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Bioactive Molecule Composition of Natural Egg Membrane Concentrate (NEMC^{TM}) vs. Soluble Egg Membrane (SEM)

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Abstract

The main objective of this study is to compare and evaluate the active molecule composition between Natural Egg Membrane Concentrate (NEMC^{TM}) and Hydrolyzed (HEM) or Soluble Egg Membrane (SEM). The study demonstrates the differences in nutrient composition of the Natural product vs. the chemically modified Egg membrane. Egg Membrane is protein-rich membrane found between the eggshell and egg white, it has usually been regarded as waste and overlooked. Egg Membrane primarily composed of Collagen type (I,V,X), Elastin, Chondroitin sulfate, Glycosaminoglycans, membranous protein, hyaluronic acid etc and other fractional molecules that includes, growth factor β, ovocalixin, ovocleidin, ovotransferrin, desmosine, isodesmosine and sulphur-bearing amino acids as essential to maintaining healthy joint and connective tissues. Five different processing conditions has been discussed that includes Natural process (mechanical separation), Alkali digestion process, Alkaline and Methanol digestion process, EDTA+Hexane digestion process and Ozone based digestion process. Among which Microcore's NEMC^{TM} processed by the Natural processing methods has effected high nutrient profile with integration of the bimolecules resulted 96% protein, 17% Collagen, 27% Elastin (16.2% Desmosine, & 10.8% Isodesmosine), 28.3% Chondroitin sulphate, 20.5% Glycosaminoglycan and 5% Hyaluronic acid whereas the Hydrolyzed (or) Soluble Egg Membrane produced by 20% NaOH at extreme conditions has resulted 71% protein, 8% Collagen, 12% Elastin (7% Desmosine & 5%

Isodesmosine), 20.19% Chondroitin sulphate, 10.9% Glycosaminoglycan and 4% Hyaluronic acid and Hydrolyzed or soluble egg membrane by combined treatment 5% Methanol and 20% NaOH digestion has resulted 64.5% protein, 8% Collagen, 12% Elastin (7.2% Desmosine & 4.8% Isodesmosine), 9.73% Chondroitin sulphate, 8.18% Glycosaminoglycan and 3.5% Hyaluronic acid, whereas 10 % EDTA and 5% hexane treated Egg Membrane has affected 65% protein, 8% Collagen, 8% Elastin (4.8% Desmosine & 3.2% Isodesmosine), 15.2% Chondroitin sulphate, 14.8% Glycosaminoglycan and 3.8% Hyaluronic acid. where as 10 % O_3 treated egg membrane has affected 35% protein, 5% Collagen, 6% Elastin (3.6% Desmosine & 2.4% Isodesmosine), 7.25% of Chondroitin sulphate, 8.4% of Glycosaminoglycan and 1.5% Hyaluronic acid. The study concludes the significant reduction of nutrient profiles in above mentioned chemically modified Egg Membrane or soluble or hydrolyzed egg membrane. The Characterization of Egg membrane for NEMC and HSEM were investigated with elemental analysis and FTIR studies also support the above results.

Keywords: Natural Egg Membrane, Soluble Egg Membrane, Hydrolyzed Egg Membrane Collagen, Elastin, Chondroitin sulfate, Glycosaminoglycan, Hyaluronic acid.

1. INTRODUCTION

Chicken egg shell and membrane are byproducts of eggs. The egg shell membranes are true source of valuable bioactive materials, including collagen that has many applications in medical, health and cosmetics. The eggshell membrane is a natural ingredient obtained from the inner membrane that covers the shell of the egg. Eggshell membranes are an abundant raw material that are a novel source for naturally occurring bioactive compounds such as glucosamine **(Picard *et al.*, 1973)** chondroitin sulfate **(Baker and Balch, 1962**), hyaluronic acid **(Long FD *et al.*, 2005),** fibrous proteins such as collagen Type I **(Wong M *et al.*, 1984)** and sulfur-rich proteins **(Tsai *et al.*, 2006)** and other components including lysozyme, ovotransferrin, ovocalixin and desmosine and isodesmosin. Glycosaminoglycan and proteins essential for maintaining healthy joint and connective tissues.

Arthritis describes around 200 conditions that cause pain in the joints and the tissues surrounding the joints. The two most common and best known types of arthritis are osteoarthritis (OA) and rheumatoid arthritis (RA) **(Bagchi D *et al.*, 2002)**. OA is more common than RA, and pain associated with both is often unrelated to the degree of physical/ structural damage to the joint. "Pain is a multidimensional and complex challenge that is as much related to biomechanical damage as it is to mindset and environment within which that pain exists.

Osteoarthritis (OA) is a disease of cartilage degradation, which results pain in major joints, especially in knee joint. Globally OA ranks eighth in all diseases and covers around 15% proportions among all musculoskeletal problems. Clinical symptoms and

radio-diagnosis are the basis of diagnosis used for OA characterization **(Chandra Shekhar Azad-2018).** It is estimated that 140 million adults in the United States (US) suffer from some form of joint or connective tissue (JCT) disorder (i.e., arthritis, lupus, gout, fibromyalgia, neck or back pain, etc.) **(Helmick CG *et al.*, 2008).** According to the Arthritis Foundation, Arthritis is more common in women, 26% of whom have it as opposed to 18% of men. A 2017 Centers for Disease Control (CDC) analysis of 435,331 American adults estimated that one in four have arthritis, while nearly 27% of those who do said they experience severe joint pain **(Guglielmo D *et al.*, 2019).** India has higher proliferative rate of OA among world and expected to be at top rank in chronic diseases till 2025. Andhra Pradesh has highest prevalence among India. In Andhra Pradesh and Bihar, exceptionally males are highly affected than females **(Chandra Shekhar Azad-2018).**

Increased longevity has the medical and holistic communities focused on joint health preservation solutions. According to National Center for Health Statistics, 48% of individuals ages 65 and experience joint pain. And as adults become more active, working out and participating in sports, these fitness activities tend to accelerate wear and tear of joint cartilage and synovial fluid, the result is inflammation, pain and stiffness of the joints.

