Original bioactive complexes rich in glycosaminoglycans obtained from small fish

NATALIA ROSOIU[1], ROXANA NITA[2], LAURA OLARIU[2], VERONICA DRUMEA[2], DIANA MANUELA ENE[2]
[1] *OVIDIUS University Faculty of Medicine, Constanta, Romania, 58 Ion Voda Street, RO 900573, p*

[2] *S.C. BIOTEHNOS S.A., Otopeni, Romania*

Abstract

An original technology was developed to obtain a bioactive complex from small fish species Engraulis encrassicolus ponticus, Sprattus sprattus sprattus, Odontogadus merlangus euxinus) which contains: glycosaminoglycans, essential amino acids, essential fatty acids (linoleic, linolenic and arachidonic acids), eicosanoids, vitamins (myoinositol), glycerolphosphates, microelements: Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si, etc. The bioactive complex was analyzed by gas chromatography coupled with mass-spectrometry, gel electrophoresis in different systems (SDS-poliacrilamide, cellulose acetate), potentiometric titration, and high performance liquid chromatography. The content of glycosaminoglycans (from which 44% represent sulfated glycosaminoglycans) make the bioactive complex useful to prevent the un unsettlement of the macromolecular structure of background substance and keep the functionality of the extracellular matrix from conjunctive, cartilaginous and bone tissue. Antioxidant activity and regenerative of collagen fibrils was investigated in vitro. The chemical composition provides multiple therapeutically properties: anticoagulant, antithrombotic, antilipidemic, tissue restoration, a strong antioxidant and anti-inflammatory activities. The complex obtained is rich in biological active compounds and could be used to make pharmaceutical preparations with high efficiency and minimal side effects.

Keywords: glycosaminoglycans, essential aminoacids, essential fatty acids, antioxidant activity, pharmaceutical purposes

Introduction

Glycosaminoglycans (GAGs) or mucopolysaccharides are long unbranched polysaccharides consisting of a repeating disaccharide unit. This unit consists of an N-acetyl-hexosamine and a hexose or hexuronic acid, either or both of which may be sulfated. Members of the GAG family vary in the type of hexosamine, hexose or hexuronic acid unit they contain (e.g. glucuronic acid, iduronic acid, galactose, galactosamine, glucosamine). GAGs represent the major component of proteoglycans, that associated with collagen and chondrocytes form the cartilage.

The proteoglycans are extracellular compounds that form the fundamental substance of all types of conjunctive tissues. They are predominant in the structure of the conjunctive tissue, the cellular membranes, the epiphysis of long bones, cartilages and ligaments, epithelial mucus, vitreous fluid, cornea, mucinous substances with lubricant activity, mucine if the intestinal mucosa, saliva, milk, plasma.

In the degenerative processes, acidic GAGs (hyaluronic acid, chondroitin sulfates) are intensely broken by hyaluronidase, the extracellular matrix – fundamental substance – of the conjunctive tissue and cartilage being destroyed.

GAGs are biopolymers industrially extracted from various animal organs. There are patent applications, which disclose obtaining methods of some GAGs derivatives of heparinoids from bacteria *Escherichia coli* [1] or heparin and heparinoids from fish (carp, mackerel, cod, herring, anchovy, shark, salmon) and others marine organisms with anticoagulant and anti-thrombotic activity exclusively [2,3,4]. Another patent application discloses a pharmaceutical composition with anti-inflammatory properties, which contain sulfated hexosamines and a protein extract from *Perna canaliculis* [5]. Other inventors isolate GAGs from cartilaginous fish that present anti-tumor effect [6]. These products do not capitalize the whole therapeutic potential of the GAGs.

This research aim to obtain a bioactive complex of substances from marine organisms with a predominant content of GAGs, which can take various pharmaceutical forms, for human or veterinary use, with large therapeutically applications, having tissue restitutive, anti-proliferative, bio-stimulating, anticoagulant, antithrombotic, anti-lipemic, anti-inflammatory, antioxidant activity. Thus, they determine the restructuring of the macromolecular components of the conjunctive, cartilaginous and bone tissue.

The authors have a vast experience in this domain, as they launched on the national and international market a valuable anti-rheumatic injectable drug, obtained from marine organisms (small sea fish) with commercial name of ALFLUTOP^{R} [7-12]. The marine organisms present a major interest for the extraction of biological active substances with multiple and valuable therapeutically applications, with notable results worldwide (M. SERBAN & N. ROSOIU [7]), (J.M. KORNPROBST [13]).

