CODEX ALIMENTARIUS COMMISSION E

Food and Agriculture Organization of the United Nations

World Health Organization

Viale delle Terme di Caracalla, 00153 Rome, Italy - Tel: (+39) 06 57051 - E-mail: codex@fao.org - www.codexalimentarius.org

Agenda Item 3 **CX/MAS 23/42/3**

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JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING

42nd Session

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ENDORSEMENT OF METHODS OF ANALYSIS AND SAMPLING PLANS FOR PROVISIONS IN CODEX STANDARDS

1. This document contains the methods of analysis (Appendices I, II, III, IV and V) proposed by the following Committees:

- Codex Committee on Nutrition and Foods for Special Dietary Uses
    - Amend the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) to include new entry "product for young children" with the “follow-up formula”.
    - Methods for vitamin B12; total amino acids (excluding taurine and tryptophan), and tryptophan in the *Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants* (CXS 72-1981).
- Codex Committee on Spices and Culinary Herbs
    - Methods of analysis for provisions in the *Standard for Dried Roots, Rhizomes and Bulbs – Dried or Dehydrated Ginger* (CXS 343-2021)*, Standard for Dried Floral Parts – Cloves* (CXS 344-2021) and *Standard for Dried Leaves - Dried Basil* (CXS 345-2021) (standards adopted by CAC44 (2021) at Step 8, REP21/CAC44, para. 38 and Appendix III).
    - Methods of analysis for provisions in the *Standard for Dried Floral Parts – Saffron* (CXS 351-2021) and *Standard for Dried Seeds - Nutmeg* (CXS 352-2022) (standards adopted by CAC45 (2022) at Step 8, REP22/CAC45, para. 94(i)-(ii) and Appendix II).
    - Methods of analysis for provisions in the *Standard for Dried or Dehydrated Chilli Pepper and Paprika* (CXS 353-2022) (standard adopted by CAC45 at Step 5/8, REP22/CAC45 para. 94(iii) and Appendix II).
    - Methods of analysis for provisions in the draft Standard for Dried Small Cardamom, draft Standard for Spices derived from Dried Fruits and Berries (Part A - Allspice, Juniper berry and Star anise) (standards adopted by CAC45 at Step 5, REP22/CAC para. 97 and Appendix III, submitted to CAC46 (2023) for adoption at Step 8).
- FAO/WHO Coordinating Committee for Africa
    - Methods of analysis for provisions in the *Standard for Dried Meat* (CXS 350-2022)) (adopted by CAC45 at Step 8, REP22/CAC45, para. 149 and Appendix II).
- FAO/WHO Coordinating Committee for Asia
    - Methods of analysis and sampling for provisions in the draft Regional Standard for Soybean Products Fermented with Bacillus Species and draft Regional Standard for Cooked Rice Wrapped in Plant Leaves (submitted for adoption at Step 5/8 by CAC46).
- FAO/WHO Coordinating Committee for North America and Southwest Pacific
    - Methods of analysis for provisions in the draft Regional Standard for Fermented Noni Fruit Juice (submitted for adoption at Step 8 by CAC46).
    - the revised Standard Operating Procedure (SOP) for the identification of kavalactones and flavokavains in fresh and dried kava products by High Performance Thin Layer Chromatography (HPTLC) in the *Regional Standard for Kava Products for Use as a Beverage when Mixed with Water* (CXS 336R-2020) (adopted by CAC43 (2020) at Step 5/8).

CODEX COMMITTEE ON NUTRITION AND FOODS FOR SPECIAL DIETARY USES (CCNFSDU43, 2023)

***Methods of analysis for provisions in the Standard for Follow-Up Formula* (CXS 156-1987)[1]**

2. CCNFSDU43 agreed to inform CCMAS to include a new entry titled "product for young children" within the "follow-up formula" section of CXS 234.

Methods for vitamin B12; total amino acids (excluding taurine and tryptophan), and tryptophan in the Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (CXS 72-1981)[2]

3. CCNFSDU43 agreed to forward the methods of analysis for vitamin B12; total amino acids (excluding taurine and tryptophan), and tryptophan to CCMAS for in CXS 234. CCNFSDU also agreed that a note should be inserted in CXS 234 to clarify that the provisions are methods for testing total amino acids (excluding taurine and tryptophan) and tryptophan, i.e., for use according to Section 3.1.3a footnotes 3 and 4 of CXS 72.

4. CCMAS **is invited to endorse** the methods in Appendix I.

CODEX COMMITTEE ON SPICES AND CULINARY HERBS (CCSCH5[3], CCSCH6[4])

Methods of analysis for provisions in the Standard for Dried Roots, Rhizomes and Bulbs – Dried or Dehydrated ginger (CXS 343-2021), Standard for Dried Floral Parts-Cloves (CXS 344-2021), Standard for Dried Leaves - Dried Basil (CXS 345-2021), Standard for Dried Floral Parts – Saffron (CXS 351-2021), Standards for Dried Seeds – Nutmeg (CXS 352-2022), Standard for Dried or Dehydrated Chilli Pepper and Paprika (CXS 353-2022), draft Standard for Dried Small Cardamom, draft Standard for Spices Derived from Dried Fruits and Berries (Part A - Allspice, Juniper berry and Star anise)

5. CCMAS **is invited to endorse** the methods of analysis in Appendix II.

FAO/WHO COORDINATING COMMITTEE FOR AFRICA (CCAFRICA24)[5]

Methods of analysis for provisions in the Standard for Dried Meat (CXS 350-2022)

6. CCMAS **is invited to endorse** the methods of analysis in Appendix III.

FAO/WHO COORDINATING COMMITTEE FOR ASIA (CCASIA22)[6]

Methods of analysis and sampling for provisions in the draft Regional Standard for Soybean Products Fermented with Bacillus Species, draft Regional Standard for Cooked Rice Wrapped in Plant Leaves

7. CCMAS **is invited to endorse** the methods of analysis in Appendix IV.

FAO/WHO COORDINATING COMMITTEE FOR NORTH AMERICA AND SOUTHWEST PACIFIC (CCNASWP16)[7]

Methods of analysis for provisions in the draft Regional Standard for Fermented Noni Fruit Juice

8. CCNASWP15 (2019) noted that relevant methods of analysis and sampling provided by the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) shall be used. The Committee further agreed to forward the provisions for methods of analysis and sampling to CCMAS for endorsement.[8]

9. CCMAS41 (2021) did not endorse:[9]

- the AOAC 983.17 / EN 12143 / IFUMA 8 / ISO 2173 as the appropriateness of extending the methods to noni juice needed further evaluation by CCMAS; and noted the offer of IFU to do a small single or inter-laboratory study to determine its fitness for purpose in noni juice;
- the methods for the identification of scopoletin and deacetylasperulosidic acid noting that changes needed to be made to the methods to give a clear indication of the solid phase extraction separation mode needed and agreed to request CCNASWP to provide clarification.

1 REP23/NFSDU43, para. 51
2 REP23/NFSDU43, paras. 122, 123 and Appendix VI
3 REP21/SCH05 paras 36, 65, 81, 115, 149 and Appendices II, III, IV, V, VI
4 REP22/SCH06 paras 39, 59, 80, 107, 121(i) and Appendices III, IV, V, VI, VII Part A
5 REP22/AFRICA24, para. 40 (i) and Appendix IIIV
6 REP23/ASIA22, para. 50 (ii) 83 (ii) and Appendix V, VII
7 REP23/NASWP16, para. 28 (iii) 73 (i) and Appendix III, VII Part A
8 REP20/NASWP15, paras. 80, 83(ii) and Appendix II
9 REP21/MAS41, paras. 13-14.

10. CCNASWP16 (2023) considered the request from CCMAS41 and reviewed the methods of analysis including Annex A (identification of scopoletin) and B (identification of deacetylasperulosidic acid) and agreed[10] to task the Regional Coordinator of CCNASWP to work with the Members in the NASWP region to resolve outstanding issues related to methods of analysis and sampling (specifically specification of the solid-phase extraction cartridge and the HPLC method to identify scopoletin and deacetylasperulosidic acid) in order to forward methods of analysis and Annexes A and B to CCMAS42 for endorsement.

11. Further information on updated methods of analysis for the identification of scopoletin and deacetylasperulosidic acid is provided in conference room document CRD05 submitted by the Regional Coordinator for CCNASWP in collaboration with Members of the NASWP region which is available on the CCMAS42 webpage[11].

12. CCMAS **is invited to endorse** the methods of analysis in Appendix V as follows:

    i. Endorse then SPE-TLC method as provided in Annex A (updated) as Type IV for the Identification of Scopoletin in Fermented Noni Fruit Juice.

    ii. Endorse the TLC method as provided in Annex B (updated) as Type IV for the Identification of deacetylasperulosidic acid in Fermented Noni Fruit Juice.

        Where the Procedural Manual states a Type IV typing reflects a recently introduced method but for which the criteria required for acceptance by CCMAS have not yet been determined.

        Note 1: The modification to Annex A (compared to CX/MAS 21/41/3 Appendix II) are highlighted in red and include additional instruction for the SPE cartridge type and steps in SPE processing. Further refinements suggested is use of a 0.10 mg/mL Scopoletin standard in the Scopoletin TLC instead of 1.0mg/mL, as the later concentration is excessively higher than the typical Noni Juice sample.

