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Effect of water activity and temperature on the stability of creatine during storage

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Abstract

Creatine degradation to creatinine, which has no biological activity, in combinations of glycerol and pH 4.0 buffer solutions followed first-order kinetics up to a point where degradation started to level off, generally beyond the first half-life. Practical data are reported for a wide range of water activity (a_w) values (0.31–0.983) at 4°C, 23°C, and 35°C. Creatine degradation did not exhibit a dilution effect, that is a decrease in rate about an a_w of 0.7, as is found for both microbiological growth and chemical reactions in semisolid food matrix systems. The temperature dependence obeyed the Arrhenius relationship with an energy of activation of about 20 kcal/mol at $a_w \geq 0.68$ increasing to 23 kcal/mole below that a_w. In addition, a semilog plot of half-life as a function of a_w at each temperature follows a predicted straight line. The pH and assumed liquid viscosity increase through increased glycerol concentration were not able to completely explain the decrease in rate of degradation as a function of a_w. Furthermore, we confirmed that creatine stability in the crystal form is very high as long as it does not reach the deliquescence state.

Key words: *Creatine; first-order kinetics; glycerol; storage stability; water activity*

Introduction

Creatine (2-(carbamimidoyl-methyl-amino) acetic acid) is a nonprotein amino acid that occurs naturally in vertebrates and takes part in the recycling of the high-energy phosphate in ATP to form ADP and phosphocreatine. Phosphocreatine can be catalyzed by creatine kinase to form ATP. Creatine kinase is typically located in cells that undergo high-energy changes such as muscle cells and is a key enzyme in cellular energy homeostasis[1]. Thus, creatine was suggested in several studies to improve muscle function by improving the ability to produce energy during high-intensity exercise as well as improve the speed of recovery. In addition, creatine supplementation was related to increased lean body mass of athletes although some argued that the measured weight gain occurring in the first days of practice is accounted for creatine-induced water retention[2]. In addition to the suggested usefulness in athletes' performance, creatine was demonstrated to have a positive effect on isometric strength and lean body mass following strength exercise training in older adults[3].

Because of this, creatine (mostly in the monohydrate form) is widely used as a dietary supplement for athletes and body builders. One of the main obstacles in creatine usage as a dietary supplement is the fact that creatine is a weak base that is easily degraded to creatinine, a nonactive metabolite, especially in acid environments[4]. Many efforts are being taken by the dietary supplement industry to create either of semi-solid (e.g., 'gummy bears') vehicle systems. Many of these include the use of water activity reduction to help reduce reaction rates and get the system below a water activity where microbes cannot grow, that is <0.65. Labuza[5] has outlined the effects of water activity reduction on lowering of rates of chemical reactions. The aim of this research was to study the effect of water activity control on the rate of creatine degradation and to evaluate the degradation kinetics.

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Materials and methods

Standards and reagents

Creatine monohydrate (Fluka 27900), creatine anhydrous (Fluka 27890), creatinine (Fluka 27910), and 4-(2-aminoethyl) benzene sulfonamide (Aldrich 275247) were all purchased from Sigma-Aldrich (Allentown, PA, USA). Ammonium sulfate as well as HPLC grade methanol and water were purchased from Fisher Scientific. Glycerol and the buffer solutions in which creatine was dissolved (certified, pH 4.0, 4.5, 5.0) were purchased from Fisher Scientific (Fisher Chemicals).

Sample preparation

Solutions of glycerol and buffer pH 4.0 were prepared at different ratios (Table 1) to obtain desired water activities (a_w), as measured by an Aqualab instrument (Series 3; Decagon Devices Inc., Pullman, Washington, DC). About 125 mg of anhydrous creatine was weighed in 25-mL volumetric flasks. Creatine was dissolved in the above solutions using a magnetic stirrer for 1–3 hours, and the flasks were filled to volume with the same solutions. Each flask was divided to vials that were placed in incubators at 4°C, 23°C, or 35°C. Periodically (according to sampling program), 200-µL samples were taken from each vial and kept in 0.5-mL Eppendorf vials at –30°C until HPLC analysis. Because the pH measurement of solutions was affected by the presence of glycerol (Table 1), the above procedure was repeated with buffer solution (without glycerol) at the same range of pH (4.0–5.0) to separate the effect of pH.

