Characterization of natural monatin isomers, a high intensity sweetener from the plant *Sclerochiton ilicifolius* from South Africa

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Highlights

- Marfey's derivatization method and HPLC MS was used to separate monatin enantiomers.
- Both the 2*S*,4*S*; 2*R*,4*R* enantiomeric pair was present in extracts of *S. ilicifolius*.
- The 2*S*,4*S*; 2*R*,4*R* isomers were present in *S. ilicifolius* collected at different seasons.

ABSTRACT

The objective was to establish the natural occurrence of the various isomers of monatin in extracts of *Sclerochiton ilicifolius* plant material harvested from different growing regions in South Africa. The natural occurrence of the 2*S*,4*S* isomer has been reported as well as the synthesis of the 2*R*,4*R* isomer. The 2*R*,4*R* is reported as the most intense sweetness however its natural occurrence has not been fully reported, as a result it was not possible to establish whether these isomers are indeed already present in the plant or come from racemisation during the processing of the plant. The presence of the monatin isomers 2*S*,4*S*; 2*R*,4*R* in aqueous extracts of *S. ilicifolius* root bark was demonstrated in each sample harvested at two different time points. The *2R,4R*, *2S,4S*, *2R,4S*, and *2S,4R* monatin isomers were absent in the aqueous extracts of *S. ilicifolius* stem and leaf samples, however was shown to be present in the root bark, and root core samples. This report confirms previous findings which suggested that the 2*S*,4*S* and 2*R*,4*R* monatin isomers occur naturally in *S. ilicifolius*.

KEY WORDS

Monatin, Natural, Isomeric Distribution, *Sclerochiton ilicifolius*, South Africa, Plant Characterization, Chiral

1. INTRODUCTION

Monatin, (Indol-3-yl)-2-amino-4-carboxy-4-hydroxypentanoic acid (Figure 1), is a naturally occurring high intensity sweetener isolated from the bark of the roots of *Sclerochiton ilicifolius*, a spiny-leafed hardwood shrub growing in the rocky hills of the Limpopo Province in South Africa (Vleggaar et al., 1992). The same authors also assigned the 2*S*,4*S* absolute configuration to the natural levorotatory compound based on NOE NMR experiments on a cyclic derivative and the application of the empirical Clough–Lutz–Jirgenson rule. The crystal structure of synthetic 2*R*,4*R* monatin potassium salt dehydrate was only recently determined by single crystal X-ray structure analysis (Amino et al., 2016). The relative sweetness of the 2*S*,4*S* monatin isolated from the natural plant was reported in 1992 to be 1200 to 1400 fold more intense than that of sucrose however synthetic 2*R*,4*R* monatin, was reported much later to have a more intense sweetness than the 2*S*,4*S* isomer *i.e.* up to 2700 times that of 5% sucrose (Patent No. WO2003059865 A1, 2003). Monatin is compatible with other sweeteners and forms acceptable blends for example with aspartame. Extensive *in vivo* toxicology studies for 2*R*,4*R* monatin have also been completed moving it closer towards commercialization (Brathwaite et al., 2011, Brathwaite et al., 2014, Brathwaite et al., 2016, Brathwaite et al., 2016). The low concentration of monatin in the root bark has challenged scientists to develop processes to produce natural monatin in sizeable quantities for commercial use. While the synthesis of all four monatin stereo isomers has been reported the natural occurrence of the three remaining stereo isomers was demonstrated in an extract of the plant (Bassoli et al., 2005). However these scientists have referenced that owing to the small amounts available and the impossibility of obtaining a larger sample or information about the exact origin and detailed extraction methodology, it was not possible to establish whether these isomers are indeed already present in the plant or come from some racemisation during the processing of the sample and suggested that this should be further investigated. This study focused on determining the natural occurrence of monatin stereo isomers in minimally processed extracts through appropriate, sustainable, controlled harvesting procedures, extraction and analysis.