Non-steroidal anti-inflammatory drugs (NSAIDs) are widely recommended and prescribed to treat pain in osteoarthritis. Non-steroidal anti-inflammatory drugs are one of the most widely used drugs in OA: over 50% of patients with OA in USA are prescribed NSAIDs, and among patients with OA across Europe using prescription medications (47%), 60% of those received NSAIDs. While measured to have a moderate effect on pain in osteoarthritis, NSAIDs has been associated with wide-ranging adverse events affecting the gastrointestinal, cardiovascular, and renal systems **(Chou R *et al.*, 2011).** Methotrexate [MTX] is a standard treatment for rheumatoid arthritis [RA], but long-term use could be a concern for some users. In case of major joint degradation (osteoarthritis [OA] of the knees and hips), corticosteroid injections may do more harm than good. Thousands of intra-articular corticosteroid injections are done daily, and researchers found that in 2018, 8% of the individuals receiving these injections had complications, with 10% in the hips and 4% in the knees. Co-author Ali Guermazi, M.D., Ph.D., chief of radiology at VA Boston Healthcare System and professor of radiology at Boston University School of Medicine, observed, "We are now seeing these injections can be very harmful to the joints with serious complications such as osteonecrosis, sub chondral insufficiency fracture and rapid progressive osteoarthritis"**(Kompel AJ *et al.*, 2019).**

This knowledge, combined with the recent study news about the harm of intra-articular corticosteroid injections, may bring many more consumers (and caregivers) to use more natural means of joint support. According to the U.S. Population Reference Bureau, "With a disease-treatment health care model, and soaring costs, many consumers have turned to natural, preventive solutions to address major concerns like muscle loss and joint pain." This recent research is a clarion call for further development of natural joint

health products. The consumers are still buying the majority of joint health products from the natural and specialty channel, which garnered 36% market share, reaching $693 million in sales in 2017.

The discovery of eggshell membrane as a natural source of combined glucosamine, chondroitin, and hyaluronic acid has prompted the evaluation of this material as a potential treatment for OA **(Ruff KJ *et al*., 2009)**. Anti-inflammatories such as glucosamine sulfate and chondroitin sulfate are very effective at lessening inflammation in the joints and relieving pain **(Shaw J *et al*., 2017).** In the U.S. alone, an estimated 600,000 tons of eggshells are produced annually as a by-product of the poultry industry. Disposal of these eggshells creates an environmental and financial burden and, therefore, alternative uses for these materials would be of obvious benefit **(Ruff KJ *et al*., 2009)**.

Eggshell membrane is a bilayered barrier between egg white and egg shell. It has an abundant, cost-effective resource from the waste materials of food industry [4]. Egg shell membranes are primarily composed of fibrous proteins such as collagen type I, V, X, Osteoprotein and siloprotein. Both the outer and inner membranes are composed of interwoven protein fibers, while the inner membrane is comparably thicker and more compact [5]. The fibers appear to be a network or scaffold predominantly containing Type I collagen fibers that are encapsulated in a continuous mantle of proteoglycans and other macromolecules **(Dale P D & Frank D L., 2007).** These further includes naturally occurring glycosaminoglycans (GAGs), such as dermatan sulfate and chondroitin sulfate, hexosamines, such as glucosamine, as well as hexoses and fucose **(Sandson J *et al.*, 2009)**. More recently, significant amounts of hyaluronic acid have been detected in egg membrane. Other components identified in egg membrane include sialic acid, desmosine and isodesmosine, ovotransferrin, lysyl oxidase and lysozyme **(Ruff KJ *et al.*, 2009)**. Eggshell membrane permits gaseous exchange, protects the chicken embryo just as the human amniotic membrane does to human foetus and specifically plays a key role in the biomineralization of egg shell, which only takes less than 24 h and is the fastest biomineralization process we have ever known. In Chinese traditional medicine, ESM was formally named as “Phoenics cloth” and frequently used for treating the chronic ulcer and bone fractures since many centuries.

Natural Egg Membrane contains the same nutrients that make up human joints and are important for joint health and flexibility. In NEM is a 100% natural, renewable and sustainable source of healthy joint nutrients. This once daily formula supplies key nutrients to help decrease joint pain and improve joint flexibility and stiffness. A recently published study in the Journal of Medicinal Food indicates NEM’s ability to reduce joint pain and stiffness may be a result of its ability to reduce Tumor Necrosis Factor-alpha (TNF-α) **(Ruff KJ *et al.*, 2009)**. Tumor necrosis factor-α (TNF-α) plays an important role in inflammatory processes **(Benson KF *et al.*, 2012)**. The processed eggshell membrane reduced plasma pro-inflammatory cytokines, typically associated with inflammation and pain **(Dale P D & Frank D L., 2007).**

Natural eggshell membrane mainly consists of protein in the form of collagen. Proteins found in egg membranes help to reduce inflammation and nourish the joints such as *collagen* a fibrous protein critical to cartilage strength and elasticity. It serves as essential and functional food to strengthen the cartilage and synovial fluids. The details of the fractional molecules of Egg Membrane are followed by,

Collagen Type (1,V,X) is the most important protein produced by the human body, it is mainly formed by the amino acid glycine (33%), proline and hydroxyproline (22%) (primary structure) in a triplex helix which is formed by three α chains**(Sorushanova A. *et al.*, 2019).** Each alpha chain is composed for 1014 amino acids approximately with a molecular weight around 100 kDa. These chains are coiled into a left-handed helix with three amino acid (secondarystructure). The chains are twisted around each other into a triple helix to form a rigid structure (tertiary structure). The super helix represents the basic collagen structure (quaternary structure) **(Gelse K. *et al.*, 2003)**. This collagen structure is very stable because of the intramolecular hydrogen bonds between glycine in adjacent chains. The collagen molecule is formed for a triple helical region and two non-helical regions at either end of the helix structure with ~300 kDa molecular weight, 280 nm in length, and 1.4 nm in diameter **(Schrieber R & Gareis H, 2007).**

Elastin is a protein critical to skin, cardiovascular, cartilage and spinal column health. As its name suggests, elastin gives tissue the elastic tension and ability to resume their shape after stretching. Desmosine and isodesmosine - two little known amino acids that is responsible for elastin's elastic and rubbery properties. Transforming growth factor – a protein that plays a critical role in tissue repair, cellular differentiation and immune function help to reduce inflammation and nourish the joints **(Kevin J R *et al.*, 2009)**

Chondroitin sulfate is a sulfated glycosaminoglycan (GAG) composed of a chain of alternating sugars (N-acetylgalactosamine and glucuronic acid). It is usually found attached to proteins as part of a proteoglycan. Chondroitin sulfate is an important structural component of cartilage and provides much of its resistance to compression. Along with glucosamine, chondroitin sulfate has become a widely used dietary supplement for treatment of osteoarthritis. Chondroitin is used in dietary supplements as an alternative medicine to treat osteoarthritis **(Gianni B, 2016)**.

