Determination of Incubator-Reject Eggs in Egg Products by means of the 3-Hydroxybutyric Acid Content. 1. Improved Enzymatic and Gas Chromatographic Assays

Henri L. Elenbaas,* Bart G. Muuse, Willem Haasnoot, Ben Rutjes, Piet Stouten, Theo G. Uijttenboogaart, and A. Tom G. Steverink

The use of incubator-reject eggs in egg products intended for human consumption is legally prohibited or strongly restricted in many countries. Because of the need for a reliable method of control, enzymatic and gas chromatographic methods for the analysis of 3-hydroxybutyric acid (3-HBA)—an indicator of embryonic development in eggs—have been improved to lower the detection limits of the former and shorten analysis time for the latter. The two methods have been tested and compared at low levels of 3-HBA (<6 mg/kg). Statistical analysis revealed no significance between results obtained by the two methods. The limit of reliable detection of the enzymatic method is estimated as 0.5 mg of 3-HBA/kg. With both methods about 15 samples of liquid whole egg or egg products can be analyzed daily by one analyst.

INTRODUCTION

In the U.S. and Canada the use of incubator-reject eggs in egg products for human consumption is prohibited. In the European Economic Community (EEC) some members prefer the American and Canadian legislation, while others want to maintain the present arrangement of permitting the processing of *clear unfertilized incubator-reject eggs held in incubators for less than 6 days (EEC, 1975)*.

The enforcement of any regulation requires a reliable method of control. Several methods have been described in the literature to distinguish between incubated and nonincubated eggs. These methods can be divided into two groups: one group based on the presence or development of 3-hydroxybutyric acid (3-HBA), the other on the denaturation of proteins as a result of incubation heat. The denaturation can be shown by electrophoretic means (Csuka et al., 1973; Harwalkar, 1968) and by measuring the increase of reducing proteins (Cattaneo et al., 1979).

The desired method has to be reliable for egg products as well. Since normal commercial processing with pasteurization of the egg products or other treatments will denature some of the egg protein, neither the electrophoretic method nor the method for analyzing the reducing proteins is suitable.

The development of 3-HBA by a growing embryo and the use of this acid as an indicator for the presence of incubator rejects were first described by Salwin et al. (1972). The usefulness of this indicator has been confirmed (Heaney and Curtis, 1976; Littmann et al., 1982; Parry et al., 1980; Robinson et al., 1975).

The development of 3-HBA can be determined by gas chromatography and by an enzymatic assay. The proposed limit of 2 mg of 3-HBA/kg in liquid whole egg as a concentration to preclude adulteration of fresh liquid whole egg with incubator-reject eggs (Heaney and Curtis, 1976) makes the enzymatic method, described by Parry et al. (1980), suitable for screening only, as the reliable detection limit of this method is 2 mg/kg.

The existing gas chromatographic procedures (Heaney and Curtis, 1976; Littmann et al., 1982; Robinson et al., 1975; Staruszkiewicz et al., 1970) are too time consuming for routine application. This paper describes modifications to improve the detection limit of the enzymatic method and to shorten analysis time of the gas chromatographic procedure. Results obtained with both methods are compared.

State Institute for Quality Control of Agricultural Products (RIKILT), 6700 AE Wageningen, The Netherlands (H.L.E., B.G.M., W.H., B.R., P.S.), and Spelderholt Centre for Poultry Research and Extension, 7361 DA Beekbergen, The Netherlands (T.G.U., A.T.G.S.).

MATERIALS AND METHODS

Equipment. *Enzymatic.* A Sorvall RC2-B centrifuge (Du Pont, Newton, CO) was used to centrifuge samples at 20000*g*. A DU-8 UV–Vis spectrophotometer (Beckman Instruments International, Geneva, Switzerland) equipped with a six-position cell and sample transport mechanism was used to follow the reaction at 340 nm at a temperature of 30 °C.

