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Studies on the inhibitory effect of *Graptopetalum paraguayense* E. Walther extracts on mushroom tyrosinase

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Abstract

This study was aimed to evaluate the kinetic properties and capacities of 95% ethanolic (GE95), 50% ethanolic (GE50) and water (GWE) extracts from *Graptopetalum paraguayense* for their potential to inhibit mushroom tyrosinase activity. The results showed that GE95, GE50 and GWE showed potent inhibitory effects on L-3,4-dihydroxyphenylalanine (L-Dopa) oxidation catalyzed by tyrosinase. It was found that the tyrosinase inhibitory activities of all the extracts increased with the increase of their concentrations. The inhibition kinetics, analyzed by Lineweaver–Burk plots, revealed that *G. paraguayense* extracts showed a mixed-type inhibition for mushroom tyrosinase when L-Dopa was used as substrate. A comparison of the IC_{50} and K_i values showed that GE95 exhibited the most effective inhibition of tyrosinase among the extracts.

1. Introduction

Tyrosinase (monophenol, dihydroxyphenylalanine: oxygen oxidoreductase EC 1.14.18.1) is a multifunctional copper-containing enzyme present in microorganisms, plants and animals. This enzyme is mainly involved in the first two steps of melanin biosynthesis, which consist of the hydroxylation of L-tyrosine (monophenolase activity) and the oxidation of the product of this reaction, the L-Dopa (diphenolase activity), to the corresponding *O*-quinone (Seo, Sharma, & Sharma, 2003). Tyrosinase is responsible for enzymatic browning in plants, producing undesirable changes in colour, flavour and nutritive values of plant-derived foods and beverages (Friedman, 1996; Sánchez-Ferrer, Rodríguez-López, García-Cánovas, & García-Carmona, 1995). In addition, tyrosinase is responsible for the pigment melanin biosynthesis in human skin. Clinically, various dermatological disorders, such as melasma, freckles and age spots, result in epidermal hyperpigmentation (Mosher, Pathak, & Fitzpatrick, 1983). Recently, safe and effective tyrosinase inhibitors have become important for their potential applications in improving food quality and preventing pigmentation disorders and other melanin-related health problems in human beings (Maeda & Fukuda, 1991; Mosher et al., 1983; Seo et al., 2003). Furthermore, tyrosinase inhibitors are also important in cosmetics for skin-whitening effects because lighter skin colour is preferred by many countries and races (Dooley, 1997). As plants are rich sources of bioactive chemicals, which are mostly free from harmful side-effects, there is an increasing interest in finding natural tyrosinase inhibitors from them. Some potent tyrosinase inhibitors, such as cuminaldehyde (Kubo & Kinst-Hori, 1988), oxyresveratrol (Shin et al., 1998), kaempferol (Kubo & Kinst-Hori, 1999), quercetin (Chen & Kubo, 2002) and gallic acid derivatives (No et al., 1999) have been isolated from various plants. In addition to higher plants, a fungal metabolite, kojic acid [5-hydroxy-2-(hydroxymethyl)-*r*-pyrone], has been demonstrated to be a potent tyrosinase inhibitor and is extensively used as a cosmetic agent with an excellent whitening effect (Chen et al., 1991b; Kahn, Ben-Shalom, & Zakin, 1997).

Graptopetalum paraguayense E. Walther is a traditional Chinese herbal medicine-that belongs to the

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Crassvlaceae family. In folk medicine, it has several health benefits, such as lowering of blood pressure, alleviating hepatic disorders and diuretic effects; furthermore, it is considered to have a skin-whitening effect. However, little information is available about the effects of *G. paraguayense* against mushroom tyrosinase activity. Therefore, the objectives of this study were to investigate the tyrosinase inhibitory effect of the water (GWE), 50% ethanolic (GE50) and 95% ethanolic (GE95) extracts from *G. paraguayense*. In addition, the results were compared with those of the well-known tyrosinase inhibitor, kojic acid.

2. Materials and methods

2.1. Chemicals

Mushroom tyrosinase (6050 units/mg), L-3,4-dihydroxyphenylalanine (L-Dopa) and kojic acid were purchased from Sigma Chemicals Co. (St. Louis, MO). All other chemicals were reagent grade or purer.

