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Enzymatic determination of choline in milk using a FIA system with potentiometric detection

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The development of a FIA system for the determination of total choline content in several types of milk is described. The samples were submitted to hydrochloric acid digestion before injection into the system and passed through an enzymatic reactor containing choline oxidase immobilised on glass beads. This enzymatic reaction releases hydrogen peroxide which then reacts with a solution of iodide. The decrease in the concentration of iodide ion is quantified using an iodide ion selective tubular electrode based on a homogeneous crystalline membrane. Validation of the results obtained with this system was performed by comparison with results from a method described in the literature and applied to the determination of total choline in milks. The relative deviation was always $< 5\%$. The repeatability of the method developed was assessed by calculation of the relative standard deviation (RSD) for 12 consecutive injections of one sample. The RSD obtained was $< 0.6\%$.

Introduction

Choline is an amino alcohol that performs many functions in the body. It is one of the fundamental constituents of cell membranes, a major constituent of phospholipid (phosphatidylcholine) and a precursor of acetylcholine, one of the most important neurotransmitters. It is part of the diet and is also synthesised in the body as necessary. It is present in free and combined forms.[1]

In milk, choline exists principally in the combined form as phosphatidylcholine (lecithin) and in a smaller part as acetylcholine, although it may also be present in other forms,[2] which complicates quantitative determination. Some analytical methodologies have been developed for the determination of this constituent in several types of milk, first subjecting the samples to hot acid hydrolysis to liberate free choline, followed by colorimetric determination at 505[2] or 600 nm,[3] depending on the chromogenic reagent used. These methods are very time consuming because apart from the need for hot acid hydrolysis it is also necessary to have 'blank' tests[2] or to remove colour using activated carbon.[3] Recently, Matsuzawa and Kawai[4] proposed a method for the determination of choline using high-performance liquid chromatography (HPLC) with electrochemical detection.

To reduce the cost of choline determination, and particularly to reduce the amounts of reagents and the length of time required for the determinations, several flow injection analysis (FIA) systems have been developed in the last few years to allow the quantification of choline in various biological matrices. Some methods use an enzyme immobilised on the surface of the electrode so that the enzymatic reaction occurs when the flow contacts the membrane of the electrode and the reaction product is detected amperometrically.[5,6] The alternative strategy is to immobilise the enzyme on a suitable support in a small reactor and to detect the reaction product by chemiluminescence.[7–10].

In this work, we tried to develop an automatic system for the determination of choline in several types of milk. It was necessary to pre-digest the samples in order to release choline, which is present in a bound form, resulting in a digestate whose turbidity prevented the direct use of colorimetric detection and whose composition complicates amperometric detection.

We describe a FIA system for the enzymatic determination of choline in several types of milk in which detection of the reaction product is by means of a tubular electrode with an iodide selective membrane constructed according to Ferreira *et al.*[11] The tubular configuration allows the detector to be attached rigidly to the FIA system, making it possible to use potentiometric analysis as a routine procedure.[12,13]

The enzyme choline oxidase was immobilised according to a procedure described by Masoom,[14] based on covalent linking of the enzyme to the surface of the support, aminoalkylated glass beads, using glutaraldehyde. The glass beads were then put into a column located in the FIA system after the injection valve.

The digests were injected into the system and passed through a small reactor containing the glass beads. The choline present in the sample was oxidised by the immobilised choline oxidase and hydrogen peroxide was liberated. The hydrogen peroxide was then reacted with an iodide solution at low pH and a stoichiometric decrease in concentration occurred. This reaction was catalysed by certain metals in their highest oxidation state, *e.g.*, molybdenum and tungsten.[15] The concentration of choline in the samples was quantified potentiometrically from the decrease in the iodide concentration.

Experimental

Reagents and solutions

All chemicals were of pro analysi grade from Sigma (St. Louis, MO, USA). Standard solutions used for construction of the calibration curves were prepared by rigorous dilution of a stock standard solution containing 0.10 mol l^{-1} choline, obtained by careful weighing of choline bitartrate previously dried to constant weight at 100 °C.[2] The dilutions were made with a 5×10^{-2} mol l^{-1} tris(hydroxymethyl)aminomethane hydrochloride (tris–HCl, pH 8.0) prepared by dissolution of 6 g Tris (hydroxymethyl) aminomethane in distilled water, adjusting the

pH to 8.0 with 3 mol l^{-1} hydrochloric acid and making up to a volume of 1000.0 ml with distilled water. In the comparison method, 0.5 g phenol was also added.[2]

