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Vanilla curing under laboratory conditions

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Abstract

A laboratory model curing is described in which the cured vanilla beans are analysed for enzyme activity and aroma. The activity of the enzymes was highest in green beans. β-Glucosidase (β-Glu) could not be detected after 24 h of autoclaving. Peroxidase (PER) and protease (PROT) activity decreased, but were still present (20%) after 29 days. Phenylalanine ammonia lyase (PAL) survived autoclaving, but was not detected later in the process. Beans that were scalded for 20 min at 80 °C showed no detectable β-Glu and PAL activity, but PROT and PER were still active. Under traditional curing conditions glucovanillin (GV) and glucovanillic acid (GVA) were hydrolysed to vanillin and vanillic acid, respectively. Upon scalding for 20 min at 80 °C the concentration of glucosides was still high (after 16 day: GV 2000 ppm, GVA 700 ppm). This may be an indication that the normal scalding leads to inactivation of a non-specific glucosidase, while the prolonged scalding also inactivates a specific glucosidase.

Keywords: Orchidaceae; *Vanilla planifolia*; β-Glucosidase; Peroxidase; Protease; Curing; Aroma analysis; Vanillin

1. Introduction

Vanilla beans are the fruits of *Vanilla planifolia* Andrews (Orchidaceae). The curing of green vanilla beans to obtain the well-appreciated vanilla aroma, is a very laborious process. Every vanilla cultivating country has developed its own curing process, but it generally consists of four steps: scalding, sunning/sweating, drying and conditioning. Usually, scalding is followed by an autoclaving step. This means that the beans that have become hot after scalding, are put into an airtight container overnight, to retain as much heat as possible. It can be regarded as the first sweating step. The whole process normally takes more than 6 months (Dignum, Kerler, & Verpoorte, 2001a).

The main component of the vanilla aroma is vanillin, which was first isolated and identified in vanilla by Gobley (1858). In the green bean, important phenolic aroma compounds are present as glucosides. The curing process is meant to release the aglycons to set free the aroma compounds. The exact reactions during curing are not known, but apparently enzymes play an important role in flavour formation (Arana, 1943, 1944; Jones & Vicente, 1949); β-glucosidase being the most important enzyme. Also the influence of microorganisms on the final product has been investigated (Röling, Kerler, Braster, Apriyantono, Stam, & van Verseveld, 2001).

In the last century considerable research was devoted to the vanilla curing process. Many experiments have been conducted to determine the optimal conditions to obtain a good quality of cured vanilla (Broderick, 1956; Theodose, 1973), what compounds are important (Kanisawa, Tokoro & Kawahara, 1994; Odoux, 2000; Pu, Zhang, Zhang, & Jiang, 1998) and what enzymes are involved (Dignum, Kerler, & Verpoorte, 2001b; Hanum, 1997; Jiang, Pu, Xie, Hu, & Li, 2000; Ranadive, Szkutnica, Guerrera, & Frenkel, 1983; Wild-Altamirano, 1969).

Since we investigated the problems of retaining the β-glucosidase activity in bean extracts (Dignum et al., 2001b), it seemed important to analyse beans as fresh as possible. As analysis of cured beans is difficult to perform at the curing stations, a laboratory model curing process was set up where the influence of the curing

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Nomenclature

β-Glu	β-glucosidase
PER	peroxidase
PROT	protease
PAL	phenylalanine ammonia lyase

conditions on enzymes and the release of aroma compounds in the vanilla beans was investigated.

2. Materials and methods

2.1. Set-up of the experiment

The curing conditions were adapted from the curing process as used by the company Djasula Wangi in their curing station in Singaraja, Bali, Indonesia (Fig. 1 a and b; Tiollier, 1983). In 1998 the curing was followed and the temperature and the relative humidity were registered continuously using Testo 650 equipment (Testo, Almere, the Netherlands). In the present study the curing was performed in a climate room, where the temperature and relative humidity could be controlled (Fig. 1c and d).

Approximately 20 kg of green beans (Djasula Wangi, Jakarta, Indonesia) were used for a model curing experiment under traditional conditions (as in Table 1). The beans were scalded batchwise in a waterbath and they were put in a climate room throughout the experiment. Autoclaving was done with all beans put together in a plastic bag in order to keep humidity as high as possible. After the autoclaving procedure the beans were spread onto trays, to facilitate the drying process (sunning).

