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Research Article

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Development and validation of a normal phase HPLC method for separation of anacardic acid isomers in cashew nut shell liquid

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ABSTRACT

A simple normal phase HPLC method was developed and validated for the separation and determination of anacardic acid isomers present in cashew nut shell liquid. A Chiralpack, IA Chiral column (250 mm x 4.6 mm, packed with 5 microns) is used as stationary phase. An isocratic mode with mobile phase consists of 0.1% trifluroacetic acid in n- Hexane and IPA in ration of 1:1000:10 (v/v/v), at a flow rate of 1 mL/min. and the eluent was monitored at a wavelength of 210 nm. The result obtained from method validation showed a good agreement with the declared content. The method shows good linearity in the range of 0.06-0.24 mg/mL with correlation coefficient 0.9997-0.9999. Analytical recovery was between 99.0 -102.0 %. The proposed method successfully applied for separation of anacardic acid isomers i.e. saturated, monoene, diene and triene present in cashew nut shell liquid. The established normal phase HPLC method is precise, accurate and selective, because of its sensitivity and reproducibility.

Key words: Anacardicacid; Cashew nut shell liquid; HPLC; Validation.

INTRODUCTION

Anacardic acid is chemically known as 2-hydroxy-6-[(8Z, 11Z)-pentadeca-8, 11, 14-trienyl] benzoic acid [8]. The term "anacardic acid" is used to describe the mixture of 6-n-alkylsalicylic acids which constitute the major part of the extract of cashew nut shells (*Anacardium occidentale*). Its principal components, 6-pentadecy1, 6-(pentadec-8-enyl), 6-(pentadec- 8, ll-dienyl) and 6-(pentadec-8, 11, 14-trienyl) salicylic acids are accompanied by small amounts of the C_{13} to C_{17} side chain homologues. As they are closely related to urushiol, they also cause an allergic skin rash on contact, known as urushiol-induced contact dermatitis. Anacardic acid [1-8] is a yellow liquid. It is partially miscible with ethanol and ether, but nearly immiscible with water. Chemically, anacardic acid is a mixture of several closely related organic compounds. Each consists of a salicylic acid substituted with an alkyl chain that has 15 or 17 carbon atoms. The alkyl group may be saturated or unsaturated; anacardic acid is a mixture of saturated and unsaturated molecules. The exact mixture depends on the species of the plant of which the 15 carbon unsaturated side chain found in the cashew plant is very lethal to Gram positive bacteria. There is also suspected that anacardic acid may arrest the growth of cancer tumors such as breast cancer. The anacardic acid aliphatic side chain isomers {$C_{15}H_{31-n}$, where n = 0 (Saturated), n = 2 (Monoene), n = 4 (Diene), n = 6 (Triene)}. The chemical structure is shown in figure 1.

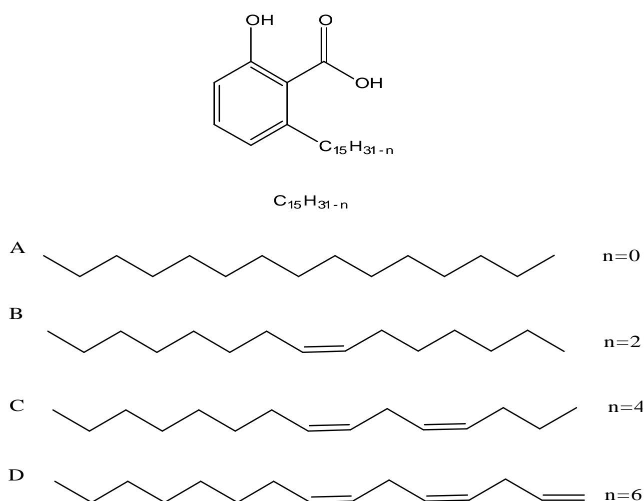

Figure 1: Structure of anacardic acid (A) saturated, (B) monoene, (C) diene and (D) triene

The analytical method was validated [9-11] as per current International Conference on Harmonization (ICH) guidelines [12].

