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Review: The In-Line Biosensors as Detectors for Chromatography

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Abstract

The review mainly describes the in-line HPLC biosensor for analytical work as a promising detector/method of detection, its presentation, application, and progress in use. The unique ability of the biosensor detector is selectivity reaction on some pheromones, alarm substances, phosphor organic insecticides, toxins such as cholinesterase inhibitors among which are chemical warfare substances such as Tabun, Sarin, Chlorosarin, Soman, Ethylsarin, and Cyclosarin. The olfactory system of fish *Carassius carassius* L. is used as an in-line HPLC biosensor for analytical work. The olfactory system is built to be a highly selective and sensitive detection device with incorporated sensory-, primary filter-, primary multiplier- and selection regions for odorants (by LOT, IMOT and *m*MOT tracts). Olfactory neurons are nature's constructed sensors. The spectrum of efficiency to relevant substances varies greatly between the different species and is determined by the olfactory receptors expressed in the sensory neurons. The detection thresholds for the olfactory-relevant substances are commonly in or below picomolar/L concentrations due to the integrative operation of the secondary neurons in the olfactory bulb. In our laboratory, we have used the olfactory system in the fish *Carassius carassius* L., as an in-line detector for HPLC for recordings of the alarm substances and sex pheromones under chromatographic separation. This approach was useful for detection of some insecticides, xenobiotics, and toxins as cholinesterase inhibitors among which are chemical warfare substances. In the publication (Brondz, 2015) High-Performance Liquid Chromatograph (HPLC) Equipped with a Neurophysiological Detector (NPD) as a Tool for Studying Olfactory System Intoxication by the Organophosphate (OP) Pesticide Diazinon and the Influence of OP Pesticides on Reproduction. *International Journal of Analytical Mass Spectrometry and Chromatography*, 3, No. 1, pp. 14-24. http://dx.doi.org/10.4236/ijamsc.2015.31002 was shown experimental work. However, in this paper the explanations, discussion and conclu-

sion were not sufficiently elaborated, and they were conceptual to only the professionals and were directed to a narrow spectrum of scientists. This review presents answers and explanations that will be understandable and useful for a broad spectrum of scientists and researchers as environmentalists, medics, biologist, toxicologist, specialists in military intelligence and everyone interested in nature.

Keywords

Biosensors, Chromatography, HPLC, Electroolfactography, Electroantennography, Organophosphates (OPs), Pesticides, Warfare Toxins, Endocrine and Sexual Activity Disruptors, Behavioral Modifiers, Pheromones, Alarm Substances, Neurophysiological Detector

1. Introduction

The in-line biosensor for analytical work as a Chromatographic detector was first described by D. Schneider and Z. Vergl, in Schneider & Vergl (1957), as depolarization across an insect antenna in response to stimulation by volatile compounds. Several other in-line biosensors for analytical gas chromatography (GC) were described and some of these are in use up to today, however, the potential of in-line biosensors for analytical works is strongly underestimated.

Biosensors are often used in cases when other methods are not available or need complicated and unwieldy equipment or consume a relatively long time. In submarines for the detection of CO (carbon monoxide), the toxic gas was used a small bird *Melopsittacus* undulatus from family *Psittaculidae*. These birds are very sensitive to CO poisoning.

The electroolfactogram (EOG) and electroantennogram (EAG) are still used with Gas Chromatography (GC). Depolarization across an insect antenna in response to stimulation by volatile compounds was first shown by D. Schneider and Z. Vergl, in 1957 (Schneider & Vergl, 1957). The basis for these experiments was the investigations by Autrum (1936). In 1964 was described the periodical exposure of frog olfactory membrane to gas flow from a GC for the separation of physiologically active substances (Ottoson & von Sydow, 1964). Ottoson and von Sydow have published the use of physiologic detection methods in connection with GC. However, the method described by Ottoson and von Sydow has not been extensively used. The frog's olfactory membrane was difficult to maintain in proper physiological conditions. The high gas flow and elevated temperature from GC are leading to the non-physiological dryness in the frog olfactory membrane. These disadvantages were possible to overcome in neurophysiologic detectors in connection with high-performance liquid chromatography (Brondz, Hamdani, & Døving, 2003). The GC detection method for insect hormones, pheromones and repellents is a popular method (Wadhams, 1990). Arn et al.

described the electroantennographic detector which was proposed in 1975 (Arn, Städler, & Rauscher, 1975).