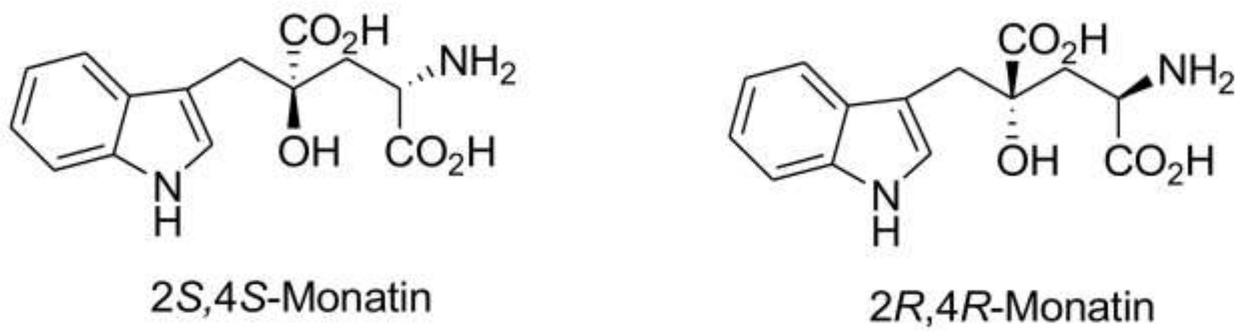

Figure 1. 2*S*,4*S* and 2*R*, 4*R* stereoisomers of monatin

As a member of the plant family Acanthaceae, *S. ilicifolius* has been referred to as 'Molomo monate' from the Sepedi name meaning “mouth nice” (Vleggaar et al., 1992). Based on actual herbarium specimens housed in the South African National Biodiversity Institute (SANBI) in Pretoria, information on plant availability is limited. The ‘epicentre’ of the distribution of *S. ilicifolius* is the Waterberg area in the Limpopo province of South Africa. The second ‘main’ area of distribution is the Zoutpansberg, a mountain range in northern Limpopo, and a third area in the Blyde River Canyon Nature Reserve near the Abel Erasmus Pass in the Mpumalanga Province.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

The reagents ammonium acetate, sodium bicarbonate and hydrochloric acid were purchased from Saarchem Univar, Marfey's reagent purchased from Pierce, Rockford, IL, USA, Cat # 48895). HPLC grade acetone and acetonitrile was purchased from Burdick and Jackson (ACS/HPLC grade).

2.2 Plant material harvesting

Initial collections in September 2002 focused on the harvesting of representative samples of the root bark at each of the collection sites for the main purpose to establish the presence of the isomers, while representative portions of roots, stems, and leaves were also harvested in later collections (February 2005) to also establish the presence of monatin isomers in the other plant parts. All samples were delivered to the Council for Scientific and Industrial Research (CSIR) for further processing. Plant identification was done at the time of the collection of the research material by Hans Vahrmeijer, a registered professional Botanist (Reg. No. 400182/83 South African Council for Natural Scientific Professions). A voucher specimen was deposited in the herbarium of the SANBI (Voucher No. 00675).

2.3 Processing of plant material.

All samples harvested were separately processed. For the 2002 collections, samples were immediately dried in an oven preset at 60°C. After 48 hours the samples were removed for further processing and the root samples were debarked while the root bark retained for further processing. Each sample (10-15 g) was separately ground in a high speed blender. The dried ground root bark was extracted by adding 75 ml of purified water to each of the samples. The mixture was allowed to stand at ambient temperature for 4 hours and shaken manually by hand every 30 minutes. Each of the mixtures was separately centrifuged and the supernatant decanted. The water layer was transferred to freeze drying tubes and separately freeze dried overnight. The dried powder was transferred to pre-weighed labelled vials and stored at 4 °C prior to analysis.

For the 2005 collection, the stems, leaves samples and root samples were transferred to trays and immediately dried in an oven preset at 60°C. After 48 hours the samples were removed for further processing. The leaves were stripped and separately retained for further processing. The roots were debarked and the root bark and roots retained for further processing. Each sample was separately ground in a high speed blender. The sample was extracted by adding 200 ml of de-ionized water and the mixture was allowed to stand at ambient temperature for 4 hours and shaken manually by hand every 30 minutes. Each of the mixtures was separately filtered through filter paper, the water layer transferred to freeze drying tubes and separately freeze dried. The dried powder was transferred to pre-weighed labelled vials and stored at 4°C prior to analysis.

2.4 Preparation of standards

Monatin synthetic standards, *2S,4S*; *2R,4R* and mixture of RS/SR stereoisomers used for the characterization in the plant materials were prepared prior at the CSIR as described in literature (Rousseau et al., 2011). The standards were available at the CSIR for this research.

2.5 Chiral chromatography for separation of Monatin enantiomers using Marfey's derivatization procedure (Marfey et al., 1984).

100 μl of a monatin standards in 10 mg/ml solution was mixed with 200 μl of 1 % Marfey's reagent and 40 μl of 1 M sodium bicarbonate. The mixture was shaken and reacted at 40 ^{O}C for 1 hour. After cooling to room temperature 20 μl of 2 M hydrochloric acid was added. For analysis, the derivatization mixture was diluted 100:1 (20 μl : 2 ml) in water.

Approximately 15 mg of freeze-dried extract was weighed into a vial. 100 μl of water, 200 μl of 1% Marfey's reagent and then 40 μl of 1 M sodium bicarbonate was added in that order. The mixture was mixed vigorously using a vortex mixer and ultrasonication, and then reacted at 40 ^{O}C for 1 hr. After cooling to room temperature 20 μl of 2 M hydrochloric acid was added. 200 μl of the derivatized mixture was diluted with 1800 μl of water and filtered for analysis.

The Chiral Method also referred to as "Marfey's", was done employing Waters Alliance 2690 HPLC, with a Quattro Micromass mass spectrometer. Compound separation was accomplished using a 250mm x 4.6mm reversed phase C_{18} column (Phenomenex Luna; 5µm particle) protected by a guard column under binary gradient elution conditions (0.05% ammonium acetate/H_2O and 100% acetonitrile). The flow rate was set to 1.0mL/Min, and the column temperature set to 40ºC. Mass spectra were collected across the *m/z* range of 400-600 in negative-ion electrospray mode, and UV spectra were collected from 193-400nm. Peaks on HPLC-MS chromatograms from different plant extracts were identified as Marfey's derivatives of monatin if their retention times and mass were the same as those of standards, and if the $(M - H)^-$ ion at *m/z* 543 as well as the $(M{-}H{-}H_2 0)^-$ at *m/z* 525 were present. The elution times of the isomers were established by analyzing the two set standards of *2S,4S* and *2R,4R* enantiomers together, and the *2R,4S* and / *2S,4R* enantiomers together.

