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Pitch Problems in Making Japanese Papers (III) Separation and Identification of Acidic Components and Their Contribution to Speak Formation

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Acidic compounds from pokasa (*Broussonetia papyrifera*) bark methanol extractives containing fatty acids, triterpene acids and phenolics were separated and identified. Certain neutral substances were assumed to remain with the acidic fraction, as both fractions might have certain characteristics that make their separation difficult.

Fatty acids separated included a series of n-fatty acids with carbon number ranging from 10 to 22. Among them, palmitic, stearic and myristic acids were the major components. Unsaturated fatty acids, myristoleic acid, palmitoleic acid, oleic acid, and linoleic were also identified. Triterpene acid fraction is made up of a complex mixture, from which only betulinic acid and ursolic acids were identified. Among the phenolic acids, vanillic, syringic, ferulic, and sinapic acids were identified.

Each fraction of fatty acid, triterpene acid, and phenolic acid with or without the addition of neutrals was submitted to the turbidity test to examine its interaction with calcium. In cases where neutrals were not added, triterpene acids showed the highest At values. But with addition of neutrals, the phenolic acid fraction displayed the highest synergistic effect.

The $\Delta\tau$ values of the neutral fractions were also analyzed. The results show that $\Delta\tau$ values decrease in the order of triterpene alcohol, triterpene acetate, steroid alcohol, fatty alcohol, and alkanes, regardless of the presense of total acids. However, aggregation of the neutral components with calcium was highly accelerated by a mixture of fatty acids, but only weakly by the pure acids. Unsaturated fatty acids, although minute in quantities, also showed great effects in turbidity changes. On the other hand, the $\Delta\tau$ values due to pure and combined phenolic acids were not as high. When neutrals were added, however, they all showed equal enhancement. In conclusion, any combination of one or more neutrals with one or more acids demonstrated some synergistic effects. Among them, triterpene esters, phenolic acids and unsaturated fatty acids exhibited the greatest effects.

INTRODUCTION

In previous papers (Su et al., 1984,1986,) major resinous materials responsible for ink-repellent specks were considered to be the neutrals which containing a number of triterpenoids and steroids. Both the acidics and the phenolics were smaller fractions compared to the neutrals, but were shown to have be quite important in speck

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formation. In readditon tests and turbidity tests, co-existence of small amounts of acids and phenols with neutrals caused significant interactions to increse the speck formation and $\Delta\tau$ values, respectively. (Su et ***al.,*** 1984,1986). These acids and phenols were therefore, assumed to act as flocculation aids in speck formation. (Su et ***al.,*** 1986: Takayama et ***al.,*** 1984). Similar phenomena were also found in the sequences of kraft pulping and bleaching. For example, in pulping and bleaching of tropical woods'such as apiton (Yoshihara et *al.*,1963) sepetir paya (Tachibana and Sumimoto, 1980), rengas (Ohtani and Sumimoto, 1981), deposition and flocculation of neutral resins were accelerated greatly by the presence of acids or phenolic substances. In this paper, separation and identification of the acids were conducted, and through characteristics of these compounds, interactions among the acidic components, the neutrals and calcium cation are discussed.

MATERIALS AND METHODS

Extraction and Fractionation

Thirteen kg of pokasa bark chips were extracted thrice with MeOH at boiling temperature for 8 h. After evaporation, 530 g of extractives were obtained and were fractionated in a routine manner. Twenty grams of the resulting acidic components were submitted to a silica-gel column chromatography by using a developing solvent of benzene : EtOAc : HCO_2H = 50 : 4 : 1 to 5 : 4 : 1 to separate in into six fractions (frs. l-6). Their TLC chromatograms and the amounts of each fraction were shown in Fig. 1 and Table 1, respectively. Turbidity tests were run on these fractions to determine the $\Delta\tau$ values by the method described in a previous paper (Su ***et al.,*** 1986).