Glycosaminoglycan (GAG) is anionic polysaccharides composed of repeating disaccharide units of hexosamine glucosamine or galactosamine. Glycosaminoglycan is also found in the chicken eggshell. Glycosaminoglycan essential for maintaining healthy articular cartilage and the surrounding synovium. Glycosaminoglycan has a wide range of applications in the pharmaceutical, cosmetic, and food industries **(Schaefer L & Schaefer RM, 2010).**

Egg Membrane remains is the key protecting membranous layer for the Albumin and Yolk and on hatching egg membrane serves as the natural skin and organs for the development of the birds. Naturally Egg Membrane has low solubility index as it is has a solubility of 10 to 12 %. In recent days many commercial egg membrane products are available with the claim of hydrolyzed or highly soluble egg. In order to

achieve highest degree of solubility the egg membrane are chemically modified under extreme conditions. In this paper we discuss about the several methods of modified egg membrane concentrate vs. the natural egg membrane concentrate and their difference in the molecular profiles.

2. MATERIALS & METHODS

2.1. Preparation of NEMC

Natural Egg Membrane Concentrate NEMC^{TM} is produced from chicken eggs of Strain called BV-300 is collected from Microcore Research Labs India Pvt Ltd, the FSSAI Approved manufacturing facilities. NEMC^{TM} is produced through natural process that does not involve any chemicals and solvents. The process enables to produce pure ingredient that do not contains artificial additives, chemical preservatives, pesticides or chemicals residues of any kind and it serves as natural joint health support. The pure egg membrane is separated by sequence of physical methods by membrane peeling technologies and further refined to achieve the appropriate product of interest. The isolated membrane is then converted in to nano particle of 50 microns (Ball milling machine, Retsch, Model PM100) and further analyzed for the nutrient compositions by several analytical methods as per AOAC.

2.2 Preparation of Hydrolyzed (or) Soluble Egg Membrane

Method -I

Hydrolyzed (or) Soluble Egg Membrane is made by digestion with 1:10 of egg membrane with 20% NaOH treatment at 70-100 deg C for 60 minutes further neutralized and spray dried to produce Hydrolyzed or soluble egg shell membrane. The neutralization process release high degree of H_2S gas is handled by trapping methods.

Method-II

Hydrolyzed (or) Soluble Egg Membrane is made by digestion with 1:10 of egg membrane with 20% NaOH and 5 % Methanol treatment at 70-100 deg C for 60 minutes further neutralized and spray dried to produce Hydrolysed or soluble egg shell membrane. The neutralization process release high degree of H2S gas is handled by trapping methods.

Method-III

Hydrolyzed (or) Soluble Egg Membrane is made by digestion with 1:10 of egg membrane with 5% EDTA, 5 % Hexane and 20% NaOH at 70-100 deg C for 60 minutes further neutralized and spray dried to produce Hydrolysed or soluble egg shell membrane. EDTA acidifies the Egg Shell and created Caclium leaching and it is separated by centrifugation process.

Method-IV

Hydrolyzed (or) Soluble Egg Membrane is made by digestion with 1:10 of egg membrane with 10% O_3 (Ozone Liquid) at 70-100 deg C for 60 minutes, O_3 breaks the protein bonds and makes a milky extract further separated by centrifugation and filtration methods further neutralized and spray dried to produce Hydrolysed or soluble egg shell membrane.

2.3 Solubility index

Sample derived from above methods are dried at over at 60 deg C and at 4 % moisture the samples are taken for the solubility test of 3 gram in 10 ml RO water. Then the mixture was kept for settling for 1 hr and based on the dry mater the solubility value is determined.

2.4 Nutrient profile analysis

2.4.1 Protein by Lowry's etal method (George W & Latimer JR, 2019)

Protein for value was analyzed Protein by Lowry's etal method with Bovine serum albumin as the standards by AOAC official method (AOAC method 2017.11.)

2.4.2 Chondroitin sulphate (George W & Latimer JR, 2019)

Chondroitin sulphate was analyzed by AOAC official method (AOAC 2015.11.)

2.4.3 Glycosoaminoglycan (George W & Latimer JR, 2019)

Glycosoaminoglycan was analyzed by AOAC official method (AOAC 2005.01).

2.4.4 Collagen by Sircol method (George W & Latimer JR, 2019)

The Sircol™ Collagen Assay is a quantitative assay for measurement of both acid-soluble and pepsin-soluble collagens and analyzed by AOAC official method (AOAC 2003.09).

2.4.5 Elastin (George W & Latimer JR, 2019)

The elastin was estimated by the AOAC official method (AOAC 2005).

2.4.5.1 Desmosine and Isodesmosine (Norman T Soskel, 1987)

Desmosine and isodesmosine are building blocks of elastin an it is called as major crosslink of elastin was analyzed by using high-performance liquid chromatographic separation and absorbance detection that is rapid (21–35 min) and sensitive (accurate linearity from 100 pmol to 5 nmol).

Chemicals: Acetonitrile (Burdick and Jackson, Muskegon, Mich) was glass-distilled and of HPLC grade. Triethylamine was from Aldrich Chemical Company (Milwaukee, Wise.). Hydrochloric acid (Ultrex) and phosphoric acid were from Baker (Philipsburg, N.J.). Water was HPLC grade generated from an Millipore (Milford, Mass.) purification system. Desmosine and isodesmosine standards are from Elastin Products (St. Louis, MO.; Rockford, III.). All other chemicals were of reagent grade.

HPLC system: All HPLC equipment was from Waters Associates (Milford, Mass.) and included two M510 pumps and automatic sample injector (WISP 7 10B), an 840 controller, an M490 uv-vis absorbance detector and a mechanical guard column in series with the analytical column.

HPLC column: Analysis was accomplished using a 4 X 300-mm (i.d.) MicroPak AX 10 anionic exchange column which is packed with 10 micrometer particles (Varian Instruments, Sunnyvale, Calif. Part No. 03-9 12 154-44). Chromatographic conditions varied as described below, and the total flow rate always remained at 1 ml/min. HPLC solvents. Buffer A: 8 1% acetonitrile, 19% 0.0 125 M KH_2P0_4.

HPLC solvents: Buffer A: 8 1% acetonitrile, 19% 0.0 125 M KH_2P0_4. Buffer B: 0.025 M KH_2P0_4 with triethylamine (12 or 1 ml/ liter). The pH was adjusted to 6.3 using phosphoric acid. Solvents were degassed by helium displacement prior to use and were kept under 5 psi of helium during analysis.

Sample preparation: Standards. Stock solutions of 100 nmol/pl of desmosine or isodesmosine were mixed 1: 1 and then serially diluted with water to 100 pmol of total crosslinks/pl. Test sample such as Microcore NEMC^{TM}, Hydrolysed or soluble egg membrane, Chemically refined egg membrane and O_3 egg membrane are taken as per the standard procedure. All standards and test samples contain equal amounts of desmosine plus isodesmosine (DID).