To evaluate the role of viscosity/mobility on the rate of reaction, solid gels of the same glycerol–buffer solutions were prepared by the following procedure. Fifty milliliters of the above solutions was placed in a 100-mL beaker; 1 g of agar-agar powder was slowly added at room temperature while stirred on a magnetic stirrer. The solution was heated to 80°C to obtain a clear solution and then moved to a nonheated stirring plate. When the solution temperature decreased to 55°C, 250 mg of anhydrous creatine was added and stirred until creatine was fully dissolved. The obtained solution was poured to 3 cm diameter Petri dishes and stored at 4°C to solidify. After 24 hours at 4°C, samples were taken for initial creatine content and the rest was stored at 23°C for periodical sampling.

Table 1. Composition of glycerol–buffer solutions used for water activity control.

Water activity	0.31	0.49	0.68	0.81	0.9	0.983
Glycerol (% wt/wt)	88	78.6	64.8	47.9	31.5	0
Buffer pH 4 (% wt/wt)	12	21.4	35.2	52.1	68.5	100
Measured pH	5	4.76	4.46	4.26	4.15	4.05
Estimated viscosity from data at 25°C (cp)[11]	~305	~50	~33	~6	~3	~1

Chromatography

The HPLC analysis method for creatine was adapted from Dash and Shawney[6]. In a borosilicate 10-mL vial, 120 µL of internal standard solution (3 mg/mL, 4-(2-aminoethyl) benzene sulfonamide in methanol) was dried with N_2 at 45°C. To this vial, 50 µL of the analyzed sample was transferred and 4.95 mL of mobile phase added. The resulting sample was vortexed, filtered (13 mm, 0.22 µm; Millex, USA), and 30 µL was injected to HPLC. An HPLC system (AKTA Purifier; Pharmacia Biotech, USA) connected to a UV detector (UV-900; Pharmacia Biotech) was used with a C_{18} column (Betabasic; Thermo, USA) equipped with a C_{18} guard column (AJ0-7597; Phenomenex, Torrance, CA, USA). HPLC conditions: isocratic 0.045 M ammonium phosphate (5.9 g in 1000 mL water) at 1.2 mL/min, UV detection at 205 nm. External standards of creatine and creatinine at concentrations of 10, 20, 50, and 100 µg/mL were injected to the HPLC (30 µL) for calibration curve. The intercept of calibration curves were set to zero and showed high linearity over the whole range. The calibration curve equations were as follows: creatine (µg/mL) = 1.9367 × peak area (mAU × min), R^2 = 0.9996; creatinine (µg/mL) = 4.4176 × peak area (mAU × minute), R^2 = 0.9996.

Results and discussion

Significant degradation to creatinine which has no biological activity occurred during storage at all temperatures as shown in Figure 1a (23°C). In this case at the highest water activity (0.983), the half-life was 43 days while at the lowest a_w the half-life was 182 days. The degradation rate of creatine followed an apparent first-order pattern at all tested temperatures (Table 2) up to a certain time where it starts to level-off as seen at 35°C (Figure 1b). Table 2 also shows the calculated half-life based on the calculated rate constant. As seen, if refrigerated at 4°C, the half-life is more than 1 year at any water activity, whereas at room temperature the half-life was less than 3 months at $a_w \geq 0.68$. Temperature abuse at 35°C had a significant effect on stability with no composition having a half-life of more than a month. It also should be noted that in only one case (4°C and a_w 0.68 and 0.81) were the differences in k not significant. As creatine degradation to creatinine is a reversible equilibration chemical reaction, the rate of loss decreased more than expected from a first-order beyond the first half-life[6].