Material and methods

Small sea fish species (*Engraulis encrasicolus ponticus, Sprattus sprattus sprattus, Odontogadus merlangus euxinus*) were the raw material for obtaining bioactive complexex using original technologies.

The four extracts obtained by some different methods were analyzed chemical and *in vitro*, by the modern methods included in US Pharmacopoeia 30 [13].

The identification of GAGs – is performed using horizontal electrophoresis on cellulose acetate sheet. The migration uses a buffer of Ba $(CH_3COO)_2$ 0.1M, pH 5, at 60 V, for 2 hours. Coloring use toluidine blue 0.1% and discoloring CH_3COOH 5% solution. The apparatus is MINI-SUB CELL GT (Figure 1 and 2).

Dosing GAGs. The method consists of sulfated GAGs turbidimetric dosing using an automated titrator with photometric sensor, based on the reaction of sulfated groups with cetyl pyridine.

Principle: sulfate groups from the sulfated GAGs react with cetyl pyridine forming a complex whose turbidity is proportional with the amount of sulfated GAGs in solution. The quantitative report is based on interpolation on the basis of the quantitation curve, drown using standards of known purity, in identical conditions of procedure with those used for the samples: dynamic titration at equivalence point, with measurement of pH.

Equipment: automated titrator with photometric sensor (λ= 660 nm).

Reagents:

- titrant : solution of cetylpyridine chloride 1mg/ml in distilled water

- diluter: 297 mg of monobasic potassium phosphate, 492mg dibasic potasium phosphate and 250mg polysorbate 80 are dissolved in distilled water in a glass of 1 L; the pH is adjusted to 7.0±0.2; it is prepared daily.

- standard solution: an accurate amount of standard solution of chondroitin sulfate is weighted and diluted with water, obtaining the stock solution; it is used to prepare three standard solutions with water:0.5, 1.0 and 1.5 mg/ml. The standard solutions are prepared each time when the titration solution is changed.

- Dialysis bags.

Procedure: the sample is dialyzed against distilled water, for 24 hours, and then is diluted to 5 ml with distilled water; 59mL of diluter are added (phosphate buffer solution pH 7) and it is titrated with cetyl pyridine 1mg/mL. The amount of GAGs is calculated by means of interpolation using the linear equation of the regression obtained after titration of three known concentrations of CS (e.g. 0.5mg/ml, 1.0 mg/ml, 1.5 mg/ml.)

1. **Identification and dosing of amino acids and other compounds** is performed using gas chromatography coupled with mass spectrometry. The sample is derived with BSTFA in acetonitrile and injected in GC/MS. The identification of the compounds used the NIST library and dosing uses the method of external standard (for the components for which we have reference substances).

Identification and quantization of amino acids:

- Gas chromatograph Agilent Technologies 6890N coupled with mass spectrometer MSD Agilent 5973:
  - split/splitless injector
  - detector: mass spectrometer with quadrupol and electronic impact ionization
  - capilary column DB5-MS: 60m x 0,25 mm x 0,25 µm (stationary phase: 5% fenyl-methyl-polysiloxan)
- Computer: ChemStation specialized soft, with NIST library of spectres

The sample is derived with bis-trimethylsilyl-trifluoracetamide (BSTFA) to transform the compounds in trimethyl silylated derivatives (TMS). The reaction of derivatization is performed as it follows: in the sample vial, 0.5 ml of BSTFA reagent and 0.5 ml acetonitrile are added; it is gently homogenized and closed, and incubated at 90˚C for 1 hour.

Procedure parameters:

- gas helium 1.2ml/min
- splitting ratio: 1:20
- temperature program:

$$100^{\circ}C \xrightarrow[4\ min]{12\ ^{\circ}C/min} 170^{\circ}C \xrightarrow{3^{0}C/min} 190^{0}C \xrightarrow[10min]{10^{0}C/min} 280^{0}C$$

- time for solvent elimination 7.5 min
- mass domain: 35 - 400 u.a.m.
- injector temperature: $250^{0}C$
- interface temperature: $280^{0}C$
- source temperature: $230^{0}C$
- quadrupol temperature: $150^{0}C$

2. **Identification of fatty acids** is performed by using gas chromatography with detector with flame ionization. Into a reflux flask 1g lipid, 10 ml methanol, 0.2 ml potassium hydroxide 60 g/l are introduced; it is maintained to reflux for 15 minutes, then it is cooled and

transferred into a separation funnel with 5 ml heptane and 10 ml NaCl solution 200 g/l. The organic layer is passed on sodium sulfate into a volumetric flask of 20 ml. It is diluted 1:50 and it is injected.

