

# Catalytic Determination of Cobalt in Liver by Flow-Injection Spectrophotometry

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Um sistema de análise por injeção em fluxo foi projetado visando a determinação espectrofotométrica de cobalto em fígados. O método fundamenta-se no efeito catalítico do cobalto na oxidação do reagente Tiron pelo peróxido de hidrogênio. Alguns parâmetros tais como pH da reação, força-iônica, temperatura, vazões, adição de mascarantes químicos, possibilidade de discriminação cinética e concentração de reagentes, foram investigados. O sistema processa 40 amostras por hora, fornecendo resultados com precisão superior a 99%. A curva analítica mostrou-se linear entre 0,00 e 2,00  $\mu\text{g Co l}^{-1}$ , o limite de detecção foi determinado como 2 ng  $\text{Co l}^{-1}$ , o consumo de Tiron foi calculado como 0,13 mg por amostra e a exatidão analítica foi comprovada empregando-se espectrometria de absorção atômica com forno de grafite. Procedimentos para melhoria em seletividade são enfatizados.

A flow-injection procedure is proposed for the catalytic spectrophotometric determination of cobalt in liver. The method exploits the catalysis of this metal on Tiron oxidation by hydrogen peroxide in alkaline medium. Influence of pH, reagent concentrations, temperature, ionic strength, flow rates, feasibility of kinetic discrimination and presence of potential interfering ions were studied. With a sampling rate of about 40 samples per hour, the system yields precise results (r.s.d. usually < 1% within the 0.20 – 2.00  $\mu\text{g Co l}^{-1}$  range) in agreement with graphite furnace atomic absorption spectrometry. The detection limit is 2 ng  $\text{Co l}^{-1}$  and the consumption of Tiron is only 0.13 mg per sample. Additional procedures for selectivity enhancement are suggested.

**Key words:** catalysis, flow-injection, cobalt, liver.

## Introduction

Since cobalt is required for growth of grazing animals, the demand for the determination of this nutrient in feed materials<sup>1</sup> and animal tissues<sup>2</sup> has been ever increasing. In this context, liver analysis providing information on cobalt deficiency<sup>3</sup> has often been requested. Sensitive techniques should be employed because the contents in plant and animal tissues are usually below<sup>2</sup> 0.3 mg  $\text{Co l}^{-1}$ . Therefore, catalytic methods have received more and more attention, especially when combined with flow-injection analysis<sup>4-6</sup>. Precise results and reduced possibility of sample contamination<sup>4,5</sup> are inherent to this combination.

Several catalytic procedures have been proposed for the spectrophotometric determination of cobalt<sup>4-8</sup>. In general, they are based on the catalytic effect of this metal on the oxidation of reagents such as SPADNS<sup>7</sup>, Stilbazo<sup>8</sup>, gallic acid<sup>9</sup>, protocatechuic acid<sup>10</sup>, Alizarin red S<sup>11</sup>, Quinalizarin<sup>12</sup> and Tiron<sup>13-15</sup>. Since most of these reagents yield colorless oxidation products, color fading is usually quantified. In flow-injection analysis, this means a high baseline which becomes noisy with fluctuations in flow rates. As Tiron leads to the formation of a colored unstable product when oxidized by hydrogen peroxide<sup>13</sup>, direct spectrophotometry can be used. High sensitivity is achieved because the uncatalyzed Tiron oxidation proceeds at a very slow rate<sup>15</sup>.

This paper reports the development of an automated procedure for the spectrophotometric determination of cobalt in liver using a simple flow-injection manifold. The Tiron/hydrogen peroxide indicator reaction was chosen after considering selectivity and sensitivity aspects.

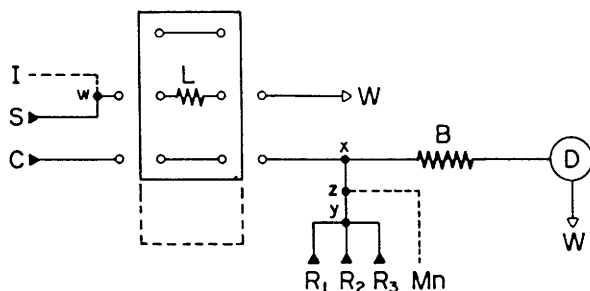
## Experimental

### Standards, reagents and samples

All solutions were prepared with analytical quality chemicals and distilled/deionized water. Spectrographically pure ferric oxide from Johnson Matthey Chemicals Ltd. was used in interference studies.