To assess the response of the iodide ion selective tubular electrode based on a homogeneous crystalline membrane, a series of iodide solutions was prepared by dilution in KNO_3 (0.1 mol l^{-1}) of a stock standard solution of 0.10 mol l^{-1} KI, prepared daily and stored in the dark. Both solutions were prepared by weighing the solid compound and the concentration of iodide was confirmed by potentiometric titration with $AgNO_3$. The solutions of hydrogen peroxide used in the optimisation of the reaction with iodide were prepared from a 30% w/w stock standard solution. The 1.0×10^{-3} mol l^{-1} iodide solution used in the FIA system was prepared in 0.25 mol l^{-1} HNO_3 and contained Mo(VI) as catalyst, obtained from $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$.

Choline oxidase (C-4405, 10 mg = 100 units), peroxidase (P-8250 Type II, 1 mg = 200 units), 4-aminoantipyrine (A-4382), aminoalkylated glass beads (200–400 mesh, 75 Å id (pore size) and glutaraldehyde solution (25%) were obtained from Sigma.

The choline oxidase solution used in the immobilisation was prepared by dissolving 2 mg of the enzyme in 1.00 ml of 5×10^{-2} mol l^{-1} Tris–HCl, pH 8.0.

In the comparison method, the colour development reagent was prepared by dissolving 100 units of choline oxidase, 250 units of peroxidase and 15 mg of 4-aminoantipyrine in Tris–HCl, pH 8.0, in accordance with the method used by Woollard and Indyk.[2]

Immobilisation of the enzyme

Immobilisation of choline oxidase was effected on pre-prepared aminopropylglass (CPG) following a procedure already described.[14]

Apparatus

The solutions were transported through the FIA system by a Gilson (Worthington, DH, USA) Minipuls 3 peristaltic pump using pump tubing of the same brand. Samples and standards were introduced into the carrier stream through a six-port Rheodyne (Cotati, CA, USA) Type 5020 injection valve. Omnifit (Cambridge, UK) PTFE tubes (0.8 mm id) connected by Gilson end-fittings and joints were used in the construction of the manifold. Several Perspex components, confluences and supports for the reference and indicator electrodes, were constructed according to Alegret *et al.*[16]

A Crison Model 2002 (Barcelona, Spain) digital decivoltmeter (sensitivity ±0.1 mV) coupled to a Kipp & Zonen (Delft, The Netherlands) BD 111 recorder was used for the determination of potential differences between the indicator and reference electrodes. An Orion (Cambridge, MA, USA) 90-00-29 double-junction electrode was used as the reference electrode. The indicator electrode comprised a tubular electrode fitted with a homogeneous crystalline membrane selective for iodide.[11] In addition, the set-up included a stainless-steel tube (about 10 mm long × 0.8 mm i.d.) as grounding electrode and which was intended to eliminate electrical interference. A Hitachi (Tokyo, Japan) U-200 spectrophotometer was used for absorbance measurements in the comparison method.

Comparison method

As the reference method for the determination of total choline in milks we used a technique developed by Woollard and Indyk.[2] Choline was determined following acid digestion: to 20.0 ml of milk were added 10 ml of 3 mol l^{-1} HCl and the mixture was heated in a water-bath at about 70 °C for about 3 h. The mixture was then allowed to cool to ambient temperature and the pH adjusted to 3.8–4.0 with a 50% w/w solution of NaOH, made up to a volume of 20.0 ml and finally filtered. To 0.10 ml of the filtrate were added 3.00 ml of chromogenic reagent. The colour reaction was allowed to proceed at 37 °C in a thermostatically controlled water-bath for 10 min before measuring the absorbance at 505 nm against a colour reagent blank. The remaining filtrate was used for choline determinations in the FIA system. The mineralization process did not cause loss of choline, as was demonstrated by Woollard and Indyk in comparison with the Reineckate method[17,18] or using spiking techniques.

FIA system

The set-up developed for the determination of choline concentration in several milks is shown schematically in Fig. 1.

Following the acid digestion described above, 200 μl of hydrolysate were injected into the carrier stream (C) (5×10^{-2} mol l^{-1} Tris–HCl, pH 8.0) and passed through the enzyme reactor (E), which was maintained at 30 °C in a water-bath (TB). During this passage, the carrier stream maintained the pH of the hydrolysate at a level suitable for enzymatic reaction and in the reactor (E) choline was oxidised with the production of H_2O_2. At the following confluence (X) a solution of iodide (R_1) [1.0×10^{-3} mol l^{-1} prepared in 0.25 mol l^{-1} HNO_3 and containing 0.82 g l^{-1} Mo(VI)] was added to the flow. In the reactor L_1 the H_2O_2 reacted with the iodide anions in an acidic environment and the diminution of the concentration of iodide was quantified in the iodide selective crystalline membrane affixed to the tubular electrode. Because the operating range of the electrode is understood to be from pH 2.5 to 11.0,[11] a solution of 0.2 mol l^{-1} K_2HPO_4 (R_2) was added to the flow at confluence Y to raise the pH of the solution and simultaneously to adjust the ionic strength before passage through the detector.

