NMR and ESR approaches of biophysical properties of wild 'Sockeye' salmon oil

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ABSTRACT

Sockeye salmon oil (SSO) is one of the richest natural sources of Omega 3. This oil also contains high amounts of astaxanthin, a strong antioxidant molecule. In the present work the physico-chemical properties of wild Alaskan Sockeye Salmon Oil were explored by NMR. The antioxidant properties were assessed by ESR in old mice brain (24 month) after 4 weeks feeding 100 μl/mouse, 5 Days/Week with Sockeye Salmon Oil. SSO exhibits interesting physicochemical and antioxidant properties: in aqueous medium, SSO forms unilamellar or multilamellar droplets, and exhibits specific interactions with sphingomyelin (lowering of transition phase) and phosphatidylserine (fluidizing effect) membranes. Preliminary tests performed on aged mice showed a noticeable enhancement in health status (shinier fur, bite marks, weight, explorative activity ...) of chronically SSO fed subjects, while ESR analysis found a decrease in the oxidative stress. The possible link between health status and a decrease of the local oxidative stress in brain upon SSO supplementation is discussed in terms of nutriprevention in Alzheimer disease.   © 2011 Trade Science Inc. - INDIA

INTRODUCTION

The concepts of nutritherapy and nutriprevention have only been relatively recently recognized as of significant interest in the prevention or complementary treatment of some pathologies by eating specific foods or food supplements. However, traditional empirical medicine had early identified potential benefits of fish meat or oil ; for instance, those obtained from Greenland shark have been extensively used in the past for healing of wounds, physical stress tolerance, and also immune stimulation and antitumor properties[1,2]. Such natural products are known to be rich in specific molecules (e.g. alkylglycerols...), mainly omega-3 fatty acids. Omega-3 fatty acid family is one of the precursory and most extensively used structures in these therapeutic concepts. Omega-3 are unsaturated fatty acids which cannot be synthesized by human body. Hence, during the last decades, many basic and clinical research have pointed out the role of omega-3 fatty acids as nutritional factors exhibiting a protective effect on the development of cardiovascular diseases. Omega-3 fatty

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acids are thought to prevent cardiovascular diseases by their role in lowering levels of serum lipid[3], and by their anti-inflammatory, antithrombotic, anti-plateled-derived growth factor and antiarhythmic properties. It was also shown that an increased intake of dietary fish or fish oil rich in long-chain polyunsaturated n-3 fatty acids (PUFAs), eicosapentanoic acid (EPA) and docosahexaenoic acid (DRA), could decrease the incidence of cardiovascular diseases[4]. Omega-3 neuroactive properties have also been evoked, e.g.in the treatment of Alzheimer's disease[5]. Fatty fishes such as salmon or tuna are a well known source omega-3[6] and are the only concentrated source of eicosapentaenoic acid (EPA; 20:5 omega-3) and docosahexaenoic acid (DHA; 22:6 omega-3)[7]. The farmed fish present one of the highest omega 3 level whereas contain also a higher levels of organohalogen pollutants (OHP) which could limit the benefits of consuming fish[8] and lead to focused on wild species.

According to such observations, wild Sockeye salmon oil (SSO) appeared as one of the most interesting natural sources of omega-3. This oil also contains a high amounts of astaxanthin, a strong antioxydant molecule, implicated in UV-light protection, anti-inflammatory, and other properties possibly of interest in the treatment of diverse human health problems, especially immunitary or neurodegenerative process[9]. Other mechanisms of action have also been evoked, such as interactions with natural membranes[10,11] or particular physic-chemical properties[12,13]. However, no precision about the mechanism involved was clearly proposed at the molecular level. This led us to investigate the biophysical properties of Sockeye Salomon oil (SSO), in its commercial form (Neuromer®), by using both NMR and ESR spectroscopies, and to undertake preliminary tests on aged mice chronically submitted to dietary supplemented with SSO.

EXPERIMENTAL

Materials

Oil extract from Sokeye salmon (SSO) was purchased by NUTRILYS® Company (Divonne les bains, France) and used without further purification. As this extract is a natural mixture, the amounts of SSO are better expressed in this paper in mg rather than in mM concentrations (even if the apparent density was esti-

mated at 0.79).

This product was characterized by NMR (see figure 1 for peak assignment) ^{1}H-NMR peak integration and ^{13}C direct spectra and DEPT data, a very coarse estimation of the mean apparent molecular weight M#760 was proposed SSO. The corresponding molar ratios were not considered whereas W/W ratios were used (molecular mass for Dimyristoylphosphatidylcholine, DMPC, is 678).