2.2. Preparation of the water (GWE), 50% ethanolic (GE50) and 95% ethanolic (GE95) extracts from G. paraguayense

The *G. paraguayense* were planted in pots and kept at room temperature. The plant was harvested until the leaves grew up to 5 cm long. Each 20 g of *G. paraguayense* was extracted with 700 ml of 95% ethanol at 75 °C, 50% ethanol at 85 °C, or with distilled water at 100 °C for 3 h. The decoction was filtered and then dried by a vacuum freeze-dryer. The extraction rates of the GWE, GE50 and GE95 were 3.84%, 1.95% and 2.50%, respectively. The extracts were sealed in plastic bottles and stored at −70 °C until use.

2.3. Enzymatic assay of tyrosinase

The tyrosinase activity, using L-Dopa as substrate, was measured according to the method of Kubo and Kinst-Hori (1998) with slight modifications. First, 0.3 ml of 2.25 mM L-Dopa solution was mixed with 0.27 ml of 25 mM phosphate buffer (pH 6.8) and incubated at 25 °C for 10 min. Then, 0.3 ml of each sample solution and 0.03ml of 400 units/ml mushroom tyrosinase in aqueous solution (added last) were added, in that order, to the mixture. This solution was immediately monitored for the formation of dopachrome by measuring the linear increase in optical density at 475 nm. One unit (U) of enzymatic activity was defined as the amount of enzyme increasing 0.001 absorbance per min at 475 nm in the experimental condition.

Inhibition effects on the enzyme activity by test samples were represented as % of inhibition. % inhibition $= (1 - B/A) \times 100$, where $A = \Delta OD_{475}$/min without test samnle and $B = \Delta OD_{475}$/min with test sample. Means of triplicates were determined. The 50% inhibition (IC_{50}) of tyrosinase activity was calculated as the concentrations of test samples that inhibited 50% of tyrosinase activity under experimental conditions.

2.4. Kinetic analysis

The reaction mixture consisted of five difference concentrations of L-Dopa (0.75–7.5 mM) as substrate and mushroom tyrosinase in 25 mM phosphate buffer (pH 6.8). Three different concentrations of GWE, GE50 or GE95 (0.25–2.0 mg/ml) were added to the reaction mixture. Michaelis–Menten constant (K_m) and maximal velocity (V_{max}) of the tyrosinase were determined by Lineweaver–Burk plots.

2.5. Statistical analysis

All the assays to determine enzyme activity, the tyrosinase inhibitory effect of *G. paraguayense* and kojic acid and the enzyme kinetics, were conducted at least three times with three different sample preparations. All data were expressed as means ± SD. Analysis of variance was performed by using SPSS (SPSS Inc. USA). Duncan's new multiple range test was used to determine the difference of means, and $p < 0.05$ was considered to be statistically significant.

3. Results and discussion

3.1. Effect of G. paraguayense extracts on the activity of mushroom tyrosinase

Mushroom tyrosinase has been widely used as the target enzyme for screening and characterizing potential tyrosinase inhibitors. Because the mode of inhibition depends on the structures of both the substrate and inhibitor, L-Dopa was used as the substrate in this study. Therefore, the activity studied in this paper was concerned with *O*-diphenolase inhibitory activity of mushroom tyrosinase.

Fig. 1 shows the dose-response curve for the tyrosinase inhibitory effect of the *G. paraguayense* extracts and a well-known tyrosinase inhibitor, kojic acid. It was found that all *G. paraguayense* extracts had potent inhibitory effects on Dopa oxidase activity of mushroom tyrosinase and the inhibitory activities increased with increase of extract concentrations. The results showed that GE95 had the highest tyrosinase inhibitory activity among the extracts, followed by GE50, and GWE had the lowest. On the basis of the half-inhibition concentration (IC_{50}) for the extracts, the GE95 had the highest tyrosinase inhibitory ability as shown by the lowest