The plots of half-life versus $1/T$ (Arrhenius plot) in all cases had an $R^2 > 0.99$ at all water activities for the three

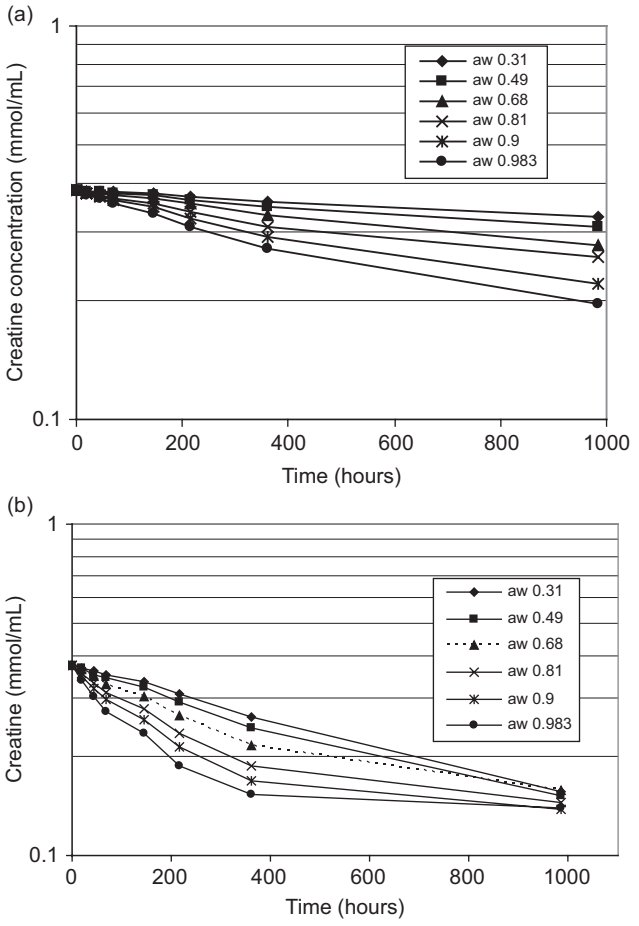

Figure 1. (a) Creatine degradation during storage at 23°C in glycerol-buffer solutions, (b) Creatine degradation during storage at 35°C in glycerol–buffer solutions.

temperatures tested with an example at an a_w of 0.49 shown in Figure 2. Figure 3, using the data at a_w of 0.49, shows a semilog plot of half-life versus temperature. This is a simple method to visually represent the data in an understanding format. The calculated activation energy (E_a) and Q_{10} (rate increase for a 10°C increase in temperature) from these plots shows an increase of about 15% in the value at the two lower water activities (Table 3). The values are typical for many chemical degradation reactions found in food[5]. The increased temperature sensitivity at the lower water activity would be one disadvantage of lowering the a_w to increase creatine stability, as the formulation would be impacted more by temperature fluctuations.

Decreasing water activity by the addition of glycerol slowed the rate of creatine degradation in a logarithmic fashion over the whole a_w range as seen in Figure 4, which is a plot of half-life versus a_w for each temperature, making it easy to visualize. This logarithmic dependence has been found for many chemical reactions in food systems[5,7]. However, in most food degradation studies, the rate generally increases as a_w is lowered from 0.98 reaching a maximum at around an a_w of 0.8 and then decreasing to zero at a_w = 0.2–0.3, even in complex reactions such a the Maillard browning reaction, a problem in some liquid drug formulations[8,9]. It has been proposed that for semisolid matrix systems prepared by drying or combining dry ingredients and then humidification to different water activities, a 'dilution effect' occurs, where above a certain a_w in the range of 0.6 to 0.8 all the reactant is dissolved, and thus increasing the water content decreases the rate as the total liquidus volume increases dramatically at high a_w. The absence of a 'dilution effect' in a single-reactant no-solid matrix system was demonstrated in another study using a liquid system of water–glycerol mixtures[10] such as we did here. They found that anthocyanin loss at 80°C followed a increase in rate as a_w increased over the whole range. However, in this study, data were only collected at one point in time and only at one temperature (80°C) at each a_w, and thus we cannot do any true kinetic analysis of the results. But in these two cases without a solid matrix, there is no dilution effect. Another aspect of using a glycerol–buffer solution to control a_w is the obvious change in liquidus phase viscosity. Water goes from about 1.4 cp (1.4 mPa·s) at 4°C to about 0.72 cp at 35°C, over twofold decrease, whereas pure glycerol at 25°C has a viscosity of ~934 cp. Based on direct measurements by Segur and Oberstar[11], the system at 25°C goes from ~1 cp at the highest a_w to an estimated 305 cp at the lowest a_w, a 300-fold difference across the whole concentration range (Table 2). Using the values from Table 2, at 4°C there is a sevenfold change in rate constant over the whole a_w range, a 4.3-fold change at 23°C and a 2.4-fold change at 35°C over this whole range. Thus, the change in rate is much less than the estimated viscosity change. It also decreases as temperature increases, so some other factors must control the reaction rate, including ionic strength and matrix effects.

