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Short communication

Effect of gamma-radiation on major aroma compounds and vanillin glucoside of cured vanilla beans (*Vanilla planifolia*)

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ABSTRACT

Cured vanilla beans were irradiated (5, 10, 15, 20 and 30 kGy) for a possible enhancement of vanillin content by radiolysis of vanillin glucoside. The vanillin content of control and irradiated (30 kGy) samples were 26.6 ± 0.85 and 26.9 ± 0.70 mg/g (dw), respectively, while vanillin glucoside were 7.74 ± 0.2 and 7.35 ± 0.35 mg/g (dw). Radiation caused no significant changes ($p \leqslant 0.05$) in aroma constituents. In a pulse radiolysis experiment, vanillin glucoside on reacting with ·OH radical gave transients with absorption peaks at 360 nm and 410 nm. The species absorbing at 410 nm has been interpreted to be a hydroxyl radical adduct. It decayed at the same rate in the presence of oxygen, while the absorption at 360 nm did not. Results obtained revealed that the more stable one absorbing at 360 nm is aldehydic radical. Hence the highly stable oxygen–carbon linkage between vanillin and glucose limits the possible enhancement of aroma quality of irradiated beans.

1. Introduction

Vanilla (*Vanilla planifolia*) is a commercial crop widely cultivated as a source of natural vanillin. The compound is obtained from cured vanilla beans and is extensively used in flavouring food, beverages and confectionery (Ranadive, 1994). Worldwide total demand for vanillin is more than 10,000 tons per year, of which less than 0.5% is met out by vanillin isolated from vanilla beans (Priefert, Rabenhorst, & Steinbuchel, 2001). The rest is produced synthetically, mostly from petrochemicals, such as guaiacol and lignin (Clark, 1990). The price of natural vanillin extracted from beans is between US$1200/kg and US$4000/kg, while that of synthetic vanillin is less than US$15/kg (Serra et al., 2005). Thus there is a need for improving the yield of natural vanillin obtained from vanilla beans for economic reasons.

Cured vanilla beans have moisture content in the range of 25–30%. They are generally kept in cartons or wooden boxes for conditioning (3–6 month's period) and subsequent storage. The conditions prevailing in these boxes are conducive for fungal growth. Thomas and Bindumol (2005) reported microbial spoilage of cured vanilla beans by fungal species belonging to the genera *Aspergillus* and *Penicillium*.

Radiation processing of food materials by gamma-radiation is a well established method for microbial decontamination and insect disinfestation. Irradiation of spices at doses ranging from 10 to 30 kGy has been reported to result in complete elimination of microorganisms with negligible changes in the flavour quality (Farag, Aziz, & Attia, 1995). The effect of gamma-radiation on micro flora and vanillin content of cured vanilla beans in the dose range of 5–50 kGy has been investigated by Bachman, Pietka, and Zegota (1995), but its effect on other major aroma compounds and vanillin glucoside (vanillin aroma precursor) remaining after curing have not been studied so far.

Recent studies have demonstrated the role of radiation processing in improving the sensory quality of spices, such as saffron (Zareena, Variyar, Gholap, & Bongirwar, 2001) and nutmeg (Ananthakumar, Variyar, & Sharma, 2006). Radiolytic breakdown of glycosidic precursors of aroma constituents and consequent release of free aroma was shown to result in the enhancement of aroma quality of these products. Since a considerable amount of vanillin exists as its glycosidic precursor (7.74 ± 0.20 mg/g) in cured vanilla pods, a possible enhancement in yield of vanillin by radiation processing is thus expected. Results with enzymatic preparations have demonstrated that as much as half of the

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amount of vanillin sequestered in the complex cellulose matrix is in either free or bound glycosidic forms, and can be liberated by enzyme assisted procedures (Ovando, Waliszewski, & Pardio, 2005; Ruiz-Teran, Perez-Amador, & Lopez-Munguia, 2001).

Hence, the present work was aimed to study the effect of gamma-radiation processing on the major aroma compounds of cured vanilla beans and also to investigate possible enhancement in vanillin content by the radiolytic breakdown of vanillin glucoside present already.

