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Application of Kohonen neural network for evaluation of the contamination of Brazilian breast milk with polychlorinated biphenyls

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ABSTRACT

Due to the tendency of polychlorinated biphenyls (PCB) to accumulate in matrixes with high lipid content, the contamination of the breast milk with these compounds is a serious issue, mainly to the newborn. In this study, milk samples were collected from breastfeeding mothers belonging to 4 Brazilian regions (south, southeast, northeast and north). Twelve PCB were analyzed by HS-SPME-GC-ECD and the corresponding peak areas were correlated to the answers to a questionnaire of general habits, breastfeeding and characteristics of the living places. To realize this exploratory analyze, self-organizing maps generated applying Kohonen neural network were applied. It was possible to verify the occurrence of different PCB congeners in the breast milk relating to the region of the Brazil that the breastfeeding lives, the proximity to an industry, the proximity to a contaminated river or sea, the type of milk (colostrum, foremilk and hindmilk) and the number of past pregnancies.

1. Introduction

Polychlorinated biphenyls (PCB) are an important class of widespread contaminants due to their high persistence in the environment and to their tendency to accumulate on soil, plants and animals and to propagate in the trophic level of food chain [1,2]. People are exposed to PCB primarily from the dietary intake (ca. 90% of the cases) [3], as well as by the contaminated atmosphere.

Children are exposed to PCB in the same way as adults but because of their smaller weight, the corresponding intake PCB per kilogram of body weight may be greater. In the case of fetuses and toddlers, the transference from mother happens via placenta blood exchange and breast milk, respectively [4], and poses a serious risk to health, since their enzymatic and metabolic systems are not yet mature [5]. The PCB concentration in breast milk is higher when compared with maternal and cord serum [6], and therefore it is the preferred matrix to evaluate human background exposure [7,8].

Taylor et al. [9] compared some data obtained from women that work in capacitor manufacturing facilities (a typical environment where PCB exposition may be high) with women that work in lower PCB exposition area. The babies whose mothers work in the first situation weighed significantly less at birth than the babies from the other mothers. Also, this high exposure to PCB was also associated with shortened pregnancy period. A survey of 128 children known to have been in uterus during or after Yu-Cheng exposure found that mean birth weight was decreased by approximately 15% compared to a control group of 115 non-exposed children [10].

Other studies concluded that neurotoxic effects of PCB and dioxins in prenatal exposure may persist into school age and may result in cognitive and motor developmental subtle delays [11,12]. Rylander et al. [13] related that the consumption of contaminated fish by girls during infancy and adolescence increases their risk of generating below-average weight babies. Some studies suggest as well that the immune system may be affected in children born and nursed by mothers exposed to increased levels of PCB [14].

In response to their adverse effects, the production and use of PCBs were restricted and banned in Europe and North America during 1970s; in Brazil, they were prohibited only in 1981 [15].

Among the principal factors that lead to the high levels of PCB in human breast milk, the most important are the lipid content of this matrix, the time delayed from the beginning of nursing, the age, the weight and number of previous pregnancies of the mother, her origin, her living place, her alimentary and smoking habits, as well as seasonal and occupational factors [2,16]. Vaclawik et al. [17] showed that age, body mass index (BMI), lactation and the consumption of fish with high fat content were all

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consistently associated with high PCB levels in adipose tissue on Danish women. LaKind et al. [18] concluded that the levels in women today can be attributed principally to accumulation during lifetime, so there are no immediate actions that women can take to reduce the exposure of their toddlers during breastfeeding breastfed infants' exposure.

Information regarding women lifestyle can be correlated to analytical data to monitor and point out possible sources of PCB contamination. However, the evaluation of such large number of possible influencing variables in the PCB content in breast milk can be difficult or even impossible without proper data treatment tools. Chemometric resources such as self-organizing maps (SOM) generated through Kohonen neural network [19] can be appropriate to these exploratory analysis, by the possibility of evaluating distribution of samples and influence of variables on a bi-dimensional graphics (maps) [20,21].

In this paper, twelve PCB in breast milk from 193 voluntary donating lactating mothers from 10 cities and towns spread through Brazil were detected, identified and quantified by headspace solid-phase microextraction (HS-SPME) combined to gas chromatography with electron capture detection (GC-ECD), and the resulting data matched to general habits, breastfeeding and environmental conditions of the living place using maps generated by Kohonen neural networks.

