CODEX ALIMENTARIUS COMMISSION

Food and Agriculture Organization of the United Nations

World Health Organization

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Agenda Item 3 **CRD05**

ORIGINAL LANGUAGE ONLY

JOINT FAO/WHO FOOD STANDARDS PROGRAMME

CODEX COMMITTEE ON METHODS OF ANALYSIS AND SAMPLING

42nd Session
Budapest, Hungary

13 – 16 June 2023
with report adoption on 20 June 2023 (virtual)

SUBMISSION OF METHOD FOR ENDORSEMENT OF METHODS OF ANALYSIS AND SAMPLING

(Prepared by the Regional Coordinator on behalf of CCNASWP)

Executive Summary

After a CCNASWP15 (2019)[1] referral to CCMAS41 (2021)[2], the Committee review of the identification methods for Scopoletin and Deacetylasperulosidic acid in fermented Noni Juice (as proposed in CX/MAS 21/41/3 Appendix II - Annex A & B) stated they required a clear indication of the specific details as to what type of SPE-OASIS® cartridge to be used as well as volumes (mLs) of aqueous solutions used in the method(s).

CCNASWP16 (2023)[3] considered the request from CCMAS41 and agreed to task the Regional Coordinator to work with the Members in the NASWP region to resolve outstanding issues in the draft Standard for Fermented Noni Juice i.e. Section 10 - methods of analysis and sampling (specifically specification of the solid-phase extraction cartridge and the TLC method to identify scopoletin and deacetylasperulosidic acid) in order to forward the section and Annex A and B to CCMAS42 for endorsement.

Following this decision, further work titled 'Verification of the Thin-Layer Chromatography Identification of Scopoletin and Deacetylasperulosidic Acid for Fermented Noni Fruit Juice was undertaken. However, an OASIS-SPE for both Scopoletin and Deacetylasperulosidic acid could not be found, with the best path forward considered to be to continue with the SPE-TLC with UV visualisation for the Scopoletin using an OASIS® HLB SPE column based on the West & Deng 2010[4] procedure. However modifying the DAA identification procedure by removing the SPE extraction step and just processing using TLC on a 1:1 v/v juice:MeOH sample mixture. As an identification test the analytical procedure performance characteristics to be demonstrated was only the specificity, which we completed by showing increasing TLC spot intensity with compound of interest concentration, analysis of 35 fermented Noni juice samples from a range of Pacific Island countries, verification of negative identifications for other juices, plus verification of 'positive' and 'negative' compound of interest identifications by an orthogonal HPLC method.

This is a new provision for a commodity where the draft standard and methods are in process of endorsement.

[1] REP20/NASWP para. 83 ii.
[2] REP21/MAS para. 13.
[3] REP23/NASWP para. 73 ii.

Agenda Item #3: Endorsement of Methods of Analysis Provisions and Sampling Plans in Codex Standards

Codex Committee for North America and the South West Pacific.

Methods of analysis for provisions in the draft regional standard for fermented noni fruit juice (CXS to be determined)

Attribute – Identification of Scopoletin	***Method - ANNEX A***
Matrices, samples used in SLV studies	In study on which method is based[4] used 4 commercial juices. In Annex C study 35 fermented Noni Juice from 9 different Pacific Island countries; plus 6 other fruit juices.
Concentration range of matrices validated	Study from which method is based reported typical range as 0.0037-0.0212 mg/mL[4], while Annex C study showed 'positive' results for all 35 samples. All 6 other fruit juices show 'negative' results.
Repeatability (RSD_r or s_r)	Not required for IDs.
Reproducibility (RSD_R or s_R)	Not required for IDs.
Recovery range from SLV/MLT	Not required for IDs.
Accuracy (Certified materials)	Not required for IDs.
Limit of Quantitation	Standards suggest a LOD=0.001, thus LOQ=0.003 mg/mL based on standards responses, verifying study[4] which reported 0.0037mg/L for SPE-TLC.