        Note 2: The modifications to Annex B (compared to CX/MAS 21/41/3 Appendix II) are highlighted in red and include the removal of the SPE instruction and a simple dilution of Noni juice samples with Methanol. Plus, the addition of instruction for Preparation of para-anisaldehyde solution to the DAA identification (Annex B). Note, the included p-anisaldehyde solution preparation uses less sulfuric acid (4%) compared to the percentage originally specified by West and Deng (i.e.,10%); we believe the excess acid posed a heating and safety concern for preparation and use. The resultant p-anisaldehyde solution suffered no defect in its ability to visualize the desired analytes.

    iii. The Single Laboratory Verification/Validation for Identification of Scopoletin by SPE-TLC and Deacetylasperulosidic Acid by TLC in Fermented Noni Fruit Juice in Annex C.

    iv. The TLC methodologies recommended above to facilitate progression of the draft Regional Standard for Fermented Noni Juice to Step 8 noting that a HPLC-DAD method will be available in the future.

Revised SOP for the Identification of Kavalactones and Flavokavains in Fresh and Dried Kava Products by HPTLC in the Regional Standard for Kava Products for Use as a Beverage when Mixed with Water (CXS 336R-2020)

13. CCNASWP15 (2019) agreed[12] to forward provisions for methods of analysis and sampling to CCMAS for endorsement.

14. CCMAS41 (2021) noted that the review of the references did not produce a clear procedure for determining kavalactone or flavokavins, and that it appeared there were different sections within each reference that needed to be followed and that the 2016 reference may not be required for flavokavins.

15. CCMAS41 agreed to request CCNASWP to consider producing a single stepwise method or SOP which would capture the necessary steps for each provision in one easy to follow document.[13]

16. CCNASWP16 (2023) considered the request from CCMAS41 and revised the SOP accordingly. The Committee further agreed forward the revised SOP for the identification of kavalactones and flavokavains in fresh and dried kava products by HPTLC to CCMAS for endorsement.[14]

17. CCMAS **is invited to endorse** the revised SOP in Appendix V.

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10 REP23/NASWP16, para. 73(ii) and Appendix VII, Part B
11 https://www.fao.org/fao-who-codexalimentarius/meetings/detail/en/?meeting=CCMAS&session=42
12 REP20/NASWP15, para. 96(iii)
13 REP21/MAS41, paras. 15-17
14 REP23/NASWP16, para. 28(iii), Appendix III

APPENDICES
ORIGINAL LANGUAGE ONLY

APPENDIX I

CODEX COMMITTEE ON NUTRITION AND FOODS FOR SPECIAL DIETARY USES (CCNFSDU43)

Methods of analysis for provisions in the Standard for Infant Formula and Formulas for Special Medical Purposes Intended for Infants (CXS 72-1981)

All additions are shown in **bold underlined** font.

Commodity	Provision	Method	Principle	Type
Infant Formula	**Vitamin B12**	**AOAC 2014.02**	**LC-UV**	**III**
	Total amino acids (excluding taurine and tryptophan) **For use according to Section 3.1.3 (a) footnotes 3 and 4 of CXS 72-1981**	**AOAC 2018.06 / ISO 4214 | IDF 254 /AACC 07-50.01**	**UHPLC-UV**	**II**
	Tryptophan **For use according to Section 3.1.3 (a) footnotes 3 and 4 of CXS 72-1981**	**AOAC 2017.03**	**HPLC**	**II**

APPENDIX II

CODEX COMMITTEE ON SPICES AND CULINARY HERBS (CCSCH5)

Methods of analysis for provisions in the Standard for Dried Roots, Rhizomes and Bulbs – Dried or Dehydrated Ginger (CXS 343-2021)

Parameter	Method	Principle	Type[1]
Moisture	ISO 939	Distillation	I
Total Ash on dry basis	ISO 939 and ISO 928	Distillation and Gravimetry	I
Acid Insoluble Ash on dry basis	ISO 939 and ISO 930	Distillation and Gravimetry	I
Volatile Oil on dry basis	ISO 939 and ISO 6571	Distillation followed by Volumetry	I
Extraneous Matter	ISO 927	Visual Examination followed by Gravimetry	I
Foreign Matter	ISO 927	Visual Examination followed by Gravimetry	I
Insect Damage	Method V-8 Spices, Condiments, Flavours and Crude Drugs (Macroanalytical Procedure Manual) MPM: V-8. Spices	Visual Examination	IV
Whole dead insect	ISO 927	Visual examination	I
Mammalian/ Other Excreta	MPM V-8 Spices, Condiments, Flavours and Crude Drugs (Macroanalytical Procedure Manual) MPM: V-8. Spices (For whole)	Visual Examination followed by Gravimetry	IV
Mould visible	Method V-8 Spices, Condiments, Flavours and Crude Drugs (Macroanalytical Procedure Manual) MPM: V-8. Spices	Visual examination	IV
Live Insect	ISO 927 AOAC 960.51	Visual Examination Visual Examination	IV IV
Calcium (as oxide) on dry basis	ISO 1003, Annex A	Chemical reaction followed by gravimetry	IV
SO_2	AOAC 963.20	Colorimeter	II

[1] According to the definition of “types of method of analysis” as per Codex Procedural Manual Section II

Methods of analysis for provisions in the Standard for Dried Floral Parts-Cloves (CXS 344-2021)

Parameter	Method	Principle	Type[1]
Moisture	ASTA 2.0	Distillation	I
Volatile oil	ISO 6571	Distillation Volumetry	I
Total ash (dry basis)	ISO 928	Gravimetry	I
Acid Insoluble Ash	ISO 930	Gravimetry	I
Extraneous matter	ISO 927	Visual Gravimetry	I
Foreign matter	ISO 927	Visual Gravimetry	I
Insect damage	ISO 927 Method V-8 Spices, Condiments, Flavors and Crude Drugs	Visual Examination Visual Examination	IV IV
Insects/Excreta/Insect fragments	ISO 927	Visual Examination	IV
Crude fibre	ISO 5498	Gravimetry	I
Mould visible	Method V-8 Spices, Condiments, Flavours and Crude Drugs	Visual Examination	IV
Live insect	ISO 927	Visual Examination	IV
Mammalian or/and Other excreta	Method V-8 Spices, Condiments, Flavours and Crude Drugs	Visual Examination	IV

[1] According to the definition of “types of method of analysis” as per Codex Procedural Manual Section II

*Latest edition or version of the approved method should be used

Methods of analysis for provisions in the Standard for Dried Leaves - Dried Basil (CXS 345-2021)

Parameter	Method	Principle	Type
Moisture	ISO 939	Distillation	I
Total Ash	ISO 928	Gravimetry	I
Acid Insoluble Ash	ISO 928 and ISO 930	Gravimetry	I
Volatile Oil	ISO 6571	Distillation Volumetry	I
Extraneous Matter	ISO 927	Visual Examination followed by Volumetry	I
Foreign Matter	ISO 927	Visual Examination followed by Volumetry	I
Insect Damage	Method V-8 Spices, Condiments, Flavours and Crude Drugs (Macroanalytical Procedure Manual, FDA Technical Bulletin Number 5)	Visual Examination	IV
Insects/Excreta/Insect Fragments	Method appropriate for particular spice from AOAC Chapter 16, subchapter 14	Visual Examination	IV
Mould damage	Method V-8 Spices, Condiments, Flavours and Crude Drugs (Macroanalytical Procedure Manual, FDA Technical Bulletin Number 5)	Visual examination (for whole)	IV
Mammalian Excreta, And Other Excreta	Method V-8 Spices, Condiments, Flavours and Crude Drugs (Macroanalytical Procedure Manual, USFDA, Technical Bulletin V.39 B) (For whole)	Visual Examination	I

* Latest edition or version of the approved method should be used.

[2] According to the definition of “types of method of analysis” as per Codex Procedural Manual Section II.

CODEX COMMITTEE ON SPICES AND CULINARY HERBS (CCSCH6)

Methods of analysis for provisions in the Standard for Dried Floral Parts – Saffron (CXS 351-2021)

Provision	Method	Principle	Type
Moisture	ISO 3632-2	Gravimetry	I
Total Ash	ISO 3632-2 and ISO 928	Gravimetry	I
Acid Insoluble Ash	ISO 3632-2 and ISO 930	Gravimetry	I
Soluble extract in cold water	ISO 3632-2 and ISO 941	Extraction	I
Taste strength (expressed as picrocrocin) $A_{1cm}^{1\%}$ 257 nm	ISO 3632-2	Absorbance	IV
Aroma strength (expressed as safranal) $A_{1cm}^{1\%}$ 330 nm	ISO 3632-2	Absorbance	IV
Coloring strength (expressed as crocin) $A_{1cm}^{1\%}$ 440 nm	ISO 3632-2	Absorbance	IV
Extraneous Matter	ISO 3632-2	Visual Examination followed by Gravimetry	I
Foreign Matter	ISO 3632-2	Visual Examination followed by Gravimetry	I
Insect Damage	ISO 927	Visual Examination followed by Gravimetry	I
Whole dead Insects /Insect Fragments	ISO 927	Visual Examination	I
Visible mould	Method V-8 Spices, Condiments, Flavors and Crude Drugs (Macro analytical Procedure Manual, FDA Technical Bulletin Number 5) http://www.fda.gov/Food/FoodScienceResearch/Laoratory Methods/ucm084394.htm#v-32	Visual Examination followed by Gravimetry	I
Mammalian Excreta	Macro analytical Procedure Manual, USFDA, Technical Bulletin V.39 B (For whole)	Visual Examination followed by Gravimetry	I
Other Excreta	AOAC 993.27 (For Ground)	Enzymatic Detection Method	IV
Rodent filth	ISO 927	Visual Examination	I

1 Latest edition or version of the approved method should be used

2 The methods of analysis will be included in CXS 234-1999 after endorsement by CCMAS and the following text replace the Table

"For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used."

