

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

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**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<sub>1</sub> (retinol) and vitamin A<sub>2</sub> (dehidroretinol) whereas the liver oils of pirarucu and cuiu-cuiu have preferently the vitamin A<sub>2</sub>. 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 diseases. 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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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 accuracy 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 difficult 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; Frolík *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  $\beta$ -carotene have been determined in vegetable oils, dietetic foods, infant formulas and milk powders by Mankei (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 wavelength 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, 7 $\mu$ m (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 extracted 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 20 $\mu$  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 amber 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<sub>2</sub> (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<sub>2</sub> and  $\alpha$ -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<sub>1</sub> (retinol), A<sub>2</sub> (dehydroretinol), D and  $\alpha$ -tocopherol. The separation of the vitamins A<sub>1</sub> and A<sub>2</sub> is important because vitamin A<sub>2</sub> has only 40% of the biological activity in comparison with vitamin A<sub>1</sub>. Fresh water fish-liver is the only natural source of vitamin A<sub>2</sub> (Neumüller, 1977). The vitamins D<sub>2</sub> (ergocalciferol) and D<sub>3</sub> (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<sub>2</sub> and D<sub>3</sub> are very similar and the biological activity is the same. Therefore, it is not necessary to separate the two forms of vitamin D.  $\alpha$ -tocopherol is biologically the most active naturally occurring 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<sub>2</sub> 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  $\alpha$ -tocopherol before homogenization, and taking these samples through the entire procedure. The mean recoveries were 102% for retinol and 77% for  $\alpha$ -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<sub>1</sub> and A<sub>2</sub> varied considerably from species to species and even between the specimen analyzed. This is in accordance with Goswani & Barua (1981) who found that the ratio vitamin A<sub>1</sub>/A<sub>2</sub> 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 length 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 length 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<sub>1</sub> (retinol) e vitamina A<sub>2</sub> (dehidroretinol), enquanto que nos óleos de fígados do pirarucu e cuiu-cuiu ocorre, preferentemente, a vitamina A<sub>2</sub>. 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 <sub>1</sub>	A <sub>2</sub>	D	E
<i>Colossoma macropomum</i>	Tambaqui	1	2220	1300	46	112
		2	n.d. <sup>1</sup>	n.d. <sup>1</sup>	10	88
		3	1200	960	14	50
		4	1310	1060	13	n.d. <sup>1</sup>
<i>Colossoma macropomum</i>	Tambaqui	Liver Oil	2850	4900	403	2300
<i>Arapaima gigas</i>	Pirarucu	1	tr <sup>2</sup>	303	3	24
<i>Oxydoras niger</i>	Cuiu-cuiu	1	649	2420	146	854
Cod-liver oil <sup>3</sup>		1	tr <sup>2</sup>	2780	183	995
		2	300	-	3	33

<sup>1</sup>Not determined

<sup>2</sup>Trace

<sup>3</sup>Mean Values as cited by Belitz and Grosch (1982)

