CHAPTER 2

EXTRACTION AND SPECIFICATION OF Baccaurea motleyana hook f. FRUIT'S EXTRACT

2.1 Introduction

Baccaurea motleyana hook f. fruits contains lots of juice. Since we are only interested to obtain the juices, the technique used to extract it is by expression. According to Scalia et. al. (1998), the current chromatographic methods used to analyse short-chain carboxylic acids are based on gas chromatography (GC) and high performance liquid chromatography (HPLC). In this study, HPLC technique is used to identify and quantify the type of AHAs present in Baccaurea motleyana hook f. fruit's extract.

Product specification is important since it helps us to assess whether the product is stable over a period of time or any changes that have occurred is within the acceptable range as set in the product's specification. There are many factors that should be considered when setting product specifications. Among them are

- (a) Only to set specifications for product attributes which are meaningful for the particular product concerned such as those related to the sensory properties, efficacy and safety of the products.
- (b) Product specifications should be set such that natural variances which occur in the raw materials used to make the product can be accommodated.
- (c) Product specification should also be set such that the inherent process capability during manufacturing the product can be accommodated.

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(d) Any product specifications set should be measurable, either qualitatively or quantitatively.

Thus, product's specification should have a range of values. The product's specification should be set at the time of manufacturing the product. This is important to ensure that the product meets the required standards of quality. However, the product attributes are also likely to change when the product is subsequently distributed in the market place. It is therefore important for a manufacturer to set two different specifications for product's manufacturing which is more rigid and the product's shelf-life specification which is to accommodate the likely changes in the product during its shelf-life; but not to jeopardise the product's safety, functionality and quality. In this study, Baccaurea *motleyana hook f.* fruit's extract's specification is determined for consistency of the extract used throughout the study.

2.1.1 Quality Assessment of *Baccaurea motleyana hook f.* Fruit's Extract's

It is usually difficult to maintain the properties of any plants or fruits extracts since they are easily attacked by microrganismns, oxidised and become unstable. This might change the extract physical and chemical properties such as its colour, pH, viscosity and appearance. Therefore, the extract need to be preserved and a stability study need to be performed.

2.1.2 Objective of the Study

The objective of this study is

 to identify and quantify the types of AHAs in *Baccaurea molleyana hook f.* fruit's extract.

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(2) to study the stability of Baccaurea motleyana hook f. fruit's extract under different stability conditions and prepare its specification.

2.2 Materials and Method

2.2.1 Preparation of *Baccaurea motleyana hook f.* Fruit's Extract Specification

Baccaurea motleyana hook. f fruits used are from Jelebu, Negeri Sembilan. Its extract is obtained by pressing the washed full-ripe fruits using a pressing machine. The fruits are manually washed a few times with tap water until the soaked water is clean. The fruits were then poured onto the filter tray to remove the water and were left dried at room temperature (30-33°C) for about 20 – 30 minutes. Then the fruits were tied in a cloth bag where they were later put in the pressing machine. These fruits are then manually squeezed by tightening the pressing machine handle until all the extract was extracted out. This whole process took about 4 hours to produce about 30 kg of the Baccaurea motleyana hook. f fruit's extract from about 60 kg of Baccaurea motleyana hook. f fruits. The photographs of the extraction technique were shown in Figure 2.1.

The extract was then stored in a freezer (-10°C). It was thawed at room temperature overnight to let it melted and later filtered using Whatman filter paper No. 52 (7 microns) to filter any small particles present in the extracts during extraction process. The filtered extract was preserved with Euxyl K100 which is a commercial preservative and kept at 4°C. The filtered *Baccaurea molleyana hook f.* fruit's extract are further analysed for its specification development. The types of analysis and instrument used to analyse the extract are shown in Table 2.1. The extract's viscosity is measured at RT condition using Brookfield Viscometer, Spindle LV1 at 6 rpm, 12 rpm, 30 rpm and 60 rpm.