Further Separation of Acidic Fractions

The fr.1(1.5g) and fr.2(1.2g) were successively separated by methods of chromatography and preparative TLC. Several compounds were isolated and some of these were identified as: 1) alkanes: 0.05g of yellowish oil was obtained. Elemental analysis and IR spectra suggested that it is a mixture of n- and iso- alkanes. The GLC analyses of this fraction indicated carbon numbers ranging from 10 to 22. This was confirmed by an elemental composition of C 85.24% and H 14.65%. IR absorption peaks were cm^{-1}: 2920, 2860, 1458, 1260, 1032, 1020, 860. 2) β-sitosterol : Appeared as colorless plates with mp of 141-143°C and MS (M, 414), suggesting a molecular formula of $C_{29}H_{50}O$, which showed positive in Liebermann-Burchard test. Direct comparison of IR of the specimen with a mixed sample containing authentic β-sitosterol confirms the identity. 3) α-amyrin acetate: Obtained as a colorless plague with mp of 183-186°C, its acetate had a mp of 228-230°C and was positive in Liebermann-Burchard reaction. Mass spectrum showed m/e 426 as the molecular ion and a base peak at m/e 218. Comparison of its IR and a mixed specimens containing authentic sample confirmed its identity. Based on the aforementioned facts, both β-sitosterol and α-amyrin acetate were neutrals dissolved in the acidic mixture.

Fraction 3 : Separation of 2g of fr.3 by preparative TLC gave 1.25g of a yellowish oil for further analyses. It has IRν_{max}^{film} cm^{-1} : 3350, and 1740, This fraction showed yellow specks when sprayed with bromophenol blue on a TLC plate, indicating a mixture of caboxylic acids. Methylation of this oil with diazomethane at low temperature produced 1.40g of crystalline powders with mp of 78-80°C. GC analysis of this crystal using a Hitachi gas chromatograph 163 equipped with a FID and a temperature programmer, 2mm×2m glass columns packed with 1.5% SE-30 and 1.5% OV-17 on chromosorb WAW (100-120 mesh) were programmed from 150-300°C by 5°C/min. Calibratioin curve was prepared by using authentic samples of n-fatty acids'having carbon number of $C_{12}, C_{16}, C_{18}, C_{20}$. By comparing retention time the n-fatty acids were proven to have carbon numbers ranging from C_{10} to C,,. Percentages of the n-fatty acids to total fatty acids were determined by the peak areas which were shown in Figure 2. Moreover, GC-MS of the methly esters of these compounds were also conducted and their molecular weights ranging from 186 ($C_9H_{19}COOMe$) to 354 ($C_{21}H_{43}COOMe$) were detected. Some minor peaks which have the molecular weights of 240, 254, 266, and 268, suggested the existence of unsaturated fatty acids. Comparison of these peaks with authentic samples of myristoleic ($C_{14:1}$), palmitoleic ($C_{16:1}$), oleic ($C_{18:1}$), and linoleic acid ($C_{18:2}$) confirmed the identity of these unsaturated fatty acids.

Fraction 4 : 0.85g of this fraction was methylated and acetylated, and separated sequentially with preparative TLC and HPLC. The GC and HPLC of this fraction seemed to indicate the presence of many triterpenes. Unfortunately, only two compounds yielded enough substances to be identified. The fraction was kept thereafter only for further turbidity tests.

Compound 4-l : Appeared as colorless needles with mp above 300oC and showed positive in Liebermann-Burchard test. IRν_{max}^{film} cm-' : 3405(-OH), 3050 (-CH), 1680(C=0), 1635(C=C), 885(=CH). Comparing TLC, mixed mp, mixed IR, with authenetic sample showed its identity as betulinic acid (I).

Compound 4-2 : Appeared as colorless powder crystals with mp of 283-285°C and showed positive in Liebermann-Burchard test. IR ν_{max}^{film} cm^{-1}: 3420(-OH), 1690(C=0), 1400 (tert-dimethyl carbon). Comparing IR spectrum and mixed mp, IR with authenetic sample confirmed its identity as ursolic acid (II).