Chemicals. Dimyristoylphosphatidylcholine (DMPC), Dimyristoylphosphatidy1ethanolamine (DMPE), Dimyristoylphosphatidylserine (DMPS), bovine brain Sphingomyeline (SPM), and deuterated solvents were purchased from Sigma (La Verpillère, France) and were used as received.

Multibilayers (MLV): The liposomes for 31P-NMR experiments were prepared in pure deuterated water by successive freezing and thawing cycles[1] until an homogenous milky sample was obtained[2]. The suspensions were degassed under nitrogen gas then introduced into NMR tubes and sealed. The final lipid concentration was 60 mM (in $500\mu L$ samples), while SSO/DMPC in mixed systems was ranged 10 µL (12.8mg) and 60mM, respectively.

Methods

NMR experiments: All NMR experiments were recorded on a Bruker AM-400 spectrometer. ^{1}H-NMR spectra in D_2O were acquired at 298K using a presaturation of the water resonance and a spectral width of 10 ppm. The chemical shifts were referenced by setting the water resonance at 4.75 ppm. ^{1}H-NMR control spectra were recorded using classical 1D and 2D (COSY, TOCSY[3]) experiments at 300K, 2mg, in perdeuterated di methyl sulfoxide (DMSO-d6).

In ^{1}H-NMR T_1 and T_2 measurements in water preparation (lmg, D_2O, 298K) used standard inversion recovery and cCPMG sequences[4].

^{31}P-NMR experiments were performed at 162MHz. Phosphorus spectra were recorded using a dipolar echo sequence ($\pi/2$-t-π-t)[17] with a t value of 12 µsec and a broadband two levels proton decoupling $\pi/2$ pulse was 4.8µsec, recycling delay of 5sec. Phosphoric acid (85%) was used as extemal reference.

^{13}C-NMR experiments were performed at 100.62 MHz, using a composite proton decoupling, $\pi/3$ pulses

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(4μsec) and 2s recycling delay. 10,000 scans were collected for each spectrum. A spectral width of 250ppm was used, with 64K acquisition points. A line broadening of 3Hz was applied before Fourier transformation.

Mass Spectroscopy: the ES+ control spectra were acquired in CH_2Cl_2 as solvent with 0.1% formic acid, using a VG. Quatro II spectrometer from Micromass/Waters, and treated with the Masslink 4.00 software. The capillary tension was 4.5kV and the cone tension and ion energy 74V and 0.9V, resolution values were set to 14.8, and the multipliers 1 and 2 set to 650V.

RESULTS AND DISCUSSION

Structure and physico chemical properties

SSO structure evaluation in solution and in water samples: NMR experiments.

Chloroformic solution: The ^{1}H-NMR of the 10μL/mL solution of SSO is presented on the top of figure 1 (proton nomenclature is also placed here). Owing to very good solubility of SSO, very resolved lines were detected on such a spectrum (line widths of less than 0.5Hz). The control and attribution of SSO resonances could be easily obtained from standard 1D and 2D ^{1}H-NMR and ES-MS experiments (not shown). Especially, no other hydrophobic components such as phospholipids, sterols and squalene were detected and the resonances of glycerol moiety (around 5.4 and 4.1-4.3ppm) and those of the chain (see the nomenclature on the figure 1) were clearly identified. Homogenous relaxation times (T_1 and T_2 very close and an exceeding 1sec[1,2]) thus allowed the use of peak integrals to propose an estimation of main SSO characteristics in terms of average length and insaturation of the chains by building indexes from ^{1}H-NMR peak integrals as follows (considered that the integral of a given peak is directly proportional to the number of corresponding protons, e.g a methylenic CH_2 resonance gives an integral twice this of a methynic group, or 2/3 that of a methyl group). For each group, the value of the integral was divided by the corresponding number of protons (3, for methyl, 2 for methylen) to allow a count of the number of groups.

