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A GAS-CHROMATOGRAPHIC HEADSPACE METHOD FOR THE DETERMINATION OF ACETONE IN BOVINE MILK, BLOOD AND URINE

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ABSTRACT

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An automated headspace gas-chromatographic method has been developed for the determination of acetone in the milk, blood and urine of dairy cows. Five mℓ samples were saturated with 2 g of sodium chloride and equilibrated for 30 min at 90 °C in a Hewlett-Packard HP 19395A automatic headspace sampler. The headspace volatiles were transferred without splitting to a 25 m × 0,3 mm × 0,4 µm Carbowax column in a Shimadzu GC 9A gas chromatograph, operating isothermally at 50 °C. The coefficients of variation for the determination of acetone were 1,5–4,4 % for urine, 10,0–24,9 % for milk and 2,0–19,6 % for blood. The detection limits were 0,0055 mg/100 mℓ for milk, 0,0072 mg/100 mℓ for blood and 0,0080 mg/100 mℓ for urine. The analysis time of 5 min per sample provided an adequate rate of throughput for routine monitoring.

INTRODUCTION

Increases in the concentration of acetone and other so-called 'ketone bodies' in the body fluids are symptomatic of clinical ketosis, a metabolic disorder of lactating dairy cows, ewes in late pregnancy, and goats in late pregnancy or early lactation (Schultz, 1979; Collier, 1985). Clinical ketosis in dairy cattle is economically costly because milk production and quality decline, the animal's general condition and fertility deteriorate and veterinary support is expensive (Shaw, 1956; Payne, 1989). Sub-clinical ketosis is important in that it may remain undetected, and, hence, untreated, and yet have adverse effects on health and performance which parallel those of clinical ketosis (Fox, 1971; Baird, 1982). The concentrations of ketone bodies in the body fluids determine the degree of ketosis and are of direct clinical importance as practical indicators of the severity of the metabolic disturbance (Kronfeld, 1972; Schultz, 1974; Hradecky, Jagos & Janak, 1978; Van der Walt, Hunter & Procos, 1981).

Andersson (1984) reported high correlations between acetone plus acetoacetate in milk and ketone bodies in blood. These results supported those of Steger, Girschewski, Piatkowski & Voigt (1972, cited by Andersson & Lundström, 1984). Since the ratio between milk acetoacetate and milk acetone plus acetoacetate did not vary significantly, Andersson (1984) proposed that milk acetone alone can be used to monitor the level of ketone bodies.

The volatility of acetone, to which all other ketone bodies may be converted, makes it a good candidate for headspace gas chromatography. This method can be applied to a wide variety of sample materials, which do not need to be deproteinized. Separate determinations of micromolar concentrations of oxidized, reduced and total ketone bodies have been reported, using this technique (Eriksson, 1972; Hradecky *et al.*, 1978; Hradecky & Kudlac, 1984; Lopez-Soriano & Argilés, 1985). Whether it can be successfully applied to milk samples had not previously been tested.

The aim of this study was, therefore, to develop a practical method for monitoring ketone bodies by determining headspace volatiles, such as acetone, in cow's milk. For this application the method was required to be:

- simple and straightforward, with the least possible chemical manipulation,
- automated in order to provide a high sample throughput,
- able to produce results from blood and urine specimens directly comparable to those from milk,
- sensitive enough to detect normal levels of ketone bodies,
- quantitatively precise enough to detect sub-clinical variations in ketone body concentrations.

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METHODS

Instrumentation

A Shimadzu GC-9A gas chromatograph[1] with a flame ionization detector (FID) was connected to a Shimadzu C-R3A Chromatopac[1] integrator and a Hewlett-Packard 19395A headspace sampler[2]. The headspace sampler and the injection system of the gas chromatograph were modified to provide "splitless" transfer of sample to the capillary column (Apps & Winterbach, 1991). The temperatures of the injector and detector were set at 220 °C and 230 °C respectively. Hydrogen supply to the FID was adjusted to 0,9 kg/cm^2 pressure, the synthetic air to 0,5 kg/cm^2 and the make-up gas (nitrogen) flow rate to 55 mℓ/min. A sensitivity of 10^{-11} A/mV was selected for the flame ionization detector.

