DETERMINATION OF FAT SOLUBLE VITAMINS FROM AMAZONIAN FRESH-WATER FISHES.1. HPLC ANALYSIS OF TAMBAQUI, PIRARUCU AND CUIU-CUIU LIVERS.

F. Marx (*)
J. G. S. Maia (**)

SUMMARY

It was found that fish livers from the Amazon have considerable amounts of vitamins A, D and E compared with the values of the standardized cod-liver oil. Tambaqui liver oil has high concentration of vitamin A_1 (retinol) and vitamin A_2 (dehidroretinol) whereas the liver oils of pirarucu and cuiu-cuiu have preferently the vitamin A_2. The contents of the vitamins D and E observed in the liver oils of tambaqui and cuiu-cuiu was extremely high.

INTRODUCTION

More than 2000 different species of fishes live in the waters of the Amazon region. The actual yield of the commercial fishery from the middle and upper Amazon is about 100,000t (Saint-Paul, 1981). Generally the livers of these fishes are thrown away. But it is well known that fish livers may have a considerable amount of fat soluble vitamins. Cod-liver oil, for instance, is rich in vitamins A, D and E. This oil is used worldwide prophylactically against rickets and other deficiency deseases. Therefore it would be of great interest to the pharmaceutical industry of Brazil, if fish-liver oil from the Amazon could substitute cod-liver oil.

In the relevant literature we could not find any publication about the vitamin contents of Amazonian fish. Therefore, a few years ago we initiated a survey of the content of vitamins A, D and E in the livers of the principal fish species commercialized in the Manaus Markets. Colorimetric estimation and U. V. absorption were used as a preliminary analysis of these vitamins (Mourão **et alii**, 1976). Thereon we chosed two of the most important and largest edible scaled fish, tambaqui (**Colossoma macropomum**) and pirarucu (**Arapaima gigas**) besides cuiu-cuiu (**Oxidoras niger**) an armoured catfish, for an accurate vitamin determination. The pirarucu of the Brazilian Amazon, was probably the

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(*) Institute of Food Science, University of Boon, Germany.
(**) Instituto Nacional de Pesquisas da Amazônia, CNPq, Manaus, Brazil.

most important commercial fish species of inland Amazônia until about 1970, when it was replaced by the tambaqui (Goulding & Carvalho, 1982). Catch data of reasonable accurate were collected for the first time in 1976, and in that year the tambaqui accounted for about 44 percent of total catch of 30,800 tons landed at the principal Market of Manaus (Petrere, 1978; Smith, 1979).

There exist well-established methods for the determination of fat-soluble vitamins by separate operations, but generally they are time-consuming and in many cases are dificcult to perform. The new method of high-performance liquid chromatography (HPLC) has made the analysis of fat soluble vitamins faster, easier, more reliable and there are some recent publications about the determination of vitamin A (Ranfft & Rückermann, 1978; Frolik **et alii**, 1978), of vitamin D (Vanhaelen-Fastri & Vanhaelen, 1978; Cohen & Lapointe, 1979) and of vitamin E (Gertz & Herrmann, 1982; Carpenter, 1979) using the HPLC method. With the aid of reversed phase (RP-HPLC) it is possible to separate and quantify each of the fat soluble vitamins, simultaneously. Vitamin A,D,E and β-carotone have been determined in vegetable oils, dietetic foods, infant formulas and milk powders by Mankel (1979), using the RP-HPLC method. Therefore the choice of the RP-HPLC method for our vitamin studies in fish livers was highly recommended.

EXPERIMENTAL

The HPLC instrument was a 8500 series (Varian), equipped with a loop injector(Valco), a variable wavelenght UV detector Variochrom (Varian) and a dual-pen recorder coupled with a microprocessor CDS-111 (Varian). The chromatographic column was a stainless steel tube (25cm x 4mm I.D.) packed with ODS/C18, 7µm (Latek, Heidelberg) protected by a precolumn (5cm x 4mm I.D.) packed with the same material. The mobile phase consisted of methanol-water (85:15). Three minutes after sample injection a linear gradient program was initiated at 1%/min to 100% methanol. The flow-rate was 100ml/h. The UV-detector was set at 280nm.