For the identification of fatty acids, there were used two standard mixtures of C_{14}-C_{22} fatty acids saturated and unsaturated.

Conditions for chromatography:

Gas chromatograph Agilent Technologies 6890N

- injector split/splitless
- detector with flame ionization (FID)
- column DB-WAX 30m x 0.32mm x 0.15µm (stationary phase: polyethylene-glycol)
- gaz: nitogen, 0.8 ml/min
- splitting ratio: 1:50
- injection volume 1 µl
- oven temperature: 200˚C / 15 minute
- injector temperature: 250^0C
- detector temperature: 260^0C

3. Determination of total antioxidant activity *in vitro* – method with DPPH

Reagents:

- Solution of 1,1-diphenyl-2-picrylhydrazyl (DPPH) 0.004% in methanol
- Methanol
- Distilled water
- Ascorbic acid 0.01M

Procedure: The following reagents are added: 1ml solution DPPH, 0.5 ml methanol, X ml sample, 0.5 –X ml distilled water. The absorbence of the samples is read at 517nm after they have been stored at room temperature, in a dark place, for 30 minutes. To eliminate the interference of the substances in the mixture, for each sample a blank is prepared of methanol, distilled water interest mixture.

The results are presented as % inhibition calculated with the following formula:

$\%Inhibition = 100x(A_{DPPH} - A_{sample})/A_{DPPH}$

where A_{DPPH} = absorbance of DPPH in the absence of the analysed substance

A_{sample} = absorbance of the sample – absorbance of blank

Results and discussion

It is noticed that worldwide there is an acute need for developing new therapeutic products, based on bio-complexes that may allow to improve the efficiency/toxicity ratio of some therapy means, for patients with major pathology. Research in biotechnological domain and the resulted products with application in pharmacy respond to the need to improve and develop new therapy strategies. Thus, our research propose a study that aims biotechnology that produce new bioactive complexes, in order to identify, using *in vitro* and *in vivo* analyses, with respect to research ethics, new therapy agents with significant antioxidant, anti-inflammatory, anticoagulant, antithrombotic, anti-atherosclerotic, tissue restitutive, bio-stimulating activity.

The analyses, performed on various samples obtained in laboratory, revealed that the obtained extract is a complex of biological active substances.

In figures 1 and 2, GS-MS chromatograms of the bioactive complex showing their amino-acid content are presented.

