Identification of Vertebrate Type Steroid Hormones in the Shrimp *Penaeus Japonicus* by Tandem Mass Spectrometry and Sequential Product Ion Scanning

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The identification of testosterone, pregnelonone, and 17α-hydroxyprogesterone by tandem mass spectrometry and of progesterone by sequential product ion scanning in the shrimp gonads of *Penaeus japonicus* is described. The identification of these substances in biological samples is usually done by gas chromatography–mass spectrometry and involves several liquid chromotographic purification steps followed by derivatization. The utilization of tandem mass spectrometry in this analysis has simplified considerably the sample pretreatment and provided a very simple method of screening these substances in complex mixtures. (J Am Soc Mass Spectrom 1997, 8, 365–370) © 1997 American Society for Mass Spectrometry

The physiological role of "vertebrate type" steroid hormones (progesterone, pregnenolone, and male and female sexual hormones) in arthropods (insects and crustaceae) is still unclear [1]. Their identification by unequivocal methods [gas chromatography–mass spectrometry (GC/MS)] in several species of crustaceae [2] and their titre variation during important physiological processes are important prerequisites to establish these compounds as invertebrate hormones [1].

Some of us are studying the process of ovary maturation in the shrimp *Penaeus japonicus* in which progesterone, 17α-hydroxyprogesterone, and various estrogens have been implicated [3]. The identification of these compounds is usually done by GC/MS and involves several liquid chromatographic purification steps followed by high-performance liquid chromatography HPLC separation in small groups of steroids, derivatization, and GC/MS analysis. Tandem mass spectrometry (MS/MS) [4] has proved to be a good alternative to GC/MS analysis, because it greatly simplifies the treatment of the sample prior to analysis. In fact several applications of this technique to the identification of steroids in natural mixtures have been described in the literature [5, 6]. The development of hybrid instruments (sector and quadrupole combinations) has expanded considerably the type of experiments that can be performed, in particular, by allowing the possibility of sequential product ion scanning (MS/MS/MS) [7, 8]. In this article we will present the results of the application of MS/MS and MS/MS/MS to the identification of the vertebrate type hormones shown in Scheme I in the shrimp gonads of *Penaeus japonicus*.

Experimental

Animals

Penaeus japonicus shrimp were reared by Eurodáqua S.A., Algarve, Portugal, where the ovary samples were taken, transported to the laboratory in dry ice, and lyophilized.

Materials

Lipidex 1000 is a trademark of Pharmacia Fine Chemicals (Uppsala, Sweden) and was obtained from Sigma Chemical Co. (St. Louis, MO) as hydroxyalkoxypropil dextran 10%, type I.

Pregnelonone (M_r = 316), testosterone (M_r = 288), progesterone (M_r = 314), and 17α-hydroxyprogesterone (M_r = 330) were used as purchased from Sigma Chemical Co. Solvents were of Pro Analysis grade and were obtained from E. Merck (Darmstad, Germany).

Sample Treatment

Lyophilized gonads (1 g, corresponding to 10–20 shrimps in all states of maturation) were treated with methanol (20 mL, 4°C), the suspension was centrifuged

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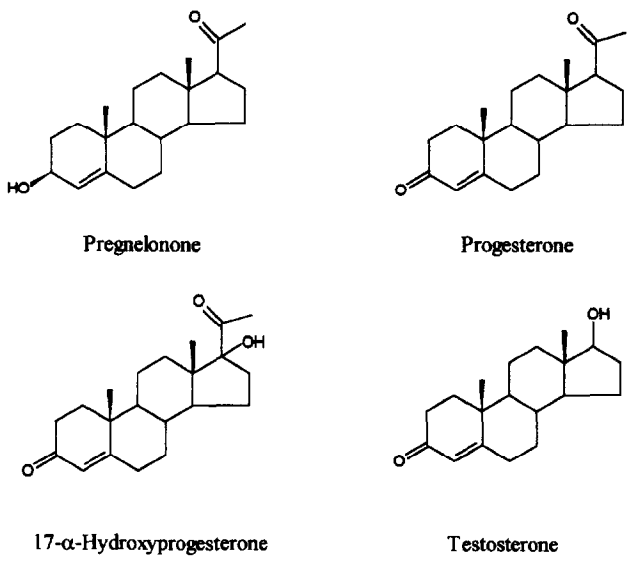