2. Experimental

2.1. Reagents and material

Twelve PCB standards (IUPAC numbers 28, 52, 74, 101, 118, 128, 138, 153, 156, 170, 180 and 187) were obtained from AccuStandard (New Haven, CT, USA). Methanol was obtained from Merck (Darmstadt, Germany), reagent grade NaCl from Ecibra (São Paulo, Brazil) and pesticide grade isooctane from Mallinckrodt (Kentucky, USA). Deionized water was purified through a Milli-Q system (Millipore, Bedford, MA, USA). Helium (99.999% purity) and nitrogen (99.999%) were supplied by White Martins (Rio de Janeiro, Brazil).

SPME fiber coated with 100 μm polydimethylsiloxane (PDMS) fiber were supplied by Supelco (Bellefonte, PA, USA) and fit in the appropriate holders (Supelco). Septum-sealed 16 mL glass vials were obtained from Pierce (Rockford, IL, USA). All glassware were silanized with a 10% solution of chlorotrimethylsilane in toluene as described by Potter et al. [22].

2.2. Gas chromatograph

All analyzes were performed on an AutoSystemXL GC-ECD system (Perkin-Elmer, Norwalk, CT) fitted with a HP-1MS column (30 m × 0.32 mm × 0.25 μm) and ^{63}Ni electron-capture detector. The split-splitless injector was operated in splitless mode and fitted with a suitable liner for SPME. Injector and detector temperatures were set to 280 °C and 320 °C, respectively. Helium was used as carrier gas with a flow rate of 1.3 mL/min and nitrogen was used as detector make-up gas. The column oven temperature was programmed as follows: 40 °C for 2 min, then 30 °C/min to 190 °C, hold for 5 min, then 5 °C/min to 220 °C, hold for 5 min, 20 °C/min up to 300 °C and hold for 1 min.

2.3. SPME procedure

The samples were kept frozen until use. To a 5.00 mL aliquot of sample, 1.80 g of NaCl and 210 μL of methanol were added; the sample suspension was magnetically stirred at 1200 rpm for 10 min to sample/headspace equilibration and then a 100 μm PDMS SPME fiber exposed to the sample headspace for 60 min. During sample/headspace equilibration and extraction, the vial was kept at 95 °C. All the extraction conditions were performed as defined in previous studies [23]. Immediately after extraction the fiber was withdrawn and the analytes immediately desorbed directly in the GC-ECD injection port at 280 °C for 5 min.

2.4. Study subjects

A cohort of 193 breast-feeding women from different parts of Brazil was evaluated, according to Fig. 1. The samples (mean of 20 mL) were frozen immediately after collection and kept at −20 °C until the analysis be performed.

The National Commission on Ethics in Research (CONEP) and the Ethics Committee of the Local Health Units approved the study protocol. In each Mother Milk Bank a nurse was orientated to explain and obtain informed consent from each voluntary allowing the use of samples of their milk on this work. Short questionnaires were also applied to each mother including questions related to personal features and habits, lifestyle, economic and social status, occupation and residence history, consume of water on the immediate period before breastfeeding and type of milk produced at that moment (colostrum, transition milk or mature milk).

2.5. Kohonen neural network processing

All calculations were performed using MATLAB 7.9 programming environment (the MathWorks, Natick, MA) and the public domain SOM toolbox [24].

Samples were organized according their city of origin and an exploratory analysis was performed for each resulting group in order to point out samples with a profiles. This preliminary proccessing was used to reduce the size of the data set (193 samples), selecting only samples representative the data variability; it was found the 50 samples that could be used to represent the whole original data set.

Fig. 1. Brazilian map: 1 – Florianópolis/Santa Catarina; 2 – Curitiba/Paraná; 3 - Santo Amaro/São Paulo; 4 – Belenzinho/São Paulo; 5 – Vitória/Espírito Santo; 6 – Araxá/Minas Gerais; 7 – Aracajú/Sergipe; 8 – Natal/Rio Grande do Norte; 9 – São Luis/Maranhão and 10 – Rio Branco/Acre.

This reduced sample set was organized as a matrix and the SOM was applied to the evaluation of the profile of PCB contamination in different Brazilian regions. Finally, the correlation with the maternal lifestyle was performed.

The data sets were auto-scaled along all the variables in order to have the variances of the variables normalized and their means redefined as zero. Scaling of variables is of special importance for Kohonen neural network application, since this algorithm computes the Euclidian distances between vectors. If a particular variable is much higher than its counterparts, it will dominate the map organization due to its greater impact on the measured distances. The pre-processing procedure warrants that all variables have the same importance in the map, allowing users to evaluate their significance in sample qualification [25].