As shown on Figure 1 the resonance labeled (4) at 5.2-5.4 ppm is representative of methynic group; however, this resonance is completely overlapped by glycerol signal (G2), leading to use the unambiguous reso-

nance of the methylenic groups (3) neighbouring methynic peaks (4). The resonances (5) were representative of polyunsaturation, and terminal methyle peaks (7) of the number of chains. Also, 2D ^{1}H- NMR experiments easily identified **ω3** related groups, as la-
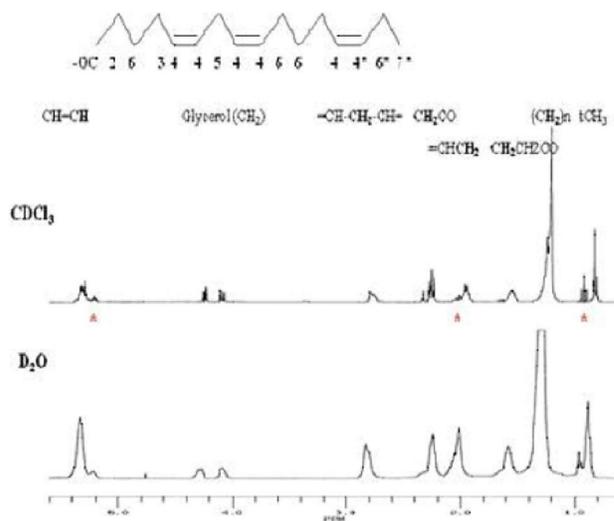
Figure 1. (top) : proton nomenclature (middle trace) : ^{1}H-NMR (400.13MHz) spectrum of SSO (10μL/mL), 298K, in $CDCl_3$. (bottom trace) : ^{1}H-NMR spectrum of SSO (10μL/mL) in D_2O, 298K. The resonances specifies of (ω-3 chains are labeled with a star.

beled on figure 1.

Finally, an estimation of the chain length reference, **A**, was obtained by adding all the weighed resonances 1,2,3,4,5,6 and 7, with subtraction of half the contribution of G1 methylenic group of glycerol (at 4 ppm) to overcome the G2 (CH) contribution at 5.2 ppm. Within the different samples controlled, no variation exceeded 10% from the following values:

Number of groups per chain; A/(7) = 19 +/- 2

Chain insaturation index: (3)/ A ~15% +/-1 %

Chain polyinsaturation index: **(5)/A** = ~9+/-0.5%, indicating a good homogeneity within the different samples used.

Proportion of ω3 (labeled with a star on the spectrum and nomenclature figure 1):

(7*)/[(7)+(7*)]= 22%+/- 2

in good agreement with commercial specifications and MS spectra (not shown). MS spectra confirmed this homogeneity by giving a dominant line centered at m/z=901.94, (in fact a group of lines splitted between 823 and 1013; another line was found at m/z=577 and four

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minor components at m/z= 603, 549, 369 and 285 (577/2). If one considers the major component as possibly related to triglycerids, by subtracting the mass values corresponding to glycerol (89), dividing by 3 (3 chains) and assuming the each building block is (very roughly) mainly composed of methylenic groups (M=14), the latter division gives, for M/Z=901 a number of groups centered on N=19.4, (in fact spread from 18.4 to 22), which is in good agreement with both NMR datas and the supposed composition of SSO, consisting of eicosanoïc acids (and also docosahexanoïc acid). Similar observations would also be performed on the other groups of peaks whereas assuming that only two side chains would be present (e.g. for M/Z=603, N would be approximatively 18.4).

Under these experimental conditions no peak corresponding to astaxanthin (M=596.84) was observed.

Aqueous samples. From the estimated composition, chain length and insaturation, of the hypothesis of a partial solubilization in the water could not be ruled out and was thus tested[3]. Hence, a milky solubilization was obtained up to 15 (V/V), allowing to record ^1H-NMR spectra, as shown on figure 1 (bottom). However, whether the overall lineshape was still recognizable, the typical line widths measured (from 30 to 60Hz) precluded the existence of a true solution, while suggesting the presence of supramolecular assemblies, such as micelles or droplets. This led to measure T_1 and T_2 relaxation times. As mentioned above[14,15], these parameters are closely related to the correlation time τc, and the volume of the system as classically described following the Solomon's relations[16]:

$$1/T_1 = R_1 = B.\ \tau c[(1/1+\omega^2\tau c^2)+4/(1+4\omega^2\tau c^2)] \qquad (1)$$

$$1/T_2 = R_2 = 6B.\ \tau c[4+9/(1+\omega^2\tau c^2)+6/(1+4\omega^2\tau c^2)] \qquad (2)$$

with $\omega = 400$ MHz; $B = \gamma^4.(h/2\pi)^2/r^6$; γ the gyromagnétic factor, $(h/2\pi)$ Planck's constant and r the inter spins distance.