Methods of analysis for provisions in the Standard for Dried Seeds - Nutmeg (CXS 352-2022)

For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used.

Provision	Method[1]	Principle	Type
Moisture content	ISO 939	Distillation	I
Total ash	ISO 939 and ISO 928	Distillation Gravimetry	I
Acid-insoluble ash	ISO 939 and ISO 930	Distillation Gravimetry	I
Water-insoluble ash	ISO 939 and ISO 929	Distillation Gravimetry	I
Volatile oil content	ISO 939 and ISO 6571	Distillation Distillation	I
Extraneous matter	ISO 927	Visual examination followed by gravimetry	I
Foreign matter	ISO 927	Visual examination followed by gravimetry	I
Visible mould	ISO 927	Visual examination followed by gravimetry	I
Insect defiled/infested	MPM V-8 Spices, Condiments, Flavours and Crude Drugs A. General methods for spices herbs and botanicals (V 32)	Visual Examination followed by gravimetry	I
Dead insect, insect fragments, rodent contamination	ISO 927	Visual examination	I
Live insect	ISO 927	Visual examination	I
Mammalian and or other excreta	Macroanalytical Procedure Manual (MPM) USFDA technical bulletin V.41	Visual examination followed by gravimetry	I
Piece of mace	ISO 927	Visual examination followed by gravimetry	I

The methods of analysis will be included in CXS 234-1999 after endorsement by CCMAS

For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used.

[1] Latest edition or version of the approved methods should be used.

Methods of analysis for the provisions off size, when sized and broken/damaged among the whole to be developed.

Methods of analysis for provisions in the Standard for Dried or Dehydrated Chilli Pepper and Paprika (CXS 353-2022)

Provision	Method[1]	Principles	Type[2]
Moisture	ISO 939	Distillation	I
Total Ash	ISO 939 and ISO 928	Distillation Gravimetry	I
Acid-insoluble ash	ISO 939 and ISO 930	Distillation Gravimetry	I
Pungency Scoville Heat units	ASTA 21.3	Chromatography	IV
	ISO 3513	Sensory evaluation	I
Colour value	ISO 7541	Spectrophotometry	IV
Mammalian excreta	ISO 927	Visual examination followed by Gravimetry (whole)	I
Mould damage	MPM V-8 Spices, Condiments, Flavours and Crude Drugs A. General methods for spices herbs and botanicals (V 32)	Visual Examination (for whole)	I
	AOAC 945.94	Visual Examination (for Ground)	I
Insect Damage	MPM V-8 Spices, Condiments, Flavours and Crude Drugs A. General methods for spices herbs and botanicals (V 32)	Visual Examination followed by Gravimetry	I
Extraneous matter[3]	ISO 927	Visual Examination followed by Gravimetry	I
Foreign matter[4]	ISO 927	Visual Examination followed by Gravimetry	I
Live insect	ISO 927 / AOAC 960.51	Visual Examination	I
Insect filth	ISO 927	Visual Examination	I
Insect fragments	ISO 927	Visual examination counting	I
Rodent hair	AOAC 978.22 (Ground chilli) AOAC 977.25 B (Ground paprika)	Microscopic examination Microscopic examination	I I

[1]Latest edition or version of the approved method should be used.

[2]According to the definition of "types of method of analysis" as per Codex Procedural Manual Section II

[3] Vegetative matter associated with the plant from which the product originates but not accepted as part of the final product.

[4] Any visible/detectable objectionable foreign matter or material not usually associated with the natural components of the spice plant, such as sticks, stones, burlap bagging, metal, etc.

The methods of analysis will be included in CXS 234-1999 after endorsement by CCMAS and the following text shall replace the Table

"For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used

Methods of analysis for provisions in the draft Standard for Dried Small Cardamom

Provision	Method[1]	Principle	Type [2]
Moisture	ISO 939	Distillation	I
Total Ash	ISO 939 and ISO 928	Distillation and Gravimetry	I
Acid Insoluble Ash	ISO 939 and ISO 930	Distillation and Gravimetry	I
Volatile Oil	ISO 939 and ISO 6571	Distillation followed by Volumetry	I
Extraneous Matter	ISO 927	Visual Examination followed by Gravimetry	I
Foreign Matter	ISO 927	Visual Examination followed by Gravimetry	I
Insect defiled/infested	Method V-8 Spices, Condiments, Flavors and Crude Drugs (Macroanalytical Procedure Manual) MPM: V-8. Spices	Visual Examination followed by Gravimetry	I
Immature and shrivelled capsules	ISO 927	Visual Examination followed by Gravimetry	I
Mammalian or/and other excreta	Method V-8 Spices, Condiments, Flavors and Crude Drugs (Macroanalytical Procedure Manual) MPM: V-8. Spices	Visual Examination followed by Gravimetry	I
Mould visible	Method V-8 Spices, Condiments, Flavors and Crude Drugs (Macroanalytical Procedure Manual) MPM: V-8. Spices	Visual Examination followed by Gravimetry	I
Empty and malformed capsules	IS 1907:1984	Visual Examination followed by Gravimetry	I
Whole insect Live/dead	ISO 927	Visual examination followed by Gravimetry	I
Light seeds	ISO 927	Visual examination followed by Gravimetry	I

1 Latest edition or version of the approved method should be used

2 According to the definition of “types of method of analysis” as per Codex Procedural Manual Section II

***** The methods of analysis will be included in CXS 234-1999 after endorsement by CCMAS and the following text replace the Table

“For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used”

Methods of analysis for provisions in the draft Standard for Spices Derived From Dried Fruits and Berries (Part A - Allspice, Juniper berry And Star anise)

Sl. No	Spices	Provision	Method[1,2]	Principles	Type
1	Dried Allspice Dried Juniper Berries Dried Star Anise	Moisture	ISO 939	Distillation	I
		Total ash	ISO 939 and ISO 928	Distillation followed by gravimetry.	I
		Acid- insoluble	ISO 939 and ISO 930	Distillation followed by gravimetry.	I
		Volatile oils	ISO 939 and ISO 6571	Distillation followed by gravimetry.	I
		Extraneous matter	ISO 927	Visual examination followed by gravimetry	I
		Foreign matter	ISO 927	Visual examination followed by gravimetry	I
		Mould visible	ISO 927	Visual examination followed by gravimetry	I
		Mammalian excreta	MPM V-8 Spices, Condiments, Flavors and Crude Drugs A. General methods for spices herbs and botanicals (V 32) *https://www.fda.gov/food/laboratory- methods-food/mpm-v-8-spices-condiments- flavors-and- crude-drugs* (Applicable to whole form of the spices)	Visual examination followed by gravimetry	I
		Whole dead insect	ISO 927	Visual examination	I
			AOAC 969.44	Flotation method	IV
		Insect fragments	ISO 927	Visual examination counting	I
			AOAC 975.49	Flotation method	IV

Sl. No	Spices	Provision	Method[1,2]	Principles	Type
		Insect damage	MPM V-8 Spices, Condiments, Flavours and Crude Drugs General methods for spices herbs and botanicals (V 32) (Applicable to whole form of the spices)	Visual examination followed by gravimetry or counting	I
		Mould damage	MPM V-8 Spices, Condiments, Flavours and Crude Drugs General methods for spices herbs and botanicals (V 32) (Applicable to whole form of the spices)	Visual examination followed by gravimetry or counting	I
2	Allspice (whole, cracked/ pieces)	Filth (list all the filth here-for example - mammalian excreta)	AOAC 965.40	Flotation	I
	Allspice (Ground/ powdered)	Light filth (list all the filth here-for example- mammalian excreta)	AOAC 981.21	Flotation	I
3	Juniper Berries, Star Anise, (cut/broken, ground/ powdered)	Light filth (list all the filth here-for example- mammalian excreta)	AOAC 975.49	Flotation	I

[1] Latest edition or version of the approved method should be used

[2] The methods of analysis will be included in CXS 234-1999 after endorsement by CCMAS and the following text replace the Table

"For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used."

