*Journal of Chromatography*, 577 (1992) 215–220
*Biomedical Applications*
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 6327

# Simple and sensitive determination of diacetyl and acetoin in biological samples and alcoholic drinks by gas chromatography with electron-capture detection

Masato Otsuka and Shinji Ohmori*

*Faculty of Pharmaceutical Sciences, Okayama University, Tsushima-Naka-1-1, Okayama 700 (Japan)*

(First received October 23rd, 1991; revised manuscript received February 18th, 1992)

ABSTRACT

Acetoin was quantitatively oxidized into diacetyl by $Fe^{3+}$ in 1 *M* perchloric acid. The reaction of diacetyl with 4,5-dichloro-1,2-diaminobenzene afforded 6,7-dichloro-2,3-dimethylquinoxaline (DCDMQ), which was extracted by benzene containing aldrin (25 ng/ml) as an internal standard, and determined by gas chromatography with electron-capture detection. The method is very simple and sensitive. The detection limit of DCDMQ (either diacetyl or acetoin) was 10 fmol/μl of the benzene extract, and the determination limit of DCDMQ (either diacetyl or acetoin) was 50 fmol/μl of the extract. Both acetoin and diacetyl could be determined in 0.1 ml of normal human urine or blood, and both were found in rat liver, kidney and brain. The method was also applied to the determination of acetoin and diacetyl in alcoholic drinks.

## INTRODUCTION

Extensive studies of diacetyl and acetoin have been made in the fields of fermentation technology and food chemistry. However, little is known about the biochemistry of these two simple substances, especially in animals, although they have been well known for a long time. Our aim was to study the formation and degradation of diacetyl and acetoin in animals, and their biological role in yeast. For this purpose, we required a method for determining these two compounds.

This paper describes a simple and sensitive determination of diacetyl and acetoin in biological samples by gas chromatography (GC) with electron-capture detection (ECD), based on the reactions shown in Fig. 1.

## EXPERIMENTAL

### *Chemicals*

Diacetyl (2,3-butanedione), acetoin (3-hydroxy-2-butanone), 2,3-pentanedione, aldrin (standard-material grade), ethyl acetate and benzene for GC–ECD were purchased from Wako (Osaka, Japan). 4,5-Dichloro-1,2-diaminoben-

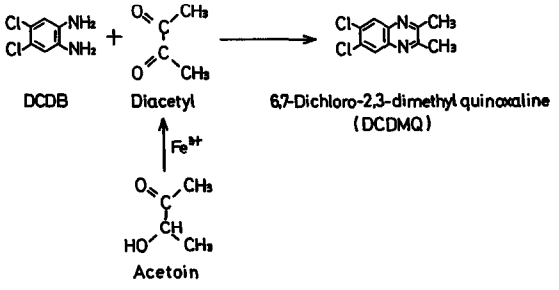


Fig. 1. Reaction scheme for the determination of diacetyl and acetoin.

zene (DCDB) was obtained from Aldrich (Milwaukee, WI, USA) and purified by recrystallization from hydrochloric acid after charcoal treatment. The purchased DCDB (free form) was black, whereas the recrystallized DCDB was colourless platelets. 6,7-Dichloro-2,3-dimethylquinoxaline (DCDMQ) was prepared from 0.25 g of DCDB and 0.1 g of diacetyl in 30 ml of 1 *M* HCl at 40°C for 2 h. The reaction mixture was allowed to stand overnight at room temperature, and then extracted with 30 ml of ethyl acetate twice. The extract was dried with $Na_2SO_4$, and evaporated to dryness under reduced pressure. The residue was recrystallized from benzene in 92.9% yield, m.p. 190–193°C (191–192°C [1]), as yellowish brown needles. 6,7-Dichloro-2-ethyl-3-methylquinoxaline (DCEMQ) was obtained from 0.5 g of DCDB and 0.25 g of 2,3-pentanedione in 50 ml of 1 *M* HCl at 40°C for 2 h. The reaction mixture was extracted and the residue was recrystallized as for DCDMQ: 85.0% yield, m.p. 144–145°C, pale yellow needles.