Fraction 5 : After methylation and acetylation, 1.5g of acetate methyl ester of fr. 5 was submitted to column chromatography by using a developing solvent of benzene: EtOAc=20:1 to 5 : 1. Five further fractions were obtained. Each fraction was a complicated mixture and was proceded with HPLC by eluting with the solvent of hexane : isopropanol= 100 : 1.5 to 100 : 5. Although 12 compounds could be isolated, only four compounds had enough quantities to achieve identification as follows.

Compound 5-l : Appeared as colorless needles with mp of 118-120°C. Elemental analysis and mass spectrum showed its molecular formula to be $C_{12}H_{14}O_5$.^{1}H-NMR($CDCl_3$) : 2.32(3H,$_s$), 3.76(3H,$_s$), 3.93(3H,$_s$), 6.28(1H, d, J= 16), 6.84(1H, d, J= 2.5), 6.96(1H, d, J =8.0), 7.04(1H, d, J = 16). The data suggested that this compound was ferulic acid methyl ester acetate. Comparing IR spectra with authentic sample and mixed mp confirmed the identity.

Compound 5-2 : Appeared as colorless needles with mp of 120-123°C. Elemental and MS analyses showed that the molecular formula was $C_{13}H_{16}O_6$.$_1$H-NMR($CDCl_3$): 2.36(3H,$_s$), 3.98(6H,$_s$), 3.80(3H,$_s$), 6.92(2H,$_s$), 6.84(1H,$_s$, J=16.0, 7.0), 7.3(1H, d, J=16.0). The data suggested that the compound was sinapic acid methyl ester acetate. Comparing this compound with authentic sample by IR spectra and mixed mp confirmed its identity.

Compound 5-3 : Appeared as colorless plate with mp of 186°C. Elemental analysis and MS spectrum showed that its molcular formula was $C_{11}H_{13}O_6$. ^{1}H-NMR($CDCl_3$): 2.36(3H,$_s$), 3.90(3H,$_s$), 3.94(3H,$_s$), 6.88(1H,$_s$), 7.55(1H, d, J=16.0), 7.64(1H, dd, J=12.0, 6.0). The data mentioned above were similiar to those of vanillic acid methyl ester acetate. Direct comparison of this compound with the authentic sample in IR and mixed mp confirmed the identity as such.

Compound 5-4 : Appeared as colorless needles with mp of 195°C. Elemental analysis and MS spectrum suggested a molecular formula of $C_{11}H_{13}O_6$. ^{1}H-NMR($CDCl_3$) : 2.38(3H,$_s$), 3.74(3H,$_s$), 3.90(6H,$_s$), 7.36(1H,$_s$). The data indicated this compound to be syringic acid methyl ester acetate. Comparison of IR spectra with those of authentic sample and mixed mp confirmed the identity.

Turbidity Tests of Individual Acidic Fractions and Combined Fractions

Turbidity tests were conducted as described in a previous paper (Su et al., 1986). Only that the organic fractions were replaced by various acidic fractions, acidic compounds, combined fractions in which 20% of α-amyrin were added or 20% of the total acids were added to the neutrals, respectively.

RESULTS AND DISCUSSIONS

Fractionation of Acidic Components and Turbidity Changes

Interactions among organic compounds and inorganics, especially calcium, were discussed in a previous paper (Su et al., 1986). The acids showed a more synergistic effects on turbidity change than those of the phenols. This can be assumed as a reflection of acceleration of flocculation and deposition of resin. However, flocculation or aggregation of neutrals with the aid of phenolic or acidic substance were reported to be greatly dependent on their mutual characters and the amounts of the interacting compounds. For this reason, separation of the acidic components accompanied by the turbidity tests were proceeded. First, separation was made by subjecting the acidic components (0.15% based on chips) to a silica-gel column to separate it into six fractions. Their TLC chromatograms and yields were shown in Fig. 1 and Table 1, respectively. The $\Delta\tau$ values, as an indication of the degree of flocculation of organic extractives and inorganic metallic ions, were also determined. Results shown in Table 1 indicate that acidic components with higher polarity have higher $\Delta\tau$ values.