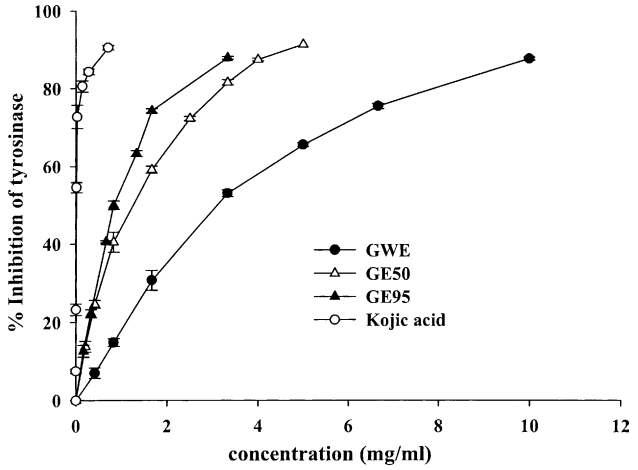

Fig. 1. Dose-dependent inhibition of mushroom tyrosinase by extracts from G. paraguayense and kojic acid. Tyrosinase activity was measured using L-Dopa as the substrate. Each value represents mean ± SD ($n = 3$).

value of IC_{50}, while GWE showed the least ability (Table 1). The GE95 is approximately 1.4 times and 3.5 times more effective than GE50 and GWE, respectively. From a comparison of the IC_{50} values, the tyrosinase inhibitory activity of kojic acid was found to be significantly more pronounced than those of *G. paraguayense* extracts. To obtain 75% tyrosinase inhibitory activity, the concentrations needed for GE95, GE50, GWE and kojic acid were 1.76, 2.38, 7.07 and 0.03 mg/ml, respectively. In other words, to reach a similar extent of tyrosinase inhibitory effect, the concentration required for *G. paraguayense* extracts was significantly higher than that required for kojic acid. Although the anti-tyrosinase abilities of the extracts were significantly less than that of kojic acid, it was evident that the *G. paraguayense* extracts did have potent tyrosinase inhibitory activities.

3.2. Determination of the inhibition type of G. paraguayense extracts on mushroom tyrosinase

The kinetic behaviour of mushroom tyrosinase during the oxidation of L-Dopa was studied. Under the experimental conditions, the apparent K_m and V_{max} of the L-Dopa oxidation reaction, catalyzed by the tyrosinase, were 0.56 mM and 243 U, respectively. The K_m value was lower than the values reported by Chen and Kubo (2002); however, it was higher than that for the L-Dopa oxidation observed by Yu (2003). Different enzyme preparations and assay methods could have contributed to these differences in K_m values (Chen et al., 1991b).

The inhibition kinetics for the GE95, GE50 and GWE were analyzed by Lineweaver–Burk plots as shown in Figs. 2–4, respectively. The four lines, obtained from the unhibited enzyme and from the three different concentrations of *G. paraguayense* extracts, intersected

Table 1
Inhibition constants of the *G. paraguayense* extracts and kojic acid for mushroom tyrosinase

Inhibitor	IC_{50} (mg/ml)	K_i	Inhibition type
GE95	$0.80 \pm 0.02^{a,b}$	0.59 mg/ml	mix
GE50	1.14 ± 0.05^{c}	1.11 mg/ml	mix
GWE	2.83 ± 0.05^{d}	1.84 mg/ml	mix
Kojic acid	0.01 ± 0.00^{e}	3.28 μg/ml	mix

[a] Values are given as means ± SD ($n = 3$).
[b–e] Means in the same column followed by different letters are significantly different ($p < 0.05$).

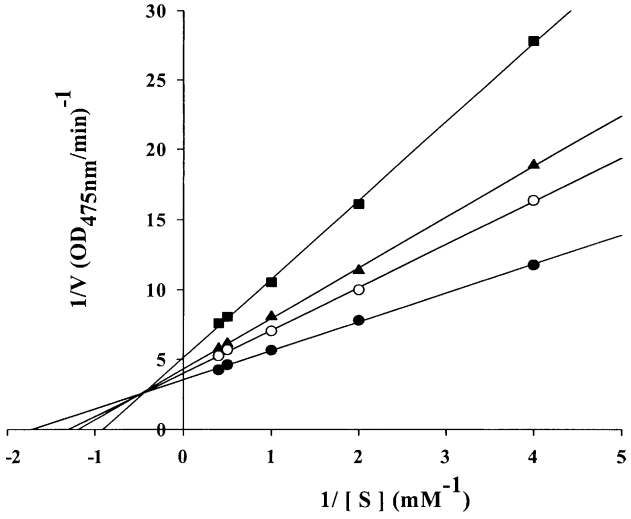

Fig. 2. Lineweaver–Burk plots of mushroom tyrosinase and L-Dopa without (●) and with [(◯) 0.25 mg/ml, (▲) 0.5 mg/ml and (△) 1.0 mg/ml] 95% EtOH extracts from *G. paraguayense*. 1/V:1/ΔOD_{475} nm/min.

