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Article

Saponin contents in the starfish *Echinaster sepositus*: Chemical characterization,
qualitative and quantitative distribution

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Abstract

In this study, we report the inter-organ, the sexual and the seasonal variability, of saponins contained in the common Mediterranean starfish *Echinaster (Echinaster) sepositus*. Saponins were extracted from five distinct body components namely the stomach, the pyloric caeca, the gonads, the oral body wall and aboral body wall. Of both sexes (males and females) collected at different seasons, the saponins mixtures were analyzed by Mass spectrometry (HR-ESI-MS and HR-ESI- MS/MS). Semi-quantitative approach was performed to estimate the variability of the saponin amounts. Our results demonstrated that the diversity of saponins in *E. sepositus* is higher than previously reported. We highlighted 11 different saponins, including 9 new congeners. Presumptive molecular structures are proposed for 6 molecules on the basis of key-fragmentations identified by HR-ESI-MS/MS. The comparison of the saponin contained in the five different body components revealed that minimum 3 saponins are common in all tissues. In addition, qualitative and quantitative variability of saponins compounds were linked to the organ, sex and the collecting season. The relative highest level of saponins was found in the stomach on the period of active feeding (winter). The significant higher levels of saponins were found in the gonads and oral body wall on the spawning period (summer). Generally, a great inter-organ, sexual and seasonal variability was found in both sexes. These results suggest that saponins probably fulfill several biological functions in *E. sepositus*.

Keywords

Asteroidea; *E. sepositus*; saponins; electrospray ionization spectrometry; qualitative distribution; quantitative distribution.

1. Introduction

Asteroids (sea stars) are one of the few marine organisms (sea cucumber and sponge) that contain saponin, in their organs. Numerous studies on starfish saponins reported that these compounds possess a wide variety of pharmacological activities (antibacterial, antifungal, antiviral and anti-tumor activities) (Kalinin et al., 1996; Stonik et al., 1999; Kicha et al., 2015; Malyarenko et al., 2017); and biological functions as inter-specific chemical communication and chemical defense (Mackie et al., 1968; Demeyer et al., 2014). These compounds have a large chemical diversity, including, polyhydroxylated steroids, with carbohydrate chain consisting of one to three sugars; steroid oligoglycosides (asterosaponins) with five or six sugars in a carbohydrate chain; cyclic glycosides with a trisaccharide chain, cyclized between C-3 and C-6 of the aglycone moiety, $\Delta^{7,8-3\beta,6\beta}$ -dihydroxysteroid.

Cyclic saponins are discovered in the two species of the genus *Echinaster* (*E. sepositus* and *E. luzonicus*) (De Simone et al., 1981; Riccio et al., 1981; Riccio et al., 1982). Since more than 30 years, these saponins have been used as chemotaxonomic markers of the genus *Echinaster*. Recently, cyclic saponins were isolated from tropical starfish *Leiaster* sp. This finding indicates that cyclic are more widely distributed in starfish than the two species of the genus *Echinaster* (Malyarenko et al., 2016). To date, nine representatives of cyclic saponins have been discovered from the starfish *E. sepositus* (De Simone et al., 1981; Riccio et al., 1981) and *E. luzonicus* (Riccio et al., 1982; Kicha et al., 2015) (Fig.1).

Seven saponins have been identified in *E. sepositus* common sea star living in the Mediterranean, including four cyclic saponins. Sepositoside A is a major cyclic saponin from *E. sepositus*, containing glucuronic acid, glucose and galactose in the glycone part chain.

This trisaccharide chain is attached to the steroid aglycone by a glycosidic bond at C-3 and by ether bond at C-6.

Three minor cyclic saponins were also isolated from *E. sepositus*. They differ from sepositoside A only in the structures of the aglycone moiety, characterized by 22, 23-epoxy functionalities in the side chains (**Fig.1**). The three other compounds are isomers of minor's cyclic ones that lose the ether bond between the C-6 of the aglycone and C-6 of Glc of the trisaccharide chain, leading to appearance of a double bond between the C-7 and C-8 of the aglycone (**Fig.1**) (**De Simone et al., 1981; Riccio et al., 1981**).

De Simone et al. (1981) and Riccio et al. (1981), when investigating *E. sepositus*, they extracted saponins from whole specimens, without comparison between different body components. Indeed, authors have not dealt with saponins in the various body components of *E. sepositus*. Several studies on starfish species other than *E. sepositus* have suggested that each organ would possess its own saponin mixture, and highlighted remarkable qualitatively and quantitatively differences among the saponin mixtures (**Mackie et al., 1977; Voogt and Van Rheenen, 1982; Kisha et al., 2001; Demeyer et al., 2014; Popov et al., 2019**). This indicates that starfish saponins would be involved in chemical defense, digestion and reproduction.

Since 1981, no further investigation of *E. sepositus* saponins has been reported. In this study, we investigated the specific distribution of saponins compounds in the various body components of *E. sepositus* collected on the Algerian coast. Our approach was to determinate the heterogeneity inter-organs, sexual difference and seasonal variation of the saponins distribution, by analyzing separately saponins extracts of five different body components (stomach, pyloric caeca, gonads, oral body wall and aboral body wall) from both sexes (males and females) collected at different seasons (winter, spring, summer and autumn).

Recent studies have demonstrated that mass spectrometry (MS and MS/MS analysis) procedures represent very valuable techniques in respect to fragmentation mechanism and rapid determination of native saponins in extract mixture (**Van Dyck et al., 2009, 2010a, 2010b; Demeyer et al., 2014; Grauso et al., 2019**). Electrospray ionization tandem mass spectrometry

(ESI-MS) has been established as a powerful tool for the profiling of saponin mixtures, which may somewhat avoid the time-consuming steps in the isolation of saponins (**Schoepke et al., 1996**).

In this study, high resolution mass spectrometry experiments (HR-ESI-MS and HR-ESI-MS/MS) were used to detect and analyze saponins. A semi-quantitative study was performed to compare total saponin contents between different organs, sexes and seasons.

2. Materials and methods

2.1. Biological materials

Individuals of *Echinaster (Echinaster) sepositus* (Retzius, 1783) were sampled monthly (September 2017 to September 2018), between 5 and 10 m in depth, on the rocky shore the Algerian coast. The site (36°49'17" N, 3°0'37" E) is located in the Bay of Algiers (Pointe Pescade). The individuals of starfish were separately transported in plastic bags containing sea water. At the laboratory, they were rapidly dissected. Identification of sexes was performed according to **Riesgo et al. (2011)**, based on a color of the gonads being dark red in females and yellow in males. The organs of both sexes (stomach, pyloric caeca, gonads, oral body wall and aboral body wall) were recovered, weighted, freeze-dried, ground and stored in dark waterproof tubes inside a dissector until use.

2.2. Saponins extraction and purification

The saponins were extracted from the different organs of both sex (stomach, pyloric caeca, gonads and bodywall: aboral face and oral face) of the starfish *E. sepositus* by microwave assisted extraction using a multimode microwave oven (NN-S674MF, Samsung, Malaysia). The operating conditions selected for microwave-assisted extraction were: 25 min, 60% methanol, liquid-solid ratio 44mL.g⁻¹, and microwave power 177 W (**Dahmoune et al., 2019**). The extract obtained at the end of microwave irradiation was filtered with a Büchner flask through No. 1 Whatman paper under vacuum. The filtrate was evaporated at low pressure in a

double boiler at 30 °C using a rotary evaporator (RE301 – Stuart). The residues were recovered and dissolved in milli-Q water (aqueous crude extract) to undergo different purification steps. The extracts were purified according to the method reported in the literature (**Van Dyck et al., 2009**). The aqueous crude extract was partitioned by using the liquid-liquid extraction method. Three organic solvents, *n*-hexane (0.09 D), dichloromethane (1.60 D) and chloroform (1.04 D) were conducted respectively to partition crude extract. Saponins were maintained in methanol phase (1,70 D) for each one of these extractions (**Lide, 2009**). In fact, the aqueous crude extracts were dried by using rotary evaporation (RE301 – Stuart, UK). Then dry extracts were solubilized in 20 ml of methanol 90% followed by liquid-liquid partitioning in *n*-hexane (v/v) to eliminate lipids and fatty acid. The methanol fractions were recovered and adjusted with water corresponding to 20% of the methanolic volume. After that, this later was mixed with an equal volume of dichloromethane. After decantation, the dichloromethane fraction was removed and the hydromethanolic phase was adjusted with 40% of water. Then, the liquid-liquid partitioning procedure for a remaining hydromethanolic phase was continued by adding in a separatory funnel a chloroform (v/v) that was stirred and left at rest for three hours minimum. After the sample decantation process, the lower phase was removed and the resulting hydromethanolic upper phase was evaporated under rotary evaporation to evaporate the solvents. Then, the obtained dry extracts were dissolved in 5 ml distilled water, which was transferred to chromatography column packed with Amberlite XAD-4 (Sigma- Aldrich St. Louis, MO, USA). The column was then washed with 100 ml of distilled water to remove any organic salts. The absorbed fraction saponins were eluted with 100 ml of methanol.