Gas Chromatographic. The analyses were performed on two gas chromatographs, Model 3700 (Varian, Palo Alto, CA), equipped with capillary columns and flame ionization detectors. One apparatus was equipped with a Grob splitter, and the other, with a Varian cold on-column injector. The fused silica capillary column was 20 m × 0.22 mm i.d. The liquid phase was CP Wax 57 CB (Chrompack, Middelburg, NL). For cleanup purposes Extrelut columns (Merck, Darmstadt, FRG) were used. A 0.45-μm Acrodisc CR cartridge (Gellman Science Inc., Ann Arbor, MI) was employed for filtering the methylated solutions.

Reagents and Buffers. *Enzymatic.* The enzyme D-3-hydroxybutyrate dehydrogenase (EC 1.1.1.30 from *Rhodopseudomonas spheroides*; 3-HBDH) was purchased from Sigma (St. Louis, MO) (Type III, 2 mg of protein/mL). The calibration standard, the sodium salt of DL-3-hydroxybutyric acid, was also purchased from Sigma. Nicotinamide adenine dinucleotide (NAD^+, grade I) was supplied by Boehringer (Mannheim, FRG). All other chemicals used were reagent grade.

The Tris buffer (0.1 M), pH 8.4, consisted of tris(hydroxymethyl)aminomethane (0.2 M), hydrochloric acid (0.2 M), and water (3:1:2). Hydrazine hydrate, 5% (w/v), was prepared from hydrazine hydrate, hydrochloric acid (1.0 M) and water (2:5:3) and adjusted to pH 8.4 with KOH solutions.

Gas Chromatographic. Glutaric acid (GC grade), used as an internal standard, was purchased from Merck (Darmstadt, FRG). As an external standard, 3-hydroxybutyric acid was used and supplied by Fluka (Buchs, Switzerland). The methylating reagent was methanolic hydrochloric acid (2 M); the neutralizing reagent after methylation was methanolic ammonia (5 M). Both reag-

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ents were prepared according to Vogel (1964).

Procedures. *Enzymatic.* Forty grams of liquid whole egg or liquid egg product was mixed with 25 mL of 30% w/v perchloric acid. In the case of egg powder, 10 g was mixed with 30 mL of water and then with 25 mL of 30% w/v perchloric acid. The mixture was centrifuged at 20000 *g* for 15 min; the clear supernatant was then filtered. Twenty-five milliliters of filtrate was adjusted to pH 8.4, first with 30% w/v and then with 3% w/v potassium hydroxide. After cooling to room temperature and filling up to 50 mL with water, the mixture was cooled in ice for 15 min. The insoluble potassium perchlorate was removed by centrifuging or filtering. From the clear supernatant, 2.70 mL (preheated to 30 °C) was put into a 1-cm plastic cell. A 0.25-mL portion of Tris buffer, 0.25 mL of 5% w/v hydrazine hydrate, 0.20 mL of 20 mM NAD^+, and 10 μL of enzyme suspension were added and mixed. The total increase in absorbance at 340 nm was measured against a similar mixture without enzyme. The time required to complete the reaction at 30 °C was 30–50 min depending on the 3-HBA present. The amount of 3-HBA was calculated from the total change in absorbance using the NADH molar absorption coefficient of 6.3 l $mmol^{-1}$ cm^{-1}.

Gas Chromatographic. Sample Preparation. Twenty-five grams of liquid whole egg or an equivalent amount of dry matter suspended in water was mixed with 10 mL of 20 mg/L glutaric acid and 50 mL of water. The suspension was cleared by mixing with 20 mL of 2 M sulfuric acid and 40 mL of 10% w/v phosphotungstic acid, followed by centrifuging (2500*g* for 10 min). Twenty milliliters of the clear supernatant was put on an Extrelut column. After 15 min the compounds were eluted with 160 mL of ethyl acetate in portions of 40 mL. The eluate was concentrated by rotary evaporation under low pressure at 40 °C, transferred to a 5 mL vial, and evaporated until dry.