[Note: SLV refers to Single Laboratory Validation. MLT refers to Multi-Laboratory Testing studies (i.e. collaborative studies).]

Attribute – Identification of Deacetylasperulosidic acid	***Method - ANNEX B***
Matrices, samples used in SLV study	35 Different fermented Noni Juice from 9 different Pacific Island countries. Plus 6 other fruit juices.
Concentration range of matrices validated	The different studies show commercial Noni juices to contain, 1.08 to 1.52 mg/mL (4 samples)[5], 0.2-1.7 mg/mL(4 samples)[4], and 0.23-2.43 mg/mL(7 samples)[6]. While Annex C study showed 'positive' results for 30 of 35 samples. All 6 other fruit juices show 'negative' results.
Repeatability (RSD_r or s_r)	Not required for IDs.
Reproducibility (RSD_R or s_R)	Not required for IDs.
Recovery range from SLV/MLT	Not required for IDs.
Accuracy (Certified materials)	Not required for IDs.
Limit of Quantitation	Standards suggest a LOD=0.1, thus LOQ=0.25 mg/mL based on standards responses, verifying study[4] which reported 0.2mg/L for TLC.

Summary of proposed changes in CXS 234, including retyping of existing methods and recommendations to CCMAS

[4] West, Brett & Shixin, Deng. (2010). Thin Layer Chromatography Methods for Rapid Identity Testing of Morinda citrifolia L. (Noni) Fruit and Leaf. Advance Journal of Food Science and Technology 2(5): 298-302.
[5] Bittová M, Hladůkova D, Roblová V, Krácmar S, Kubán P, Kubán V. Analysis of Organic Acids, Deacetyl Asperulosidic Acid and Polyphenolic Compounds as a Potential Tool for Characterization of Noni (Morinda citrifolia) Products. Nat Prod Commun. 2015 Nov;10(11):1817-20. PMID: 26749805.
[6] Potterat O, Felten RV, Dalsgaard PW, Hamburger M. Identification of TLC markers and quantification by HPLC-MS of various constituents in noni fruit powder and commercial noni-derived products. J Agric Food Chem. 2007 Sep 5;55(18):7489-94. doi: 10.1021/jf071359a. Epub 2007 Aug 14. PMID: 17696360.

Table 1. Recommended Methods of Analysis and Sampling (CXS 234-1999)

Commodity	***Provision***	***Method***	***Principle***	***CXS***	***Proposed Type***
fermented noni fruit juice	Identification of scopoletin	Annex A*	Solid phase extraction and thin layer chromatography	TBA	IV
	Identification of deacetylasperulosidic acid	Annex B*	Thin layer chromatography	TBA	IV

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

Recommendations to CCMAS

CCNASWP recommends CCMAS to take the following actions:

1. Endorse SPE-TLC method as provided (Annex A updated) as Type IV for the Identification of Scopoletin in Fermented Noni Juice.
2. Endorse TLC method as provided (Annex B updated) as Type IV for the Identification of deacetylasperulosidic acid in Fermented Noni Juice.

   Where the procedural manual states a Type IV typing reflects a recently introduced method but for which the criteria required for acceptance by the Committee on Methods of Analysis and Sampling 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.
3. Accept the following Single Laboratory Verification / Validation for Identification of Scopoletin by SPE-TLC and Deacetylasperulosidic Acid by TLC in Fermented Noni Juice Annex C.
4. That CCMAS endorses the TLC methodologies recommended above to facilitate progression of the Draft Fermented Noni Juice standard to stage 8. But with the expectation that a HPLC-DAD method will be available in the future.

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)[1] 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.

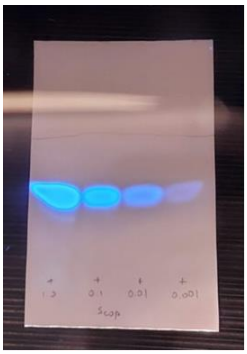

Figure 1 TLC for Scopoletin standards at 1.0, 0.1, 0.01 and 0.001 mg/mL in MeOH at 365nm.