2. Materials and methods

Cured and fresh green vanilla beans were procured from a curing centre in South Canara district of Karnataka State, India. Beans were stored at 4 °C until analysis. Standard compounds were procured from Sigma–Aldrich Chemical Company, St. Louis, MO. All solvents including HPLC grade were purchased from E. Merck India Ltd., Mumbai. Solvents were distilled before use. Other chemicals and reagents of AR grade were obtained from Thomas Baker, Mumbai.

2.1. Moisture content and water activity

Moisture content of the samples was determined using a moisture metre (Sartorius MA100, Goettingen, Germany). Water activity (a_w) of samples was measured at fixed temperature (27 ± 1 °C), using an Aqualab water activity metre (Model Series 3 TE, Decagon Devices, Pullman, WA) with temperature control.

2.2. Irradiation of the vanilla beans

Ten different doses (0, 1, 2, 3, 4, 5, 10, 15, 20 and 30 kGy) of gamma-radiation were given to cured vanilla beans samples of 250 g each. All the treatments were performed in triplicate. Irradiation was carried out on a GC5000 Gamma irradiator (BRIT, Mumbai) using ^{60}Co as source, with dose rate of 7.881 kGy/h.

2.3. Isolation of free aroma constituents

The procedure developed by Ranadive (1992) was followed with some modifications. Cured vanilla bean samples (25 g) from each treatment were ground using a pestle and mortar with liquid nitrogen. The resultant powder was made into a slurry by mixing it thoroughly with 100 ml of 45% ethanol and kept overnight at room temperature. Before filtering, the slurry was stirred using an Omni Mixer (2–3 min; Omni International, Kennesaw, GA) and then filtered under suction using a Buchner funnel. The residual cake was again extracted (100 ml × 2) as above and the extracts were pooled and centrifuged at 8500*g* for 45 min at room temperature to remove any suspended particles. Exactly 50 ml of the supernatant were taken and dried completely under vacuum and the residue was made up as a 10% solution in 45% ethanol. These solutions were analysed by HPLC for qualitative and quantitative changes in aroma profile of cured vanilla beans.

2.4. Isolation of vanillin glucoside

Vanillin glucoside from vanilla beans (both fresh green and cured) was isolated following the method described by Dignum, Kerler, and Verpoorte (2002). In a 100-ml volumetric flask, 65 ml of acetate buffer (pH 5.0) and 5 g of ground vanilla bean sample were mixed thoroughly for 5–10 min. Then the suspension was heated in microwave oven to deactivate enzymes. Finally the volume was made up to 100 ml using distilled methanol. This whole extract was concentrated under vacuum to remove methanol completely and the resultant aqueous portion was suitably diluted with distilled water. Total glycosides from this aliquot were separated using an XAD-16 (Sigma) column (13 cm bead × 3 cm ID), following standard procedures (Gunata, Bayonove, Bumes, & Cordoinnier, 1985). The methanol fraction (100 ml) eluting from the XAD-16 column was evaporated to dryness under vacuum and the residue was dissolved in an appropriate volume of methanol to obtain a final concentration of 10 mg/ml. Individual glycosides from this extract were separated by preparative TLC, using ethyl acetate:isopropanol:water (65:30:15) as the mobile phase. Vanillin glucoside, the most abundant glycoside reported (Odoux, 2006) in vanilla beans, visualised after exposure of plates to iodine vapours (R_f = 0.7) was scraped from TLC plates and eluted with methanol. The eluate was dried under vacuum and made up to a 1% solution in methanol. A part of this solution was subjected to acid hydrolysis (2N HCl, 1 h, 100 °C) and extracted with diethyl ether. The remaining aqueous portion was neutralised with 1N KOH, dried under vacuum and the residue was dissolved in methanol. This methanol solution along with standard glucose was subjected to TLC (isopropanol:acetone:water, in ratio of 45:30:25, as mobile phase) and the spots were detected by 10% sulphuric acid spray, followed by heating at 110 °C for 15 min, in order to identify the sugar moiety.