The in-line HPLC biosensor made from the olfactory system of the fish *Carassius carassius* L. first was presented in (Brondz, Hamdani, & Døving, 2003). The complete text was published in *Journal Chromatography B*: *Biomedical Sciences and Applications* (Brondz, Hamdani, & Døving, 2004a) with description of the experimental method and technical data. The use of human olfactory organs for qualitative control in food, flavors, parfums and vine production was known for centuries. However, the connection of HPLC to the fish olfactory system was a significant step forward with additional possibilities and opportunity to study the influence of insecticides on fish's population and possibly beyond this the influence of insecticides on mammals and even on human population.

The fish *Carassius carassius* L., used as donor of neurophysiologic biosensor is shown in **Figure 1**.

The method is based on the registration of nervous activity from the secondary neurons in the olfactory bulb recorded during exposure of the olfactory sensory epithelium to the effluent from the HPLC column.

Experiments were performed with an HPLC HP 1100 model equipped with a diode array detector, fluorescence detector and ChemStation software. An adsorbosphere nucleotide-nucleoside column was used to separate the substances in the *Carassius carassius* L., skin extract. In the *Carassius carassius* L., the secondary neurons of the posterior part of the medial olfactory bulb responded specifically to alarm substances in the skin extracts (Brondz, Hamdani, & Døving, 2003; Brondz, Hamdani, & Døving, 2004a; Brondz, Hamdani, & Døving, 2004b; Brondz, Karaliova, & Ekeberg, 2006) and to some insecticides (Brondz, 2015), xenobiotics, and toxins as cholinesterase inhibitors (Brondz, 2015) among which are chemical warfare substances (unpublished results). By this means, we attempt to identify the R_t of alarm substances. This biological HPLC detector is unique because it takes advantage of the integrative properties of the olfactory system and responds to distinct substance and differs from the majority of HPLC detectors that are bulk or solute detectors.

Figure 1. The fish *Carassius carassius* L. **Figure 1** is copied from Brondz, Hamdani, & Døving (2003) provided from archives of Jupiter AS, Norway.

There are few analytical detectors that can detect the physiological activity of the substances passing through the detector cell. We made an in-line detection cell from the nasal cavity of *Carassius carassius* L., and the sensor unit from the olfactory epithelium. The use of the olfactory system in HPLC studies seems a promising tool for recording and isolating substances with physiological activity such as sex attractants, alarm substances, food odors, environmental pollutants, pharmaceutical drugs, or toxins. Once the detection and isolation of the active components are performed the identification can be pursued by standard chemical techniques such as nuclear magnetic resonance (NMR) spectrometry, mass spectrometry (MS), or different spectrometric measurements.

2. Preconditions of Use NPD to Monitoring of Toxicology Studies of Organophosphate (OP) Pesticides as Endocrine and Sexual Activity Disruptors

Organophosphates (OPs) pesticides such as diazinon, malathion and others are widely used in agriculture as potent insecticides, however, they have significant impact on mammal and human fecundity as disruptors of sexual activity and reproduction (Brondz, 2015). The other group of OPs is warfare agents such as Tabun, Sarin, Chlorosarin, Soman, Ethylsarin and Cyclosarin, and some others. Both groups have multiple physiological impacts on human health as impairment of cognitive abilities and post toxic syndromes. The main toxic mechanism of OPs is acetylcholinesterase inhibition. *Acetylcholinesterase degrades the neurotransmitter acetylcholine. Preventing acetylcholinesterase activity through exposure to OP leads to an accumulation of a high concentration of acetylcholine in the synaptic cleft or the synaptic junction. Nerve impulses are continually transmitted to muscles, excretory cells, and the brain* (Brondz, 2015). The axoplasmic transport of different neuroactive substances to the brain through the Olfactory Nerve (ONs) was described by Gross, and Kreutzberg in Gross & Kreutzberg (1978). The axoplasmic transport of OPs direct to the brain is obvious and was supported in publications (Benmoyal-Segal, Vander, Shifman, Bryk, Ebstein, Marcus, Stessman, Darvasi, Herishanu, Friedman, & Soreq, 2005; Miranda-Contreras, Gómez-Pérez, Rojas, Cruz, Berrueta, Salmen, Colmenares, Barreto, Balza, Zavala, Morales, Molina, Valeri, Contreras, & Osuna, 2013; Racciatti, Vecchiet, Ceccomancini, Ricci, & Pizzigallo, 2001; Behan, 1996; Abou-Donia, 2010). The intoxication with OPs includes impairment of the hypothalamic-hypophyseal-adrenal axis, and endocrine system, and cognitive functions (Benmoyal-Segal, Vander, Shifman, Bryk, Ebstein, Marcus, Stessman, Darvasi, Herishanu, Friedman, & Soreq, 2005; Miranda-Contreras, Gómez-Pérez, Rojas, Cruz, Berrueta, Salmen, Colmenares, Barreto, Balza, Zavala, Morales, Molina, Valeri, Contreras, & Osuna, 2013; Racciatti, Vecchiet, Ceccomancini, Ricci, & Pizzigallo, 2001; Behan, 1996; Abou-Donia, 2010). The OPs and warfare analogs and their degradation products are difficult to analyze, due to their instability. The analysis of OPs and degradation products are described in Brondz, Kolmo-