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

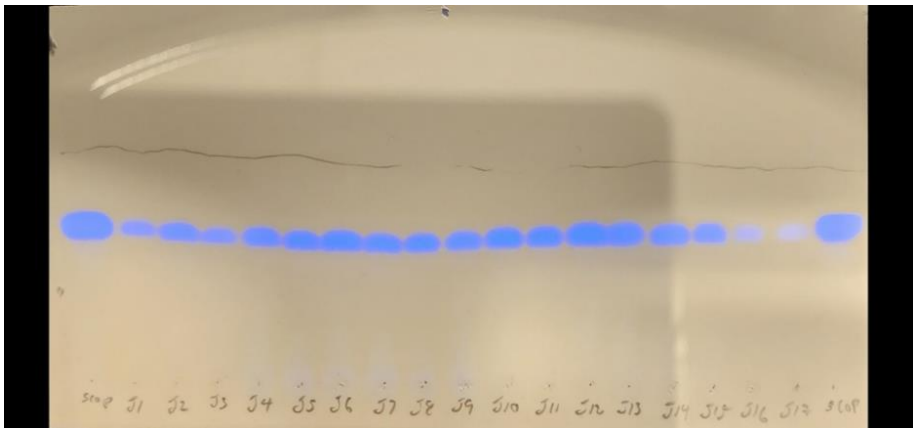

Figure 2. Scopoletin standard plus SPE extraction & TLC with UV@365nm visualization of fermented Noni juice samples, with left to right, standard; fermented Noni juice samples J1-17; standard.

Standard and Pacific Island samples J18-J19.

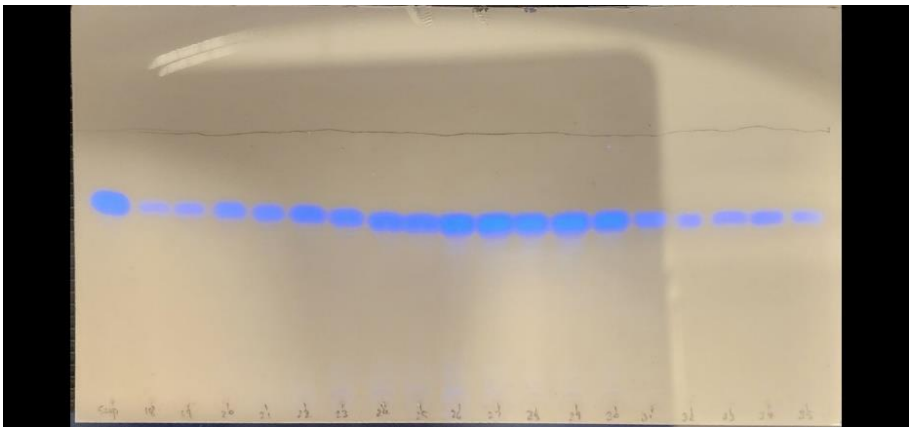

Figure 3. Scopoletin standard plus SPE extraction & TLC with UV@365nm visualization of fermented Noni juice samples, with left to right, standard; fermented Noni juice samples 18-35.

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.

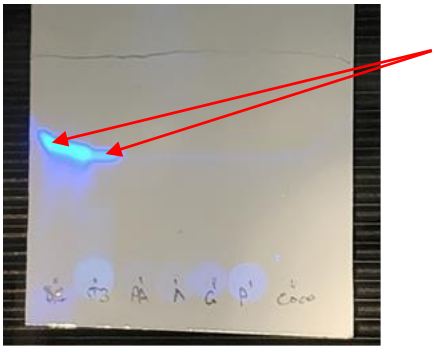

Figure 4. Scopoletin ID TLC for a Noni juice plus various other juices; from left to right, Scopoletin (0.1mg/mL), Noni Juice#3(J3), Pineapple juice (PA), Apple and Blackcurrant juice(A), Grape juice(G), Pear juice(P), and Coconut juice (Coco). Scopoletin band for standard and Noni Juice#3 indicated 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)[1] 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.