Alternatively, a mild curing process was used. After scalding, the autoclaving was carried out for 3 h at 50 °C, followed by 3 h at 45 °C and finally 18 h at 40 °C, all at 95% humidity.

Each time samples of 1 kg were used. Two hundred grams of this batch were cut into 1-cm pieces and used for enzyme analysis in duplicate. The rest was frozen in liquid nitrogen and ground. Two hundred grams of frozen material was freezedried in order to measure the moisture content of the sample. The freeze-dried material was used later for glycoside analysis. Six hundred grams of sample were used for aroma analysis.

2.2. Enzyme analysis

Samples were extracted with 0.15 M BisTris–Propane (BTP) (Sigma, St. Louis, MO) buffer pH 8 for the assays of the following enzyme activities: β-glucosidase

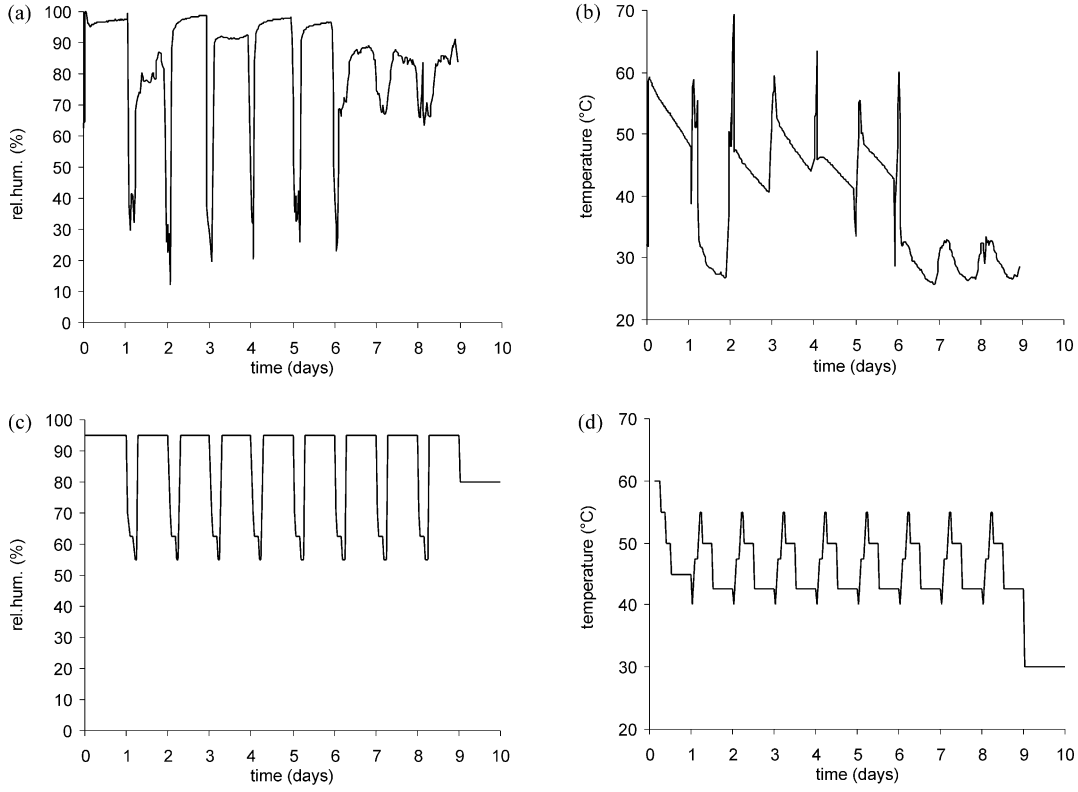

Fig. 1. (a) relative humidity during vanilla bean curing in Djasula Wangi curing station, Bali; (b) temperature during vanilla bean curing in Djasula Wangi curing station, Bali; (c) relative humidity during model curing; (d) temperature during model curing.