Finally, the obtained methanolic fractions were evaporated to remove the methanol solvent; the dry extracts were then dissolved in 5 ml of distilled water. The resulting aqueous solution obtained previously was mixed in a separatory funnel with 5 mL iso-butanol solvent that was blended and left at rest for 2 h. After this period, the upper saponins-rich phase was separated

from lower phases, then, the *iso*-butanol solvent was evaporated in a rotary evaporator. Finally, the dry recovered extract was dissolved in distilled water and lyophilized.

2.3. Mass spectrometry analysis

High resolution mass spectrometry experiments (HR-ESI-MS and HR-ESI-MS/MS) were performed on a Thermo Fisher Scientific Q-Exactive mass spectrometer, in both positive and negative ionization modes by direct infusion using Electrospray ionization source (ESI+/-).

MS and MS/MS experiments were performed at the higher resolution of the instrument (140,000 at m/z 200). Elementary composition of the ions was determined using instrument software (Xcalibur). The saponin extracts were prepared using a concentration of 10-20 $\mu\text{g.mL}^{-1}$ and the flow rate was 5-10 $\mu\text{L.min}^{-1}$.

ESI conditions parameters were as follows: spray voltage 3-4 kV, capillary temperature 300 °C, sheath gas flow rate 10-80, auxiliary gas flow 0-10, SLens RF Level 100.

The single-stage ESI-MS spectra were recorded by scanning 150-2000 m/z mass range.

For the ESI-MS/MS experiments, the interest ions were mass-selected with a window of 0.4 D. The $[M+Na]^+$ of each ion was selected as a precursor and they were submitted to collision energy. Optimized conditions were obtained by decrease of 2/3 intensity of the parent ion to obtain the better MS/MS fragment ions.

The relative intensities are calculated by comparing the peak intensity of each saponin ion to the most intense saponin ion signal (set at 100%).

2.4. Semi-quantitative study

A semi-quantitative approach has been realized in order to obtain data on the total saponin content in *E. sepositus*. The aim of this approach was to quantify the concentration of the whole saponin mixture, for each body compartment of each sex collected in different seasons. The saponins amount was determinate according to the Food Standard Code (**Kwon et al., 2003**). The round bottom flask was dried at 105 °C, cooled in desiccators, and weighed to a constant weight before its freeze-drying. Once the lyophilization was completed, the flasks were

weighed again. The difference in weights corresponds to the total saponin amount of the sample ($\text{mg } T_{\text{sap}}$). The saponins were recovered in the form of white residues.

The total saponins compound yield (TSC) was expressed in $\text{mg}\cdot\text{g}^{-1}$ and calculated from the total saponin amount (T_{sap}) and starfish powder dry, according to the following equation (Eq. (1)):

$$\text{TSC (mg}\cdot\text{g}^{-1}) = \frac{\text{mg } T_{\text{sap}}}{\text{g starfish powder}} \quad (1) .$$

Saponin concentrations have been expressed as means \pm standard deviation (SD). The Data obtained was statistically assessed by ANOVA and Tukey's post hoc test for means discrimination (95% confidence level). The difference in saponins amounts was taken significant and at $p < 0.05$. The Minitab software was used to analyze all the results.

3. Results and discussion

3.1. Qualitative study

3.1.1. HR-ESI-MS analysis

HR-ESI-MS technique was used for direct detection and analysis of saponin mixtures. The major peaks in mass spectra were in accordance in ESI (+) ionization mode with $[\text{M}+\text{Na}]^+$ ions which makes them easier to analyze. The full-scan HR-ESI-MS mass spectrum of the saponins extract obtained from different organs of *E. sepositus* showed intense signals in the m/z 800-1000 range and corresponding potentially to ionized saponins. As a typical example, the ESI mass spectrum of the saponin extract obtained from the oral body wall of female's individuals is presented in (**Fig. 2**), and the accurate mass data are shown in **table 1**. The mass accuracies obtained for all full-scan MS were within 1 ppm (≤ 1 ppm in most cases).

This spectrum displays 6 peaks that could correspond to ionized $[\text{M}+\text{Na}]^+$ saponins. The 6 ions were detected at m/z 891.4354, 907.4298, 921.4455, 935.4612, 939.4564, and 953.3867.

The molecular Formulae of these peaks (Table 1) were obtained by analyzing its high-resolution mass.

The isotopic mass profiles were utilized to deduce the molecules weights (M.Ws) of peaks. For instance, the 1Da mass difference and the descending intensities for ions peaks (921.4451,

922.4488, 923.4517) (**Fig.S1**) agree well with the properties of the isotopic ions. Consequently, m/z 921.4451 with a higher intensity was preliminarily determined as the real pseudo-molecular ion of this peak.

The relative intensities of the peaks were different, suggesting that saponins are present in different proportions in the organ (**Table 1**). The most intense signal is detected at m/z 921.4455 as a sodium cation adduct and seems to be the most abundant saponins in the oral body wall. The less intense signals are detected at m/z 907.4298 and 939.4564.

The saponin detected at m/z 921.4455 and 907.4298, and marked by a black dot on the HR-ESI-MS spectrum are likely to correspond to the compositions already identified in *E. sepositus* by **Minale et al. (1978)**, **Minale et al. (1979)**, **Desimone et al. (1981)** and **Riccio et al. (1981)**. The m/z 921.4455 $[C_{45}H_{70}O_{18} Na]^+$ may correspond to the sepositoside A, the major saponin of *E. sepositus*, while the mass m/z 907.4298 $[C_{44}H_{68}O_{18} Na]^+$ could correspond to the minor saponin of *E. sepositus* already described by **Riccio et al. (1981)**, which provisionally named sepositoside B (**Fig. 1**). It should be noted that the two molecules sepositoside A and B are different only at the level of the lateral chain of the aglycone (**Fig. 1**).

The ions marked by gray could be related to new saponins. For example, the signal m/z 891.4354 $[C_{44}H_{68}O_{17} Na]^+$ could be considered as a saponin never observed for *E. sepositus*. This saponin possesses the same mass (m/z) and raw formula as the luzonicoside A saponin isolated from *E. Luzonicus* (**Riccio et al., 1982; Kicha et al., 2015**) (**Fig. 1**).

3.1.1.1. Distribution of saponins ions in body components of the starfish *E. sepositus*

The starfish saponins are not distributed uniformly among body components. Each organ would possess its own saponin mixture (**Demeyer et al., 2014; popov et al., 2019**).

In addition to the saponins detected in oral body wall at m/z 891.4354, 907.4298, 921.4455, 935.4612, 939.4564, and 953.3828, three other ones were obtained from the pyloric caeca,

gonads and aboral body at m/z 909.4455, 919.4298 and 937.4397. **Tables 1, 2 and table S1** in supplementary data features the detailed distribution of these saponins ions in the different organs of different animals, i.e. females vs males, collected at different moments (winter, spring, summer and autumn). It appears that saponins ions detected at m/z 921.4455, 891.4354, and 907.4298 are common to all tissues in both sexes at different seasons. Although these congeners are found in all organs, some saponins are restricted to one or two organs. For instance, the m/z 935.4612 and 953.3828 saponins ions are associated to the saponins composition of gonads and oral body wall.

Although these saponins ions were detected at the same m/z ratios, the relative intensities in the different spectra showed some difference between organs sex and season.

Fig. 3 describes the distribution and the relative intensity of the saponin ions (m/z) of the five selected organs for males and females collected at different seasons.

Considering inter-organs variability, the relative intensity of the saponin ions (m/z) indicated a significant difference among the five studied organs, except the saponins ions m/z 921.4455, which is the most represented saponin ions in all organs. These results confirm the inter-organ heterogeneity of the saponin contents in *E. sepositus*.

When comparing the composition and the relative abundance of the saponins ions of each body compartment between male and female individuals, we observed that the only organs in which there is important sexual variation are the gonads collected in winter and autumn. In fact, saponin ions that detected at m/z 919.4298 were restricted to female gonads saponins, while that detected at m/z 939.4564 characterized the male gonads. In addition, the relative abundance of saponin associated to the m/z 891.4354 and 953.3828 appear different in female and male gonads.