The wall-coated, open-tubular 25 m × 0,3 mm × 0,4 µm Carbowax column[3] was operated isothermally at 50 °C. The optimized headspace sampling conditions were as follows: bath temperature, 90 °C; valve/loop temperature, 110 °C; equilibration time, 30 min; sampling interval, 5 min; pressurization time, 0 s; venting time, 1 s and injection time 5 s (Winterbach, 1989).

Animals

Milk was obtained from Friesian cows in the dairy herd maintained at the Veterinary Research Institute (VRI), Onderstepoort, on a semi-intensive, zero-grazing system, under uniform conditions of feeding and management. The herd was machine-milked twice a day. The cows were generally healthy

[1] Ilsa (Pty) Ltd, Process House, Charles Crescent, Eastgate, 2103, R.S.A.

[2] Hi-Performance Systems, 9, Eastern Service Road, Sandton, 2146, R.S.A.

[3] Institute for Chromatogrphy, University of Pretoria, Pretoria, 0002, R.S.A.

and varied in age, number of lactations, daily milk yield and stage of lactation.

Samples

Milk

Milk samples were obtained from the pooled herd milk in the bulk tank.

Blood

Approximately 300 mℓ of blood was collected into a heparinized glass bottle directly from the heart of a slaughtered cow. Since pilot experiments had shown that freezing and thawing did not affect acetone determinations the blood was frozen overnight before analysis.

Urine

Approximately 300 mℓ of urine was collected from the bladder of the cow which provided the blood. It was frozen for storage, and thawed before analysis.

Sample preparation

Each sample consisted of 5 mℓ of either milk, blood or urine in a 10 mℓ vial[4] with a Teflon-faced silicone rubber septum[4].

Salting-out was used to improve the partitioning of volatiles into the headspace (Ioffe & Vitenberg, 1983 pp. 24–25); each sample was saturated with 2 g of sodium chloride[5] (Winterbach, 1989).

Reproducibility and calibration standards

To calibrate the FID response in terms of acetone concentration and to estimate the precision of the determinations, volumes of 1 % acetone in distilled water ranging from 1 µℓ to 200 µℓ were added to 3 replicate series of 11, 5 mℓ samples of each body fluid. Each sample was saturated with 2 g of sodium chloride and analysed under the standard conditions. Coefficients of variation of acetone peak area were calculated for each fluid at each level of acetone addition. The line of best, least squares fit, and the corresponding regression equation for acetone peak area against level of added acetone was calculated for each fluid.

Mass spectrometer-gas chromatography

One sample of milk which was known, on the basis of quantitive analysis, to contain a high level of acetone, was used to confirm the identity of the acetone peak by mass spectrometer-gas chromatography. The milk sample was equilibrated for 30 min at 120 °C. Using a 5 s injection, the headspace volatiles were transferred, to a Carbowax 25 m × 0,3 mm ID × 0,4 µm film thickness column in a Varian 1200 gas chromatograph with an oven temperature of 50 °C isothermal. The gas chromatograph was connected by an open-split interface to a VG Micromass 16F mass spectrometer, operating in the electron impact mode with an electron energy of 70 eV.

RESULTS

Resolution

For all three body fluids the acetone peak on the chromatogram was sharp and symmetrical and clearly resolved from any interfering peaks (Fig. 1). With an analysis time for each sample of 5 min and equilibration time of 30 min, 2,5 h was required to analyse the 24 samples in a fully-loaded headspace sampler carousel.