Table 1
Curing scheme

Stage	Temp (°C)	RH (%)	Time
Scalding	70	–	1.5 min
Autoclaving	60	95	3 h
	55	95	3 h
	50	95	3 h
	45	95	12 h
	40	70	1 h
Sunning (7–8 times)	47.5	62.5	3 h
	55	55	2 h
Sweating (7–8 times)	50	95	6 h
	42.5	95	12 h
Slow drying	30	80	3 weeks

(β-Glu), peroxidase (PER) and protease (PROT). For the analysis of the phenylalanine ammonia lyase (PAL) activity, samples were extracted with BTP buffer at pH 6. Extraction was performed by adding buffer (with 2 mM EDTA and 3 mM DTT) to beans cut into 1-cm pieces, followed by addition of PVPP. The mixture was ground in a Waring blender (Dignum et al., 2001b). All assays were standardised using McIlvaine buffers to make sure that the differences between assays were minimal. The enzymes were assayed at the pH optimum for the specific enzyme. The assays were performed in duplicate at 30 °C. Enzyme activities are expressed as International Units (IU)/gram dry weight (1 IU = 1 μmol/min).

β-Glucosidase assay was according to Luijendijk, Stevens, and Verpoorte (1998). To 25 μl of enzyme extract, 475 μl of 2.1 mM *p*-nitrophenyl-β-D-glucopyranoside in McIlvaine buffer pH 7.0 were added. After approximately 30 min 800 μl of 1 M sodium carbonate were added and the absorbance was read at 400 nm.

The activity was calculated using $\varepsilon_{400nm} = 18{,}500$ $M^{-1}cm^{-1}$.

Phenylalanine ammonia lyase was assayed according to Moreno, Poulsen, van der Heijden, and Verpoorte (1996). To 200 μl of enzyme extract, 400 μl of 43 mM phenylalanine in McIlvaine buffer pH 8.0 were added. The mixture was incubated for 2 h and the reaction stopped by adding 50 μl of 5 M TCA. Samples were analysed on an HPLC (water/methanol/acetic acid: 50/50/1, 275 nm) using a Phenomenex ODS-Hypersil column. For calibration a standard curve was prepared of 0–1 mM *t*-cinnamic acid.

Peroxidase activity was determined by using guaiacol as substrate (Maehly & Chance, 1954). To a cuvette 0.6 ml of 1.5 mM H_2O_2 in McIlvaine buffer pH 5.0 and 0.3 ml of 40 mM guaiacol were added. The reaction was started by adding 20 μl of enzyme extract. Absorbance was read at 470 nm after exactly 3 min. Peroxidase activity was calculated from the amount of tetraguaiacol formed using $\epsilon_{470nm} = 26{,}600$ $M^{-1}cm^{-1}$.

To determine the protease activity, the procedure of Boehringer Mannheim was used. Fifty microliters of Universal protease substrate (resorufin-labelled casein, Boehringer Mannheim No. 1734 334; 4 mg/ml), 50 μl of McIlvaine buffer pH 7.0 and 100 μl of sample were mixed and incubated for a suitable amount of time. The reaction was stopped by adding 480 μl of 5% w/v TCA. After 10 min of incubation, the samples were centrifuged for 5 min. The supernatant (400 μl) was transferred into a cuvette and 600 μl of 0.5 M Tris pH 8.8 were added and mixed well. Absorbance was read at 574 nm. Protease activity was calculated from the amount of resorufin-labeled peptides released using $\epsilon_{574nm} = 66{,}000$ $M^{-1}cm^{-1}$.

2.3. Protein determination

The protein concentration was determined in triplicate by using the assay according to Bradford (1976), modified accordingly for use in microtiter plates. Bovine serum albumine (BSA) was used as a standard for plotting the calibration curve.

2.4. Aroma analysis

Beans were frozen in liquid nitrogen, and ground in a blender to a fine powder. Fifty grams of sample were suspended in 200 ml of water (pH 4) and stirred for 10 min. Internal standard (100 μl octanal) was added and 100 ml organic solvent to extract the volatiles (pentane:dichloromethane 60:40). This mixture was stirred for half an hour. The organic layer was separated, dried over Na_2SO_4 and concentrated by high vacuum distillation. The extract was then analysed on a GC [Interscience GC8000 (Breda, the Netherlands), split/splitless injector 250 °C, FID 280 °C, column HP5 50 m, 0.25 mm, 0.25 μm (Agilent, Amstelveen, the Netherlands)] with a temperature programme 3 °C/min from 40 to 100 °C, followed by 6 °C/min to 300 °C. Injection volume was 1 μl splitless. As the carrier gas, helium is used at a flow rate of 15.4 cm/s. The results were calculated by the use of an external standard (calibration curve of compound to be measured). The internal standard (octanal) was used for determination of extraction efficiency.