Scheme I

(4500 rpm, 25 min, 4°C), and the supernatant was decanted. The residue was mixed and centrifuged with methanol (2 × 10 mL). The methanolic solution (40 mL) was evaporated to dryness and the residue was taken up in aqueous phosphate buffer (0.2 M, pH 7.4, 40 mL). The suspension was left overnight at 4°C, filtered, and the lipidic material extracted with *n*-hexane: isopropanol (3:2, 2 × 25 mL). The organic phase was dried and evaporated to dryness. The residue was taken up in *n*-hexane: isopropanol (3:2, 1 mL) and mixed with 2 mL of Lipidex 1000 gel [formed by mixing 1 g of Lipidex 1000 with *n*-hexane: isopropanol (3:2, 3.5 mL) with overnight equilibration at 4°C]. The solvent was removed, the remaining solid was introduced into a chromatography column (i.d. 1 cm × 2.5 cm), and the extract was eluted sequentially with H_2O (15 mL), CH_3OH : H_2O (9:1, 10 mL), CH_3OH (10 mL), and $CHCl_3$ (10 mL). The CH_3OH : H_2O (9:1) fraction was evaporated and analyzed by tandem mass spectrometry.

Mass Spectrometry

Tandem mass spectrometry analyses were performed by a VG Analytical (Manchester, UK) Autospec Q of EBEqQ by using geometry. The samples and standards were introduced with an unheated direct insertion probe and ionized by electron impact at 70-eV electron energy and source temperature of 200 °C. First-generation product ion spectra [mass-analyzed ion kinetic energy (MIKE) spectra] were obtained by adjusting the magnetic field strength to select the precursor ion at a resolution of 1000 with subsequent scanning of the second electric sector to detect the product ions formed in the third field-free region. Precursor ion analysis was performed through a B^2/E linked scan. Second-generation product ion spectra were obtained by adjusting the magnetic field strength to select the precursor ion, followed by adjustment of the second electric sector to select the first-generation product ion formed, by unimolecular decomposition, in the third field-free region. This ion species was then subjected to collision activation with argon in the rf-only quadrupole (65-eV collision energy). The product ions thus formed were analyzed with the quadrupole mass filter operated at 1-u resolution. The spectra presented represent accumulation of single scans and, in some instances, have been smoothed to different extents. For the MS/MS/MS analysis an accumulation of 5 scans was needed to obtain the spectrum of the standard, whereas for the sample, accumulation of 40 scans yielded the spectrum presented in Figure 5b. A limit of detection for the standard (progesterone) was established by directly introducing decreasing amounts of a methanolic solution of progesterone into the mass spectrometer and recording the MIKE spectra of the molecular ion. The limit of detection was estimated to be on the order of 0.2 pg. The time of volatilization of each steroid depended on the steroid itself, and especially on the matrix. For example, in the case of the MS/MS/MS analysis for progesterone identification, the spectra shown resulted from an accumulation of 5 scans over 70 s for the standard and an accumulation of 40 scans over 4 min for the sample.

Results and Discussion

The strategy used in this analysis involved a very simple and quick pretreatment of the shrimp gonads, followed by introduction of the extract thus obtained into the mass spectrometer by using the direct insertion probe. In Figure 1 are shown the mass spectra of the methanolic extract of the sample gonads (Figure 1a) and of the same extract purified by Lipidex 1000 liquid chromatography (Figure 1b). A comparison of spectra shows the efficiency of the liquid chromatography purification step, particularly in the removal of cholesterol, whose peaks, present in the first spectrum (m/z 386, 368, 353, 301, 275), are practically absent in the second. It is also worth mentioning that the intensities of the peaks corresponding to the molecular ions of the steroids under study are, in general, less than 3% of the base peak.

The general mass spectrometric approach for steroid identification was the comparison of the first-generation product ion spectra of the molecular ion of each pure steroid with the spectra obtained for each putative steroid molecular ion formed in the sample by electron impact. Figures 2 and 3 show the spectra (standard and sample), obtained for the molecular ions of pregnelonone and 17α-hydroxyprogesterone, respectively, which provide a positive identification of the presence of the mentioned steroids in the shrimp gonads.