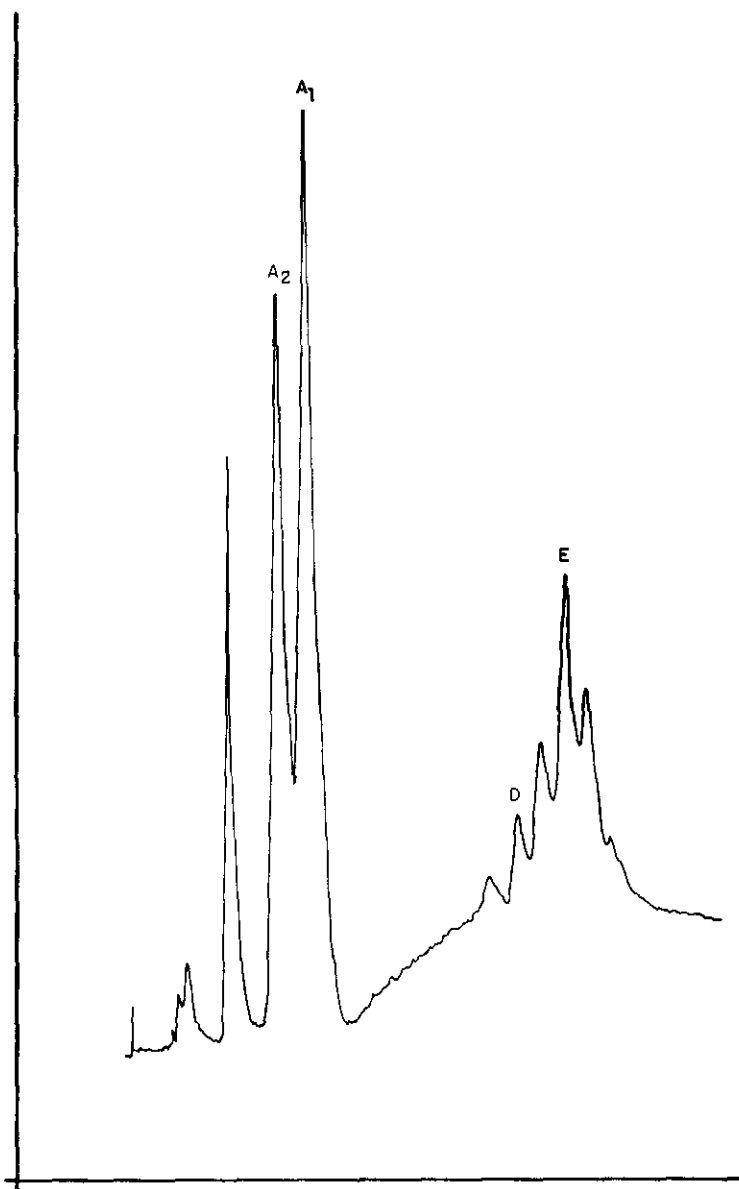


Fig. 1 - HPLC chromatogram of fat-soluble vitamins in tambaqui liver (conditions see Experimental)