To test the influence of the matrix effect, we evaluated the creatine degradation rate in the same solutions but after making them into a solid gel using agar-agar. The gelled solutions showed almost exactly the same degradation rates at most of the water activities (Figure 5). The effect of matrix presence on reaction rate was very slightly apparent only at a_w 0.9 and 0.983, where the viscosity differences are the highest. Anyway, the degradation retardation caused by glycerol addition is most likely not the consequence of a matrix effect or a local viscosity increase and must be directly related to the method of preparation, that is, dissolving the reactants into a totally liquid solution versus combining dry ingredients and/or drying a solution or combination to low moisture and then followed by humidification to

Table 2. Influence of water activity and temperature on degradation kinetics of creatine in glycerol water solutions (rate constants in hour^{-1}).

T°C	a_w	k-first order	Upper 95% CL	Lower 95% CL	R^2	Significance[a]	$t_{1/2}$ days
4°C	0.31	1.142E-05	1.382E-05	9.019E-06	0.986	A	2529
	0.49	2.050E-05	2.592E-05	1.507E-05	0.895	B	1409
	0.68	3.941E-05	4.430E-05	3.452E-05	0.980	C	733
	0.81	5.461E-05	6.261E-05	4.662E-05	0.928	C	529
	0.90	6.742E-05	7.534E-05	5.949E-05	0.953	D	428
	0.98	7.842E-05	8.646E-05	7.038E-05	0.963	E	368
23°C	0.31	1.584E-04	1.633E-04	1.534E-04	0.999	A	182
	0.49	2.193E-04	2.267E-04	2.120E-04	0.997	B	132
	0.68	3.284E-04	3.395E-04	3.173E-04	0.993	C	88
	0.81	3.942E-04	4.127E-04	3.758E-04	0.965	D	73
	0.90	5.577E-04	5.758E-04	5.397E-04	0.984	E	52
	0.98	6.766E-04	6.983E-04	6.549E-04	0.977	F	43
35°C	0.31	9.901E-04	1.022E-03	9.586E-04	0.989	A	29
	0.49	1.184E-03	1.211E-03	1.157E-03	0.996	B	24
	0.68	1.551E-03	1.576E-03	1.526E-03	0.998	C	19
	0.81	1.916E-03	1.956E-03	1.876E-03	0.992	D	15
	0.90	2.201E-03	2.253E-03	2.149E-03	0.983	E	13
	0.98	2.400E-03	2.510E-03	2.369E-03	0.957	F	12

[a]Same letter within each temperature denotes not significantly different at $P < 0.05$. All rate constants at each water activity were significantly different between each temperature at $P < 0.05$.

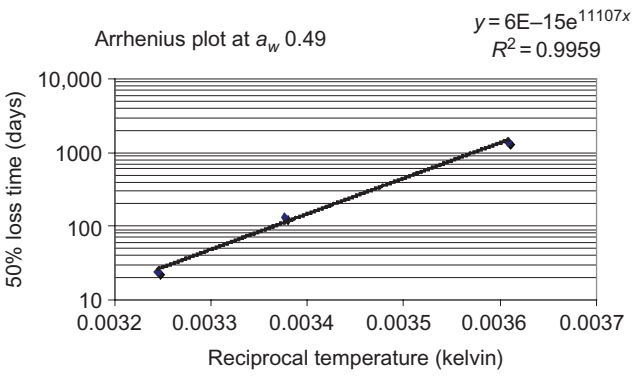

Figure 2. Arrhenius plot (log time for 50% loss versus reciprocal absolute temperature) for creatine in a liquid medium at $a_w = 0.49$.