For the identification of testosterone we could not select the molecular ion (m/z 288) as the precursor ion because estriol, which might also be present in the

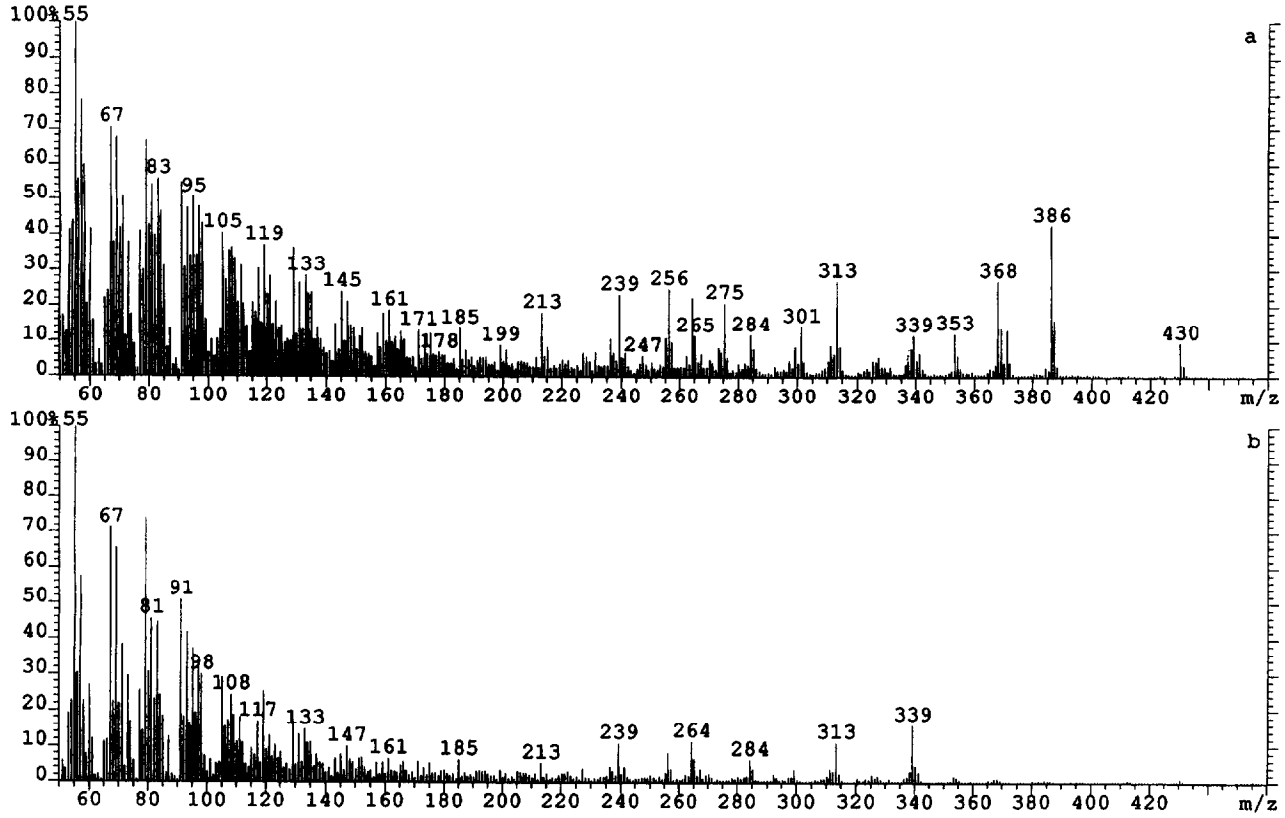

Figure 1. (a) Electron impact mass spectra of the methanolic extract of shrimp gonads and (b) of the same extract purified by Lipidex 1000 chromatography.