din-Hedman and Løfvenius (1989). The warfare analogs have relatively short time of persistence in a humid environment. The monitoring of warfare analogs and their degradation products in water and soil can give (and gave (unpublished results)) a clue of production sites and possible use of chemical OPs in warfare. Uses of those substances are prohibited by international law.

3. General Description of Methodology

The artificial pond water (APW) was used as a mobile phase to HPLC, because of this it could be applied directly and continually to the olfactory epithelium. Single unit recordings were made from the posterior part of the medial region of the olfactory bulb in *Carassius carassius* L., where the secondary neurons are responsive to alarm substances and phosphor organic cholinesterase inhibitors. Recordings were made from the lateral region of the olfactory bulb for examination of the specificity of responses.

Samples of 200 μL of physiological active substances were injected to the HPLC. The substances in the skin extract or other substances of significance were separated on an Adsorbosphere Nucleotide-Nucleoside 7 μ column to record R_t and the nervous activity. The flow from the outlet of the HPLC was applied directly and continuously to the olfactory epithelium. The temperature was between 20˚C and 24˚C, this prevented overheating and dryness of the biosensor.

Biological Material and Materials and Methods Used

Crucian carp, *C. carassius* L. (20 - 35 g) were caught in small lake (Tjernsrud) just outside Oslo city borders, Norway. They were transported to the aquaria facilities at the Department of Biology, University of Oslo where they were fed three times a week. Fish were initially anaesthetised with benzocaine (45 mg/L) and immobilised by intra-peritoneal injection of Saffan (Schering-Plough Animal Health, Welwyn Garden City, UK) 24 mg/kg body weight. To prevent drying and avoid any unforeseen movement during the experiment, fish were wrapped in a wet cloth and fixed by two steel rods, which fastened to the upper parts of the orbital bones. Care was taken not to disrupt the tissue around the olfactory epithelium. The damage to the skin causes release of alarm substances. Fish was continuously irrigated through the mouth and over the gills by pound water during the experiment with APW at 20˚C. Materials and methods were described in (Brondz, Hamdani, & Døving, 2003) and later in ((a) Brondz, Hamdani, & Døving, 2004a). A schematic drawing of the experimental set-up of HPLC with NPD and the details are shown in **Figures 2-6**.

4. Materials and Methods as They Were Described in (Brondz, 2015)

The detailed description of preparation of biosensors, columns, and the experimental set-up of HPLC with NPD is shown in (Brondz, 2015) and on **Figure 6**.