PRESSING MACHINE



RAMBAI FRUITS IN PRESSING MACHINE BOWL



RAMBAI FRUITS AFTER BEING PRESSED



RAMBAI EXTRACTS -FILTERED & UNFILTERED

Figure 2.1: Extraction of Baccaurea motleyana hook f. fruit's extract

No.	Type of Analysis	Instrument
1.	Appearance	Visual Observation
2.	pH	Mettler Toledo 320 pH Meter
3.	Colour	Minolta Colour Spectrophotometer CN-3500d
4.	Density	SG Bottle, Volumetric 25 cm ³
5.	Specific Gravity	Hydrometer, 1.0 – 1.2 gm/ml
6.	wt/wt% Dry Content	Mettler Toledo HG53 Halogen Moisture Analyser
7.	Clarity	Visual Observation
8.	Viscosity	Brookfield Dial Viscometer, Model LVF, Spindle LV1
9.	Fruit Acids Content	High Performance Liquid Chromatography, SHIMADZU LC 10- AS
10.	Total Acidity	Metrohm 716 DMS Titrino Detector

Table 2.1: T	ypes of	analysis	and	instrument	used
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2.2.2 Identification and Quantification of AHAs in Baccaurea motleyana hook f. Fruit's Extract by HPLC

(a) Reagents

The reagents used as standards to identify AHAs compounds in *Baccaurea motleyana hook f* fruit's extract were presented in Table 2.2 below. Water was purified by Elgastat Maxima-HPLC (Elga Ltd., U.K.).

No	Reagents	Purities/Grade	Origin
1	DL-Malic acid	≥ 99.5%	Merck, Germany.
2	Oxalic acid dihydrate	> 99.5%	Merck, Germany.
3	L(+)-Tartaric acid	Min. 98%	Sigmaultra, Sigma, U.S.A.
4	Glycolic acid	Min. 98%	Sigmaultra, Sigma, U.S.A.
5	Citric acid	AR Grade, Assay 99 - 102%	Ajax Lab Chemicals, Australia.
6	Perchloric acid, HClO ₄	HPLC Grade	BDH Laboratory Supplies, UK.
7	Bromothymol Blue (BTB)	HPLC Grade	Ajax Lab Chemicals, Australia.
8	Methanol	HPLC Grade	BDH Laboratory Supplies, UK.
9	Sodium Hydroxide, NaOH	A.C.S. Reagent, 98.7%	J.T.Baker, U.S.
10	Di-Sodium Hydrogen Phosphate, Na ₂ HPO ₄	DAC, USP, 99.25%	Merck, Germany.

Table 2.2 : Reagents used to identify AHAs compound in *Baccaurea* motleyana hook f. fruit's extract

(b) HPLC Analysis Method

The HPLC apparatus consists of a modular SHIMADZU chromatographic system (LC-10AS Liquid Chromatograph (Pump A) and LC-10AS Liquid Chromatograph (Pump B) linked to an injection with a 50 μ L sample loop. The wavelength for the UV/VIS Spectrofluorometric detector (Model: SPD-10A) was set at 439 nm. Data acquisition and processing were accomplished with an interface CBM-10A communicator bus module and Shimadzu Class LC10 software, using Post Column Detection Method for organic acid analysis (Shimadzu, Japan). Sample injections were effected with a Model SIL-10A autoinjector.

Separations were performed on a Shodex RSpak KC-811column (8mm I.D. x 300 mm Length, Showa Denko K.K., Japan) fitted with a guard column, Shodex RSpak KC-G (6 mm I.D. x 50 mm Length, Showa Denko K.K., Japan) and eluted with 3 mM HClO4. The mobile phase was filtered through 0.45 µm membrane filter (Millipore, Bedford, USA) and deaerated on-line by an automatic solvent degasser (Model : DGU-14A, Shimadzu, Japan). The column temperature was maintained at 40°C using a column oven (Model : CTO-10A Shimadzu, Japan).

Chromatography analysis was performed under isocratic conditions at a flow rate of 0.8 ml/min. The identity of the AHAs compounds in *Baccaurea motleyana hook f.* fruit's extract will be determined by comparing the chromatography peak obtained in the extract with the standards chromatography peak. Quantification of the AHAs compound present in the extract was carried out by integration of the peak areas using the external standardization method and quantification by Least Squares Method. This organic acids analysis system by HPLC using a pH indicator Bromothymol Blue were also used by Tanemura *et. al.* (1994) and Raof *et. al.* (1999) in anaerobic treatment of wastewater that contains volatile fatty acids.

