Lebensm.-Wiss. u.-Technol., 33, 531–537 (2000)

Influence of Dissolved Carbon Dioxide on the Growth of Spoilage Bacteria

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(Received February 9, 2000; accepted September 19, 2000)

Growth of bacteria is, for most strains, influenced by CO_2 in the atmosphere. The effect of the concentration of dissolved CO_2 ($[CO_2]_{diss}$ in mg/L) was quantified for different Gram-negative (Pseudomonas fluorescens, Photobacterium phosphoreum, Shewanella putrefaciens, Aeromonas hydrophila) *and Gram-positive* (Lactobacillus sake, Brochothrix thermosphacta, Bacillus circulans) *spoilage bacteria at 7 °C. A linear relationship between $[CO_2]_{diss}$ and the maximum specific growth rate μ_{max} as well as between $[CO_2]_{diss}$ and the inverse of the lag phase λ was established. The growth parameters (λ as well as μ_{max}) of Gram-negative bacteria were more influenced by $[CO_2]_{diss}$ in comparison to the growth parameters of Gram-positive bacteria.*

When CO_2 is inserted in a food package, it will begin to dissolve in the water phase of the food. The dissolving rate of CO_2 was determined for gas packaged cooked ham at 7 °C. CO_2 showed a high dissolving rate as, for an initial CO_2 concentration in the headspace of 40 and 80 mL/100 mL, respectively 78% and 87% of the CO_2 dissolved at equilibrium was already dissolved after 60 min.

The $[CO_2]_{diss}$ at 7 °C in the aqueous phase of several food products, packaged in a realistic gas mixture and at two different gas/product volume ratios (1/1 and 2/1), was determined as well. The levels of $[CO_2]_{diss}$ varies between 152 and 898 mg/L while the average ratio of $[CO_2]_{diss}$ (mg/L) over the initial CO_2 concentration (mL/100 mL) in the headspace amounted to 8.98 ± 1.41 ([mL/L]/[mL/100 mL]). The importance of the gas/product volume ratio was demonstrated as $[CO_2]_{diss}$ increased significantly ($P < 0.001$) with increasing gas/product volume ratio (G/P). In average, an increase of 42% of $[CO_2]_{diss}$ was achieved when the G/P was increased from 1/1 to 2/1.

Keywords: dissolved CO_2; modified atmosphere; lag phase; growth rate; spoilage bacteria

Introduction

Modified atmosphere packaging (MAP) is well introduced in the Western food industry to prolong the shelf-life of perishable food products. Literature regarding MAP focuses mainly on two aspects: (a) effect of MAP on the growth of specific food pathogens and (b) shelf-life prolongation of specific food products by using MAP. Only a few publications deal with the effect of MAP on specific spoilage microorganisms. The effect of CO_2 on the growth of some fresh meat spoilage bacteria has been compared before (1) but at a temperature (30 °C) that is not of practical interest for refrigerated modified atmosphere packed food products. In another publication (2) the resistence of a number of food-related bacteria to CO_2 was also determined. Because of different experimental set-ups, it is very difficult to compare inhibition of spoilage organisms by CO_2 on the basis of the results reported in different scientific papers. Earlier research (3) has indeed demonstrated that the concentration of dissolved CO_2 ($[CO_2]_{diss}$) determines the growth inhibition of microorganisms in a modified atmosphere. Several intrinsic (pH, water activity, fat content, type of fat), extrinsic (temperature) and process parameters (initial CO_2 concentration in the head space, gas/product volume ratio (G/P)) determine the final $[CO_2]_{diss}$ in the aqueous phase of a food product. Especially the importance of the G/P is often neglected. An increase of the G/P from 0.3 to 2.0 (at 8 °C and for an initial CO_2 concentration in the gas-phase = 50 mL/100 mL) resulted in an increase of $[CO_2]_{diss}$ in broth of 145% (402 mg/L to 989 mg/L) (3). Predictive models using $[CO_2]_{diss}$ as an independent variable were developed in the past to predict the spoilage and the safety of cooked gas packed meat products (4–6).

The aim of this work was to quantify the effect of $[CO_2]_{diss}$ on the growth of different spoilage microorganisms and thus provide a tool for better understanding the shelf-life prolongation of MAP utilizing CO_2. Because it was felt essential to quantify the $[CO_2]_{diss}$ in the

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0023-6438/00/080531 + 07 $35.00/0
doi:10.1006/fstl.2000.0705

aqueous phase of MAP food products in packaging configurations used in practice, the second part of this study addresses this issue.

