# A RAPID QUANTITATIVE COLORIMETRIC DETERMINATION OF BLOOD ACETONE APPLIED TO THE ASSESSMENT OF KETOSIS IN FASTED PREGNANT EWES

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## ABSTRACT

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A simple, accurate, colorimetric method for determining blood acetone as an adjunct to the enzymic method of estimating the other ketones was developed and tested on a group of fasted pregnant ewes. Acetone reacted with 2-hydroxybenzaldehyde to form a stable coloured complex that followed Beer's Law up to an acetone concentration of at least 4 mg/100 mℓ of the test solution at 490 nm. While the optimum incubation time of the reaction mixture was found to be 3 h at 40 °C, it could also be left to incubate overnight at room temperature. When tested in a blood matrix, the method gave a mean within-batch coefficient of variation of 0,7%, and a day to day variation of 0,3–1,2%, while an overall recovery of 100,6±1,4% was achieved over 5 concentration ranges (2,86–10,53 mg/100 ml). The values obtained from this method corresponded closely to those from the diffusion technique previously employed and it considerably simplified the procedure. A direct linear relationship, $y = 2,594x + 2,917$ with a coefficient of determination $r^2 = 0,958$ for 49 pairs of data, was found between the acetone ($= x$ mg/100 mℓ) and total ketone ($= y$ mg/100 mℓ) concentrations in blood samples drawn from fasted pregnant sheep. This relationship can therefore be used to estimate accurately the degree of ketosis from the blood acetone concentration alone.

### Résumé

### UNE DÉTERMINATION COLORIMÉTRIQUE QUANTITATIVE RAPIDE DE L'ACÉTONE SANGUIN APPLIQUÉE POUR L'ÉVALUATION DE LA CÉTOSE CHEZ DES BREBIS PLEINES À JEUN

*Une méthode colorimétrique simple et précise pour déterminer l'acétone du sang en tant qu'adjointe à la méthode d'estimation enzymique des autres corps cétoniques, a été développée et mise à l'epreuve sur un groupe de brebis pleines à jeun. L'acétone réagit avec 2-hydroxybenzaldehyde pour former un complexe coloré stable qui suivit la Loi de Beer jusqu'à une concentration acétonique d'au moins 4 mg/100 mℓ de la solution test à 490 nm. Tandis que le temps d'incubation optimum du mélange réactif s'avéra être de 3 h à 40 °C, on trouva qu'on pouvait aussi le laisser incuber pendant la nuit à la température ambiante. Quand la méthode fut appliquée dans un milieu sanguin, elle donna un coefficient moyen de variation dans le lot de 0,7% et une variation de jour à jour de 0,3–1,2% tandis qu'un rétablissement d'ensemble de 100,6±1,4% fut réalisé sur 5 échelles de concentration (2,86–10,53 mg/100 mℓ). Les valeurs obtenues à partir de cette méthode correspondirent étroitement à celles de la technique de diffusion antérieurement utilisée et elle simplifia considérablement le procédé. Une relation linéaire directe, $y = 2,594x + 2,917$ avec un coefficient de détermination $r^2 = 0,958$ pour 49 paires de données fut trouvée entre l'acétone ($= x$ mg/100 mℓ) et le total ($= y$ mg/100 mℓ) des concentrations cétoniques dans les échantillons de sang retiré des brebis pleines à jeun. Cette relation peut donc être utilisée pour estimer avec précision le degré de cétose de la concentration acétonique seule du sang.*

## INTRODUCTION

Fasting, diabetes and pregnancy ketosis are all characterized by high concentrations of the ketones (acetone, aceto-acetic and 3-hydroxybutyric acids in the blood). The concentration of these metabolites determines the degree of ketosis and hence of metabolic acidosis and is, therefore, of great clinical importance.

