.

Spectrophotometric Analysis of Na-Cu-Chlorophyllin

The concentration of each of the prepared samples solutions and their colour strength were calculated spectrophotometrically, for comparative evaluation of the applied methods. The measurements were carried out at wavelength 405 nm. The unknown concentration of Na-Cu-Chlorophyllin solution was obtained by correlation of their absorbances from the constructed calibration curve.

Standard Calibration

0.250 g of pure standard Na-Cu-chlorophyllin (Sigma Chemical Company) was accurately weighed and dissolved in 50 ml of a mixture of 0.1N NaOH/MeOH (1:10) as stock solution (1). 0.5 ml from this stock solution was taken into measuring flask 50 ml and diluted it with the same solvent mixture as stock solution (2). A series of dilutions were made by taking 0.5, 1, 1.5, 2, 2.5, 3, 3.5, and 4 ml from stock solution (2) and each was diluted to 50 ml. The absorbances of each solution were measured at 405 nm. The results obtained are summarized in Table 1. A graphical relationship between the concentration against the absorbances were plotted (Fig. 1) which revealed a straight line passing through the origin.

Table 1
Absorbance of known concentration of Na-Cu-Chlorophyllin (Purity 100 %)*

Concentration (mg l)	Absorbance at 405 nm
0.5	0.02
1.0	0.11
1.5	0.18
2.0	0.22
2.5	0.27
3.0	0.34
3.5	0.38
4.0	0.44
4.5	0.52

*Correlation coefficient = 0.99639

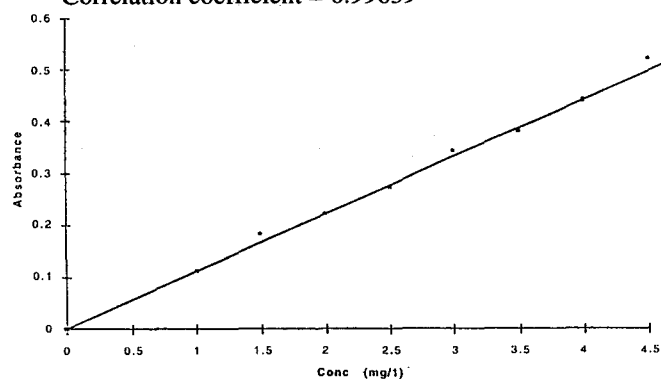


Fig. 1. Standard Curve of Na-Cu Chlorophyllin

Sample preparation

The Na-Cu-chlorophyllin solution which prepared in the previous methods were adjusted to 500 ml with distilled water, then 0.1 ml from each of the stock water soluble chlorophyllin were diluted until the reading in spectrophotometer ranged from 0.1 to 0.6. The obtained absorbances at 405 nm for each sample were extrapolated to the X-axis and the corresponding concentration was determined (Table 2).

Table 2
Comparative evaluation of applied methods

Methods	Powdered plant material (Kg)	Oil soluble form (g)	Water soluble form (g)	Spectrophotometric evaluation of Na-Cu-Chlorophyllin Complex (water soluble form)***			
				A*405	Conc.** mg/l	Colour strength	% on the basis of dry wt
Modified							
Ia	2	44.2	5.5	0.13	1.26	0.52%	0.13%
Ib***	2	95	8.3	0.16	1.62	0.85%	0.25%
Classical							
II	2	25	6	0.10	1.00	0.62%	0.15%
III***	2	10.2	8.1	0.17	1.50	0.80%	0.25%

* Absorbance at 405 nm.

** From standard curve after dilution (1: 6250)

*** Calculation based on dry powdered plant material

RESULTS AND DISCUSSION

Several trials for the preparation of chlorophyll pigments and their derivatives namely Cu-chlorophyll complex from *Spinacia oleracea* Linn. have been carried out.

These trials include some modifications concerning processing of plant materials, type of extracting solvent, procedures of chemical reactions and purification. This was followed by carrying out the application of two of the classical methods previously reported[1,2]. Upon applying

the classical method (II)[1] which involves the use of large quantities of solvent including petroleum ether for defatting, acetone for extraction followed by partitioning with petroleum ether then with aqueous methanol. These steps seemed to be tedious and cannot be applied on industrial scale. Although, the classical method (III)[2] gave the highest yield of purified chlorophyll pigments, yet, specific solvents like dioxane which is highly expensive solvent seemed to be non-economic method for large scale production. This method could be only recommended as an appropriate analytical technique.

According to the results obtained (Table 2) applying application of both methods (II & III), as previously discussed, it was suggested to carry out further trials during which some modifications were introduced.

In the first modified method (Ia), the dried powdered plant material was extracted with 80% acetone. During concentration of the combined acetone extracts *in vacuo* the replacement of magnesium atom naturally occurring in chlorophyll molecule by copper atom was carried out by the addition of copper sulfate solution in slightly acidic medium (pH 3-4). Celite as an inert carrier, was early added to the mixture to facilitate the agglomeration of the fine precipitated copper chlorophyll complex on the surface of its particles upon evaporation of acetone. The obtained mixture (Celite + Cu-chlorophyll complex) after drying was extracted with acetone which gave after the removal of the solvent the oil-soluble form as Cu-pheophytin complex. The water soluble form was carried out by removal of the insoluble unsaponifiable materials. by centrifugation followed by acidification to obtain the Cu-chlorophyllic acid. The Na-Cu-chlorophyllin was prepared by neutralization of Cu-chlorophyllic acid with NaOH.

The second modified method (Ib) was carried out following the same procedure in method (Ia) on fresh blanched plant material. Comparative evaluation of the four methods revealed that the modified method (Ib) gave reproducible results and a higher yield of the end product as Na-Cu-chlorophyllin.

The commercially natural green colour derived from chlorophyll is copper complexes of both pheophytin and pheophorbide which have the metal very firmly bound and it is not liberated even by the action of concentrated hydrochloric acid. This particular aspect renders the copper complexes safe for food colouration and it has been

established that in such use the copper is not absorbed by the body but totally excreted in a complex form[5]. Such complex forms are permitted as food colours by Food and Drug Administration[7] and the current UK law.

Therefore scaling up of the achieved modified method (Ib) on an experimental scale (50 Kg of plant material) followed by a semi pilot scale experiments (300 Kg) of plant material were carried out. On the other hand the experimental application of the different forms of chlorophyll pigments prepared as natural colour additives as colourants for some food industrial forms such as candy, jellies, pudding, ice cream, chewing gum and toilet soap products were carried out and the obtained results were successful. Furthermore, evaluation of the obtained products in comparison with the corresponding products of Hansen Co. in the market proved its identity.

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