Derivatization. To the dry residue was added 1 mL of 2 M methanolic hydrochloride, and the compounds were methylated at 60 °C for 30 min. The hydrochloric acid was neutralized by adding 0.25 mL of 5 M methanolic ammonia and about 0.5 g of crystalline sodium hydrogen carbonate. After mixing intensively, the sample was filtered through an acrodisc 0.45-μm cartridge before split injection.

Chromatographic Conditions. For methyl esters on CP Wax 57 CB: Injection port 280 °C; 1-μL injection in Grob splitter with split ratio 1:7; detector 280 °C; helium carrier gas constant at 100 kPa; temperature program 70–200 °C with a 10-min hold at the initial and final temperature and a program rate of 5 °C/min.

The 3-HBA content was calculated by means of the internal standard glutaric acid (GA) according to the equation

$$\text{mg/kg of 3-HBA} = \frac{\text{peak area 3-HBA}}{\text{peak area GA}} \frac{\text{mass GA}}{\text{mass sample}} \times \text{Rf} \times 10^6$$

Rf is the response factor of glutaric acid relative to 3-HBA (GA/3-HBA).

Comparison of the Enzymatic and GC Methods. The two methods were compared by analyzing six samples with a 3-HBA range of 0.8–5.5 mg/kg. These samples were prepared by mixing fresh eggs with an increasing amount of a sample of incubator-reject eggs containing a high concentration of 3-HBA. The 3-HBA content of the latter was determined by the enzymatic method only. Each sample was divided into 16 subsamples. After randomly numbering, eight subsamples of each sample were enzymatically analyzed and the other eight subsamples were analyzed with the gas chromatograph. The subsamples were analyzed once only on four different days over a 2-week period in such a way that in each day two whole addition ranges were analyzed (12 subsamples). All analyses were conducted by the same person.

The results were statistically evaluated according to ISO 5725 (1981).

Table I. Influence of Enzyme Activity and Purity on the 3-Hydroxybutyric Acid Content of Liquid Whole Egg Samples

	3-hydroxybutyric acid in eggs, mg/kg			3-hydroxybutyric acid in eggs, mg/kg	
sample	enzyme 1[a]	enzyme 2[b]	sample	enzyme 1[a]	enzyme 2[b]
1	0.41	0.82	5	1.11	1.60
2	0.44	0.91	6	1.37	1.83
3	0.77	1.20	7	1.80	2.14
4	0.82	1.23			

[a] Sigma enzyme type III: 3-HBDH sp act. ~25 U/mg (37 °C), maximum 0.05% lactate dehydrogenase (LDH) and 1% malate dehydrogenase (MDH); 2 mg of protein/mL. [b] Boehringer enzyme grade II: 3-HBDH sp act. ~3 U/mg (25 °C), maximum 0.1% LDH and 5% MDH; 5 mg of protein/mL. Less active, more impure.

RESULTS AND DISCUSSION

Enzymatic Method. For determining incubator-reject eggs in egg products the reliable 3-HBA detection limit of the kinetic method, published by Parry et al. (1980), is still too high. To lower the detection limit, the sample extract was concentrated and the end point method was applied. It was essential to measure the overall increase in absorbance of the sample solution since the enzymatic reaction was a two-substrate reaction and neither of the substrates (3-HBA, NAD^+) was present in a very high concentration in relation to its Michaelis constant (Bergmeyer, 1978). The total reaction time depended on the type of egg sample. Salt, which if often added to liquid whole egg, usually increased the reaction time. Sugar can give significantly reduced 3-HBA values. The cause of this phenomenon has not yet been found.

