ISSN: 2581-5466, Vol. I, Issue. I, Dec-2017

International Journal of Scientific Research Letters

Journal Home page: http://cphfs.in/research.php

Comparison of TLC and GCMS techniques for steryl glucoside analysis

Ashutosh Singh
Department of Biochemistry, University of Lucknow, Lucknow, UP, India.
singh_ashutosh@lkouniv.ac.in

Nitin Bhardwaj
Government Post Graduate College, Karanprayag, UK, India.

Madri Kakoti
Department of Linguistics, University of Lucknow, Lucknow, UP, India.

Taruni Lalchandani
Dr. Ram Manohar Lohia Institute of Medical Sciences, Lucknow, UP, India.

Abstract: ***Steryl glucosides are a complex class of glycolipds, which in recent times, has gained importance due to their significant roles in modulating the immune system. Therefore, a much emphasis has been put towards their accurate identification and quantification in laboratory settings. In the present study, we have compared the thin layer chromatography and gas chromatography – mass spectrometry techniques, the two most commonly used methods employed in analysis of sterols and sterol derivatives. Our data clearly shows that while gas chromatography – mass spectrometry is a better technique to analyze steryl glucosides, thin layer chromatography does provide sufficient information that may be useful in comparative studies. Here, the advantages and disadvantages of these techniques in their ability to analyze steryl glucosides have been experimentally demonstrated.***

Key words: Lipids, chromatography, mass spectrometry.

Abbreviations: TLC, thin layer chromatography; GCMS, gas chromatography mass spectrometry; BSTFA/TMCS, (N,O-bis(trimethylsilyl) trifluoroacetamide/TMCS (trimethylchlorosilane).

Running title: Chromatographic approaches for analysis of lipids.

Contribution to the manuscript: AS performed the experiments and data analysis. AS, NB, MK and TL provided significant scientific inputs, wrote and edited the manuscript.

I. INTRODUCTION

Steryl glucosides are a complex class of glycolipids produced in a wide variety of organisms including plants, animals, fungi and bacteria [1,2]. Plants have been shown to contain as much as 80% of their phytosterols as steryl glucosides [3,4]. In humans, several studies have reported that steryl glucosides improve immune response [5-9]. For example, *in vitro* steryl glucosides have shown to improve cytokine secretion in mice [5]. Several studies have shown that immuization with steryl glucosides resulted in improved survival of mice infected with lethal fungal titres [9,10]. Considering the importance of the steryl glucosides, much emphasis has been made to develop robust analytical methods for their accurate detection and quantification.

In earlier days, researcher used alkaline saponification or the acid hydrolysis methods to detect steryl glucosides [11]. However, these methods showed limited detection and poor estimation accuracy, and are now considered obsolete. Qualitative detection of steryl glucosides could be easily achieved using the thin layer chromatography (TLC) approach, as appropriate standards are commercially available [10]. It is important to note that TLC is not the method of choice for quantitative analysis of steryl glucosides. More recently, direct

detection of intact steryl glucosides using gas chromatography mass spectrometry (GCMS) methods has allowed fast, reliable and accurate detection of these glycolipids [2,10,12,13].

In the present study we have compared the TLC and GCMS methods for their quantitative efficiencies of steryl glucoside detection. The aim is to develop a simple yet precise method for steryl glucoside analysis.

II. MATERIALS AND METHODS

Reagents: Lipid standards were purchased from Avanti polar lipids Inc., US. All solvents and chemicals, unless specifically mentioned, were LCMS (liquid chromatography mass spectrometry) grade purchased from Sigma Aldrich, US and Fisher Scientific, UK.

TLC analysis: Pre-coated silica plates (Merck) were activated by heating at 80°C for 30 min. Dry plant steryl glycoside standards (Avanti Polar Lipids, Inc.) were dissolved in 20 uL chloroform and were applied in band-form using a Hamilton syringe. The TLC plates were resolved in a tank containing chloroform/methanol/water (65/25/4 by volume) [10]. TLC plates were then dried and stained with iodine vapour for the visualization of steryl glucoside band. To semi-quantify the band area, $MnCl_2$ charring of the TLC plate was performed using a solution containing: 0.63 g $MnCl_2.4H_2O$, 60 mL H_2O, 60 mL CH_3OH, 4 mL conc. H_2SO_4 [14]. TLC plates were photographed using Gel Doc EZ System (BioRad) and band densities calculated using the Image Lab™ Touch software.