Considering seasonal variations in both sexes, the composition and the proportion of the saponins ions of stomach and oral body wall in both sexes do not show real variation. While,

the pyloric caeca, the gonads and the aboral body wall have two homogeneous compositions, the first one is associated to winter and autumn, the second one is associated to spring and summer (**Tab. 2**).

The peak of the highest intensity in all organs was always detected at m/z 921.4354 and closely followed by the saponin ions at m/z 891.4354 (**Fig.3**). Accordingly, the saponin ions detected at m/z 921.4354, 891.4354, were the most abundant molecules whatever the body component considered. These m/z 891.4354 ions were more intense in the stomach and pyloric caeca extracts than in the other body compartments.

De Simone et al. (1981) and Riccio et al. (1981) who studied saponins from *E. sepositus* described 7 saponins for this species (**Fig.1**) without considering the differences among body components. The reported saponins were: the major sepositoside A ($[M+Na^+]$: m/z 921) as the major one with its four isomers, and the sepositoside B of *E. sepositus* ($[M+Na^+]$: m/z 907) and its own isomer. Considering only the m/z value of the ions detected in the first-order ESI mass spectrum (**Fig. 2**), it appears that the saponins reported by **De Simone et al. (1981) and Riccio et al. (1981)** were also observed in our saponins extract at m/z 921.4354 and 907.4298. The saponin molecule associated with the m/z 891.4354 ions is identified as luzonicoside in the starfish of the same genus *E. luzonicus* (**Riccio et al., 1982; Kicha et al., 2015**).

De Simone et al. (1981); Riccio et al. (1981); Riccio et al. (1982) and Kicha et al. (2015) described the molecular structures of sepositoside and luzonicoside. These studies were taken as a starting point to identify saponins in the present work. To validate that signals detected in the full- ESI mass spectrum really correspond to saponins ions, HR-ESI- MS/ MS analyses were undertaken for each m/z ratio.

3.1.2. HR-ESI-MS/MS analysis and identification of new saponins

Exact structures cannot be deduced from mass spectrometry data only. Detailed fragmentation analysis with accurate mass measurement allowed for characterizing and to proposing tentative structures for the detected compounds.

On the basis of HR-ESI-MS analyses we observe 9 new saponin congeners that were until now not reported in the literature for *E. sepositus*. The corresponding saponins are detected at following ions signals m/z 891.4354, 907.4298, 909.4455, 919.4298, 935.4612, 937.4397, 939.4564 including two isomers at 907.4298 and 919.4298 m/z ration.

Based on MS/MS analysis, fragmentation patterns were built according to the collision-induced dissociation (CID) mass spectrum of each parent saponins ions. Saponin structures were elucidated or proposed corroborating the following literature (**Minale et al., 1978; Minale et al., 1979; De Simone et al., 1980 De Simone et al., 1981; Riccio et al., 1981; Riccio et al., 1982 and Kicha et al., 2015**). When identification was not possible, only molecular masses are reported as a support for further investigations (**Tab. 2**). The global results, i.e. merging all the animals, are summarized in **Table 2** and **Fig. 6**.

Before starting to analyze the MS/MS, and based on the potential structure of the m/z 921 ions, i.e. sepositoside A and the m/z 981 ions, i.e. luzonicoside A reported in the literature (**Fig. 1**), it is important to remind some dissociation rules that will be relevant to the present discussion. The negative charge is localized on the glucuronic acid group attached on the sugar moiety. Therefore, all the expected collision-induced dissociation (CID) processes must provide fragment ions containing the glycone moiety or part of the glycone moiety. The sugar sequence in the oligosaccharide chains can be defined by following the successive losses of the monosaccharide residues from the mass-selected parent ions. Characteristic losses of 176 Da, 162 and 132 Da can be associated to the presence of glucuronic acid, Galactose or Glucose and arabinose residues, respectively. Consequently, characteristic fragment ions from consecutive loss of sugar groups can provide information for the distinction of different aglycone.

The ions at m/z 921.4455 and m/z 891.4354 were selected to HR-ESI- MS/MS analysis, in order to reconstruct the collision-induced fragmentation pattern of the parent confirming that ions detected at m/z 921.4455 and m/z 891.4354 correspond to the sepositoside A and luzonicoside A, respectively (**Fig. 4**).

The HR-ESI-MS/MS mass spectrum for the ion detected at m/z 921.4455 in the oral body show two competitive dissociation pathways. First, black lines in **Fig.4 (A)** and **Fig.S2**, demonstrate consecutive losses of glucuronic acid, galactose residues and the aglycone part producing ions detected at m/z 745.4136, 583.3608, and 185.0423, respectively. This sequence of decomposition confirms the proposed sepositoide A (structure **2** in **Fig.6**).

In the second fragmentation pathways, represented by a dotted black line (**Fig.4 (A)**), the fragmentation of the parent ions is initiated by the loss of the aglycone moiety, as well as the occurrence of formal successive eliminations of the monosaccharide residues (glucuronic acid and galactose). Ultimately, the fragmentation process leads to signals detected at m/z 347.0951 and 185.0432 and unambiguously reveals the presence of sepositoside A ions (**Fig. S2**).

The MS/MS spectrum of m/z 891.4354 detected upon ESI ionization of the oral body wall extract shows the co-occurrence of two fragmentation pattern as m/z 921.4455 ions had did. The first fragmentation pathway represented by black lines, where the consecutive losses of glucuronic acid, arabinose and the aglycone part afford ions detected at m/z 715.4034, 583.3610 and 185.0432, respectively. This sequence of fragmentation is exemplified in **Fig. S3**. The second fragmentation pathways represented by a dotted black line, gave rise to fragment ion peak m/z 511.1276 resulting from the loss of aglycone and corresponding to the tri-saccharide chain m/z 511.1276 ion $[C_6H_8O_6 + C_5H_8O_4 + C_6H_{12}O_6Na]^+$. After dehydration this chain loses a glucuronic acid $[511.1276 - H_2O - C_6H_8O_6]^+$ to form the di-saccharide chain at m/z 317.0847 $[C_{11}H_{18}O_9 Na]$. The elimination of arabinose (-132 Da) from the di-saccharide ultimately leads to the glucose at m/z 185.0423 $[C_6H_{10}O_5 + Na]^+$. The complete dissociation pathway is

described in **Fig. S3**. Indeed, these two fragmentations gave signals consistent with those obtained by analyzing the luzonicoside of *E. luzonicus* by ESI-MS/MS in positive mode (**Kicha et al., 2015**).

This result suggests that the CID spectra of the m/z 891.4353 are in agreement with the presence of ions corresponding to luzonicoside.

The collision-induced dissociation (CID) mass spectrum of the m/z 921.4458 and 891.4353 ions allows us to identify the key signals of two types of glycosidic chains. The m/z 541.1379, m/z 523.1274 [541-18] and m/z 374.0951 peaks would correspond to the signature of the GlcA-Gal-Glc ([GlcA-Gal-Glc+Na]⁺) oligosaccharide chain, and the m/z 511.1276, m/z 493.1168 [511-18] and m/z 317.0847 peaks as a signature of the GlcA-Ara-Glc ([GlcA-Ara-Glc+Na]⁺) oligosaccharide chain.

The fragmentation sequence exemplified in **Fig.4, Fig.S2 and Fig.S3** established the molecular formula C₂₇H₄₀O of the aglycone part for m/z 921 and 891, which confirm that the fragment ions originate from sodium-cationized sepositoside and luzonicoside.

The collision-induced dissociation (CID) mass spectrum of the m/z 907.4306 ions detected in the oral body wall is presented in **supplementary data Fig. S4 and Fig.5**. It shows the co-occurrence of three fragmentation patterns (**Fig.5**): two fragmentation routes represented by black lines (solid and dotted lines (**A**)) can be associated to sepositoside B of *E. sepositus* (**structure 1 in Fig.6**), whereas the third fragmentation pathways, identified by the gray lines (dotted lines (**B**)), are characteristic of luzonicoside F ions (**structure 7 in Fig.6**).

As far as the solid black line, the mass differences between the fragment ions are attributable to the losses of glucuronic acid, galactose and aglycone moiety ultimately leading to the m/z 185.0423 ions. When following the dotted black line in the scheme, i.e. m/z 907.4306 \rightarrow m/z 541.1381 \rightarrow m/z 523.1275 \rightarrow m/z 347.0953 \rightarrow m/z 185.0423, it is interesting to note that the ultimate mass transition corresponds to m/z 523.1275 and m/z 347.0953. This can

unambiguously reveal the presence of GlcA-Gla-Glc oligosaccharide chain and confirm that the fragment ions are associated to sepositoside B of *E. Sepositus*. The complete dissociation pathway is described in **Fig.5 (A)**.