All commercially obtained 3-HBDH suspensions are more or less contaminated with the enzymes lactate dehydrogenase (LDH) and malate dehydrogenase (MHD). Since relatively high contents of lactic and malic acid are present in eggs (Littmann et al., 1982) and large amounts of enzyme must be used for complete conversion of small concentrations of 3-HBA in a reasonable time, the contribution of the contaminating dehydrogenases can be substantial. Table I shows the differences in results obtained by using 3-HBDH suspensions with relatively low and high LDH and MDH activities per milliliter of Sigma type III and Boehringer grade II enzyme preparations, respectively. The former enzyme, which had also a 3 times higher specific activity per milliliter, gave significant ($\alpha < 0.01$) lower 3-HBA values (0.43 mg/kg average) than the latter.

Another Boehringer 3-HBDH preparation, grade I, which had the same relative LDH and MDH activities but half the specific activity of the Sigma enzyme, gave not significantly ($\alpha < 0.05$) higher 3-HBA values (0.09 mg/kg average) than the Sigma enzyme.

As we will show later in the Results and Discussion, the enzymatic method is equal to the gas chromatographic assay. Consequently, the Sigma type III suspension of 3-HBDH meets the requirements. Although the Boehringer grade I enzyme preparation can be used, resulting in slightly but not significantly higher results, we recommend the use of a 3-HBDH suspension with at least the same properties as the Sigma type III enzyme. The ac-

Table II. Comparison between Enzymatic and GC Methods for Determination of 3-Hydroxybutyric Acid in 1–6 mg/kg Range: Mean Content, Standard Deviation (SD), and Coefficient of Variation (CV) of 3-HBA[a] Added to Fresh Egg

	3-hydroxybutyric acid, mg/kg											
day of anal.	fresh egg (E)		E + 1.0 mg/kg 3-HBA[a]		E + 2.0 mg/kg 3-HBA[a]		E + 3.0 mg/kg 3-HBA[a]		E + 4.0 mg/kg 3-HBA[a]		E + 5.0 mg/kg 3-HBA[a]	
	enzymic	GC	enzymic	GC	enzymic	GC	enzymic	GC	enzymic	GC	enzymic	GC
1	0.7; 0.8	0.9; 0.7	1.9; 1.8	2.0; 2.0	2.6; 2.7	2.6; 2.6	3.7; 3.4	3.4; 3.6	4.4; 4.7	4.1; 4.3	5.6; 5.4	5.2; 5.2
2	0.6; 0.7	0.8; 0.9	1.5; 1.6	1.8; 1.6	3.0; 2.6	2.4; 2.4	3.7; 3.9	3.5; 3.4	4.8; 4.4	4.2; 4.2	5.4; 5.5	5.2; 5.2
3	0.7; 1.3	1.3; 0.9	3.8; 2.1[b]	1.8; 2.0	2.6; 2.7	2.9; 2.6	3.5; 3.6	4.5; 4.0	4.8; 4.5	5.8; 4.4	5.7; 5.4	5.2; 5.3
4	0.6; 0.8	1.7; 0.9	1.7; 1.6	2.8; 2.3	2.8; 2.6	3.0; 2.8	4.5; 3.8	4.2; 3.9	4.6; 4.6	4.4; 3.1	5.4; 6.0	5.8; 5.7
x[c]	0.775	1.01	1.68	2.04	2.70	2.66	3.76	3.81	4.60	4.31	5.55	5.35
SD[d]	0.225	0.327	0.147	0.370	0.141	0.220	0.338	0.405	0.160	0.734	0.214	0.251
CV[e]	29	33	8.8	18	5.2	8.3	9.0	11	3.5	17	3.9	4.7

[a] Addition of a standard egg sample of a high 3-HBA content to fresh egg (E). Each of the six samples was divided into 16 subsamples: eight for enzymatic and eight for GC analysis. [b] Cochran outlier according to ISO 5725 (1981). [c] Mean content 3-HBA (mg/kg). [d] Standard deviation (mg/kg). [e] Coefficient of variation (%).