(c) Sample and Standards Solution Preparations

All the samples were weight using Satorious Analytical Balance and make up to the desired amount (ml) with water taken from Elgastat Maxima – HPLC (Elga Ltd., UK) instrument and mixed homogenously in a volumetric flask. The volume is calculated in

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mg/L. For this study, the standards prepared were about 5000 mg/L. If dilution of the solution is needed, then it will be calculated by using the standard dilution method.

(d) Identification and Estimation of AHAs Compound in Baccaurea motleyana hook f. Fruit's Extract

A few analysis were performed to identify types of AHAs compounds present in *Baccaurea motleyana hook f*. fruit's extract. The results obtained are confirmed by spike test analysis using the presumed identified standard compounds. The *Baccaurea motleyana hook f*. fruit's extract is homogenously mixed with the standard compound in the ratio of 1:1. The result acquired will be used as a rough estimation to prepare the linear calibration curve graph for actual quantification of AHAs compounds in the extract.

(e) Quantification of AHAs Compounds in *Baccaurea motleyana hook f.* Fruit's Extract

A linear calibration curve analyses with at least three points need to be plotted for a more accurate quantification of AHAs compounds in *Baccaurea motleyana hook f.* fruit's extract. Most analytical methods require a calibration step in which standards containing known amounts of analyte (x) are used to quantify the presence of the same analyte in the samples used to be identified. For this method, the experimental quantity measured (y) is plotted against x to give a calibration curve, Figure 2.2, which typically approximate a straight line.

In this study, the Least-Squares Method is used to generate this line. In order to used this method, two assumptions are made as follows:

(a) A linear relationship does exist between the amount of analyte (x) analysed with the magnitude of the measured variable, that is y = mx + c

whereby m is the slope of the line and c is y value when x is zero.

(b) Any deviation of individual points from a straight line is entirely the consequence of undetermined error in the measurement of the variable (y), that is no significant error exists in the composition of the standards.



Figure 2.2: Calibration curve for least squares method determination

A standard calibration curve that consist of three acid concentrations (three points) were then generated by using the known amount of standards solution identified present in the extract so that the estimated amount of AHAs compound present in the extract will fall within this calibration range.

2.2.3 Stability Study of Baccaurea motleyana hook f. Fruit's Extract

In this study, *Baccaurea motleyana hook f.* fruit's extract is subjected to three stability conditions, namely at room temperature (RT), 4°C and freeze-thaw. The extract's samples were kept in clear glass universal bottle covered with aluminium foil for 5

weeks and kept at RT and at 4°C; whilst for freeze-thaw test, the extract was subjected until 5 cycles were completed. Freeze-thaw test is a condition where the extract is placed into a freezer and allowed to freeze for 24 hours and then re-equilibrated to room temperature (thaw) for another 24 hours before any product attributes are measured. This operation is known as one (1) freeze-thaw cycle. The successful completion of 5 freeze-thaw cycles without physical product separation or degradation normally indicates that the product is stable (Knowlton, 1996e).

For stability testing condition at RT and at 4°C, the extract's samples were taken out each week to be analysed for its pH, total acidity content and colour. For freeze-thaw stability testing condition, the extract's samples were analysed after completion of 5 freeze-thaw cycles. The analysis carried out were pH, total acidity content and colour. For the assessment of *Baccaurea molleyana hook f.* fruit's extract, the analysis carried out are wt/wt% dry content, clarity, colour, pH, viscosity, density, specific gravity, total acidity and fruit acids content. All the test samples were prepared triplicate. The results presented for the extract's specification were taken from the lowest to the highest value obtained whereas the stability data result presented were averaged of triplicate data obtained.

2.3 Result and Discussions

- 2.3.1 Identification and Quantification Types of AHAs in *Baccaurea motleyana hook f*. Fruit's Extract
- 2.3.1 (i) Identification Types of AHAs in *Baccaurea* motleyana hook f. Fruit's Extract

The standards chromatogram of AHAs compounds used in this study was shown in Figure 2.3. A few analyses conducted showed that *Baccaurea motleyana hook f.* fruit's extract contains two types of AHAs that is tartaric acid and malic acid. The chromatogram presented in Figure 2.4 below clearly showed an overlapping of the tartaric acid and malic acid when the analysis is carried out for the standards together with *Baccaurea motleyana hook f.* fruit's extract.