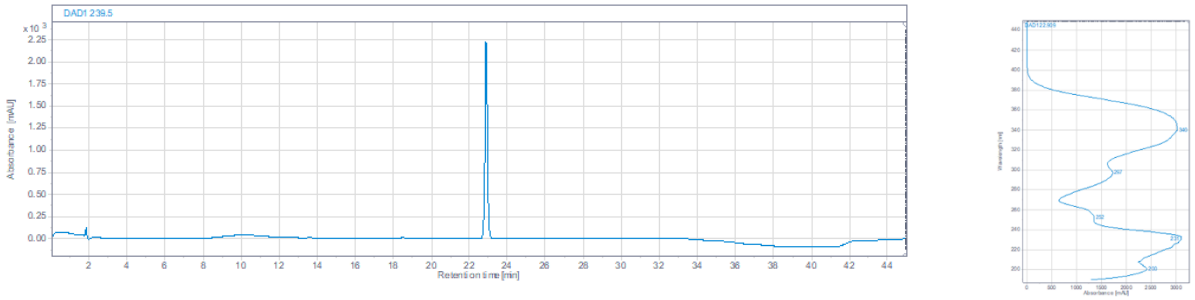

Figure 5. Scopoletin standard, HPLC-DAD chromatogram, 10µL injection, @ 239.5nm and peak UV spectra.

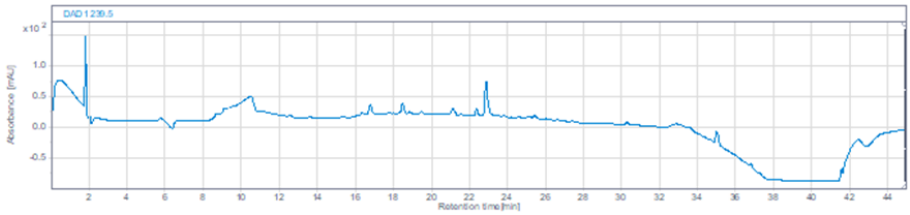

Figure 6. Juice#3, filtered, HLB-SPE 100% MeOH elution solution and injected 10µL on HPLC-DAD @ 239.5nm

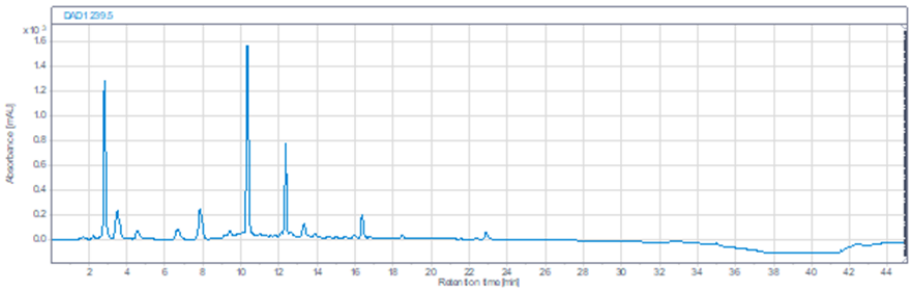

Figure 7. Juice#3, filtered 0.45um, no SPE, and direct injected 10µL on HPLC-DAD @ 239.5nm.

See section f) for HPLC-DAD conditions.

For Deacetylasperulosidic acid Identification

a) Colour response with 2% anisaldehyde / 4% 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.

