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Nutritional Components and Safety of Purified Pufferfish (*Lagocephalus gloveri*) Liver Oil

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The safety of pufferfish (*Lagocephalus gloveri*) liver oil and the contents of some nutritional components were examined to obtain important information on their use as high valued functional foods. Pufferfish liver oil was extracted by the hot-water method using 1% NaOH solution to remove toxic compounds, and then purified using a general purifying method of fish oil. Any extraordinary clinical symptoms were not observed from all groups administrated with pufferfish liver oil throughout the test period. None of the rats died when administrated the highest concentration of 10 mL/kg of the pufferfish liver oil. Vitamin A content was 114.2 ppm, as a retinal equivalent in the oil extracted using hot-water, but the content was higher (169.3 ppm) in oil extracted using n-hexane. Vitamin D and E were not detected in ppm in oil extracted using hot-water. Vitamin D in the pufferfish liver oil extracted using n-hexane was also undetected, but vitamin E was at 32.5 ppm. Among the 18 minerals detected, the sodium content was the highest at 253.5 ppm, followed by 13.9 ppm of potassium, 1.5 ppm of calcium, 0.2 ppm of magnesium, and other trace minerals. The contents of EPA and DHA were 0.8% and 14.8%, respectively, in the pufferfish liver oil extracted using hot-water. Considering these results, there is potential that pufferfish liver oil could be used as a functional food.

Key words: Pufferfish liver oil, Acute toxicity, DHA, EPA

Introduction

Although pufferfish belonging to the order Tetraodontiformes has substances of peculiar toxicant, the demand is increasing on a national scale as of a singular fish species. It therefore takes large importance on the earnings of fishermen and marine industry related workers. In the mean time, the study of pufferfish liver oil has not often taken place as a source of fish oil even though the liver of pufferfish contains large amount of fatty substances. Cod liver oil contains large amounts of fatty acids. Usually omega-3 fatty acids like as EPA and DHA are found in the meats of mackerel, salmon, herring, sardines, black cod, anchovies, and tuna (Sidhu, 2003). The health benefits from consuming fish or fish oil containing omega-3 are described in a lot of literatures (Mori et al., 1986; Kinsella, 1987; Neuringer and Conner, 1987; Singh and Chandra, 1988). Although diacylmonoalkylglycerol in shark liver oil is used widely as a functional food, pufferfish liver oil could not be known as like that. If there exist useful components unknown in pufferfish liver oil, it would also be a greatly commercial product. It is the reason why the pufferfish is a unique fish containing potent tetrodotoxin. Paradoxically, toxin sometimes reverses drug when it is only used adequately, and the pufferfish liver oil might bear a greatly latent value. Pufferfish usually goes through pretreatment process to separate edible and inedible parts by professional wholesalers or fish processing factories in Korea owing to its toxicity. At pretreatment process, the amount of the separated pufferfish liver is comparatively large to those of other fishes. But there are no proper ways to exploit these sources. This paper investigated nutritional components and safety of the pufferfish liver oil to seek for possibility of its utilization as a functional food.

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Materials and Methods

Materials

The liver of pufferfish (*Lagocephalus gloveri*, black

milbog in Korean), was separated during treatment process at pufferfish processing factory (Dongnam Food Developing Corp., Kijang, Busan) and kept in freezer (-45℃) until used.

Approximate composition and VBN content of pufferfish liver

Moisture content was measured by heat-drying method in normal pressure, crude protein content by Kjeldahl method, crude lipid content by Soxhlet extraction, and ash content by laying directly in a furnace at about 525℃ (AOAC, 1990). pH was measured by pH meter (DP-135 M) with 5.0 g of liver sample mixed with 25 mL of distilled water followed by homogenization and filtering. VBN content was measured by micro-diffusion method using Conway unit (KFDA, 2002).