2.5. Glucoside analysis

To 5 g of the ground material from aroma analysis in a volumetric flask of 100 ml, 65 g of a 30 mM acetate buffer pH 5 were added and mixed well for 5 min. Enzymes were inactivated by heating the suspension to

90 °C in a microwave oven. The flask was filled up to 100 ml with methanol. Extracts were analysed in duplicate on an HPLC (HP 1090, column Waters Spherisorb 5 μm ODS2 4.6×250 mm at 35 °C, PDA detection). The gradient system consisted of eluent A (3% HOAc, 1% methanol in water) and eluent B (3% HOAc, 90% methanol in water) starting with 0% B. Then B was increased to 25% in 40 min, to 60% after 60 min and to 100% after 63 min The injection volume was 25 μl and the flow was set on 1 ml/min. The concentrations of vanillin, vanillic acid, glucovanillin, glucovanillic acid and glucoguaiacol were calculated from calibration curves of the respective compounds.

3. Results

Fig. 2 shows the results of the enzyme analysis of the model curing. The initial enzyme activity for all enzymes was the highest in green beans. Upon scalding β-Glu, PER and PROT lost 30, 11 and 9% of their activities, respectively. After autoclaving however, β-Glu activity was lost completely. PROT was still present in high amounts, and PER activity was reduced by 75%. Throughout the curing process β-Glu could not be detected anymore. PROT decreased gradually in two weeks of curing and PER activity remained at the same level as after autoclaving.

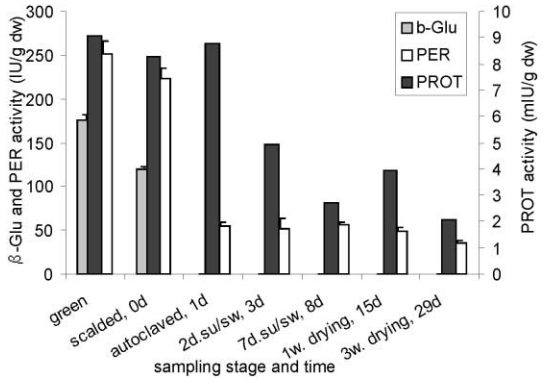

Fig. 2. β-Glucosidase, protease and peroxidase activity during model curing experiment. Green: green beans; scalded: immediately after scalding; autoclaved: after autoclaving (day 1); 2d. su/sw: after 2 days sunning/sweating (day 3); 7d. su/sw: after 7 days sunning/sweating (day 8); 1w. drying: after 1 week slow drying (day 15); 3w. drying: after 3 weeks slow drying (day 29).

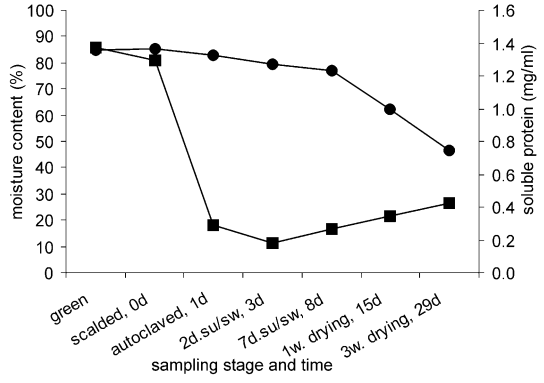

Fig. 3. Moisture content and extractable protein during model curing experiment. ● = Moisture content; ■ = protein content in extract; legend *x*-axis as in Fig. 2.

The moisture content in the beans decreased slowly during the first steps of the process (Fig. 3). Starting after one week of slow drying, the moisture content decreased. Compared to the process suggested by Tiollier (1983) this is too slow. He suggests that after sunning and sweating (2 weeks) the moisture content should be 45–50% and after drying (30 days) the final concentration of 25–30% should be reached.

The decrease in soluble protein (Fig. 3) is in agreement with the results of Ranadive et al. (1983).

The next step was to investigate the influence of the scalding temperature (Table 2). It seemed that PER activity was not much affected by the scalding method used. β-Glu on the other hand showed a complete loss of activity after scalding at 70 and 80 °C. Scalding at 50 or 60 °C was the best option to retain much of the β-Glu activity. After autoclaving, however, β-Glu activity was completely lost in all batches.