Sample preparation: 1g liver oil or 10g well ground fish liver (both ice maintained was accurately weighed into a 250ml amber roundbottom flask. 30ml ethanol, 1ml of a 10% ascorbic acid solution and 2ml of a 50% KOH solution were added and the mixture was refluxed under nitrogen during 30min. The mixture still hot, was mixed with 10 ml water and transferred to an amber separating funnel. After rewashing with 20ml of cold water and cooling to room temperature the mixture was extraxted three times with 25 ml light petroleum b.p. 40-60°C. Which contained 0,02% BHT. The combined light petroleum phases were washed with water until neutral, dried in the presence of anhydrous sodium filtered and evaporated with nitrogen to dryness. A standard solution with exactly 5ml of methanol was made before injection of 20µ onto the HPLC column. Because of the light sensitivity of the fat soluble vitamins, the extraction procedures were carried out in a laboratory with dimmed light. The samples and standards were kept in ambar glassware,

for no more than a week, in a refrigerate environment, in order to avoid possible vitamin degradation, solvent evaporation or other adverse effects.

Vitamin A_2 (3-dehydroretinol) was identified by its mass spectrum after collecting the relevant HPLC eluent-fraction of the fish liver samples. The amount of vitamins were calculated by comparing the heights of the standard peaks with the corresponding sample peaks in the HPLC chromatogram and by integration data from the microprocessor used.

The vitamin standards used were retinyl acetate, vitamin D_2 and α-tocopherol (Merck, biochemical grade). Retinol was prepared by saponification of retinyl acetate. The reagents used were 2,6-di-tert-butyl-4-methylphenol (BHT) (Aldrich) and potassium hydroxide (Merck, p.a.). All the solvents used were analytical grade (Merck, Darmstadt).

RESULTS AND DISCUSSION

The HPLC conditions used permit the desired separation of the vitamins A_1 (retinol) A_2 (dehydroretinol), D and α-tocopherol. The separation of the vitamins A_1 and A_2 is important because vitamin A_2 has only 40% of the biological activity in comparison with vitamin A_1. Fresh water fish-liver is the only natural source of vitamin A_2 (Neumüller, 1977). The vitamins D_2 (ergocalciferol) and D_3 (cholecalciferol) were not distinguished under these conditions, but in nature the source of vitamin D is almost exclusively cholecalciferol. The UV absorption maxima of vitamins D_2 and D_3 are very similar and the biological activity is the same. Therefore, it is not necessary to separate the two forma of vitamin D. α-tocopherol is biologically the most active naturally occuring isomer of vitamin E and it was the only isomer quantified in our studies.

Figures 1 and 2 show HPLC chromatograms of typical sample analysis of fish livers. Between the elution of vitamins A_2 and D the recorder-sensitivity was increased fourfold in order to obtain reliable peak heights for vitamins D and E.

The relationships of peak height and the amounts of the standard vitamins were linear in the concentration rangers used. Recoveries were estimated by spiking liver samples with adequate amounts of retinol and α-tocopherol before homogenization, and taking these samples through the entire procedure. The mean recoveries were 102% for retinol and 77% for α-tocopherol.

Table 1 shows the quantities of vitamins found in the analyzed fish samples. It demonstrates that fish livers from the Amazon really have considerable amounts of vitamins compared with the values of the standardized cod-liver oil. The concentration ratios of vitamin A_1 and A_2 varied considerably from species to species and even between the spcimen analyzed. This is in accordance with Goswani & Barua (1981) who found that the ratio vitamin A_1/A_2 depends upon many factors like the species, the colour, the state of nutrition, the migration behaviour, etc.