GCMS analysis: Steryl glucoside samples were derivatized using 100 µl BSTFA/TMCS (N,O-bis(trimethylsilyl) trifluoroacetamide/TMCS (trimethylchlorosilane) reagent (Sigma Aldrich, US) at 85°C for 90 min [10,15]. Next, 40 µl *n*-hexane was added to the derivatized sample and vortexed. The samples were then analyzed using 30 mt. (0.25 µm) DB5-MS column on Agilent 7890 GCMS (Agilent Technologies, CA, US). Steryl glucosides were analyzed using the initial column temperature of 270°C ramped at 2°C/min to 315°C, with hold of 20 min at 315°C. All EI-mass spectra were recorded at 70 eV with an ion source temperature of 230 °C. The mass spectral patterns of plant steryl glucoside standard (Avanti Polar Lipids, Inc.), published earlier were used a reference (Table 1) [12].

TABLE 1. Detection of steryl glucosides by GCMS. * ND represents 'no detection'. 'Q.C.', quality control.

Plant SG species	Peak#	Estimated Abundance (%)	m/z	Q.C. Abundance (%)
β-Campetseryl glucoside	1	29.04 ± 3.46	383.3	25
β-Stigmasteryl glucoside	2	15.34 ± 1.85	395.4	18
β-Sitosteryl glucoside	3	55.62 ± 5.21	397.4	56
β-Δ5-Avenosteryl glucoside	ND	ND	ND	1

III. RESULTS

Analysis of steryl glucosides by TLC: To evaluate the detection efficiency of TLC technique, standard concentrations of the standard steryl glucosides ranging from 1 – 32 µg were loaded and TLC analysis was performed as described in methods. The standard mix of steryl glucoside containing β-Campetseryl glucoside, β-Stigmasteryl glucoside, β-Sitosteryl glucoside and β-Δ5-Avenosteryl glucoside (in % ratio of 25:18:56:1; represented as % QC abundance in Table 1) were resolved as a single band on the TLC plate (Figure 1A). The TLC separation buffer used was unable to resolve the steryl glucoside mixture into the respective species (Figure 1A). The densitometry analysis of the observed bands showed that the area density (a.u.) of these bands has a linear relationship up to 32 µg loadings (Figure B). To test the quantitative efficiency, 3 control unknowns, of 5, 10 and 20 µg steryl glucosides, were also analyzed by the TLC (Figure 1A). The densitometry based estimations showed that we can quantify the % concentrations of these unknown bands with < 4% variation compared to the expected known concentrations (Figure 1C).

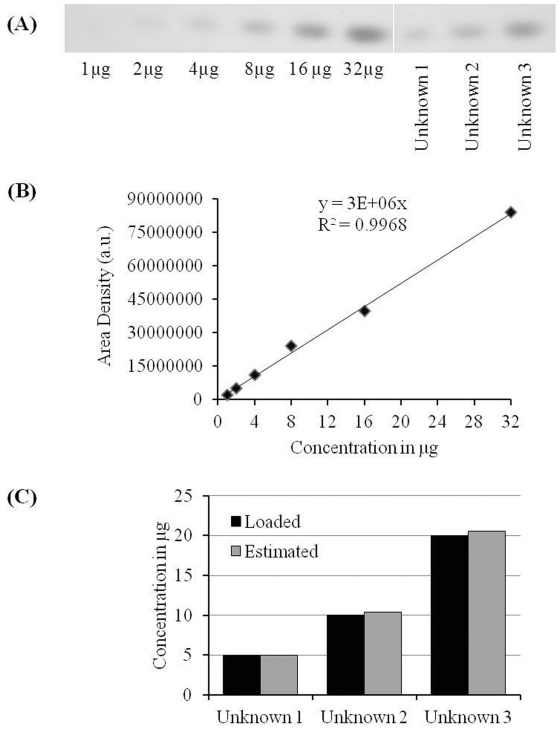

Figure 1. Quantification of steryl glucosides by TLC. (A) Detection of varying concentrations of plant steryl glucoside standard and the 3 unknown loading control concentrations. (B) Calibration curve showing area densities obtained for the corresponding band on TLC. (C) Comparison of estimated concentration of sterol glucosides to the loading controls.