The individual concentrations of the ketones may be accurately determined by the fairly lengthy acid dichromate oxidation of the aceto-acetic and 3-hydroxybutyric acids to acetone which is assayed colorimetrically in a Conway unit (Procos, 1961). Alternatively, the 2 acids may more rapidly be estimated by an enzymatic technique (Williamson, Mellanby & Krebs, 1962) which does not measure acetone. Since the semi-quantitative estimate of acetone alone has often been used to gauge the degree of clinical ketosis (Procos & Clark, 1963), we felt that the relationship between acetone and the total ketones needed to be quantified. To accomplish this we developed a simple, accurate, quantitative test for acetone as a complement to the enzymatic determination of aceto-acetic and 3-hydroxybutyric acids in blood, and use the method to demonstrate a simple stoichiometric relationship between acetone and total blood ketones in fasted pregnant sheep.

## MATERIALS AND METHODS

### Animals

A group of adult pregnant Merino ewes were kept in individual pens with free access to water. Once daily they were given 1 500 g of lucerne hay plus 30 g of mineral salt[1] mixture. They were fasted for 8 days from the 135th day of gestation.

### Sample collection and preparation

Exactly 2 mℓ of blood was collected without suction from a jugular vein at 08h00 on each day of the fasting period and immediately added to 2 mℓ perchloric acid (30% w/v) kept at 4 °C. The mixture was thoroughly shaken and centrifuged at 10 000 g for 20 min. A 2 mℓ aliquot of the supernatant was neutralized to pH 8, using first 4 N and then 0,1 N potassium hydroxide to minimize volume change. After standing at 4 °C for 30 min, the mixture was centrifuged at 10 000 g for 20 min and aliquots of the supernatant were used for the various ketone determinations.

### Reagents

*Chemicals.* All the reagents were of AR grade and were obtained from Merck, Darmstadt, unless otherwise specified. Boehringer, Mannheim, supplied the enzyme 3-hydroxybutyric acid dehydrogenase (EC 1.1.1.30) suspended in 3,2 M ammonium sulphate

[1] Kimtrafos 25, Kynoch Feeds, Kimberley, RSA

at a concentration of 5 mg/ml and with an activity of 3 U/mg, as well as the coenzyme nicotinamide adenine dinucleotide in the oxidized ($NAD^+$) and reduced ($NADH+H^+$) forms.

*Standard solutions.* The colour reagent consisted of a well-mixed solution of 1 ml of 2-hydroxybenzaldehyde (GR) in 80 ml of 4 N potassium hydroxide, prepared on the day of use and kept in an amber bottle. Reference solutions of acetone, aceto-acetic and 3-hydroxybutyric acids were prepared as previously described (Procos, 1961).

*Procedures*

*Enzymatic analysis of aceto-acetic and 3-hydroxybutyric acids.* The method of Williamson, Mellanby & Krebs (1962) was used, with the following modifications. Absorbance was monitored in a Zeiss PMQ II spectrophotometer, and the cuvettes containing the aceto-acetic acid reaction mixture were sealed with Parafilm[2] to prevent loss of acetone during the incubation period.

*Direct analysis of acetone.* At the completion of the enzymatic analysis, the entire contents (3 ml) of each sealed aceto-acetic acid cuvette were added to 5 ml of colour reagent in a 15 ml tissue culture tube, recapped, mixed thoroughly on a Vibromixer and incubated for 3 h at 40 °C. The absorbance was read in a Zeiss PM 2 D spectrophotometer with a 10 mm lightpath at 490 nm. Suitable blank and calibration standards were prepared by adding either water or an aliquot of appropriately diluted standard solution respectively to a blank aceto-acetic acid reaction mixture and treating them in parallel with the unknown samples. After the spectrophotometer was set to the zero reference point, using the blank solution for this, the upper calibration point was adjusted to the concentration value of the standard solution. The concentration of each unknown sample was read directly from the instrument.

Standard curves were produced by the addition of 3 ml aliquots of appropriately diluted stock solution to 5 ml of colour reagent in a 15 ml tissue culture tube and then treated as above.