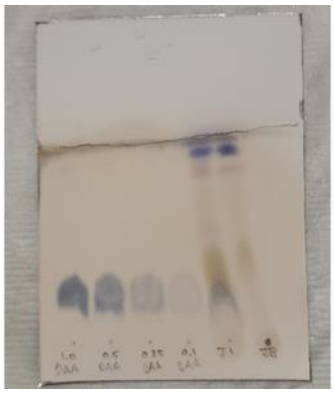

Figure 8. TLC standard solutions spots 1.0 mg/mL, 0.5 mg/mL, 0.25 mg/mL, 0.1 mg/mL; Juice 1; Juice 8.

b) Colour response with 2% anisaldehyde / 4% sulfuric acid-ethanol (EtOH) solution then heating for visualisation, and R_f relative to standard Deacetylasperulosidic acid for various Pacific Island samples.

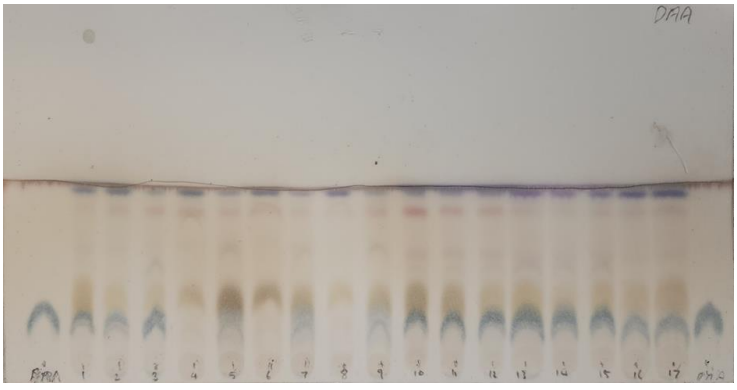

Figure 9. From left to right, DAA standard; fermented Noni juice samples 1-17; DAA standard; with TLC visualised with 2% anisaldehyde / 4% sulfuric acid-ethanol (EtOH) solution then heating.

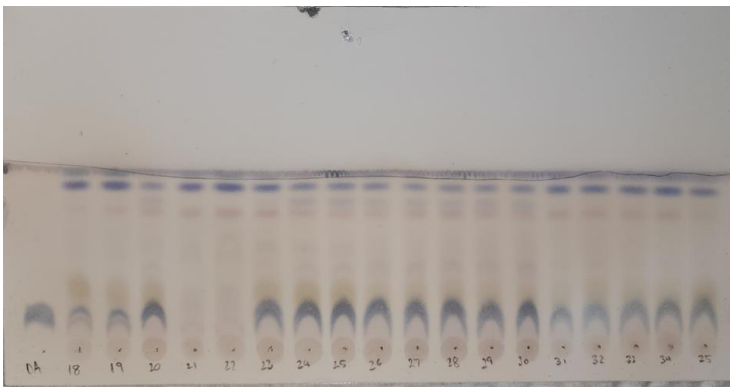

Figure 10. From left to right, DAA standard, fermented Noni juice samples 18-35; with TLC visualisation with 2% anisaldehyde / 4% sulfuric acid-ethanol (EtOH) solution then heating.

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.

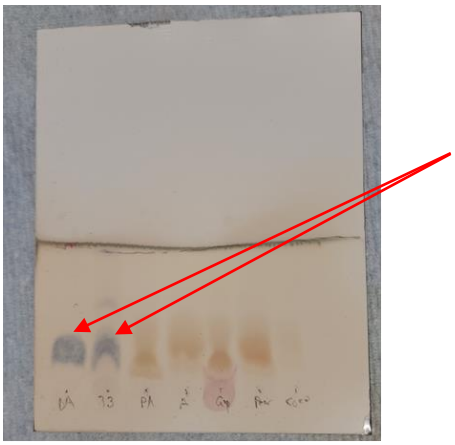

Figure 11. Deacetylasperulosidic acid ID TLC for a Noni juice plus various other fruit juices; from left to right, Deacetylasperulosidic acid (0.5mg/mL DA), Noni Juice#3(J3), Pineapple juice (PA), Apple and Blackcurrant juice(A), Grape juice (Gp), Pear juice (Pear), and coconut juice (Co). Deacetylasperulosidic 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.

An orthogonal HPLC technique based on Choi et al (2022)[1] 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.