Analysis of steryl glucosides by GCMS: To evaluate the detection efficiency of GCMS technique, standard mix of steryl

glucoside was derivatized and analyzed by GCMS as described in methods. In the GC chromatogram, we could see a clear separation and detection of 3 major steryl glucoside species, namely β-Campetseryl glucoside, β-Stigmasteryl glucoside and β-Sitosteryl glucoside (Figure 2A, Table 1). The β-Δ5-Avenosteryl glucoside species remained below detection in our analysis (Figure 2A, Table 1). Respective peaks were identified using the characteristic m/z ions reported for these species (Table 1) [12]. The calibration curve between the observed mass spectral signal versus the loaded concentrations remained linear up to 16 µg concentration (Figure 2B). The estimated % abundance of β-Campetseryl glucoside, β-Stigmasteryl glucoside and β-Sitosteryl glucoside species was quite similar to the expected % abundance that is reported for this mixture (Table 1).

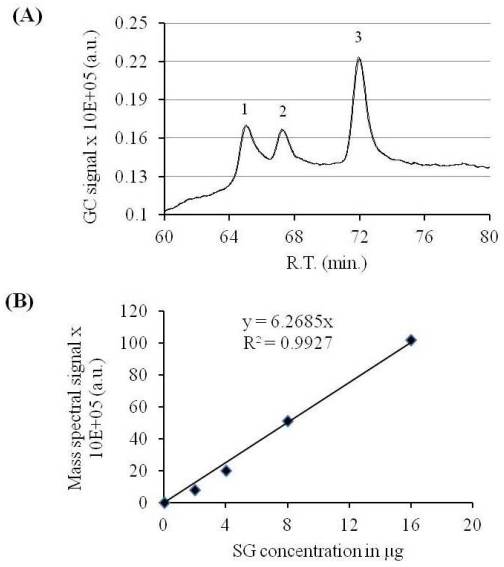

Figure 2. Quantification of steryl glucosides by GCMS. (A) Representative chromatogram for the detection of plant steryl glucoside standard on GC. Peak 1, 2 and 3 represent β-Campetseryl glucoside, β-Stigmasteryl glucoside and β-Sitosteryl glucoside, respectively. The x-axis has been depicted starting at 60 min for clarity. (B) Calibration curve showing peak area intensities (represented as the 'mass spectral signal') obtained for the corresponding peak on GC.

IV. DISCUSSION

In the present study, we compared the detection and quantification efficiency of steryl glucosides by TLC and GCMS techniques. In our TLC analysis we could easily resolve the steryl glucoside as a clear band. However, TLC failed to separate the steryl glucoside mixture into the respective species. Nonetheless, TLC showed good semi-quantitative efficiency, cost effectiveness, required less sample preparation time (no derivatization required), and therefore remains a useful method of detecting steryl glucosides. Evidently, TLC should be useful technique to analyze steryl glucoside in exploratory research work and when dealing with large sample sets. GCMS on the other hand provides more accurate analysis of steryl glucosides in terms of clear separation of individual steryl glucoside species, quantitative measurements and confirmed identity of the resolved peaks based on the mass spectrum. However, a few limitations to GCMS include long sample preparation time (requires derivatization by BSTFA/TMCS), higher sample running cost and expertise in solving structures using mass spectrum. Based on this study, we can conclude that both TLC and GCMS, with their advantages and disadvantages, are robust techniques for identification and quantification of steryl glucosides. The efficiency of these analyses could be improved by comparing and cross-validating the results obtained from both of these techniques.

ACKNOWLEDGEMENTS

AS thanks University Grant Commission, Government of India for financial assistance.

FINANCIAL AND COMPETING INTEREST DISCLOSURE

Author has no financial and competing interests with the subject matter or materials discussed in the manuscript.

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CORRESPONDING AUTHOR: ASHUTOSH SINGH

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