Preparation of pufferfish liver oil

Crude pufferfish liver oil was extracted in 1% NaOH solution by heating with hot-water to remove toxic compounds. Two liters of water containing 1% (w/w) of NaOH was added to 3 kg of pufferfish liver and heated in hot water bath for 2 hrs. Crude oil was obtained through pressured filtering of liver and hot water mixture in cotton wool bag. The crude pufferfish liver oil was kept in freezer (-25℃) and used for examination. The purification of crude pufferfish liver oil was carried out in order of degumming, deacidification, decolorizatin and deodorization which is common purifying methods for fish oils. Solvent extraction with n-hexane was also carried out to compare the differences of available nutrient contents between in pufferfish liver oils prepared by different methods, hot-water and solvent extraction. Lipid was extracted from pufferfish liver by the method of Bligh and Dyer (1959). 50 g of sample added with 150 mL of n-hexane was homogenized for 2 min. To this solution, another 50 mL of n-hexane was added and homogenized for 30 sec. The homogenized solution was suction-filtrated with Büchner funnel using Toyo (No. 5) filter paper. The eluate was filtrated again with Büchner funnel using active charcoal (Wako chemicals) to remove. Then eluate was transferred to separating funnel. Diethylether was poured and shaked to extract pure lipid and anhydrous Na_2SO_4 was used to remove any moist. Solvents used in lipid extraction were removed with vacuum evaporator under nitrogen gas stream. The purified lipids were melted with diethylether and filled up with nitrogen gas. It was kept in a freezer (-20℃) until used.

Acute toxicity testing for pufferfish liver oil

To verify the safety of purified pufferfish liver oil prepared by hot-water extraction, the only acute toxicity test was conducted (KFDA, 1999) because pufferfish toxin is known to show acute toxicity, and so subacute toxicity and chronic toxicity testing were not performed.

Animal tests

Five weeks old male and female rats of SPF SD origin were purchased from Samtako Co. (O San, Korea). The healthy ones were used for testing after one week adaptation period. Twenty rats with body weight of male 167.1±7.5 g and female 144.1±3.6 g were administrated for 2 weeks. Separation of test group and identification of rats were done by weighing healthy rats that are used in testing. Also test group was separated, so that the mean weight of each test group would almost be the same. Pelage dye marking of rats and TAG marking of cages were used for individual identification.

The temperature and relative humidity of animal chamber was kept at 22±2℃ and 53±2%, respectively. Light was given from 9 a.m. to 6 p.m., and intensity of illumination was 150-300 Lux. Rats were raised in polycarbonate cage during acclimation and inspection period, and administration period. Five of the rats were used during acclimation and inspection period, and another five of the rats were used during administration period. Solid type of animal feed was purchased from Purina Korea Ltd.. Filtered faucet water was used for drinking water and corn oil was used as control.

The oral administration was conducted with Sonde on the basis of acute toxicity test method. Amount and concentration of administration was based on "Standard Manuals of Toxicity Test of Drug etc." by Korea Food and Drug Administration (KFDA, 1999). The test material was in liquid form and maximum dosage was set up as 10 mL/kg of body weight, and the same amount of corn oil was used in control group. Separation of test group, materials and amount of administration were shown in Table 1. On the day of administration, the general symptoms were observed every hour for 12 hours. From 1st day after the administration to 14th day, the change in general symptoms, toxic symptoms, appearance, autonomic nerve, and mortality of animals were observed. All the tested animals were weighed on

Table 1. Separation of animal group for acute toxicity test

Group	Administered materials	Sex	Number of animals	Serial No. of animals	Administered amounts (mL/kg body weight)
I	Pufferfish liver oil	Male	5	1101-1105	10
		Female	5	2101-2105	10
II	Corn oil	Male	5	1201-1205	10
		Female	5	2201-2205	10

7th days and 14th day after administration. When the test was over, the survived rats were etherized and killed by bloodletting, and all internal organs were observed with unaided eyes.

Vitamins, Minerals, DHA and EPA

Vitamin A, D, and E were determined by HPLC methods in Food Official Regulation (KFDA, 2002). For analysis of minerals, the pretreatment of sample was conducted by dry-ashing method in Food Official Regulation (KFDA, 2002). The sample solution was injected into argon plasma, the concentration of target element was measured by ICP-MS (inductively coupled plasma mass spectrometry, Perkin-Elmer Sciex ELAN 6000) method. DHA and EPA was analyzed by GC (Hewlett Packard Series II 5890) on the basis of Food Official Regulation (KFDA, 2002).

Statistical analysis of the data

Significance on rat weight was verified by one-way analysis of variance (ANOVA).