APPENDIX III

FAO/WHO COORDINATING COMMITTEE FOR AFRICA (CCAFRICA24)

Methods of analysis and sampling for provisions in the Regional Standard for Dried Meat (CXS 350-2022)

1. METHODS OF ANALYSIS AND SAMPLING

8.1 Methods of Analysis[15]

Provision	Method	Principles	Type
Moisture Content	AOAC 950.46B	Gravimetry	I
Total Fat	ISO 1443	Gravimetry	I
Nitrogen*	ISO 937*	Titrimetry	II
Chloride as Sodium Chloride (≥ 1.0%)	ISO 1841-1	Volhard method	III
Chloride as Sodium Chloride (≥ 0.25%)	ISO 1841-2	Potentiometry	II
Ash	ISO 936	Gravimetry	I
Water Activity	ISO 18787	Electrometry	II
*nitrogen-to-protein conversion factor = 6.25			

8.2 Sampling

Sampling shall be in accordance with the *General Guidelines on Sampling* (CXG 50-2004).

[15] After adoption, the table containing the Methods of Analysis will be removed and replaced with the following Text, as per the requirements of the Procedural Manual:
"For checking the compliance with this standard, the methods of analysis and sampling contained in the Recommended Methods of Analysis and Sampling (CXS 234-1999) relevant to the provisions in this standard, shall be used."

APPENDIX IV

FAO/WHO COORDINATING COMMITTEE FOR ASIA (CCASIA22)

Methods of analysis and sampling for provisions in the draft Regional Standard for Soybean Products Fermented with Bacillus Species

9. METHODS OF ANALYSIS AND SAMPLING[16]

For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used.

9.1. Determination of Moisture Content

Natto: According to AOAC 925.09.(Type I Gravimetry (vacuum oven))

Cheonggukjang: According to AOAC 934.01. (Type I Gravimetry)

Thua Nao: According to AOAC 925.09. (Type I Gravimetry (vacuum oven))

9.2. Determination of Protein Content

Natto: According to AOAC 988.05. (Type I Titrimetry, Kjeldahl digestion)

(Nitrogen factor 5.71)

Cheonggukjang: According to AOAC 988.05. (Type I Titrimetry, Kjeldahl digestion)

(Nitrogen factor 5.71)

Thua Nao: According to AOAC 988.05. (Type I Titrimetry, Kjeldahl digestion)

(Nitrogen factor 5.71)

9.3. Determination of Lipid Content

Natto: According to AOAC 963.15. (Type I Gravimetry (Soxhlet Extraction))

(Quantity of sample:4g)

Cheonggukjang: According to AOAC 963.15. (Type I Gravimetry (Soxhlet Extraction))

(Quantity of sample:5g)

[16] The analytical methods will be removed when the standard is adopted by CAC and included in CXS 234-1999.

ANNEX I

Sampling Plans (AQL=6.5)

Sampling plan 1 – Normal sampling

Lot size (N)	Sample size (n)	Acceptance number (c)
4,800 or less	6	1
4,801-24,000	13	2
24,001-48,000	21	3
48,001-84,000	29	4
84,001-144,000	38	5
144,001-240,000	48	6
More than 240,000	60	7

Sampling plan 2 – Dispute, enforcement or need for better lot estimate

Lot size (N)	Sample size (n)	Acceptance number (c)
4,800 or less	13	2
4,801-24,000	21	3
24,001-48,000	29	4
48,001-84,000	38	5
84,001-144,000	48	6
144,001-240,000	60	7
More than 240,000	72	8

Methods of analysis and sampling for provisions in the draft Regional Standard for Cooked Rice Wrapped in Plant Leaves

10. METHODS OF ANALYSIS AND SAMPLING[17]

For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used.

10.1. Determination of Peroxide Value

10.1.1. Extraction of Oils from the Product

10.1.1.1. Apparatus

(a) Rotary evaporator

(b) Water bath

10.1.1.2. Extraction

Remove the product package and plant leaves, etc., take out the edible part of the representative sample, crush it and put it in a homogenizer or glass mortar, and grind it continuously to make the sample fully mashed and mixed well, and then put it in the wide-mouth bottle, and add 2 to 3 times the sample volume of petroleum ether (boiling range: 30°C-60°C). After fully mixing, stopper the bottle and leave for more than 12 hours. Filter all the solution with a funnel filled with anhydrous sodium sulphate into a round-bottom flask. Rinse the residue in the wide-mouth bottle with petroleum ether. Repeat the filtration once with a new anhydrous sodium sulphate funnel, if the filtrate is not clear enough. Evaporate the petroleum ether in the round-bottom flask under reduced pressure on a rotary evaporator at below 40°C, and the residue is the test sample. A sufficient number of representative samples should be selected to ensure that not less than 8 grams of the test sample can be obtained. The test sample should be tested as soon as possible.

10.1.2. Determination

According to ISO 3960 or AOCS Cd 8b-90 (03) (Type I Titrimetry (Colorimetric)).

[17] The analytical methods will be removed when the standard is adopted by CAC and included in CXS 234-1999.

APPENDIX V

FAO/WHO COORDINATING COMMITTEE FOR NORTH AMERICA AND SOUTHWEST PACIFIC (CCNASWP16)

Methods of analysis and sampling for provisions in the draft Regional Standard for Fermented Noni Fruit Juice

10. METHODS OF ANALYSIS AND SAMPLING

For checking the compliance with this standard, the methods of analysis and sampling contained in the *Recommended Methods of Analysis and Sampling* (CXS 234-1999) relevant to the provisions in this standard, shall be used.

10.1 Methods of Analysis

Provision	Method	Principle	Type	Notes
Brix value (Soluble solids)	AOAC 983.17 EN 12143 IFUMA 8 ISO 2173	Refractometry	I	Adopted for fruit juices and nectars
pH value	NMKL 179 / AOAC 981.12	Potentiometry	II	Adopted for fruit juices and nectars
Ethanol	IFUMA 52 AOAC 2017.07	Enzymatic determination	IV	
	AOAC 2016.12	Headspace GC-FID	IV	
Identification of scopoletin	Annex A*	Solid phase extraction and thin layer chromatography	IV	
Identification of deacetylasperulosidic acid	Annex B*	Thin layer chromatography	IV	

** In compliance with the general criteria for testing laboratories laid down in ISO/IEC Guide 17025:2017*

ANNEX A

IDENTIFICATION OF SCOPOLETIN

1. PREPARATION OF SAMPLES

Noni fruit juice is filtered through a 0.45 µm membrane filter and then purified by solid-phase extraction (SPE) with Waters OASIS® HLB 6cc 200 mg extraction cartridges (or similar solid-phase extraction cartridge), after first equilibrating with methanol (5 mL) followed by deionized water (5 mL). The filtered juice samples (3 mL) are then loaded onto the equilibrated cartridge and washed with 5% methanol (MeOH) in deionized water (5 mL). The cartridges are allowed to dry under flow of air for 5 mins and then, eluted with MeOH (3mL). The MeOH eluate is retained for TLC analysis. The SPE flow rates of equilibration, wash and elution solvents through the cartridge is approximately 1 drop per second.

2. PREPARATION OF REFERENCE STANDARD

2.1 A reference standard is prepared by dissolving 0.1 mg Scopoletin in 1 milliliter of methanol.

2.2 Alternately, certified *Morinda citrifolia* reference plant material may be prepared in the same manner as the samples to be analyzed. The certified *Morinda citrifolia* reference material should be from the same part of the plant as the samples to be analyzed.

3. IDENTIFICATION

3.1 THIN LAYER CHROMATOGRAPHY

Spot 5 microliters of sample solutions and reference standard solution on a silica gel 60 F254 thin layer chromatography (TLC) plate. After spotting the plates are dried at 110°C for 15 minutes in a drying oven. Develop the plate with a mobile phase of dichloromethane:methanol (19:1, v/v). View bright fluorescent blue colours on developed plate under UV lamp, 365 nm. Identify Scopoletin in samples by comparing Rf values and colours to the standard.

REFERENCES

1. Deng S, West BJ, Jensen J. A Quantitative Comparison of Phytochemical Components in Global Noni Fruits and Their Commercial Products. Food Chemistry 2010, 122 (1): 267-270.

2. Potterat O, et al. Identification of TLC markers and quantification by HPLC-MS of various constituents in noni fruit powder and commercial noni-derived products. Journal of Agricultural and Food Chemistry 2007, 55(18):7489–7494.

3. Basar S, Westendorf J. Identification of (2E, 4Z, 7Z)-Decatrienoic Acid in Noni Fruit and Its Use in Quality Screening of Commercial Noni Products. Food Analytical Methods 2011, 4(1):57-65. DOI: 10.1007/s12161- 010-9125-9.

4. Chan-Blanco Y, et al. The ripening and aging of noni fruits (*Morinda citrifolia* L.): microbiological flora and antioxidant compounds. Journal of the Science of Food and Agriculture 2007, 87:1710 – 1716.

5. West BJ, Deng S. Thin layer chromatography methods for rapid identity testing of *Morinda citrifolia* L. (noni) fruit and leaf. Advance Journal of Food Science and Technology 2010, 2(5):298-302.

ANNEX B

IDENTIFICATION OF DEACETYLASPERULOSIDIC ACID

1. PREPARATION OF SAMPLES

Noni fruit juice is filtered through a 0.45 µm membrane filter and diluted 1:1 with MeOH ~~and then purified by solid-phase extraction (SPE) with Waters OASISS® extraction cartridges, or similar solid-phase extraction cartridge. [SPE cartridges is first equilibrated with water, followed by methanol. The samples are then loaded onto the cartridge and washed with 5% MeOH, followed by 100% MeOH. The MeOH eluate is retained for TLC analysis.]~~.