2.5 Elemental analysis of Egg membrane (Tsai W.T *etal*, 2006)

Carbon/hydrogen/oxygen/nitrogen/sulfur (C/H/O/N/ S) contents were used as a means of examining the remaining residues in the preparation of the eggshell products. The elemental analysis of the sample (1–3 mg) was made using an elemental analyzer (model CHN-O-RAPID, Heraeus Co., Germany). For each analysis, the standard sample (i.e., sulfanilic acid) was first analyzed for checking the experimental error within ±1%. All measurements were carried out in duplicate.

2.6 Fourier transforms infrared spectroscopy (FTIR) Analysis (Tsai W.T *etal*, 2006)

FTIR has been used for the examination of functional groups on the surface of activated clays or carbon materials previously. FTIR spectra analysis was performed with 1.5 mg test samples in approximately 150 mg potassium bromide (KBr). Discs (12.7 mm ID and 1 mm thick) were prepared in a manual hydraulic press (model 15.011, Perkin Elmer Co., USA) at about 10 tonnes for a

pressing time of 30–60 s. All spectra were obtained from 4000 to 400 cm^{-1} at a data acquisition rate of 4 cm^{-1} by using a FTIR spectrophotometer (FTIR-8400S, Shimadzu, Japan).

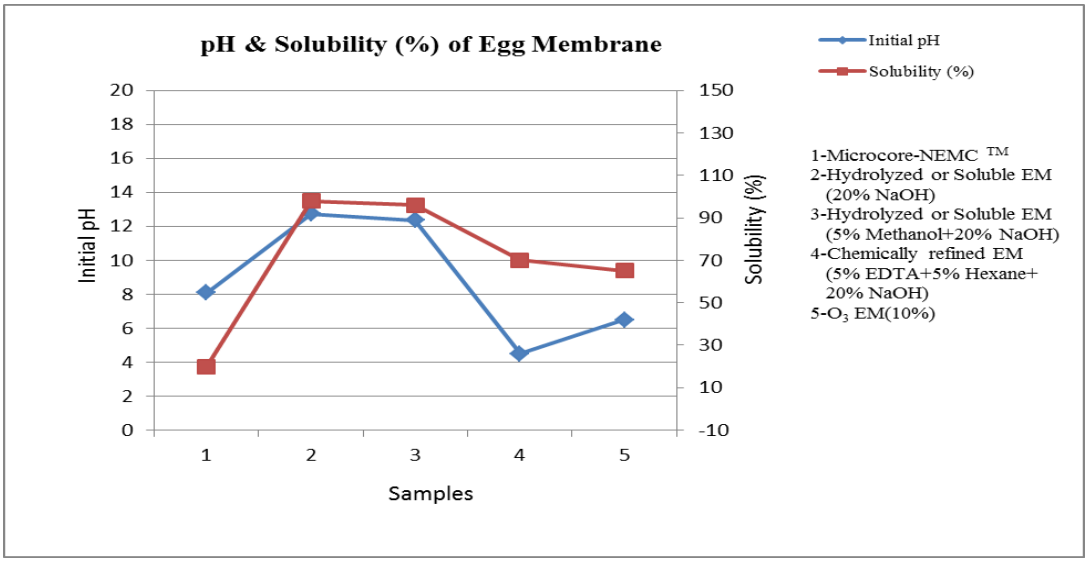
3. RESULTS AND DISCUSSION

3.1 Solubility index of egg membrane

Five different processing conditions include Natural process, Alkali digestion process, Alkaline Methanol process, EDTA + Hexane process and O_3 process. During the different process conditions releasing of H_2S gas and byproducts are separated was shown in the Table 1. pH and Solubility index of five different processing condition of Egg membrane concentrate was shown in the Graph 1. From this analysis, Solubility (%) was decreased in Natural Egg Membrane Concentrate (NEMC^{TM}) due to its insoluble property than Hydrolyzed (HEM) or Soluble Egg Membrane (SEM).

Table 1: pH and Solubility Index

S.No	Sample	Initial pH	Neutralized pH	Solubility (%)	H_2S	By products
1	Microcore-NEMC^{TM}	8.1	8.1	20	NO	Nil
2	Hydrolyzed or soluble EM(20%NaOH)	12.72	8.1	98	YES	Sodium Acetate
3	Hydrolyzed or soluble EM(5% Methanol+ 20%NaOH)	12.35	8.1	96	YES	Sodium Acetate
4	Chemically refined EM (5% EDTA+5% Hexane+20% Alkali)	4.5	8.1	70	NO	Sodium Acetate
5	O_3 EM (10%)	6.5	8.1	65	NO	Nil



Graph 1: pH and Solubility Index

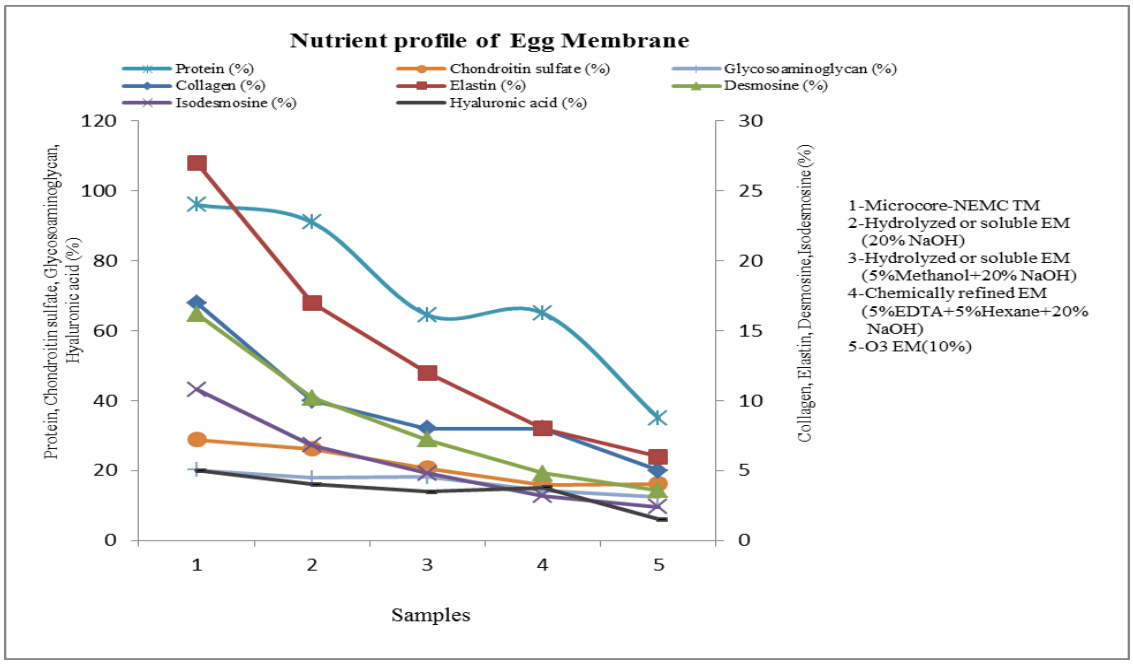
3.2 Nutrient profile analysis of egg membrane