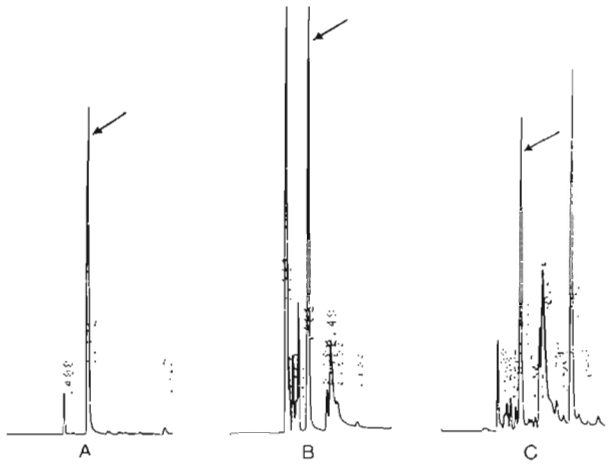

FIG. 1 Chromatograms of headspace volatiles from; A, milk; B, blood and C, urine of a mildly ketotic cow. The acetone peak is arrowed. For analytical conditions see text

Reproducibility

The coefficient of variation of acetone peak areas at the various levels of added acetone were between 1,5 % and 4,4 % for urine, between 10,0 % and 24,9 % for milk and between 2,0 % and 19,6 % for blood. The highest values for blood and milk were from unspiked samples.

Calibration

Fig. 2 shows the linear, least-squares regressions between acetone peak area and volume of 1 % acetone standard added for the 3 body fluids.

The corresponding calibration equations were:

$$\text{for milk, } c = \frac{a}{18337\ (\pm\ 1244)}$$

$$\text{for blood, } c = \frac{a}{13884\ (\pm\ 202)}$$

$$\text{for urine, } c = \frac{a}{12511\ (\pm\ 330)}$$

where: c = acetone concentration in mg/100 mℓ
a = peak area,
and figures in brackets are 95 % confidence limits

The regressions were significant at the $P < 0{,}0001$ level for all 3 fluids (Table 1).

TABLE 1 The sample sizes (n), coefficients of determination (r^2) and confidence levels (P) for the linear regression of headspace acetone peak area *vs* added acetone for cow's milk, urine and blood

	n	r^2	P
Milk	30	0,9687	0,0001
Blood	30	0,9985	0,0001
Urine	33	0,9946	0,0001

With a working peak detection threshold of 100 area counts the detection limits in mg/100 mℓ were 0,0055 for milk, 0,0072 for blood and 0,0080 for urine.

[4] Hi-performance Systems, 9, Eastern Service Road, Sandton, 2146, R.S.A.

[5] Merck (SA) (Pty), Federated Park, Midrand, R.S.A.