Based on these results, an experiment was done in which beans were either not scalded, or scalded at 60 °C or at 50 °C for 1.5 min. Scalding was followed by the mild curing method. The aim was to find conditions to retain as much β-Glu activity as possible during the first curing steps. The results of this experiment are shown in Table 3.

If the results of Table 3 are compared with the results of Table 2, the conclusion is that even the mild curing is not mild enough to retain β-Glu activity. Beans that were not scalded kept the β-Glu activity only in the first hours, but after 24 h of autoclaving, all β-Glu activity was lost in all cases. Again, the influence of scalding and

Table 2
Influence of scalding temperature on enzyme activities during standard curing

		β-Glu (IU/g dw)		PER (IU/g dw)	
	Green bean	89±6			45±4
Scalding temp. (°C)	Scalding time (min)	After scalding	After autoclaving	After scalding	After autoclaving
80	10	0±0	0±0	21±1	11±1
80	1.5	0±0	0±0	17±1	60±1
70	1.5	2±0	0±0	36±2	56±2
60	1.5	85±6	0±0	60±7	73±2
50	1.5	140±2	0±0	71±2	63±2

autoclaving on PER was less expressed. PER activity was even detected after autoclaving in samples that were scalded for 10 min at 80 °C.

A final longterm curing experiment was performed. In this experiment, one batch was not scalded, but was subjected to the mild curing immediately. Another batch was scalded for 20 min at 80 °C in order to try to inactivate all enzymes and was then also subjected to the mild curing. The results of these experiments are shown in Fig. 4.

The β-Glu activity decreased rapidly. For PROT and PER it is interesting to see that they even survived at 80 °C. PAL on the other hand seemed to be less stable. In non-scalded beans, this enzyme survived the autoclaving step but was not detected anymore after 3 days of sunning. In the 80 °C treated beans PAL did not survive the scalding.

The results shown in Figs. 2 and 4 show that there were no significant differences between the standard or the mild curing method.

Fig. 5 shows the soluble protein and moisture contents in the beans during curing. The soluble protein for non-scalded beans followed the same trend as the beans in Fig. 3. The protein content of scalded beans (20 min,

Table 3
Influence of scalding temperature on enzyme activities during mild curing

	β-Glu (IU/g dw)			PER (IU/g dw)		
Green bean	83±1			71±1		
Scalding temp. (°C)	After scalding	After 4 h autoclaving	After 24 h autoclaving	After scalding	After 4 h autoclaving	After 24 h autoclaving
Not scalded		4±0	0±0		79±0	74±0
60	72±1	0±0	0±0	64±4	50±0	33±1
50	78±10	0±0	0±0	69±2	61±0	49±0