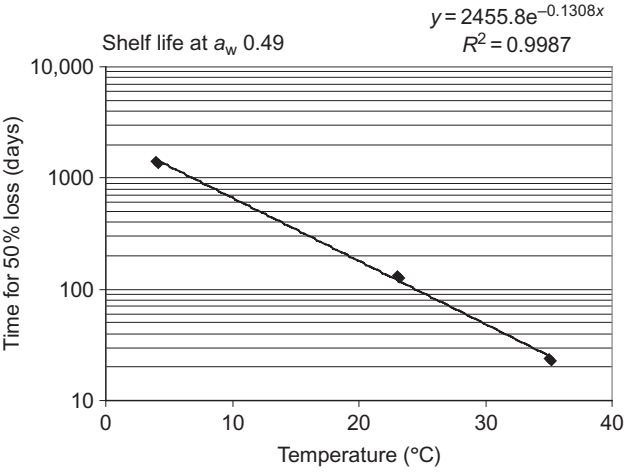

Figure 3. Shelf life plot for creatine loss (50%) in a liquid medium at $a_w = 0.49$ as a function of temperature.

the respective a_w levels. This needs further study as it is important to formulation.

Creatine degradation is also highly affected by pH, being highly unstable at acidic conditions and fairly stable at near-neutral pH and room temperature[4]. While preparing the buffer–glycerol solutions, we noticed that the addition of glycerol increased the measured pH. It is not clear whether this pH increase is a real change in acidity or an artifact caused by the fact that a significant amount of nonaqueous liquid is present. Bell and Labuza[12,13] showed that the pH is increased in solid and liquid systems, when the a_w is lowered by following the degradation rates of methycillin and aspartame. Thus, systems made to lower water activity but starting at higher pH gave reaction products only when formed

Table 3. Water activity affect on the temperature sensitivity for creatine degradation in water–glycerol solutions at different water activities.

a_w	E_a (kcal/mole)	R^2	Q_{10} for 10°C–20°C
0.31	24.18	0.999	2.98
0.49	21.96	0.999	2.70
0.68	19.86	0.999	2.45
0.81	19.16	0.999	2.36
0.90	18.94	0.999	2.33
0.983	18.67	0.999	2.30

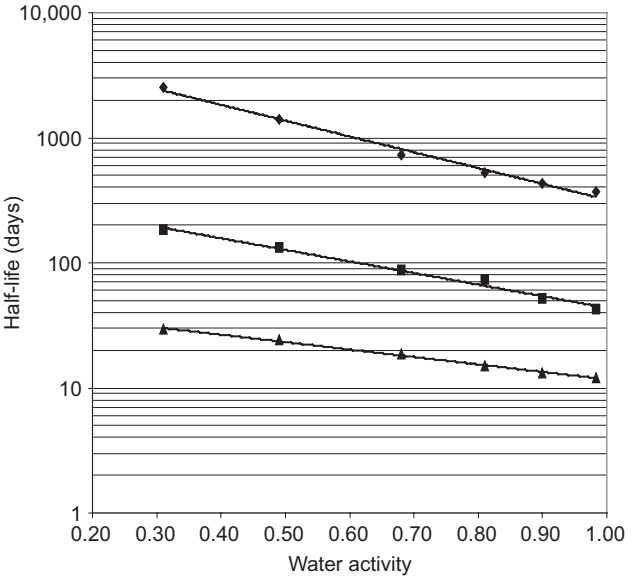

Figure 4. Effect of water activity on half-life (50% loss) of creatine at three temperatures. ✦, 4°C; ■, 23°C; ▲, 35°C.