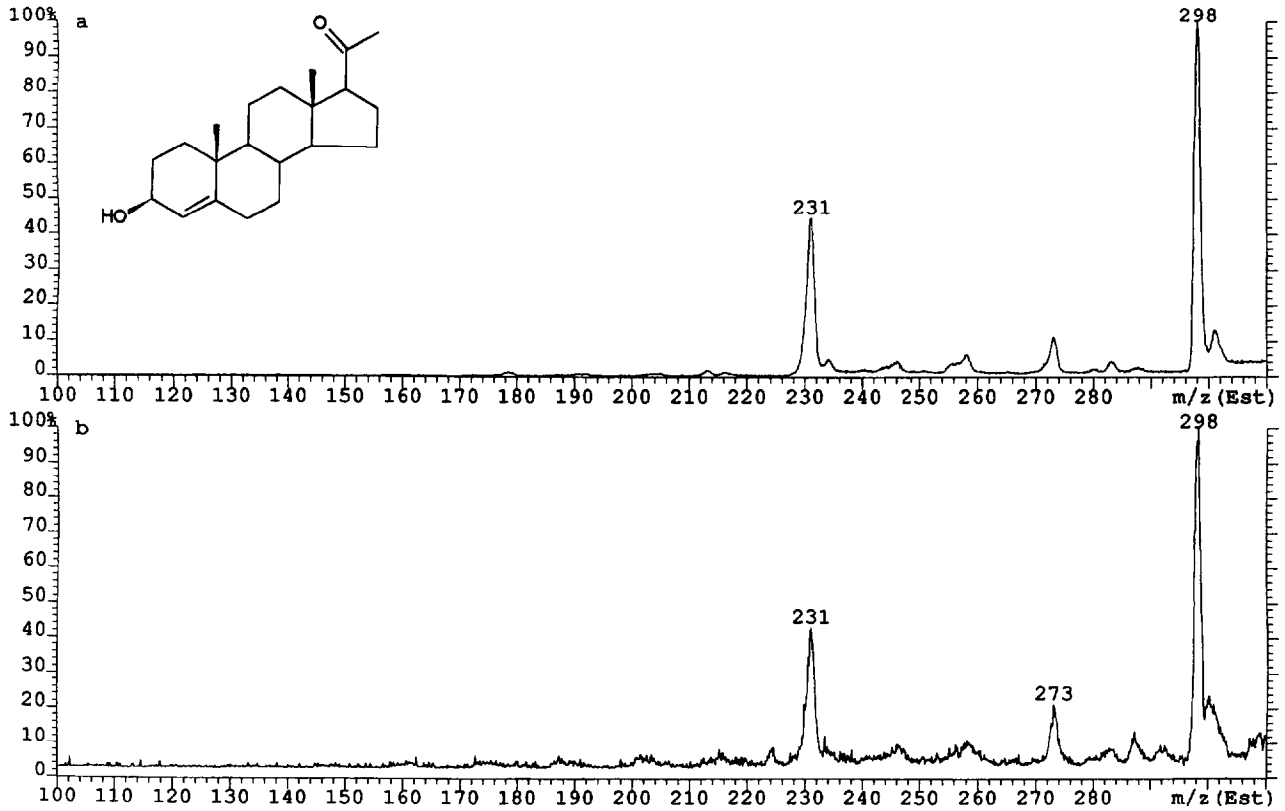

Figure 2. (a) MIKE spectra of the molecular ion (m/z 316) of pregnelonone in the standard and (b) in the shrimp sample.