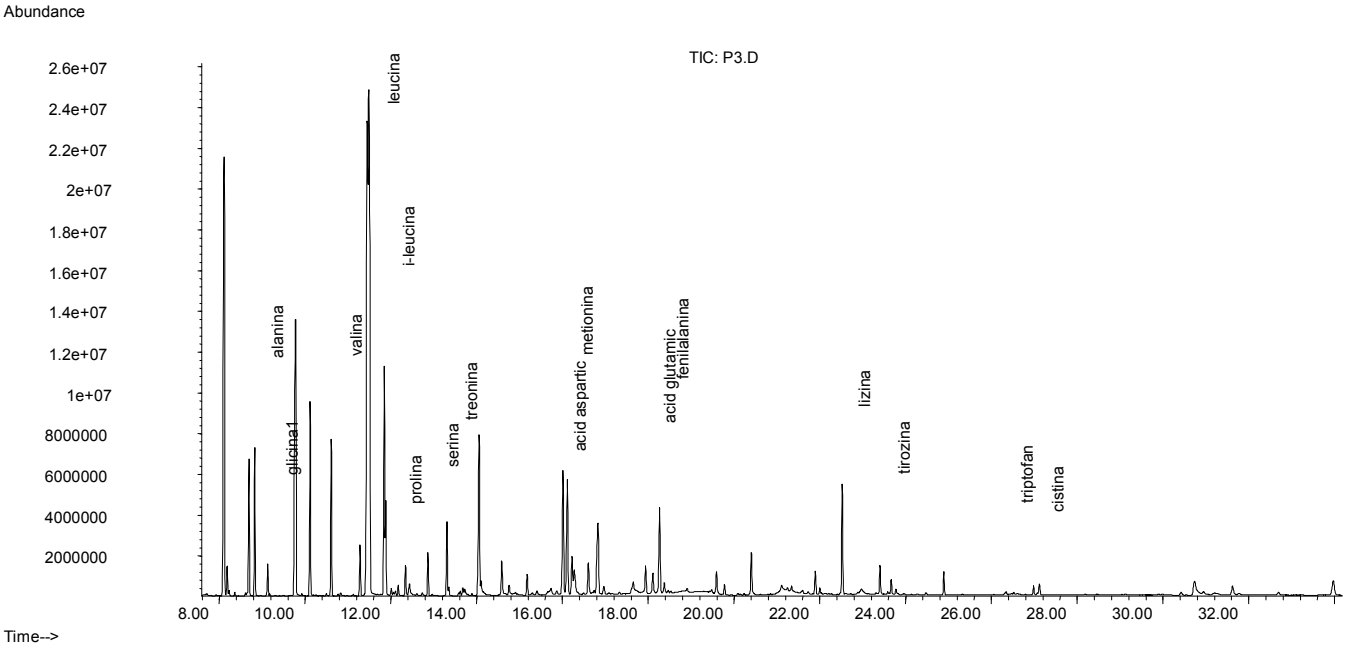

Figure 1. GC-MS chromatogram of the bioactive complex – content of amino acids

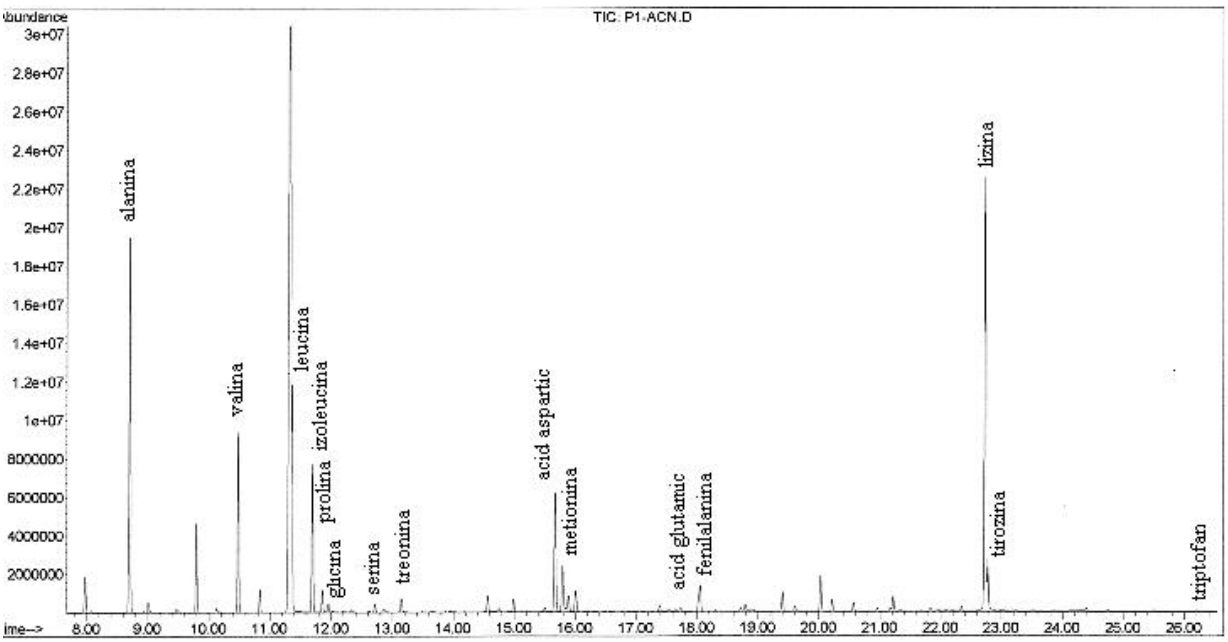

Figure 2. GC-MS chromatogram of the bioactive complex – content of amino acids

The content of amino acids calculated in % is presented in table 1.

Table 1. Content of amino acids (in %)
* Essential amino acids

	Amino acid	**Content (%)**
1	Glycine	0.01 – 0.05
2	Alanine	0.30 – 0.45
3	Valine *	0.20 – 0.50
4	Leucine *	0.80 – 1.50
5	Isoleucine *	0.30 – 0.80
6	Proline	0.01 – 0.03
7	Hyroxyproline	0.01 – 0.04
8	Serine	0.05 – 0.25
9	Threonine *	0.10 – 0.30
10	Aspartic acid	0.10 -0.15
11	Methionine *	0.20 – 0.90
12	Glutamic acid	0.25 – 0.50
13	Phenylalanine *	0.15 – 0.60
14	Lyzine*	0.40 – 1.90
15	Tyrozine	0.10 -0.30
16	Tryptophan*	0.03 -0.08
17	Cystine	0.10 – 0.30
18	Cysteine	0.001 -0.003
19	Glutamine	0.40 – 0.65
20	Asparagine	0.25 – 0.35
	Total amino acids	**3.7 – 12 %**

Other compounds: 2-aminobutyric acid, phosphoric acid, malic acid, 3-amino-2-piperidone, oxo-proline, 4-aminobutanoic acid, 2,3,4-trimethoxymandelic, glycero-phosphoric acid, citrulline, ornithine, creatinine, mezoinositol etc. were identified.