3. RESULTS AND DISCUSSION

3.1 Plant harvesting and processing

A summary of the locations of root samples harvested during September 2002 and that for the recollections from same locations during February 2005 is shown in Table 1. Even though there was a three year period between the collections, sufficient material was still available for the recollections.

Table 1: Summary of harvested material during September 2002 and February 2005

Ethno botanist identification	Sample ID - Name / description of site	Harvest date
Sclerochiton ilicifolius	Mon I-38 , Schoongelegen in Vaalwater; Limpopo province of South Africa, single plant on rocky outcrop	September 2002
Sclerochiton ilicifolius	Mon I-29, Weidehoek in Ellisras, Limpopo province of South Africa	September 2002
Sclerochiton ilicifolius	Mon I-35, Buffelshoek in Thabazimbi, Limpopo province of South Africa	September 2002
Sclerochiton ilicifolius	Mon I-32, Schoongelegen in Vaalwater, Limpopo province of South Africa,	September 2002
Sclerochiton ilicifolius	Mon I-47A1-2, Buffelshoek in Thabazimbi, Limpopo province of South Africa	February 2005
Sclerochiton ilicifolius	Mon I-48A1-2, Schoongelegen in Vaalwater, Limpopo province of South Africa	February 2005
Sclerochiton ilicifolius	Mon I-50A1-2, Weidehoek in Ellisras Limpopo province of South Africa	February 2005

3.2 Analysis of separated diastereomers (*RR/SS* and *RS/SR).*

The separated *2S,4S* and *2R,4R* isomers and the mixture of the *2R,4S* and *2S,4R* isomer standards were analysed using the Marfey's chiral method. The separated *2S,4S* and *2R,4R* isomers gave chromatograms with single major peaks corresponding to each of the separated isomers while the *2R,4S* and *2S,4R* isomer standards yielded chromatograms with two major peaks corresponding to each of the isomers and only minor peaks at the retention times of any of the other isomers (*i.e. 2S,4S* and *2R,4R*). (Figures 2A, 2B, 2C, 2D, 2E, and 2F). The mass spectra of the Marfey's derivatives of monatin agreed with the known molecular weight ($C_{23}N_6O_{10}H_{24}$, MW = 544, $(M – H)^-$ at *m/z* 543) and showed a neutral loss of 18 (H_2O) confirming the presence of the quaternary hydroxyl group in the molecule. The retention times for the Marfey's derivatives of the *2R,4S* and *2S,4R* isomer standards is shown in Table 2. This is the first chromatographic separation of monatin isomers using Marfey's derivatization procedure as most reported separation of the monatin stereoisomers has generally been done through the use of chiral HPLC (Bassoli et al., 2005, Amino et al., 2016)

Table 2: Retention times of derivatized monatin standards

Monatin isomer	Retention time of Marfey's derivative of monatin
2R,4*R*,	23.06 min.
2*S*,4*S*	20.05 min.
2*R*,4*S* and 2*S*,4*R* mixture	21.31 min and 25.82 min.*