The maps were linearly created and initialized. The Kohonen neural network was trained with the input data set using the batch training algorithm; in this algorithm, the whole set is presented to the map before any adjustments. The neighborhood function used in the training was a gaussian; the lattice structure was hexagonal and the map shape was planar. Two-dimensional maps with different architectures were tested, since the capacity of discrimination depends on the number of units selected to define the map. Samples assigned to the same neuron can be considered as equivalent towards the property described by the input data set; also, individuals in neighboring neurons can be designed as forming groups of samples with similar properties. The larger the distance between the neurons more different, in respect to the measured property used as data input, are the samples associated with these neurons [25].

3. Results and discussion

3.1. Kohonen treatment

The Kohonen neural network was initially applied to sample groups coming from each evaluated region to allow detection of similarities and differences within of each group. The network architecture was optimized varying the number of neurons. The most suitable combination for interpretation of sample distribution consisted on 25 neurons in 5×5 square arrangement.

Fig. 2 shows the resulting groups for each of the studied regions. It was possible to notice that many samples representing Belenzinho/ São Paulo, Paraná, Rio Grande do Norte and Santa Catarina were located in a unique neuron, where PCB were not detected. Also, in none of samples from Acre, Maranhão and Minas Gerais PCB were found; therefore, it was not necessary to build maps to these regions.

Considering that many samples have either very similar chromatographic profiles or even absence of PCB, it was possible reduce the dimension of the data matrix, retaining only 50 out of the 193 original samples for further work. Additionally, four of the targeted PCB (IUPAC numbers 28, 101, 118 and 156) were not detected in any sample. As a result, the original 193×12 matrix was reduced to 50×8 without loss of information.

The chosen samples were codded M01 to M50 (M01–M06: Belenzinho/SP; M07–M17: Santo Amaro/SP; M18–M29: Vitória/ES; M30–M37: Aracajú/SE; M38–M42: Florianópolis/SC; M43: Rio Branco/AC; M44–M46: Curitiba/PR; M47–M48: Natal/RN; M49: Araxá/MG and M50: São Luís/MA). New architectures were obtained aiming their informative distribution. Network architectures from 5×5 to 12×12 neurons were tested, being chosen a 7×7 arrangement, which allowed the best distribution of samples in the network.

Fig. 3 presents the distribution of the samples representing all regions studied, as well as the formation of the sets. The formation of these groups was analyzed taking in account the distribution maps of individual variables PCB represented in Fig. 4.

Through the scale intensity indicated on the lateral of each map in Fig. 4, it can be seen that neurons with whitish tones correspond to ones which contain samples with the higher values of that PCB. For example, the neuron in Fig. 3 which contains sample M17 suggests that this sample has high levels of PCB 74 and 153 which is consistent with the determined concentrations (2.08 μg/L and 18.4 μg/L, respectively). For this sample it is also possible to notice intermediate values for levels of PCB 52 and 180.

This procedure was done for the 50 samples studied, being possible to find those who are contaminated with specific PCB. From these results, eight distinct sample sets are evident in Fig. 3, considering the levels for each PCB as shown in Fig. 4:

- Group I: all samples containing PCB 153, as well as sporadic contamination by PCB 52, PCB 74, PCB 138 and PCB 180;
- Group II: samples contamined by PCB 180; also, some samples with PCB 153 in moderate concentrations;
- Group III: samples containing only PCB 128, PCB 138 and PCB 187;
- Group IV: higher concentrations of PCB 138 and moderate amounts of PCB 52, PCB 74 and PCB 153;
- Group V: low levels of PCB 52;
- Group VI: no PCB detected;
- Group VII: samples contaminated with PCB 52, PCB 74 and/or PCB 170; and
- Group VIII: samples with PCB 170, as well as sporadic contamination with PCB 52 and PCB 74.

Despite the incomplete separation of groups VII and VIII in this 7×7 architecture, it is interesting to maintain a compromise between the number of neurons in the architecture and the number of samples. In this case, there are 49 neurons for 50 samples. Architectures with more neurons were able to separate these two groups but, on the other hand, many well-defined groups in a 7×7 structure were divided because of the greater number of neurons to allocate the samples.

Information from Figs. 3 and 4 were cross-related to data from the questionnaires filled by volunteer milk donors. It was verified that the 5 out of 35 topics covered by the questionnaire could be related to PCB distribution and groups on the maps: type of milk, number of children, the region of origin of the sample and the industrial activity/ hydrographic basins near the place of residence. Fig. 5 is a representation of Fig. 3 considering these factors.