In figure 3 and 4 the horizontal electrophoresis on cellulose acetate of GAGs are presented and in figure 5, potentiometric titration curve of sulfated glycosaminoglycans.

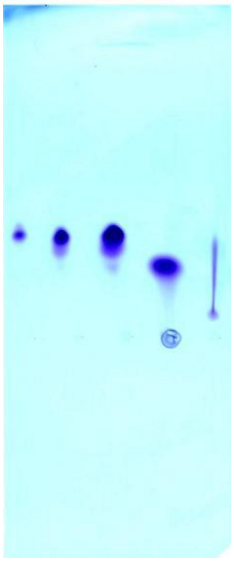

Figure 3. Horizontal electrophoresis on cellulose acetate

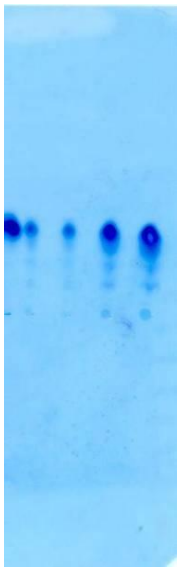

Figure 4. Horizontal electrophoresis on cellulose acetate

- In figure 4, from left to right: (1) standard chondroitin sulfates (CS), sample GAGs (2 spots– different volumes), standard heparin sulfate and Na hyaluronate
- In figure 5, from left to right: standard chondroitin sulfates (CS) and sample GAGs (4 spots – different volumes)

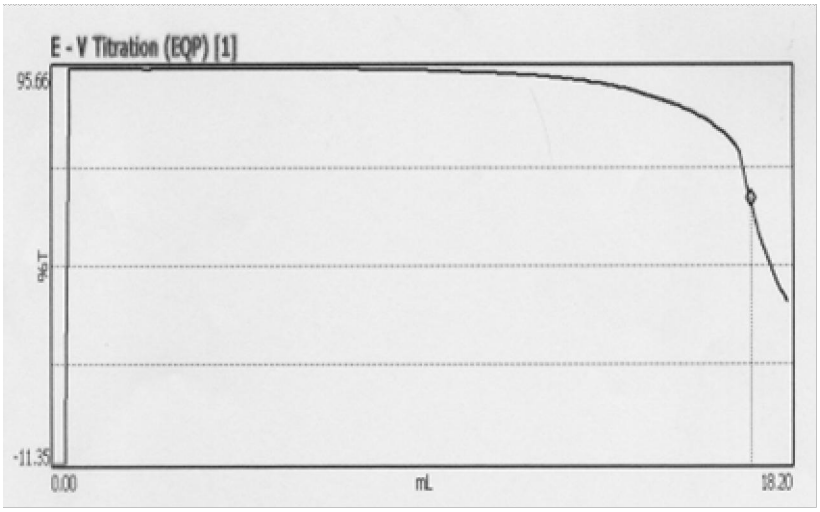

Figure 5. Potentiometric titration curve of sulfated glycosaminoglycans

The fatty acids analyzed by GC-FID (sample PUFA) are presented in figure 6 and in figure 7, the fatty acids analyzed by GC-FID (Standard PUFA Supelco).