This allowed to calculate the range of correlation time by using the ratio R_1/R_2, as follows

$$R_1/R_2 = [1/1+ \omega^2\tau c^2)+4/(1+4\omega^2\tau c^2)] /$$

$$6.[4+9/(1+\omega^2\tau c^2)+6/(1+4\omega^2\tau c^2)] \qquad (3)$$

Relation in $\omega^2\tau c^2$ simplified in a single second degree equation, finally giving $\omega\tau c$ limits (from 1 to 3).

As the relaxation values clearly differed between groups within the molecule (T_2 from 30 to 60ms ; T_1 from 280 to 700ms), and, by assuming a spherical approximation for the molecular assemblies, the Stockes-Einstein relation allows an coarse approximation of the apparent volume distribution :

$$\tau c = \eta\ V/\ kT \qquad (4)$$

where $\eta = 0.9$ x $10^{-3}P$, (N.s/m² at 298 K), k = 1.38 x 10^{-23} J/kg, T = 297K and V the volume (m³) Finally, the volumes appeared ranged from 500nm³ to 0.5μm³ approximatively (6 to 60nm radius). Such assemblies are of the same size order than model membranes, i.e. small unilamellar vesicles of phospholipids, SUV (typically of 10-20 nm radius, with T_1, in the 450-900 ms range and line widths of 40 to120 Hz)[4].

From these features, the use of such aqueous dispersions appeared suitable to study the collective properties of SSO in biological systems, for which organic solutions cannot easily be used. By considering the great importance of interfacial systems in biology, such as cell surfaces, complementary physico-chemical tests were then performed.

^{13}C-NMR approach of supramolecular organization: Paramagnetic broadening experiments

The figure 2 (A) shows the aliphatic part of the ^{13}C-NMR spectrum of SSO (2mg/mL, D20). As classically described[5], typical resonances of lipid groups are found around l5ppm (terminal methyl group) and in the 20-35ppm region (methylenic groups). The same acquisition conditions were used to obtain the spectrum 2B on the same sample, after addition of 4μL $MnCl_2$ 0.lM in the medium. It is here important to recall some basic properties of paramagnetic ions (i.e. Mn2+)[6]. In the presence of such ions, paramagnetic relaxation of all accessible (geometric vicinity) nuclei is so dramatically increased that the resulting extreme resonance broadening makes it undetectable[7]: this nuclei give no contribution to the spectrum. Conversely, the resonances of the nuclei that are not close to the paramagnetic ion remain unaffected both in relaxation characteristics and in their contribution to the main spectrum. As Mn^{2+} does not cross the membranes[8], in the presence of membranes, all the resonances of a monolayer completely vanish, and similarly only the superficial layer of bi- or multi-layers are affected ; thus the remaining contrition is attributed to the nuclei of the internal layer (s). From this, the intensity of the spectrum presented on the figure 2B directly represents the rela-

tive proportion of the nuclei that are not accessible to Mn^{2+}. One can observe that the main line shape remains unaffected where their intensity is reduced by a factor of 4 by comparison with the spectrum 2A. This feature reveals that, at least, SSO is not only consisting of monolayers or single droplets, and that supramolecular assembly (bi-multilayers) has been formed.

Such a feature is all in the favour of interactions with biological membranes[9]. These interactions are the purpose of the following section, by using the classical phospholipids multibilayer model[10].

Interactions with MLV: ^{31}P-NMR experiments

Phospholipid dispersions (MLV, 60mM) were used to observe the structural and dynamics consequences of the presence of SSO at the polar head (^{31}P-NMR) by recording NMR spectra on the 290-320K temperature range. Polar head group specificity of SSO interaction was also tested by using different phospholipids, i.e. DMPC, Sphingomyeline, DMPE and DMPS.