EXPERIMENTAL SECTION

Apparatus and Chromatographic Conditions: Agilent 1200 series HPLC, G1331A Quaternary pump connected with G1314B Variable Wavelength detector, G1316A Thermostatted Column Compartment, G1329A ALS autosampler. The data acquisition was performed by Agilent Chemstation software. The chromatographic separation was performed using a Chiralpack, IA Chiral column (250 mm x 4.6 mm, packed with 5 microns) is used as stationary phase. An isocratic mode with mobile phase consists of 0.1% trifluroacetic acid in n- Hexane and IPA in ration of 1:1000:10 (v/v/v), at a flow rate of 1 mL/min. and the eluent was monitored at a wavelength of 210 nm. The column temperature was maintained at 25 °C temperature and injection volume 5 µL was used. The total runtime was 30 min. The mobile phase was filtered through 0.45 µm nylon membrane (Millipore) prior to use.

Reagents: n-Hexane (HPLC grade, MERCK), Isopropyl alcohol (IPA) (HPLC grade, Thomas Baker) and trifluoroacetic acid (AR grade), saturated, monoene, diene and triene anacardic acid.

Preparation of Mobile Phase:
The mobile phase consists of 0.1% trifluroacetic acid in n- Hexane and IPA in ration of 1:1000:10 (v/v/v), mix well. Mobile phase was filtered through a 0.45 µm nylon membrane (Millipore) and degassed in an ultrasonic bath (Branson-5510). Mobile phase used as diluent for standard and sample preparations.

Preparation of Standard Solution:
The standard stock solution 2 mg/mL of saturated, monoene, diene and triene was prepared in diluent. The resulting solution was transferred to standard analytical HPLC glass vials and injected into the HPLC.

Preparation of Sample Solution:
To prepare a stock solution of sample, 20 g CNSL were weighed mixed and extracted. An aliquot of sample equivalent to the weight of 100 mg sample was accurately weighed and transferred to 50 mL volumetric flask and dissolved in 20 mL of diluent and the mixture was sonicated for 30 min. The contents of the flask were then left to return to room temperature and volume was adjusted with the same solvent mixture. This solution 20 mL was filtered through a 0.45 µm nylon syringe filter. The concentration obtained is 2 mg/mL of anacardic acid.

RESULTS AND DISCUSSION

Method Development:

The object of this work was to develop and validate a simple, rapid and sensitive isomer separation method for the determination of saturated, monoene, diene and triene anacardic acid. To achieve the object, different options were evaluated. Optimization of the chromatographic conditions is intended to take into account that various goals of method development like good resolution, runtime sensitivity, peak symmetry, theoretical plates, tailing factor, etc. The chromatographic conditions were optimized by changing the mobile phase composition and buffers used in the mobile phase. Different ratios were experimented to optimize the mobile phase. Finally a mixture of IPA and 0.1 % trifluroacetic acid in n-Hexane in the ratio of 10:1000 was found to be suitable. 5 µL of the assay preparation using the optimized chromatographic condition was injected in to the NP-HPLC. The retention time of anacardic acid isomers is Saturated (~13.941 min), Monoene (~14.701 min), Diene (~16.432 min) and Triene (~18.462 min) and the typical chromatogram is shown in figure 2.