The vanillin glucoside thus isolated in pure form was used as authentic standard for quantitative estimation of vanillin glucoside by HPLC. The diethyl ether extract was washed free of acid with distilled water and then dried over sodium sulphate. The organic layer was concentrated by passing over it a mild stream of nitrogen gas and then analysed by GC–MS.

2.5. GC/MS analysis

GC/MS analysis was carried out on a Shimadzu GC/MS instrument (Shimadzu, Kyoto, Japan) equipped with a GC-17A gas chromatograph and provided with a DB-5 (J&W Scientific, Folsom, CA) capillary column (length, 30 m; i.d., 0.25 mm and film thickness, 0.25 μm). The operating conditions were: column temperature programmed from 60 to 200 °C at the rate of 4 °C/min, held at initial temperature and at 200 °C for 5 min and further to 280 °C at the rate of 10 °C/min, held at final temperature for 20 min; injector and interface temperatures, 210 and 230 °C, respectively; carrier gas helium (flow rate, 0.9 ml/min); ionisation voltage, 70 eV; electron multiplier voltage, 1 kV. Samples (0.5 μl) were injected in splitless mode. Peaks were identified by comparing their mass fragmentation pattern with that of standard compounds as well as with the data available in the spectral library (Wiley/NIST Libraries) of the instrument.

2.6. HPLC analysis

HPLC separation of compounds was performed using a JASCO HPLC (Model PU 980; JASCO International Co. Ltd., Tokyo, Japan) fitted with C18 reverse-phase column (250 mm × 4.6 mm, Thermo Hypersil-Keystone; Thermo Fisher Scientific Inc., Waltham, MA) and 3-cm guard column. Compounds were detected by UV–Vis detector (UV 975) set at 275 nm and data were interpreted using Jasco Borwin-PDA Software (Version 1.50). The mobile phase used was methanol:acetonitrile:acetic acid (50:100:10 ml) finally diluted to 1 l with filtered (0.45-μm nylon membrane) deionised water (Milli-Q), at a flow rate of 1 ml/min (Archer, 1989). Samples were filtered through 0.45-μm nylon membrane before HPLC analysis.

Standard solutions of vanillin, vanillin glucoside, vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were prepared at appropriate concentrations in methanol and analysed by HPLC.

Standard graphs were prepared by plotting peak area against concentration using the above standards.

2.7. Pulse radiolysis

Use of pulse radiolysis system (7 MeV electrons) for generation and studies on the free radical reactions have been described earlier (Adhikari & Mukherjee, 2001). Dosimetry was carried out using an air-saturated aqueous solution containing 5×10^{-2} mol/dm KSCN, assuming a *G*-value for $(SCN)_2^-$ of 23.889 dm^3/mol/cm per 100 eV at 500 nm (Buxton & Stuart, 1995). The width of the electron pulse was 50 ns and the dose per pulse was 15 Gy. The kinetic spectrophotometric detection system covered the wavelength range from 250 to 800 nm. High purity (99.9%) N_2O, from British Oxygen Co. (Kolkata, India) was used as per requirement. The following reactions occur after irradiation in the reaction medium used.

$$H_2O \longrightarrow e_{aq}^-, H^\cdot, {}^\cdot OH, H_2, H_2O_2 \quad (1)$$

$$e_{aq}^- + N_2O + H_2O \Rightarrow {}^\cdot OH + OH^- + N_2 \quad (2)$$

The bimolecular rate constants were calculated by plotting the pseudo-first order rates of formation of the transients against the concerned solute concentrations. The uncertainty in the measurement in bimolecular rate constant is ±10%.

2.8. Statistical analysis

All analyses were performed in triplicate. One-way ANOVA was done to evaluate the differences between treatments at the 5% significance level (Sokal & Rohlf, 1987).