*may be interchanged for the isomers

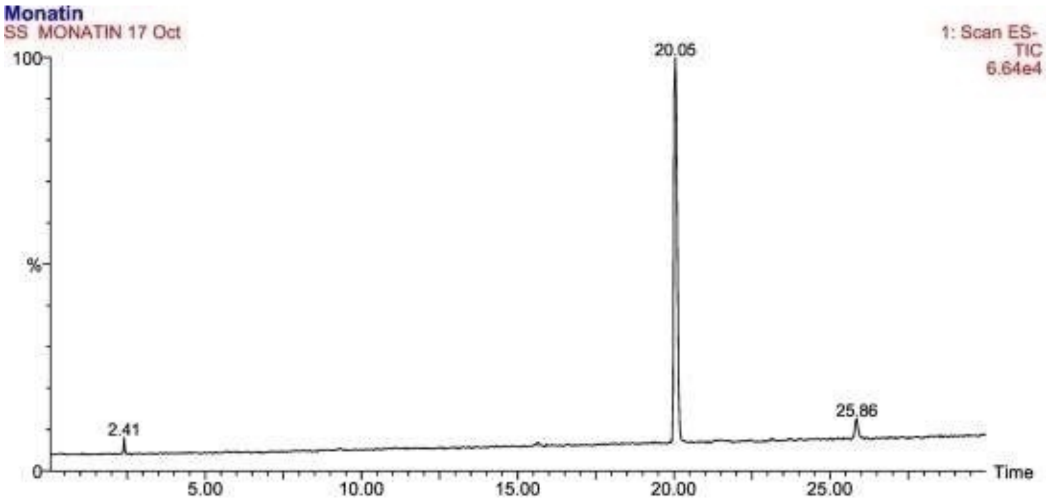

Figure 2A. Total ion chromatogram of 2*S*,4*S* monatin derivative

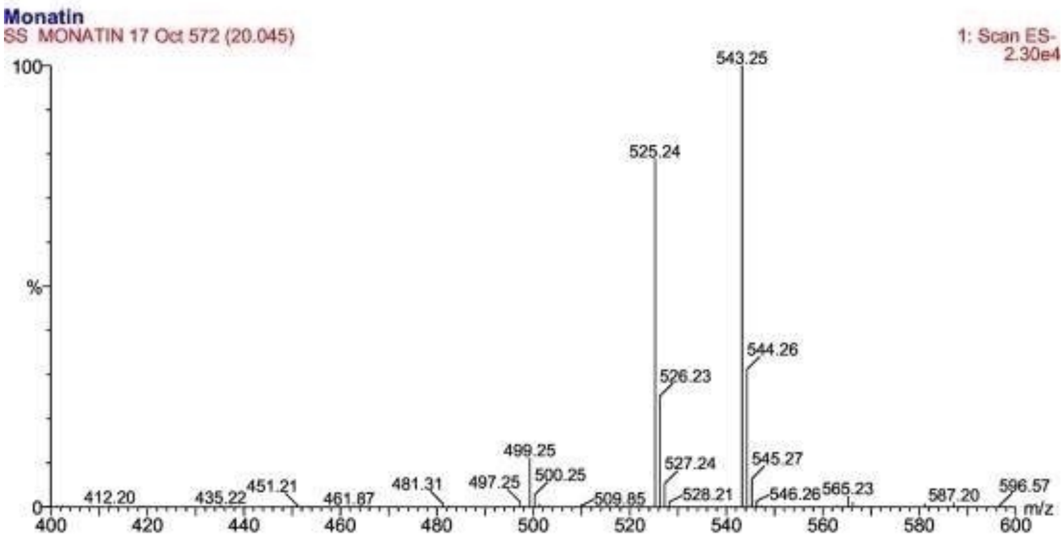

Figure 2B. Mass spectrum of 2*S*,4*S* monatin derivative

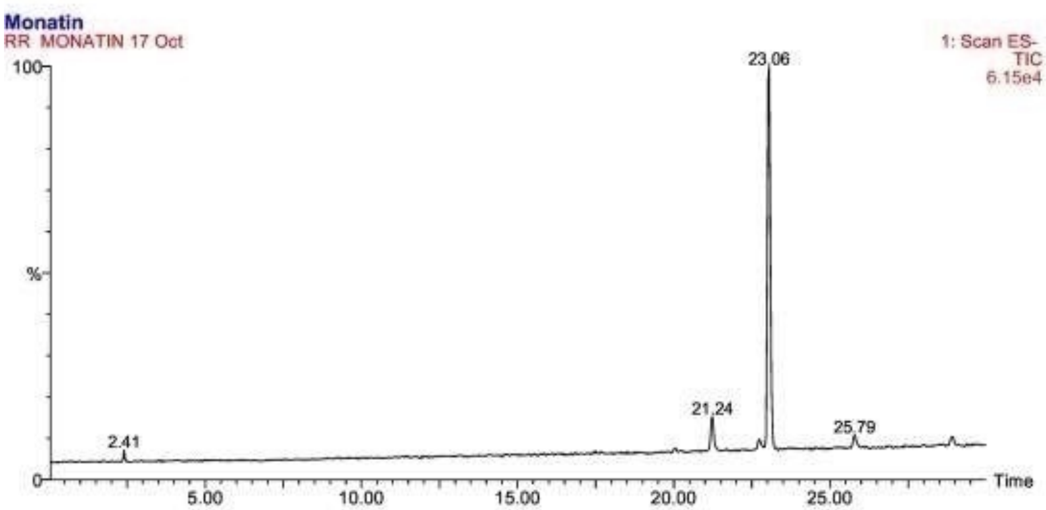

Figure 2C. Total ion chromatogram of 2*R*,4*R* monatin derivative

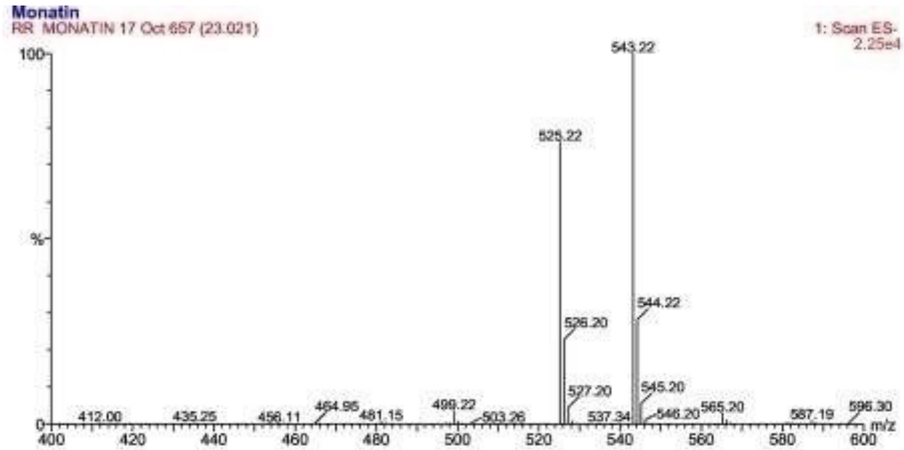

Figure 2D. Mass spectrum of 2*R*,4R monatin derivative

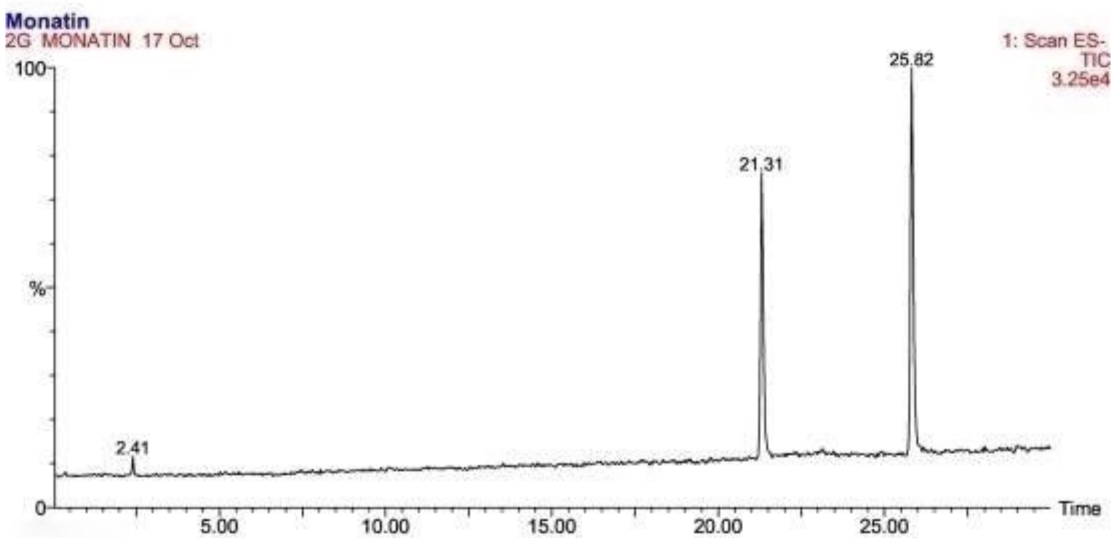

Figure 2E. Total ion chromatogram of 2*S*,4*R*/2*R*,4*S* monatin derivatives