Further Separation of Acidic Fractions

As shown in Table 1, an acidic component contribute more or less to $\Delta\tau$ values. Further separation and identification was conducted as shown in Fig. 2.

Fraction 1 and 2 : Using column chromatography and preparative TLC, several neutral compounds were isolated and identified as n- and iso-alkanes,α-amyrin and β-sitosterol. Even with repeated extraction using 3% aqueous $NaHCO_3$ with ethyl acetate in a separatory funnel, a few of the neutral components still remained in the aqueous phase. Neutral components were presumably surrounded by hydrophobic acid salts to form micells in the aqueous layer (Bergmann, 1975). In separation of the neutral components, some of the acids were invaribly contaminating the neutral part. Presumably, these acids and the neutral components in pokasa bark have certain inseparable characters. This character also has great implications in speck formation.

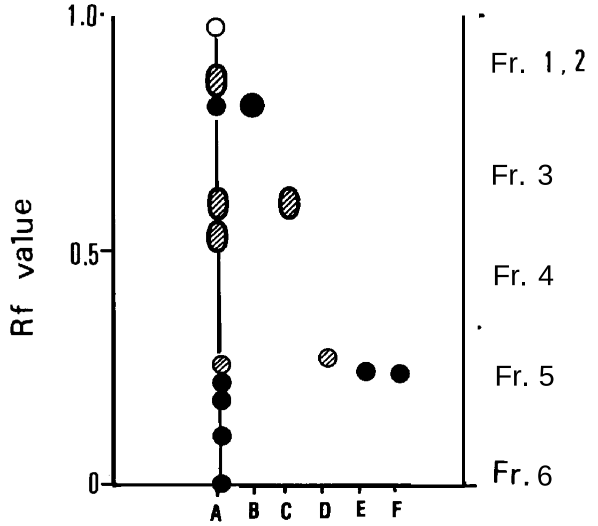


Fig. **1.** TLC chromatograms of aidic components.
Note : A : acids, B : β-sitosterol, C : fatty acids, D : vanillin acid, E : ferulic acid, F : syringic acid. Solvent ; benzen : EtOAc : HCO_2H 20 : 4 : 1

Table 1. Yields and $\Delta\tau$ values of acidic fractions.

Fractions	Yields*	( )**	$\Delta\tau$
1	0.0168	(11.6)	40
2	0.0175	(12.1)	42
3	0.0519	(36.0)	60
4	0.0324	(22.5)	80
5	0.0234	(16.2)	82
6	0.0024	(1.7)	70
Total	0.1443	(100)	40

*: % on chips, **: % on acids

Further details of the two fractions were not investigated.

Fraction 3 : In purification of fr. 3 by preparative TLC, a yellowish oil was obtained. Its IR spectrum showed absorption bands at 3350 and 1740 cm-‘, indicating it as a mixture contained carboxylic acids. Esterificaiton of this oil with diazomethane gave crystalline powders with mp of 78-80°C. The powders were subjected to GC and GC-MS analyses. Results showed that this mixture contained a series of fatty acids with carbon number ranging from 10 to 22. Based on this finding, comparison of the retention time and peak areas with the calibration curves of standard n- fatty acids of carbon number C_{12}, C_{16}, C_{18}, and C_{20} was shown in Table 2. Molecular weights of the unsaturated fatty acids were determined by GC-MS. Direct comparison of retention time of authentic samples by GC in retention times, using OV-17 and SE-30 columns,