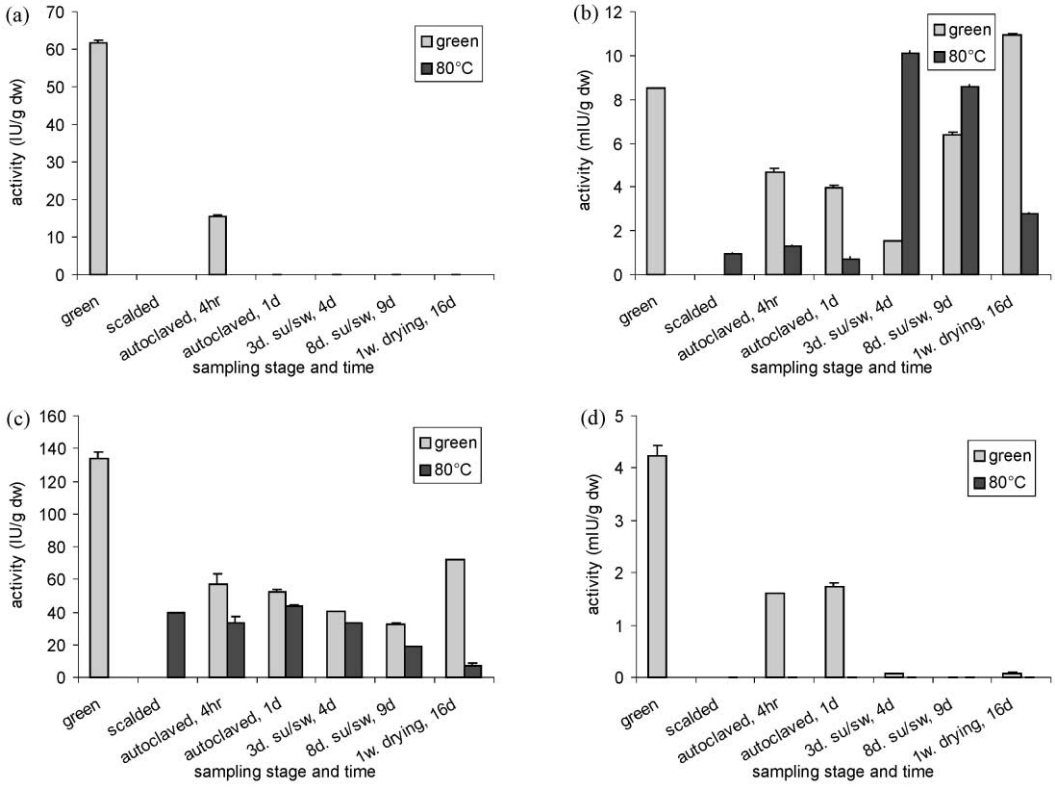

Fig. 4. β-Glucosidase (a), protease (b), peroxidase (c) and PAL (d) activity during model curing experiment (mild curing method). The series called green, were not scalded before curing. The series called 80 °C were scalded at 80 °C for 20 min. green: green beans; scalded: immediately after scalding; autoclaved, 4 hr: after 4 h of autoclaving: autoclaved, 1d: after autoclaving; 3d. su/sw: after 3 days sunning/sweating (day 4); 8 d. su/sw: after 8 days sunning/sweating (day 9); 1w. drying: after 1 week slow drying (day 16).

80 °C) is low immediately after scalding. This is in contrast to scalding for 1.5 min at 70 °C (Fig. 3) where the decrease in protein started after autoclaving.

The moisture content in non-scalded beans decreased less than that in beans scalded for 20 min at 80 °C (Fig. 5). When compared with Fig. 3 the moisture content in non-scalded beans decreased less than beans scalded for 1.5 min at 70 °C. This effect might be caused by the amount of cell damage due to scalding. In non-scalded beans all cells are intact before the curing starts, so the evaporation of water will be restricted. After scalding for 20 min at 80 °C, there will be a lot of damaged cells, which will loose water more easily.

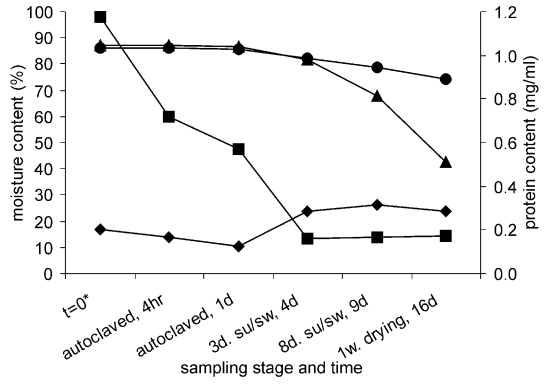

Fig. 5. Moisture content and extractable protein during model curing experiment (mild curing method). ● = Moisture content non scalded beans; ▲ = moisture content in beans scalded at 80 °C for 20 min; ■ = protein content in extract of non scalded beans; ◆ = protein content in extract of beans scalded at 80 °C for 20 min; legend *x*-axis as in Fig. 4) * $t = 0$ for non scalded beans: green beans; $t = 0$ for beans scalded at 80 °C for 20 min: after scalding.

The results of the analysis of aroma compounds (Table 4) show that for 3-methylbutanal, hexanal, phenylacetaldehyde, *p*-cresol, phenylethanol, 4-methylguaiacol and methylcinnamate the differences between non-scalded and scalded beans were less obvious. For guaiacol and glucoguaiacol, however, it is notable that their concentration was higher in scalded beans than in non-scalded beans. This could indicate that the release of aroma compounds is not strictly due to β-Glu activity. Glucovanillin decreased to form vanillin in non-scalded beans. In beans scalded for 20 min at 80 °C, however, there was only little vanillin formed and the decrease of glucovanillin was small. Vanillic acid and glucovanillic acid followed the same pattern, but the formation of vanillic acid from glucovanillic acid is slow compared to that of vanillin from glucovanillin.