Figure 5 B is characterized by the key signals of AGlc -Ara-Glc oligosaccharide chain (m/z 493.1168 and m/z 317.0847 respectively). The detection of this sequence of fragmentations allows the identification of the m/z 907.4306 as luzonicoside F already identified in *E. lozonicus* (**Kicha et al., 2015**).

This result indicates that isomeric saponins can be highlighted by running ESI-MS/MS analyses, which provided key-fragmentations for all the isomeric saponin ions that have been previously identified.

Similar mass spectrometry analyses were performed for all the ions detected upon ESI ionization of the oral body wall saponins (m/z 935.4612, 939.4564 and 953.3828). On the basis of the recorded MS/MS spectra, three new saponins provisionally named saponin A, C and D detected at m/z 935.4612, 939.4567 and 953.3828 respectively, were identified (**Fig. 6**). These saponins are straightforward characterization since their CID spectra share the common m/z 523.1274 and 374.0951 key signals as a signature of the oligosaccharide chain GlcA-Gal-Glc. Consequently, all those congeners differ by their aglycone structure.

Based on molecule weight obtained from ESI-MS/MS analysis and the literature survey that highlight different saponins in *Echinaster* genus (**Minale et al., 1978; Minale et al., 1979; De Simone et al., 1981; Riccio et al., 1981; Riccio et al., 1982; Kicha et al., 2015**), the aglycone structures were proposed and saponins structures were tentatively elucidated (**Fig.6**).

The molecular mass difference of 32 Da between m/z 907.4306 and 939.4564 (also between m/z 921.4354 and 953.3828) suggested the presence of an additional hydroxy group and one epoxy group in the steroid nucleus of compounds (**5**) and (**6**) compared to compounds (**1**) and (**2**), respectively (**Fig.6**).

Saponins ions observed at m/z 921.4354 and 935.4612 are separated by 14Da, which agreed with the presence of an additional methylene group added in the side chain of the aglycone of compounds **(3)** in comparison with compound **(2)** (**Fig.6**).

The mass spectrometric analyses were performed for all the organs saponin ions extracts (stomach, pyloric caeca, gonads and tegument aboral).

For each analysis, fragmentation patterns were built and saponin structures were elucidated or proposed based on their elemental compositions, on their CID fragmentations and on common building parts of known saponins (**Fig.1**). Interestingly, the spectra of saponins at m/z 907.4298, 891.4349, 921.4453, 935.4607, 939.4558 and m/z 953.4607 obtained from other organs was found to be similar to the oral body wall saponin ion spectrum. Consequently, all the product ions identified in the oral body wall MS/MS spectrum were also detected in the corresponding MS/MS spectrum of the saponins ions obtained from other organs (**Table. 2**).

MS/MS analysis has been carried out for the m/z 909.4455, 919.4298, 939.4558 signals. The results of the whole analysis are summarized in **Table 2**.

The CID spectra of the m/z 919.4292 ions are presented in **Fig. S5** and are clearly in agreement with the presence of two isomers. Among the m/z 919.4292 parent ions, at least two isomeric ions are present which, in their dissociation products, possess the m/z 523.1267 and 374.0948 product ions (in accordance with GlcA-Gal-Glc oligosaccharide chain) for the first isomer (**Fig.S4**); and m/z 493.1161, 317.0847 (characteristic of the GlcA-Ara-Glc oligosaccharide chain) for the second one. Consequently, the two molecules are differing at the level of the glycone and aglycone chains.

Similar fragmentation patterns were obtained for m/z 937.4167 observed in the stomach, the pyloric caeca and the aboral body wall, presenting all the common m/z 523.1267 and m/z 374.0948 keys as a signature of the GlcA-Gal-Glc oligosaccharide chain. This reveals that the carbohydrate chain of these saponins is structurally related to that of sepositoside A (**compound**

(2), **Fig.6**). Hence, the structure of this saponin was proposed as **compound (8) (Fig.6)**, and its aglycone differs from that of **compound (2)** only in the structure of ring B with an additional oxygen atom (16 Da).

We noted that the MS/MS spectrums, obtained for the gonads and pyloric caeca, m/z 919.4299 saponin ions were similar as well as the MS/MS spectra of m/z 909.4455 ions saponins of stomachs and pyloric caeca.

3.2. Quantitative comparison

The mean concentration of saponins in different organs is presented in (**Fig.7**) and **Table 3**. Quantitative differences of saponins in the organism as a whole and each of its body parts were highlighted depending on sex individual and season.

3.2.1. Inter-organs variability

An examination of the organ distribution of saponins in *E. sepositus* (**Fig.7**) showed that these substances were present in all the organs examined, and each organ its own saponins amount, this may have some general functional significance in the development cycle.

The content saponins was found to be statistically different between the various organs by ANOVA and Tukey's post hoc test (**Table. 3**). Total saponins concentrations in the corresponding organ were increasingly or decreasingly changed as a function of developmental stage of *E. sepositus*.

In early development stage, in autumn, the maximal content of the saponins was observed in the stomach when compared with other body components of *E. sepositus*. The smallest total amount of saponins in this period was recorded in the gonads.

In advanced development stage, in winter, the amount saponins in different organs of female individuals varied in the following order: Gonads > Stomach = aboral body wall > pyloric Caecab > oral body wall. Whereas the variability inter-organs in male individuals of saponins

amount show the following order: stomach = aboral body wal >pyloric caeca > oral body wal>gonads.

In starfish the stomach saponins are implicated in the solubilization of cholesterol accelerating its absorption (Mackie et al., 1977); This explains the higher saponin levels in stomach than in other body components during the early stage of growth (autumn and winter). In addition, it is known that starfish have the so-called “external” nutrition, they evert the stomach from the organism’s body and engulf the food, and then it can be assumed that saponins protect the stomach against predators at the nutrition time, and immobilize or kill living creatures and pathogenic microorganisms in food (Minale et al., 1993; Ivanchina et al., 2017).

In maturation stage, in spring, the largest quantities of saponin were found in the gonads and in the oral and aboral body wall. The presence of high levels in body wall is consistent with a role of saponins as a chemical defense system against predators, parasites and microorganisms.

In summer, the spawning season in starfish *E. sepositus*, the maximum saponin content was in the gonads for both sexes, indicating that saponins may have a role in reproduction. Naruse et al. (2010) reported that saponins of *Asteria. amurensis* are cofactor for acrosome reaction during fertilization in *A.amurensis*. According to our results, the amount of saponins in the gonads showed a strong seasonal variation, which hypothesizes the intervention of these molecules in the reproduction of *E. sepositus*.

3.2.2. Sex and seasonal variability

The data relative to the sex and seasonal variability of TSC in *E. sepositus* (Fig.7, table. S2 and table S3; respectively) highlighted the following results:

- Among body components, The maximal content of saponins was observed in the female gonads (19.4 ± 0.5 mg/g), and the minimal contents was observed in male gonads (09.1 ± 0.1 mg/g);

- The significantly different rates of gonads saponins observed in autumn and winter between male and female, created substantial differences in the total saponins compounds (TSC) in whole male and female organism (**Fig.7**);
- Stomach saponins had important amount all over the year indicating that this species is actively feeding throughout the life cycle;
- The seasonal variation trend of saponins amounts in gonads of both sexes is in agreement with their maturation stage. According to seasonal variation of gonadosomatic index (GSI) (**Fig. S5**) we noted a variation in the timing of gonad maturation between female and male individuals. The female gonads maturation of *E. sepositus* is precocious; begin in autumn and finishing in early summer with only one spawning event in late summer. According to **riesgo et al., 2012** oogenesis was a continuous process in *E. sepositus*, with only one spawning event in August–September. These data justify the presence of important amount of saponins in the female gonads throughout the maturation. In male individuals, the GSI values indicate that spermatogenesis was restricted to 5–6 months in spring-summer. This result concord with the result obtained by **Riesgo et al., 2012** and can explain the low gonads content of saponins observed in male individuals in early development stage (autumn and spring);
- The seasonal variation of saponins amount in whole body, gonads and oral body wall of both sexes was significant, with higher rates of saponins in spawning season over the others.

Conclusion

HR-ESI-MS and HR-ESI-MS/MS were applied to study the diversity and body distribution of saponins in *E. sepositus*. This technique was used to monitor the parent saponins and their mass fragmentation patterns in the MS and MS² experiments.