Nutrient composition of the Natural product vs the chemically modified Egg membrane was shown in the Table 2. In Microcore's NEMC^{TM} resulted 96% protein, 17% Collagen, 27% Elastin (16.2% Desmosine, & 10.8% Isodesmosine), 28.3% Chondroitin sulphate, 20.5% Glycosaminoglycan and 5% Hyaluronic acid whereas the Hydrolyzed (or) Soluble Egg Membrane produced by 20% NaOH at extreme conditions has resulted 71% protein, 8% Collagen, 12% Elastin (7% Desmosine & 5% Isodesmosine), 20.19% Chondroitin sulphate, 10.9% Glycosaminoglycan and 4% Hyaluronic acid and Hydrolyzed or soluble egg membrane by combined treatment 5% Methanol and 20% NaOH digestion has resulted 64.5% protein, 8% Collagen, 12% Elastin (7.2% Desmosine & 4.8% Isodesmosine), 9.73% Chondroitin sulphate, 8.18% Glycosaminoglycan and 3.5% Hyaluronic acid. whereas 10 % EDTA and 5% hexane treated Egg Membrane has affected 65% protein, 8% Collagen, 8% Elastin (4.8% Desmosine & 3.2% Isodesmosine), 15.2% Chondroitin sulphate, 14.8% Glycosaminoglycan and 3.8% Hyaluronic acid, whereas 10 % O_3 treated egg membrane has affected 35% protein, 5% Collagen, 6% Elastin (3.6% Desmosine & 2.4% Isodesmosine), 7.25% of Chondroitin sulphate, 8.4% of Glycosaminoglycan and 1.5% Hyaluronic acid were shown in the graph 2. Reduction in chondroitin sulphates shows that it is not stable under Hydrolyzed or soluble egg membrane than

Microcore's NEMC^{TM} and also Microcore's NEMC^{TM} has the GAG tolerance under Hydrolyzed or soluble egg shell membrane. Effect of the chemicals and processing conditions and its effects on the nutrient profile, the Natural processing methods resulted high nutrient profile with integration of the bimolecules. This study reveals that Microcore's NEMC^{TM} has the higher content of protein, Collagen, Elastin (Desmosine, Isodesmosine) Chondroitin sulphate, Glycosaminoglycan essential for treating joint pain and connective tissue disorder.

Table 2: Nutrient profile with respect to the process conditions

S.No	Sample	Protein (%)	Collagen (%)	Elastin (%)	Desmosine (%)	Isodesmosine (%)	Chondrointin sulfate (%)	Glycosaminoglycan (%)	Hyaluronic acid (%)
1	Microcore-NEMC^{TM}	96	17	27	16.2	10.8	28.83	20.15	5
2	Hydrolyzed or soluble EM(20%NaOH)	91	10	17	10.2	6.8	26.19	17.9	4
3	Hydrolyzed or soluble EM (5% Methanol + 20% NaOH)	64.5	8	12	7.2	4.8	20.73	18.18	3.5
4	Chemically refined EM (5% EDTA+5% Hexane+20% Alkali)	65	8	8	4.8	3.2	15.8	14.2	3.8
5	O_3 EM (10%)	35	5	6	3.6	2.4	16.25	12.4	1.5



Graph 2: Nutrient profile with respect to the process conditions

3.3. Elemental analysis of egg membrane (Tsai W.T *etal*, 2006)

The results of ultimate elemental analysis of five different processed samples were given in Table 3. The elemental contents (including C, H, N and S) of eggshell membrane sample are obviously larger. It is reasonable that the composition of fibers in eggshell membrane has been determined to be approximately 95% protein with a small amount of polysaccharide (Parsons, 1982). Therefore, the eggshell membrane particle would be expected to contain positively charged functional groups such as $–NH_3^+$ and $–CO–N^+H_2^–$, which are dependent on the pH of the aqueous solution.

Table 3: Elemental analyses of egg membrane

Sample	C (wt.%)	H (wt.%)	N (wt.%)	O (wt.%)	S (wt.%)
Microcore-NEMC^{TM}	46.50 ± 0.05	6.72 ± 0.05	15.34 ± 0.03	12.10 ± 0.01	3.00 ± 0.02
Hydrolyzed or soluble EM(20%NaOH)	37.46 ± 0.04	5.81 ± 0.05	11.41 ± 0.02	11.18 ± 0.02	2.10 ± 0.02
Hydrolyzed or soluble EM (5% Methanol+20% NaOH)	34.21 ± 0.05	5.76 ± 0.02	11.16 ± 0.03	11.16 ± 0.01	2.0 ± 0.01
Chemically refined EM (5% EDTA+5% Hexane+20% Alkali)	29.35 ± 0.02	4.72 ± 0.01	10.34 ± 0.01	10.05 ± 0.03	1.82 ± 0.02
O_3 EM (10%)	42.52 ± 0.01	5.91 ± 0.04	13.34 ± 0.02	11.45 ± 0.02	1.41 ± 0.03

3.3 FTIR analysis (Tsai W.T *etal*, 2006)

The FTIR analysis for Natural Egg Membrane Concentrate (NEMC^{TM}), Hydrolyzed Egg Membrane (HEM) or Soluble Egg Membrane (SEM), chemically refined Egg membrane (5% EDTA+5% Hexane+20% NaOH) and 10% O_3 egg membrane were shown in the Figure 1-5. The absorption peaks at different frequency (cm^{-1}) was shown in the Table 4-8. Different significant peak of intensity of NEMC^{TM}, Hydrolyzed or soluble egg membrane (20% NaOH), Hydrolyzed or soluble egg membrane (5% Methanol+5% NaOH), chemically refined Egg membrane (5% EDTA+5% Hexane+20% NaOH) and 10% O_3 egg membrane at 1419.66, 1401.56, 1421.58, 1432.38, 406.99cm^{-1} were the characteristics of aromatic, C-F (alkyl halides), CH_3 bend (alkanes), CH_3 bend (alkanes) and C-Br (alkyl halides).