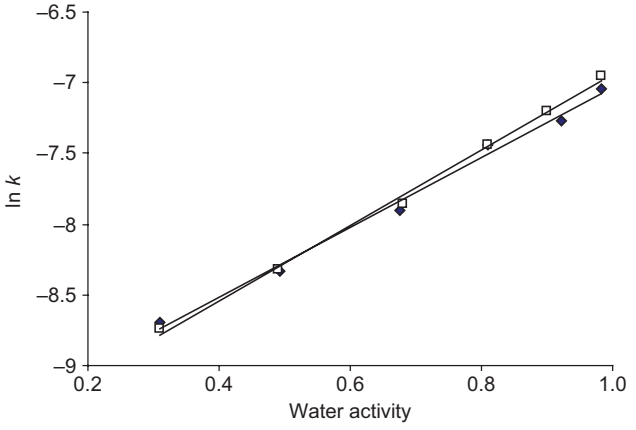

Figure 5. Influence of water activity on creatine degradation rate at 23°C in glycerol–buffer solutions (□) as compared to glycerol–buffer gels (◆).

at higher pH in dilute solution. Nevertheless, using buffer-only solutions at parallel pH values, we found (Figure 5) that a change in one pH unit reduced the rate by 2.6-fold while the same measured pH change caused by glycerol decreased the rate by 6.0-fold. Thus, it is clear that the effect of degradation reduction by lowering water activity is complex.

Assuming that the water was at pH 7 and extrapolating our data to pH 7 (Figure 6 in buffer only), a rate of 1.1×10^{-3} would be expected. Actually, the rate reported by Dash and Shawney[6] is almost identical to the one that we measured at pH 4. Because we used exactly the same analysis method and the same source of creatine, the only way we

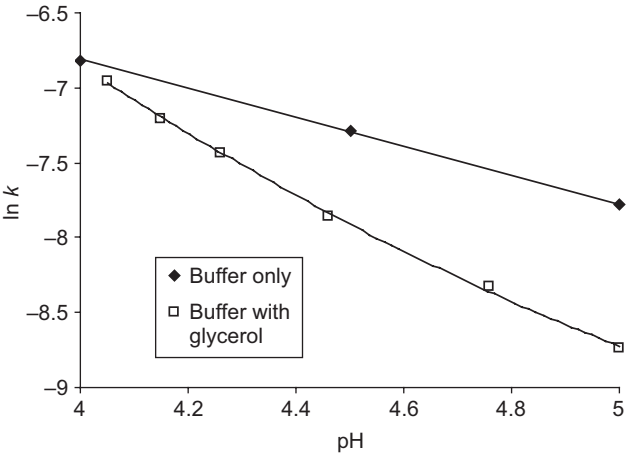

Figure 6. Influence of solution pH with and without glycerol on rate loss constant for creatine.

can explain the difference is the fact that their solution was shaken throughout the experiment while our samples were standing. The slight temperature difference (2°C) and possible pH deviation (not reported) cannot explain the large difference. The noted difference is probably a more pronounced expression of the mobility effect or may be due to trace mineral catalysis. Anyway, for a conclusive proof, this needs to be compared in a single study.

As expected, the degradation of creatine was negligible when in the crystalline state. Storage of creatine crystals in desiccators using saturated salt solutions to create about the same range of water activities and held for 3 months at 23°C resulted in no degradation at all up to a_w = 0.68 and only 0.03% and 0.11% degradation at a_w = 0.75 and a_w = 0.9, respectively. The sorption isotherm of creatine is shown in Figure 7. Calculation of the addition of water on a molar basis above an a_w of 0.4 shows that this change is the transition from creatine anhydrous to creatine monohydrate. At these higher

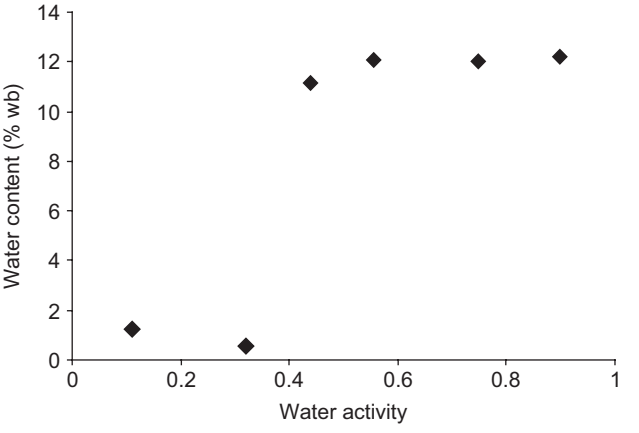

Figure 7. Sorption isotherm for crystalline creatine.

levels of humidity, the water is bound in the crystal form, and thus no dissolution occurs as the deliquescence point must be above an a_w of 0.91. Thus, crystalline creatine is stable, whereas solutions are not unless lowered in water activity and held cold.