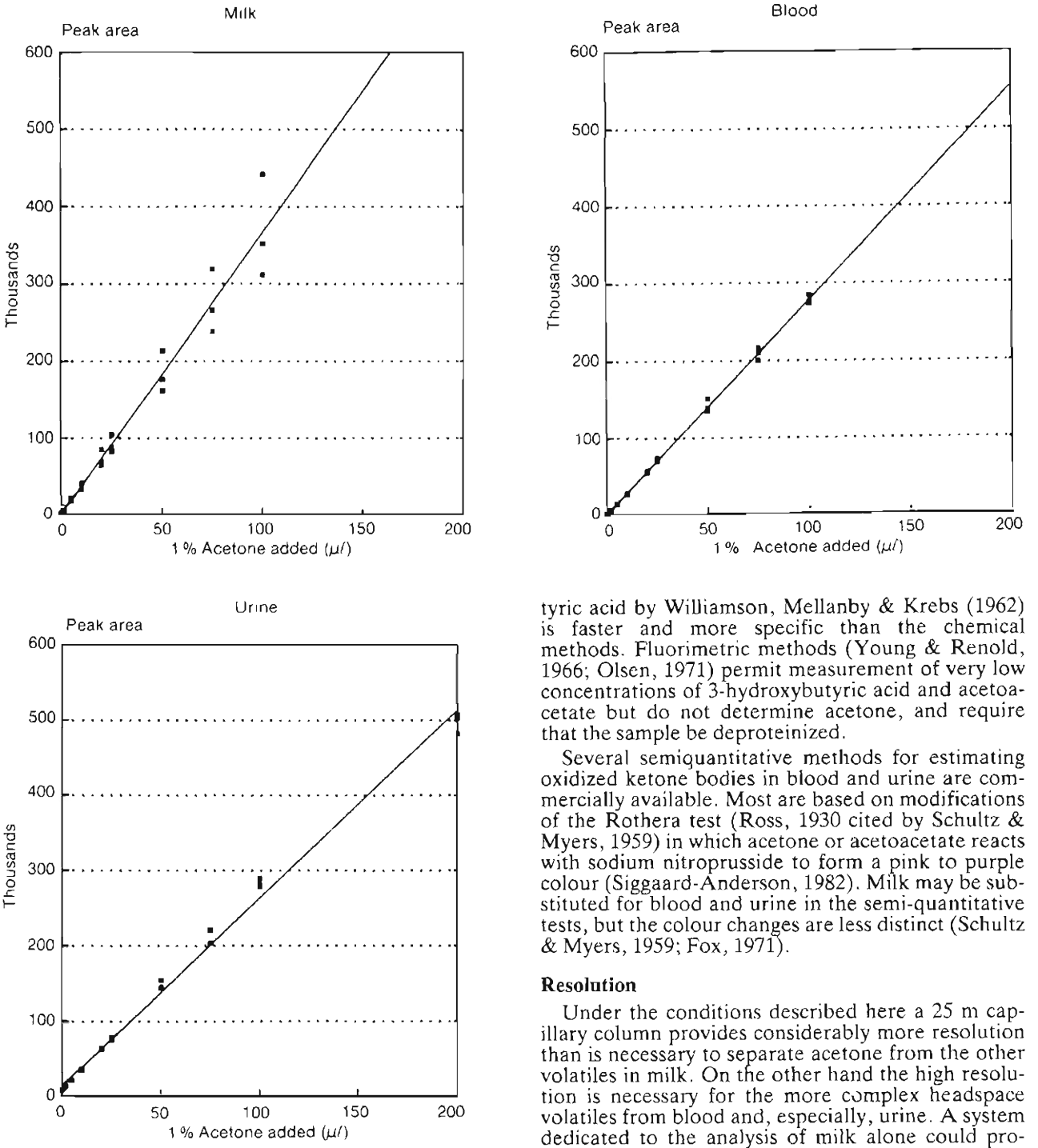

FIG 2. Linear regressions of acetone peak area on volume of 1 % acetone standard added for milk, blood and urine

DISCUSSION

Acetoacetate, 3-hydroxybutyric acid and acetone may be assayed colorimetrically as acetone (Thin & Robertson, 1952; Procos, 1961; Van der Walt *et al.* 1981), although the methods are cumbersome and, as a consequence, are generally unsuitable for routine determinations on large numbers of samples. Furthermore, these techniques have not been used to assay milk samples. Flow injection analysis (Marstorp, Anfält & Andersson, 1983) is fast, but it is not particularly specific and it requires pre-heating and homogenization of milk samples. The enzymatic determination of acetoacetic acid and 3-hydroxybutyric acid by Williamson, Mellanby & Krebs (1962) is faster and more specific than the chemical methods. Fluorimetric methods (Young & Renold, 1966; Olsen, 1971) permit measurement of very low concentrations of 3-hydroxybutyric acid and acetoacetate but do not determine acetone, and require that the sample be deproteinized.

Several semiquantitative methods for estimating oxidized ketone bodies in blood and urine are commercially available. Most are based on modifications of the Rothera test (Ross, 1930 cited by Schultz & Myers, 1959) in which acetone or acetoacetate reacts with sodium nitroprusside to form a pink to purple colour (Siggaard-Anderson, 1982). Milk may be substituted for blood and urine in the semi-quantitative tests, but the colour changes are less distinct (Schultz & Myers, 1959; Fox, 1971).

