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2 **under different light conditions**

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12

13 *Running title*

14 *Photostability of beverages containing microemulsions*

15 **ABSTRACT**

16 *Background*

17 Microemulsions have been proposed as delivery systems for different lipophilic substances in

18 transparent water-based systems. The chemical stability of the delivered compounds is a key factor

19 to broad the application of microemulsions in the food sector. The stability of a model beverage

20 containing microemulsions delivering β-carotene and lemon oil was tested under increasing light

21 intensity up to 6000 lux at 20 °C.

22 *Results* Transparent microemulsions resulted physically stable during storage indicating that no

23 coalescence phenomenon occurred. On the contrary, both colour and flavour of the microemulsion

24 degraded as a consequence of limonene and β-carotene oxidation. Kinetic data obtained at

25 increasing light were used to estimate the light dependence of beverage spoilage and the

26 mathematical relationship obtained was used to predict spoilage rate under different light

27 conditions. Finally, a shelf life predictive model was proposed.

28 *Conclusions*

29 Transparent microemulsions can be successfully used to deliver into beverages flavour oil and

30 colorants. However, the photostability of the delivered compounds should be carefully studied to

31 estimate product shelf life. To this aim, the availability of models predicting shelf life as a function

32 of light conditions could largely contribute to speed up the process.

33

34 **KEYWORDS**

35 Microemulsion, lemon oil, β-carotene, beverages, phostability, shelf life

36

37

38   **INTRODUCTION**

39   Microemulsions are thermodynamically stable aqueous dispersions of oil droplets with mean radii

40   in the 2 – 100 nm range[1]. This reduced particle size confers transparent appearance to the system.

41   Microemulsions are usually produced by low energy emulsification methods allowing the

42   spontaneous self-assembly of components under appropriate compositional and environmental

43   conditions[1,2]. Based on these unique features, microemulsions have been proposed in literature as

44   delivery systems for different lipophilic substances (e.g. essential oils, bioactive compounds and

45   drugs) in transparent water-based systems[1].

46   Recently, Valoppi et al.[3] developed transparent microemulsions stabilized with Tween 80 and

47   delivering large amount of lemon oil, which is one of the major flavor oil used by the beverage

48   industry. These microemulsions were formed by applying the phase inversion temperature method

49   (PIT). The latter relies on coarse emulsion heating above the surfactant PIT, followed by rapid

50   cooling. The abrupt temperature change induces phase inversion thanks to the ability of the

51   surfactant to recovery its original molecular geometry[1]. To obtain a high load of lemon oil in

52   microemulsions, lemon oil was mixed with peanut oil[3]. This strategy allowed to include in

53   transparent systems up to 15 % lemon oil. It was suggested that the presence of an oil rich in long-

54   chain fatty acids beside lemon oil promoted Tween 80 optimum curvature increasing the lemon oil

55   loading capacity.

56   In the food sector, microemulsions can be considered as stock emulsions containing high levels of

57   the target substances, which are generally lipophilic flavors and/or colorants. Microemulsions are

58   intended for dilution with water to prepare the final beverages[4]. Since mosy microemulsions are

59   formed at limited combinations of their constituents, their dilutability needs being tested to be

60   potentially exploited as delivery systems of lipophilic compounds in transparent beverages.

61   Moreover, being the latter generally packed in see-through materials and displayed on highly

62   enlighten shelves to attract consumers, liposoluble compounds delivered by microemulsions might

63   undergo oxidation during beverage storage. The chemical stability of the delivered

3

64 flavour/colourants is thus an additional key factor to be considered to broad the application of

65 microemulsions. However, up to now, there is limited information on this aspect.

66 Three main concomitant events leading to quality depletion during storage of transparent beverages

67 delivering flavour and colors might be expected: i) physical instability with loss of transparency due

68 to oil droplets coalescence and flocculation, ii) color modifications due to pigment depletion and iii)

69 flavour changes due to the degradation of volatile compounds. The challenge is to understand the

70 relative rate of the spoilage events to determine the most critical for the product stability and shelf

71 life.