Conclusions

The advantage of glycerol-controlled water activity lowering was demonstrated to decrease the rate of creatine degradation in solution and in agar gel. Creatine degradation in glycerol–buffer solutions demonstrated apparent first-order kinetics up to a certain level of degradation when equilibrium in solution occurred. At all a_w values, the degradation rates as a function of temperature followed the Arrhenius equation and E_a increased by 15% as a_w was lowered below 0.68. At higher a_w, E_a remained fairly constant at about 20 kcal/mole and only slightly increased at $a_w = 0.983$. Being a single-reactant reaction in a no-solid matrix system, degradation of creatine in glycerol–buffer solutions did not demonstrate any 'dilution effect' as one might have expected at high water activities. The reduction of a_w by glycerol was accompanied by an increase in measured pH and calculated viscosity. However, using different buffer solutions at parallel pH values, we showed that the rate reduction obtained by glycerol addition could not be explained by the change of pH alone. Additionally, we showed that glycerol-mediated rate reduction is not directly related to the calculated viscosity increase or solid matrix introduction, but increasing mobility by stirring may possibly increase the degradation rate of creatine in solution. Finally, we showed that creatine in the form of either anhydrous or monohydrate crystals were stable at all humidity environments at ≤0.9 as they did not deliquesce.

Declaration of interest: The authors report no conflicts of interest.

References

1. Stryer L. (1995). Biochemistry. 4th ed. New York: W.H. Freeman.
2. Williams MH, Branch JD. (1998). Creatine supplementation and exercise performance: An update. J Am Coll Nutr. 17:216–34.
3. Brose A, Gianni P, Tarnopolsky MA. (2003). Creatine supplementation enhances isometric strength and body composition improvements following strength exercise training in older adults. J Gerontology. 58A(1):11–9.
4. Nigel JF, Marinos E. (1998). Factors influencing the production of creatinine: Implications for the determination and interpretation of urinary creatinine and creatine in man. Clinica Chimica Acta. 175(3):199–210.
5. Labuza TP. (1980). The effect of water activity on reaction kinetics of food deterioration. Food Technol. 34:36–41, 59.
6. Dash AK, Shawney A. (2002) A simple LC method with UV detection for the analysis of creatine and creatinine and its application to several creatine formulations. J Pharm Biomed Anal. 29: 939–45.
7. Lee S, Labuza TP. (1975). Destruction of ascorbic acid as a function of water activity. J Food Sci. 40:370–3.
8. Sherwin C, Labuza TP. (2003). Role of moisture in Maillard browning reaction rate in intermediate moisture foods: Comparing solvent phase and matrix properties. J Food Sci. 68(2):558–94.
9. Sherwin C, Labuza TP. (2006). Beyond water activity and glass transition: A broad perspective on the manner by which moisture can influence reaction rates in foods. In: Pilar M, editor. Water properties of food, pharmaceutical and biological materials. New York: Taylor and Francis; [chapter 20].
10. Katsaboxakis K, Papanicolaou D, Melanitou M. (1998). Stability of pigmented orange anthocyanins in model and real food systems. Ital J Food Sci. 10:17–25.
11. Segur B, Oberstar HE. (1951). Effect of temperature and concentration on viscosity of glycerol solutions. Ind Eng Chem. 43: 2117–20.
12. Bell LN, Labuza TP. (1991). Potential pH implications in the freeze dried state. Cryo Letters. 12:235–44.
13. Bell L, Labuza TP. (1991). Kinetics of aspartame degradation as a function of water activity, pH and temperature. J Food Sci. 56:17–20.