The industrial exploration of tambaqui-liver oil to obtain vitamin concentrates

could be of some interest, because this species reaches at least one meter in total lenght and 30kg in weight. Extraction of a tambaqui liver (44g in media) with chloroform yielded an oil content of 7,7%. As can be seen in Table 1, the vitamin contents of this oil were extremely high. The concentration of the vitamins A and E in the cuiu-cuiu liver was very high too. This species reaches 1.20m in total lenght and 30kg in weight.

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RESUMO

Foi descoberto que os fígados de peixes da região Amazônica possuem quantidades apreciáveis das vitaminas A, D e E, em paralelo com os valores padronizados para o óleo de fígado de Bacalhau. O óleo do fígado de tambaqui apresentou elevada concentração de vitamina A_1 (retinol) e vitamina A_2 (dehidroretinol), enquanto que nos óleos de fígados do pirarucu e cuiu-cuiu ocorre, preferentemente, a vitamina A_2. O teor em vitaminas D e E observado nos óleos de fígados de tambaqui e cuiu-cuiu foi extremamente alto.

Table 1. Fat soluble vitamins in fish-livers from the Amazon.

SPECIES	Common Name	Sample	Vitamins (µg/g) A_1	A_2	D	E
Colossoma macropomum	Tambaqui	1	2220	1300	46	112
		2	n.d.[1]	n.d.[1]	10	88
		3	1200	960	14	50
		4	1310	1060	13	n.d.[1]
Colossoma macropomum	Tambaqui	Liver Oil	2850	4900	403	2300
Arapaima gigas	Pirarucu	1	tr[2]	303	3	24
Oxydoras niger	Cuiu-cuiu	1	649	2420	146	854
Cod-liver oil[3]		1	tr[2]	2780	183	995
		2	300	-	3	33

[1]Not determined

[2]Trace

[3]Mean Values as cited by Belitz and Grosch (1982)