Cobalt standards, 0.25 M in  $\text{HClO}_4$  and covering the 0.00 – 2.00  $\mu\text{g l}^{-1}$  range, were prepared by dilution of a 100 mg  $\text{Co l}^{-1}$  stock solution. This solution was prepared by weighting 0.2385g  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$ , dissolving it in about 400 ml water, adding 2 ml concentrated  $\text{HNO}_3$  and making the volume up to 500 ml with water. The establishment of undesirable concentration gradients along the sample zone was avoided by using a 0.25 M  $\text{HClO}_4$  solution as sample carrier stream (C – Fig. 1).

The R1 reagent was a 0.5 M NaOH plus 0.01 M  $\text{Na}_4\text{P}_2\text{O}_7$  solution. The R2 reagent (0.001 M Tiron) was prepared by dissolving 0.03142 g 1,2-dihydroxybenzene-3,5-disulfonic acid disodium salt in 100 ml water. The R3 reagent was a freshly prepared 0.0005 M  $\text{H}_2\text{O}_2$  solution. Bovine liver tissues preserved in formaldehyde were supplied by Empresa Brasileira de Pesquisa Agropecuária, S. Carlos, SP (Brazil) and mineralized as follows: 0.2 g dried sample (60°C to constant weight) was accurately weighted and placed in 75-ml digestion tubes; 2.0 ml concentrated  $\text{HNO}_3$  were added and the samples were allowed to rest overnight. Thereupon, 1.0 ml concentrated  $\text{HClO}_4$  was added and the tubes were placed on a digester block for heating (100°C) during about two hours until almost dryness. The residue was solubilized in 5 ml 0.1 M  $\text{HClO}_4$  and filtered through a Whatman #1 paper. The



**Figure 1.** Flow Diagram. S: sample ( $2.0 \text{ ml min}^{-1}$ ); L: sampling loop (15 cm, about  $60 \mu\text{l}$ ); C: carrier stream ( $0.25 \text{ M HClO}_4$  at  $1.0 \text{ ml min}^{-1}$ ); R1, R2 and R3:  $2.0 \text{ M NaOH}$  plus  $0.1 \text{ M Na}_2\text{P}_2\text{O}_7$ ,  $0.005 \text{ M Tiron}$  and  $0.0005 \text{ M H}_2\text{O}_2$  at  $0.21 \text{ ml min}^{-1}$ ; B: reactor (150 cm); D: detector (426 nm); x, y, w e z: confluence points; W: waste. Black arrows indicate where pumping is applied. Boxed components are linked to the movable portion of the commutator with next commutating state specified by the dashed line. I and Mn – optional streams for studying interference effects and for enhancing selectivity.

volume was completed to 50 ml with water. The mean acidity of the so prepared digest was about  $0.25 \text{ M}$  in perchloric acid.

#### The flow-injection system

Peristaltic pump, spectrophotometer with flow-cell, injector-commutator, recorder, tubing and accessories of the flow-injection manifold have already been described<sup>16</sup>.

The flow-injection system is outlined in Fig. 1. Since preliminary tests indicated that the order of reagents addition was not relevant, it was decided to combine the R1, R2 and R3 reagents (point y) in order to get only one confluence point in the analytical path (single reagent configuration). After loop-based injection into the carrier stream, the sample selected volume was pushed towards the x confluence where the combined reagents were added. Tiron oxidation by hydrogen peroxide under alkaline conditions proceeded inside the following reactor, producing a greenish unstable complex monitored at 426 nm. In this way, the baseline reflected the extent of uncatalyzed reaction, whereas the peak recorded during the passage of the sample zone through the detector was proportional to the cobalt catalytic activity, hence the cobalt content in the sample.

#### Procedure

Since sensitivity was not a limiting factor, the system was designed to provide large dispersion thus avoiding the manipulation of too dilute solutions. The sampling loop was therefore chosen as 15 cm and the reactor length as 150 cm. With a  $1.0 \text{ ml min}^{-1}$  carrier stream flow rate,  $0.21 \text{ ml min}^{-1}$  flow rate was selected for the confluent streams in order to avoid the addition of confluent reagents in a pulsed fashion and to guarantee better mixing conditions and system stability<sup>17</sup>.

To provide concentrations inside the main reactor similar to those recommended<sup>13</sup>, the Tiron and hydrogen peroxide concentrations were chosen as  $0.001$  and  $0.0005 \text{ M}$ . Preliminary tests confirmed the interdependency of these concentrations<sup>13</sup> and showed only minor modifications in peak heights after variations in these concentrations.