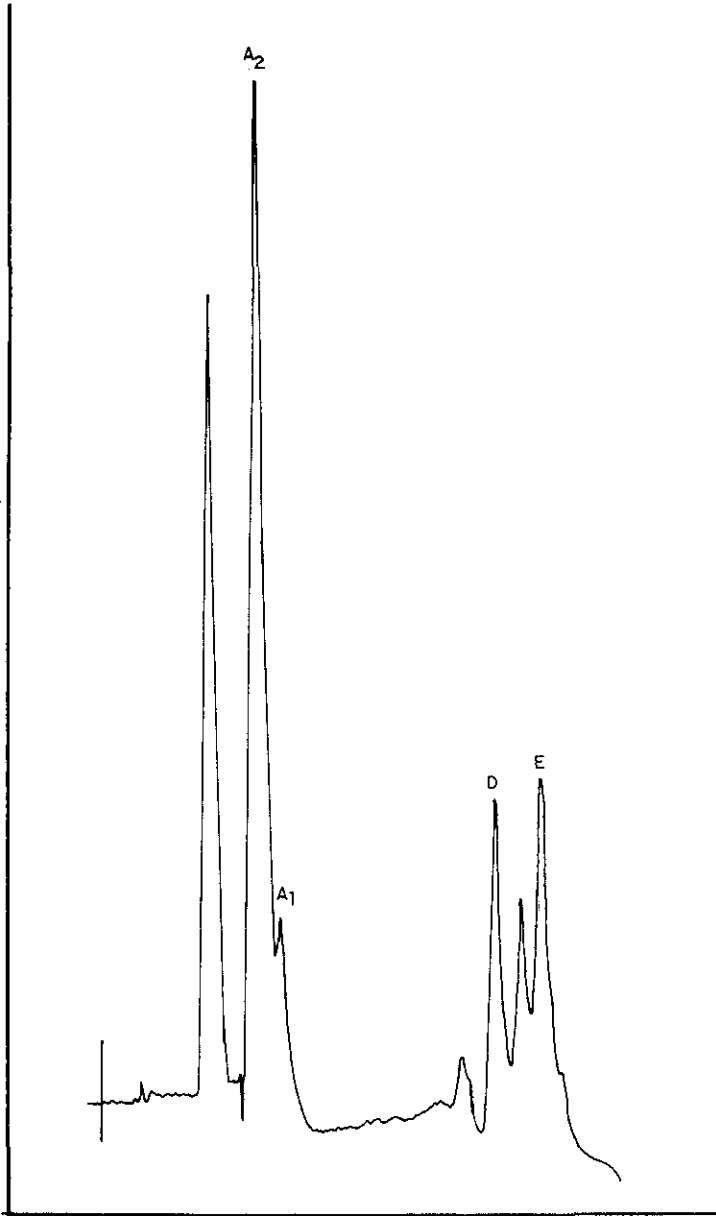


Fig. 2 - HPLC chromatogram of fat-soluble vitamins in cuiu-cuiu liver (conditions see Experimental)

## Referências bibliográficas

- Belitz, H. D. & Grosch, W. - 1982. **Lehrbuch der Lebensmittelchemie**. Berlin, Springer Verlag.
- Carpenter, A. P. - 1979. Determination of tocopherols in vegetable oils. **J. Am. Oil Chem. Soc.**, 56: 668-671.
- Cohen, H. & Lapointe, M. - 1979. Quantitative analysis of vitamin D<sub>3</sub> in a feed using normal phase high performance liquid chromatography. **J. Chromatogr. Sci.**, 17: 510-513.
- Frolic, C. A.; Tavela, T. E.; Sporn, M. B. - 1978. Separation of the natural retinoids by high-pressure liquid chromatography. **J. Lipid Research**, 19: 32-37.
- Gertz, C. & Herrmann, K. - 1982. Analysis of tocopherols and tocotrienols in foods. **Zeitschrift für Lebensmitteluntersuchung und-forschung**, 174: 390-94.
- Goswami, W. C. & Barua, A. B. - 1981. Distribution of retinol and dehydroretinol in freshwater fish. **Indian J. Biochem. & Biophys.**, 18: 383-385.
- Goulding, M. & Carvalho, M. L. - 1982. Life history and management of the tanbaqui (*Colossoma macropomum*, Characidae): An important Amazonian food fish. **Rev. Bras. Zool.**, 1 (2): 107-133.
- Mankel, A. - 1979. Quantitative Bestimmung fettlöslicher Vitamine durch Hochdruckflüssigkeits-chromatographie. **Deutsche Lebensmittelrundschau**, 75: 77-85.
- Mourão, A. P.; Maia, J. G. S.; Albuquerque, H.; Wolter Filho, W. - 1976. **Analysis of the fat soluble vitamins in liver oils of 27 fish species of the Amazon**. Unpublished results.
- Neumüller, O. A. - 1977. **Römpps Chemie-Lexikon Francksche Verlagsbuchhandlung**. Stuttgart.
- Petrere Jr., M. - 1978. Pesca e esforço de pesca no Estado do Amazonas. II. Locais de pesca, aparelhos de captura e estatísticas de desembarque. **Acta Amazonica**, 8(3)Supl. 2: 1-54.
- Ranfft, K. & Rückermann, H. - 1978. Methods for determination of vitamins by high performance liquid chromatography. II. Determination of vitamin A. **Z. Lebensm. Untersuch. Forsch.**, 166: 13-14.
- Saint-Paul, U. - 1981. Fischzucht in Amazonien. **Naturwissenschaftliche Rundschau**, 34: 58-64.
- Smith, N. J. H. - 1979. **A pesca no Rio Amazonas**. Manaus, INPA.
- Vanhaelen-Fastri, R. & Vanhaelen, M. - 1978. High performance liquid chromatographic determination of potencial content of vitamin D<sub>2</sub> (ergocalciferol) and vitamin D<sub>3</sub> (cholecalciferol) in resins, oils, dry concentrates and multivitamin formulas. **J. Chromatogr.**, 153: 219-226.

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