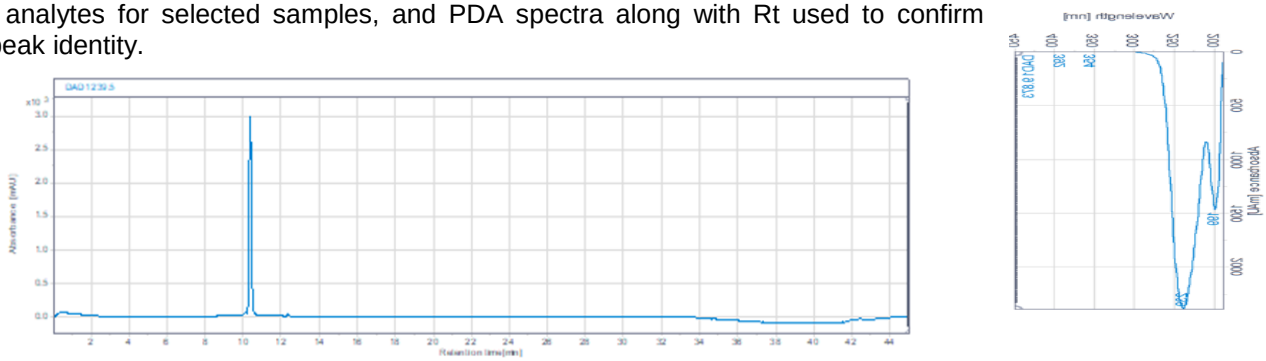

Figure 12. Deacetylasperulosidic acid 2 mg/mL; HPLC-DAD chromatogram, 10µL injection, @ 239.5nm; and peak UV spectra.

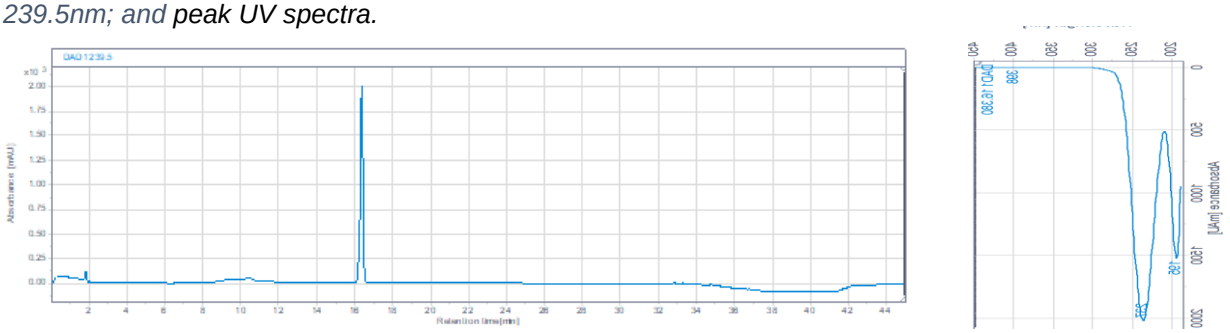

Figure 13. Asperulosidic acid; HPLC-DAD chromatogram, 10µL injection, @ 239.5nm; and peak UV spectra.

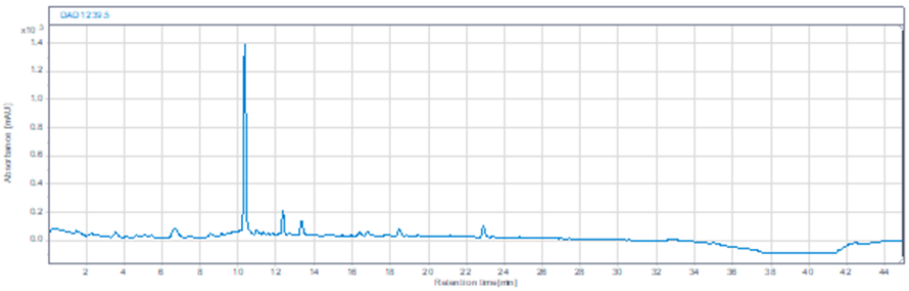

Figure 14. Juice 1, 0.45um filtered & no SPE, direct injected 10µL on HPLC-DAD @ 239.5nm

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.