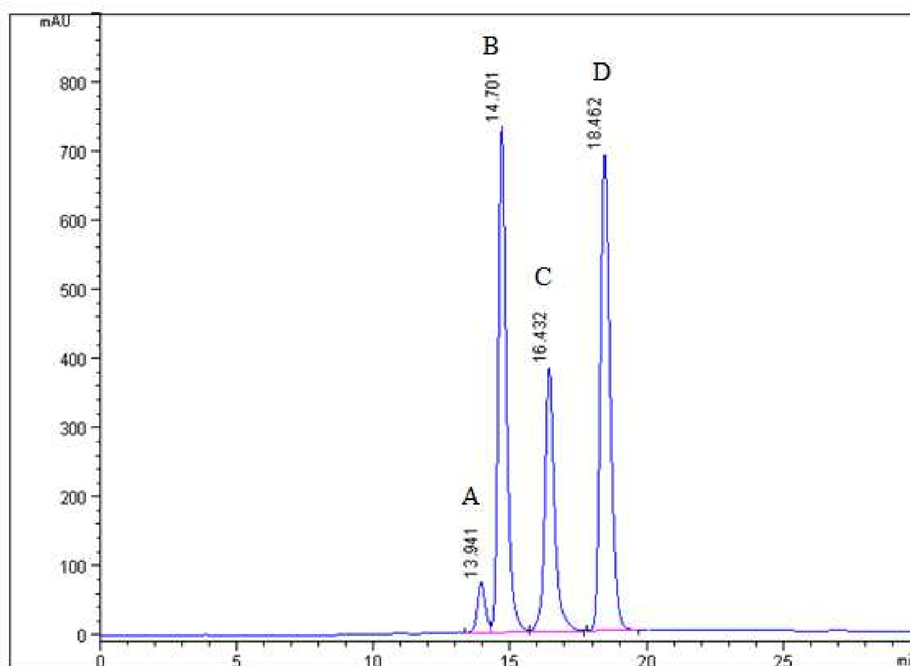

Figure 2: A typical chromatogram showing the peaks of anacardic acid (A) saturated, (B) monoene, (C) diene and (D) triene

Accuracy and Precision:

Accuracy of developed method was confirmed by doing recovery study as per ICH norms. A known quantity of the pure compound was added to the test sample at three different concentration levels 50 %, 100 % and 150 % by replicate analysis (n=3). From the recovery study it was clear that the method is very accurate for quantitative estimation of anacardic acid isomers in CNSL as all the statistical results were within the range of acceptance between 99. 0 -102.0 %, which indicates accuracy of the method. The precision of method was ascertained from the peak area response obtained by actual determination of six replicates of a fixed amount of drug. The percent relative standard deviations were calculated for anacardic acid isomers and presented in the table 1.

System Suitability:

The system suitability test ensures the validity of the analytical procedure as well as confirms the resolution between different peaks of interest. All critical parameters tested met the acceptance criteria on all days. The following system suitability criteria were fulfilled in the chromatograms-viz., % RSD of anacardic acid isomers less than 2.0, tailing factor is between less than 2.0 and theoretical plates more than 2000. As shown in the chromatogram, all the analytes are eluted by forming symmetrical single peaks well separated from the solvent front. Results are tabulated in table 2.

Table 1: Precision study of anacardic acid

Parameter: Precision		
Set No.	Anacardic acid isomers	
	Method	Intermediate
1	99.27	99.24
2	99.18	99.16
3	99.31	99.56
4	99.68	99.47
5	99.18	99.19
6	99.61	99.74
Mean	99.37	99.39
SD	0.22	0.23
RSD %	0.22	0.24

Linearity:

The linearity of the method was tested from 0.06 - 0.12 mg/mL for saturated, 0.12- 0.24 mg/mL for monoene, 0.06-0.12 mg/mL for diene, and 0.12- 0.24 mg/mL for triene anacardic acid. Linearity solutions were injected in triplicate and the calibration graphs were plotted as peak area of the analyte against the concentration of the sample in mg/mL. In the simultaneous determination, the calibration graphs were found to be linear for both the analytes in the mentioned concentrations and the correlation coefficients for the regression line were 0.9999, 0.9997, 0.9998 and 0.9997 for saturated, monoene, diene and triene anacardic acid respectively see figure 3.