Resolution

Under the conditions described here a 25 m capillary column provides considerably more resolution than is necessary to separate acetone from the other volatiles in milk. On the other hand the high resolution is necessary for the more complex headspace volatiles from blood and, especially, urine. A system dedicated to the analysis of milk alone could probably use a 5 m column, with a reduction in analysis time to only 1 min per sample, but then sample preparation would become the limiting step in analytical productivity.

Reproducibility

The variability in acetone peak areas can be ascribed to the effects of the differing times for which the samples were queued for automated analysis. The differences between the fluids were probably due to their different chemical compositions. The greater variability of acetone peak areas for the milk samples could have been due to a higher conversion rate of acetoacetate to acetone. Both blood and urine in cattle are somewhat alkaline while milk is slightly acidic, with a pH of 6,6–6,7. Acetoacetate readily decomposes to acetone in hot acid solutions (Eriksson, 1972). It is unlikely that 3-hydroxybutyric

acid had any influence on the acetone peak areas for any of the body fluids, since its conversion to acetone requires a strong oxidative reagent.

The variability in acetone peak areas for milk was less than the biological variation arising from subclinical ketosis (Winterbach, 1989), and therefore did not pose any practical problem in using this method to monitor metabolic changes in dairy cattle.

Calibration

The differences among the regressions of peak area on acetone concentration for milk, blood and urine are probably due simply to different solubilities of acetone in the three fluids. The lower coefficient of determination for milk (0,9687) was probably due to a higher, non-quantitative conversion rate of acetoacetate to acetone in milk. Better precision could, perhaps, have been obtained with a longer equilibration time, but a balance had to be struck between analysis time and precision. The 30-min equilibration time provided a usefully rapid sample throughput and sufficient analytical precision to detect both day-to-day, and inter-quarter, variations in milk acetone concentrations (Winterbach, 1989).

Detection limit

The headspace technique's detection limits for acetone are in the microgram per 100 mℓ range for all three body fluids, which is 2–3 orders of magnitude below normal, physiological acetone concentrations (Knodt *et al.*, 1942; Schultz, 1974; Andersson & Lundström, 1984; Kelly & Whitaker, 1984). Marstorp *et al.'s* (1983) flow infection analysis of acetone in milk has a detection limit of 0,56 mg/100 mℓ. Other workers have not reported the detection limits of their methods.

The fulfillment of the requirements for sensitivity and precision in relation to physiological, sub-clinical levels of acetone, and the application of this method to the diagnosis of subclinical ketotic changes in dairy cows will be discussed in a subsequent paper.

The localized, intramammary effects of ketosis, and its udder health implications, have received less attention than its "whole body" metabolic aspects. Concentrations of ketone bodies in blood and milk are highly correlated (Andersson, 1984), and are increased during ketosis (Schultz & Myers, 1959; Fox, 1971; Schultz, 1974). Clinical, infectious mastitis is most prevalent during early lactation, when lactation stress increases (Giesecke, 1985) and freshening cows are in a state of increased ketogenesis. White & Rattray (1968) have shown that, *in vitro*, increased lacteal concentrations of isopropanol and acetoacetic acid reduce the killing of staphylococci by leucocytes whose function is to protect the udder from infection. Potezny, Atkinson, Rofe & Conyers (1981) have found that the growth of gram negative bacteria is inhibited *in vitro* by 3-hydroxybutyrate. Further research on the udder health implications of ketone bodies in milk would be particularly interesting.

Conclusion

Automated, headspace gas-chromatography provides a rapid, precise method of determining the concentration of acetone in the milk, blood and urine of dairy cows. Its detection limit is 2–3 orders of maqnitude below normal, physiological concentrations of acetone in the three fluids.

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