72 This work was planned to study the potential exploitation of microemulsions as delivery systems of

73 flavour and colours in transparent beverages. To this aim, microemulsions delivering β-carotene

74 and lemon oil were used to prepare beverages. The latter were submitted to a storage stability test

75 under different light intensity (from 800 to 6000 lux) at 20 °C. Turbidity, β-carotene and limonene

76 content - chosen as indicator of lemon flavour degradation - were monitored during storage under

77 different light conditions to compute kinetic rate constants. Kinetic data obtained at increasing light

78 were used to estimate the light dependence of beverage spoilage with the final aim to develop a

79 predictive stability model exploiting light as acceleration factor[5,6]. The mathematical relationship

80 between beverage spoilage rate and light intensity was used to predict spoilage rate under different

81 light conditions and estimate shelf life.

82

83 **MATERIALS AND METHODS**

84

85 *Materials*

86 Peanut oil and sucrose were purchased in a local market. Essential lemon oil was kindly provided

87 by Enrico Giotti S.p.A. (Scandicci, Italy). Tween 80, citric acid monohydrate and (*R*)-(+)-limonene

88 were purchased from Sigma-Aldrich (Milan, Italy). NaCl, and sodium benzoate were purchased

89 from Carlo Erba (Milan, Italy). All solutions were prepared using milli-Q water.

90

Microemulsion preparation

91

92 The emulsion was prepared following the methodology proposed by Valoppi et al [3]. The aqueous

93 phase consisted of 30% (w/w) Tween 80 dispersed in NaCl aqueous solutions (0.8 M). The mixture

94 was stirred overnight at 300 rpm in order to dissolve the surfactant before use. The lipid phase was

95 composed of a mixture of lemon oil and peanut oil (3:1) containing 0.6 mg/g of β-carotene.

96 The aqueous phase and lipid phase were mixed at 600 rpm for 10 min at ambient temperature to

97 form a coarse emulsion. Aliquots of 8 g of the coarse emulsion were transferred into 10 mL vials,

98 sealed and heated for 30 min at 90 °C in a water bath. Samples were then hand shaken until a

99 homogeneous system was obtained and finally cooled in an ice bath until reaching 20 °C.

100

Beverage preparation

101

102 To simulate commercial beverage, an aqueous solution containing citric acid (8.4 g/L; pH 2.1),

103 sucrose (100 g/L) and sodium benzoate (0.8 g/L) use as preservative was prepared. Finally, 5 g/L of

104 the microemulsion was added to the water phase. This concentration was chosen considering the

105 Acceptable Daily Intake (ADI) of Tween 80, as reported by EFSA[7]. Since the surfactant has

106 a maximum ADI of 25 mg/kg die, the calculation was performed considering an average man of 70

107 kg consuming a maximum amount of 1 L beverage/day[3].

108

Beverage storage

109

110 Aliquots of 8 mL of beverage were introduced into 10 mL capacity clear glass vials and

111 hermetically sealed with butyl septa and metallic caps with air in the headspace (Carlo Erba,

112 Milano, Italy). The vials were stored into an incubator (Climacell 222, MMM Medcenter,

113 Einrichtungen GmbH, Graefling, Germany) at different distances from the SLI Activa-172

114 fluorescent tubes (34.2 W, Sylvania, SLI Lighting, Raunheim, Germany) positioned vertically in the

115 internal part of the door of the incubator. Irradiance of the fluorescent tubes was 1.199 mW/cm^2 and

116   their emission spectrum was within 250 and 780 nm. Temperature was set at 20 °C and no

117   temperature changes were observed as a consequence of lightning. A control sample was stored

118   inside the incubator in a black box (dark conditions). Accordiing to the enlightening level measured

119   by a portable luminometer (HD-2102.2 Delta Ohm, Padova, Italy), samples resulted to be exposed

120   to 0, 800, 200 and 6000 lx.

121

122   *Analytical determination*

123

124   *Particle size*

125   Particle size was determined using a Particle Sizer 380 ZLS analyzer (PSS NICOMP Particle Sizing

126   system, Goleta, USA). Before analysis, emulsions were diluted with deionized water in order to

127   avoid multiple scattering effects. Mean particle diameter was expressed as volume weighted mean

128   diameter ± standard deviation (SD).