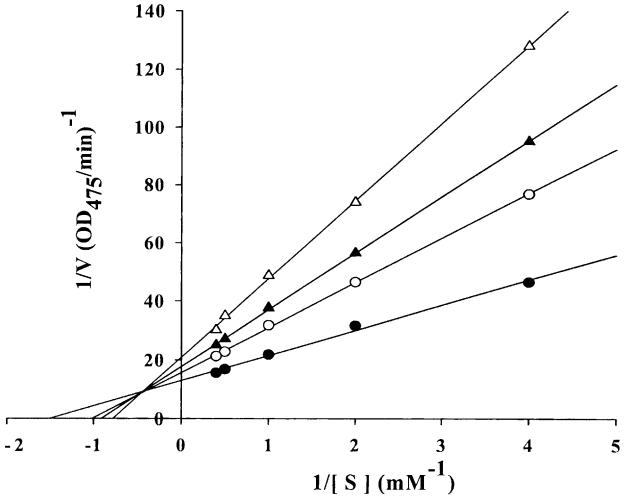

Fig. 3. Lineweaver–Burk plots of mushroom tyrosinase and L-Dopa without (●) and with [(◯) 0.5 mg/ml, (▲) 1.0 mg/ml and (△) 2.0 mg/ml] 50% EtOH extracts from *G. paraguayense*. 1/V:1/ΔOD_{475} nm/min.

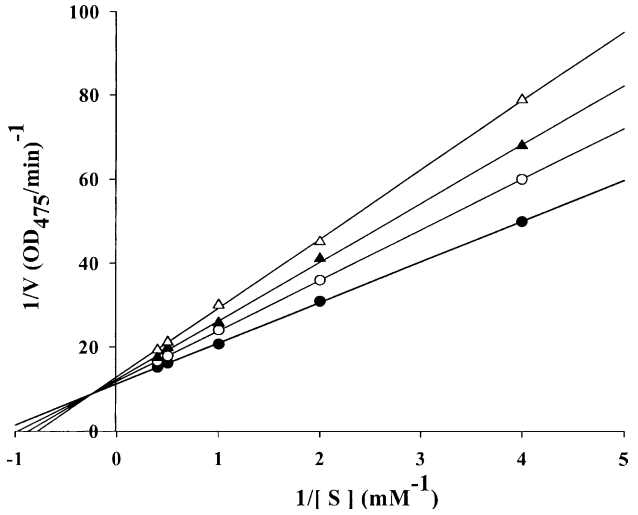

Fig. 4. Lineweaver–Burk plots of mushroom tyrosinase and L-Dopa without (●) and with [(◯) 0.3 mg/ml, (▲) 0.6 mg/ml and (△) 1.2 mg/ml] water extracts from *G. paraguayense*. 1/V:1/ΔOD_{475} nm/min.

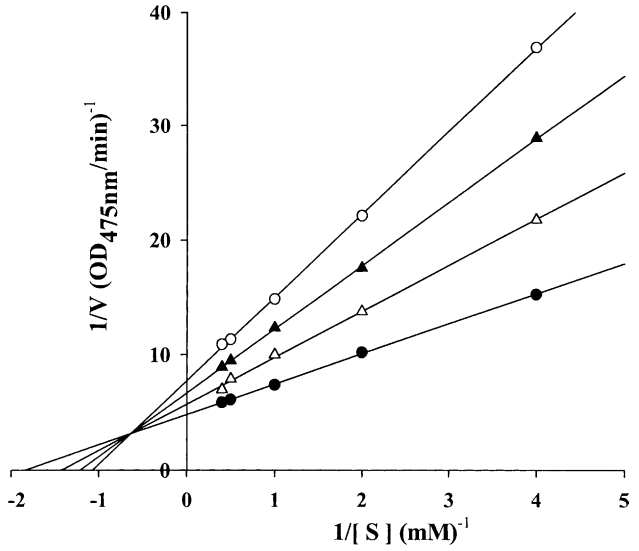

Fig. 5. Lineweaver–Burk plots of mushroom tyrosinase and L-Dopa without (●) and with [(◯) 6.0 μg/ml, (▲) 4.0 μg/ml and (△) 2.0 μg/ml] kojic acid. 1/V:1/ΔOD_{475} nm/min.