The influence of pH in the oxidation reaction was in-

vestigated within the 7 – 14 range by adding  $0.1 \text{ M NaH}_2\text{PO}_4$  plus suitable amounts of NaOH into the R1 reagent. In this experiment, NaCl was used in the R1 stream to provide ionic strengths within the 1 to 4 range. For each alkalinity, further development of the involved reactions was observed by placing a  $7.0 \mu\text{g Co l}^{-1}$  solution instead of the sample carrier stream<sup>17</sup> and stopping the peristaltic pump after the system reached the steady state situation.

The influence of temperature was studied within the  $10\text{--}50^\circ\text{C}$  range by immersing the main reactor in a thermostated water bath. Whenever necessary, the sample residence time was varied by changing the rotation of the peristaltic pump.

The merging stream approach<sup>16</sup> was used for interference studies to avoid the preparation of an excessive set of standard solutions. A cobalt standard and a solution with a potential interfering species merged together (w – Fig. 1) before reaching the sampling loop. Since the convergent streams were pumped with identical flow rates ( $2.0 \text{ ml min}^{-1}$ ), the concentrations inside the loop were halved. These concentrations are well above the expected contents in the liver digests<sup>3</sup>. As masking agents,  $0.1 \text{ M NaH}_2\text{PO}_4$ ,  $\text{Na}_4\text{P}_2\text{O}_7$ , NaF or EDTA were used and the pH was kept at 13.0.

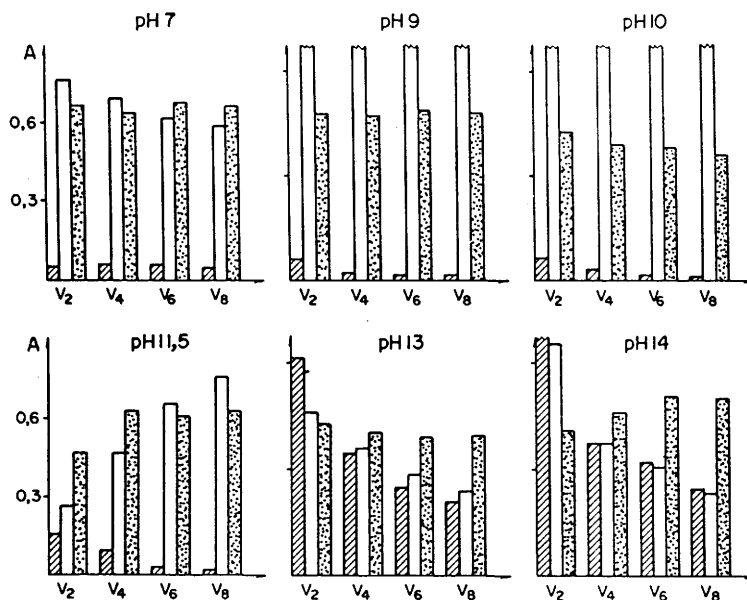
After system dimensioning, the I stream was removed and the analytical characteristics of the proposed procedure were evaluated. Precision was estimated as the standard deviation of results obtained after 10 successive analysis of a typical sample and accuracy was assessed by running samples already analyzed by graphite furnace atomic absorption spectrometry<sup>18</sup>. In addition the NBS-1577a bovine liver sample was also employed.