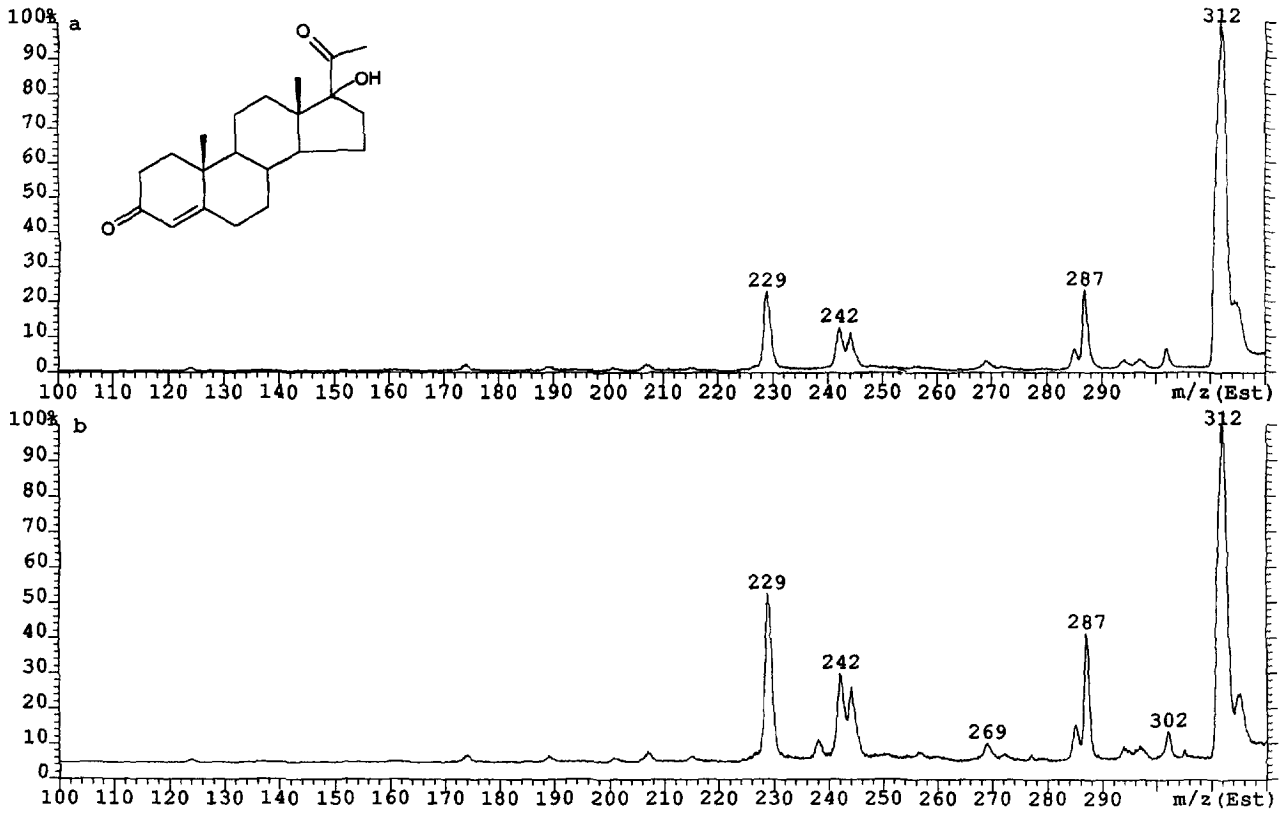

Figure 3. (**a**) MIKE spectra of the molecular ion (m/z 330) of 17α-hydroxyprogesterone in the standard and (**b**) in the shrimp sample.

shrimp gonads, has the same molecular weight. The second most abundant ion in the mass spectrum of testosterone is the fragment ion formed by loss of ketene from the molecular ion as confirmed by a precurson ion scan (linked B^2/E scan) of ion $[M\text{-}42]^+$ in the standard and the sample, which matched very well with each other. In Figure 4 we show the first-generation product ion spectra of ion m/z 246 (mentioned previously) present in the sample and in testosterone, which provide evidence for the presence of the hormone in the shrimp gonad.

For progesterone the MS/MS analysis by using the molecular ion (m/z = 314) as the precursor ion was inconclusive because there was a strong interference from another ion of the same mass-to-charge ratio in the mixture. Although we could identify in the sample product ion spectrum the main fragment ions of the pure steroid, their abundance was quite low and, on the other hand, we observed quite strong new signals that could only be explained by the presence of another ion at m/z 314. The second most abundant ion in the mass spectrum of progesterone, m/z 272, has the same mass-to-charge value as the molecular ion of estradiol, which might also be present in the shrimp, so we could not select it as a precursor ion for the identification of progesterone. To overcome this difficulty we explored the ability of a hybrid instrument to perform MS/MS/MS analysis, which can increase the specificity of the analysis. In the first-generation product ion spectrum of the molecular ion of progesterone the most abundant fragment ion corresponds to loss of 123, yielding an ion of m/z 191, whose structure and mode of formation have been described fully [9]. In the MS/MS/MS experiment we selected the precursor ion (m/z 314) with the EB part of the instrument and adjusted the electric sector voltage of the second electric sector to select the first-generation product ion (m/z 191) formed in the third field-free region. This ion was then subjected to collision activation with argon in the rf-only quadrupole (65-eV collision energy) and the product ions thus formed were analyzed with the quadrupole mass filter. The spectra obtained for sample and standard are shown in Figure 5. Because the absolute signal in Figure 5b is very low, as compared to Figure 5a, only peaks at m/z 147 and 92 can be attributed confidently to ions. In the MS/MS/MS data, the similarity between the signals representing ions provides a strong indication of the presence of progesterone in the shrimp gonads. Loss of acetaldehyde is a possible explanation for the most abundant ion (m/z 147) in the second-generation product ion spectra.