The comparison has been made in respect to body, sex and seasons components. Our results reported the presence of twelve different saponin congeners, which highlights the diversity of saponins identified in a Mediterranean starfish *E. sepositus*. With the analytical techniques used, only 8 structures have been elucidated. The distribution of these saponins in stomach, pyloric caeca, gonads, oral body wall and aboral body wall of the starfish *E. sepositus* showed qualitative and quantitative differences based on MS analysis and semi-quantitative study. The saponin mixtures in each organ were rich and complex (5 to 7 different saponins) with usually one or two saponins differing from one body compartment to the other and from sex's individuals to others. Sepositoside A and luzonicoside A were the most abundant saponins in the five studied organs.

The obtained data indicate high significant inter-organs variability of saponin amounts in both sexes at different seasons. It is likely to conclude that the level of saponins in each organ seems to depend on the season in accordance with the stage of development.

High content of gonads saponin contributed to the maximal saponin content of whole body in summer (spawning season).

Saponins congeners were located mainly in gonads and oral body during spawning period, but were located at early developmental stage in stomach when starfish are actively feeding. The presence of saponins in the reproductive and digestive organs in such manner suggests that these compounds are involved in the reproduction and digestion of food.

At this point of the work, HR-ESI-MS and HR-ESI-MS/MS analyses seems to be efficient for a direct screening of saponin distribution within the organ extracts of *E. sepositus*. The identification of key-fragmentations can be very useful to highlighted isomeric saponins by running HR-ESI-MS/MS analyses. However, an additional characterization step based on a chromatographic separation prior to the mass spectrometry analyses is required to distinct isomeric saponins.

In the future, chromatography methodologies will be used to separate and purify different saponins from these saponin extracts in order to clarify the biological roles of each saponin in *E. sepositus*.

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Declaration of competing interest

The authors declare no conflict of interest.

Author contributions

Fouzia Houma Bachari, Didier Hauchard, Mohamed Chibane and Vincent Ferrières designed the study. Bouchra Dahmoune, Jean-Paul Guégan, and Philippe Jéhan performed the experiments. Farid Dahmoune and Lotfi Mouni participated in study design, data collection and analysis. Bouchra Dahmoune and Farid Dahmoune wrote the manuscript. Cherifa Akrou Aissou with all the authors revised the manuscript.

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Table1

HR-ESI-MS data of saponin extract obtained from the oral body wall of female's individuals of *E. sepositus*.

Saponins	compositions	Theoretical mass (<i>m/z</i>)	Measured mass (<i>m/z</i>)	Mass error (ppm)	Relative intensity (%)
Luzonicoside A	C ₄₄ H ₆₈ O ₁₇ Na	891.4349	891.4354	1	19
Sepositoside B	C ₄₄ H ₆₈ O ₁₈ Na	907.4298	907.7298	0	7
Luzonicoside F	C ₄₅ H ₇₀ O ₁₈ Na	921.4454	921.4455	0	100
Saponin A	C ₄₆ H ₇₂ O ₁₈ Na	935.4610	935.4621	1	10
Saponin C	C ₄₆ H ₇₂ O ₁₉ Na	939.456	939.4568	1	8
Saponin D	C ₄₅ H ₇₀ O ₂₀ Na	953.4352	953.4351	0	15

Table2

Saponin congeners detected in the different organs of *E. sepositus* (STO: stomach; CAE: pyloric caeca; GON: gonads; OBW: oral body wall; ABW: aboral body wall) from different sexes (♂: males individuals; ♀: females individuals) collected at different seasons (a: all seasons; Wn: winter; Sp: spring; Sm: summer; At: autumn).(S: molecular structure; MW: molecular weight).

Saponins	MW (Da)	[M+Na] ⁺	composition	S	CID Key signals				Body distribution				
					523	374	493	317	STO	CAE	GON	OBW	ABW
Luzonicoside A	868	891	C ₄₄ H ₆₈ O ₁₇ Na	4			X	X	♀♂, a	♀♂, a	♀♂, a	♀♂, a	♀♂, a
Sepositoside B	884	907	C ₄₄ H ₆₈ O ₁₈ Na	1	X	X			♀♂, a	♀♂, a	♀♂, a	♀♂, a	♀♂, a
Luzonicoside F				7			X	X	♀♂, a	♀♂, a	♀♂, a	♀♂, a	♀♂, a
Unidentified	886	909	C ₄₄ H ₇₀ O ₁₈ Na						♀♂, a	♀♂, Sp,Sm			
Unidentified	896	919	C ₄₅ H ₆₈ O ₁₈ Na		X	X				♀♂, Wn,Au	♀, Wn, Au ♀♂, Sp, Sm		♀♂, Sp, Sm
Unidentified							X	X		♀♂, Wn,Au	♀, Wn, Au ♀♂, Sp, Sm		♀♂, Sp, Sm
Sepositoside A	898	921	C ₄₅ H ₇₀ O ₁₈ Na	2	X	X			♀♂, a	♀♂, a	♀♂, a	♀♂, a	♀♂, a
Saponin A	912	935	C ₄₆ H ₇₂ O ₁₈ Na	3	X	X					♀♂, a	♀♂, a	
Saponin B	914	937	C ₄₅ H ₇₀ O ₁₉ Na	5	X	X			♀♂, a	♀♂, a			♀♂, a
Saponin C	916	939	C ₄₄ H ₆₈ O ₂₀ Na	6	X	X			♀♂, a	♀♂, a	♂, Wn, Au	♀♂, a	♀♂, a
Saponin D	930	953	C ₄₅ H ₇₀ O ₂₀ Na	8	X	X					♀♂, a	♀♂, a	

Table 3

ANOVA analysis of total saponins amounts ($\text{mg}\cdot\text{g}^{-1}$, dry weight) in the different organs of *E. sepositus* as a function of sex in different seasons .Wsp: Whole specimens; STO: stomach; CAE: pyloric caeca; GON: gonads; OBW: oral body wall; ABW: aboral body wall.

For each season and each sex same letter in the same column refer to means considerate not statistically different (between the different organs) according to ANOVA and Tukey's test.

	Winter		Spring		Summer		Autumn	
	♀	♂	♀	♂	♀	♂	♀	♂
Wsp	46.6±0.7	36.8±0.1	58.8±0.8	58.0±0.5	61.7±1.4	60.6±0.3	32.3±0.3	24.6±0.9
STO	10.3±0.3 ^B	9.4±0.2 ^A	9.8±0.3 ^B	9.1±0.4 ^C	8.5±0.3 ^D	8.2±0.1 ^D	9.2±0.1 ^A	8.6±0.4 ^A
CAE	7.6±0.5 ^C	7.1±0.1 ^B	7.7±0.4 ^C	7.1±0.2 ^D	7.3±0.3 ^E	7.1±0.2 ^E	6.6±0.5 ^B	5.6±0.5 ^c
GON	11.5±0.4 ^A	3.2±0.2 ^D	17.4±0.5 ^A	16.3±0.2 ^A	19.4±0.5 ^A	19.1±0.5 ^A	5.2±0.2 ^C	0.9±0.1 ^E
OBW	5.4±0.4 ^D	5.0±0.3 ^C	11.3±0.4 ^B	11.3±0.3 ^B	14.1±0.1 ^B	14.3±0.1 ^B	2.7±0.5 ^D	1.8±0.3 ^D
ABW	9.7±0.3 ^B	9.1±0.3 ^A	11.7±0.3 ^B	11.1±0.3 ^B	10.3±0.1 ^C	10.0±0.2 ^C	6.8±0.5 ^B	6.8±0.3 ^B