2. PREPARATION OF REFERENCE STANDARD

2.1 A reference standard is prepared by dissolving 1 mg deacetylasperulosidic acid in 1 milliliter of methanol.

2.2 Alternately, certified *Morinda citrifolia* reference plant material may be prepared in the same manner as the samples to be analyzed. The certified *Morinda citrifolia* reference material should be from the same part of the plant as the samples to be analyzed.

3. PREPARATION OF p-ANISALDEHYDE SOLUTION

Anisaldehyde solution was prepared by dissolving 2g of p-anisaldehyde in 96 mL of ethanol with stirring. The solution was then acidified through dropwise addition of concentrated sulfuric acid (4 mL).

4. IDENTIFICATION

4.1 THIN LAYER CHROMATOGRAPHY

Spot 5 microliters of sample solutions and reference standard solution on a silica gel 60 F254 thin layer chromatography (TLC) plate, previously dried at 110 °C for 15 minutes in a drying oven. After spotting samples are again dried at 110°C or through application of heat via a heat gun for a period of 8-10 seconds. The TLC plates are developed with a mobile phase of dichloromethane: methanol: water (13:6:1, v/v/v). Upon completion of elution, the plate is air dried and developed by spraying with 2% anisaldehyde / 4% sulfuric acid in ethanol (EtOH) solution and then heat in oven at 110 °C for 1-5 minutes to reveal and maximise the blue colour. Identify deacetylasperulosidic in samples by comparing spot Rf values and colour with reference standard solution on same TLC plate.

REFERENCES

1. Potterat O, et al. Identification of TLC markers and quantification by HPLC-MS of various constituents in noni fruit powder and commercial noni-derived products. Journal of Agricultural and Food Chemistry 2007, 55(18):7489–7494.

2. Deng S, et al. Determination and comparative analysis of major iridoids in different parts and cultivation sources of *Morinda citrifolia*. Phytochemical Analysis 2011, 22(1):26-30.

3. West BJ, Deng S. Thin layer chromatography methods for rapid identity testing of *Morinda citrifolia* L. (noni) fruit and leaf. Advance Journal of Food Science and Technology 2010, 2(5):298-302

ANNEX C

SINGLE LABORATORY VERIFICATION / VALIDATION FOR IDENTIFICATION OF SCOPOLETIN AND DEACETYASPERULOSIDIC ACID IN FERMENTED NONI JUICE

The performance characteristics validation for an 'Identification test' is usually limited to 'Selectivity'. Where the capability of an analytical procedure to identify an analyte can be confirmed by obtaining positive results comparable to a known reference material with samples containing the analyte, along with negative results from samples which do not contain the analyte. In addition, the identification test can be applied to materials structurally similar to or closely related to the analyte to confirm that an undesired positive response is not obtained. Specificity/selectivity can be verified by demonstrating that the measured result of an analyte is comparable to the measured result of a second, well characterized analytical procedure (e.g., an orthogonal procedure).

Thus,

a) the colour response with the TLC visualization technique with standards, and a relative response for increasing standard concentration tested was confirmed,

b) the coloured TLC spot with samples with a Rf similar to the standard was confirmed for different Fermented Noni juices from a range of pacific island locations (supplied by Scientific Research Organisation of Samoa (SROS)-Apia),

c) various juices observed mixed in commercial Noni products were tested along with a Noni Juice by TLC to confirm a negative result for other juices.

d) an orthogonal HPLC technique based on Choi et al (2022)[11] was used to measure concentrations or absence of the identity analytes for selected samples, and PDA spectra along with Rt used to confirm HPLC peak identity.

For Scopoletin Identification

a) Colour response under UV@365nm and relative intensity/response for Scopoletin TLC standards at 0.001, 0.01, 0.1 and 1.0 mg/mL in MeOH. We thus suggest that a 0.10 mg/mL Scopoletin standard may be more appropriate in the Scopoletin TLC identification.

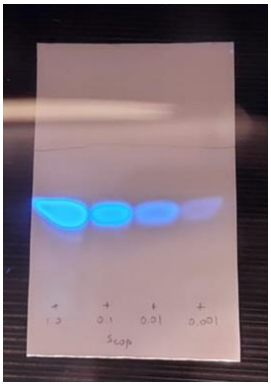

Figure 1 TLC for Scopoletin standards at 1.0, 0.1, 0.01 and 0.001 mg/mL in MeOH at 365nm.

b) Colour response under UV@365nm and R_f relative to standard Scopoletin for various Pacific Island samples.

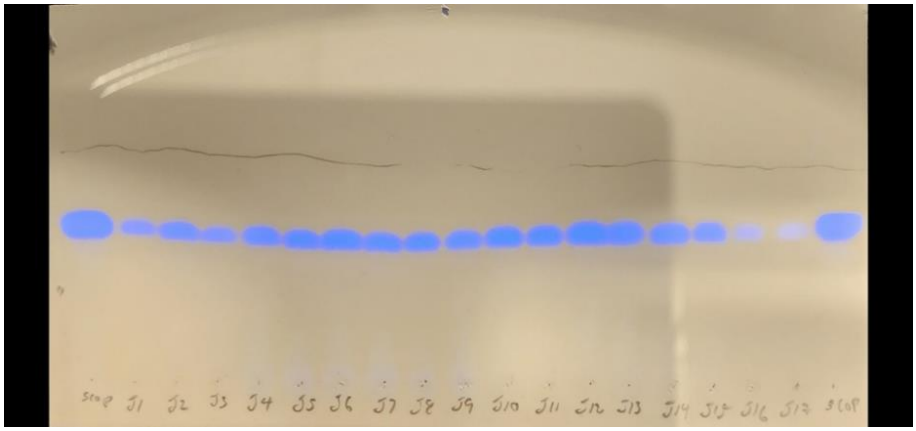

Figure 2. Scopoletin standard plus SPE extraction & TLC with UV@365nm visualization of fermented Noni juice samples, with left to right, standard; fermented Noni juice samples J1-17; standard.

Standard and Pacific Island samples J18-J19.

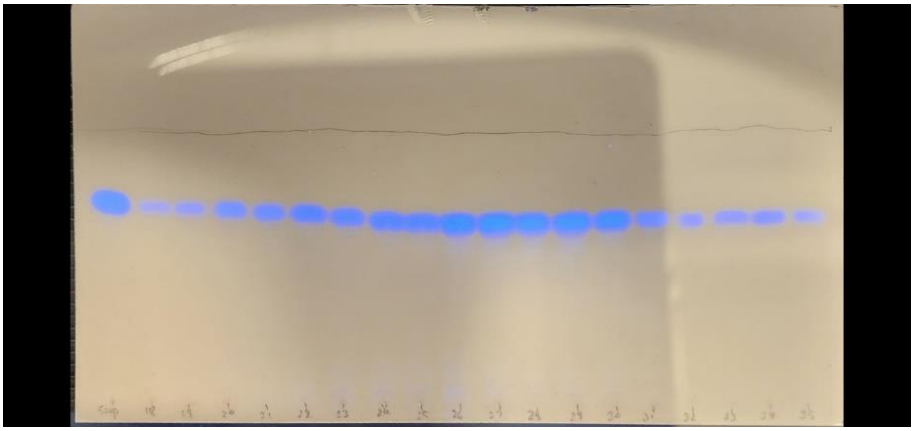

Figure 3. Scopoletin standard plus SPE extraction & TLC with UV@365nm visualization of fermented Noni juice samples, with left to right, standard; fermented Noni juice samples 18-35.

c) Following is the Scopoletin TLC Identification test applied to various juices observed mixed in commercial Noni products, including commercial pineapple juice, apple and blackcurrant juice, grape juice, pear juice, and coconut juice.

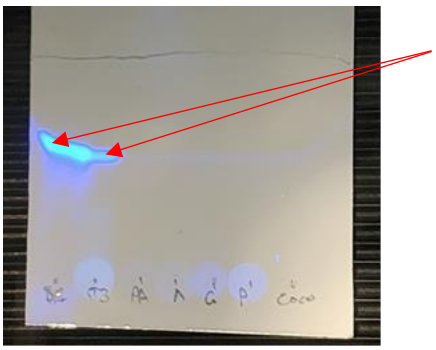

Figure 4. Scopoletin ID TLC for a Noni juice plus various other juices; from left to right, Scopoletin (0.1mg/mL), Noni Juice#3(J3), Pineapple juice (PA), Apple and Blackcurrant juice(A), Grape juice(G), Pear juice(P), and Coconut juice (Coco). Scopoletin band for standard and Noni Juice#3 indicated by red arrows, where the absence of similar band for the other samples gives a negative Scopoletin Identification.

a) An orthogonal HPLC technique based on Choi et al (2022)[11] used to confirm 'presence' or 'absence' of the identity analytes for selected samples, and PDA spectra along with peak at Rt=22.8min(approx.) used to confirm HPLC peak identity.