129

130   *Turbidity*

131   Aliquots of 2 mL of simulated beverages were introduced in glass cuvette and turbidity was

132   measured using a UV-2501 PC UV-VIS (Shimadzu, Kyoto, Japan) spectrophotometer recording the

133   absorbance at 600 nm.

134

135   *β-carotene*

136   The changes of sample absorbance at 450 nm (Cary 1E UV/VIS spectrophotometer, Varian, Palo

137   Alto, California) were taken as an index of β-carotene degradation. Data were elaborated as relative

138   percentage of β-carotene as a function of storage time.

139

140   *Limonene*

141 Limonene was determined by SPME-GC-MS on a GC-17A gas chromatograph, coupled with a QP-

142 5000 mass spectrometer (both from Shimadzu, Kyoto, Japan). Solid-phase microextraction was

143 carried out on 0.5 mL of beverage, at 10 °C, by using a 2 cm 50/30 μm

144 divinylbenzene/carboxen/polydimethylsiloxane fiber (Supelco, Bellefonte, PA, USA), with a

145 sampling time of 1 min. Vials were pre-conditioned for 10 min before microextraction to allow their

146 thermal equilibration.

147 Volatile compounds were separated on a SLB-5ms capillary column (30 m x 0.25 mm i.d., 0.25 μm

148 film thickness), purchased from Supelco (Bellefonte, PA, USA), with the following operating

149 conditions: initial temperature 40 °C, 4 °C /min up to 180 °C, then 25 °C/min up to 260 °C and a

150 final holding of time of 10 min. Injection was performed in split mode (split ratio 1:200) and

151 temperatures of injection port and transfer line were set at 260 °C. Carrier gas was helium, at a

152 linear flow rate of 36 cm/s. Detector voltage was set at 1.4 kV. Electron impact mass spectra were

153 recorded at 70 eV and the identification of limonene was carried out by comparison of mass spectra

154 and retention times with those of a commercial standard.

155

156 *Data analysis and modelling*

157 All determinations were expressed as the mean ± standard error (SE) of at least two measurements

158 from two experiment replicates (n ≥ 4).

159 β-carotene and limonene degradation data were fitted to a pseudo-first order kinetic reaction and

160 apparent reaction rate constant (k_L, hours^{-1}) at each light intensity (L) were calculated by linear

161 regression:

162

163 $\ln\left(\frac{c}{c_0}\right) = -k_L t$ (1)

164

165 Where C is β-carotene or limonene content at storage time *t* and C_0 is their content at time zero.

166 Regression significance was evaluated by considering determination coefficients (R^2) and

167 probability value (p).

168 Shelf life equation based on the pseudo first order was as follows:

169 $$SL_L = \frac{\ln a - \ln b}{k_L} \quad (2)$$

170 where SL_L is the shelf life at the selected light intensity (*L*), *a* and *b* are the final (corresponding to

171 the end of shelf life) and the initial quality index value, respectively.

172 **RESULTS AND DISCUSSION**

173 *Microemulsion physical stability upon beverage preparation*

174 Starting from our previous research[3], beverage prototypes were prepared by diluting transparent

175 microemulsion loaded with lemon oil (15% w/w) as flavouring ingredient and β-carotene as

176 bioactive/colorant compound. In these experiments, β-carotene was solubilized in a 3:1 lemon oil-

177 peanut oil mixture before microemulsion preparation. The resulted microemulsions had a mean

178 particle diameter lower than 30 nm, giving reason of yellow transparent systems. This transparency

179 was maintained upon microemulsion dilution to obtain a beverage with pH 2.1. This was

180 demonstrated by the very low turbidity value (~ 0.1 O.D. at 600 nm) of the beverage, confirming

181 data previously reported[3].

182

183 *Light-induced quality depletion of beverage*

184 The beverage delivering β-carotene and lemon oil was stored at 20 °C under different light

185 intensities for increasing time up to 30 days. Transparency, taken as an indicator of emulsion

186 stability, color changes due to β-carotene degradation and flavor profile modifications associated to

187 limonene changes were monitored.