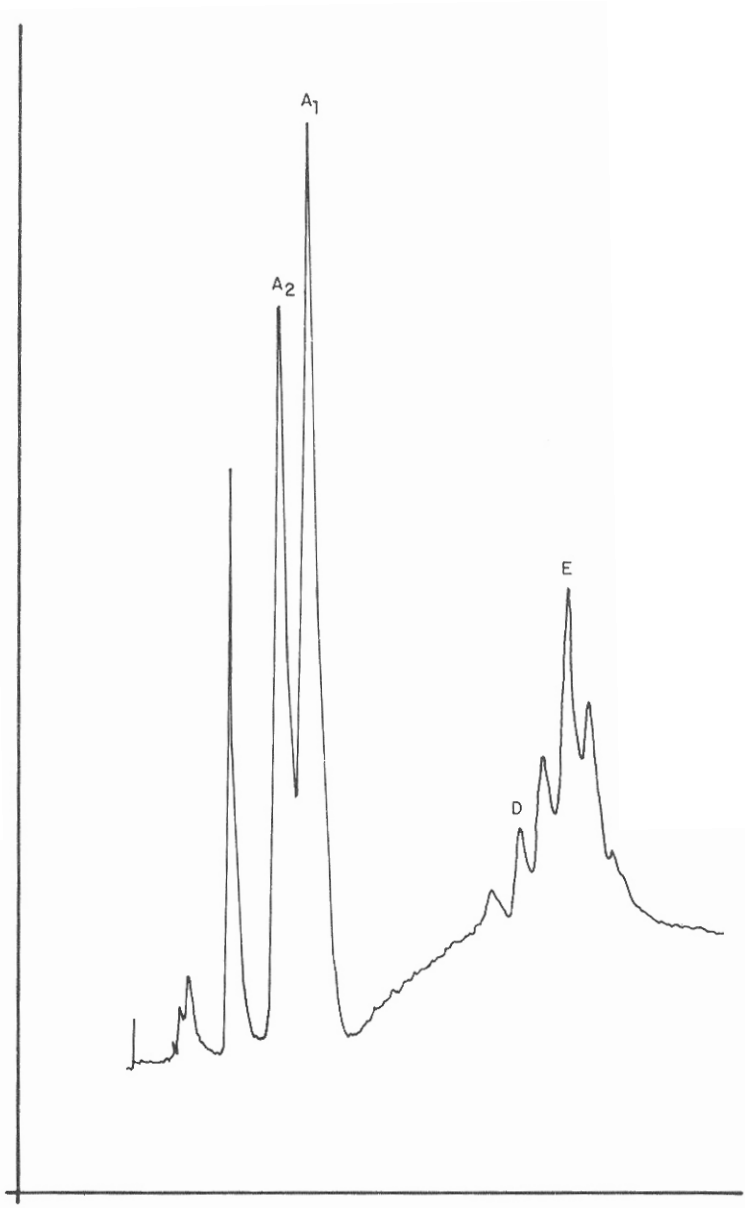

Fig. 1 - HPLC chromatogram of fat-soluble vitamins in tambaqui liver (conditions see Experimental)

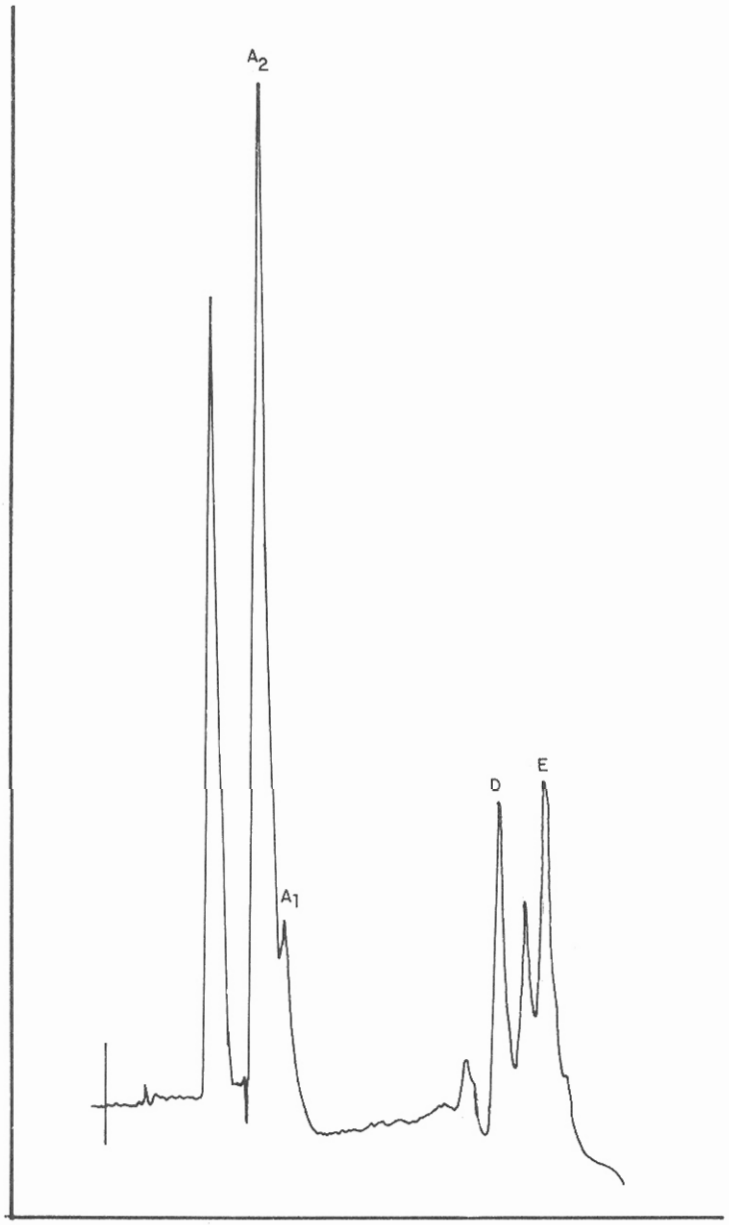

Fig. 2 - HPLC chromatogram of fat-soluble vitamins in cuiu-cuiu liver (conditions see Experimental)

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