*Gas chromatography*

We used two types of GC–ECD. One was a Hewlett-Packard Model HP5890 gas chromatograph (Hewlett-Packard, Avondale, PA, USA), with an electron-capture detector. A Hi-Cap CBP1-M25-025 capillary column (25 m × 0.25 mm I.D.) (Shimadzu, Kyoto, Japan) was used. The electron-capture detector was maintained at 300°C. The column temperature was raised from 60 to 200°C at 30°C/min, and from 200 to 260°C at 10°C/min. The injector block temperature was adjusted to 230°C. The flow-rate of carrier gas (helium) was 65.6 ml/min and its pressure was 105 kPa.

The other system was a Shimadzu Model GC-4CM gas chromatograph (Shimadzu), equipped with a $^{63}Ni$ electron-capture detector (ECD-4C). The glass column (2 m × 3 mm I.D.) was packed with 1.5% Silicon OV-17 on Shimalite W, 80–100 mesh (Shimadzu). The temperature of the detector and the injector block was 270°C, and that of the column was 185°C. The nitrogen gas flow-rate was 30 ml/min. The electron-capture detector was operated at a pulse frequency of 10 kHz, and the electrometer was set at an attenuation of $10^2$.

*Determination of diacetyl and acetoin in water*

Various amounts of diacetyl (10–200 μl of 0.1 m*M* diacetyl) were placed into 10-ml test-tubes with screwed caps, and 1 ml of 12 m*M* DCDB in 1 *M* HCl was added. After reaction at 40°C for 90 min, the DCDMQ formed was extracted with 2.5 ml of benzene containing 25 ng/ml aldrin as an internal standard. The benzene layer was transferred to a 5-ml vial and dried with sodium sulphate, and an aliquot (1 μl) was injected into the GC column. For acetoin analysis, various amounts of acetoin aqueous solution (10–200 μl of 0.1 m*M* acetoin) were placed into 10-ml test-tubes with screwed caps. After addition of 1 ml of 1 *M* $FeCl_3$ in 1 *M* perchloric acid, the test-tubes were heated at 90°C for 2 h and the diacetyl formed was determined as described above. The amounts of acetoin were obtained by subtracting the values obtained for endogenous diacetyl.

*Determination of diacetyl and acetoin in rat tissues*

Male Wistar albino rats weighing 200–250 g were injected with 0.2 ml of 5% pentobarbital. After *ca.* 5 min the abdomens were opened and *ca.* 4 ml of blood were drawn through cannulation of abdominal aorta. The blood samples were centrifuged at 1700 *g* for 15 min. From the same rats, the livers, kidneys and brains were immediately excised and rinsed in ice-cold physiological saline. Livers and kidneys were perfused with ice-cold saline. These organs were homogenized at 4°C for 1 min in a glass PTFE homogenizer. The homogenate (1 ml) was placed into a 5-ml test-tube, and an equal volumes of 1.2 *M* perchloric acid was added. The mixture was allowed to stand at 0°C for 30 min, then centrifuged at 700 *g* for 5 min. The supernatant was analysed for acetoin and diacetyl.

*Determination of acetoin and diacetyl in alcoholic drinks*

Three kinds each of beer, sake (rice wine), wine, shochu (distilled sake), whisky and brandy as distilled liquor were purchased from stores in the city and analysed without any pretreatment.

## RESULTS

### *Extraction of DCDMQ*

DCDMQ (1 μg) was dissolved in 1 ml of 1 *M* $NaHCO_3$, 10 m*M* phosphate (pH 4–7), 1 *M* HCl or water. These aqueous solutions were extracted with 1 ml of benzene containing 25 ng/ml aldrin, and the benzene layer was analysed. The extraction efficiencies were 97.7% (1 *M* $NaHCO_3$), 98.3% (pH 7.0), 94.5% (pH 6.0), 93.2% (pH 5.0), 92.0% (pH 4.0), 98.7% (1 *M* HCl) and 100% (water). These data indicate that DCDMQ should be extracted under acidic conditions, because DCDB is then eliminated.

### *Reaction conditions for the formation of DCDMQ from diacetyl*

When 10 nmol of diacetyl were treated with various concentrations of DCDB in 1 ml of 1 *M* HCl, a molar ratio of DCDB to diacetyl of greater than 20 gave the best yield. The reaction of 10 nmol of diacetyl with 1 ml of 0.2 m*M* DCDB–1 *M* HCl solution at different times and temperatures revealed that the reaction should be carried out at 40°C for 90 min.