Conclusions

These results illustrate the utility and versatility of MS/MS and MS/MS/MS techniques in the rapid identification of minor amounts of steroid hormones in complex mixtures. The simplicity of sample pretreat-

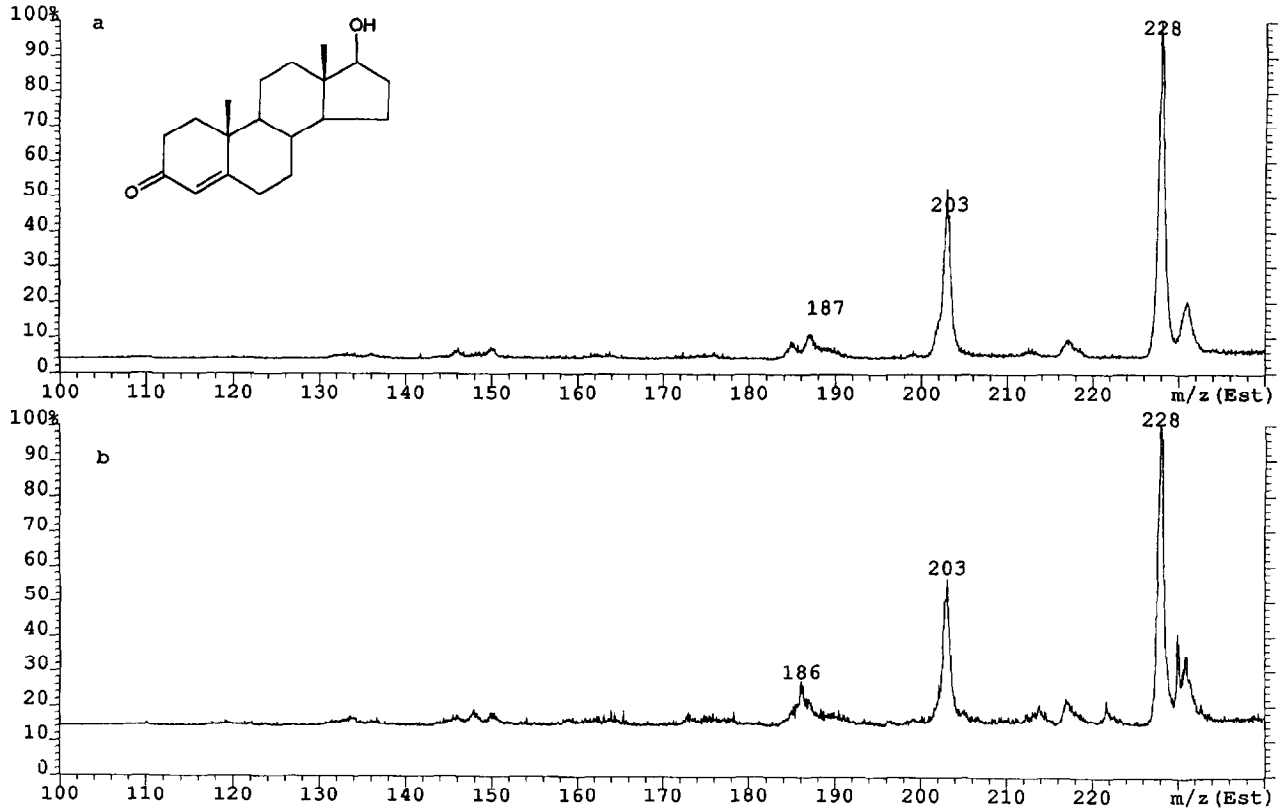

Figure 4. (**a**) MIKE spectra of the fragment ion (m/z 246) of testosterone in the standard and (**b**) in the shrimp sample.