*Analysis of acetone by diffusion.* The method, based on the diffusion of acetone in a Conway unit from the reaction mixture to the colour reagent, described by Procos (1961), was used without modification. The absorbance was read at 490 mm in a Zeiss PM 2 D spectrophotometer with a flow-through glass cell of 10 mm lightpath.

## Results

*Direct analysis of acetone*

*Optimum conditions.* The investigation of the time-temperature relationship of the 2-hydroxybenzaldehyde-acetone reaction yielded the following results. When incubated at room temperature (20–22 °C), the colour reaction required more than 5 h to reach maximum absorbance. By raising the incubation temperature to 40 °C, maximum colour production was achieved in 3 h and remained stable for at least another 3 h. The reaction followed Beer's Law from zero to an acetone concentration of at least 4 mg/100 ml in the neutralized supernatant, even when the reaction was incomplete. Accordingly, the neutralized supernatant was suitably diluted so as not to exceed 4 mg/100 ml (equivalent to a blood concentration of

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[2] Marathon Products, Neenah, Wisconsin, USA

9 mg/100 ml), and a standard was always included to set the slope of the standard curve on the spectrophotometer.

*Precision.* About 150 ml of blood was withdrawn 2 h after feeding from a Merino sheep and used to prepare 3 subsamples containing 2, 5 and 8 mg/100 ml acetone respectively. Each subsample was further divided into 7 aliquots which were analysed by the direct colorimetric method, yielding a mean within-batch coefficient of variation of 0,7% with a range of 0,3–1,2% over a period of 3 days. A day to day precision value was not directly measured, as blood samples containing acetone could not be stored for any length of time. For instance, in a batch of 8 replicate samples, the acetone concentration declined at room temperature from 7,95±0,05—6,08±0,04 mg/100 ml in 2 h.

*Accuracy.* About 200 ml of blood, collected from a well-fed Merino sheep, was subdivided into 5 ml portions to which suitably diluted acetone standards (0,1 ml) were added to form a concentration series ranging from 0–10 mg/100 ml. The results of the analysis for acetone, presented in Table 1, show an overall recovery of 100,6±1,4% (SEM) across the range of tested values.

TABLE 1 Recovery of acetone standards added to blood samples (mean ± standard error of 4 samples)

| Acetone (mg/100 ml) | | % Recovery mean ± SEM |
|---|---|---|
| Added | Found ± SEM | |
| 2,86 | 2,98±0,06 | 104,2±1,6 |
| 4,29 | 4,23±0,06 | 98,6±1,5 |
| 5,72 | 5,68±0,03 | 99,3±0,4 |
| 7,85 | 8,16±0,09 | 103,9±1,1 |
| 10,53 | 10,23±0,29 | 97,2±2,8 |
| Overall recovery... | | 100,6±1,6 |

This method was compared to the Conway diffusion technique (Procos, 1961) by analysing a series of 80 samples differing in acetone concentration in parallel. The results are summarized in Fig. 1. A regression analysis of the data yielded a straight line with a slope insignificantly different from unity (0,987±0,014), which passed essentially through the origin (0,025±0,057).

The coefficient of determination $r^2=0,986$ and the standard error of x on y=0,279 indicated that there was a close correspondence between the 2 sets of data.

