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Cacao Polyphenolic Substances

2. CHANGES DURING FERMENTATION

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(*Received* 6 *September* 1951)

Fresh cacao beans are prepared for marketing by heaping in boxes so that their covering of mucilaginous pulp can be broken down through the action of yeasts and acetic acid bacteria (Knapp, 1937). After this fermentation the beans are sun-dried. During both processes various changes take place in the cotyledons of the beans, which result in a product suitable for the manufacture of chocolate. The most striking chemical changes taking place during processing of fresh cacao beans are the alterations in the polyphenolic substances of the beans, the simpler polyphenols yielding insoluble complexes (Hallas, 1939, 1949).

From analogy with tea fermentation, it has generally been assumed (Knapp, 1937) that the initial change in the cacao bean fermentation is one of oxidation by atmospheric oxygen activated by a polyphenol oxidase system. Oxidases are certainly present in abundance (Brill, 1915; Ciferri, 1931).

In previous work in this series (Forsyth, 1949, 1952) it has been shown that the polyphenols extracted from fresh Forastero cacao with dilute acid consist mainly of catechin and cyanidin compounds. Both anthocyanins and leucoanthocyanins are present. Of the catechins, epicatechin is present in by far the greater proportion.

By quantitative paper chromatography of extracts of beans removed from the fermentation heap throughout the process, the destruction and conversion of the main polyphenols has now been followed. The changes during artificial treatments of the beans have also been investigated.

METHODS

Commercially fermented beans. Twenty beans were taken at random from a fermentation 'sweat box'. This number is required to give a representative sample. The beans were peeled and the cotyledons (about 20 g.) immediately extracted with 0·3 N-HCl as described in Part 1 (Forsyth, 1952).

Chromatographic estimation of polyphenols. The extract (0·2 ml.) was streaked across the starting line (16 cm.) of a Whatman no. 1 paper sheet 20 cm. wide. Two lanes at the side were used to spot on marker solutions of fresh bean extract. The sheets were then chromatographed with amyl alcohol-acetic acid-water for 18 hr. (Forsyth, 1949, 1952) by descending chromatography. Before drying the paper the anthocyanin pigments were cut out and extracted to pre-

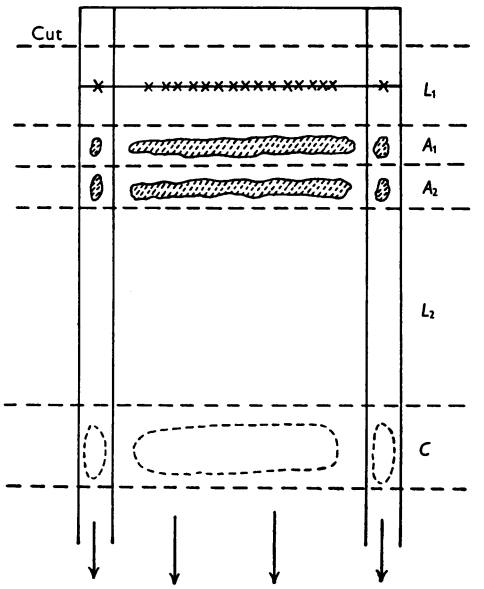

Fig. 1. Quantitative paper chromatography of 0·3 N-HCl extract of fresh cacao beans. L_1, complex leucocyanidins; L_2, leucocyanidin; A_1, cyanidin monoglucoside; A_2, cyanidin arabinoglucoside; C, epicatechin.

vent fading. The remainder of the paper was allowed to dry at room temperature. The marker strips were cut out and passed through vanillin-HCl to detect the epicatechin spot. The sheets were cut as shown in Fig. 1. In all, five components were estimated which make up the bulk of the soluble polyphenols of Forastero cacao beans.