### *Oxidation of acetoin to diacetyl*

A 1-ml volume of 5 m*M* acetoin was heated with 1 ml of 1 *M* $FeCl_3$ in 1 *M* $HClO_4$ at 90°C for various time intervals. The diacetyl formed was analysed as described above. Acetoin was perfectly oxidized after 2 h, and the yield gradually fell to 80% after 5 h owing to further oxidation. $Cu^{2+}$ did not oxidize acetoin at pH 10 under heating at 40°C for 24 h, and $Mn^{2+}$ did not catalyse the oxidation by $O_2$.

### *ECD gas chromatograms of DCDMQ derived from acetoin and diacetyl*

Fig. 2a and c show gas chromatograms of DCDMQ derived from diacetyl in rat urine and liver, respectively. Fig. 2b and d show chromatograms of acetoin in rat urine and liver, respectively, oxidized to diacetyl. Fig. 2e and f show blank chromatograms. The peaks of DCDMQ and aldrin (internal standard) appeared at 11.2 and 12.9 min, respectively.

### *Intra- and inter-assay precision and accuracy*

Five aliquots (1 ml) of a rat urine sample were determined for diacetyl and acetoin on the same day. Diacetyl was measured as 1.75 ± 0.04 nmol/mg of creatinine (coefficient of variation, C.V. = 2.3%) and acetoin was measured as 2.77 ± 0.10 nmol/mg of creatinine (C.V. = 3.6%).

The same determination procedure was used for the same sample on other days and gave values of 1.73 ± 0.04 nmol/mg of creatinine (C.V. = 2.3%) for diacetyl and 2.71 ± 0.12 nmol/mg of creatinine (C.V. = 4.4%) for acetoin.

### *Calibration curves and limits of determination*

Various amounts of diacetyl were treated with DCDB, and the DCDMQ formed was extracted with benzene containing aldrin. An aliquot of the benzene layer was injected into the GC column. When diacetyl was analysed in the range 0.1–1 nmol/ml, the peak-height ratio ($y$) was directly proportional to the diacetyl concentration ($x$, nmol/ml): $y = 1.91x + 0.01$ ($r = 0.9998$, $n = 5$). When diacetyl was analysed in the range 1–100 nmol/ml, the peak-height ratio equation was $y = 1.82x + 0.46$ ($r = 0.9998$, $n = 5$). The detection limit of DCDMQ, based on diacetyl, was 10 fmol/μl of the extract, and the determination limit of DCDMQ, based on diacetyl, was 50 fmol/μl of the extract.

A plot of the peak-height ratio ($y$) *versus* the acetoin concentration ($x$, nmol/ml) gave straight lines: $y = 1.92x + 0.01$ ($r = 0.9999$, $n = 6$), $0.1 < x < 1.0$ nmol/ml; $y = 1.83x + 1.40$ ($r = 0.9999$, $n = 5$), $1.0 < x < 100$ nmol/ml.

The detection and determination limits for acetoin were the same as those for diacetyl.

### *Recovery of diacetyl and acetoin from normal human urine and rat liver homogenate*

Various amounts (0.5–10 nmol) of diacetyl were added to 1 ml of normal human urine or the supernatant obtained from rat liver after centrifugation at 700 *g* for 15 min, and determined as described above. The recoveries were 95.8 ± 3.5 and 96.3 ± 2.7% ($n = 5$), respectively. The recoveries of acetoin were 93.9 ± 2.1% ($n = 5$) for urine and 95.8 ± 2.2% ($n = 5$) for rat liver homogenate.