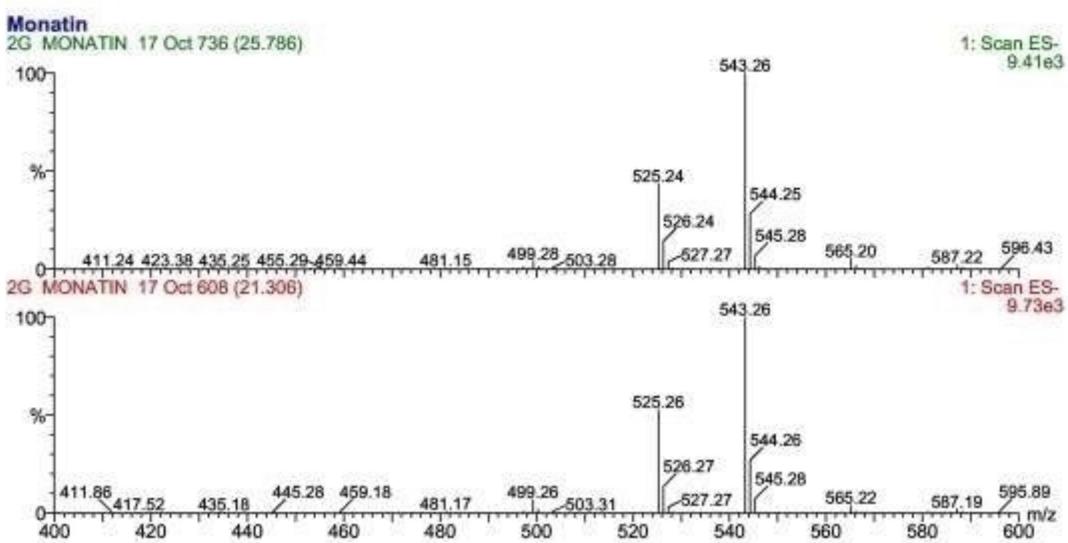

Figure 2F. Mass spectrum of 2*S*,4*R*/2*R*,4*S* monatin derivatives

3.3 Sample extracts.

Those compounds in the single ion chromatograms at *m/z* 543 which showed the same molecular ion and a neutral loss of 18 (H_2O) as the derivatized monatin standards, were identified and their retention times compared to the derivatized standards. Results of the analysis of samples from the initial harvesting and recollection are shown in Tables 3 and 4, respectively.