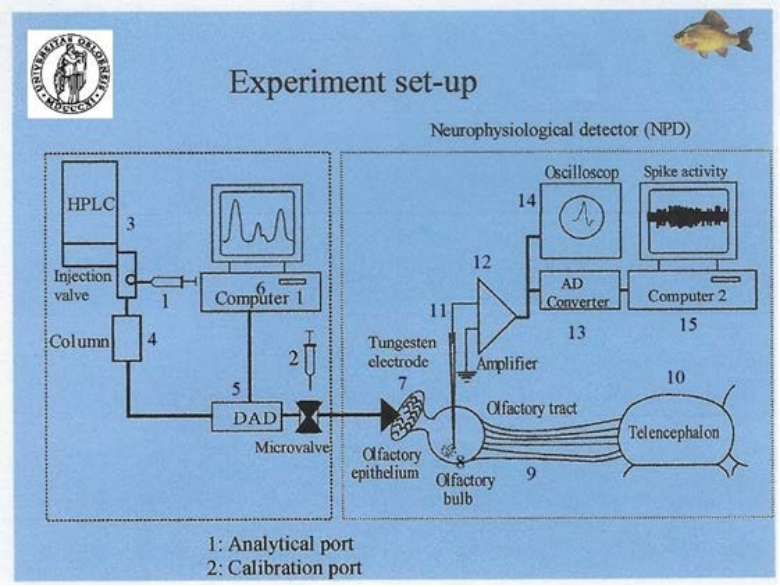

Figure 2. On the picture a schematic drawing of the experimental set-up of HPLC with NPD. (1) analytical port; (2) calibration port; (3) HPLC pumps; (4) an adsorbosphere nucleotide-nucleoside 7 μm column; (5) a diode array detector; (6) a PC; (7) an olfactory epithelium; (8) an olfactory bulb; (9) olfactory tract; (10) a telencephalon; (11) a tungsten electrode; (12) an amplifier; (13) an AD converter; (14) an oscilloscope; (15) a PC. **Figure 2** is copied from Brondz, Hamdani, & Døving (2003) provided from archives of Jupiter AS, Norway.

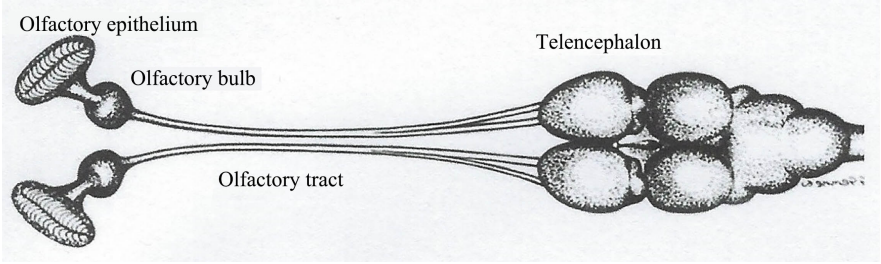

Figure 3. The schematics presentation of olfactory organs: olfactory epithelium, olfactory bulb, olfactory tracts, and telencephalon. **Figure 3** is copied from Brondz, Hamdani, & Døving (2003) provided from archives of Jupiter AS, Norway.

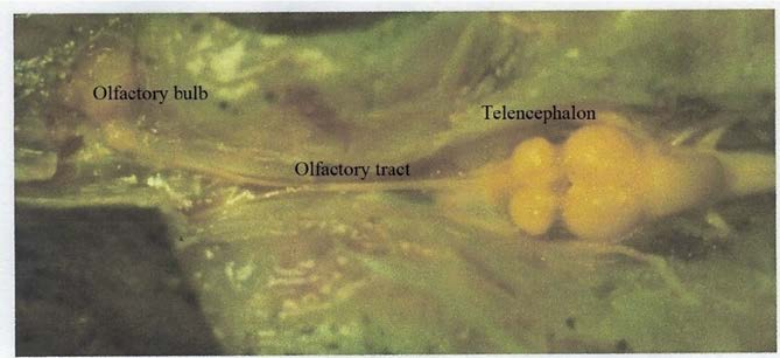

Figure 4. The Olfactory organs are shown in the photo. **Figure 4** is copied from Brondz, Hamdani, & Døving (2003) provided from archives of Jupiter AS, Norway.

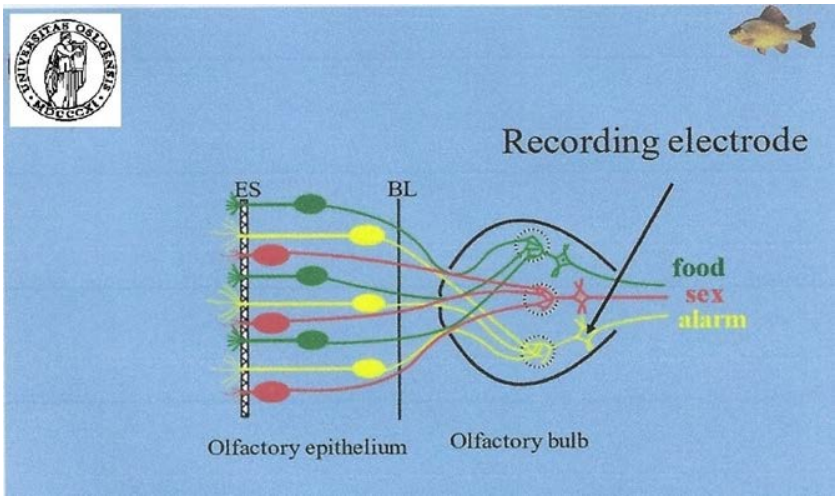

Figure 5. The scheme of neurons responsible for nervous signals and the point of recording electrode. **Figure 5** is copied from Brondz, Hamdani, & Døving (2003) provided from archives of Jupiter AS, Norway.