Results and discussion

Optimisation of the FIA system

Optimisation of the FIA system was performed using the univariant method, always taking into account the best compromise between reproducibility, sensitivity, sampling rate and reagent economy. It was effected in two stages: first, optimisation of the iodide–hydrogen peroxide reaction, and second, optimisation of the choline–choline oxidase reaction.

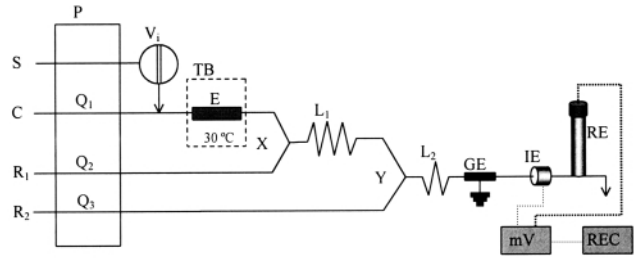

Fig. 1 Flow injection system: P = peristaltic pump; S = sample; V_i = injection volume (200 μl); E = enzymatic reactor; C = carrier stream; TB = thermostatic bath; X, Y = confluence points; L_n = tube length (L_1 = 400 cm; L_2 = 30 cm); Q_n = flow rates ($Q_1 + Q_2 = Q_3$ = 0.5 ml min^{-1}) GE = grounding electrode; IE = indicator electrode; RE = reference electrode; mV = decivoltmeter and REC = recorder.

(a) Optimisation of the iodide–hydrogen peroxide reaction. This optimisation was effected having fixed the injection volume at 200 μl and the concentration of hydrogen peroxide at about 1.0×10^{-2} mol l^{-1}.

The H_2O_2 was prepared daily in 5×10^{-2} mol l^{-1} Tris–HCl, pH 8.0. The choice of this concentration was made because of the known concentrations of choline in different kinds of milks and the stoichiometry of the reaction (H_2O_2:I^- = 1:2 I^-). To ensure that the ionic strength was sufficient and that the pH of the solution was above 2.5 on arrival at the detector a solution of 0.2 mol l^{-1} K_2HPO_4 at a flow rate of 1 ml min^{-1} was introduced at confluence Y. This flow rate was also used initially for the Tris–HCl and iodide streams. Under these conditions the effects of pH and of the concentrations of iodide and Mo(VI) on the intensity of the analytical signal were studied and optimised.

For the evaluation of the pH effect on the iodide–hydrogen peroxide reaction, iodide solutions of 1.0×10^{-3} mol l^{-1}, containing 0.54 g l^{-1} Mo(VI), were prepared in nitric acid with concentrations ranging 1×10^{-2} to 3×10^{-1} mol l^{-1} and in distilled water. After injection into the FIA system of the 1×10^{-2} mol l^{-1} solution of H_2O_2, the iodide solution prepared in water gave no signal, whereas the acidified solutions promoted an increase in the potential readings, up to a concentration of 0.25 mol l^{-1} HNO_3. At higher nitric acid concentrations the signal remained almost unaltered. Accordingly, the concentration of HNO_3 was fixed at 0.25 mol l^{-1} for the subsequent preparations of iodide solutions. Subsequently, the concentration of the iodide solution to be added at confluence X was optimized in order to guarantee complete reaction of the H_2O_2. Iodide solutions with concentrations between 5.0×10^{-5} and 5.0×10^{-3} mol l^{-1} were tested, and it was observed that the analytical signal increased up to 1.0×10^{-3} mol l^{-1} and diminished thereafter. For concentrations lower than 1.0×10^{-3} mol l^{-1}, although the detection limit was lower it was not possible to quantify concentrations of hydrogen peroxide $\geqslant 1.0 \times 10^{-2}$ mol l^{-1} because the amount of iodide was insufficient. For concentrations higher than 1.0×10^{-3} mol l^{-1} the detection limit was too high and this did not permit the detection of lower concentrations of H_2O_2. Therefore, the concentration of I^- was fixed at 1.0×10^{-3} mol l^{-1}. Having determined the above concentrations, the level of Mo(VI) was studied. The concentrations tested ranged from 0.0 to 2.7 g l^{-1} and the maximum detection signal was observed at 0.82 g l^{-1}, decreasing slowly at higher concentrations.