## Results and Discussion

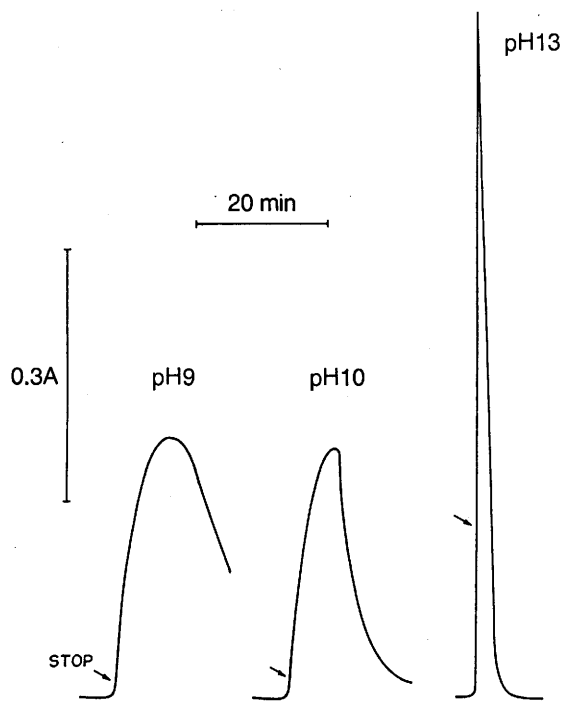
The pH for Tiron oxidation is a very important parameter, as increase in alkalinity inside the B reactor improved both sensitivity and selectivity (Fig. 2). In contrast with the literature, which recommends a pH usually within the 9.5 – 10.5 range, better analytical signals were obtained under more alkaline conditions without increasing the signals related to the main interferents. The effect was more pronounced for lower flow rates. The progress of the chemical reaction is shown in Fig. 3: for all the investigated pH values, the monitored absorbance increased after stopping the peristaltic pump, reached a maximum value, then decreased, confirming the formation of the unstable colored semiquinone radical<sup>19</sup>. Since the formation and degradation rates increased with alkalinity, this parameter should be as high as possible to improve sampling rate and sensitivity. However, precipitation of some metals yielding insoluble hydroxides (Ca, Mg, Fe in concentrations higher than 500, 50 and  $30 \text{ mg l}^{-1}$ ) and air bubbles formation by hydrogen peroxide solution was sometimes observed when the sodium hydroxide concentration in the R1 reagent was higher than  $3.0 \text{ M}$ . This concentration was then selected as  $2.0 \text{ M}$ , which provides a pH of about 13 for the development of Tiron oxidation.

Among the investigated masking agents, sodium pyrophosphate was the most effective (Tab. 1) regardless of its concentration. Since its masking efficiency was almost unaffected by variation in alkalinity,  $0.01 \text{ M Na}_4\text{P}_2\text{O}_7$  was added to the R1 reagent. Although already employed<sup>15</sup>, sodium tetraborate should be avoided because hydrogen peroxide complexation by borate in alkaline medium suppress Tiron oxidation. In fact, when  $0.1 \text{ M}$  sodium tetraborate was added to R1, no analytical signal was detected.

For  $4.0 \mu\text{g Co l}^{-1}$  and ionic strengths of 1, 2, 3 and 4,



**Figure 2.** Influence of pH on Tiron oxidation and of peristaltic pump speed. Bars correspond to peak heights recorded after injections of  $2.0 \mu\text{g Co l}^{-1}$  (dashed),  $1.0 \text{ mg Mn l}^{-1}$  (white) and  $10.0 \text{ mg Fe l}^{-1}$  (dotted) solutions:  $V_2$ ,  $V_4$ ,  $V_6$  and  $V_8$  refer to 50, 100, 150 and 200% of the pumping speed related to flow rates of Fig. 1.



**Figure 3.** Trends in the involved chemistry. Absorbance plateaux correspond to  $7.0 \mu\text{g Co l}^{-1}$  flowing in the infinite volume situation<sup>17</sup>. Measurements recorded for different pH values obtained by stopping the peristaltic pump. Arrows indicate the instant of pump stoppage.

peak heights of 0.25, 0.17, 0.1 and 0.1 A were observed, reflecting the dependence of the proposed procedure on the sample ionic strength. Therefore, care should be taken during sample preparation, in order to get digests with similar acidity. Although reducing sensitivity and/or sam-

pling rate, the effect can be circumvented by adding 3 M NaCl to the R1 reagent.

**Table 1.** Influence of masking agents. Data are peak height absorbances measured after injections of different I solutions converging with a  $2.0 \mu\text{g Co l}^{-1}$  S solution. The R1 reagent is 2.0 M NaOH alone (M1) or with 0.01 M  $\text{H}_2\text{PO}_4$  (M2), NaF (M3) or  $\text{Na}_2\text{P}_2\text{O}_7$  (M4).

I solution	M1	M2	M3	M4
blank	0.12	0.14	0.15	0.11
10.00 mg Zn $\text{l}^{-1}$	0.13	0.15	0.17	0.11
50.00 mg Mg $\text{l}^{-1}$	0.02	0.07	0.02	0.08
0.50 mg Ti $\text{l}^{-1}$	0.12	0.14	0.15	0.11
10.00 mg Al $\text{l}^{-1}$	0.13	0.15	0.16	0.11
0.50 mg V $\text{l}^{-1}$	0.12	0.14	0.14	0.11
5.00 mg Cr $\text{l}^{-1}$	0.21	0.24	0.23	0.20
0.50 mg Ni $\text{l}^{-1}$	0.11	0.13	0.12	0.11
5.00 mg Mn $\text{l}^{-1}$	0.69	0.74	0.70	0.72
5.00 mg Cu $\text{l}^{-1}$	0.15	0.17	0.17	0.13
500.0 mg Ca $\text{l}^{-1}$	0.01	0.05	0.01	0.05
30.00 mg Fe $\text{l}^{-1}$	0.23	0.30	0.26	0.29