4. Discussion

This research has demonstrated that β-Glu is not detectable within a few hours after scalding, in agreement with the data reported by Ranadive et al. (1983). Hanum (1997) on the other hand concluded that β-Glu is still active at least till the end of the sunning/sweating session. Another point of discussion is the β-Glu itself. In these experiments we have only looked at the non-specific β-Glu activity. Upon scalding this activity was not detectable anymore. The results suggest that there is

Table 4
Analysis of aroma compounds and glucosides at several stages in the curing process[a]

		Before autoclaving		Autoclaved, 1d[b]		3d su/sw, 4d[c]		8d su/sw, 9d[d]		1w drying, 16d[e]	
		Green	Scalded	Green	Scalded	Green	Scalded	Green	Scalded	Green	Scalded
3-Methyl-butanal	G[f]	<0.05	<0.05	0.2	<0.05	0.7	<0.05	1.1	<0.05	0.5	<0.05
Hexanal	G	2.8	<0.03	0.4	1.1	<0.03	<0.03	<0.03	<0.03	<0.03	<0.03
Phenyl-acetaldehyde	G	<0.05	<0.05	0.3	<0.05	0.4	<0.05	0.4	0.4	0.1	0.1
p-Cresol	G	<0.05	<0.05	0.2	<0.05	0.2	1.0	1.6	4.4	0.7	1.7
Phenyl-ethanol	G	<0.05	<0.05	0.7	<0.05	2.3	3.0	4.2	3.9	1.9	1.3
4-Methyl-guaiacol	G	0.3	0.3	8.9	0.3	1.4	7.3	5.4	8.2	2.3	2.5
Methyl-cinnamate	G	12	12	5.8	3.2	3.9	3.3	1.6	2.0	1.2	0.5
Guaiacol	G	<0.05	<0.05	2.9	63	48	300	96	100	39	43
Gluco-guaiacol	H	40	80	10	<10	<10	30	<10	40	<10	70
Vanillin	H	430	130	580	90	810	130	1420	170	1370	380
Gluco-vanillin	H	2400	2450	1400	1420	400	1970	190	880	180	2060
Vanillic acid	H	10	10	20	<10	30	<10	80	<10	90	20
Gluco-vanillic acid	H	200	200	160	140	110	250	150	350	160	700

[a] Samples called green were not scalded, the other samples were scalded at 80 °C for 20 min. Concentrations in ppm (dw).
[b] After autoclaving (day 1).
[c] After 3 days sunning/sweating (day 4).
[d] After 8 days sunning/sweating (day 9).
[e] After 1 week slow drying (day 16).
[f] G, analysed with GC; H, analysed with HPLC.

a β-Glu specific for glucovanillin and glucovanillic acid still present that is not detected by the non-specific assay. The release of the minor compounds seems to be via a chemical process rather than via an enzymatic one.

Kanisawa et al. (1994) have investigated β-Glu in more detail and report two β-glucosidases in vanilla: a non-specific enzyme which is present in the bean and the leaves of the plant, and an enzyme specific for glucovanillin and *p*-hydroxy benzaldehyde glucoside that was detected only in the bean. If this theory is true, this might explain the results of the aroma analysis (Table 4). After autoclaving the non-specific enzyme is inactivated. The specific enzyme could then be responsible for the release of the aroma compounds. This specific glucosidase would be inactivated when the beans are scalded for 20 min at 80 °C. This results in a large amount of glucosides still present at the end of the curing as shown in Table 4.

In this work there were still glucosides left after 16 days of curing (Table 4), similar to that reported by Ranadive et al. (1983). The latter authors found that upon addition of an external β-glucosidase the concentration of vanillin increased substantially and explained this by an unidentified enzyme inhibitor. It may be more likely that the remaining glucosides are not hydrolysed due to physical separation. Arana (1943) described that of all glucovanillin in green beans, 60–80% is present in the fleshy part of the bean. On the other hand, the β-glucosidase activity was not found in the seeds and placental tissue. If glucovanillin can not diffuse completely from the seeds to the outside of the bean to get in contact with the enzyme before the end of the curing, then there will be still glucovanillin left in the final product. Upon addition of enzyme to crushed beans, there would be further hydrolysis of glucovanillin. Consequently, the final vanilla quality depends mostly on the glucovanillin content in the green bean. This means that the quality of cured beans will be higher if the curing starts with mature beans which have the highest glucovanillin concentration. In these beans also the highest β-Glu activity is present (Arana, 1943; Jiang et al., 2000).

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