3. Results and discussion

3.1. Effect of radiation processing on aroma profile of cured beans

Natural vanilla aroma is a mixture of more than 200 volatile compounds. Among these, four major compounds collectively constitute 97% w/w of the total aroma compounds reported. They include vanillin (86% w/w), vanillic acid (6% w/w), *p*-hydroxybenzaldehyde (4% w/w) and *p*-hydroxybenzoic acid (1% w/w) (Perez-Silva et al., 2006). The aqueous ethanol extracts from treated samples (control, 5, 10, 15, 20 and 30 kGy) were analysed by HPLC as per the procedure described earlier. Presence of the above four major compounds was confirmed using authentic standards. The quantitative distribution of the four major aroma compounds in samples subjected to different radiation doses are presented in Table 1. Bachman et al. (1995) studied the effect of gamma-radiation on vanillin content. No change in the vanillin content was reported, even at a dose of 50 kGy. The results obtained in the present study are also in tandem with the report of Bachman et al. Besides vanillin, the content of other aroma compounds identified and reported in this study also showed no significant quantitative changes up to a dose of 30 kGy. Hence, gamma-radiation may be the method of choice for hygienisation of cured vanilla beans without any significant effect on the aroma profile.

3.2. Isolation and identification of vanillin glucoside

Earlier studies have shown that the vanillin glucoside content in green vanilla beans range from 10% to 15% on a dry weight basis (Odoux, Escoute, & Verdeil, 2006; Ovando et al., 2005). Aroma glycosides separated from aqueous extracts using Amberlite XAD-16 column were further separated by preparative TLC using the procedure outlined in Section 2.4. The major band corresponding to vanillin glucoside (R_f = 0.7) was isolated by preparative TLC. GC/MS analysis of the aglycone moiety obtained after acid hydrolysis confirmed it to be vanillin [*m/z* 152 (88%, M^+), 151 (100%, M^+-H), 137 (6%, $M-CH_3$), 123 (88%, M−CHO)], and the sugar moiety was identified to be glucose by TLC. The vanillin glucoside thus obtained from green vanilla beans [purity >99% on HPLC (*Rt* 4.3 min)] was used as a standard for all subsequent analyses.

Odoux et al. (2006) reported the presence of about 15 glycosides in green vanilla beans, of which vanillin glucoside was the most abundant. Fig. 1 shows the HPLC chromatogram of aqueous extracts of green and cured vanilla beans. The amount of vanillin glucoside in green beans and cured beans as estimated by HPLC in the present study was found to be 72.5 ± 0.5 mg/g and 7.74 ± 0.2 mg/g, respectively, on a dry weight basis. So, approximately 10–15% of vanillin glucoside is still present in the cured beans, compared to the green beans thanks to incomplete hydrolysis of glucovanillin during the curing process. Thus there is scope for further enhancing the vanillin content of cured beans by an appropriate technology which can release vanillin from the remaining glucovanillin.

3.3. Effect of radiation processing on vanillin glucoside content

Irradiation has been successfully used for the quality improvement of many agricultural products. Ananthakumar et al. (2006) reported the radiolytic breakdown of aroma glycosides in nutmeg. A linear increase in antioxidant isoflavones with radiation dose resulting from a breakdown of glycosidic precursors, was also noted in soybean (Variyar, Limaye, & Sharma, 2004). In a recent work on the stability of phenol-β-D-glucopyranosides in fenugreek, Chatterjee, Adhikari, Variyar, Gupta, and Sharma (2009) have demonstrated a radiolytic breakdown of glycosidic linkage, *via* a carbon-centred radical using pulse radiolysis studies. Thus carbon–oxygen linkages in the glycoside are susceptible to radiolysis. It was of interest to understand the stability of vanillin glucoside during radiation processing, with a view to improve the vanillin content in cured beans. Contents of vanillin and its glycoside as estimated by HPLC in cured beans samples subjected to various doses of gamma-radiation, is provided in Table 1. No difference in either vanillin or glucovanillin content was noted in the samples subjected to different radiation doses up to 30 kGy. Strong resis-

Table 1
Contents of major aroma compounds and vanillin glucoside of cured vanilla beans irradiated at different doses of gamma-radiation (mg/g).