Materials and Methods

Influence of dissolved CO_2 on the growth of spoilage microorganisms

Strains. Different types of strains were used for this study. *Shewanella putrifaciens* (LMG 2250), *Pseudomonas fluorescens* (LMG 1794) and a cocktail of three strains of *Photobacterium phosphoreum*) (LMG 4231, 4232, 4233) were supplied by the Laboratory of Microbiology, University of Ghent, Belgium. *Bacillus circulans* was isolated, after 10 min pasteurization at 80 °C from baked mushrooms which were part of a ready-to-eat meal. The psychrotrophic character (growth at 7 °C) of the strain was confirmed and identification was performed by means of API CHB (Biomérieux, France). *Lactobacillus sake* subsp. *carnosus* was isolated out of gas packed cooked ham and identified by SDS-PAGE (7). A cocktail of five strains of *Aeromonas hydrophila*, isolated out of chilled fresh food products (respectively halibut, shark, thornback, haricots and sorrel (8)) was also used. Five strains of *Brochothrix thermosphacta* were isolated from vacuum packed and gas packed cooked meat products (cooked ham, cooked turkey fillet and black pudding) on STAA medium (9). The identity of the strains was confirmed based on Gram staining, morphology, catalase, oxydase, glucose fermentation and API 50CHL (Biomérieux, France) tests. A mixture of the five strain were used as an inoculation cocktail.

Experimental set-up. To assess the sensitivity of the different spoilage microorganisms for CO_2, growth curves for each microorganism were determined at different concentrations of dissolved CO_2. Growth curves were determined in buffered (0.1 M disodium phosphate) modified BHI (3). The appropriate amount of broth was placed in 600 mL glass jars, provided with a Teflon valve and a central opening which is closed with a silicone septum (3). The pH of the broth was adjusted to 6.1 after autoclaving.

Each strain, except for *P. phosphoreum* and *B. thermosphacta*, was subcultured in BHI broth (OXOID CM225) for 24 h at 30 °C. *Photobacterium phosphoreum* was subcultured at 18 °C and *B. thermosphacta* at 22 °C. A second subculture of all strains was incubated in BHI broth for 16 h at the appropriate temperature and was subsequently, before inoculation, placed for 6 h at 7 °C, to allow the test strain to adapt to the refrigerated temperature. In the case of inoculation cocktails, each strain was separately cultivated and mixed before the adaptation period. After the adaptation period, the previously cooled simulation medium in the glass jars was inoculated to a level of approximately 10^4/mL.

After inoculation, the jars were immediately gas packaged as previously described (4) and stored at 7 °C. The concentration of dissolved CO_2 in modified BHI broth at a specific packaging configuration and temperature was determined by means of a model previously developed (3). This model predicts the effect of temperature, initial CO_2 concentration in the gas-phase and gas/product volume ratio on the amount of dissolved CO_2 in modified BHI. The configurations applied to result in a specific concentration of dissolved CO_2 are mentioned in **Table 1**. CO_2 was complemented only with N_2 for the determination of the growth curves of *L. sake*, *B. circulans* and *A. hydrophila*. Fourteen mL/100 mL of O_2 was included additionally in the gas atmosphere for the growth curves of *P. fluorescens*, *P. phosphoreum* and *S. putrefaciens*. Growth curves of *B. thermosphacta* were determined in the presence as well as absence of 14 mL/100 mL of O_2. Growth curves at different $[CO_2]_{diss}$ levels (0, 500, 1000, 1500 and 2000 mg/L) were determined for each type of spoilage microorganism.

Table 1 Packaging configurations to reach specific concentrations of dissolved CO_2 ($[CO_2]_{diss}$) at 7 °C in modified BHI calculated by a previously developed model (3)

Gas/product volume ratio	Initial CO_2-concentration in the gas phase (mL/100 mL)	$[CO_2]_{diss}$ (mg/L)
1.50	0.0	0
1.50	24.0	500
1.50	60.0	1000
3.40	64.0	1500
4.00	86.0	2000

Samples (0.2 mL) were taken at regular time intervals by means of sterile disposable 1 mL syringes, diluted if necessary with Peptone Physiological Salt solution (1g/L peptone, 8.5 g/L NaCl) and plated in duplicate on MRS agar (OXOID CM359) for *L. sake*, Marine agar (Marine broth, DIFCO 0791 − 17 + 15 g/L bacteriological agar, OXOID L11) for *P. phosphoreum* and on PCA agar (OXOID CM325) for the other strains with a Spiral Plater Model D/Spiral Systems Inc., Cincinnati, U.S.A. The PCA plates and Marine Agar plates were aerobically incubated for 24 h at 30 °C. The MRS plates were anaerobically incubated at 30 °C for 3 days.