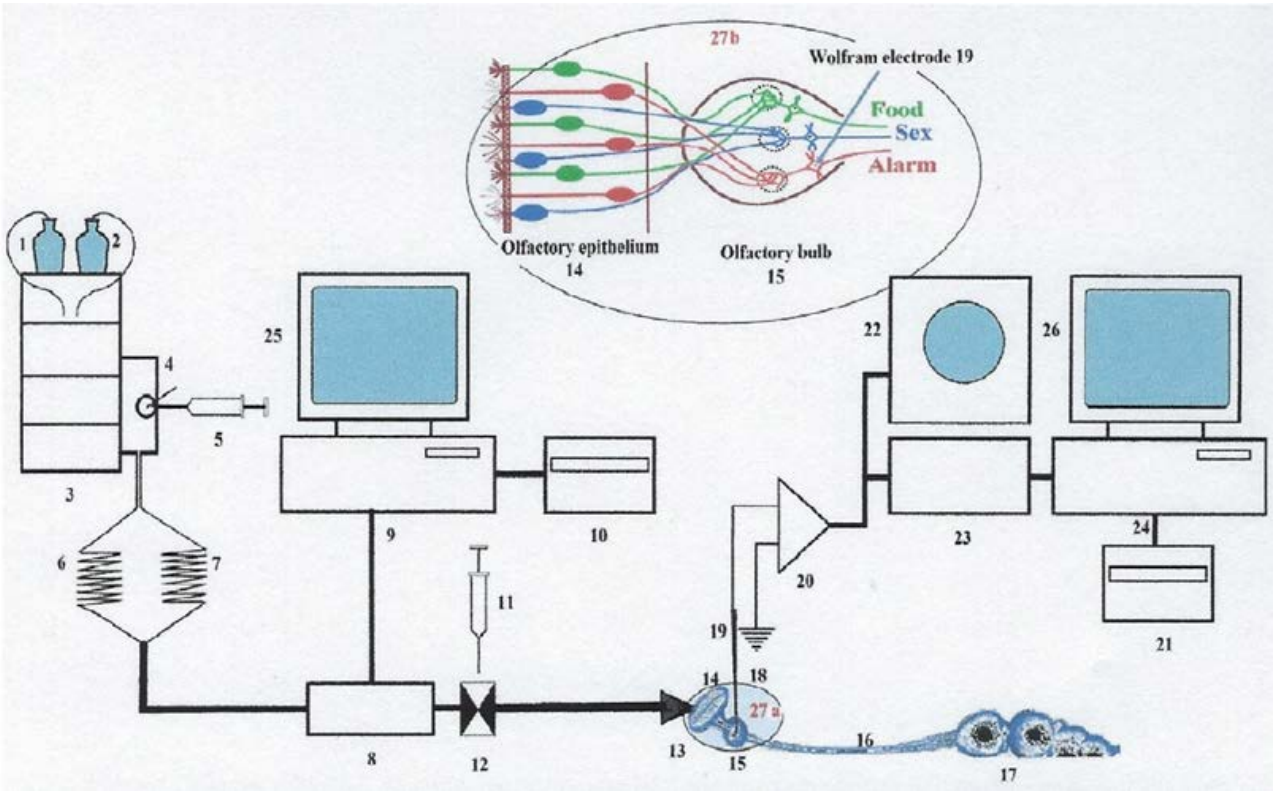

Figure 6. A schematic drawing of the experimental set-up of the HPLC equipped with DAD and NPD. The set-up included (1) reagent bottle 1 with APW; (2) reagent bottle 2 APW with 10 ppm of diazinon; (3) the HPLC system; (4) a Rheodyne injector; (5) a syringe with a mixture of sex hormones; (6) column 1; (7) column 2; (8) the DAD; (9) a data processor for the HPLC system; (10) data acquisition printer 1 for the HPLC system; (11) a syringe with fish skin extract; (12) a microvalve (13) transference tubing; (14) an olfactory epithelium (OE); (15) an olfactory bulb (OB); (16) olfactory tract; (17) telencephalon; (18) an earth ground connection; (19) a tungsten (wolf-ram) electrode; (20) an amplifier; (21) data acquisition printer 2 for the NPD; (22) an oscilloscope; (23) an AD converter; (24) a data processor for the NPD system; (25) a PC screen for the HPLC; (26) a PC screen for the NPD; (27a) the active detection part of the NPD; and (27b) the active detection part of the NPD shown in detail. **Figure 6** is copied from Brondz (2015).