to the left of the $1/V$ axis above the $1/S$ axis.The results indicated that all the *G. paraguayense* extracts exhibited mixed-type inhibition for the oxidation of L-Dopa catalyzed by mushroom tyrosinase. The mixed-type inhibition implies that *G. paraguayense* extracts affected the affinity of the enzyme for L-Dopa but did not bind at the active site (Webb, 1963). The kinetic and inhibition constants of *G. paraguayense* extracts are listed in Table 1. From the equilibrium constant for inhibitor binding, K_i of the extracts, it was clear that GE95 had the most effective binding capacity to the enzyme as shown by the lowest value of K_i, while GWE showed the least capacity. In other words, GE95 showed the highest tyrosinase inhibitory ability among the extracts, followed by GE50 and GWE, in decreasing order.

Kojic acid, a fungal secondary metabolite produced by species of *Aspergillus* and *Penicillium* (Basappa, Sreenivasamurthy, & Parpia, 1970; Manabe, Tanaka, Goto, & Matsuura, 1984; Torrey & Marth, 1977) was shown to inhibit mushroom tyrosinase activity (Chen, Wei, & Marshall, 1991a; Chen et al., 1991b; Kahn & Zakin, 1995). Kojic acid has been extensively used as a medical agent for the treatment of some skin disorders associated with hyperpigmentation. The inhibition kinetics for the kojic acid were also analyzed by Lineweaver–Burk plots as shown in Fig. 5. Kojic acid also exhibited mixed-type inhibition for the oxidation of L-Dopa catalyzed by mushroom tyrosinase, in agreement with the observations of Chen et al. (1991b). As the K_i in Table 1 indicates, kojic acid showed much more effective inhibition on tyrosinase than did *G. paraguayense* extracts. The K_i was obtained as 3.28 μg/ml for kojic acid and the value is approximately 180-times more potent than that of GE95. Because the most effective inhibitory component has not yet been isolated from *G. paraguayense* extracts, it is apparent that crude *G. paraguayense* extracts do not exhibit superior activity to kojic acid. However, safety is a primary consideration for tyrosinase inhibitors, especially for those in food and cosmetic products. From the previous data in our laboratory, it appeared that *G. paraguayense* extracts are safe in genotoxicity and exhibit antioxidative activities. It is therefore clear that the most effective tyrosinase inhibitory component in *G. paraguayense* is worthy of further studies as a potential tyrosinase inhibitor.

4. Conclusions

This study implies a depigmentation effect of *G. paraguayense* extracts and shows dose-dependent inhibitory effect on L-Dopa oxidation by mushroom tyrosinase. A study of the kinetics of inhibition of mushroom tyrosinase shows that the *G. paraguayense* extracts are mixed-type inhibitors tyrosinase of the enzyme with L-Dopa as the substrate. The isolation and the structural elucidation of the active constituents of the extracts will provide useful leads in the development of skin-whitening agents. Therefore, further research works are focussing on isolating and identifying the effective tyrosinase inhibitory component in *G. paraguayense* and its inhibitory effect on melanin synthesis with defined cultured cells in vitro.

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References

Basappa, S. C., Sreenivasamurthy, V., & Parpia, H. A. B. (1970). Aflatoxin and kojic acid production by resting cells of *Aspergillus flavus* link. *Journal of General Microbiology, 61*(1), 81–86.

Chen, J. S., Wei, C., & Marshall, M. R. (1991a). Inhibition mechanism of polyphenol oxidase. *Journal of Agricultural and Food Chemistry, 39*(8), 1891–1901.