Table 3: Summary of Initial Harvesting Results

Plant Identification and collection area	Plant Part	Retention time (min.) of peak which corresponds to the 2*S*,4*S* isomer	Retention time (min.) of peak which corresponds to the 2*R*,4*R* isomer	Retention time (min.) of peaks which corresponds to the 2S,4R/2R,4S isomers	Presence of monatin Isomers
Sclerochiton ilicifolius; *(*Schoongelegen single plant on rocky outcrop)	Root Bark	20.40	23.30	24.21 (trace)	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S*/ 2*S*,4*R* detected
Sclerochiton ilicifolius; (Weidehoek)	Root Bark	20.01	22.99	21.17 (trace)	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4/*S* 2*S*,4*R* detected
Sclerochiton ilicifolius; (Buffelshoek)	Root Bark	19.94	23.02	21.17 (trace)	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S*/ 2*S*,4*R* detected
Sclerochiton ilicifolius; *(*Schoongelegen)	Root Bark	20.33	23.30	Maybe present but in trace quantities as neutral loss of 18 not clearly detected	2*R*,4*R* and 2*S*,4*S* monatin detected

Table 4: Summary of Re-Harvesting Results

Plant Identification	Plant Part	Presence of monatin Isomers
Sclerochiton ilicifolius; Buffelshoek in Thabazimbi	Leaves	ND
	Stems	ND
	Root	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S* and 2*S*,4*R* detected
	Root Bark	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S* and 2*S*,4*R* detected
Sclerochiton ilicifolius; Schoongelegen in Vaalwater	Leaves	ND
	Stems	ND
	Root	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S* and 2*S*,4*R* detected
	Root Bark	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S* and 2*S*,4*R* detected
Sclerochiton ilicifolius; Weidehoek in Ellisrus	Leaves	ND
	Stems	ND
	Root	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S* and 2*S*,4*R* detected
	Root Bark	2*R*,4*R* and 2*S*,4*S* monatin detected Trace quantities of 2*R*,4*S* and 2*S*,4*R* detected

ND not detected

Since the results were very similar for all the sampled sites, a representative total and single-ion chromatograms and their mass spectra of compounds at *m/z* 543 for a representative sample collected from Buffelshoek in Thabazimbi (Mon I-35) is shown in Figures 3A and 3B using the Marfey's chiral method. In addition, the total and single-ion chromatograms and their mass spectra of compounds *m/z* 543 for sample Mon I-29 derivatized aqueous root extract which was spiked with 2*S*,4*S* monatin standard prior to injection on the HPLC is shown in Figures 4A and 4B. The purpose of this was to demonstrate the similarity of the compound with *m/z* 543 eluting at 20.26 minutes in the derivatized extract to the 2*S*,4*S* monatin standard as co-elution of a compound m/z 594 appeared in most of the derivatized extracts with the same retention time as the 2*S*,4*S* monatin standard.

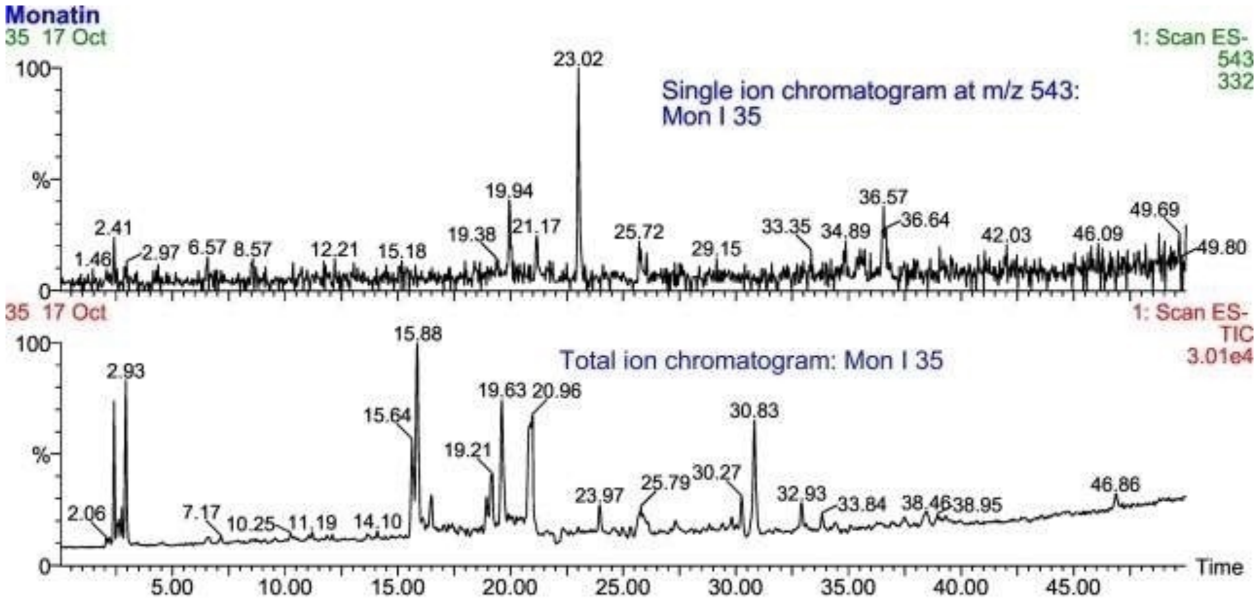

Figure 3A. Single ion *m/z* 543 and total ion chromatograms of Mon I-35 derivatized