Aroma constituents	Irradiation dose (kGy)					
	0	5	10	15	20	30
Vanillin	26.60 ± 0.85[a]	26.50 ± 0.80[a]	25.80 ± 0.73[a]	25.62 ± 0.82[a]	26.20 ± 0.85[a]	26.91 ± 0.70[a]
Vanillic acid	0.93 ± 0.08[b]	0.91 ± 0.10[b]	0.90 ± 0.08[b]	0.95 ± 0.09[b]	0.94 ± 0.10[b]	0.95 ± 0.07[b]
p-Hydroxy benzaldehyde	1.32 ± 0.12[c]	1.30 ± 0.12[c]	1.28 ± 0.14[c]	1.31 ± 0.12[c]	1.29 ± 0.14[c]	1.33 ± 0.13[c]
p-Hyroxy benzoic acid	0.45 ± 0.03[d]	0.40 ± 0.04[d]	0.43 ± 0.03[d]	0.44 ± 0.02[d]	0.41 ± 0.02[d]	0.42 ± 0.03[d]
Vanillin glucoside	7.74 ± 0.20[a]	7.40 ± 0.30[a]	7.35 ± 0.20[a]	7.45 ± 0.25[a]	7.50 ± 0.20[a]	7.35 ± 0.35[a]

[a–d] Means ± SD values sharing the same letter are not significantly different ($p < 0.05$, $n = 3$).

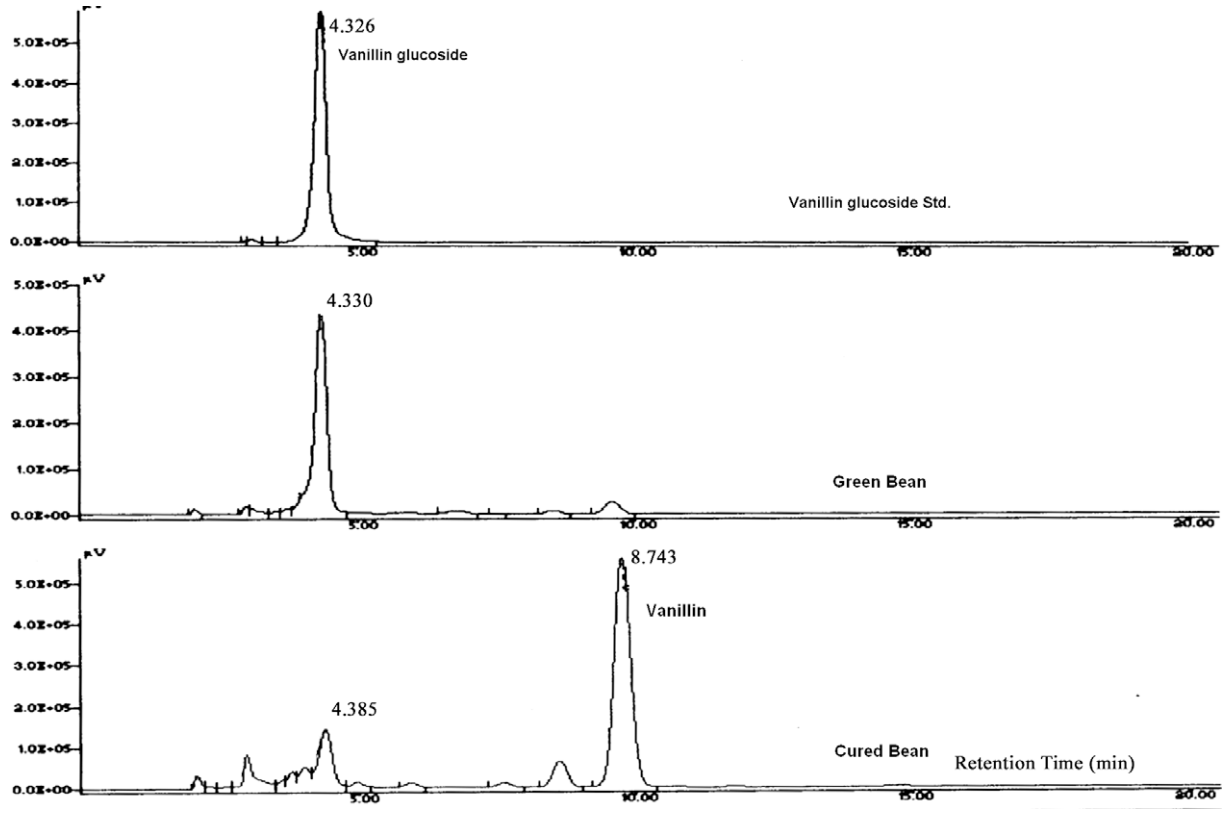

Fig. 1. HPLC chromatogram of aroma glycosides in green bean and cured bean containing vanillin glucoside.