The insert (figure 3b) shows a spectrum of pure DMPC dispersion (MLV), typical of an axially symmetric powder pattern, with a chemical shift anisotropy of 69 ppm, classical of a phospholipid (here DMPC) bilayers in their liquid crystalline phase around (297K) phase transition[11]. ^{31}P-NMR chemical shift (the resonance frequency) depends on the orientation of phosphorus nuclei in the field (shielding). The chemical shift difference between the low field and the high field edges of a ^{31}P-NMR spectrum is called Chemical Shift Anisotropy (CSA, ppm) and is directly related to the fluidity - reorientation- at the polar head level where the phosphorus nuclei are located. On such spectra a mobile phosphorus group gives a single narrow resonance (several Hz) as detected in time solution or for small structures (micelles), while phosphorus groups in solid state gives extremely broad contributions (more than 100 ppm). Note that membrane fluidity increases (and CSA decreases) with temperature, with a special jump at the transition temperature between gel phase and liquid crystal structure (e.g. around 297K for DMPC[12]). Thus the plot of CSA as a function of temperature provides a good overview of membrane dynamics at the polar head level where phosphorus nuclei are located, while the line shape allows to identify the overall membrane organization (bilayer, hexagonal, isotropic phases). Such plots are presented on the traces of the figure 3. As expected for pure DMPC dispersions a CSA de-
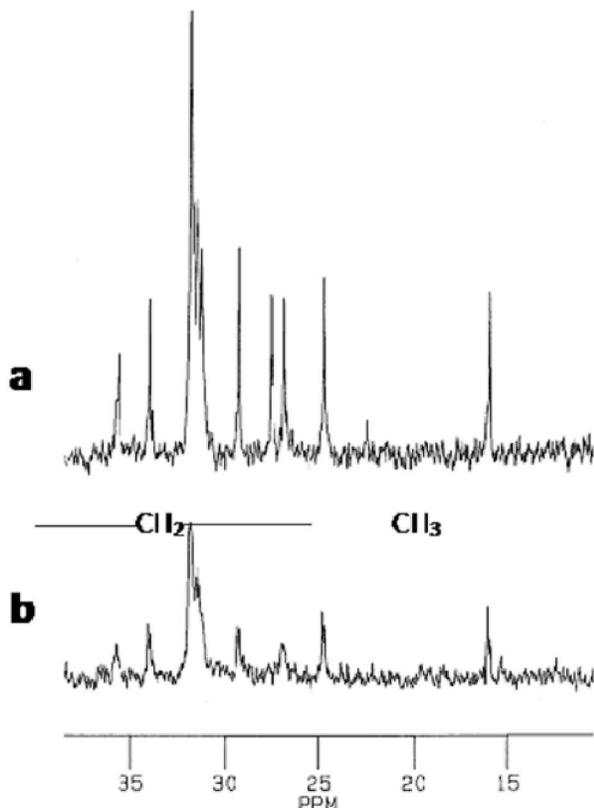
Figure 2 : (a) aliphatic part of ^{13}C-NMR (l00.62MHz) spectrum of SSO, 2mg/mL, D_2O. (b) Same conditions after addition of l0μL $MnCl_2$, 0.lM.
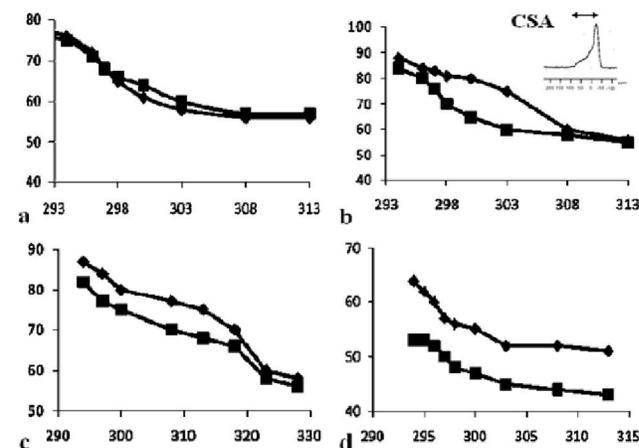
Figure 3 : (Insert): typical ^{31}P-NMR spectrum (162MHz) recorded on multibilayers of phospholipid (here DMPC) in the liquid crystal phase over temperature transition; the chemical shift anisotropy (CSA, in ppm) measured between the lowfield shoulder and the highfield peak is labeled with an arrow. (a-d) : temperature dependence of the CSA measured in pure dispersions of phospholipids (50mM, D_2O, ?) and in the presence of 10μL SSO (¦) on DMPC (a) ; SPM (b) ; PE (c) ; PS (d).

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crease (around 18-20 ppm) was observed on pure DMPC systems by increasing the temperature (and the membrane fluidity) with the transition-related jump around 297K[25]. This was also the case for SSO containing MLV (SSO/DMPC ratios of 10μL/60mM). Besides, no isotropic contribution typical of detergent effect was observed, thus finally indicating the absence of any interaction with the membrane (figure 3A). Pure sphingomyeline (SPM) dispersions exhibit quite similar temperature dependence of the membrane fluidity, with a transition temperature around 304K[13]. SSO containing MLV also present this transition, whereas at significantly lower temperature (298K, figure 3B); however, the CSA measured at high temperature (over 308K) do not differ from those obtained with pure sphingomyeline. It can be proposed that SPM-SSO interactions weaken the collective cohesion forces, thus requiring less energy -lower temperature jump- to obtain the phase transition to the stable fluid liquid crystal at higher temperatures.