188   As regard physical stability, no appreciable changes of absorbance at 600 nm were observed under

189   both dark and light conditions. This result, in agreement with Valoppi et al[3], highlighted that

190   microemulsion oil droplets were stable in the diluted systems upon storage, confirming the

191   effectiveness of microemulsions as delivery systems of liposoluble components, such as flavor oils

192   and bioactive compounds.

193   Given the beverage physical stability during storage, samples were analyzed for β-carotene and

194   limonene content (Figure 1). As the light intensity increased, the bleaching rate of β-carotene also

195   increased, indicating that light exerted a dramatic effect on β-carotene oxidation (Figure 1a). This is

196   in agreement with literature data demonstrating the photosensitivity of β-carotene[8,9]. On the other

197   hand, also limonene content progressively decreased during storage [10,11] (Figure 1b). However, the

198   effect of light appeared different from that observed for β-carotene degradation. The beverages

199   exposed to 800 and 2000 lux showed limonene content changes comparable to that observed in the

200   control sample stored in the dark. Only the exposure to the highest light intensity considered (6000

201   lux) caused an intense limonene depletion. These observations can be better highlighted by

202   observing the pseudo-first rate constants computed by fitting data shown in Figure 1 as a function of

203   storage time (Table 1). The goodness of the statistical parameters confirmed the exponential decay

204   of both selected indices. Reaction rates showed that storage under increasing light intensity

205   progressively affected β-carotene degradation rate, while a discontinuity point was observed for

206   limonene degradation rate between 2000 and 6000 lux. It could be inferred that due to the high

207   antioxidant capacity of β-carotene, the latter might progressively oxidized, protecting limonene

208   from degradation. However, this protecting effect would not be efficacious when the beverage is

209   exposed to dramatically intense light, such as 6000 lux.

210   Based on the acquired data, the colour changes associated to β-carotene degradation can be

211   considered the earliest indicator of the quality changes occurring during the beverage storage. Thus,

212   β-carotene kinetic data were used to evaluate the possibility to develop stability, and eventually

213   shelf life, prediction models.

Stability and shelf life prediction models

In order to produce a model to estimate product stability under different lighting conditions, the rate of β-carotene degradation was studied as a function of light intensity (Figure 2). This approach was previously proposed by Manzocco et al. [5,6], dealing with the development of protocols for the shelf life testing of photosensitive foods, such as beverages and oils. These authors proposed the used of light as unconventional acceleration factor in shelf life accelerated test (ASLT) to reduce the time needed to obtain a reliable shelf life estimation of photosensitive foods.

Observing Figure 2, it is evident the linear relation (R^2=0.998) between β-carotene degradation rate and light intensity. This result is consistent with those obtained by Manzocco et al. [5] studying the light dependence of crocin bleaching rate. The linear relation between β-carotene degradation rate and light intensity confirms the exploitability of light as acceleration factor in stability or shelf life tests. Thus, by measuring the bleaching rate under increasing light intensity and then extrapolating the rate at milder conditions, it can be possible to estimate the beverage degradation rate under dark but also under milder light conditions, usually experienced by the product on the retail shelves.

The knowledge of the photo-stability of the beverages could allow to generate a shelf life predicting model based on light as accelerating factor. To convert a stability model into a shelf life model, it is necessary to select the shelf life acceptability limit (that is the *b* value in the equation 2). As reported by Manzocco[12], the choice of acceptability limit value for products without compulsory indications, such as the considered beverages, is prevalently based on company policy. For the considered beverages and based on the equation reported in Figure 2, the shelf life model (eq. 3) was as follows:

238 $$SL_L = \frac{\ln a - \ln b}{1 \cdot 10^{-5} L + 0.0156} \qquad (3)$$

239 Equation 3 represents a simple model allowing prediction of the shelf life of the beverage at

240 different light intensities (L). For instance, by choosing the acceptability limit in correspondence of

241 50% β-carotene degradation and applying equation (3), the shelf life under dark resulted 250 days.

242 On the contrary, considering the typical light intensity of market shelves (about 600 lux), the shelf

243 life became about 86 days.