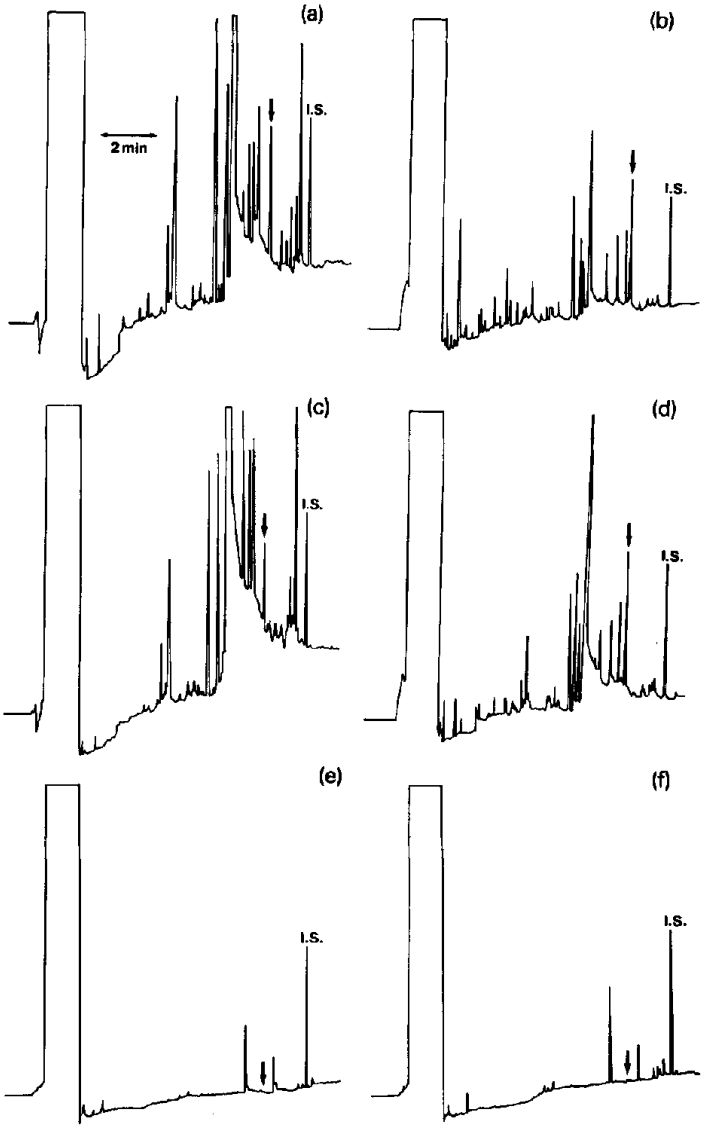


Fig. 2. Gas chromatograms of authentic DCDMQ and DCDMQ derived from acetoin or diacetyl. (a) To 1 ml of rat urine, 1 ml of a 12 m*M* DCDB solution in 1 *M* HCl was added. The mixture was heated at 40°C for 90 min, then extracted with 2.5 ml of benzene containing aldrin as internal standard. An aliquot of the benzene layer (1 $\mu$l) was analysed by capillary GC. (b) A 1-ml volume of rat urine was oxidized with 1 ml of 1 *M* $FeCl_3$ solution in 1 *M* $HClO_4$ at 90°C for 2 h. Intrinsic and formed diacetyl were determined as described in (a). (c) Rat liver (1 g) was homogenized with four volumes of physiological saline in a homogenizer at 0°C for 1 min. To 1 ml of the homogenate, 1 ml of 1.2 *M* $HClO_4$ was added, and the mixture was centrifuged at 700 *g* for 15 min. After 1 ml of 12 m*M* DCDB solution in 1 *M* HCl was added to 1 ml of the supernatant, the mixture was heated at 40°C for 90 min and extracted with 2.5 ml of benzene containing aldrin. An aliquot of the benzene layer (1 $\mu$l) was analysed. (d) The deproteinized solution (1 ml) of rat liver homogenate described in (c) was oxidized with 1 ml of 1 *M* $FeCl_3$ solution in 1 *M* $HClO_4$ at 90°C for 2 h. Acetoin was determined as described in (c). (e) To 1 ml of rat urine, 1 ml of 1 *M* HCl was added. The mixture was heated at 40°C for 90 min and extracted with 2.5 ml of benzene containing aldrin as internal standard. An aliquot of the benzene layer (1 $\mu$l) was analysed by GC as the blank of (a). (f) A 1-ml volume of rat urine was oxidized with 1 ml of 1 *M* $FeCl_3$ solution in 1 *M* $HClO_4$ at 90°C for 2 h, and 1 ml of 1 *M* HCl was added to the reaction mixture. The mixture was treated as described in (e) and analysed by GC as the blank of (b). The arrow shows the peak of DCDMQ, and I.S. is the internal standard (aldrin).