The maximum specific growth rate (μ_{max}) (h^{-1}) and the lag phase (λ) (h) of the growth curves were estimated by fitting the modified Gompertz equation (10) with the Levenberg-Marquardt algorithm of the statistical packet SPSS for Windows, version 7.5.

CO_2-measurements

Estimation of the CO_2 dissolving rate. To estimate the CO_2 dissolving rate at 7 °C, the concentration of CO_2 in cooked ham packed under two different modified atmospheres (40 mL/100 mL CO_2 and 80 mL/100 mL of CO_2 compensated with N_2, gas/product volume ratio = 4) and stored at 7 °C was followed during the time. For every measurement two glass bottles were filled and gas packed after which the internal pressure was measured after a specific time interval. The amount of dissolved CO_2 in the aqueous phase of the cooked ham was

determined by measuring the underpressure into a closed conical glass flask after a specific time interval. The underpressure was measured with an U-tube filled with mercury. The concentration of dissolved CO_2 in the cooked ham was calculated by the following formula (11):

$$A_{co_2} = \frac{44 V_H}{RTM_m}[P_i - P_f] \qquad \text{[Eqn (1)]}$$

where V_H = volume head space (m^3); R = universal gas constant (J/mol.°K); T = temperature (°K); P_i = initial pressure in the flask at the moment of packaging (Pa); P_f = final pressure in the flask after equilibrium (Pa); M_m = mass of packed product (kg).

By taking the water content of the cooked ham into account, the concentration of CO_2 in the aqueous phase of the cooked ham could be calculated. It was assumed that CO_2 was only dissolved in the water-phase of the food. This assessment will result, in the case of foods containing high amounts of unsaturated fat, in an over estimation of the CO_2-concentration in the aqueous phase of the food. It has been demonstrated however that at refrigeration conditions, pork fat does not strongly influence the amount of CO_2 dissolved in the water-phase (3). All measurements were performed in triplicate.

Determination of the concentration of dissolved CO_2 in the aqueous phase of different food products packed under modified atmospheres

The concentration of dissolved CO_2 in the aqueous phase of a food product is determining the inhibitory effect of CO_2 in a modified atmosphere (3). It is therefore essential to know the levels of CO_2 in the aqueous phase of different food products, packed under realistic packaging configurations. Several food products were therefore packed under realistic modified atmospheres (**Table 2**). To estimate the effect of the gas/product volume ratio on the amount of CO_2 dissolved in the aqueous phase of a food product, two different gas/product volume ratios were also tested (1/1 and 2/1).

The amount of dissolved CO_2 in the aqueous phase of the food products at 7 °C was determined after 24 h as mentioned above.

Results and Discussion

Influence of dissolved CO_2 on the growth of spoilage microorganisms

The pH of the buffered modified BHI decreased at maximum with 0.17 units of the case that the maximum $[CO_2]_{diss}$ (2000 mg/L) was applied. The influence of the concentration of dissolved CO_2 on the maximum specific growth rate μ_{max} (h^{-1}) and on the lag phase λ (h) of the different investigated psychrotrophic spoilage micro-organisms is presented in **Fig. 1** and **Fig. 2** respectively. The apparent linear relationship between the concentration of dissolved CO_2 $[CO_2]_{diss}$ and μ_{max} and $1/\lambda$ respectively was estimated by linear regression. In general, a good linear relationship was established between μ_{max} and $[CO_2]_{diss}$ (average $R^2 = 0.9826$). Relationships between $[CO_2]_{diss}$ and growth rate or lag phase have, to our knowledge, not been published before. Most of the published studies describe the relationship between the initial CO_2 concentration in the head space and the growth rate. Many of these experiments were performed at ambient or higher temperatures (1, 12–15). A linear relationship was identified (16) between μ_{max} and the CO_2 concentration in the gas phase for *A. hydrophila*, *Yersinia enterocolitica*, *Bacillus cereus* and *Listeria monocytogenes*, while a quadratic relationship between he initial CO_2 concentration and μ_{max} of *P. phosphoreum* was also suggested (17). In this study, quadratic equations did however lead to poorer fitting results (data not shown). An equation containing a linear and a quadratic term was proposed in (18).