The experiment was modified as it described in (Brondz, 2015). With this modification eluent and analyte were injected separately or in column 6 without directing the eluent though column 7 or was injected in column 7 without directing the eluent through column 6.

4.1. Preparation of the Support Phase (River Sand) for Columns 6 and 7

River sand was obtained from a small lake in the vicinity of the rural city Ski, Norway (the lake is a nature reservation in summer). The sand was washed with distilled water, dried, defatted with *n*-hexane p.a. quality (Merck), washed with 1 M NaOH p.a. quality (Merck) for 1 h, washed with 1 M HCl p.a. quality (Merck) for 1 h, and finally washed with deionized water. The columns were installed in the HPLC and were flushed with APW for 1 h before the experiments.

4.2. Experimental Protocol

At the start of every experiment, the basal nervous activity at the measured site was measured by passing APW from bottle 1 through the nasal cavity for 10 min. The experiment started when the nervous activity was stable. For detailed description of protocol see (Brondz, 2015).

5. Results and Discussion

5.1. Results

Figure 7 shows results of measurements of activities in biosensor connected to HPLC and appearance of active substance in the chromatogram. The explanation of results: "*The nervous activity in the OB when exposed to sex pheromones after preexposure of the fish OE to* 10 *ppm of diazinon for* 1 *min* (*Figure* 5(*d*)) *shows that, after preexposure to diazinon, injection of sex pheromones increased the nervous activity in the OB. These changes in nervous activity can be compared with those elicited by exposure of the OE to alarm substances in fish skin extract. Even the short-term preexposure to diazinon transformed the normal nervous signals from the OB to the brain to become signals about life-threatening danger. The short-term preexposure to diazinon transformed the normal nervous signals initiated by alarm pheromone from the OB to the brain to become amplified signals about life-threatening danger*" (Brondz, 2015).

5.2. Discussion

5.2.1. General Presentation of Neurons in the Olfactory Bulb

The olfactory bulb in the NPD serves as a discriminator see **Figure 8**.

Some convergence of neurons exists. It should be taken into account that addition of amino acids can give some response in the biosensor (Nikonov & Caprio, 2007). However, application of a blend of three amino acids induced activity in the lateral part of the bulb, but not in the posterior part of the medial region which is responsible for alarm substances. The activity of a single unit in

the olfactory bulb takes advantage of the specificity of the olfactory system to restricted regions of the bulb and the association of these regions to particular behavior patterns in the fish. The medial part of the medial olfactory tract (*m*MOT) mediates the alarm reaction Figure 9. These neurons are selective and sensitive to the agents eliciting the alarm reaction.