Results and Discussion

Lipid content and freshness of pufferfish liver

Table 2 shows the results of liver weight and lipid content of liver measured in 10 frozen pufferfishes. Liver weight represented about 7.1% of body weight, and lipid content was ranged in level of 30.8±2.4% in the liver. Those amounts mean that the pufferfish liver has high value to use as a material of fish oil. Liver weight to body weight of fish and lipid content of the liver show great variation among fish species. In case of shark (*Carcharhinus falciformis*), the liver weight of the whole shark body weight, and lipid content in the liver were reported to be 5.1±1.1% and 40.3±3.6%, respectively (Navarro et al., 2000). Oily fish fresh contain up to 20% oil, such as 10.4 g/100 g in mackerel, 9.1 g/100 g sardine, 8.5 g/100 g Pacific herring, while non-oily fish contain under to 1%, such as 0.5 g/100 g in Pacific cod (RNI, 1991). But these vailable data could be affected by factors

Table 2. The body weight, liver weight and lipid contents in the liver of the pufferfish (*Lagocephalus gloveri*)

Specimen No.	Total length (cm)	Body weight (g)	Sex	Liver weight (g)	Lipid (%)
1	28.2	419	M	28.5	29.5
2	29.2	425	M	30.2	30.8
3	27.5	435	M	30.4	34.4
4	30.6	465	M	34.6	36.2
5	28.7	413	M	27.4	30.9
6	27.4	456	M	33.2	36.8
7	26.8	402	M	27.9	29.2
8	31.8	437	M	31.8	32.0
9	28.3	438	M	31.8	31.8
10	29.5	431	M	32.6	31.9
Mean±SD	28.8±1.5	432±19	-	30.8±2.4	32.4±2.6

Table 3. Approximate composition and VBN of raw pufferfish liver

Component	Content*
Moisture	43.5±0.1%
Protein	21.7±0.8%
Lipid	33.1±0.8%
Ash	1.6±0.1%
VBN	17.6±0.7 mg/100 g
pH	6.4±0.1

*Mean±SD.

such as fishing season, species and location of harvest. Table 3 shows the approximate composition and freshness of pufferfish liver. That is, the content of moisture, crude protein, and crude lipid was 43.5±0.1%, 21.7±0.8%, and 33.1±0.8%, respectively. The content of VBN and pH was 17.6±0.7 mg/100 g and 6.4±0.1, respectively, which means that the freshness was fairly good.

Detoxification and acute toxicity test of pufferfish liver oil

As shown in Table 4, the death of mouse was not observed from the administration of pufferfish oil. The mortality was same as described above in which means there were no death from all orally

Table 4. Mortality in SD rats administered orally with pufferfish liver oil

Treatment		Male		Female	
		Group I	Group II	Group I	Group II
Hours	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	12	0	0	0	0
Days	1	0	0	0	0
	2	0	0	0	0
	3	0	0	0	0
	4	0	0	0	0
	5	0	0	0	0
	6	0	0	0	0
	7	0	0	0	0
	8	0	0	0	0
	9	0	0	0	0
	10	0	0	0	0
	11	0	0	0	0
	12	0	0	0	0
	13	0	0	0	0
	14	0	0	0	0
Mortaliy		0/5	0/5	0/5	0/5

administrated male and female mouse. Any extraordinary clinical symptoms were not observed from the groups that were administrated with pufferfish liver oil throughout the test period (Table 5).

Any significant changes in weight were not recognized among the groups throughout the test period as shown in Table 6. Symptoms caused by administration of pufferfish liver oil were observed in various organs such as brain, kidney, heart, lung, spleen, liver, stomach, intestine, pancreas, adrenal gland, pituitary gland, testis or ovary, and other organs. Unusal symptoms were not detected in all organs of male and female rats. Therefore, purified liver oil of the pufferfish did not induce any signs of toxicity when the highest concentration of 10 mL/kg was administrated. From these results of toxicity test, the reasons why the purified pufferfish liver oil is safe are thought to be as following facts. Although liver of most pufferfish belonging to the order Tetraodontiformes is toxic showing above 10 MU/g, the liver of the species (*Lagocephalus gloveri*) used as sample material is elucidated to be non toxic (Kanoh, 1988). Additionally, toxin of pufferfish named tetrodotoxin is known to be water soluble in tissue of living body and it is also unstable in