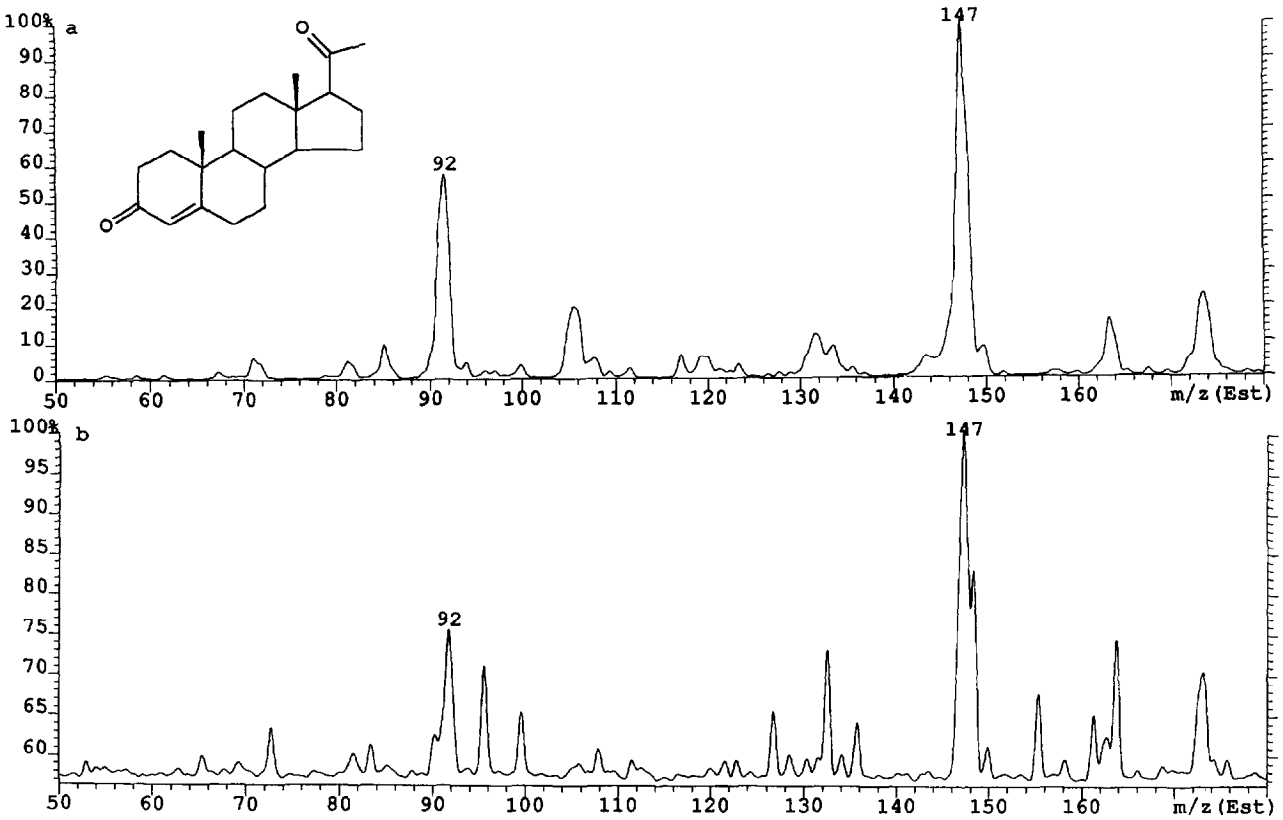

Figure 5. Second-generation product ion spectra of ion m/z 191 of progesterone in the standard and (**b**) in the sample.

ment as compared to analysis by GC/MS presents an enormous advantage of this method for screening these compounds in biological samples. Moreover, the unambiguous identification of the mentioned steroids in the shrimp *Penaeus japonicus* is also presented for the first time

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