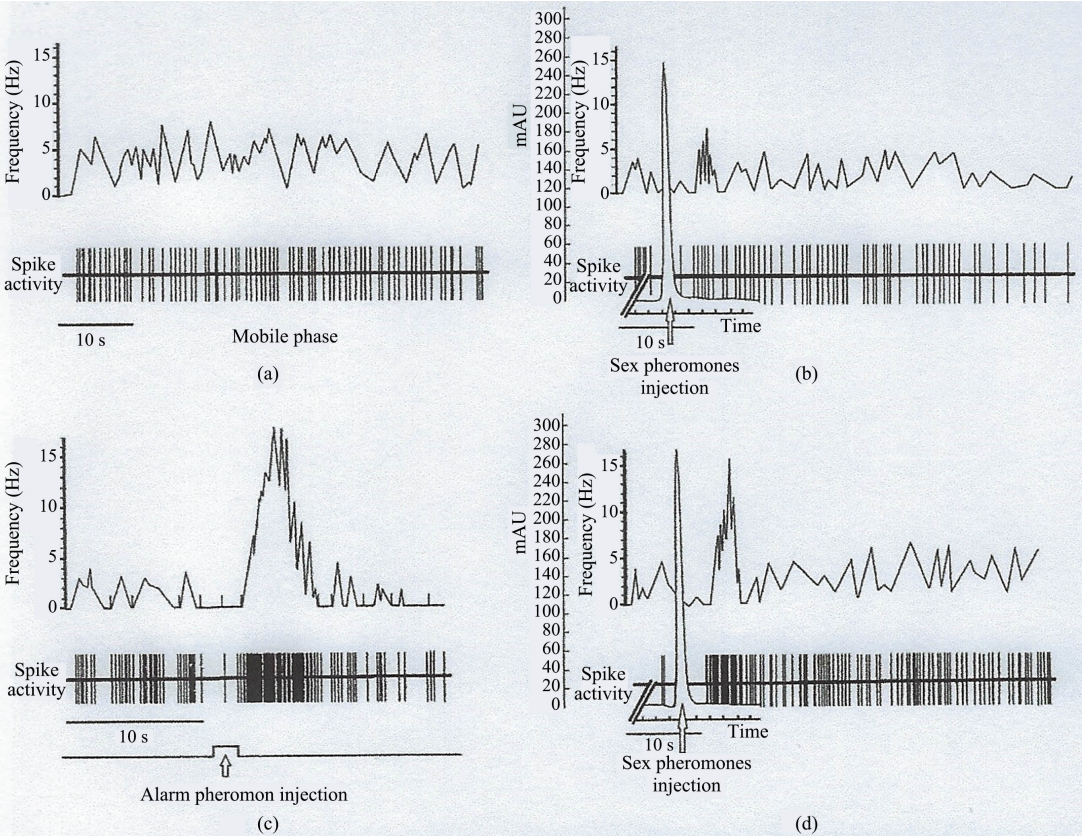

Figure 7. Nervous system activities recorded by the HPLC equipped with a DAD and an NPD. (a) A control; (b) After injection of a mixture of sex pheromones; (c) After injection of fish skin extract; (d) After injection of a mixture of sex pheromones and preexposure of the OE to 10 ppm of diazinon in APW. Figure 7 is copied from Brondz (2015).

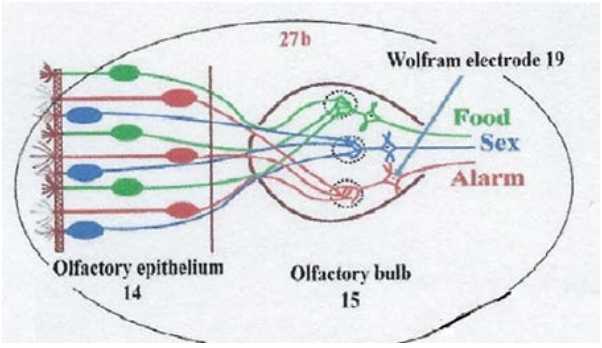

Figure 8. Bulbar neurons in the olfactory bulb (reproduced from Figure 6), the green responsible to food odorants, the blue responsible to sex pheromones, the red responsible to alarm substances. Figure 8 is copied from Brondz (2015).

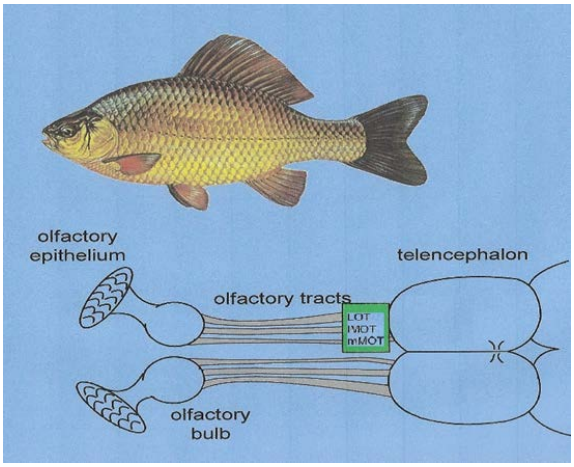

Figure 9. The olfactory tracts and differentiation by LOT, IMOT and *m*MOT is shown. The *m*MOT neurons are responsible for alarm reaction. **Figure 9** is copied from Brondz, Hamdani, & Døving (2003) provided from archives of Jupiter AS, Norway.

5.2.2. Specificity of Neurons in the Olfactory Bulb and Tracts

It was taken advantage of the specific projection of the sensory neurons to the specific regions of the olfactory bulb by recording the glomerular activity (Ressler, Sullivan, & Buck, 1994; Vassar, Chao, Siteheran, Nunez, Vosshall, & Axel, 1994; Sorensen, 1996; Nikonov & Caprio, 2001). The different nerve bundles of the olfactory tract induce different behaviors associated with feeding, reproduction, and alarm (Demski & Dulka, 1984). The olfactory tracts and differentiation by LOT, IMOT and *m*MOT are shown in **Figure 9**. The association of behavior with the nerve bundle of the olfactory tract was described in (Kyle, Sorensen, Stacey, & Dulka, 1987).