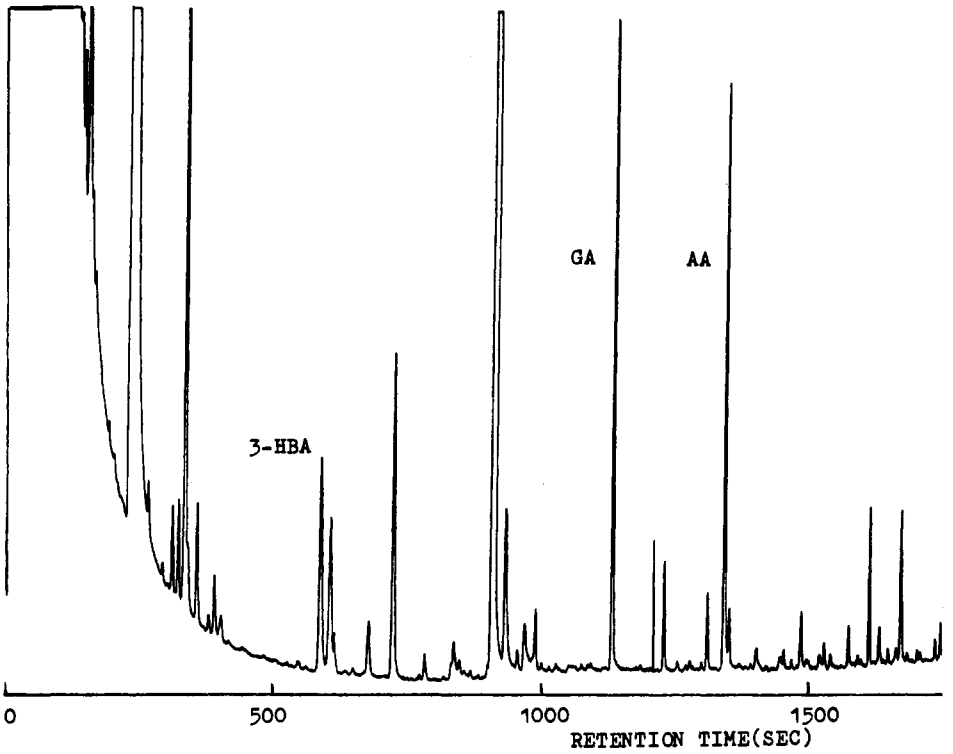

Figure 1. Gas chromatogram of a methylated egg extract. 20-m CP wax 57 CB column, split injection. 3-Hydroxybutyric acid (3-HBA, 5 mg/kg), the internal standard glutaric acid (GA), and the recovery control standard adipic acid (AA) are identified.

tivities of the enzymes can be checked by the method of Berry (1963).

Unlike Parry et al. (1980), we found that the neutralized perchloric acid extracts had a significant absorbance at 340 nm, which fluctuated in each sample preparation and sometimes decreased slowly. For liquid whole egg the absorbance was about 0.07 or 6 mg (calculated as 3-HBA)/kg sample. To correct for this background and in case of endogenous reactions after addition of NAD^+, the sample solution was also added to the reference cell while omitting the enzyme suspension. An alternative procedure is measuring the absorbance of the sample cell against a reference cell containing only reagents (A1) and subsequent addition of the enzyme to both cells and measurement of the total increase in absorbance (A2). The 3-HBA content is calculated by difference in absorbance (A2 – A1) (Williamson and Mellanby, 1974). This procedure is more time consuming and less suitable for automatic operation. When the assay is peformed as presented and with a spectrophotometer that is able to measure the absorbance to four decimals, the lower limit of reliable detection of 3-HBA in egg is of the order of 0.5 mg/kg. The measured mean concentration of 0.7 mg of 3-HBA/kg in newly laid egg corresponds to literature values.

The 3-HBA recovery, determined at the 5 mg/kg level by adding the 3-HBA calibration standard to fresh egg samples, is 97.8% (86–109%, $n = 13$). When a standard egg sample is added to fresh egg over the range of 1–5 mg of 3-HBA/kg, an average, relative 3-HBA recovery of 97.0% was obtained (Table II).