244 **CONCLUSIONS**

245 The use of transparent microemulsions can be regarded as successful strategy to deliver liposoluble

246 molecules, such as lemon oil and β-carotene, into beverages. The latter resulted physically stable

247 during storage, indicating that no coalescence phenomenon occurred during storage. On the

248 contrary, beverages resulted photosensitive showing intense colour fading and flavour depletion

249 during storage as a consequence of limonene and β-carotene oxidation. This means that in the

250 attempt to develop beverages containing microemulsions as delivery system, the photostability of

251 the delivered compounds should be carefully studied. To this aim, validated mathematical models

252 able to predict shelf life as a function of light intensity are definitively needed to speed up the

253 process.

254

255

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288

289 **Figure captions**

290 Figure 1. β-carotene (a) and limonene (b) changes as a function of beverage storage time 20 °C

291 under different light conditions.

292 Figure 2. Light dependence of first order rate constant (k) of β-carotene degradation as a function of

293 light intensity. Linear regression results and determination coefficient are also shown.

294

295 Table 1. First order degradation rate (k_L) of β-carotene and limonene in beverages stored at 20 °C

296 under different light intensity.

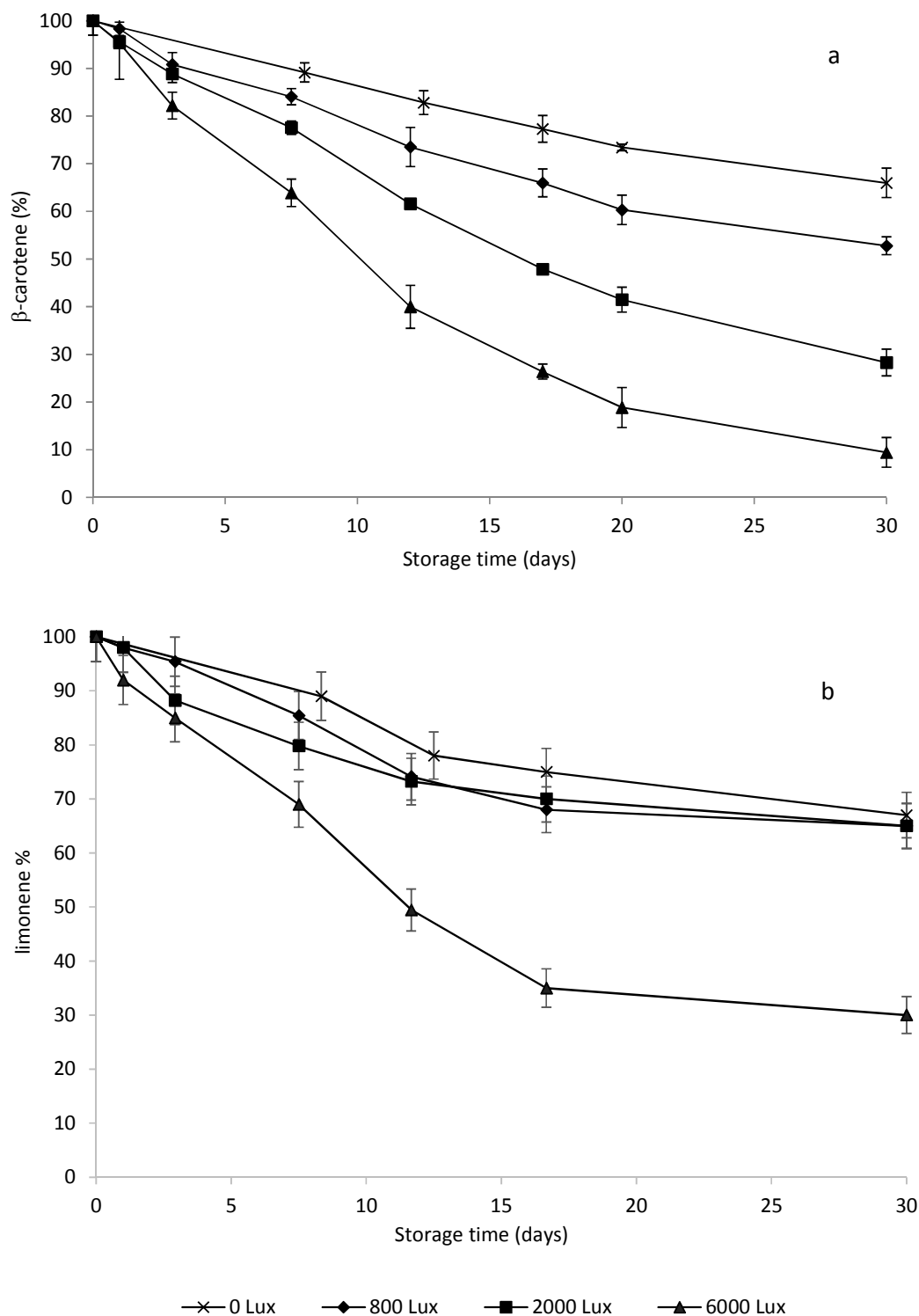| Light intensity (lux)	β-carotene		Limonene	
	k_L (day^{-1})	R^2	k_L (day^{-1})	R^2
0	0.0142±0.0010	0.996	0.0135±0.0021	0.935
800	0.0223±0.0011	0.995	0.0158±0.0026	0.879
2000	0.0430±0.0010	0.984	0.0145±0.0026	0.856
6000	0.0812±0.0023	0.997	0.0432±0.0058	0.915