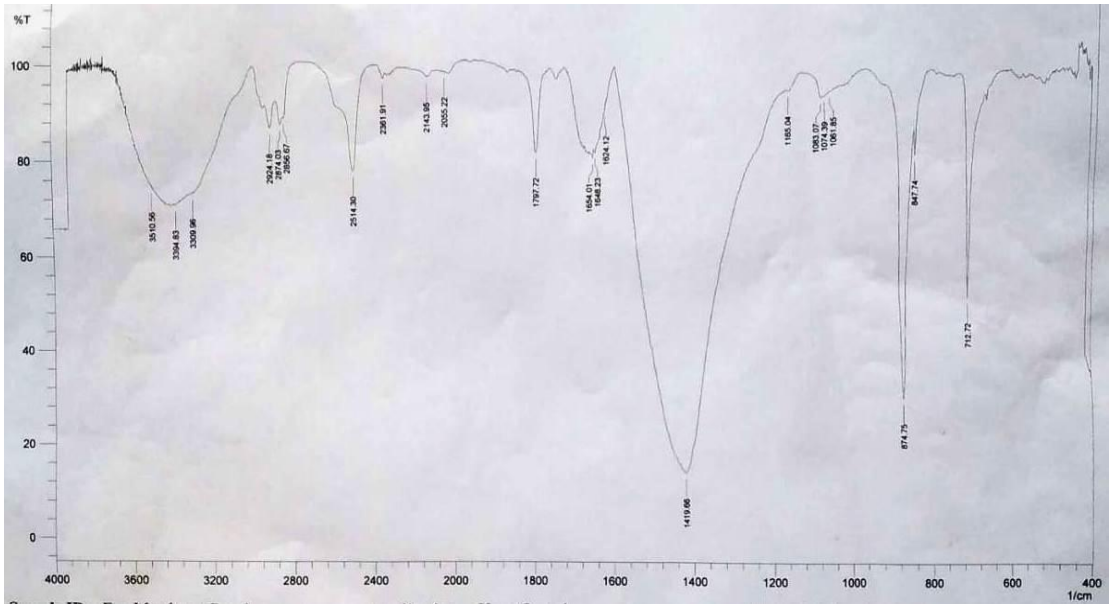


Figure 1: FTIR spectra of Microcore-NEMC^{TM}

Table 4: IR spectra table for Microcore-NEMC^{TM}

Frequency (cm^{-1})	Functional Group	Frequency (cm^{-1})	Functional Group
3510.56 (s)	water OH Stretch	1654.01 (s)	C=C amide
3394.83 (s)	alcohol OH stretch	1648.23 (s)	C=C amide
3309.96 (s)	alcohol OH stretch	1624.12 (s)	C=C amide
2924.18 (s)	carboxylic acid OH stretch	1419.66 (w)	C=C aromatic
2874.03 (s)	carboxylic acid OH stretch	1165.04 (s)	C-F
2856.67 (s)	carboxylic acid OH stretch	1083.07 (s)	C-F
2514.30 (s)	carboxylic acid OH stretch	1074.39 (s)	C-F
2361.91 (s)	C≡N stretch	1061.85 (w)	C-F
2143.95 (v)	C≡C stretch	874.75 (s)	C-Cl
2055.22 (v)	C≡C stretch	847.74 (s)	C-Cl
1797.72 (s)	C=O anhydride	712.72 (s)	C-Cl

w=weak, s=strong, v=variable, m- medium

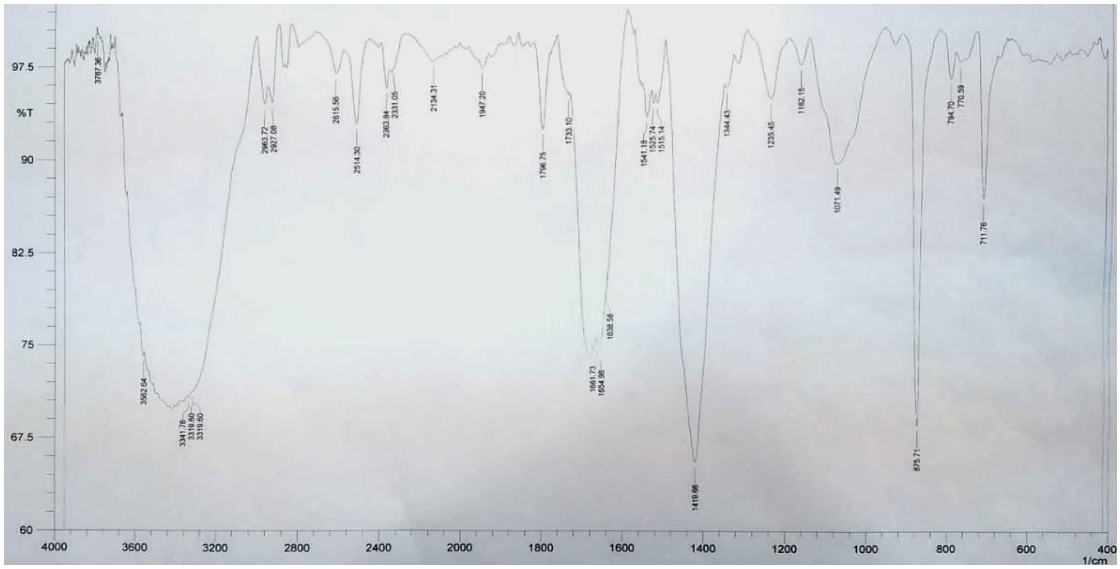


Figure 2: FTIR spectra of Hydrolyzed or soluble egg membrane (20% NaOH)

Table 5: IR spectra table for Hydrolyzed or soluble egg membrane (20% NaOH)

Frequency (cm^{-1})	Functional Group	Frequency (cm^{-1})	Functional Group
3787.36 (s)	water OH Stretch	1661.73 (w)	C=C alkene
3562.64 (s)	alcohol OH stretch	1654.98 (w)	C=C alkene
3341.78 (s)	alcohol OH stretch	1638.58 (w)	C=C alkene
3319.60 (s)	alcohol OH stretch	1541.18 (s)	NO_2 stretch
3319.60 (s)	alcohol OH stretch	1525.74 (s)	NO_2 stretch
2963.72 (s)	carboxylic acid OH stretch	1515.14 (s)	NO_2 stretch
2927.08 (s)	carboxylic acid OH stretch	1401.56 (s)	C-F
2615.56 (v)	-C-H aldehydic	1344.43 (s)	C-F
2514.30 (v)	-C-H aldehydic	1235.45 (s)	C-F
2363.84 (v)	C≡C stretch	1162.15 (s)	C-F
2331.05 (s)	C≡N stretch	1071.49 (s)	C-F
2134.31 (v)	C≡C stretch	875.71 (s)	C-Cl
1947.20 (s)	C=O anhydride	794.70 (s)	C-Cl
1796.75 (s)	C=O ketone	770.59 (s)	C-Cl
1733.10 (s)	C=O ketone	711.76 (s)	C-Cl