Table 5. Clinical signs in SD rats administered orally with pufferfish liver oil

Treatmen:		Male				Female			
		Group I		Group II		Group I		Group II	
		N[1]	Signs	N	Signs	N	Signs	N	Signs
Hours	1	5	-[2]	5	-	5	-	5	-
	2	5	-	5	-	5	-	5	-
	3	5	-	5	-	5	-	5	-
	4	5	-	5	-	5	-	5	-
	5	5	-	5	-	5	-	5	-
	6	5	-	5	-	5	-	5	-
	12	5	-	5	-	5	-	5	-
Days	1	5	-	5	-	5	-	5	-
	2	5	-	5	-	5	-	5	-
	3	5	-	5	-	5	-	5	-
	4	5	-	5	-	5	-	5	-
	5	5	-	5	-	5	-	5	-
	6	5	-	5	-	5	-	5	-
	7	5	-	5	-	5	-	5	-
	8	5	-	5	-	5	-	5	-
	9	5	-	5	-	5	-	5	-
	10	5	-	5	-	5	-	5	-
	11	5	-	5	-	5	-	5	-
	12	5	-	5	-	5	-	5	-
	13	5	-	5	-	5	-	5	-
	14	5	-	5	-	5	-	5	-

[1]Number of animals examined, [2]Number abnormality detected.

Table 6. Changes of body weight in SD rats administered orally with pufferfish liver oil

Sex	Group	Body weight (g)		
		0	7 days	14 days
Male	I	167.53±9.39*	241.69±15.76	297.90±25.54
	II	166.73±6.23	238.57±8.84	293.07±14.17
Female	I	144.59±3.54	185.36±4.9	206.17±6.12
	II	143.56±3.96	186.46±5.76	210.44±7.99

*Mean±SD (n=5).

alkali condition. Even if the toxin might have existed in raw liver, the toxin in the purified pufferfish liver oil of final product seemed to be removed toward water fraction during separation process of water and oil, or destroyed through hot water extraction by 1% NaOH solution, and also to be broken down during purifying processes including degumming, deacidification, decolorization and deodorization. These processes were carried out in the conditions of degumming by strong alkali or deodorization by heating with hot vapor at high temperature of 180℃ for 1 hour in a vacuum equipment maintained about 4 mmHg. It is concluded that there were not obvious signs of toxicity by adminstration of pufferfish liver oil for two weeks in all groups of rats, and the detoxification of the liver oil was achieved sufficiently during processes of extraction and purificaton of pufferfish liver oil.

The effect of cooking, including washing in running water and heating in about 3% sodium bicarbonate solution, on the removal of toxicity from pufferfish liver was investigated, and twenty-one samples of poisonous liver with toxicity levels from 61 MU/g to 1,270 MU/g was reduced to less than 5 MU/g (Tsubone et al., 1986). The research group also examined the effect of heat on toxin of pufferfish, tetrodotoxin, and reported that the detoxification ratio of pufferfish toxin was affected extremely by acid-alkali degree of heating solution and temperature (Fuchi, et al., 1986).

In the United States, FDA has estimated that consumption of up to 3 g/day of combined DHA and EPA in menhaden oil is safe for adult person, and Institute of Medicine (IOM) published a recommended Adequate Intakes (AI) of 0.5 g omega-3 polyunsaturated fatty acids (PUFAs, including DHA) /day for infants (Kroes et al., 2003).

Available nutrients of pufferfish liver oil

The nutritional benefits of fish consumption are due to the presence of proteins of high biological value, unsaturated essential fatty acids, minerals (calcium, iron, selenium, zinc, etc.), and vitamins, namely A, B_3 (nicotinamide), B_6 (pyridoxine), B_{12} (cobalamine), E (d-tocopherol), and D in fish tissue (Sidhu, 2003). In recent years, increasing attention has been paid to the role of fat-soluble vitamins with possible cancer-preventing activity (Luterotti et al. 1999). For example, vitamin A is important for key biological functions such as cell growth and differentiation, maintenance of epithelial integrity and normal immune function (Ross and Gardner, 1994). Consumption of fish oil enhances antioxidative defenses against the oxidative stress imposed by hypercholesterolemia, and vitamin E further enhances these beneficial effects (Hsu et al., 2001). Figs. 1-3 show the examined results of vitamin A, D, and E from purified fish oil. Vitamin A showed its peak at 5.4 min, and its content was 114.2 ppm in the pufferfish liver oil extracted hot-water and 169.3 ppm extracted using n-hexane when calculated as retinol. In the case of vitamin D, vitamin D_3 was set as standard and was calculated its content. But using both hot-water and n-hexane extraction, the contents of vitamin