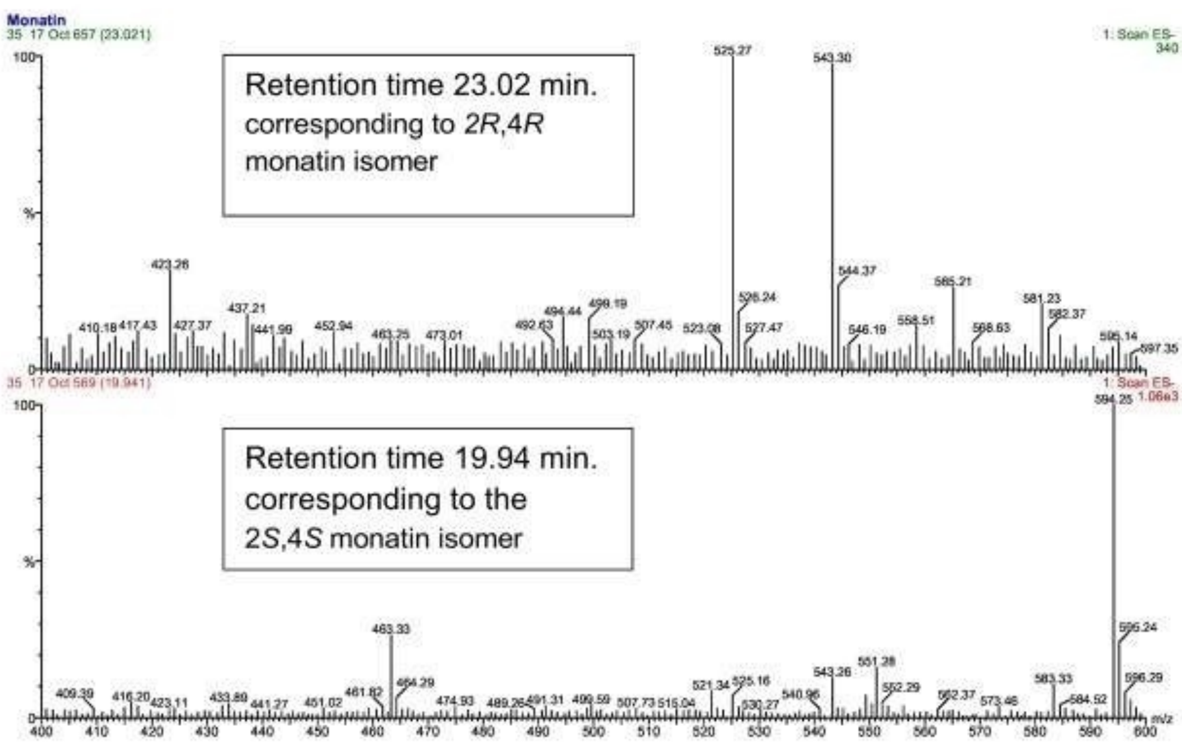

Figure 3B. Mass spectrum of compounds with *m/z* 543 in sample Mon I-35 derivatized

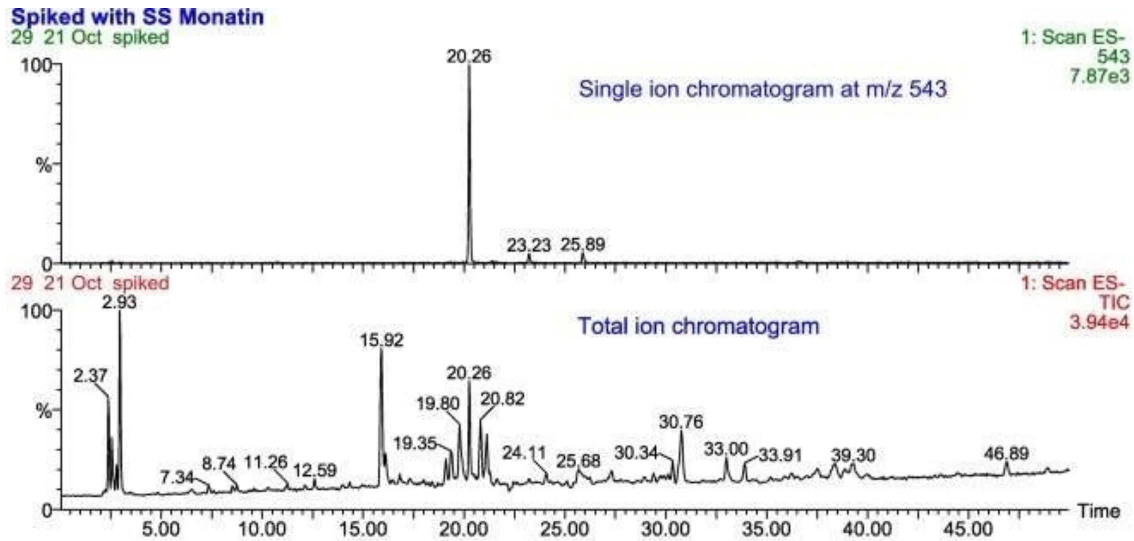

Figure 4A. Single ion *m/z* 543 and total ion chromatograms of Mon I-29 derivatized which was spiked with derivatized 2*S*,4*S* monatin prior to HPLC injection