Such was not the case for the spectra recorded under the same conditions on DMPE and DMPS systems in the presence of SSO. In both cases (figures 3C-D), a reduction of the CSA was observed on all the temperature do main explored, as a consequence of a global fluidizing effect, more markedly observed in PS (up to 8ppm at 300K and 11 ppm at 290K)[27]. Conversely, the transition temperature of DMPE (around 323K) was not significant1y affected expected for DMPE while the increase in local fluidity was less markedly noted at higher temperatures.

The presence of structural rearrangements are in the cases of DMPE as for DMPS supported by the decrease in CSA at low temperature, with a normal transition temperature (DMPE) and CSA values close to those of pure phospholipidic systems at higher temperatures. Such mechanisms had been evoked in previous works on fish oil such as those extracted from shark liver[14]. However, such a mechanism is not sufficient to explain the discrepancy of the results obtain with the choline bearing lipids, and more, within this class between SPM and DMPC. However, even if their polar head group has in common a phosphorylcholine group, DMPC contains two ester bonds whereas SPM contains an amide bond, an hydroxyle group and a *trans* double bond. These differences result in a change in the net dipole moment and the ability to form hydrogen bonds[23].

Besides, in the hydrophobic region of DMPC, the chains length and saturation is completely determined (i.e. myristic acids, C14), while natural SPM from bovine brain is a mixture of chains differing by their length an insaturation. As pointed out in other works[15], these differences are therefore expected to exert specific effects on structure and dynamics of lipids layers. Whether it is not reasonable to assume a molecular specificity of SSO-SPM interaction, it can be noted that the other models used only showed no interaction of SSO (DMPC) or a specific dynamic modifications (DMPE-DMPS).

DISCUSSION AND CONCLUSIONS

SSO was assumed to exert neuroprotective properties and well-being increase when used as a nutritional supplement to normal diet. Antioxidant properties were proposed as chemical support for such properties. The aim of the present paper was to identify the physico chemical properties of SSO, taken as a whole, and to identify possible mechanisms involved in the general properties of SSO. This was undertaken after NMR control of chain lengths and insaturation. Then, it was found that SSO self organizes in the water: rather than simple droplets, other supramolecular assemblies are also present, such as bi- or multilayers, with a size distribution roughly in the range 6 to 60nm radius. Such an organization is all the most favorable to overcome biological barriers, or simply to integrate the ubiquitous membrane systems, considered here as a target of biological action. This hypothesis is supported by the dynamics effects observed in the presence of synthetic phospholipidic membranes, and specifically those composed of SPM. Even if the biochemical effect would be supported by antioxidant properties, this effect would be present in situ -that is inside the membrane- . This is also consistent with the neurological and general distribution of SPM thus leading to general effects. Moreover the relevance of features observed in this study this study had to be tested in vivo : hence first tests were performed by using (not shown) ESR methods. This method allowed to detect specific POBN/Radical adduct (POBN is a classical spin trapping molecule,

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with a quantitative relation between local Reactive Oxygen Species (ROS) promotion and signal intensity[1]. Under these conditions, a comparison of ROS production between the control group and SSO supplemented groups (100µl/ day of SSO, 5 day a week during 4 weeks), showed a significant decrease (50.5% p<0.05) of in the ROS production (peak intensity) that would be related with a decrease in the oxidative stress.

Note that this reduction in signal intensity was quite similar as that found when vitamin E (the reference lipidic antioxidant molecule) was used following the same protocol at (20 mg per day). Other general health status tests were also performed on aged mices (12 animals, 6 per group) with a diet supplemented for a month with 100µl SSO daily significantly differing in general clinical parameters versus control group (shinier fur, recovery of exploring behavior, weight gain up to 10%, diminution of bite marks possibly related to better healing or decrease in the agressivity ...). Obviously, these preliminary results need to be confirmed and will be completed by long time behavioral tests with large series, including learning and memory abilities. Also, oxidative status will be investigated separately in different organs, and brain membrane dynamic effects of SSO directly studied by ESR experiments in vivo. Another question now to address is the precise contribution of astaxanthin present in SSO, especially when compared to pure vitamin E. Besides a single antioxidant effect, this especially leads to the existence of another mechanism of action of SSO, a biological mixture differing from the pure carotenoid of reference. This lead to investigate the contribution of astaxanthin in size, distribution, stability and membrane interactions of the SSO droplets Further experiments including composition controlled preparations are now running on.

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