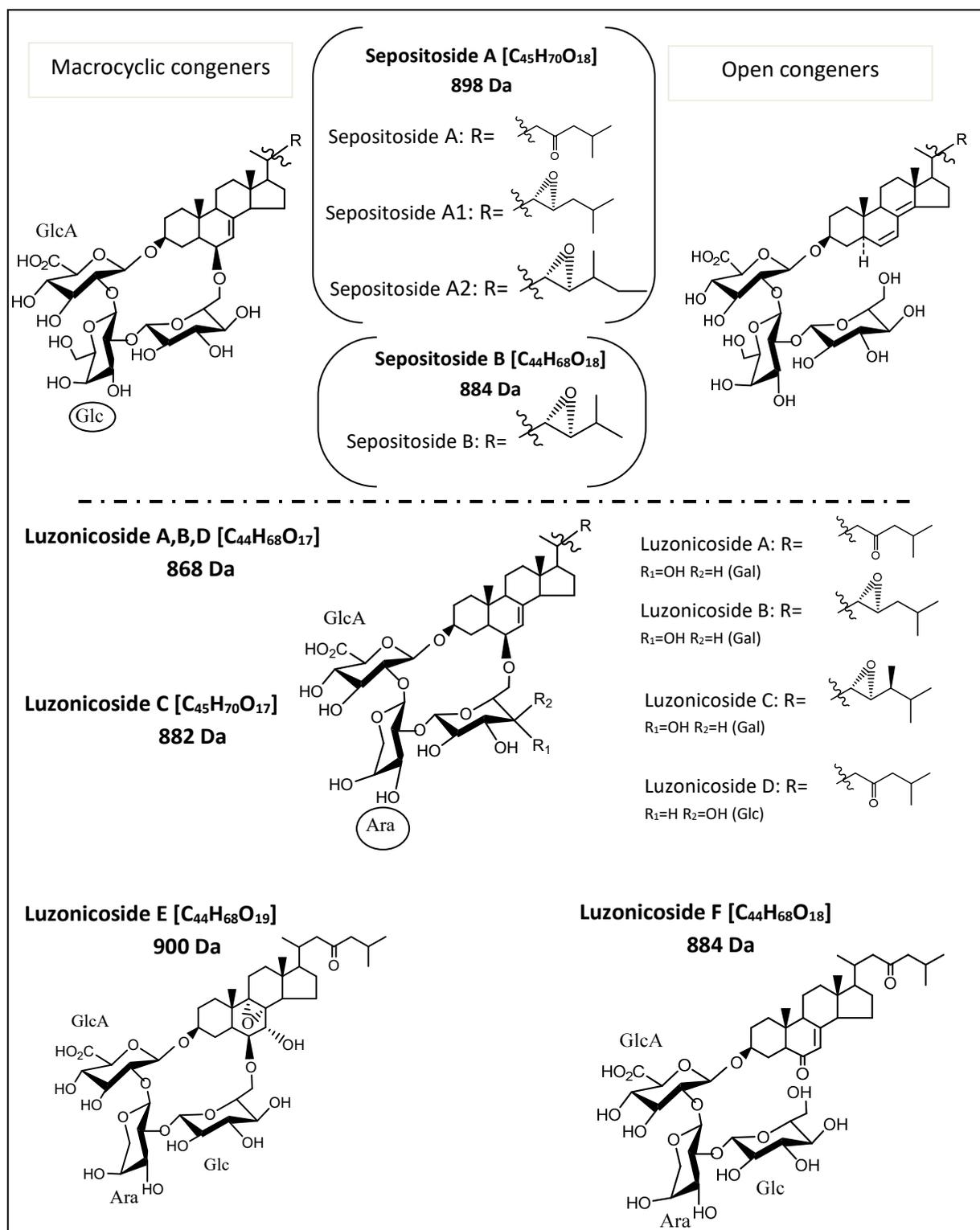


Fig.1. Sepositoside from *E. sepositus* (De Simone et al., 1981; Riccio et al., 1981) and luzonicoside from *E. luzonicus* (Kicha et al., 2015).

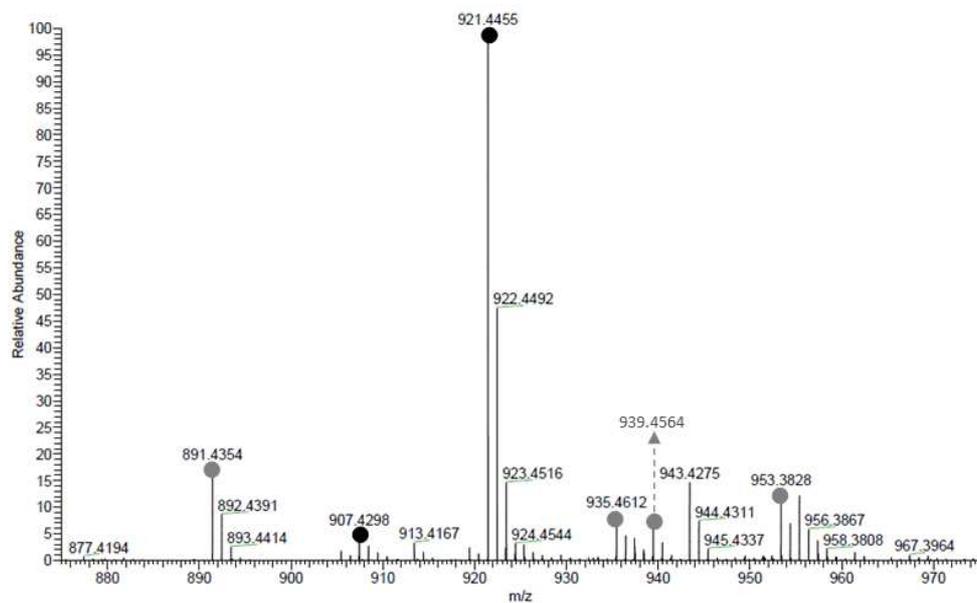


Fig.2. HR-ESI-MS analysis of saponins extracted from the oral body wall of female's individuals of *E. sepositus*. The m/z ratio of each saponin is annotated. MS signals marked by a black dot correspond to saponin congeners already identified in *E. sepositus* while signals identified by gray dots could represent new saponins for this species (see text for details). Most of the other peaks correspond to matrix adducts.

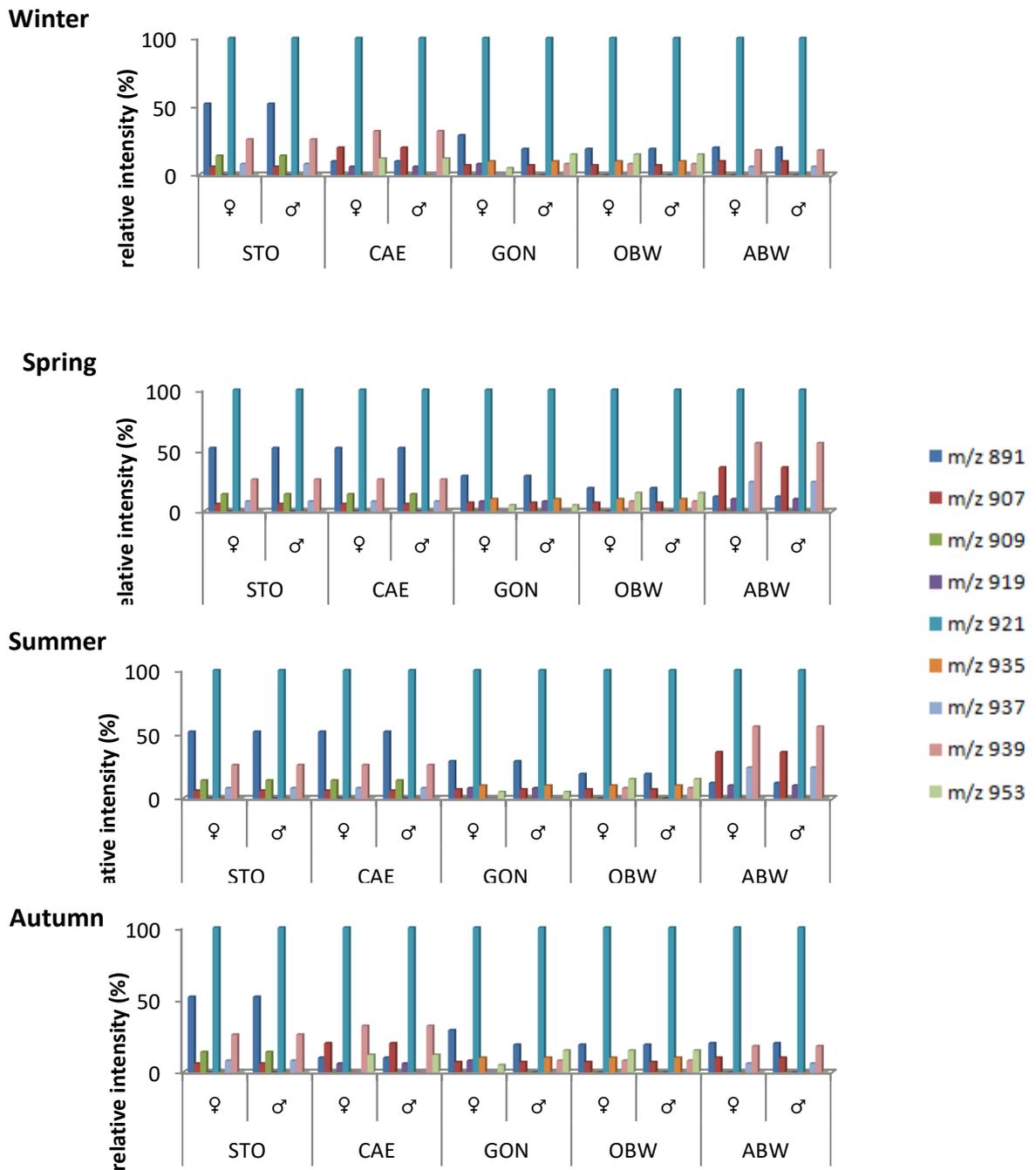


Fig.3. comparison of the distribution and the relative intensity of the saponin ions (m/z) of the five selected organs (STO: stomach; CAE: pyloric caeca; GON: gonads; OBW: oral body wall; ABW: aboral body wall) for males and females individuals collected at different seasons. In this diagram, the saponins are only identified by their compositions with no distinction between isomeric saponins.