The leucocyanidins (L_1, L_2). These compounds were not detected on the paper, but were known to be present in the selected sections of the chromatogram (Forsyth, 1952), along with traces of the other catechins. They were estimated by refluxing the paper sections for 15 min. with 10 ml. *n*-butanol and 2 ml. 10 N-HCl which converts them to cyanidin. The cyanidin solution was purified and determined colorimetrically as described by Hallas (1949). Blank paper sections gave no coloration with this treatment.

The anthocyanins (A_1, A_2). These pigments were extracted from the paper with 10 ml. methanolic HCl (0·3 N) and estimated colorimetrically (Hallas, 1949).

(−)-*Epicatechin* (*C*). The catechin was detected with vanillin-HCl and estimated by extraction with 10 ml. 1% (v/v) H_2SO_4 and titration with 0·01 N-$KMnO_4$.

As controls, fresh beans were used, extracted and chromatographed simultaneously and in an identical manner. All results are expressed as a percentage of each component as present in the fresh bean. An HCl (0·3 N) extract is stable for at least 24 hr. since, although the enzymes do not appear to be destroyed by the acid, they do not act at this low pH.

Laboratory treatment of beans for the study of oxidase activity

Grinding in air. When fresh, peeled beans are ground in a mortar and exposed to the air a rapid browning and complete disappearance of all the soluble polyphenols present takes place within 1 hr. After only 15 min. grinding over 80% of each component has been destroyed. Such a treatment is not suitable for enzyme studies. It was found, however, that similar extensive changes take place when beans are disintegrated in water, if sufficient aeration is permitted.

Grinding in aqueous solutions. Twenty fresh peeled beans were disintegrated in a Waring Blendor for 1 min. with 100 ml. water, left for 4 min., reblended for 0·5 min., left for 4·5 min., reblended for 0·5 min., left for 4·5 min., and then the acidity of the suspension made 0·3 N with conc. HCl and blended for a further minute. The final acid suspension was filtered and the polyphenols remaining determined in this filtrate by the previously described method. In this way, the beans were exposed for 15 min. to high aeration and disintegration. Boiled beans treated similarly showed no change. The pH during the blending could be made any desired value, and enzymic poisons could be introduced, to study their effect on the oxidase system. To study the effect of temperature the beans could also be blended in hot water, maintained at 50° by immersing the blender in a water bath between the short blends.

Determination of oxidase activity in whole beans. Although cacao beans contain a particularly powerful oxidase system the results from a commercial fermentation did not suggest that this system is acting in the cotyledons. This could only be due to the absence of an adequate supply of oxygen gaining access to the cotyledons during commercial fermentation. To test this, fresh pulp-free beans were fermented in anaerobic jars packed with cellulose pulp containing buffer pH 5·5 for 5 days at 48°, conditions similar to those in a sweat box but under completely sterile conditions, and in a hydrogen atmosphere.

The beans were extracted with acid in the normal manner, but the polyphenols in the extract from both peeled and unpeeled beans were estimated to determine the losses due to exudation of polyphenols, from the cotyledons to the tests.

Laboratory treatment of beans for the study of anaerobic enzyme activity

The above methods are not very suitable for a more intensive study of the enzymes since it would be difficult to obtain a disintegrated suspension of the beans without oxidation taking place. It was therefore decided to investigate whether a dry powder could be obtained containing the enzymes and substrate intact. This is feasible, since in fresh beans all the polyphenolic substances are located in the vacuoles of special isolated cells, and if the beans are washed free of pulp and dried in the sun, or dehydrated at low temperatures in a well ventilated oven, the cells are dehydrated *in situ*, and no change in the solubility of the components can be detected. The dry beans can then be ground to a fine powder without enzyme action taking place owing to their low moisture content. It was found that such a powder serves as a reasonably stable source of enzyme and substrate.

To investigate the anaerobic changes dry powder (20 g.) was submerged in water (100 ml.) sealed with liquid paraffin, for various times. The powder wetted easily and only a preliminary shake to mix was given. The suspension was then acidified to 0·3 N with HCl as before, blended for 3 min., and filtered. The polyphenols were estimated in the usual manner. The pH and temperature could be varied and enzyme poisons introduced. In no case was the catechin affected under anaerobic conditions, except where H_2O_2 was added, and the constancy of the catechin value was used as a check on the anaerobiosis. Any extract in which the catechin deviated from the control by more than 5% was discarded. Boiled beans were unaffected by this treatment.