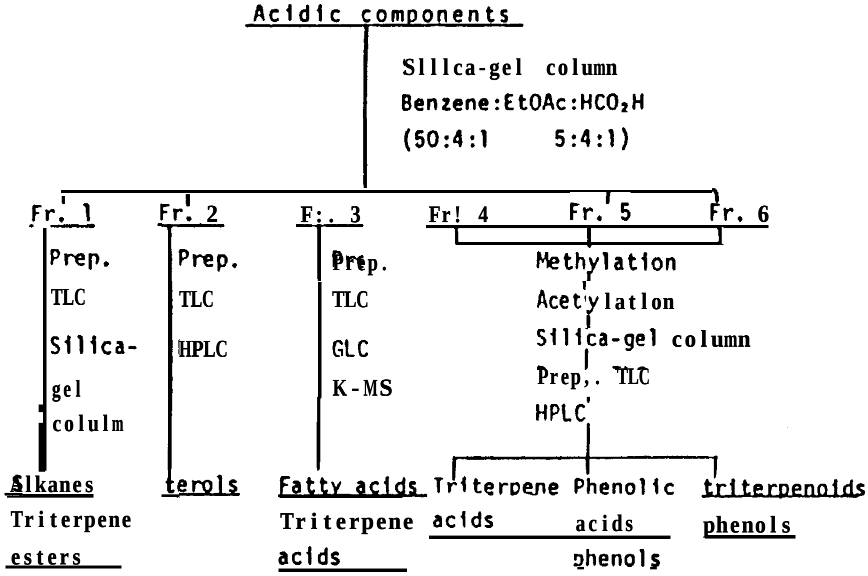


Fig. 2. Fractionation scheme of acidic components.

Table 2. Composition of fatty acids in acidic fraction.

Carbon number	Saturated	Unsaturated
10	trace	·········
12	1.42	·········
14	5.19	1.58(14:1)
16	55.43	1.72(16:1)
18	9.15	2.57(18:1) 0.70(18:2)
20	1.43	·········
22	2.52	·········

*Percentage based on fatty acids.

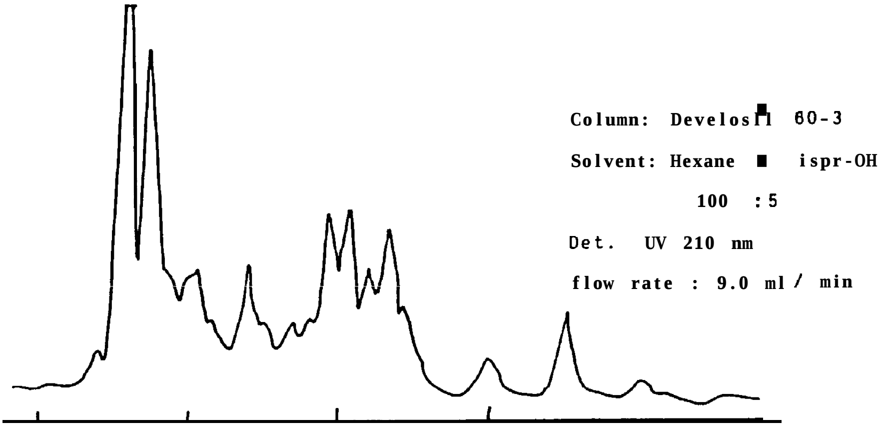
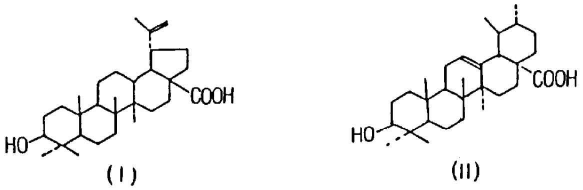


Fig. 3. HPLC chromatogram of fr 4.



(I) (II)

Fig. 4. Structure of the isolated triterpene acids.

confirmed the presence of unsaturated fatty acids with carbon number ranging from 14 to 18, namely myristoleic, palmitoleic, and linoleic acid as shown in Table 2. Among the fatty acids, palmitic acid, stearic acid, and myristic acid were the major components.

Fraction 4 : This fraction showed positive Liebermann-Burchard and Tschugaev tests at 60°C. In addition, positive bromophenol blue test confirmed that fr.4 is made up of a mixture of triterpene acids. Methylation, followed by acetylation of this fraction was made prior to purification through column chromatography, and the latter was followed by preparative HPLC. First trials of HPLC demonstrated that this fraction contained more than 20 compounds as shown in Fig. 3. The repeated separation by preparative HPLC disclosed that a representive peak shown in Fig. 3 consisted of several other compounds. Some of the pure compounds could be finally obtained, but only two compounds have enough quantities for further analyses. The rests were too small in quantities to be identified.