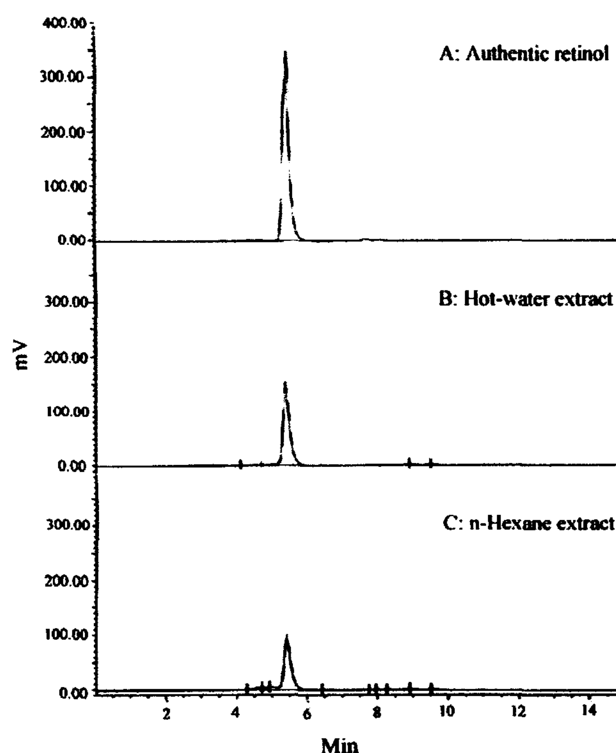

Fig. 1. HPLC chromatograms of vitamin A (retinol) in pufferfish liver oil.

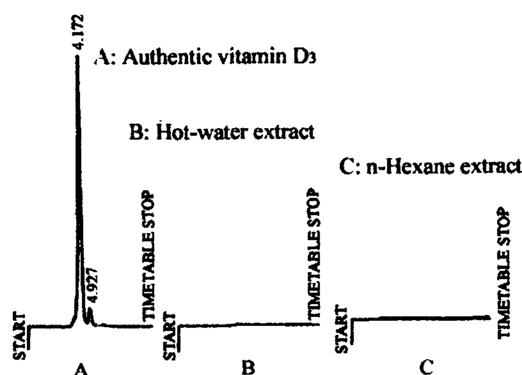

Fig. 2. Chromatograms of vitamin D_3 in pufferfish liver oil.

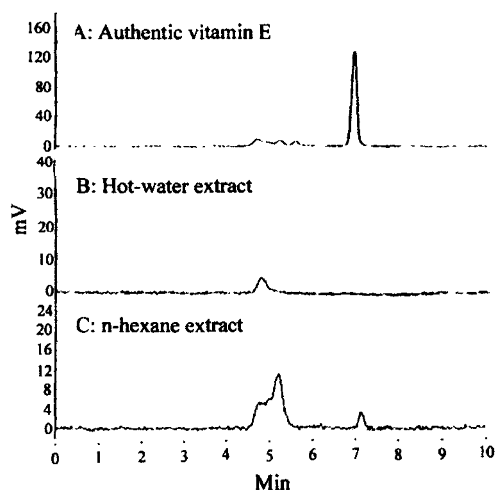

Fig. 3. Chromatograms of vitamin E in pufferfish liver oil.

D were not able to be detected at level of ppm unit. In the case of vitamin E, when the pufferfish liver oil of same sample was extracted using n-haxane as solvent, 32.5 ppm was detected. But vitamin E was extremely small amount that could not be detected in the pufferfish liver oil extracted using hot-water. Therefore, in the case of using hot-water extraction, vitamin D and E need to be added as necessity of the products. In a recently published report (Herrera, et al., 1998), calcium and fish oil supplementation significantly reduced the risk factor of pre-eclampsia in women. Contents of minerals were also determined in the pufferfish liver oil (not shown as Table). Among the 18 kinds of minerals analyzed, sodium (Na) content was highest as 253.5 ppm, followed by potassium (K), calcium (Ca), magnesium (Mg) each with 13.9 ppm, 1.5 ppm, 0.2 ppm, respectively. Contents of other 14 kinds of minerals determined (Al, Cd, Co, Cr, Cu, Fe, Mn, Mo, Ni, Pb, Ti, V, Zn, Zr) were trace.