tance to radiolysis by standard vanillin glucoside exposed directly to gamma-radiation as high as 60 kGy was also observed during this study (data not shown). Thus unlike other glycosides reported above, the carbon–oxygen linkage in glucovanillin appears to be quite stable. This observation warranted understanding the mechanism of stability of vanillin glucoside to radiolytic cleavage, so a pulse radiolysis experiment on vanillin glucoside was carried out.

3.4. Pulse radiolysis study on vanillin glucoside

In an N_2O-saturated aqueous solution, it is the ·OH radical that is produced by the passage of electron pulses. Vanillin glucoside upon reacting with ·OH radical was found to give a transient with absorption peaks at 360 nm and 410 nm (Fig. 2). The bimolecular rate constant for the reaction as determined by following the pseudo-first order rate of formation both at 360 nm and 410 nm is 8.0×10^9 dm^3/mol/s. While the absorption at 410 nm decays with time, absorption at 360 nm does not show a significant decay up to 1 ms (Fig. 3A). From this result it is clear that at least two species are produced at the initial step with one species being more stable. Further, the formation of the more stable species has no relation with the decay of the other species absorbing at 410 nm. However, both the species decay by first order kinetics, as evident from experiments carried out at different doses. It is known that a first order decay process does not depend upon the concentration of the species decaying. In this case if the concentration of the species is increased by increasing the dose of the pulse, thereby increasing the concentration of the radical, there should not be any change in the decay rate. On the contrary, a second order decay process will be faster with a proportionate increase in concentration. We have observed no effect on the decay of both the radicals by increasing the dose per pulse to 32 Gy from 16 Gy (figure not shown). In an earlier study on the reaction of hydroxyl radical with vanillin (Mahal, Badheka, & Mukherjee, 2001), it was reported that,

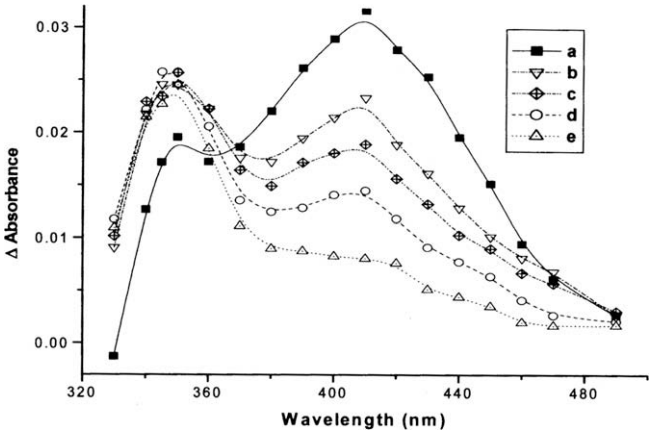

Fig. 2. Transient absorption spectra obtained from an N_2O-saturated aqueous solution (natural pH) containing 1×10^{-4} mol/dm^3 vanillin glucoside. (a) 15 μs, (b) 85 1 μs, (c) 150 μs, (d) 300 μs and (e) 850 μs after the electron pulse.

depending upon pH, two types of radicals were formed. At pH 5, a hydroxyl radical adduct (absorption maximum at 430 nm) is the predominating species while at pH 9, phenoxyl radical was observed with absorption maximum at 410 nm. In the present case, formation of phenoxyl radical is unlikely because the phenolic site is blocked by glycoside linkage. In general, phenoxyl radicals decay by second order process and become faster with increase in radiation dose, which is not the case in the present study. Thus the species absorbing at 410 nm must be a hydroxyl radical adduct. Even in the presence of oxygen this species decays at the same rate.