3.2. PCB distribution

Some considerations regarding possible correlations between environmental and personal factors and PCB distribution can be made, as follow.

3.2.1. Type of milk (colostrum × transition × mature)

When compared to mature milk, colostrum contains more lipid-soluble vitamins, minerals and approximately 10% more proteins. On the other hand, colostrum contain less lipids and water soluble vitamins: the lipid content can vary from 1–2% in colostrums compared to to 4–5% in mature milk [26]. Therefore, lipophilic compounds such as PCB are more likely to be present in the mature milk. According to Matheson et al. [27], the concentration of persistent organic pollutants (POP) increases with the amount of triglycerides in fat globules. In general, lactating women may transfer 25% of their body stocks of these substances to their children during the breastfeeding. Breastfed infants have an average daily intake of these compounds two times larger than

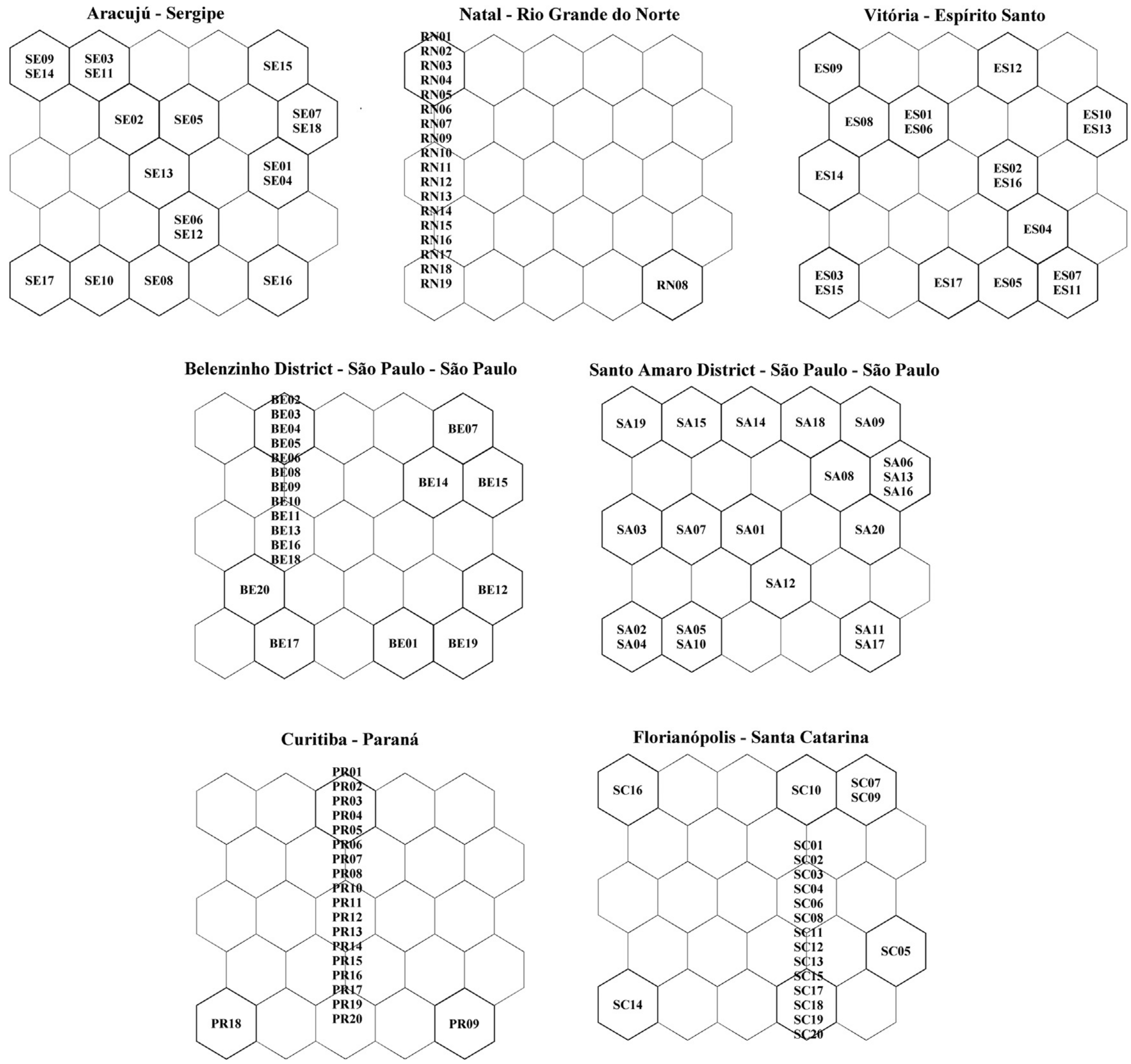

Fig. 2. Sample distribution profiles of the Brazilian cities studied according to the PCB identified and quantified, after treatment by the Kohonen neural network.