Fish oils are now regarded as excellent sources of polyunsaturated fatty acids. The health benefits from consuming fish or fish oil containing omega-3 are described in a lot of literatures. In spite that Greenland Eskimos intake a large of fat and cholesterol, the consumption of fish protected them against heart disease (Bang and Dyerberg, 1980; Dyerberg et al., 1975; Sidhu, 1993). Several studies have shown a relationship between fish oil consumption and reduced risk of heart disease (Bang and Dyerberg, 1980; Dolocek and Grandits, 1991; Stansby, 1990). Fish oil intake has been associated with a low incidence of diabetes mellitus (Rustan et al., 1997). Omega-3 fatty acids improve many metabolic squelae of insulin resistance in human by lowing hypertension and plasma triglyceride (Berry, 1997). Burdge(1998) reported that DHA may be needed for optimal neurological development and physiological functions of brain. The fish species rich in omega-3 fatty acids are mackerel, herring, salmon, sardine, anchovy, bass, bluefish, trout, and tuna (Sidhu, 2003). On the other hand, Bakes and Nicholas (1995) have reported that the liver oils from six species of deep-sea sharks collected in southern Australian waters had monounsaturated fatty acids ($C_{16:1}$, $C_{18:1}$, $C_{20:1}$, $C_{22:1}$ and $C_{24:1}$) comprised 62-84% of the fatty acids and high squalene content (50-82% of oil) in four species, while polyunsaturated fatty acids were relatively minor components (1-13%). In addition, in other paper they also reported that two kinds of sharks (*Centroscymnus crepidater* and *Etmopterus granulosus*) were found to be rich in diacylmonoalkylglycerols along with high content of monosaturated fatty acids, while DHA was in very low levels (0.6 and 0.7%) and EPA was not detected in either species. Fig. 4 shows the peaks of DHA and EPA of the purified pufferfish oils. EPA and DHA contents were 0.8% and 14.8%, respectively. KFDA (2002) specifies that DHA/EPA Foods have to containe above 12% level as total sum of DHA and EPA contents. As mentioned above, the purified liver oil from the pufferfish (*Lagocephalus gloveri*) using traditionally hot water extraction is not toxic oil, and it contains 15.6% as combined sum of DHA and EPA of oil as well as 114.2 ppm of natural vitamin A and minerals such as sodium, potassium, calcium and magnesium abundantly. In conclusion, these characteristics of pufferfish liver

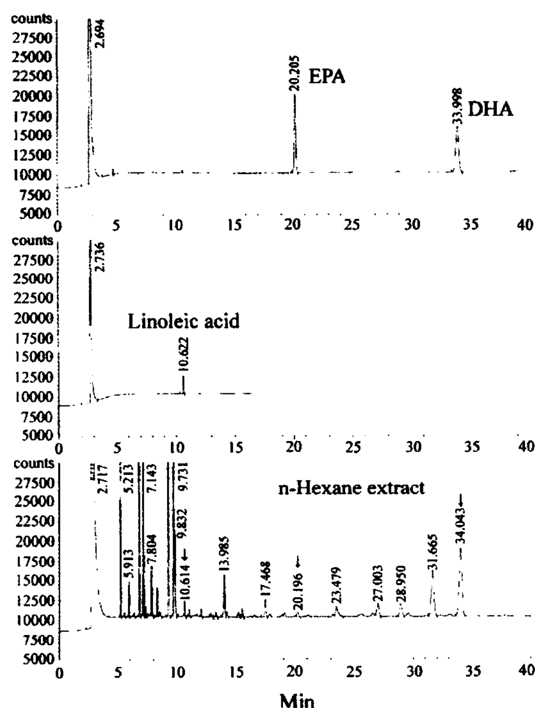

Fig. 4. GC chromatogram for the EPA, DHA and linoleic acid of puffer liver oil.

oil can be utilized to promote more efficient use of the oil as a source of functional foods.

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