The absorption at 360 nm on the other hand is susceptible to oxygen concentration (Fig. 3B). The carbon-centred radicals react with dissolved oxygen if present very quickly and the bimolecular rate constant values are in the range $2–4 \times 10^9$ dm^3/mol/s, which

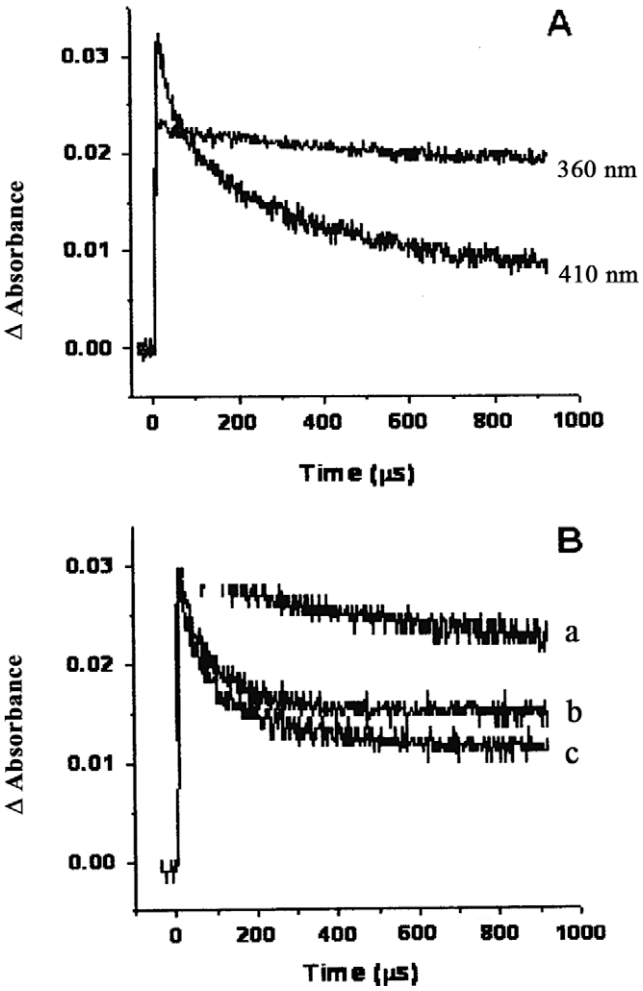

Fig. 3. Kinetic traces recorded from the same solution as for Fig. 2. (A) The decay traces at 360 and 410 nm. (B) Decay traces at 360 nm in presence and absence of oxygen.

is slightly below the diffusion controlled limit (Cooper et al., 2009, and references therein). Thus the decay rate of the carbon-centred radical becomes faster with increase in oxygen concentration in the experimental solution. It can be inferred that this absorption is due to a carbon-centred radical. The aldehydic hydrogen is weakly bonded to the carbonyl carbon, and should be highly susceptible to abstraction by ·OH radical (Nlki, Maker, Savage, & Breitenbach, 1978). Thus it can be inferred that the more stable radical absorbing at 360 nm is the aldehydic radical. In comparison to vanillin the glycoside analogue reacts in a different way. In the case of vanillin aldehyde radical formation does not occur. Thus the oxygen–carbon linkage between vanillin and glucose is less susceptible to cleavage. This explains the high stability of glucovanillin to radiolysis.

4. Conclusion

The gamma-radiation processing (up to 30 kGy) did not cause any significant changes in the aroma profile of cured vanilla beans both qualitatively and quantitatively. To our knowledge this is the first report about the effect of gamma-radiation on vanillin glucoside and major aroma compounds of cured vanilla beans. This study showed that, gamma-radiation may not be a useful alternate to β-glycosidase enzyme for the release of vanillin from its glycoside precursor, either for the purpose of aroma enhancement or for accelerated curing. But gamma-radiation may be useful in rupturing the cellulose matrix of fresh vanilla beans, thereby enhancing the contact between β-glycosidase enzyme and vanillin glucoside, which are separately located in the outer fruit wall region and the placental region, respectively. However, gamma-radiation is the best tool for the complete hygienisation of vanilla beans from spoilage and toxin-producing microbes without altering its aroma quality and market value.

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