Approximately 15 samples can be analyzed in 8 h by one analyst.

Gas Chromatographic Method. With the Extrelut column for extraction of lypophylic compounds from the aqueous solution, 160 mL of ethyl acetate was needed to elute 3-HBA. Glutaric acid (GA) was eluted within the first 40 mL. The total elution time was 20 min.

The recovery of GA was determined by means of a second internal standard, adipic acid (AA), which was added to the sample solution just before the methylation procedure. Recoveries of GA were found from 60–100% at random in the range of 0–10 mg of 3-HBA/kg. The methyl esters of both 3-HBA and GA are base line separated (Figure 1).

The identification of the 3-HBA peak in egg samples was confirmed by mass spectrometry analysis.

Under the conditions described, the method is applicable for 3-HBA when present at a concentration of at least 0.4 mg/kg of sample. The theoretical values for the response factors of 1.33 for GA and 1.09 for AA were calculated according to the principles described by Ong Kie Hong (1960) and are in close agreement with the experimental values of 1.30 and 1.15, respectively. The factors were constant over a long period of about 6 months.

The methyl ester solution could not be used for on-column injection, since this solution is saturated with salts that crystallize in the capillary column. Even with a splitter injection, it is advisable to clean or discard the first part of the column after about 100 injections. For this reason columns with chemically bound phases are preferred.

When the GC is equipped with an autosampler system about 15 analyses in 1 day can be carried out by one analyst.

Comparison of the Enzymatic Method with the GC Method. The results of the comparison over the range of 1–5 mg of 3-HBA/kg are shown in Table II. The enzymatic result of sample 2, third day, in duplicate is a Cochran outlier.

The average 3-HBA contents reveal no significant difference ($\alpha < 0.05$) between the enzymatic and gas chromatographic methods, and no level dependency was observed for either method even if the outlier is taken into account.

The standard deviations and coefficients of variation for each addition are shown in Table II.

For the GC assay the standard deviations of the repeatability (within days with the same analyst) and the reproducibility (among days with the same analyst) are calculated to be 0.27 and 0.40 mg of 3-HBA/kg, respectively. For the enzymatic assay the s_r and s_R are 0.20 and 0.23 mg of 3-HBA/kg, respectively; if the Cochran outlier is taken into account, the standard deviations will be 0.29 and 0.33 mg of 3-HBA/kg, respectively.

A 10-fold enzymatic analysis of two samples containing 0.8 and 6.7 mg of 3-HBA/kg gave a standard deviation of 0.16 and 0.31 mg of 3-HBA/kg, respectively.

Both the enzymatic method and the GC method are linear; the correlation coefficients are 0.999 and 0.994, respectively. The correlation coefficient of the GC method is a relative one, since the 3-HBA content of the incubator-reject egg sample, used as the standard sample and added to the fresh egg samples, has been analyzed by the enzymatic method only.

In conclusion, over the range of 1–5 mg of 3-HBA/kg the enzymatic method and the GC method are equal, considering the facts that there is no statistical difference, no level dependency and a good agreement between the standard deviations of both methods. The determined values of 3-HBA are method independent.

These two conclusions were confirmed when the enzymatic method was tried with commercially obtained samples. From different commercial egg producers with different flocks of laying hens, 25 samples, each containing 30 randomly choosen fresh unfertilized table eggs, were analyzed in duplicate. The average 3-HBA amount was 0.70 mg/kg (0.4–1.0 mg/kg) with a standard deviation of 0.16 mg of 3-HBA/kg (Uijttenboogaart et al., 1986).

Heaney and Curtis (1976) with their gas chromatographic method registered for unfertilized eggs from different flocks an average amount of 0.63 mg of 3-HBA/kg with a standard deviation of 0.18 mg/kg ($n = 8$). The difference in average amount between the two methods is not statistically significant ($\alpha < 0.1$; Student's t-test).

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