TABLE I

CONTENTS OF ACETOIN AND DIACETYL IN VARIOUS RAT TISSUES

All values are means + S.D. for five rats.

| Tissue | Diacetyl (nmol/g of tissue) | Acetoin (nmol/g of tissue) |
|---|---|---|
| Liver | 3.40 ± 0.95 | 3.62 ± 0.42 |
| Heart | 2.18 ± 0.45 | 1.19 ± 0.32 |
| Kidney | 2.64 ± 0.29 | 1.38 ± 0.53 |
| Muscle | 0.67 ± 0.11 | 0.42 ± 0.19 |
| Brain | 2.36 ± 0.31 | 5.76 ± 0.81 |
| Plasma | 3.29 ± 0.30[a] | 1.60 ± 0.22[a] |
| Urine | 1.78 ± 0.38[b] | 2.94 ± 0.21[b] |

[a] nmol/ml.

[b] nmol/mg of creatinine.

*Diacetyl and acetoin in rat tissues and urine*

Diacetyl and acetoin in various rat tissues and urine were determined (Table I). They were present in all the tissues, and in especially high amounts in the liver.

*Diacetyl and acetoin contents in alcoholic drinks*

Table II shows that diacetyl and acetoin were present in all the alcoholic drinks analysed. Distilled liquor contains less diacetyl and acetoin than fermented liquor. Beer was found to contain much more acetoin than other alcoholic drinks.

TABLE II

CONTENTS OF ACETOIN AND DIACETYL IN ALCOHOLIC DRINKS

All values are means ± S.D. for five samples.

| Liquor | Diacetyl (nmol/ml) | Acetoin (nmol/ml) |
|---|---|---|
| Shochu | 1.2 ± 0.3 | 3.5 ± 0.7 |
| Whisky | 2.2 ± 0.4 | 8.5 ± 0.8 |
| Brandy | 2.7 ± 0.6 | 3.9 ± 1.0 |
| Sake | 4.8 ± 0.9 | 7.5 ± 2.2 |
| Beer | 5.2 ± 0.7 | 27.3 ± 5.3 |
| Wine (red) | 9.6 ± 2.0 | 20.0 ± 9.0 |
| Wine (rosé) | 5.0 ± 0.9 | 16.5 ± 5.5 |
| Wine (white) | 6.6 ± 1.0 | 19.0 ± 1.9 |

## DISCUSSION

In 1920, Lemoigne [2] determined diacetyl as nickel dimethylglyoxime, and Van Niel [3] later improved this method. In both methods acetoin was oxidized to diacetyl by $FeCl_3$. The methods were time-consuming, and measurement was impossible by a gravimetric method below 0.1 mmol/ml acetoin. Greenberg [4] reported a polarographic method, by which 30 nmol/ml diacetyl was determined, but distillation was necessary prior to the determination. Nowadays, such apparatus is not commercially availabe. Westerfeld [5] reported a colorimetric determination of acetoin in blood, and the normal blood concentration of acetoin was reported as 10 $\mu$g per 100 ml. In this method, deproteinized solution from blood had to be distilled twice for each sample. The determination limit was 10 nmol/ml. In 1989 this colorimetric method was modified by Mattessich and Cooper [6] for the determination of diacetyl in rat tissues. However, distillation was again necessary to eliminate acetoin and inhibitors of colour reaction. The determination limit was 5 nmol/ml.

Head-space vapour GC was used for the determination of diacetyl by fermentation [7] and for the analysis of diacetyl and acetoin in cultured buttermilk [8]. The limit of determination in the former method was 47 nmol/ml for diacetyl, and in the latter it was 0.58 nmol/ml for diacetyl and 1.16 nmol/ml for acetoin.

In 1985, Bauman *et al.* [9] reported an HPLC method for the determination of diacetyl, based on triplet–triplet energy transfer from 1,5-naphthylene disulphonic acid to diacetyl. Although the limit of detection was 12 pmol/ml for authentic diacetyl, this method required the use of a pre-column prior to injection for determination of diacetyl in beer samples. In this case, 23 nmol/ml diacetyl could be determined. In comparison with the methods mentioned above, the method reported here is simple and sensitive.