5.2.3. Comparison of Electroolfactogram (EOG), Electroantennogram (EAG) with NPD-HPLC

NPD in connection to HPLC is a more useful tool (Brondz, Hamdani, & Døving, 2004a; Brondz, Hamdani, & Døving, 2004b; Brondz, 2015) than the connections of biosensors to GC as electroolfactogram (EOG) (Ottoson & von Sydow, 1964) and electroantennogram (EAG) (Wadhams, 1990; Arn, Städler, & Rauscher, 1975). Contrary to described in EOG and EAG the temperature for biosensor in the system NPD-HPLC could be adjusted close to physiological, and dryness of biosensor is avoided. The EAG approach is popular and is in use because of its sensitivity, specificity, and lack of the alternatives. The weakness of the system NPD-HPLC is in the lack of a broad choice of columns. The columns which could use artificial pond water (APW), physiological water solutes as eluents in HPLC are in shortage. The addition of organic solvents to APW is traumatizing the biosensor and negatively influencing its sensitivity, selectivity, and all performances. However, it is possible to some extent to increase the concentration of NaCl and other neutral salts, and non-polar substances to increase the column' performance without damaging the biosensors.

6. Conclusion

The experiment has demonstrated transformation of pheromone attractive signal to nervous alarm signals **Figure 7(d)**, because of cholinesterase inhibition by diazinon, which is characteristic of all OPs.

The appearance of the peak for the alarm pheromones is after 2 s and the duration is 7 s; the appearance of the peak for the sex pheromones is after 3 s and the duration is 3 s; the appearance of the peak for the sex pheromones with the preexposure of the OE to 10 ppm of diazinon in APW is after 4 s and the duration is 5 s.

The relatively limited use of biosensors in chromatography is due to the following circumstances described below. None of the biosensors used in electroolfactogram (EOG), electroantennogram (EAG) or in NPD-HPLC cannot be prepared by manufacturers; biosensors should be made in scientific laboratories. It is the reason that industry lacks the interest in this kind of instrumentation (biosensors). The special columns to use in connection to biosensors are in restricted choice. However, unicity, high sensitivity, and high selectivity in the detection of OPs in the environment as in ponds', lakes' and rivers' water is important not only for aquatic fauna, but also for humans. Most drinking water for humans is supplied from lakes, pounds and rivers. Even the tiny concentration of OPs in drinking water for a long time can have and has effects on human health (Benmoyal-Segal, Vander, Shifman, Bryk, Ebstein, Marcus, Stessman, Darvasi, Herishanu, Friedman, & Soreq, 2005; Behan, 1996; Abou-Donia, 2010; Ray, Chatterjee, Ghosh, Kabir, Pakrashi, & Deb, 1991; Miranda-Contreras, Gómez-Pérez, Rojas, Cruz, Berrueta, Salmen, Colmenares, Barreto, Balza, Zavala, Morales, Molina, Valeri, Contreras, & Osuna, 2013; Racciatti, Vecchiet, Ceccomancini, Ricci, & Pizzigallo, 2001; Poongothai, Ravikrishnan, & Murthy, 2007). The diazinon is not an exception; all OPs showed the same pattern by more or less intensity. In the environment, sulphur atoms are oxidized and substituted with oxygen atoms. The oxidation product malaoxon was 4 (four) times more toxic than the malathion itself, about the same happens to diazinon and some other OPs containing sulphur. Demographic situation in developed countries is the result of intense use of OPs. OPs are endocrine and sexual activity disruptors (Poongothai, Ravikrishnan, & Murthy, 2007; Ray, Chatterjee, Ghosh, Kabir, Pakrashi, & Deb, 1991). The neurological problems in the population also result from the same exposure to OPs. The use of some pesticides suppresses immunity and makes the human population more vulnerable to COVID viruses and other infections (Brondz & Brondz, 2011; Brondz, 2020; Brondz, 2021).

Another important advantage of use of technology described in Brondz (2015) gives possibility to control with evidence the use of OPs by despotic governments and disclose production sites of forbidden by international law chemical and warfare agents such as Tabun, Sarin, Chlorosarin, Soman, Ethylsarin, Cyclosarin and some others.

Conflicts of Interest

The author declares no conflicts of interest regarding the publication of this paper.

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