The possible interference of the ketone group of aceto-acetic acid in the 2-hydroxybenzaldehyde-acetone reaction was assessed by incubating 4 sets of acetone standards ranging from 0–4 mg/100 ml at 4 levels of aceto-acetate concentration (0–12,6 mg/100 ml). The results, given in Fig. 2, indicate that the reaction was inhibited by the aceto-acetate, although the extent of the inhibition was unaffected by the concentration of the inhibitor. The constant increase in the ordinate intercept was proportional to the increase in the inhibitor and is related to the spontaneous decomposition of aceto-acetate to acetone which is considerable at the incubation temperature and period employed (40 °C for 3 h). Therefore, to determine the acetone quantitatively, all aceto-acetate must be removed enzymatically from the neutralized supernatant, as described above, before the colour reagent is added and the mixture incubated.
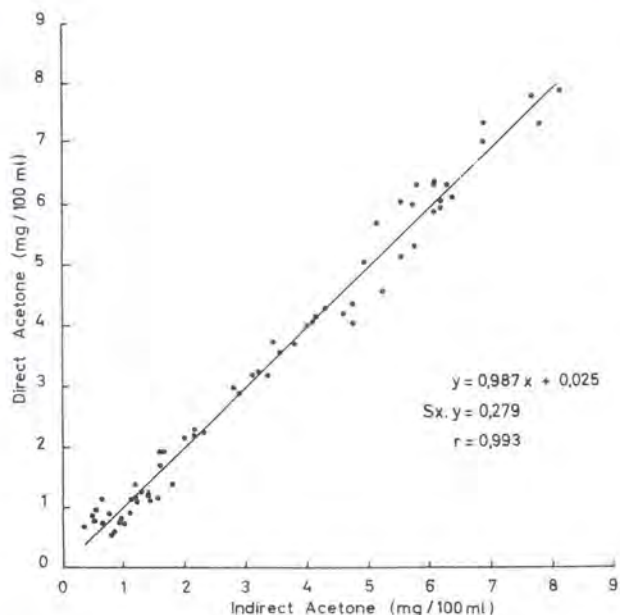
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FIG. 1 Comparison between the Conway diffusion (indirect acetone) and the colorimetric (direct acetone) techniques. Regression analysis on 80 data pairs



FIG. 2 The effect of sodium aceto-acetate on the colour reaction between acetone and 2-hydroxybenzaldehyde when incubated for 4 h at 40 °C

*Blood ketone concentrations in the fasted pregnant ewe*

A direct linear relationship was found between the acetone and total ketone concentrations in blood samples drawn from fasted pregnant ewes. A least squares regression analysis of the results, shown in Fig. 3, gave a coefficient of determination of $r^2 = 0,958$ for 49 pairs of data, with $P > 0,999$. This was a linear relationship represented by the equation $y = 2,594 x + 2,917$ with a standard error of estimation of x on $y = 1,321$ and an F factor of 1073. Thus, for an

acetone concentration of 5 mg/100 m$\ell$, the predicted total blood ketone concentration would be $15,9 \pm 0,7$ mg/100 m$\ell$ at the 99% confidence level.
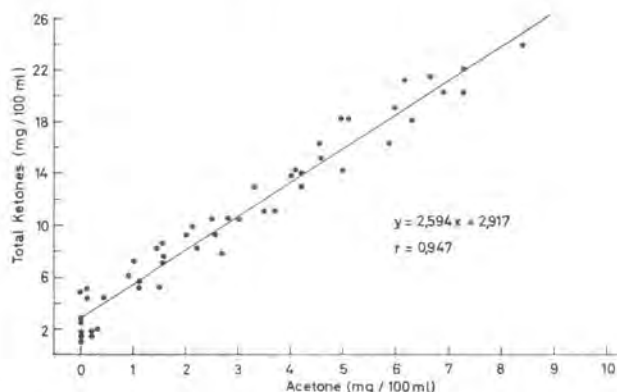


FIG. 3 Linear relationship between the concentration of acetone and total ketones in the blood of fasted pregnant ewes previously maintained on lucerne hay