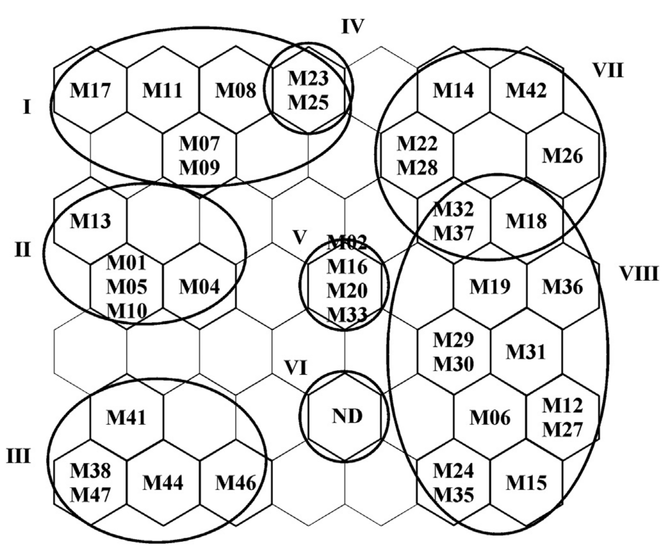

Fig. 3. General profile of distribution of all selected samples, representing all Brazilian regions studied, after treatment with Kohonen neural network. The term ND means the samples where no PCB were detected.

adults, taking in account their body weights [28]. In study with nine mothers, only two of them had decreased the concentrations of PCB 153 during the breastfeeding period. The authors suggest that the chemical concentration is mother dependent and does not appear to be related to her initial concentration [18].

In Fig. 5A, it is possible to identify how each group is composed, considering the type of milk supplied by the mother. It is important to highlight that from the 50 samples on the reduced data set, 50% consisted on mature milk, 6% on transition milk and 32% were colostrum samples (12% of the mothers did not respond the questionnaire).

Group II of Fig. 5A is composed of mature milk samples contaminated with high levels of PCB 180. This PCB has seven chlorine atoms in its molecule; it is known that the solubility on fats increases with chlorine content of the PCB. Allied to this fact, the position of the substituents makes results on steric impediment, slowing the biotransformation of this PCB compared to its correlates [29]. Therefore, the presence of higher concentrations of PCB 180 on mature milk would be expectable.

Group I is characterized by samples contaminated with PCB 153, which together with PCB 138 and PCB 180 are also commonly found in breast milk. PCB 138 and PCB 153 (groups I and IV) are chemically very

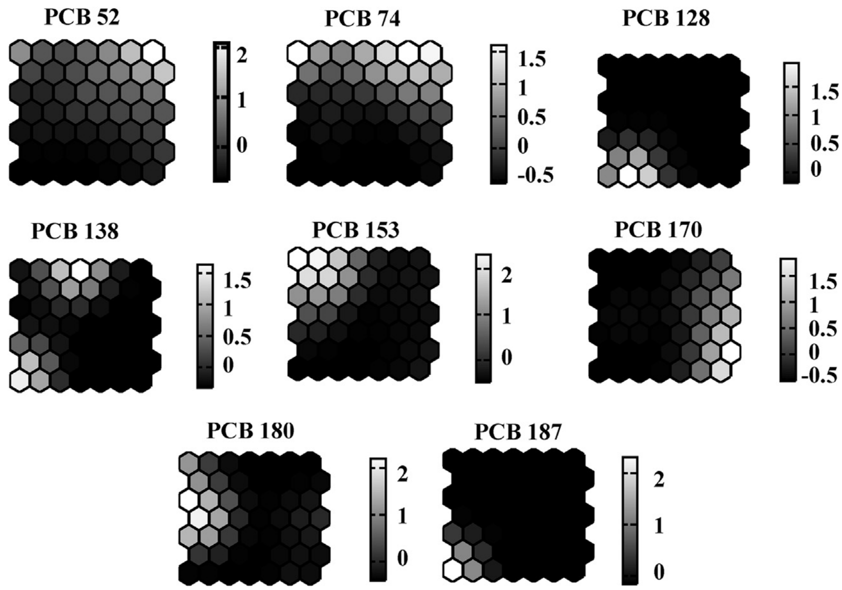

Fig. 4. Distribution maps of individual variables (PCB) obtained after treatment with the Kohonen neural network.