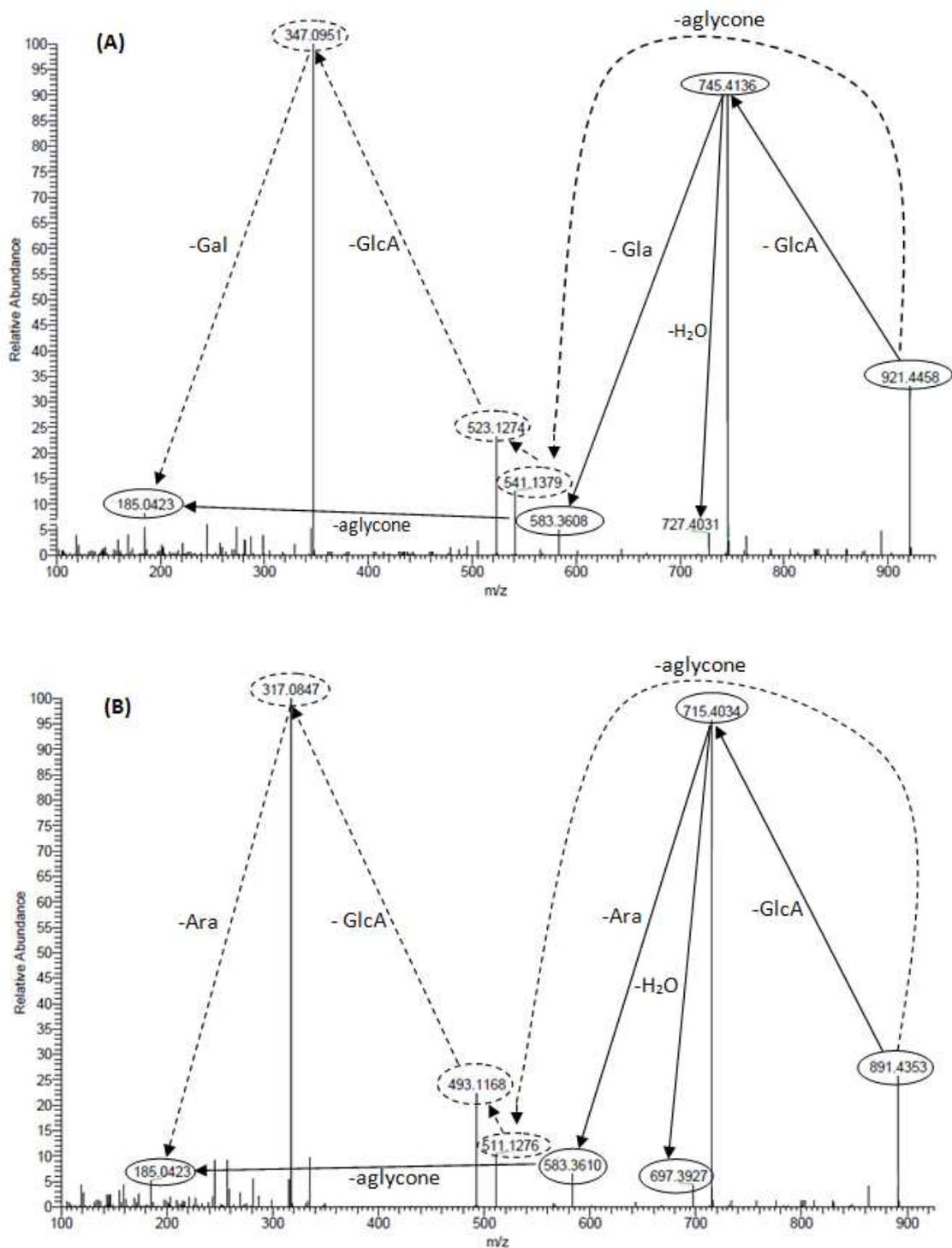


Fig.4. Comparison between the collision-induced fragmentation patterns of sepositoside m/z 921 (A) and luzonicoside m/z 891(B). Full and dotted arrows are two possible fragmentation patterns (see Fig.S2 and Fig.S3 in supplementary materials for molecular structures of these saponins and their respective fragments).

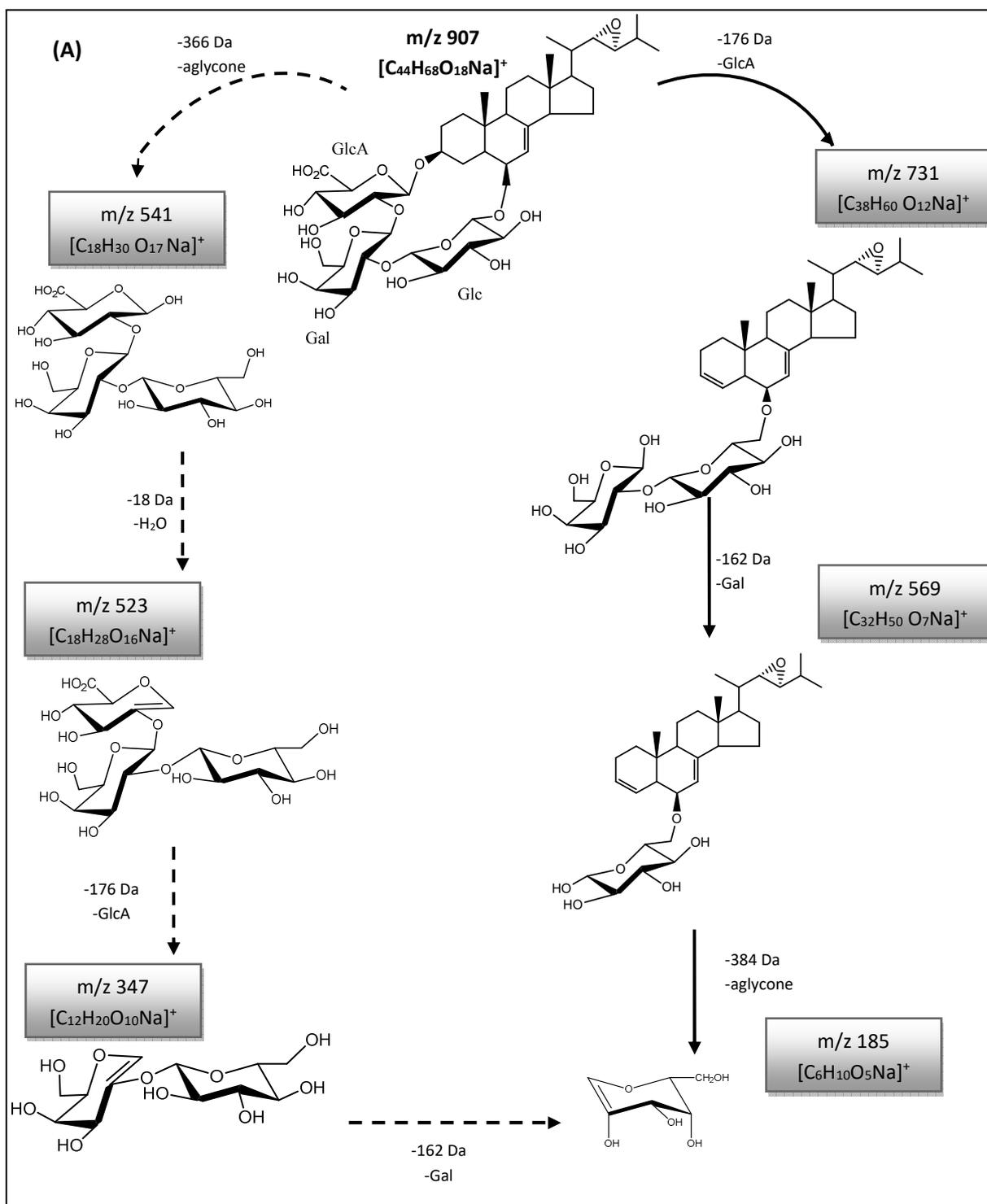


Fig.5. Collision-induced dissociation experiments of the m/z 907 ions obtained upon ESI MS/MS analysis of the oral body wall saponin extract of females Individual: fragmentation reactions of (A) sepositoside B of *E. sepositus*; and (B) lozonicoside F of *E. luzonicus*.