RESULTS

In normal Trinidad practice the fresh beans are allowed to ferment in sweat boxes for from 6 to 8 days and are then removed to the drying platform for sun-drying. A typical analysis of such beans is shown in Fig. 2.

The effects of pH and of inhibitors on the changes activated by the oxidases, during blending, are shown in Table 1. When the beans were ground at 50° at pH 5·0 the oxidase system remained active, i.e. the oxidases were not to any great extent inhibited by the highest temperature and acidity reached in commercially fermented beans in the sweat box. However, when the residual epicatechin was determined in both the cotyledons and testa of whole unpeeled beans, there was no significant difference in amount between the catechin remaining in the cotyledons of beans, fermented for 5 days in the complete absence of external oxygen, and those commercially fermented. The loss of catechin (20–30%) must be due in both cases to exudation

from the cotyledons to the testa. This exuded catechin becomes partly oxidized in the testa of commercial beans but not, of course, in beans fermented under completely anaerobic conditions.

Anaerobic enzyme activity

That the dry powder used in the following experiments can serve as a source of enzymes and substrate comparable with the fresh beans is shown in Table 2. In this table is also included a test for peroxidase which is shown to be present but which does not act under anaerobic conditions unless hydrogen peroxide is added.

It will be seen in Table 2 that some changes take place in the cyanidin compounds under anaerobic conditions even at room temperature. These changes are much more marked at 48°. This temperature was chosen as being within the range of temperature prevailing in the sweat box at the time of killing of the bean. The result of incubating the powdered beans anaerobically at 48° at various pH values are shown in Figs. 3 and 4.

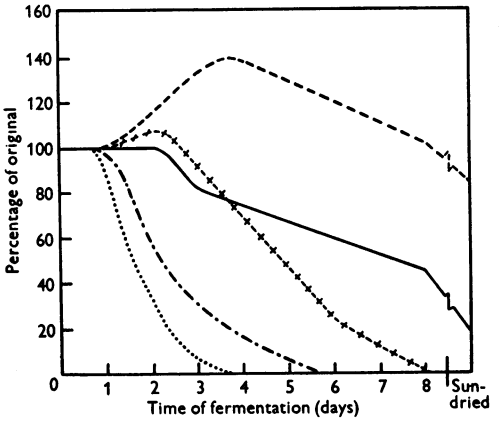

Fig. 2. Changes in soluble polyphenols during a commercial fermentation. Symbols as in Fig. 1; ....., A_1; -·-·-, A_2; ——, C; - - - -, L_1; - + - +, L_2. Sun-dried refers to beans which, after undergoing the commercial fermentation, were exposed to the sun till dry, i.e. the commercial product as shipped.

Table 1. *Oxidase activity of fresh bean homogenates in water, various buffers (MacIlvaine's) and in the presence of inhibitors*

(For conditions of blending and method of estimation see text. The inhibitor was added before disintegration of the beans. Results as percentage of original amount of each component remaining after 15 min.)

Solution	pH	Cyanidin monoglucoside	Cyanidin arabino-glucoside	Complex leuco-cyanidins	Leuco-cyanidin	Epicatechin
Water	—	<5	<5	17	<5	13
	5·5	<5	13	7	<5	18
MacIlvaine's buffer	5·0	10	8	12	<5	21
	4·5	12	10	16	10	35
	4·0	55	54	58	72	80
10^{-2}M-KCN	5·5	52	50	80	60	71
10^{-3}M-KCN	5·5	103	107	100	94	101
10^{-1}M-Ascorbic acid	5·5	72	67	101	74	102

Table 2. *Comparison between fresh beans, sun-dried beans, and sun-dried powdered beans*

(For methods of estimation see text. Results as percentage of each component in original fresh beans.)