similar and present mainly in samples of mature milk. Group III consists mainly of colostrum milk samples, being characterized by the presence of PCB 128, 138 and 187; the first two are considered as slightly soluble in water, therefore their presence in higher concentrations than other PCB also expectable in colostrum. From 16 samples of colostrum milk, 43% were assigned to groups V and VI (samples with low levels of PCB 52 and uncontaminated samples, respectively); among samples of mature milk, only 28% are located in these groups.

3.2.2. *Number of pregnancies*

Hong et al. [30] indicate that the basic factors that affect the concentration of these compounds in milk include the age of the donor, the number of children and the duration of each breastfeeding. Other studies have shown that the excretion of these compounds during lactation is much larger than the intake of these substances with the diet [31], to such an extent that lactation can be considered as one of the major processes for removal of organochlorine compounds from the mother's body [32]. Regarding the number of pregnancies, several studies show a decrease in excretion of PCB from the first to the second pregnancy [32–35]. This author also verified if the interval between pregnancies influences in the disposal of PCB and it was found that mothers that had a short interval between the first and second pregnancy (8 months) eliminated much smaller quantities of these substances in the second pregnancy than mothers whose interval between pregnancies was two years. This shows a relatively fast re-accumulation of these compounds in tissues in two years.

In our study, most of the selected samples came from primiparous mothers (78%); these samples are the main components of groups I, II, III, IV and VI on the Fig. 5B. Samples of primiparous mothers were more prone to overall PCB contamination, but it was not possible to find a relation with the presence of specific PCB congener with this factor. This is probably because the same source of PCB that contaminated the mothers in the first pregnancy continued contaminating the milk in the second pregnancy.

Mothers were also asked about the time of breastfeeding, but only 56% of them answered this question. Hence it was not possible to establish any effective relation between this information and the maps. Also, no relation was found between the PCB levels and the age of the mothers, but several studies have shown that it tends to increase with increasing maternal age [36].

3.2.3. *Region of origin of the sample*

In this study milk samples from different regions of the country were collected, to allow the determination of a comprehensive picture of the distribution of PCB contamination. Samples from places ranging from highly industrialized and populated neighborhoods (samples from São Paulo) to sparsely populated rural cities (Araxá), coastal regions (Natal) and a city in the Amazon region (Rio Branco) were included.

According to Fig. 5C, samples from São Paulo are characterized by the presence of PCB 153 and PCB 180, which due to its highest chemical stability are also the most commonly congeners found in human milk [37–39]. Two districts from the city of São Paulo were studied, and only one sample of milk from Belenzinho was not contaminated. Regarding to the 11 samples from Santo Amaro, 85% were contaminated with PCB 153, and eventual presence of PCB 52, 74, 170 and 180 were determined. As for Belenzinho, PCB 180 was also found. According to the original data it could be seen that group I (except for overlapping samples from group IV) exclusively consists on samples from Santo Amaro and the group II is predominantly composed of Belenzinho samples. These districts are geographically close to each other, being Belenzinho in the banks of Tietê Rivers and Santo Amaro in the banks of its tributary Pinheiros River. Despite sporadic revitalization efforts both streams are highly polluted, receiving domestic and industrial sewage from São Paulo city metro area [40].

In 2005, the State Company of Technology and Environmental Sanitation (CETESB) interdicted several deep wells in the region of Santo Amaro after the confirmation of the contamination of surface water and groundwater with chlorinated substances. This is more worrying when it is considered that in this region there is one of the principal dams responsible for water distribution in the metropolitan area. Almeida et al. [41] reported that a total amount of 250,000–300,000 t of Ascarel (an industrial PCB mix formerly used as dielectric fluid in power transformers) has being used through Brazil, and a significant fraction of this product was in São Paulo metro area, which resulted on several major accidental spills in recent decades, many of them on inactive power stations.