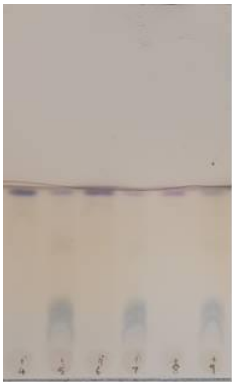

Figure 15 Cropped section of Fig 9, highlighting Deacetylasperulosidic acid ID by TLC for a selection of various Pacific Island Noni Juice samples where 'negative' and 'positive' DAA IDs were observed.

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.

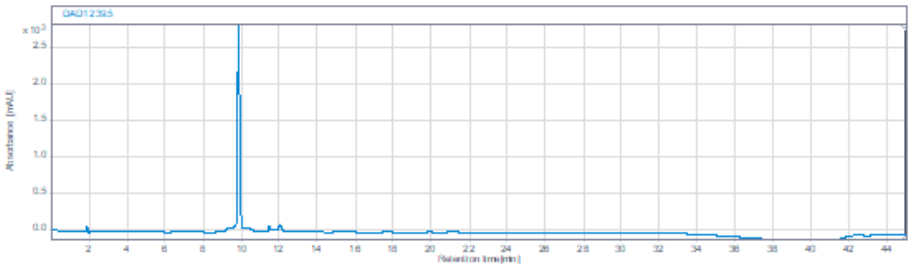

Figure 16. HPLC of DAA standard 2mg/mL with peak at 9.9mins.

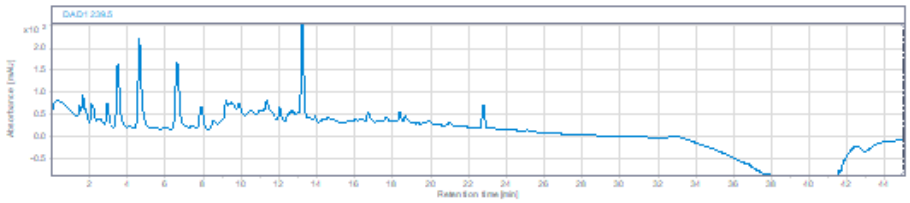

Figure 17. HPLC injection of Pacific Island juice#4, confirming 'negative' result for DAA.

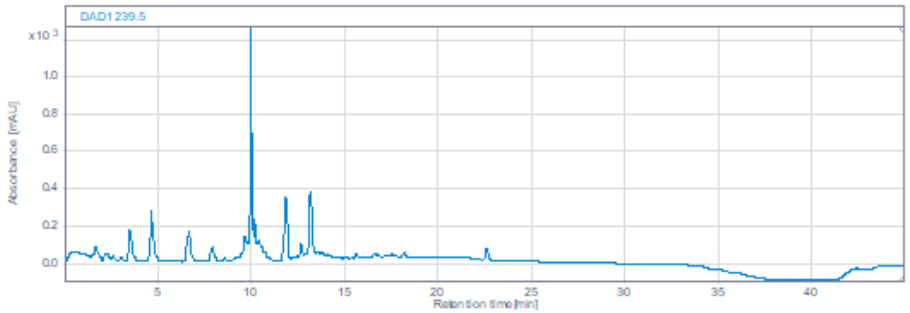

Figure 18. HPLC injection of Pacific Island juice#5, confirming 'positive' result for DAA.