Figure 2.3: Standard AHAs compounds



Figure 2.4: Standard AHAs compounds and *Baccaurea motleyana hook f.* fruit's extract sample

The spike test that contains L(+)-Tartaric and DL-Malic acid has been carried out to verify the AHAs compounds present in *Baccaurea motleyana hook f*. fruit's extract. Figure 2.5 below confirms the presence of these two acids in the extract.



Figure 2.5: Spike test - L(+)-tartaric and DL-malic acid standards with *Baccaurea motleyana hook f.* fruit's extract sample

2.3.1 (ii) Quantification of AHAs Compounds in *Baccaurea* motleyana hook f. Fruit's Extract

Baccaurea motleyana hook f. fruit's extract samples were analysed triplicates and the chromatogram areas obtained were averaged and calculated, Table 2.3. An estimation of L(+)-tartaric acid and DL-malic acid in *Baccaurea motleyana hook f.* fruit's extract was then calculated to set-up a standard calibration curve. An average of the triplicate values were also taken and calculated as follows.

No	Types of Acid	Identity	Area (Average)	Amount (Average)
1	L(+) – Tartaric Acid, T _{std}	Standard	10542881	507.6 mg/L
2	DL - Malic Acid, M _{std}	Standard	11656276	502.8 mg/L
3	Baccaurea motleyana hook f. fruit's extract	Sample		
	L(+)-Tartaric Acid, Ts	(20x D _f)	13752253	Unknown
	DL-Malic Acid, Ms		418236	Unknown

Table 2.3: Estimation of L(+)-tartaric acid and DL-malic acid in Baccaurea motleyana hook f. fruit's extract samples

Notes: $20x D_f$ means 20 times dilution factor. D_f means dilution factor.

(a) For L(+)-Tartaric Acid

 T_{std} area, 1054288 = 507.6 mg/L

So, T_s area, 13752253 = (13752253/10542881) x 507.6 mg/L

= 662.1192 mg/L

Considering the Baccaurea motleyana hook f. fruit's extract being diluted 20 times, (Df

= 20 x), thus the actual T_s value is 13242.3834 mg/L (1.32%).

(b) For DL-Malic Acid

M_{std} area, 11656276 = 502.8 mg/L

 M_s area, 418236 = (418236/11656276) x 502.8 mg/L

= 18.041 mg/L

With the *Baccaurea motleyana hook f*. fruit's extract being diluted 20 times, $(D_f = 20 x)$, the actual M_s value is 360.82 mg/L (0.036%).

From this calculation, the quantity of L(+)-Tartaric acid, T_s and DL-Malic acid, M_s in *Baccaurea motleyana hook f.* fruit's extract is about 13242.3834 mg/L (1.32%) and 360.82 mg/L (0.036%) respectively. A standard calibration curve with three acids' concentration points were set up with selected acid concentration for L(+)-Tartaric and DL-Malic acid standard selected are 200 mg/L, 400 mg/L, 800 mg/L and 10 mg/L, 20 mg/L, 40 mg/L, 40 mg/L. The results obtained were shown in Table 2.4.

Table 2.4: L(+)-tartaric acid and DL-malic acid concentrations with area

L(+)-Tartaric Acid		DL-Mali	Remark	
Concentration (mg/L)	Area	Concentration (mg/L)	Area	
200	3055858	10	154622	STD1
400	5737006	20	319621	STD2
800	10525577	40	694282	STD3

STD1, STD2 and STD3 is a mixture of the L(+)-Tartaric acid/DL-Malic acid with the concentration of 200(mg/L)/10(mg/L), 400(mg/L)/20(mg/L) and 800(mg/L)/40(mg/L) respectively. The area of the chromatogram increase almost twice with the double increase in the acid's concentration for L(+)-Tartaric acid and slightly more for DL-Malic acid. The chromatogram for the standard calibration curve for these two acids were shown in Figure 2.6 and Figure 2.7 whilst for quantification of *Baccaurea motlevana hook f.* fruit's extract is shown in Figure 2.8.

Three analyses were carried out to determine the amount of L(+)-tartaric and DL-malic acid in *Baccaurea motleyana hook f*. fruit's extract using this standard calibration curve. An average of the triplicate values obtained showed that L(+)-tartaric acid concentration in the extract is 17,058.9707 mg/L (1.71%) whilst for DL-malic acid is 261.1419 mg/L (0.026%). This value is found to be slightly higher than those estimated value obtained by using one standard calibration.