These results prompted a revaluation of the data obtained for blood ketones in previous experiments (Procos & Gilchrist, 1966) in respect of pregnant ewes fed 300 g cow meal with either 1 000 g of lucerne hay or 3 000 g of green lucerne, and then fasted for 8 days from the 135th day of gestation. The striking similarity between the results obtained from the present group of fasted pregnant ewes and those of the previous groups is presented in Table 2. The relationship in all 3 groups displayed a high degree of linearity (F = 1073, 1058 and 446 for the present, lucerne hay and green lucerne groups, respectively) and had statistically indistinguishable slopes ($2,594 \pm 0,079$, $2,637 \pm 0,075$ and $2,967 \pm 0,140$, respectively) and basal total ketone concentrations (represented by the ordinate intercept). This relationship can therefore be used to estimate accurately the concentration of total blood ketones from the acetone concentration alone, and hence the degree of ketosis.
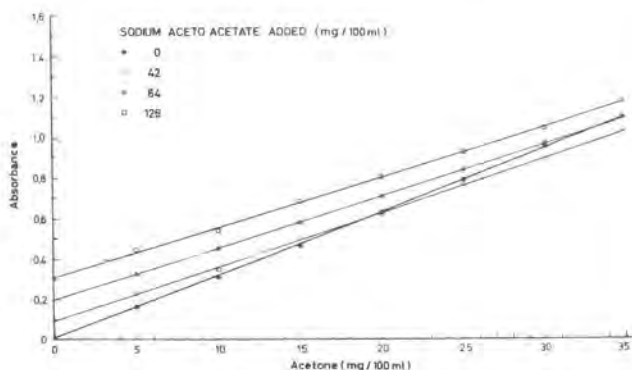
### DISCUSSION

A simple, rapid, colorimetric method for the determination of blood acetone was successfully adapted from the Conway diffusion technique previously used (Procos, 1961) without any loss of precision or accuracy. Since this direct method of estimating acetone may be directly coupled to the enzymatic method of estimating the other 2 ketones (Williamson, Mellanby & Krebs, 1962), aceto-acetic and 3-hydroxybutyric acids, there is a further saving of time and manipulation. The stability of the 2-hydroxybenzaldehyde-acetone reaction product allowed a certain

TABLE 2 A comparison of regression parameters describing the acetone-total ketone relationship in 3 groups of fasted pregnant ewes

| Group | Diet | Sample size (pairs of data) | Ordinate intercept ±SEM | Slope ±SEM | Coefficient of determination | F factor |
|---|---|---|---|---|---|---|
| 1. Present.... | Lucerne hay............. | 49 | $2,917 \pm 0,301$ | $2,594 \pm 0,079$ | 0,958 | 1073* |
| 2. Previous.... | Cow meal+green lucerne... | 45 | $2,927 \pm 0,293$ | $2,637 \pm 0,075$ | 0,966 | 1058* |
| 3. Previous.... | Cow meal+lucerne hay.... | 52 | $3,093 \pm 0,406$ | $2,967 \pm 0,140$ | 0,889 | 446* |

* Significance of $P > 0,999$

flexibility in the experimental procedure. Although the recommended protocol requires a 3 h incubation at 40 °C, the reactants may be left overnight at room temperature (20–22 °C), provided the sampling demanded by experimental conditions is carried out in the late afternoon. The inclusion of a standard with every set of samples compensates for any fluctuation in the slope of the response curve.

The striking similarity between the regression parameters obtained from the analysis of the present ($y = 2,594 \ x + 2,917$, $r^2 = 0,958$) and previous ($y = 2,637 \ x + 2,927$, $r^2 = 0,966$ and $y = 2,967 \ x + 3,093$, $r^2 = 0,889$) blood acetone ($= x$) and total ketone ($= y$) concentrations in fasted pregnant ewes (Procos & Gilchrist, 1966), means that the degree of ketosis can be estimated with confidence from the blood acetone concentration. This relationship is consistent with the known metabolism of acetone. Since aceto-acetic acid spontaneously decarboxylates to form acetone, which is very slowly metabolized, the blood acetone concentration will increase once the maximum clearance rate has been exceeded (i.e.

above a blood total ketone concentration of 2,5 mg/100 m$\ell$). Thus, since the decarboxylation is non-enzymatic, this increase must be directly proportional to the blood aceto-acetic acid and hence to the total ketone concentration, and cannot be affected by diet or the genetic background of the sheep.

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