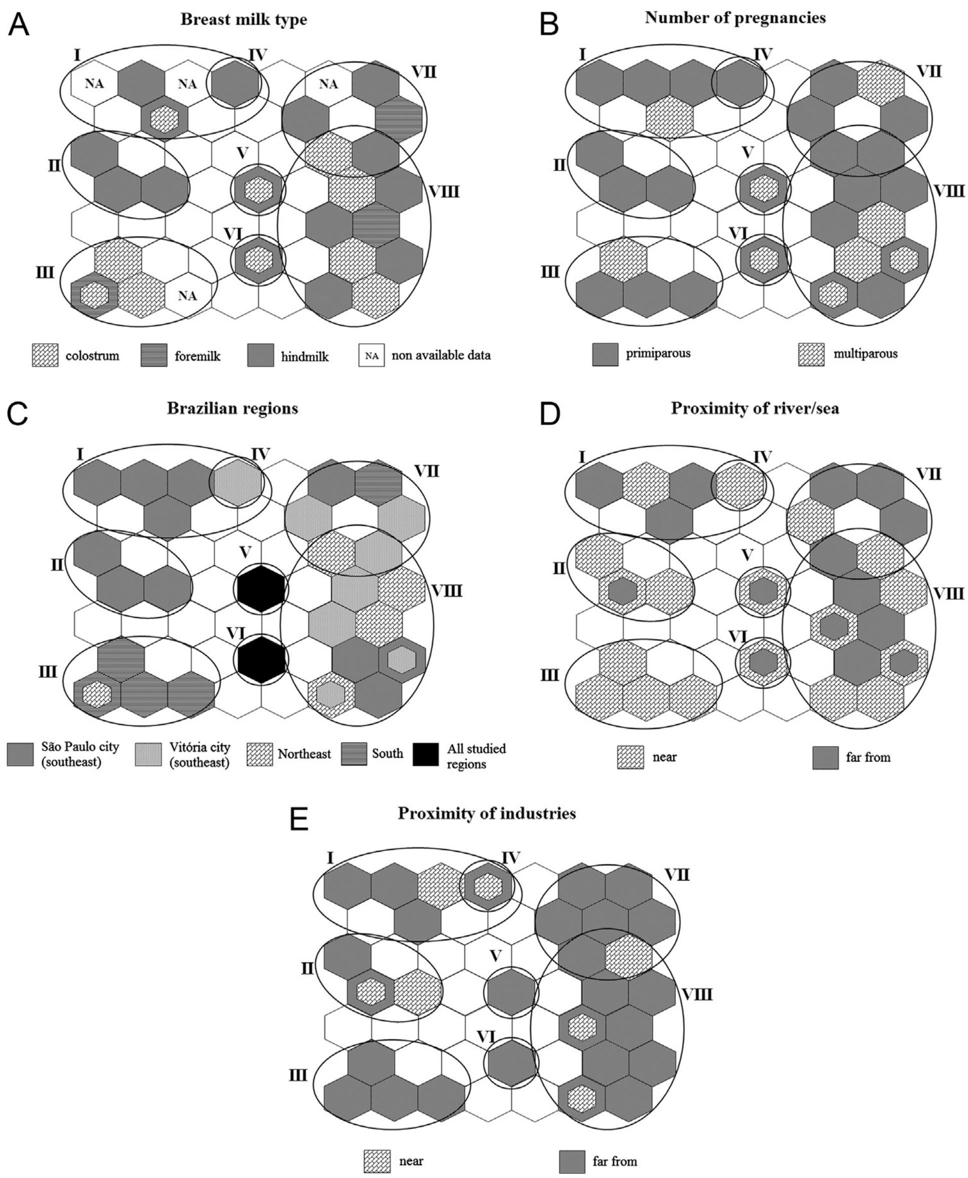

Fig. 5. Distribution profile of the answer obtained from the questionnaires on the maps of Kohonen neural network: In A, the type of milk; in B, the number of pregnancies the mother has had; in C, the region of the country where samples were collected; in D the proximity to rivers; and in E the proximity to industries.

In 1987, average PCB levels of ca. 2 mg/kg were found on samples from subcutaneous fat collected from São Paulo metro area residents, twice as much of the amount found by WHO in other industrialized cities [15]; this was attributed to the lack of efficient inspection schemes of pollution sources and inappropriate disposal of contaminated materials.

It should be pointed that, unsurprisingly, a sample from Santo Amaro (M17) was the most contaminated of the whole set, containing 28.08 μg/L of PCB 52, 74, 153 and 180. This is consistent with the correlation of the degree of industrialization and pollution level of the place and breast milk PCB contamination.