Figure 2.6: L(+)-tartaric acid standard calibration curve



Figure 2.7: DL-malic acid standard calibration curve



Figure 2.8: Quantification for Baccaurea motleyana hook f. fruit's extract

2.3.2 Specification and Stability Study of *Baccaurea* motleyana hook f. Fruit's Extract

2.3.2 (i) Specification of *Baccaurea motleyana hook f.* Fruit's Extract

The specification of *Baccaurea motleyana hook f*. fruit's extract is presented in Table 2.5 below. The *Baccaurea motleyana hook f*. fruit's extract obtained is watery, with the viscosity in the range of 3 to 5 cps. Even though the pH, density and specific gravity of the filtered and unfiltered *Baccaurea motleyana hook f*. fruit's extract is quite similar, but the percentage of its dry content, clarity and colour is slightly different. The unfiltered extract is cloudy and has darker colour as compared to the filtered one. This is probably due to the existing fibres in the crude extract that coagulate with time, forming larger and longer fibres that finally cause the cloudiness of the extract.

No.	Types of Analysis	Result
1.	Appearance	Watery
2.	%wt/wt of Dry Content (a) Filtered Rambai Extract (b) Unfiltered Rambai Extract	16wt/wt% (14wt/wt% - 18wt/wt%) 17.38wt/wt% (15.75wt/wt% - 19wt/wt%)
3.	Clarity (a) Filtered Rambai (b) Unfiltered Rambai	Transparent Cloudy with some brown sediments settle at the bottom of the container.
4.	Colour (a) Filtered Rambai By observation Colour Spectrophotometer (b) Unfiltered Rambai By observation Colour Spectrophotometer	Mild Yellowish L^* : 95.42 (91 – 95.5) a^* : -0.02 ((-0.01) – (-0.08)) b^* : 11.02 ((10.9 – 12.2)) Mild Brownish L^* : 84.5 (80 – 89) a^* : 1.13 (0.5 – 1.75)
5.	pH (a) Filtered Rambai (b) Unfiltered Rambai	b*: 19 (18 – 20) 2.95 (2.9 – 3.2) 2.97 (2.9 – 3.2)
6.	(a) Filtered Rambai (b) Unfiltered Rambai	4 (3 - 5 cps) 5 (4 - 6 cps)
7.	Specific Gravity (S.G.) - Filtered Rambai Extract - Unfiltered Rambai Extract	1.057 (1.052 – 1.062) 1.06 (1.058 – 1.062)
8.	Total Acidity (mg KOH/gm) - Filtered Rambai Extract - Unfiltered Rambai Extract	6.665 (6.550 - 6.665) 6.651 (6.579 - 6.723)
9.	Fruit Acids Content - L(+)-Tartaric Acid - DL-Malic Acid	1.71% (1.69% - 1.75%) 0.026% (0.024% - 0.028%)
10.	Density (gm/cm ³) - Filtered Rambai Extract - Unfiltered Rambai Extract	1.034 (1.030 – 1.037) 1.039 (1.038 – 1.040)

Figure 2.9 below showed filtered and unfiltered *Baccaurea motleyana hook f.* fruit's extract. The filtered *Baccaurea motleyana hook f.* fruit's extract is used in the moisturiser product development work since it has better clarity and has lighter colour. The total acidity value acquired for the extract is quite low. This is probably due to the full-ripe rambai fruits used in the study which contains more sugar as compared to those

rambai fruits which are not fully ripen and taste more sour. Further work should be explored to concentrate the extract's acquired so as to increase the total acidity value of the extract. There is also a possibility of acquiring the extract from the fruit's skin since the skin of the fruit also taste sour.



Figure 2.9: Filtered and Unfiltered Baccaurea motleyana hook f. fruit's extract

2.3.2 (ii) Stability Study of *Baccaurea motleyana hook f*. Fruit's Extract

The stability tests carried out were at RT, 4°C and at freeze-thaw conditions. The results for each of the analysis carried out were as follows:

(a) pH

There were no significant changes for the pH value for all of the extract's kept in different stability conditions within the period of stability testing. At the beginning of the stability testing period (Wk 0), the pH value for the extract was 2.95 and this pH value slightly decrease towards the 5 weeks of stability testing period to 2.90 at RT condition and to pH 2.91 at 4°C, Table 2.6. For the extracts kept at freeze-thaw stability testing condition, the pH value is 2.93 after completion of 5 cycles which is quite similar to the extract's pH before the test was conducted.