Westerfeld [5] reported that the acetoin concentration in freshly drawn blood from rat, dog, cat, fasted rabbit, pig, pigeon and cow was *ca.* 1.1 nmol/ml. Our result is close to this value. Mattes-

sich and Cooper [6] determined diacetyl in rat heart, kidney and liver, and found values of 389, 85 and 48 nmol/g of tissue, respectively; these are much higher than our values. Mills and Walker [10] found acetoin in urine samples from babies. Our literature search revealed no reports in which acetoin was measured precisely in animal tissues.

In this experiment 2,3-pentanedione, a homologue of diacetyl, was tested as a more proper internal standard than aldrin. However, it was not suitable because the DCEMQ peak was interfered with by other peaks. Since the total yield of DCDMQ derived from diacetyl and acetoin with DCDB was *ca.* 98%, there is no need for an internal standard to be used in the determination.

This method may also contribute to the quality control of alcoholic drinks in industry. For example, the content of diacetyl and acetoin in beer must be exactly controlled. In 1981, Oberman *et al.* [11] reported that the content of acetoin and diacetyl in beer was 14.8–28.4 and 0.47–3.49 nmol/ml, respectively. These values are roughly similar to our data. Nachkov *et al.* [12] determined the diacetyl and acetoin contents of white and red wines. The diacetyl concentration ranged from 2.33 to 3.02 nmol/ml in white wines and from 3.26 to 3.95 nmol/ml in red wines. The acetoin content ranged from 9.09 to 13.6 nmol/ml in white wines and from 13.6 to 38.6 nmol/ml in red wines. All these concentration values are lower than those determined by us, but are of the same order of magnitude.

Beside alcoholic drinks, diacetyl and acetoin are contained in milk [13], butter [14], yogurt [15], coffee [16], tea [17] and beef [18]. Thus almost all people have some daily intake of diacetyl and acetoin. We are studying the metabolism of diacetyl and acetoin using rat liver homogenate, perfused rat liver, and *in vivo* experiments. It is very interesting that when diacetyl or acetoin was given *per os* to rats, a large amount of acetoin accumulated in the brain. These results will be reported, elsewhere.

REFERENCES

1 J. K. Landquist and G. J. Stacey, *J. Chem. Soc.*, (1953) 2822.
2 M. Lemoigne, *C. R. Acad.*, 170 (1920) 131.
3 C. B. van Niel, *Biochem. Z.*, 187 (1927) 472.
4 L. A. Greenberg, *J. Biol. Chem.*, 147 (1943) 11.
5 W. W. Westerfeld, *J. Biol. Chem.*, 161 (1945) 495.
6 J. Mattessich and J. R. Cooper, *Anal. Biochem.*, 180 (1989) 349.
7 H. Suomalainen and P. Ronkainen, *Nature*, 220 (1968) 349.
8 R. T. Marsili, *J. Chromatogr. Sci.*, 19 (1981) 451.
9 R. A. Baumann, C. Gooijer, N. H. Velthorst and R. W. Frei, *Anal. Chem.*, 57 (1985) 1815.
10 G. A. Mills and V. Walker, *Clin. Chim. Acta*, 179 (1989) 51.
11 H. Oberman, E. Pabis and W. Szmelich, *Acta Aliment. Pol.*, 7 (1981) 93.
12 D. Nachkov, E. Kuyumdzhyan and D. Khadzhiiski, *Lozar. Vinar.*, 31 (1982) 35.
13 G. Urbach, *J. Chromatogr.*, 404 (1987) 163.
14 P. Damiani and G. Burini, *J. Assoc. Offic. Anal. Chem.*, 71 (1988) 462.
15 R. Degorce-Dumas, J. Goursaud and J. Y. Leveau, *Ind. Aliment. Agric.*, 103 (1986) 805.
16 K. Harada, O. Nishimura and S. Mihara, *J. Chromatogr.*, 391 (1987) 457.
17 M. Kawakami, A. Kobayashi and T. Yamanishi, *Nippon Nogei Kagaku Kaishi*, 61 (1987) 345.
18 G. M. Leod and J. M. Ames, *J. Food Sci.*, 52 (1987) 42.