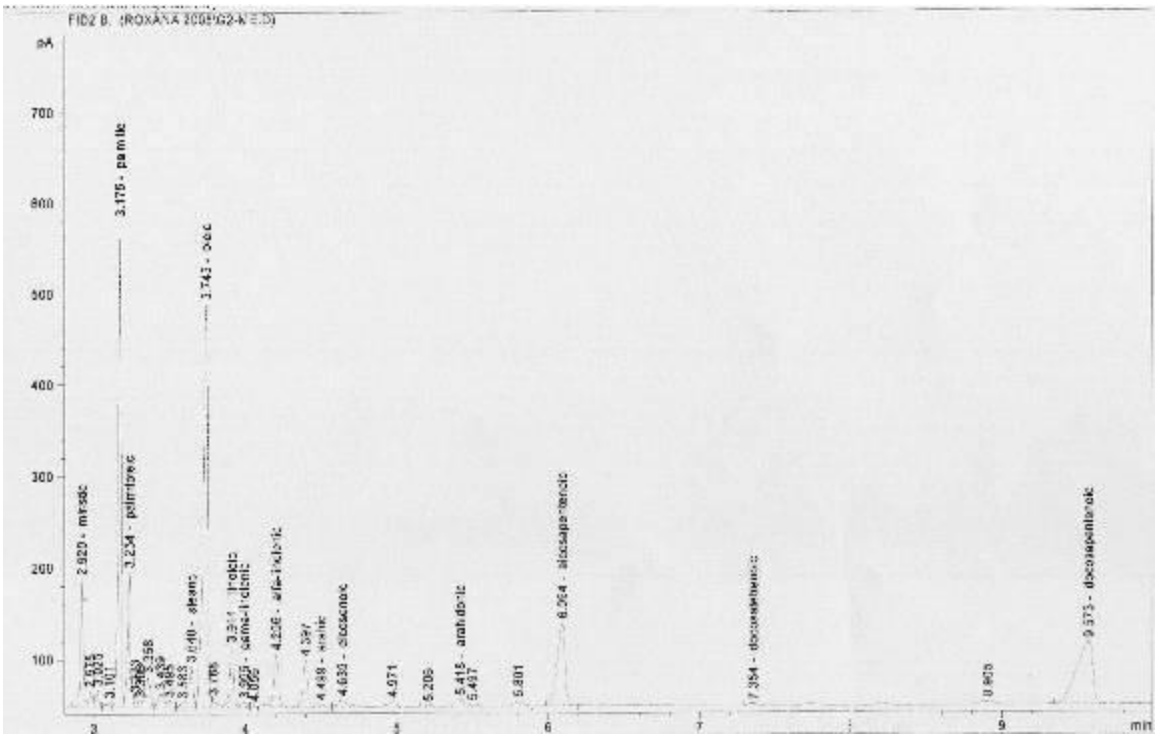

Figure 6. Fatty acids analyzed by GC-FID (sample PUFA)