w=weak, s=strong, v=variable, m- medium

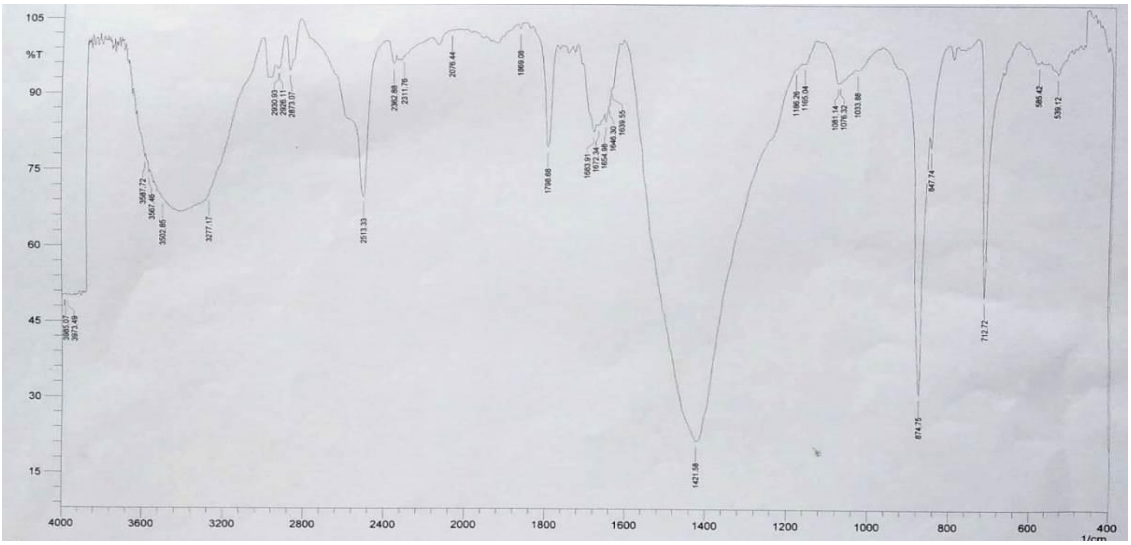


Figure 3: FTIR spectra of Hydrolyzed or soluble egg membrane (5% Methanol+5% NaOH)

Table 6: IR spectra table for Hydrolyzed or soluble egg membrane (5% Methanol+5% NaOH)

Frequency (cm^{-1})	Functional Group	Frequency (cm^{-1})	Functional Group
3985.07 (s)	water OH Stretch	1672.34 (w)	C=C alkene
3973.79 (s)	water OH Stretch	1654.98 (w)	C=C alkene
3587.72 (s)	water OH Stretch	1646.30 (w)	C=C alkene
3567.46 (s)	water OH Stretch	1639.55 (w)	C=C alkene
3502.85 (s)	water OH Stretch	1421.58 (m)	CH_3 bend
3277.17 (s)	carboxylic acid OH stretch	1186.26 (s)	C-O-C stretch
2930.93 (w)	-C-H stretch	1165.04 (s)	C-O-C stretch
2926.11 (w)	-C-H stretch	1081.14 (s)	C-O-C stretch
2873.07 (v)	-C-H aldehydic	1076.32 (s)	C-O-C stretch
2513.33 (v)	-C-H aldehydic	1033.88 (s)	C-O-C stretch
2362.88 (v)	C≡C stretch	874.75 (s)	C-Cl
2311.76 (v)	C≡C stretch	847.74 (s)	C-Cl
2076.44 (v)	C≡C stretch	712.72 (s)	C-Cl
1869.08 (w)	C=O anhydride	585.42 (s)	C-Br
1798.68 (s)	C=O anhydride	539.12 (s)	C-Br

w=weak, s=strong, v=variable, m- medium

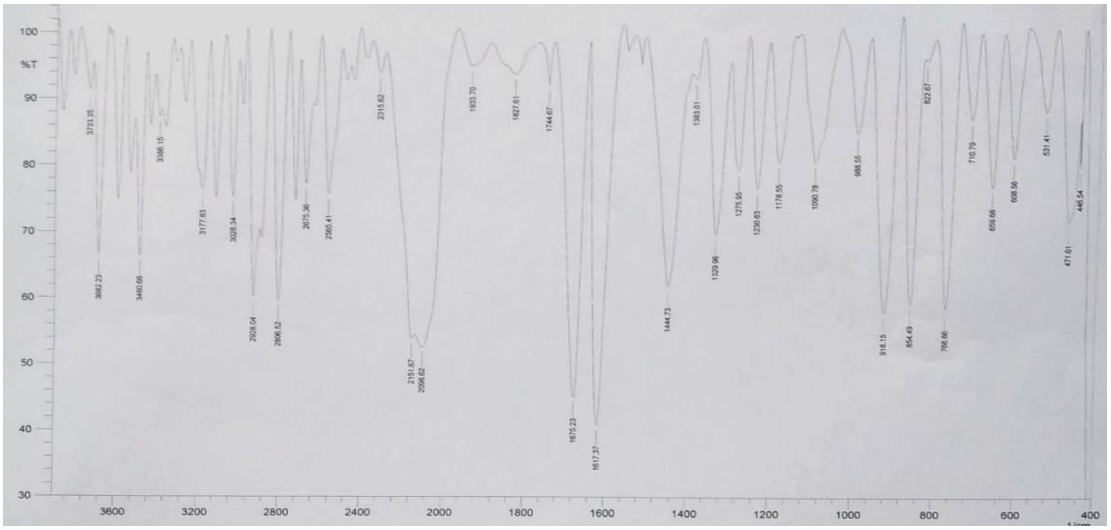


Figure 4: FTIR spectra of chemically refined EM (5% EDTA+5% Hexane+20% NaOH)

Table 7: IR spectra table for chemically refined EM (5% EDTA+5% Hexane+20% NaOH)

Frequency (cm^{-1})	Functional Group	Frequency (cm^{-1})	Functional Group
3733.35 (s)	water OH Stretch	1654.98 (w)	C=C alkene
3682.23 (s)	water OH Stretch	1646.30 (w)	C=C alkene
3480.66 (s)	water OH Stretch	1639.55 (w)	C=C alkene
3386.15 (s)	water OH Stretch	1432.38 (m)	CH_3 bend
3177.83 (s)	water OH Stretch	1186.26 (s)	C-O-C stretch
3028.34 (s)	carboxylic acid OH stretch	1165.04 (s)	C-O-C stretch
2928.04 (w)	-C-H stretch	1081.14 (s)	C-O-C stretch
2806.52 (w)	-C-H stretch	1076.32 (s)	C-O-C stretch
2565.41 (v)	-C-H aldehydic	874.75 (s)	C-Cl
2315.62 (s)	C≡N stretch	847.74 (s)	C-Cl
2151.67 (v)	C≡C stretch	712.72 (s)	C-Cl
2098.62 (v)	C≡C stretch	585.42 (s)	C-Br
1933.70 (v)	C≡C stretch	539.12 (s)	C-Br
1827.61 (w)	C=O anhydride	1654.98 (w)	C=C alkene
1798.68 (s)	C=O anhydride	1646.30 (w)	C=C alkene
1683.91 (w)	C=C alkene	1639.55 (w)	C=C alkene
1672.34 (w)	C=C alkene	1421.58 (m)	CH_3 bend