The length of reactor L_1 was selected in order to give good mixing of the sample and reagent and to ensure the highest possible analytical signal strength. Helically coiled reactors with pathlengths between 3 and 4.5 m were tested. A length of 4 m was chosen as shorter pathlengths gave weaker and less reproducible analytical signals and longer pathlengths gave better reproducibility that was not, however, adequate compensation for the lowered signal strength and sampling rate.

Sample injection volumes from 200 to 400 μl were tested and 250 μl was chosen as a good compromise between sensitivity and sampling rate. Flow rates from 1.0 to 2.0 ml min^{-1} were assessed (with $Q_1 = Q_2$) and 1.5 ml min^{-1} was selected for the same reasons as for the sample volume. Although $Q_2 < Q_1$ increased the intensity of the analytical signal by decreasing the dispersion of the sample, it was then necessary to increase the concentration of I^- and HNO_3. This would have been inconvenient, first, because in strongly acid medium iodine tends to degrade more rapidly, and second because the pump tubing would be more rapidly destroyed for the same reason.

Finally, the flow rate of the K_2HPO_4 was again studied and it was observed that increasing the flow rate from 1 to 1.5 ml min^{-1} allowed the analytical signal to return more rapidly to the baseline without compromising the signal intensity. Accordingly, several concentrations of K_2HPO_4 were tested and it was found that above 0.2 mol l^{-1} the signal amplitude was reduced, and at concentrations slightly below this level there was no significant change, so a concentration of 0.2 mol l^{-1} was therefore chosen.

For reactor L_2 the form and length selected were a coil of 30 cm.

(b) Optimization of the system relative to the enzyme reaction. The principal parameters to be optimized for this reaction were the length of the enzyme reactor, flow rate and injection volume. Reactors of 2 mm id and lengths from 2 to 8 cm were tested. Because of the resistance to the flow caused by the reactor, it was necessary to decrease the flow rates selected in the above optimization while maintaining the same proportion. For each reactor several injection volumes (200–400 μl) and flow rates (0.35–1.0 ml min^{-1}) were evaluated with regard to signal intensity and reactor efficiency (%), calculated by comparing the analytical signals obtained with those for a 5.0×10^{-3} mol l^{-1} solution of choline and a solution of 1.0×10^{-2} mol l^{-1} H_2O_2. It was observed that up to 6 cm there was a continuous increase in the efficiency of the analytical signal but above this length there was a slight diminution of the sensitivity due to dispersion effects in the reactor. Previous studies showed that the maximum efficiency (97%) and intensity of the analytical signal occurred with a 6 cm reactor, an injection volume of 200 μl and a flow rate of 0.5 ml min^{-1} (Q_1), allowing a flow rate of 1.5 ml min^{-1} at the detector. The effects of pH and temperature on the enzyme reaction were also assessed. In the case of pH, the carrier solution used was 0.05 mol l^{-1} Tris–HCl at different pHs. The maximum signal intensity was obtained at pH 8.0. Temperature effects were studied by immersion of the column in a water-bath at 25–37°C. A gradual increase in signal intensity with increase in temperature was noted, but above 30 °C gas bubbles were formed in the liquid and accordingly this value was selected as the working temperature.

Determination of choline concentration in different types of milk

To assess the quality of the results obtained from the FIA set-up, 12 samples of different kinds of milk were analysed and the results were compared with those obtained from the comparison method.[2] Following digestion, the samples were injected into the system and their concentrations interpolated from a calibration curve which had been constructed from standard solutions of choline ranging from 5.0×10^{-4} to 5.0×10^{-3} mol l^{-1} (Fig. 2). Further evaluation of the system was effected

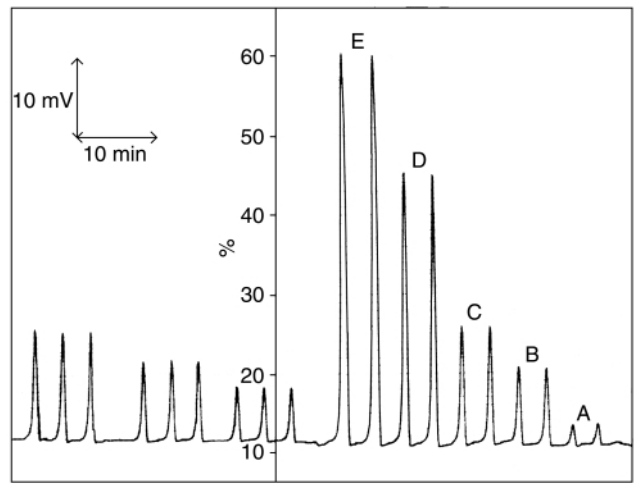

Fig. 2 Flow injection signals corresponding to the injection of a set of standards of choline and samples. A, 5×10^{-4}; B, 8×10^{-4}; C, 1×10^{-3}; D, 2.5×10^{-3} and E, 5×10^{-3} mol l^{-1}.