Test for	Preliminary treatment	Cyanidin mono-glucoside	Cyanidin arabino-glucoside	Complex leuco-cyanidin	Leuco-cyanidin	Epicatechin
Substrate	Sun-dried beans	91	94	110	97	94
	Sun-dried powdered beans	108	110	92	105	101
Oxidase activity	Fresh beans, blended water for 15 min. at 25°	<5	<5	15	<5	11
	Dry powdered beans blended as with fresh	<5	<5	32	<5	21
Anaerobic enzymic activity	Fresh beans killed by freezing and incubated in water anaerobically at 25° for 18 hr.	55	78	107	97	99
	Dry powdered beans treated as with frozen beans	45	87	115	87	102
Peroxidase	Dry powdered beans incubated anaerobically as above in 0·1M-H_2O_2	33	69	59	42	58

Whole beans killed by preliminary freezing showed the same changes when incubated anaerobically as do powdered beans. The pH of 6·2 was chosen as being about the pH prevailing at the time of the death of the bean and pH 5·5 as covering the latter part of the fermentation.

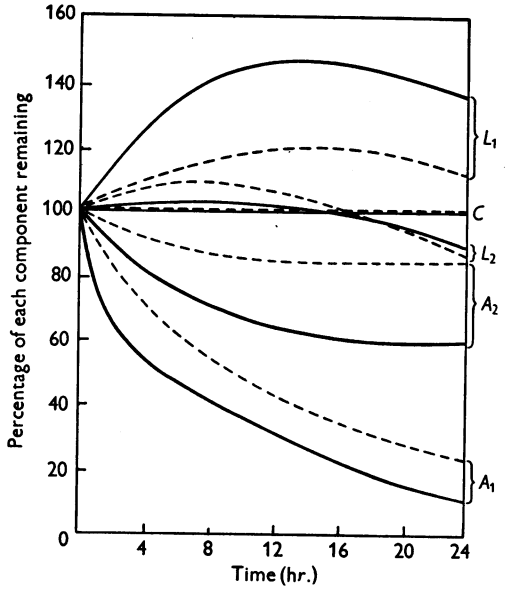

Fig. 3. Conversion of polyphenols in sun-dried powder at 48° under anaerobic conditions. Symbols as in Fig. 1; ——, pH 6·2; – – –, pH 5·5. 20 g. powder suspended in 100 ml. MacIlvaine's buffer under a paraffin seal for various times and the changes estimated as described in the text.

The effect of inhibitors on the anaerobic reactions is shown in Table 3. Sodium fluoride and copper sulphate appear to inhibit the formation of the complex leucocyanidins (L_1) while not preventing the apparent conversion of the anthocyanins to leucocyanidin (L_2).

In no case was cyanidin itself found on any of the chromatograms indicating that a simple hydrolytic mechanism was unlikely.

DISCUSSION

It has been generally assumed that the main change in the cacao polyphenols during fermentation is one of oxidation by a polyphenol oxidase system requiring external oxygen.

The beans certainly contain a particularly powerful polyphenol oxidase system capable of removing over 80 % of the total polyphenols in 15 min. when fresh beans are disintegrated and strongly aerated in buffer solutions, at the temperature and acidity prevailing in the sweat box. Peroxidase is also present. The oxidase has a high resistance to potassium cyanide in common with tea polyphenol oxidases (Lamb & Sreerangachar, 1940). However, during the commercial fermentation of the whole cacao beans no such rapid removal of the polyphenols takes place. Instead the simple cyanidin compounds are destroyed and more complex leucocyanidins are formed. The catechin is slowly removed from the cotyledons. When powdered beans are fermented under anaerobic conditions similar changes take place in the cyanidin compounds, but the catechin is completely unaffected. By fermenting whole beans under anaerobic conditions, it can be shown that the loss of catechin in commercial fermentation is due entirely to its exudation from the cotyledons to the testa.