w=weak, s=strong, v=variable, m- medium

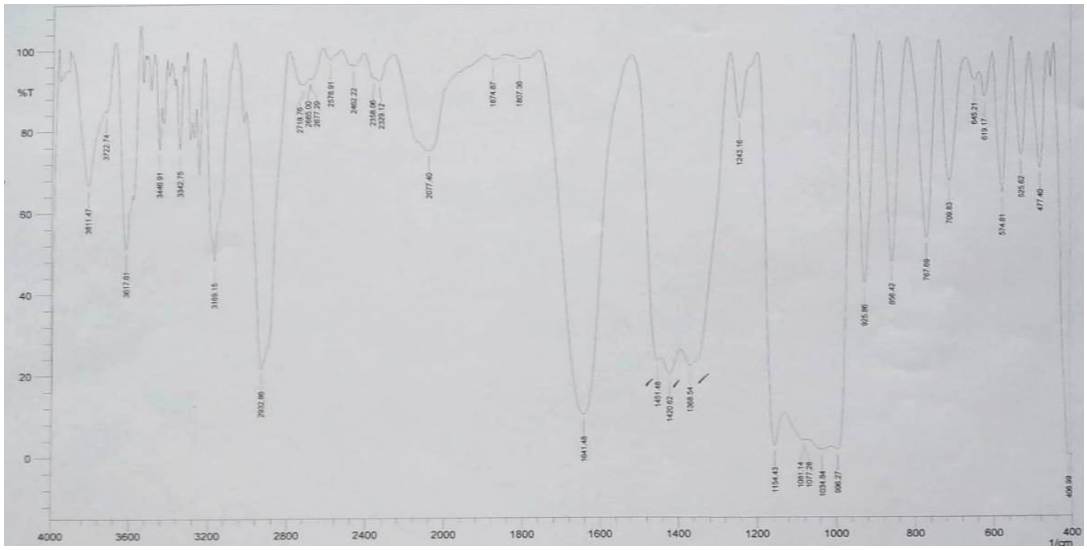


Figure 5: FTIR spectra of 10% O_3 egg membrane

Table 8: IR spectra table for 10% O_3 egg membrane

Frequency (cm^{-1})	Functional Group	Frequency (cm^{-1})	Functional Group
3811.47 (s)	water OH Stretch	1420.62 (m)	CH_2 bend
3722.74 (s)	water OH Stretch	1368.54 (m)	CH_3 bend
3617.61 (s)	water OH Stretch	1243.16 (s)	C-O-C stretch
3446.91 (s)	carboxylic acid OH stretch	1154.43 (s)	C-O-C stretch
3342.75 (s)	carboxylic acid OH stretch	1081.14 (s)	C-O-C stretch
3169.15 (w)	-C-H stretch	1034.84 (s)	C-O-C stretch
2932.86 (v)	-C-H aldehydic	996.27 (s)	C-F
2685.00 (v)	-C-H aldehydic	856.42 (s)	C-F
2677.29 (v)	-C-H aldehydic	767.69 (s)	C-Cl
2578.91 (v)	-C-H aldehydic	918.15 (s)	C-Cl
2462.22 (v)	-C-H aldehydic	854.49 (s)	C-Cl
2358.06 (s)	C≡N stretch	822.67 (s)	C-Cl
2329.12 (s)	C≡N stretch	768.66 (s)	C-Br
2077.40 (v)	C≡C stretch	619.17 (s)	C-Br
1874.87 (w)	C=O anhydride	574.81 (s)	C-Br
1807.36 (w)	C=O anhydride	525.62 (s)	C-Br
1641.48 (w)	C=C alkene	477.40 (s)	C-Br
1451.48 (m)	CH_2 bend	406.99 (s)	C-Br

w=weak, s=strong, v=variable, m- medium

In Microcore NEMC ™ , the presence of strong hydroxyl absorption at 3510.56, 3394.83 and 3309.86 cm^{-1}. Strong amide absorption peak at 1654.01, 1648.23, 1624.12 cm^{-1} was observed in Figure 1. The strong absorption peak at 2924.18, 2874.03, 2856.67 and 2514.30 cm^{-1} were the characteristic of carboxylic acid groups in Figure 1. The presence of strong hydroxyl, COOH, Amides & C=O absorption peak with 4000 cm^{-1} – 400 cm^{-1} were observed in Table 4-8.

CONCLUSION

The active molecule composition between Natural Egg Membrane Concentrate (NEMC™) and Hydrolyzed (HEM) or Soluble Egg Membrane (SEM) were analyzed and compared. Based on the nutrient profile it was found that natural processing methods has resulted high nutrient profile with integration of biomolecules than chemically treated egg membrane. Hence it is concluded that Microcore's NESM™ has the higher content of several synergy molecules to treat arthritis and osteoporosis that includes resulted 96% protein, 17% Collagen, 27% Elastin (16.2% Desmosine, & 10.8% Isodesmosine), 28.3% Chondroitin sulphate, 20.5% Glycosaminoglycan and 5% Hyaluronic acid. From the elemental analysis confirms that the positively charged functional groups such as $–NH_3^+$ and $–CO–N^+H_2^–$, which are dependent on the pH of the aqueous solution. The FTIR data illustrates the difference in significant peak of intensity of NEMC™, Hydrolyzed or soluble egg membrane (20% NaOH), Hydrolyzed or soluble egg membrane (5% Methanol+5% NaOH), chemically refined Egg membrane (5% EDTA+5% Hexane+20% NaOH) and 10% O_3 egg membrane at 1419.66, 1401.56, 1421.58, 1432.38, 406.99cm^{-1} were the characteristics of aromatic, C-F (alkyl halides), CH_3 bend (alkanes), CH_3 bend (alkanes) and C-Br (alkyl halides). In natural egg shell membrane concentrate, the presence of strong hydroxyl absorption at 3510.56, 3394.83 and 3309.86 cm^{-1}. Strong amide absorption peak at 1654.01, 1648.23, 1624.12 cm^{-1} was observed. The strong absorption peak at 2924.18, 2874.03, 2856.67 and 2514.30 cm^{-1} were the characteristic of carboxylic acid groups. The presence of strong hydroxyl, COOH, Amides & C=O absorption peak with 4000 cm^{-1} – 400 cm^{-1} were observed in SEM.

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