A similar linear relationship between $1/\lambda$ and $[CO_2]_{diss}$ was observed but a poorer correlation (average $R^2 = 0.9618$) was obtained in comparison with the relation between μ_{max} and $[CO_2]_{diss}$. No literature data are available concerning the relation between CO_2 and λ.

Table 2 Dissolved CO_2 ($[CO_2]_{diss}$) in the water-phase of different meat and fish products at 7 °C packed under modified atmospheres

Food product type	Water content (g/100 g)	CO_2 (mL/100 mL)	N_2 (mL/100 mL)	O_2 (mL/100 mL)	$[CO_2]_{diss}$ (mg/L) Gas/Product = 1/1	Gas/product = 2/1
Cooked shrimps	83.6	35	65	–	310 ± 11	561 ± 6
Cod fillets	81.1	50	20	30	417 ± 16	623 ± 45
Lean pork meat	77.2	60	–	40	420 ± 34	525 ± 7
	77.2	100	–	–	707 ± 27	842 ± 19
Chicken fillets	74.9	70	20	10	441 ± 28	704 ± 25
Lean beef	77.0	60	–	40	478 ± 24	656 ± 19
	77.0	100	–	–	737 ± 10	898 ± 32
Raw cured ham	65.8	20	80	–	152 ± 11	192 ± 21
Cooked ham	75.5	50	50	–	351 ± 6	578 ± 33
Bacon	70.0	20	80	–	131 ± 14	173 ± 15
Cooked chicken sausage	74.3	50	50	–	309 ± 7	577 ± 26

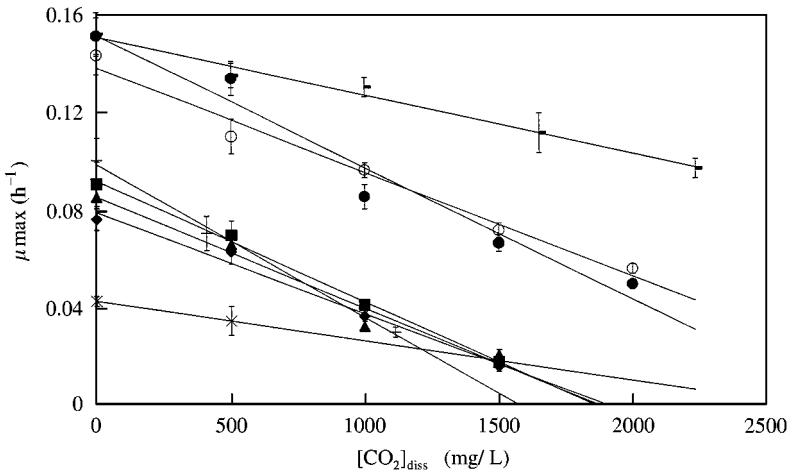

Fig. 1 Influence of $[CO_2]_{diss}$ on maximum specific growth rate μ_{max} (h^{-1}) at 7 °C of different spoilage microorganisms (◆ *P. fluorescens*, ■ *P. Phosphoreum*, ▲ *S. putrefaciens*, + *A. hydrophila*, × *B. circulans*, ● *B. thermosphacta* (aerobic), ○ *B. thermosphacta* (anaerobic), — *L. sake*)