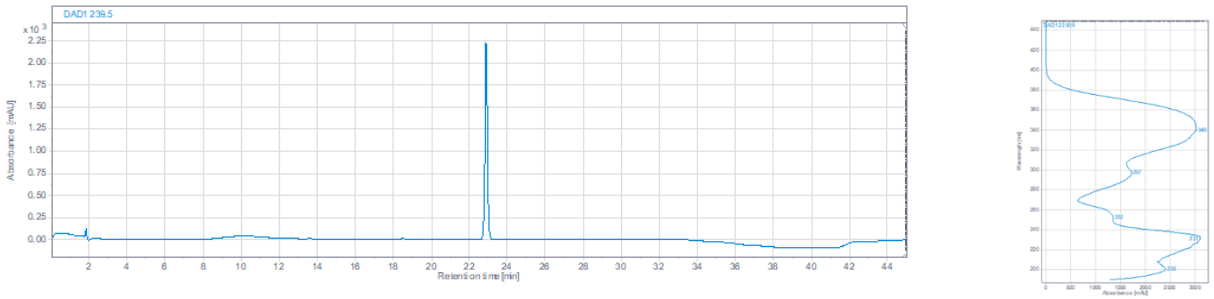

Figure 5. Scopoletin standard, HPLC-DAD chromatogram, 10µL injection, @ 239.5nm and peak UV spectra.

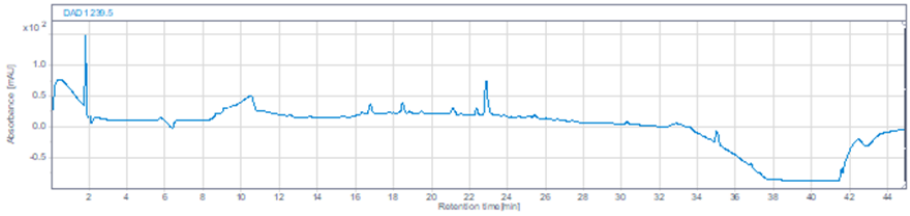

Figure 6. Juice#3, filtered, HLB-SPE 100% MeOH elution solution and injected 10µL on HPLC-DAD @ 239.5nm

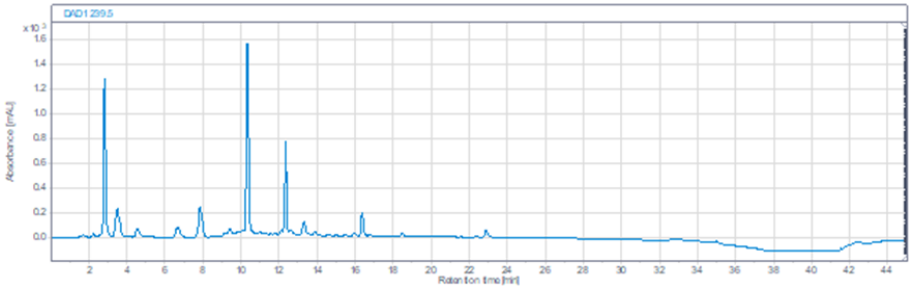

Figure 7. Juice#3, filtered 0.45um, no SPE, and direct injected 10µL on HPLC-DAD @ 239.5nm.

See section f) for HPLC-DAD conditions.

For Deacetylasperulosidic acid Identification

a) Colour response with 2% anisaldehyde / 10% sulfuric acid-ethanol (EtOH) solution then heating for visualisation, and relative intensity/response at 1.0, 0.5, 0.25 and 0.1 mg/mL Deacetylasperulosidic acid.

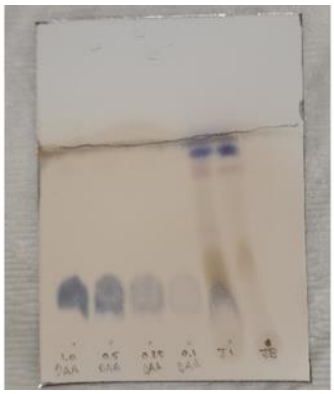

Figure 8. TLC standard solutions spots 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.1 mg/mL; Juice 1; Juice 8.

b) Colour response with 2% anisaldehyde / 10% sulfuric acid-ethanol (EtOH) solution then heating for visualisation, and R_f relative to standard Deacetylasperulosidic acid for various Pacific Island samples.

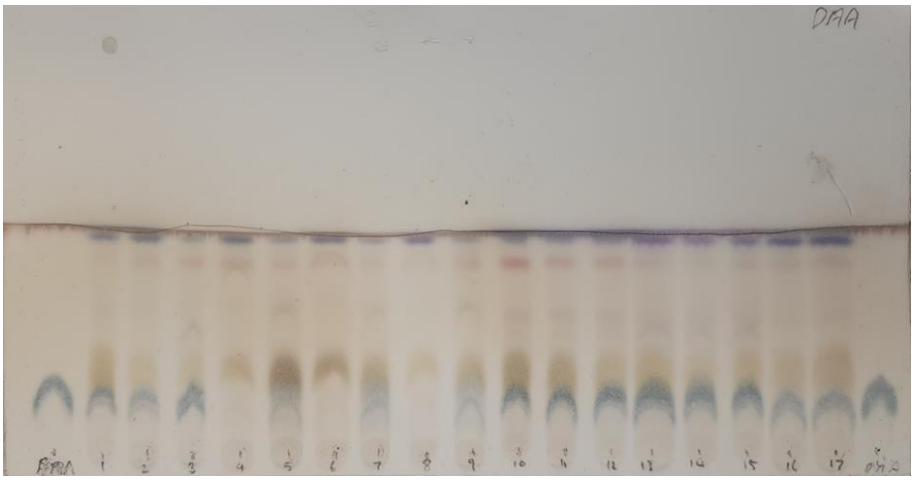

Figure 9. From left to right, DAA standard; fermented Noni juice samples 1-17; DAA standard; with TLC visualised with 2% anisaldehyde / 4% sulfuric acid-ethanol (EtOH) solution then heating.

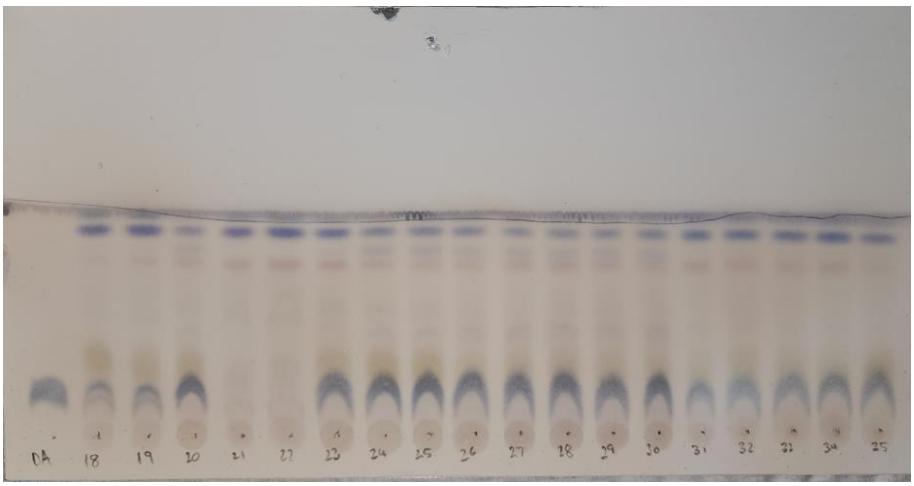

Figure 10. From left to right, DAA standard, fermented Noni juice samples 18-35; with TLC visualisation with 2% anisaldehyde / 4% sulfuric acid-ethanol (EtOH) solution then heating.

Note: Samples sourced from SROS-Apia for purpose of verification of TLC method for Scopoletin and DAA. Samples may have been subjected to adverse conditions during transport or pre-sampling prior to shipment to Australia. No conclusion can be inferred for Juices, 4, 6, 8, 21, 22 other than HPLC-DAD and TLC are in alignment in the absence or scarcity of DAA analyte. Further investigation would be required on non-compliant sample to determine the reason behind these atypical or non-compliant findings.

c) Following is the Deacetylasperulosidic acid TLC Identification test applied to various juices observed mixed in commercial Noni products, including commercial pineapple juice, apple and blackcurrant juice, grape juice, pear juice, and coconut juice.

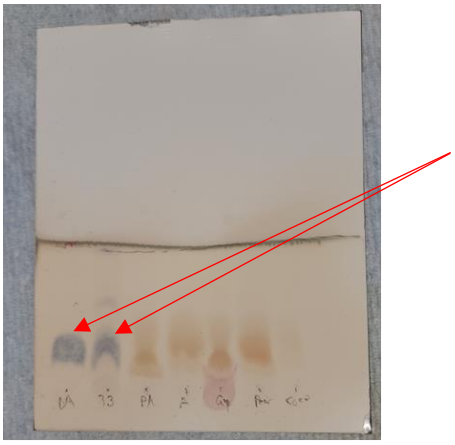

Figure 11. Deacetylasperulosidic acid ID TLC for a Noni juice plus various other fruit juices; from left to right, Deacetylasperulosidic acid (0.5mg/mL DA), Noni Juice#3(J3), Pineapple juice (PA), Apple and Blackcurrant juice(A), Grape juice (Gp), Pear juice (Pear), and coconut juice (Co). Deacetylasperulosidic acid blue band indicated by red arrow in standard and Juice#3, where the absence of similar blue bands for the other samples gives a 'negative' identification.

d) An orthogonal HPLC technique based on Choi et al (2022)[11] used to confirm 'presence' or 'absence' of the identity analytes for selected samples, and PDA spectra along with Rt used to confirm HPLC peak identity.