Table 1 Results obtained in the determination of choline concentrations in different types of milk using the FIA system and the method of comparison, showing relative standard deviations (RSD)

Sample No.	Mean concentration ± s/10^{-4} mol l^{-1} (n = 5)		
	FIA	Comparison method	RSD
1[a]	6.56 ± 0.04	6.75 ± 0.08	−2.8
2[a]	7.78 ± 0.05	7.55 ± 0.08	+3.0
3[a]	9.22 ± 0.03	9.0 ± 0.1	+2.4
4[a]	6.29 ± 0.05	6.34 ± 0.08	−0.8
5[a]	7.29 ± 0.02	7.49 ± 0.03	−2.7
6[a]	7.29 ± 0.06	7.57 ± 0.06	−3.7
7[a]	10.1 ± 0.1	10.1 ± 0.1	0
8[a]	11.1 ± 0.2	10.6 ± 0.2	+4.7
9[a]	7.58 ± 0.05	7.85 ± 0.08	−3.4
10[b]	13.9 ± 0.2	13.6 ± 0.4	+2.2
11[b]	16.2 ± 0.3	15.8 ± 0.2	+2.5
12[b]	13.0 ± 0.3	13.6 ± 0.3	−4.4

[a] Samples of powdered milk. [b] Samples of cow's milk.

by a series of recovery experiments, using some of the samples, in which the values obtained ranged from 97 to 102%.

In Table 1 the mean results of five determinations using each of the two methodologies are given, with the respective standard deviations, for each of the 12 samples. In the final column are shown the relative standard deviations (RSDs), which are all $< 5\%$.

Using the mean values obtained from the FIA method (C_F) and the method of comparison (C_R), the following regression line was established:

$$C_F = -3.0 \times 10^{-5} (\pm 7.2 \times 10^{-5}) + 1.032 (\pm 0.071) C_R$$

with a correlation coefficient $r = 0.995$. The comparison of the two methods demonstrated good agreement, as is shown by the insignificant displacement (-3.0×10^{-5}) of the origin, the slope (1.032) and the correlation coefficient ($r = 0.995$). Students' 't' test with a confidence limit of 95% gave a value of 0.938, which was below the tabulated value (2.201).

The repeatability of the FIA method was assessed by calculating the RSD for 12 consecutive injections of one sample of milk which had a choline concentration in the middle of the calibration curve. The result obtained was $< 0.6\%$. The sampling rate achieved during this evaluation was around 20 samples per hour.

Conclusions

With reference to the methods described previously for the determination of choline in milks, the following advantages are shown by the proposed system; it is automated, the analytical signal is unaffected by turbidity in the digestates (unlike the situation using colorimetric detection) and, because it is specific, it does not require separation of the reaction products, as would be necessary for amperometric detection.

The FIA methodology is a good alternative to the method of comparison, since the results obtained showed excellent agreement and the sampling rate is much higher. In the comparison method, after the addition of the chromogenic reagent to the hydrolysates and standards it is necessary to wait for 10 min to allow the reaction to proceed to completion. Moreover, owing to the technique used for detection (UV/Vis spectrophotometry), it is essential to run assay blanks not only for the samples and the standards but also for the colour development reagent itself. With the FIA system developed, because the determinations are made in the absence of physical and chemical equilibrium, it is possible to shorten considerably the time for each estimation. Additionally, the use of potentiometric detection gives the advantage of a linear analytical response over a wide range of concentrations and avoids predilution steps to adjust the concentration of the samples into the working range. The sampling rate is considerably increased because there is no need for assay blanks.

Another great advantage of the FIA system presented is the substantial economy of reagents, since peroxidase is replaced by molybdenum(VI) and the choline oxidase is immobilised. The enzyme reactor may also be used for more than 1 month, when suitably treated.

It is concluded that the FIA system which has been developed may be easily installed in routine analytical laboratories, as it is simple, economical and allows a high sampling rate, bearing in mind that it involves enzymic reactions.

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