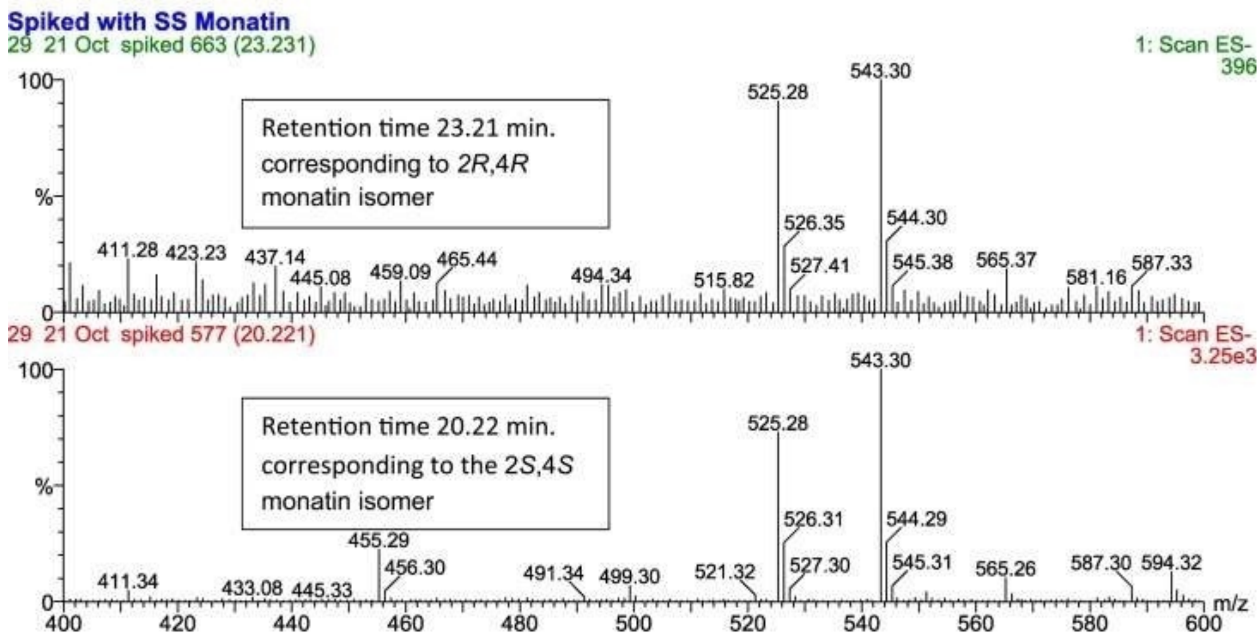

Figure 4B. Mass spectrum of compounds with *m/z* 543 in sample Mon I-29 derivatized which was spiked with derivatized 2*S*,4*S* monatin prior to HPLC injection

The monatin isomers could not be detected in the leaves and stems of *S. ilicifolius.* The identification of the presence of the monatin isomers is based on two criteria; retention time and molecular mass. The retention times of Monatin peaks from samples were always within 1.75 % (less than 22 seconds) of the retention times of 2*S*,4*S* monatin standard and 1.04% (less than 15 seconds) of the 2*R*,4*R* monatin standard. This minor variation was also seen for the spiking experiments and within the same ranges for both the isomers. The mass spectra of the $(M - H)^-$ ion with a neutral loss of 18 (H_2O) were the same for standards and samples, and the mass spectra agreed with the expected molecular mass of the monatin derivatives also indicating that the variations in retention time was minor. The results proved the presence of the 2*R*,4*R*, 2*S*,4*S*, 2*R*,4*S*, and 2*S*,4*R* monatin isomers in aqueous extracts of *S. ilicifolius* roots and root bark using the LC-MS detection of the deprotonated negative molecular ion for the Marfey's reagent and confirmed the findings of Bassoli et al. (2005).

The 2*R*,4*S* and 2*S*,4*R* isomers were present in the root and root bark extracts at trace concentrations, which excluded the production of either of the homochiral isomers due to epimerisation of the diastereomers unless mediated enzymatically. Elution of single isomer standards as single peaks demonstrated the absence of significant alteration of either the 2*S*,4*S* or 2*R*,4*R* isomer to any of the other isomers.

The results of this study has shown that the 2*S*,4*S* and 2*R*,4*R* isomers of monatin occur naturally in minimally processed aqueous extracts from the root bark of *S. ilicifolius* collected from three different sites in the Limpopo Province in South Africa. Previous studies by both Bassoli et al. (2005) and Amino et al. (2016) have shown repeatedly that the sweetness profile between these two enantiomers is vastly different and is analogous to studies in terpenoids where it has been verified that different enantiomers of the same molecule may possess different properties including odor characteristics, odor thresholds and even biological activities (Lina et.al., 2017). This finding was somewhat surprising in that only the 2*S*,4*S* isomer of monatin was found in samples in the earlier report by Vleggaar et al. (1992). The study also concludes the 2*S*,4*S* and 2*R*,4*R* isomers are also much more prevalent in the plant versus the 2*R*,4*S* and 2*S*,4*R* isomers.

ABBREVIATIONS USED

SANBI -South African National Biodiversity Institute

CSIR – Council for Scientific and Industrial Research

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