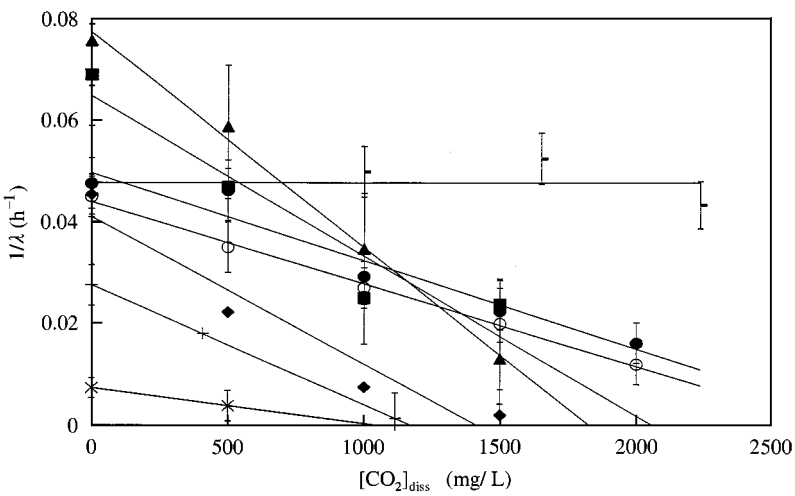

Fig. 2 Influence of $[CO_2]_{diss}$ on the inverse of the lag phase $1/\lambda$ (h^{-1}) at 7 °C of different spoilage microorganisms (◆ *P. fluorescens*, ■ *P. Phosphoreum*, ▲ *S. putrefaciens*, + *A. hydrophila*, × *B. circulans*, ● *B. thermosphacta* (aerobic), ○ *B. thermosphacta* (anaerobic), — *L. sake*)

The Gram-negative psychrotrophic strains tested (*P. fluorescens*, *P. phosphoreum*, *S. putrefaciens* and *A. hydrophila*) all showed similar inhibition patterns of CO_2 on μ_{max}. Gram-negative microorganisms are generally described as sensitive for CO_2 (19, 20). This could also be derived from the relatively steep slopes of the estimated straight lines drawn in **Figs 1** and **2**. The comparably high sensitivity to CO_2 of *P. phosphoreum* and of *S. putrefaciens* is in contradiction with published findings (17). This publication clearly demonstrated a higher inhibition by CO_2 of *S. putrefaciens* in comparison to *P. phosphoreum* and concluded that a kinetic model for spoilage of gas packed cod based on *S. putrefaciens* could result in misleading shelf-life predictions. This dissimilarity could be explained by the difference in experimental temperatures. The experiments in (17) were carried out at 0 °C while the presented experiments were performed at 7 °C.

Growth rate values of *A. hydrophila* are in accordance with previously reported values (16) which were obtained in solid surface cultures (0.12 h^{-1} at 8 °C and without CO_2). The high sensitivity of *A. hydrophila* for CO_2 has been reported by many authors (6, 16, 21–25) but is never quantified here in comparison with other spoilage microorganisms.

Lactobacillus sake showed the highest resistance for CO_2 and was clearly more resistance then *B. thermosphacta*, as reported before (2). Conceivably *Lactobacillus* strains will become dominant over *B. thermosphacta* in CO_2 containing, O_2 free atmospheres. The inhibition of *B. thermosphacta* was however not influenced by the presence of O_2 in the headspace. It has often been suggested that *B. thermosphacta* will determine spoilage of vacuum packed meat when the residual O_2-concentration and/or the film permeability is too high which results in the presence of relatively high redox potentials (9). The O_2-level in the anaerobic jars used in this study varied during the experiment between 0.5 and 1.0 mL/100 mL, except at the end of the trials were lower O_2-levels were recorded (data not shown). However, significant effects of low oxygen

atmospheres have been reported only for O_2-levels < 0.2 mL/100 mL (26). Moreover, the effect of the redox potential was shown to be water activity dependent (27). The inhibitory effect of an anaerobic atmosphere at low temperatures was not present at a high water activity ($a_w = 0.99$) but was significant at lower water activities (< 0.98). The medium applied in the current study contained only 5 g/L of NaCl and had a water activity of 0.995. The relatively high residual oxygen level in the anaerobic atmosphere and the high water activity could possibly explain the absence of a significant difference in the growth behaviour of *B. thermosphacta* in aerobic and anaerobic atmospheres.

Bacillus circulans, which was isolated from part of a chilled pasteurized prepared meal, was moderately inhibited by CO_2 but in general showed a low growth rate and a long lag phase. *Bacillus circulans* often predominates in pasteurized milk or egg products at the end of the shelf life (28–32) and has been isolated from Indian snack and lunch foods (33). Modified atmosphere packaging of pasteurized food products would apparently result in a moderate inhibition of *Bacillus* strains resulting in a slight extension of the shelf life.