As opposed to São Paulo, Vitória is a coastal city that has two important export ports, which together with steel and metallurgical industries support the local economy. Fig. 5C shows the distribution of samples from Vitória according to their PCB contamination. It was possible to verify the presence of PCB 52 (83.3% of the samples) and 74 (66.7% the samples) from this city in group VII, as well as PCB 170 (50% of the samples) in group VIII and also PCB 138 and 153 in some samples, in a contrast to samples from São Paulo, with predominance of PCB 153 and 180. This fact can be explained considering that there is a trend of less chlorinated PCB such as PCB 52 and 74 to be present in water and air, and not on soil or sediments such is the case of the most chlorinated congeners [42]. Thereby, considering that Vitória is in a coastal area, there is a greater exposure of the local population for more water soluble or volatile PCB, possibly through the skin contact and inhalation. Other factor to be considered is the large concentration of metallurgical and siderurgical industries in Vitória. The use of Ascarel in such industries occurs mainly in closed systems, as in dielectric fluids in transformers and capacitors. In this application, the commercial blends more used are Aroclors 1242, 1254 and 1260, which have higher levels of PCB 52, 74, 138 and 170. Consistent with this fact, four of the mothers whose milk was contaminated resided near to industrial areas. Two of them (M18 and M24, Fig. 3) lives near to siderurgical industries, one (M28, Fig. 3) near to a metallurgical plant and the remainder (M23, Fig. 3) near to a tanning plant. Also, all four donors cited above live near rivers possibly contaminated by effluents from these industries.

The samples from the Brazilian northeast had a behavior similar to those from Vitória. Among those collected from Aracaju, all were located in the groups VII and VIII or presented contamination with the PCB 52, 74 and 170, supporting the argument that less chlorinated PCB are more likely to be present in surface water or groundwater, i.e., the probability to find these PCB in coastal cities or cities within large hydrographic basins is greater. The only contaminated sample from Natal (M47, Fig. 3) contained PCB 138 and 187, coming from the unique donor who lives near to an industry.

Samples from the southern Brazil (Curitiba and Florianópolis) had also a very similar behavior with the presence of PCB 128, 138 and 187. The only distinct sample was M42 (Fig. 3), contaminated with the PCB 52 and 74.

As for Araxá, Rio Branco and São Luís samples, no PCB contamination was found. São Luís is a low-industrialized, seaside touristic city; Rio Branco is located in the Amazon region, where economy is driven almost exclusively by cattle farming and Araxá also a local tourism center (thermal springs), with some niobium extraction activity. None of these cities has a history of major environmental contamination or uncontrolled industrial growth.

3.2.4. Proximity to river/sea

As seen in the previous section, all samples of groups I and II (except group IV) were donated by mothers living in the city of São Paulo, and in places on the banks of polluted rivers (Fig. 5D), as mentioned before. All donors of samples allocated in the groups III and IV also said they live near a river or sea. Although the vast majority of mothers did not answer questions about the contamination of rivers and seas near their homes, it is possible to infer that there is a strong relation between the contamination of the breast milk samples and the proximity to rivers and seas.

3.2.5. Proximity to industries

Industrial activities are significant sources of environmental and occupational exposure to PCB. Bennett et al. [43] reported that the average concentration of PCB in rural areas is approximately ten lower times than in industrial regions. Few mothers surveyed live near to industries and, among these, most are from São Paulo and Vitória. Three donors from São Paulo (M01, M04 and M08, Fig. 3) reported living near to textile, metallurgical and refrigeration industries, respectively. In Vitória it was cited proximity to steel (M18 and M24, Fig. 3), metallurgical (M29, Fig. 3) and tannery industry (M23, Fig. 3). Although this was not explicitly mentioned on questionnaires, there is a high probability that the contamination of samples from Florianópolis and Curitiba, is due to the water pollution by industrial waste discharged on rivers and sea far from the cities themselves.

4. Conclusions

Exploratory analysis has been a very important tool to assess similarities and differences in a specific group of individuals. Accordingly, the efficient approach Kohonen neural network was used to obtain information about breast milk contamination by PCB in Brazil, determined by HS-SPME-GC-ECD. It was found that newborns more exposed to these contaminants during breastfeeding are those whose mothers live in large and industrialized Brazilian cities, especially São Paulo, and/or live near polluted rivers or seas. It was also verified that greater amounts of PCB are generally found in samples of mature milk and mothers who are breastfeeding for the first time, what implies that the first child is usually more exposed than others. Thus, the chemometric treatment proposed became possible to get useful information about this environmental problem in an efficient way, what could not be performed through traditional methods.

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