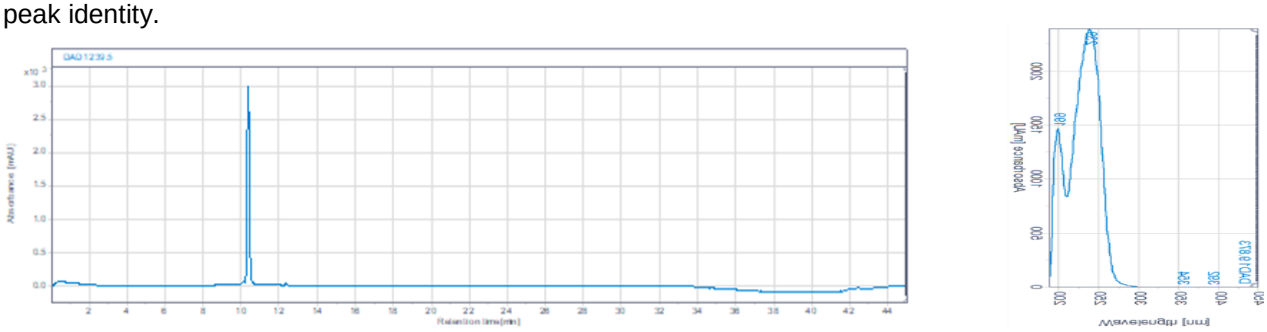

Figure 12. Deacetylasperulosidic acid 2 mg/mL; HPLC-DAD chromatogram, 10µL injection, @ 239.5nm; and peak UV spectra.

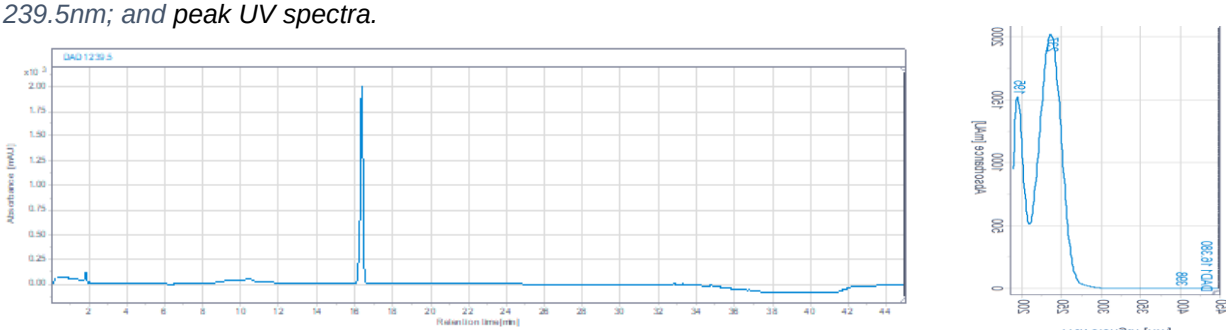

Figure 13. Asperulosidic acid; HPLC-DAD chromatogram, 10µL injection, @ 239.5nm; and peak UV spectra.

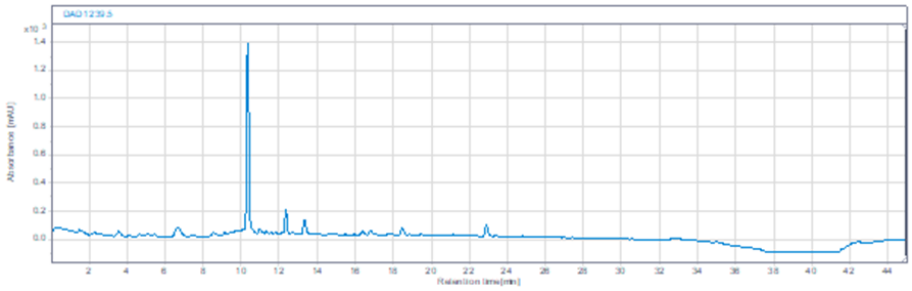

Figure 14. Juice 1, 0.45um filtered & no SPE, direct injected 10µL on HPLC-DAD @ 239.5nm

See section f) for HPLC-DAD conditions.

e) HPLC confirmation of the Deacetylasperulosidic acid ID by TLC for selected Pacific Island Noni Juice samples where 'negative' and 'positive' DAA IDs were observed.

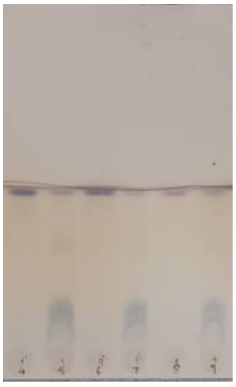

Figure 15 Cropped section of Fig 10, highlighting Deacetylasperulosidic acid ID by TLC for a selection of various Pacific Island Noni Juice samples where 'negative' and 'positive' DAA IDs were observed.

The juices in the following chromatograms were 0.45µm filtered and injected directly onto the HPLC-DAD with 10µL injection. The specific pattern to note is that according to the TLC, juices 4, 6 and 8 show a 'negative' DAA identification; while juice 5, 7, 9 show a 'positive' DAA identification. As observed in the following the HPLC-DAD chromatograms confirm the TLC results, with 'presence' or 'absence' of a sharp DAA peak at approximately 9.9 min, with 10uL injection, using 239.5 nm wavelength detection. Note, all these juices have a peak at R_t=22.8mins, thus positive ID for Scopoletin. Note the 10x reduction in absorbance scale for the negative results for DAA.

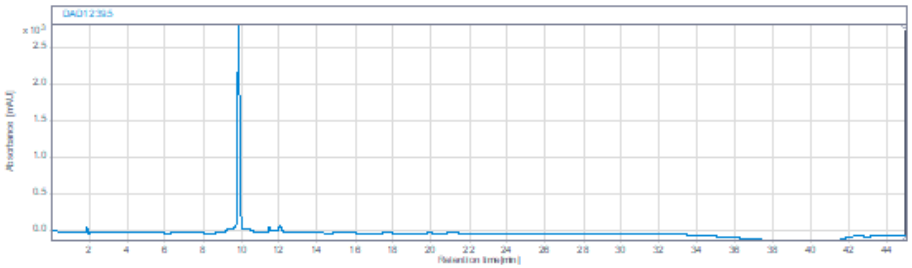

Figure 16. HPLC of DAA standard 2mg/mL with peak at 9.9mins.

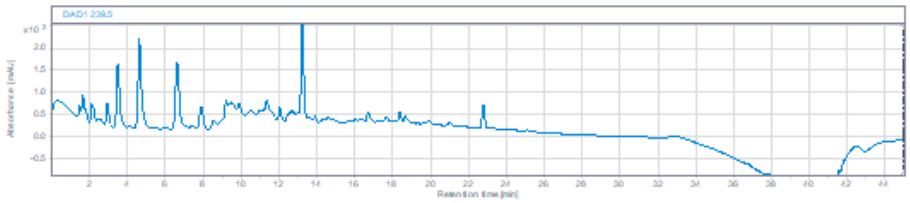

Figure 17. HPLC injection of Pacific Island juice#4, confirming 'negative' result for DAA.

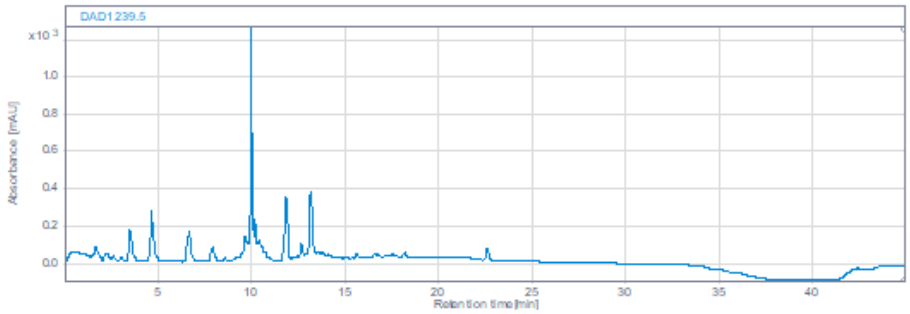

Figure 18. HPLC injection of Pacific Island juice#5, confirming 'positive' result for DAA.

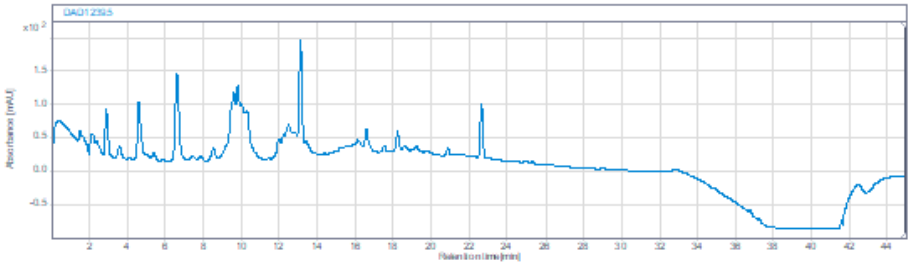

Figure 19. HPLC injection of Pacific Island juice#6, confirming 'negative' result for DAA.