297 $P < 0.01$

298

299

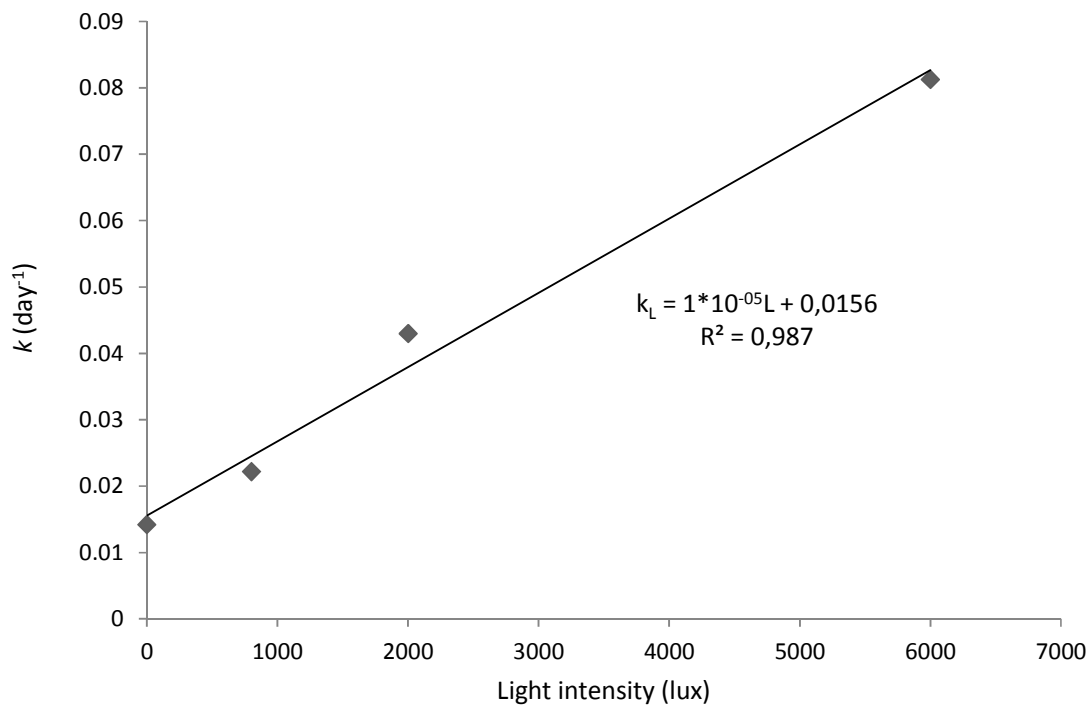Figure 1. β-carotene (a) and limonene (b) changes as a function of beverage storage time 20 °C under different light conditions.

Figure 2. Light dependence of first order rate constant (k) of β-carotene degradation as a function of

light intensity. Linear regression results and determination coefficient are also shown.