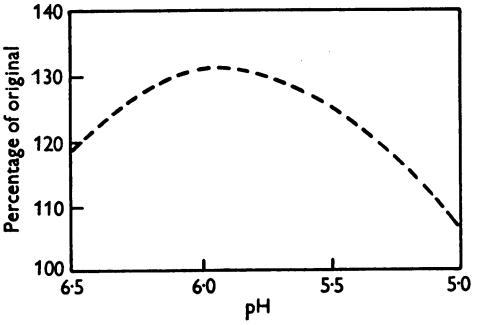

Fig. 4. The percentage of the complex leucocyanidin fraction (L_1) remaining after incubation of sun-dried powder at various pH values in MacIlvaine's buffers anaerobically for 5 hr. at 48°. (Amounts used as in Fig. 3.)

Table 3. *Effect of inhibitors on the anaerobic enzymic activity of sun-dried powdered beans*

(Powder (20 g.) was suspended in MacIlvaine's buffer pH 6·0 (100 ml.), sealed with liquid paraffin and maintained at 48° for 18 hr. then estimated as described in text.)

	Cyanidin mono-glucoside	Cyanidin arabino-glucoside	Complex leuco-cyanidins	Leuco-cyanidin
Boiled solution	92	95	100	98
Buffer	20	70	150	104
NaF (10^{-2}M)	15	64	72	130
$CuSO_4$ (10^{-2}M)	33	62	58	132
KCN (10^{-2}M)	30	67	115	96

The lack of activity of the oxidase system in the cotyledons during a commercial fermentation can only be due to a restriction in the supply of available oxygen. Such lack of oxygen is not surprising. In fermenting beans the microflora of the pulp will most certainly consume most of the oxygen introduced into the heap by ventilation. This is supported by the observation that fermenting pulp decolorizes methylene blue. Further, even in pulp-free aseptically fermented beans, the cotyledons resist oxidation. This is due to the changes which take place in the beans about the second day, when they are killed by the temperature and acidity. The free space between the cotyledons and the testa then becomes completely filled with a continuous layer of purplish juice. Paper chromatography shows that this juice contains all the soluble components of the cotyledons and that it is especially rich in epicatechin. In a commercial fermentation this juice is partly absorbed by the testa and some is even lost in the pulp and sweatings. The exudate and the testa themselves undergo browning during fermentation, but even in fully fermented beans the juice still contains considerable catechin and acts as a further barrier of oxidizable substances and prevents access of air to the cotyledons. Oxidation in the cotyledons (i.e. the part used in chocolate manufacture) only takes place during the drying period.

The significant change during fermentation would thus appear to be the conversion of the cyanidin compounds into more complex products by an enzyme system acting independently of external oxygen. This conversion has an optimum pH of about 6·0, the mean pH of the fermenting bean on the first day after the death of the bean. When the reaction is carried out in the presence of enzyme poisons the conversion of pigments to leuco compounds and the subsequent condensation of the latter can be differentiated.

It is tempting to suggest that this conversion of cyanidin compounds may be of greater importance than the previously postulated 'oxidation of tannins' in determining the flavour and aroma of the final product.

SUMMARY

1. The changes in the polyphenolic constituents of cacao cotyledons during commercial fermentation have been estimated by quantitative paper chromatography.

2. The main change is the conversion of the simple cyanidin compounds to more complex leucocyanidins.

3. Although oxidases are present they do not act in the cotyledons during fermentation due to the anaerobic conditions prevailing.

4. The catechin is partly lost by exudation and is then to some extent oxidized under the more aerobic conditions prevailing in the testa. Oxidation in the cotyledons, however, only takes place during the drying period.

5. It is possible to obtain conversion of the cyanidin compounds with sun-dried, unfermented, powdered beans, in buffers under anaerobic conditions. The reaction appears to consist of several stages.

The author is indebted to Dr A. C. Thaysen, Director, for his interest in this work, and to the Colonial Products Research Council for permission to publish.

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