Chen, J. S., Wei, C., Rolle, R. S., Otwell, W. S., Balaban, M. O., & Marshall, M. R. (1991b). Inhibitory effect of kojic acid on some plant and crustacean polyphenol oxidases. *Journal of Agricultural and Food Chemistry, 39*(8), 1396–1401.

Chen, Q. X., & Kubo, I. (2002). Kinetics of mushroom tyrosinase inhibition by quercetin. *Journal of Agricultural and Food Chemistry, 50*(14), 4108–4112.

Dooley, T. P. (1997). Topical skin depigmentation agents: Current products and discovery of novel inhibitors of melanogenesis. *Journal of Dermatological Treatment, 8*(4), 275–279.

Friedman, M. (1996). Food browning and its prevention: An overview. *Journal of Agricultural and Food Chemistry, 44*(3), 631–653.

Kahn, V., Ben-Shalom, N., & Zakin, V. (1997). Effect of kojic acid on the oxidation of N-acetyldopamine by mushroom tyrosinase. *Journal of Agricultural and Food Chemistry, 45*(11), 4460–4465.

Kahn, V., & Zakin, V. (1995). Effect of kojic acid on the oxidation of trihydroxyphenols by mushroom tyrosinase. *Journal of Food Biochemistry, 18*(6), 427–443.

Kubo, I., & Kinst-Hori, I. (1988). Tyrosinase inhibitors from cumin. *Journal of Agricultural and Food Chemistry, 46*(12), 5338–5341.

Kubo, I., & Kinst-Hori, I. (1998). Tyrosinase inhibitors from anise oil. *Journal of Agricultural and Food Chemistry, 46*(4), 1268–1271.

Kubo, I., & Kinst-Hori, I. (1999). Flavonols from saffron flower: Tyrosinase inhibitory activity and inhibition mechanism. *Journal of Agricultural and Food Chemistry, 47*(10), 4121–4125.

Maeda, K., & Fukuda, M. (1991). In vitro effectiveness of several whitening cosmetic components in human melanocytes. *Journal of the Society of Cosmetic Chemists, 42*, 361–368.

Manabe, M., Tanaka, K., Goto, T., & Matsuura, S. (1984). Producing capability of kojic acid and aflatoxin by koji mold. In H. Kurata & Y. Ueno (Eds.), *Toxigenic fungi – their toxins and health hazard* (pp. 4–14). Tokyo: Elsevier.

Mosher, D. B., Pathak, M. A., & Fitzpatrick, T. B. (1983). Vitiligo: Ethology, pathogenesis, diagnosis and treatment. In T. B. Fitzpatrick, A. Z. Eisen, K. Wolff, I. M. Freedberg, & K. F. Austen (Eds.), *Updates: Dermatology in general medicine* (pp. 373–398). New York: Mcgraw-Hill.

No, J. K., Soung, D. Y., Kim, Y. J., Shim, K. H., Jun, Y. S., Rhee, S. H., Yokozawa, T., & Chung, H. Y. (1999). Inhibition of tyrosinase by green tea components. *Life Sciences, 65*(21), 241–246.

Sánchez-Ferrer, A., Rodríguez-López, J. N., García-Cánovas, F., & García-Carmona, F. (1995). Tyrosinase: A comprehensive review of its mechanism. *Biochimica et Biophysica Acta, 1247*(1), 1–11.

Seo, S. Y., Sharma, V. K., & Sharma, N. (2003). Mushroom tyrosinase: Recent prospects. *Journal of Agricultural and Food Chemistry, 51*(10), 2837–2853.

Shin, N. H., Ryu, S. Y., Choi, E. J., Kang, S. H., Chang, I. M., Min, K. R., & Kim, Y. (1998). Oxyresveratrol as the potent inhibitor on dopa oxidase activity of mushroom tyrosinase. *Biochemical and Biophysical Research Communication, 243*(3), 801–803.

Torrey, G. S., & Marth, E. H. (1977). Isolation and toxicity of molds from foods stored in homes. *Journal of Food Protection, 40*(3), 187–190.

Webb, J. L. (1963). *Enzyme and metabolic inhibitors* (pp. 149–188). New York: Academic Press.

Yu, L. (2003). Inhibitory effects of (*S*)- and (*R*)-6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acids on tyrosinase activity. *Journal of Agricultural and Food Chemistry, 51*(8), 2344–2347.