After the two separated pure crystals which have mps 222-224°C and 160-170°C, respectively, were hydrolyzed with potassium hydroxide, two pure crystalline compounds 4-l and 4-2 obtained.

Compound 4-l appeared as needle crystals having a mp above 300°C and showed a positive Liebermann-Burchard test. By TLC comparison, mixed specimens mp, and mixed specimens IR with authenetic sample indicated that the compound 4-l was identical to betulinic acid (I) (Takana and Ohsaki, 1975), (Liang et *al.,* 1986).

Compound 4-2 appeared as powdery crystals also has positive Liebermann-Burchard test, has mp of 182-185°C. Its IR spectrum showed absorption bands at $3240cm^{-1}$ and $1690cm^{-1}$ suggested the presence of acidic carbonyl. Direct TLC comparison, IR and mixed mp: and specimens IR showed its identity with ursolic acid (II) (Yang et al., 1971), (Liang et *al.,* 1986).

Fraction 5: After methylation and acetylation of fr.5, separation were carried out by column chromatography, preparative TCL, and preparative HPLC, sequentially. Several compounds were isolated but only four of them have enough quantities for further analyses. These were identified as vanillic acid methyl ester acetate, syringic acid methyl ester acetate, ferulic acid methyl ester acetate, and sinapic acid methyl ester acetate. Quantitative analysis was also made in GC by using TMS derivative of the fr.5. Results are shown in Fig. 5 and Table 3.

Interactions between Individual Fractions and Combined Fractions of Acids and Neutrals in the Turbidity Tests

Acid fractions obtained in the previous sections were purified by the method of DEAE-Sephadex ion exchange chromatography to remove the neutral or phenolic components from each acidic fraction completely. (Zinkel, 1983). Almost pure fraction of fatty acids (B), triterpene acids (C), and phenolic acids (D) were thus obtained and submitted to the turbidity tests. $\Delta\tau$ values were determined by the latter test with or without the addition of a-amyrin as a representative of the neutral components, respectively.

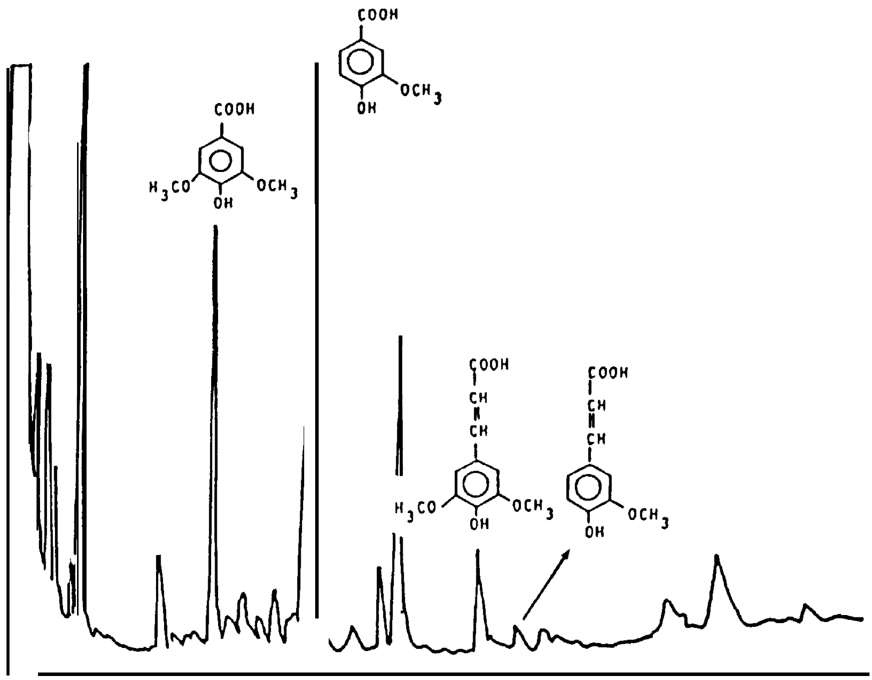


Fig. 5. GC chromatogram of phenolic acids fractions.
column : OV-17 1.5% chromosorb WAW
150-300℃, 5℃/min.