Table 2: System suitability test results of anacardic acid

Parameter: System suitability Anacardic acid isomers								
S. No.	**Saturated**		**Monoene**		**Diene**		**Triene**	
	RT	Area	RT	Area	RT	Area	RT	Area
1	13.941	1522	14.701	16236	16.432	9965	18.462	18501
2	13.943	1535	14.706	16446	16.436	10031	18.463	18661
3	13.939	1550	14.701	16634	16.431	10128	18.469	18849
4	13.946	1536	14.709	16273	16.436	10135	18.469	18736
5	13.949	1544	14.704	16392	16.431	10025	18.462	18644
6	13.946	1528	14.701	16227	16.437	10116	18.463	18598
Average	13.944	1536	14.704	16368	16.434	10067	18.465	18665
SD	0.004	10.1	0.003	157.12	0.003	69.49	0.003	119
RSD %	0.03	0.66	0.02	0.96	0.02	0.69	0.02	0.64
Tailing Factor		1.05	-	1.32	-	1.22	-	1.26
Theoretical Plates		11516	-	13223	-	13339	-	13521
Resolution		-	-	1.47	-	3.2	-	3.37

Solution Stability:

In order to demonstrate the stability of both standard and sample solutions during analysis, both solutions were analyzed over a period of 24 hours at an interval of 2 hours at room temperature. The results show that for solutions, the retention time and peak area of anacardic acid isomers remained unchanged and no significant degradation within the indicated period, this indicates that both solutions were stable for 24 hours.

Robustness:

The robustness of the method was evaluated by assaying test solutions after slight but deliberate changes in the analytical conditions flow rate (± 0.1 mL/min), the proportions of buffer: methanol (5:95 and 10:90, v/v) and detector wavelength (± 5 nm). The method was found to be robust, although small deliberate changes in method conditions did have a negligible effect on the chromatographic behavior of the solute. The results indicate that changing the detector wavelength had no large effect on the chromatographic behavior of anacardic acid isomers. The analytical method therefore remained unaffected by slight but deliberate changes in the analytical conditions.

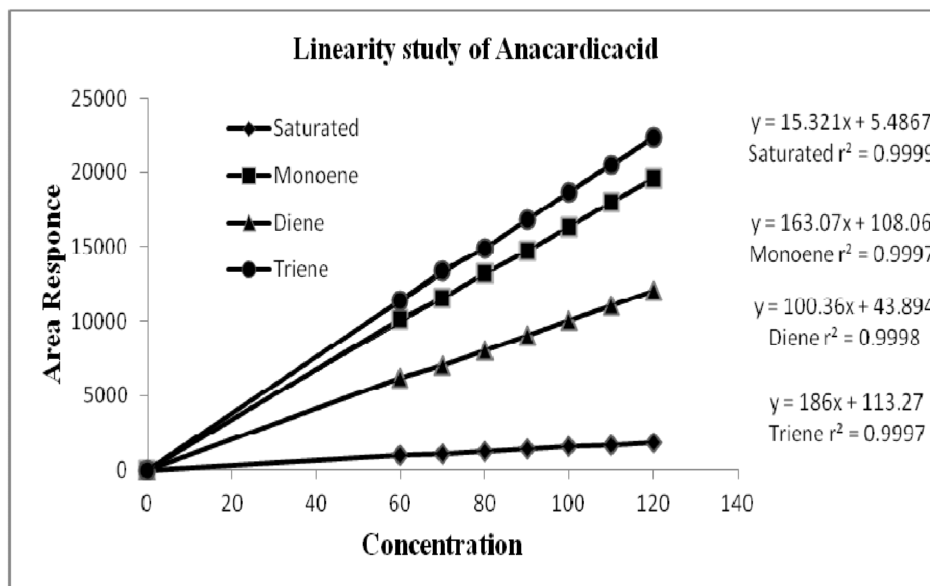

Figure 3: Graph showing linearity and range of Anacardic acid

CONCLUSION

The proposed NP-HPLC method for separation of anacardic acid isomers is simple, precise, accurate, robust and selective. The validation of the analysis proved that the method is reproducible and efficient for the determination of anacardic acid in CNSL form without any interference from the sample excipients. Hence, the method can be easily and conveniently applied for routine analysis in quality control laboratories and research institutes for the determination and separation of saturated, monoene, diene and triene anacatdic acid.

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