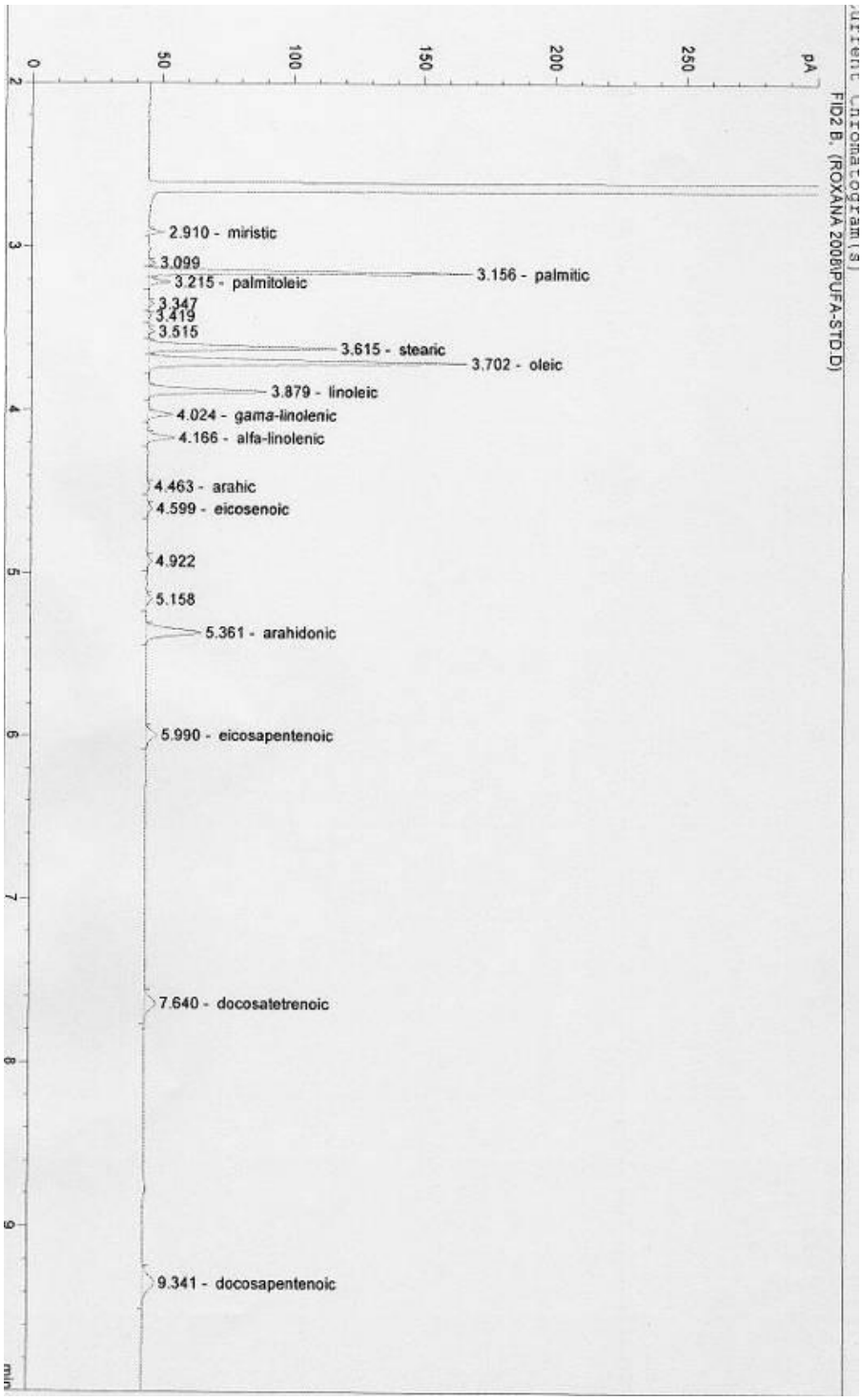

Figure 7. Fatty acids analyzed by GC-FID (Standard PUFA Supelco)

In conclusion the results show that these complexes obtained through different technological variants, are formed of glycosaminoglycans (40-60% sulfated), 3.7 ÷ 12% amino acids, of which 2 ÷ 6.5% essential amino acids (valine, leucine, isoleucine, threonine, methionine, lysine, phenylalanine, tryptophan), 1 ÷ 2% essential fatty acids (linoleic, linolenic and arachidonic acid). There were also identified: glycerophosphates, creatinine, myoinositol, mineral salts (calcium, sodium, potassium, iron, magnesium, selenium, nickel, copper, silicium). The optimal variant of technology was patented.

The quantification of the total antioxidant capacity using the stable free radical DPPH (1, 1-diphenyl-2-picrylhydrazyl) is a frequently used method for liquid and solid samples, because it is simple and rapid. During the last period of time this method was extended to reveal the antioxidant activity of the substances isolated and purified from sources other than vegetal, because it is not specific for a particular antioxidant component but it is applicable to determine the total antioxidant activity [15, 16].

The indirect spectrophotometric method for the evaluation of the total antioxidant activity using DPPH as a system of generating the stable free radical implies the measurement of the absorbance decrease at 517 nm (maximum absorption of DPPH) proportional with the concentration of the reduced free radicals in the solution. The degree of the reducing activity of the radical in the presence of the antioxidant is calculated as % inhibition. The EC50 (the concentration of antioxidant necessary to obtain the 50% of the maximum response) is calculated (Table 2 and Figure 8).

Table 2. Total antioxidant activity

		E4V1		E4V2		E3P1		E4V1R2		GAG	
	µg/ml	Abs.	%Inhibition	Abs.	%Inhibition	Abs.	%Inhibition	Abs.	%Inhibition	Abs.	%Inhibition
Sample 1	**6**	**0.390**	**4.263**	**0.402**	**0.618**	**0.654**	**17.865**	**0.360**	**14.753**	**0.361**	**14.516**
Sample 2	**13**	**0.386**	**5.062**	**0.395**	**2.349**	**0.827**	**-3.880**	**0.356**	**15.641**	**0.289**	**31.565**
Sample3	**20**	**0.374**	**8.023**	**0.387**	**4.425**	**0.821**	**-3.197**	**0.356**	**15.641**	**0.240**	**43.168**
Sample4	**36**	**0.378**	**10.466**	**0.380**	**9.957**	**0.819**	**-2.846**	**0.353**	**16.505**	**0.120**	**71.584**
Sample 5	**119**	**0.334**	**20.921**	**0.352**	**16.682**	**0.822**	**-3.239**	**0.346**	**18.139**	**0.011**	**97.395**
EC 50		**=**	**318**	**=**	**443**	**=**		**=**	**405**	**=**	**37**