Table 3. Composition of major phenolic acids.

Substance	Content(%)
Vanillic acid	22.89
Syringic acid	9.89
Ferulic acid	2.99
Sinapic acid	0.83

% on phenolic acids.

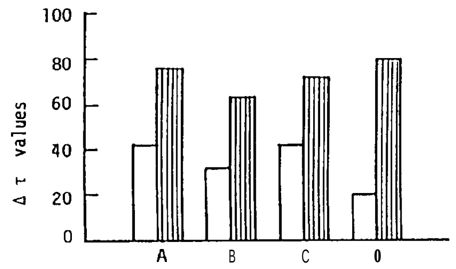


Fig. 6. A values of the acidic components.
A : total acids, B : Fatty acids,
C : triterpene acids, D : phenolic acids.
▥ represent acids + a-amyrin

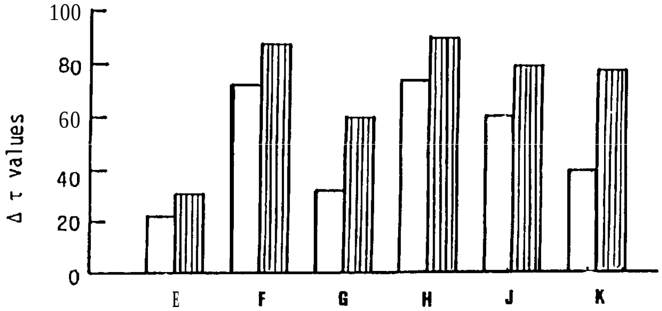


Fig. 7. A values of the acidic components.
E : alkanes, F : triterpene acetate, G : fatty alcohols,
H : triterpene alcohols, J : sterols, K : total neutrals.
▥ represents neutrals + total acids.

Among interactions due to three acidic fractions B,C,and D, the highest Az values was given by the triterpene acids (C). This was followed by fatty acids (B) and then phenolic acids (D) as shown in Fig. 6. The results without addition of a-amyrin indicated of significance of hydrophobicity of a fraction. When α-amyrin was added to the respective acid fraction, however, the phenolic acid fraction (D) demonstrated the highest synergistic effect. Total acid fraction (A) with and without a-amyrin also exhibited the high $\Delta\tau$ values almost corresponding to the latter, D with a-amyrin and to C without a-amyrin, respectively. Notably, the highly increased Az values given by both A and D with α-amyrin, respectively, are probably related to the peculiar hard to separate character between acid and neutral fractions as mentioned in section 2-2.

Neutral compounds obtained by the method mentioned in the previous paper (Su et al., 1986) were again run the turbidity test to compare the $\Delta\tau$ values. Among the six fractions as shown in Fig. 7, triterpene alcohol (H) demonstrated the highest values in pure and mixed fractions. This was followed by that of triterpene acetate fraction (F). Addition of a certain amount of total acids mentioned above to each neutral fraction increased the $\Delta\tau$ values in the order of H,F,J,K,G, and E. The order coincided with the pure fractions. However, significant differences in the $\Delta\tau$ values between aliphatic (E, G) and alicyclic compounds (F, H, J) were exhibited. Furthermore, total neutrals (K) showed the highest synergistic effect by the addition to total acid.

Further comparison of the $\Delta\tau$ values given by the pure saturated and unsaturated fatty acids with that of the parent fraction (FA) was made and shown in Fig. 8. As mentioned in Table 2, more than 90% of fatty acids are made up n-fatty acids with carbon number ranging from 12 to 22. Each of pure n-fatty acids with and without the addition of a-amyrin developed lower $\Delta\tau$ values compared to those given by the combined fatty acid fraction (FA). Generally the $\Delta\tau$ of the saturated fatty acids are larger than those of unsaturated ones.