The proposed system is remarkably stable. After an 8h working period, the slope of the calibration equation underwent only slight variations (< 5%) and no baseline drift was observed. The procedure is dependent on temperature with a 8% increase in slope per  $10^\circ\text{C}$ . Immersion of the B reactor inside a  $30^\circ\text{C}$  water bath, or the use of air-conditioning to ensure a temperature variation lower than  $1^\circ\text{C}$  is recommended. Linearity of the calibration curve ( $r > 0.999$ ;  $n = 7$ ) is observed within the  $0.20 - 2.00 \mu\text{g Co l}^{-1}$  range. Since the sample volumetric fraction<sup>20</sup> was determined as only 23%, sensitivity may undergo a 3-fold increase simply by increasing the injected volume. In addition, further sensitivity improvement may be achieved by reducing confluent stream flow rates and adjusting reagent concentrations accordingly. With a sample volume of  $56 \mu\text{l}$ , the detection limit of  $2.0 \text{ ng Co l}^{-1}$

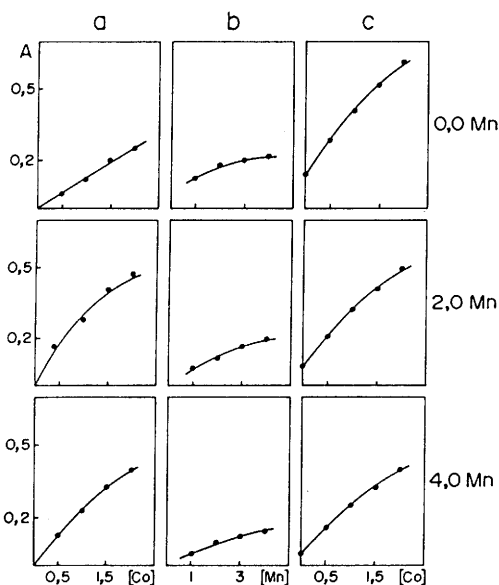
**Table 2.** Comparative results. Cobalt ( $\mu\text{g g}^{-1}$  – dry basis) in bovine liver as determined by the proposed procedure (FIA) and by graphite furnace atomic absorption spectrometry (ETA).

Sample	FIA	ETA
1	0.44	0.36
2	0.30	0.32
3	0.19	0.20
4	0.20	0.22
5	0.46	0.52
6	0.62	0.54
7 *	0.20	0.20

\* NBS – 1577a certified bovine liver, Co content =  $0.21 \mu\text{g g}^{-1}$ .

**Table 3.** Selectivity. Data express the maximum tolerance ratio of the interfering metal in the sample which causes a 10% error in the measurement related to  $1.00 \mu\text{g Co l}^{-1}$ .

Ion added	Tolerance ratio
Ca (II)	50000
Al (III)	10000
Mg (II), Zn (II), Cu (II)	5000
Cr (III), Fe (III), Mn (II)	1000
Ti (IV), Ni (II), V (V)	500



**Figure 4.** Effect of the manganese stream addition. Curves refer to addition of a  $0.21 \text{ ml min}^{-1}$  confluent stream with Mn concentrations of 0.0 (upper), 2.0 (center) and 4.0 (bottom)  $\text{mg l}^{-1}$ . Ordinates are peak height absorbances and abscisses are  $\mu\text{g Co l}^{-1}$  (a),  $\text{mg Mn l}^{-1}$  (b) or  $\mu\text{g Co l}^{-1} + 1.0 \text{ mg Mn l}^{-1}$  (c) in the injected solution.

was calculated considering a signal-to-noise ratio of three. Precise results (r.s.d. < 0.01) are obtained at a sampling rate of about 40 samples per hour. Only 0.13 mg Tiron is required per determination. Accuracy can be assessed from Tab. 2.

Selectivity data (Tab. 3) are presented as a guideline for application of the proposed procedure to other samples. The chromium signal is proportional to the color of the chromium solution, being easily circumvented by running

blank. It should be stressed that the addition of an extra manganese confluent stream (Mn – Fig. 1) diminishes this interference (Fig. 4) by the combination of two effects: a) the improvement in sensitivity due to the synergistic effect which reduces the required injected volume and therefore the manganese content in the sample zone; and b) the levelling of the manganese concentration flowing through the main reactor. Studies focusing on this point are presently in progress.

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