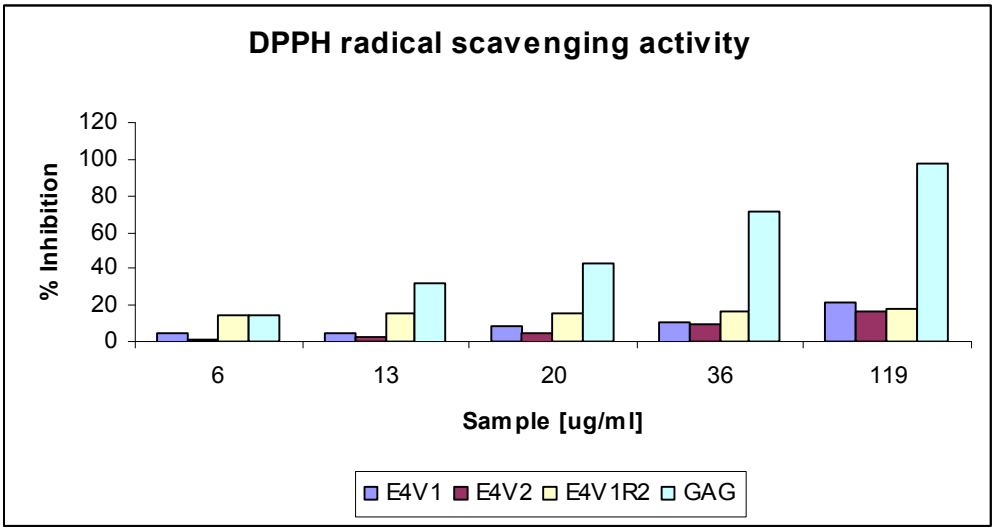

Figure 8. Scavenging capacity of the four extracts on DPPH, determined by measuring absorbance of the reaction mixture at 517 nm, 30 min after addition.

The analysis of the experimental data allows the following conclusions:

- Sample E3P1 – with a concentration higher than 6 µg/mL does not present antioxidant activity.
- As the Vitamin C, known as a powerful antioxidant, has an EC50 = 10 µg/mL, the samples E4V1, E4V2 and E4V1R2 – present a significant antioxidant activity, and the sample GAG (EC50 = 37 µg/mL) has a strong antioxidant activity.

A series of data from the references demonstrate the anti-inflammatory action of GAGs and the chondroprotective activity by modifying the cartilage structure. A two year study, on patients with erosive osteoarthritis of the hands, using radiology methods, it was shown that the oral administration of chondroitin sulfate diminished the erosions in comparison to the patients that were administered only naproxen (RAY SAHELIAN – www.raysahelian.com, [17] 2002). In another clinical study, double blind, on patients with symptomatic osteoarthritis of the knees, the inhibitory effect of chondroitin sulfate on the radiological progression of the reduction of the median femoral-tibial joint space, was revealing the induction capacity of structural modifications in the knee osteoarthritis. The clinical studies demonstrated the efficiency of chondroitin sulfate in diminishing the symptoms of degenerative rheumatic diseases, acting as the unit of building the molecules of proteoglycans. Comparing the chondroitin sulfate (administered per os) with nonsteroid anti-inflammatory drugs (NSAI), there was demonstrated that the effect of the former is installed later, but for a longer time than the latter and is better tolerated at the gastro-intestinal level.

Thus, the present study of obtaining therapeutic bio-compounds from marine organisms, with maximal efficiency and minimal secondary effects, contributes to the development of knowledge in the biotechnology domain and other related sciences; the results may be sustained at national and international level.

The proposed study is situated among the strategic objectives of the development of the sciences, top technologies and applications aiming to link to the priorities and specific objectives of the European area of research. For the application of the specific procedures/technologies of extraction and analytical studies and the *in vitro* tests for the evaluation of the effects of the biological active compounds, high performance analyses methods and techniques will be used, corresponding to the European standards of pharmaceutical research (GLP)

Conclusions

The bioactive complexes extracts from small sea fish (*Engraulis encrassicolus ponticus, Sprattus sprattus sprattus, Odontogadus merlangus euxinus*), which contain glycosaminoglycans, essential amino acids, essential fatty acids, vitamins (myoinositol), microelements (Ca, Na, K, Fe, Mg, Se, Ni, Cu, Si), obtained by a patented technology, have many therapeutically properties especially a strong antioxidant effects which was determined by *in vitro* experiments.

The bioactive complexes can be used as antithrombotics, anticoagulants, anti-atherosclerotics, anti-inflammatories, antioxidants, tissue restorative agents, etc. The bioactive complex will be useful to prevent the unsettlement of the macromolecular structure of essential substances and maintain the functionality of the extracellular matrix from conjunctive, cartilaginous and bone tissue.

The researches continue with *in vitro* and *in vivo* experiments in order to use these bioactive complexes for pharmaceutical preparations and derma-cosmetics.

Acknowledgments

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