However, each of pure unsaturated fatty acids which were small in quantity, afforded almost similar values to those by FA. Therefore, the higher $\Delta\tau$ values given by the combined fraction (FA) with and without the addition of α-amyrin could be attained only by a combination of a series of fatty acids. In other words, aggregation of the neutral components with calcium was to be highly accelerated by a mixture of fatty acids but not much by a single pure n-fatty acid, despite its larger quantity. Okagawa (1986) stated that unsaturated fatty acids and alcohol could undergo condensation at the presence of heat and saturated ones instead. This phenomena is quiet similar to the fact mentioned above, the existence of unsaturated fatty acids in combined acids fraction (FA) thus had larger $\Delta\tau$ values.

The combined phenolic acids fraction (L), ferulic acid (M), and sinapic acid (N)

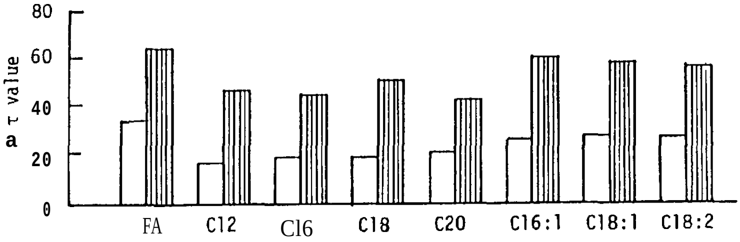


Fig. 8. A values of various saturated and unsaturated fatty acids.
☐ represents fatty acids + α-amyrin

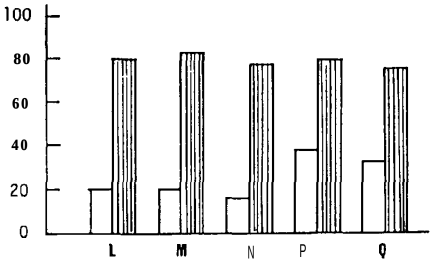


Fig. 9. A values of the phenolic acids.
L : phenolic acids, M : ferulic acid, N : sinapic acid,
P : vanillinic acid, Q : syringic acid,
☐ Phenolic acid + α-amyrin.

revealed relatively lower $\Delta\tau$ values than those of vanillic acid (P) and syringic acid (Q) as shown in Fig. 9. When a-amyrin was added to the phenolic acids fraction (L), and to pure acid (M-Q), respectively, nearly the same level of $\Delta\tau$ values were observed. Though the reason remains obscure, it may be ascribed to the characteristic properties of the phenolic acids, for example their hydrophilicity and functional groups have both phenolic hydroxyl and carboxyl in a small molecule. This concluded that any combinations of one or more neutrals with one or more acids demonstrated more or less synergistic effects. Among them, triterpene esters and phenolic acids showed the largest effects.

CONCLUSIONS

A acidic fraction of the pokasa extractives was composed mainly of fatty acids, triterpene acids, and phenolic acid. Fatty acid fraction contained mostly long chain saturated fatty acids and a small part of unsaturated fatty acids. A number of triterpene acids were found in the acid fraction of which only betulinic acid and ursolic acid were identified. The other components are too minute to be identified. Among the phenolic acid, ferulic acid, sinapic acid, vanillic acid, and syringic acid were identified.

Interaction due to the three acidic fractions are determined by turbidity test. Triterpene acids showed the highest $\Delta\tau$ values and was followed by fatty acids and then phenolic acids. These suggest the significance of hydrophobicity of the fractions. In the presence neutrals, the phenolic acid fraction, however, showed the highest synergistic effect. Among the neutrals, alicyclic compounds such as triterpenes and sterols showed greater $\Delta\tau$ values than those of the aliphatic compounds. Lower $\Delta\tau$ values were found in pure n-fatty acids as compared to either the unsturated acids or combined fatty acids. This showed that unsatured fatty acids played more important roles in flocculation and condensation.

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