The lag phase duration was variable for the different strains tested although the inoculum preparation was comparable. The lag phase of the Gram-negative strains at conditions where no CO_2 was present in the headspace varied between 13 h for *S. putrefaciens* to 36 h for *A. hydrophila*. The influence of CO_2 on λ was high for Gram-negative bacteria but was only moderate for Gram-positive bacteria. Moreover, CO_2 did not influence λ of *L. sake* as previously reported (4). Published studies do not report a consistent effect of CO_2 on the lag phase of bacteria. A significant effect of CO_2 on the lag phase of *Listeria monocytogenes* (34) and *Pseudomonas* spp. (18, 35, 36, 37) was noticed while not significant effects of CO_2 on λ were observed for *Photobacterium phosphoreum* (38), *Pseudomonas* spp. (14, 39, 40), *A. hydrophila*, *L. monocytogenes* and *Bacillus cereus* (16). In these studies (14, 16, 38–40), due to the small number of measurements in the beginning of the experiments, no significant lag phase at all was observed and therefore the effect of CO_2 on the lag phase could not been investigated.

CO_2-measurements

The dissolving rate of CO_2 in cooked ham can be derived from **Fig. 3**. 78% and 87% of the CO_2 dissolved at equilibrium was already dissolved after 60 min for an initial CO_2 concentration in the headspace of respectively 40 and 80 mL/100 mL. In contrast, equilibrium was not reached even after 12 h at 13 °C in whole chicken, chopped pork and ground beef (11). For further experiments, equilibrium was supposed after 24 h.

Table 2 tabulates $[CO_2]_{diss}$ in the water-phase of different meat and fish products at 7 °C for a gas/product volume ratio of 1/1 and 2/1 and at CO_2 concentrations applied in practice. The levels of $[CO_2]_{diss}$ varied between 152 and 898 mg/L. The average ratio of $[CO_2]_{diss}$ (mL/L) over the initial CO_2 concentration (mL/100 mL) in the headspace amounts 8.98 ± 1.41 ([mL/L]/[mL100 mL]) which reveals relatively small differences of the CO_2 solubility in the different food products investigated. In practice, flexible packaging materials are often applied. In this case, higher CO_2-concentrations in the water-phase of the food will be obtained as the dissolution of CO_2 will be compensated by a decrease in package volume.

$[CO_2]_{diss}$ increased significantly ($P < 0.001$) with increasing gas/product volume ratio (G/P). On average, an increase of 42% of $[CO_2]_{diss}$ was achieved when the G/P was increased from 1/1 to 2/1. The important effect of the G/P has been shown before in broth (3, 41) but is often ignored. For this reason, during the optimization of a packaging configuration, logistic as well as microbiological aspects have to be considered as larger G/P could lead to a higher inhibition of spoilage microorganisms resulting in considerably longer shelf-lives.

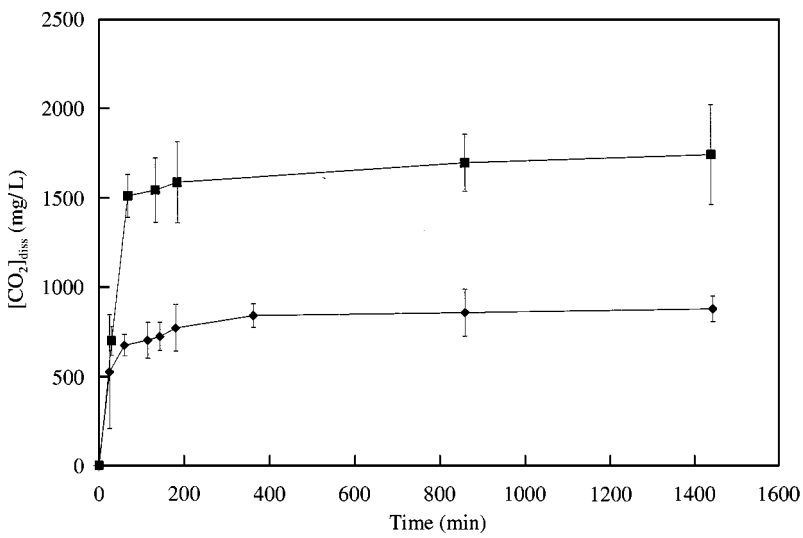

Fig. 3 Dissolving rate at 7 °C of CO_2 in the water phase of cooked ham packed under a CO_2 containing modified atmosphere (◆ 40 mL/100 mL CO_2 + 60 mL/100 mL N_2, ■ 80 mL/100 mL CO_2 + 20 mL/100 mL N_2)

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