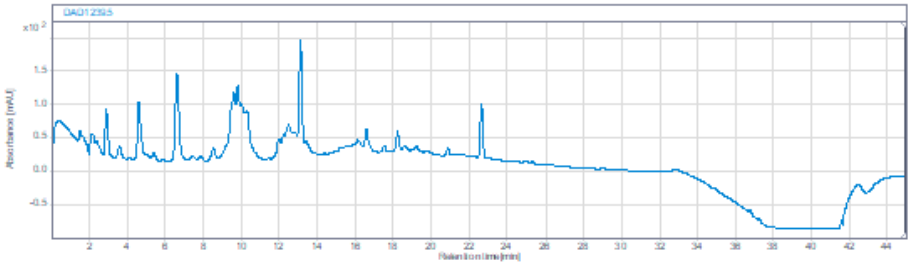

Figure 19. HPLC injection of Pacific Island juice#6, confirming 'negative' result for DAA.

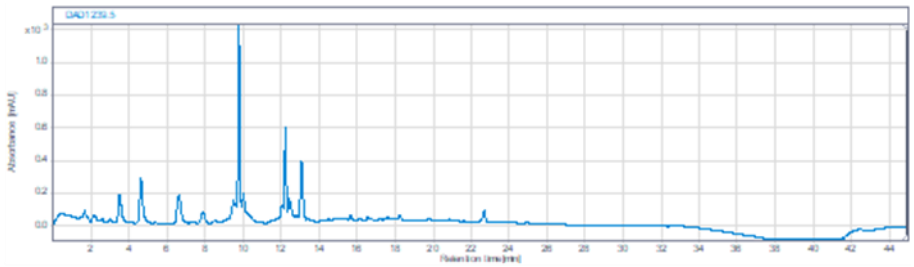

Figure 20. HPLC injection of Pacific Island juice#7, confirming 'positive' result for DAA.

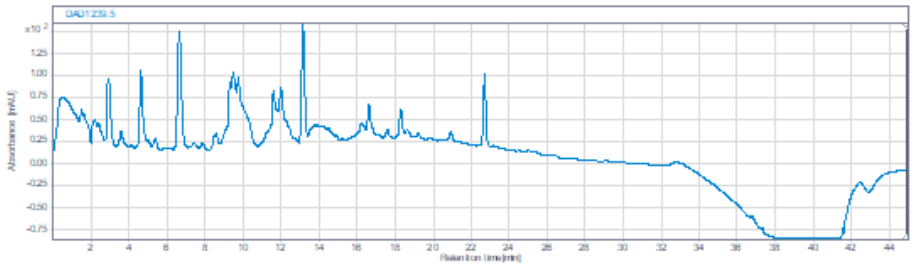

Figure 21. HPLC injection of Pacific Island juice#8, confirming 'negative' result for DAA.

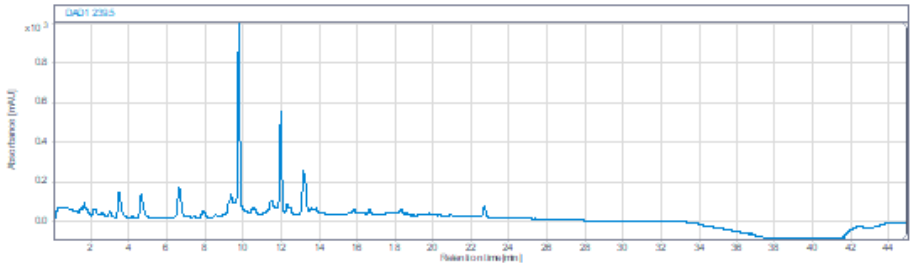

Figure 22. HPLC injection of Pacific Island juice#9, confirming 'positive' result for DAA.

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 ºC

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)

REFERENCES

[1] Choi, S.-I.; Kwon, H.-Y.; La, I.-J.; Jo, Y.-H.; Han, X.; Men, X.; Lee, S.-J.; Kim, Y.-D.; Seong, G.-S.; Lee, O.-H. Development and Validation of an Analytical Method for Deacetylasperulosidic Acid, Asperulosidic Acid, Scopolin, Asperuloside and Scopoletin in Fermented Morinda citrifolia L. (Noni). Separations 2021, 8, 80.