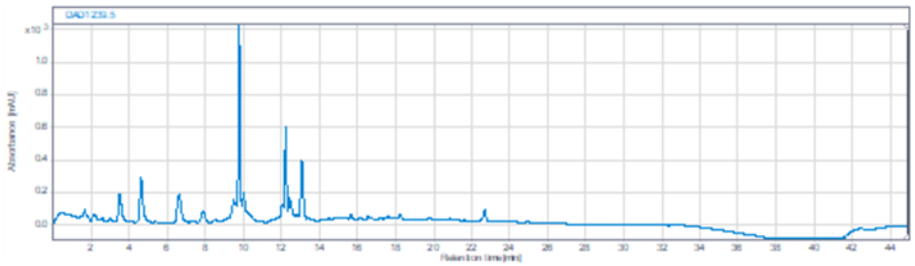

Figure 20. HPLC injection of Pacific Island juice#7, confirming 'positive' result for DAA.

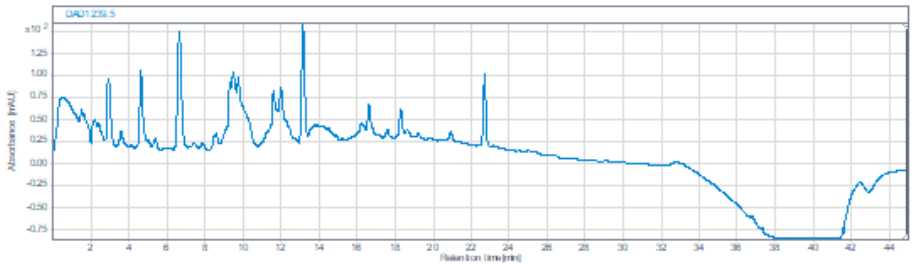

Figure 21. HPLC injection of Pacific Island juice#8, confirming 'negative' result for DAA.

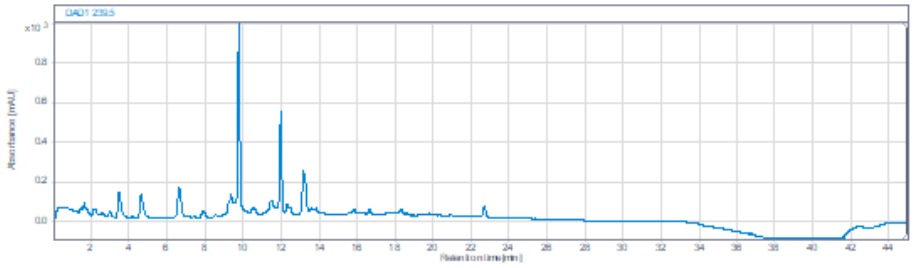

Figure 22. HPLC injection of Pacific Island juice#9, confirming 'positive' result for DAA.

f) Conditions for HPLC-DAD

HPLC-DAD was performed on an Agilent 1260 Infinity HPLC

Instrument: Agilent 1260 Infinity

Gradient:

Time(min)	0.1% Formic acid in deionised water	Acetonitrile
0	100	0
5	100	0
30	65	35
35	0	100
39	0	100
40	100	0
45	100	0

Run time: 45 minutes

Wavelengths monitored: 239.5 nm (Deacetylasperulosidic Acid); 344 nm (Scopoletin),

Peak width: >0.2 min (4s response time) 1.25Hz

Injection volume: 10µL

Mobile phase flow rate: 1.0 mLs/minute

Column temperature: 25 ^{o}C

Column: Agilent, Zorbax Eclipse Plus C18. 5um, 4.6 x 150 mm, (PN:959993-902, SN:USUXB20707, LN:B20104)

Guard Column: Agilent, Zorbax Eclipse Plus C18 2.1 x5, 1.8 micron (PN:821725-901, SN: USEDP03464)

Standard Operating Procedure for the Identification of Kavalactones and Flavokavains in Fresh and Dried Kava Products by High Performance Thin Layer Chromatography in the Regional Standard for Kava Products for Use as a Beverage when Mixed with Water (CXS 336R-2020)

1.0 Introduction

Piper methysticum G. Forst. (Piperaceae) rhizomes and roots are peeled, grinded, macerated in cold water, and pressed through a cloth strainer to prepare kava, a non-alcoholic beverage. The composition and quality of kava can be highly variable, depending on the age of the plant, the variety, and the part used to prepare the beverage: roots, rhizomes, or basal stems. The six major kavalactones (KLs: yangonin = Y, dihydrokavain = DHK, desmethoxyyangonin = DMY, kavain = K, dihydromethysticin = DHM and methysticin = M) are responsible for the physiological effect and are usually quantified with HPLC. There is a second group of molecules is flavokavins (FKs: A, B, C). The chemical composition of the kava extract is strongly influenced by the extraction solvent and extraction technique. This procedure is based on analytical procedure using High Performance Thin Layer Chromatography (HPTLC). The HPTLC is a validated procedure for 174 varieties of kava.

Scope: Identification of Kavolactones and Flavokavins by High Performance Thin Layer Chromatography

2.0 Materials and methodology

2.1 Preparation of Samples

- Wash by hand under cold running water the kava roots and peeled rhizomes.
- Cut into small pieces the kava organs with a knife.
- Sun-dry the kava pieces for 3 days (similar to traditional practises).
- Ground the dried kava matter into powder using a Forplex F00 1218 hammer mill to achieve <2 mm particle size and pack into labelled zip-log plastic bags.
- Further ground the kava powder to very fine kava flour texture using a coffee grinder.
- Weigh the kava flour sample then dry in an oven at 60°C for 6 hours.

2.2 Preparation of Reference Standard

- Make available Six kavalactone and three flavokavain standards of analytical grade possibly available from Sigma-Aldrich including standards of:

  Six kavalactones:

  - methysticin (M),
  - dihydromethysticin (DHM),
  - kavain (KAV),
  - dihydrokavain (DHK),
  - yangonin (Y),
  - desmethoyyangonin (DMY),

  Three flavokavain:

  - flavokavain A (FKA),
  - flavokavain B (FKB) and
  - flavokavain C (FKC).

- Accurately weigh 1.0mg individually the pure kava standard powder into 1ml acetone
- store in dark at 4°C if analysed later.

  Checking Purity of Standards:

- Conduct peak purity tests for the kava standards using the UV Vis spectrophotometer and compare the UV spectra.

2.3 Sample extraction

- Weigh 10g of kava powder,
- Transfer to a clean 50ml polypropylene centrifuge tube and add 30ml acetone.
- Sonicate the tubes in a water bath for 30min
- Transfer to a centrifuge instrument and set at4500 rpm for 10min.
- Transfer the supernatant to a 9mm wide opening screw thread vial of 2ml amber glass.
- Store vials in refrigerator at 4°C in dark till required for analysis.

2.4 Identification by High Performance Thin Layer Chromatography (HPTLC)

2.4.1 Chemicals and reagents for HPTLC analysis

- Analytical grade solvent (acetone, dioxane, hexane and methanol).
- Silica gel 60 F254 plates (dimension; 20 x 10cm) using Camag HPTLC system with an automatic TLC sampler (ATS 4) coupled to an automatic developing chamber (ADC 2) and a visualizer as well as a TLC Scanner 4 controlled with winCATS software.

2.4.2 Check standards and prepare Sample Run

- Prepare standards and sample solutions at bands(length of 8 mm, 250 nL/s delivery speed, track distance 8.0 mm and distance from the edge of 15 mm).
- Conduct standard linearity curve check by using the HPTLC plates. Apply different stock solutions (0.1, 0.2, 0.4, 0.6, 0.8, 1.0 µL) of the six KLs and three FKs scan at 240nm (for M, DHM, K, DHK) and scan at 355nm (for Y, DMY, FKA, FKB, FKC).
- Add 10 mL mobile phaseto develop the plates using hexane:dioxane (8:2 v/v) with a migration distance of 80 mm at room temperature after 30 s of pre-drying and no tank saturation.
- Visual documentation of the plates is carried out at 254 nm and 366 nm.
- Scan the plates in reflectance mode at 240 nm (for M, DHM, K and DHK) and at 355 nm (for Y, DMY, FKA, FKB, FKC) with D2 and W lamp slit dimension 8.00 mm × 0.20 mm, scanning speed 20 mm/s, and data resolution 100 µm/step.
- Identify the Peak area measurements (in area units, AU).
- Ensure that the total analytical time is 50 min for 20 samples and 10 mL of mobile phase (corresponding to 2.5 min and 0.5 mL per sample).

3.0 References

Lebot, V., Michalet, S., Legendre, L. (2019). Kavalctone and Flavokavins Profile Conttribute to Quality Assessment of Kava (Piper methysticum G. Forst), the Traditional Beverage of the Pacific. *Beverages*. 2019, 1-14.