This result showed that the pH value of the extract is stable when kept at all stability testing conditions within the period of stability testing. The Baccaurea motleyana hook f. fruit's extract contains fruit acids, namely tartaric acid and malic acid which are weak acids. These weak acids are not fully dissociated in water. Their reactions are reversible and the low pH value help to stabilise the extract.

Table 2.6: pH profile for *Baccaurea motleyana hook f.* fruit's extract at different stability testing conditions

Week	0	1	2	3	4	5
RT	2.95	2.95	2.95	2.91	2.89	2.90
4°C	2.95	2.95	2.96	2.93	2.89	2.91
Freeze-thaw	2.95	After completion of 5 cycles: 2.93				

(b) Colour

There are no significant colour changes for the *Baccaurea motleyana hook f*. fruit's extract kept at freeze-thaw, RT and at 4°C conditions when analysed with Minolta Colour Spectrophotometer. Although the L* value of the extract kept at 4°C and RT are slightly decreased within 5 weeks period of stability testing, but the change is very small and insignificant, Table 2.7. The slight reduction in L* value of the extract means

Table 2.7: Colour profile (L* value) for *Baccaurea motleyana hook f.* fruit's extract at different stability testing conditions

Week	0	1	2	3	4	5	
RT	95.42	95.32	95.30	95.24	94.41	94.29	
4°C	95.42	95.36	95.32	95.29	95.29	94.99	
Freeze-thaw	95.42	After completion of 5 cycles: 95.41					

the colour of the extract is slightly darker. By visual observations, all the extracts' samples kept at all different stability testing conditions were still mild yellowish in colour. It can be concluded that the colour of the extract is stable throughout the stability testing period conducted.

(c) Viscosity

The viscosity of the extract is in the range of 3–5 cps which is close to water. The extract behaves like a Newtonian fluid since its viscosity is similar when measured at different spindle speed. Since there were no changes observed on the viscosity of the extract throughout the period of stability study for all the extracts kept at different stability conditions, we can conclude that it was stable throughout the period of stability testing, Table 2.8.

Table 2.8: Viscosity profile for *Baccaurea motleyana hook f.* fruit's extract at different stability testing conditions

Week	0	1	2	3	4	5
RT	4	5	4	3	4	4
4°C	4	5	4	4	3	4
Freeze-thaw	4	After completion of 5 cycles: 5				

(d) Physical Separation

There was no physical separation of the extract observed throughout 5 weeks of stability testing period for all the extracts kept at different stability conditions, showed that they were all stable.

(e) Total Acidity

The total acidity value obtained at the end of the stability testing period for all of the extracts kept at different stability testing conditions were quite similar when compared to the value obtained at the beginning of the stability test, Table 2.9. The difference in the acidity value observed is very small and insignificant which showed that all the extracts were stable.

Week	0	1	2	3	4	5
RT	6.665	6.563	6.644	6.582	6.533	6.499
4°C	6.665	6.670	6.623	6.711	6.570	6.620
Freeze-thaw 6.665		Af	ter compl	etion of 5	cycles: 6.	670

 Table
 2.9: Total acidity profile for Baccaurea motleyana hook f.

 fruit's extract at different stability testing conditions

2.4 Conclusion

The filtered and unfiltered *Baccaurea motleyana hook f.* fruit's extract's pH, % of dry content, viscosity, density and total acidity value properties are quite similar. However, the filtered extract has better clarity and colour properties which is transparent and mild yellowish, suitable to be used in moisturiser development work. The filtered extract's pH is 2.95 and its total acidity value is 6.665 mg KOH/gm. The viscosity of the extract is close to water and it behaves like Newtonian liquid.

Baccaurea motleyana hook f. fruit's extract contains two types of alpha hydroxy acids (AHAs) that is L(+)-tartaric acid (1.71% or 17,058.9707 mg/L) and DL-malic acid (0.026% or 261.1419 mg/L). The extract is also stable when stored at 4° C, RT and freeze-thaw stability testing condition.