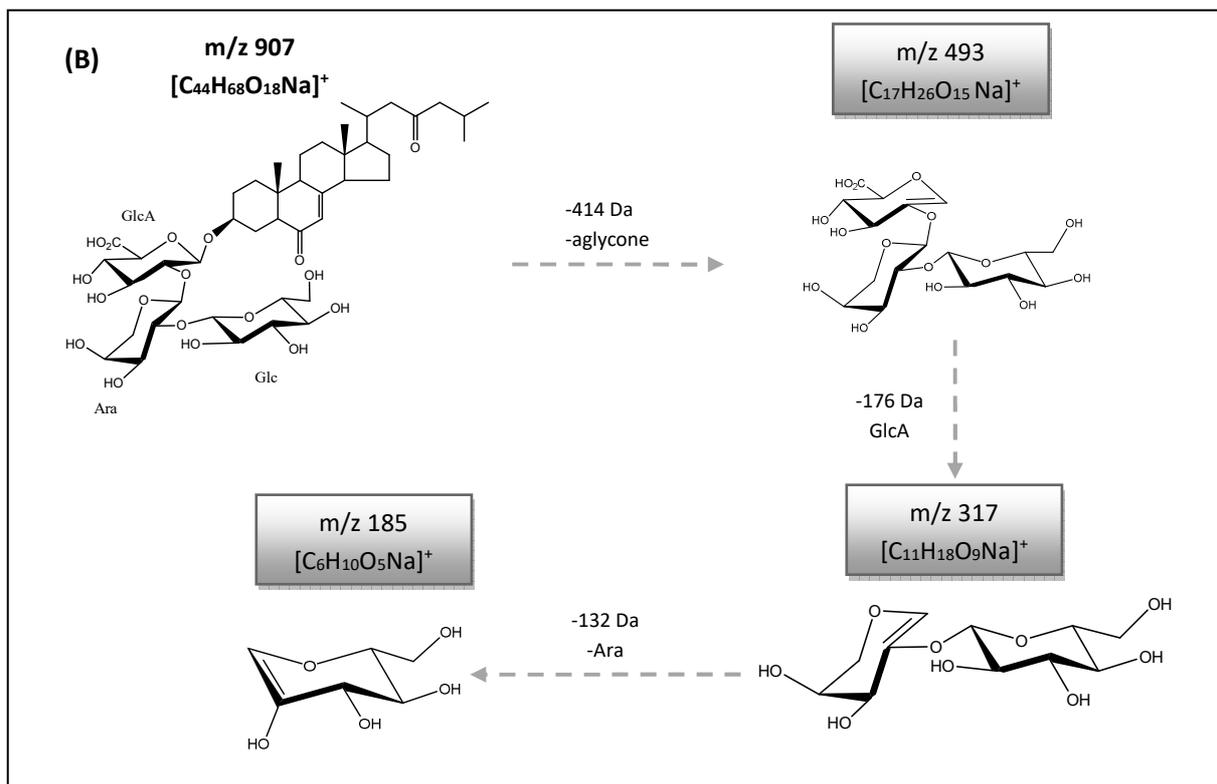
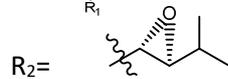
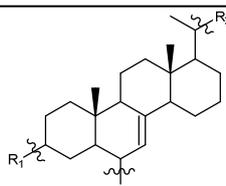
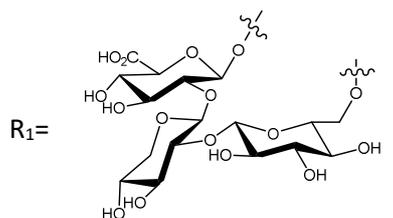
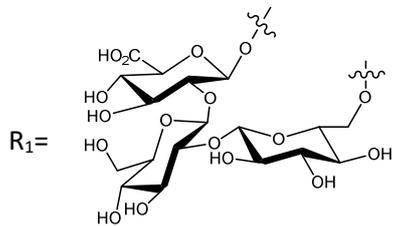
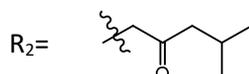


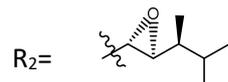
Fig.5 (continued).



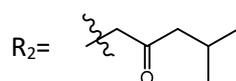
(1) Sepositoside B
884 Da



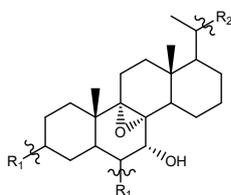
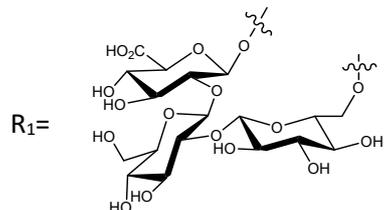
(2) Sepositoside A
898 Da



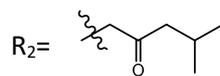
(3) saponin A (none name)
912 Da



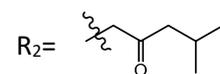
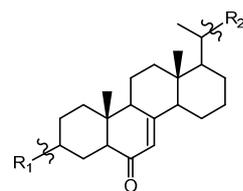
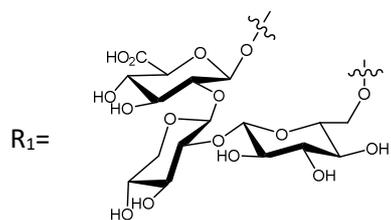
(4) luzonicoside A
868 Da



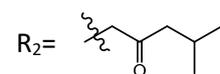
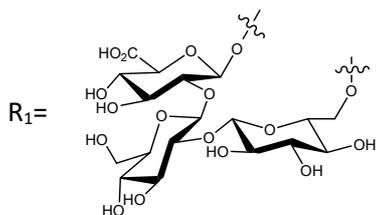
(5) saponin B (none name)
916 Da



(6) saponin C (none name)
930 Da



(7) luzonicoside F
884 Da



(8) saponin D (none name)
914 Da

Fig.6. Presumptive molecular structures of saponins detected in *E. sepositus* and described in Table1.

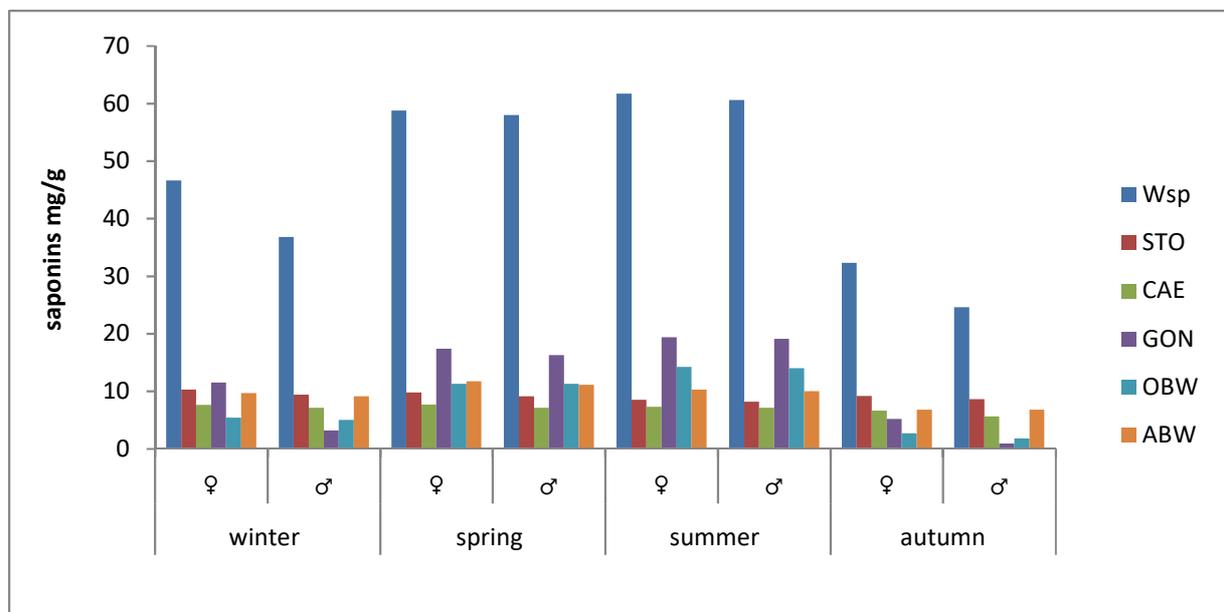


Fig.7. Total saponins amount (mg/g, dry weight) in the different organs of *E. sepositus* obtained from different sex and collected in different seasons. Wsp: Whole specimens; STO: stomach; CAE: pyloric caeca; GON: gonads; OBW: oral body wall; ABW: aboral body wall.