

Profiling of Phytosterols, Tocopherols and Tocotrienols in Selected Seed Oils from Botswana by GC–MS and HPLC

Y. C. Mitei · J. C. Ngila · S. O. Yeboah ·
L. Wessjohann · J. Schmidt

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Abstract The phytosterol, tocopherol, and tocotrienol profiles for mkukubuyo, *Sterculia africana*, manketti, *Ricinus odendron rautanenni*, mokolwane, *Hyphaene petersiana*, morama, *Tylosema esculentum*, and moretologa-kgomo, *Ximenia caffra*, seed oils from Botswana have been determined. Normal-phase HPLC analysis of the unsaponifiable matter showed that among the selected oils, the most abundant tocopherol and tocotrienol were γ -tocopherol (2232.99 $\mu\text{g/g}$) and γ -tocotrienol (246.19 $\mu\text{g/g}$), detected in manketti and mkukubuyo, respectively. Mokolwane oil, however, contained the largest total tocotrienol (258.47 $\mu\text{g/g}$). Total tocol contents found in manketti, mokolwane, mkukubuyo, morama, and moretologa-kgomo oils were 2238.60, 262.40, 246.20, 199.10, and 128.0 $\mu\text{g/g}$, respectively. GC–MS determination of the relative percentage composition of phytosterols showed 4-desmethylsterols as the most abundant phytosterols in the oils, by occurring up to 90% in moretologa-kgomo, mkukubuyo, and manketti seed oils, with β -sitosterol being the most abundant. Mokolwane seed oil contained the

largest percentage composition of 4,4-dimethylsterols (45.93%). Besides 4-desmethylsterols (75%), morama oil also contained significant amounts of 4,4-dimethylsterols and 4-monomethylsterols (15.72% total). GC–MS determination of the absolute amounts of 4-desmethylsterols, after SPE fractionation of the unsaponifiable matter, confirmed that β -sitosterol was the most abundant phytosterol in the test seed oils, with manketti seed oil being the richest source (1326.74 $\mu\text{g/g}$). The analysis showed total 4-desmethylsterols content as 1617.41, 1291.88, 861.47, 149.15, and 109.11 $\mu\text{g/g}$ for manketti, mokolwane, mkukubuyo, morama, and moretologa-kgomo seed oils, respectively.

Keywords GC–MS · HPLC · Phytosterol · Tocopherol and tocotrienol profiles · 4-Desmethylsterols · 4,4-Dimethylsterols · 4-Monomethylsterols · Mkukubuyo · Manketti · Mokolwane · Morama · Moretologa-kgomo

Introduction

Characterization of oils and fats traditionally involved determination of their bulk physicochemical properties then GC–FID or GC–MS analysis of the fatty acid composition. It soon became clear that the structure, especially the regiochemistry, of the triacylglycerol molecules has important physiological and nutritional effects on humans [1], and that the composition of the triacylglycerols could be characteristic of individual oils and fats. Thus the detailed characterization of oils and fats now also includes structural and compositional studies of the triacylglycerols, often carried out by tandem techniques such as HPLC–ESI–MS, HPLC–APCI–MS, and HPLC–FAB–MS. Other more recent techniques include direct application of high-resolution mass spectrometric techniques, for example Fourier-transform

Y. C. Mitei
Department of Chemistry and Biochemistry,
Moi University, Eldoret, Kenya

J. C. Ngila
Chemistry Department,
University of Kwa-Zulu Natal,
Durban, South Africa

S. O. Yeboah (✉)
Chemistry Department, University of Botswana,
Gaborone, Botswana
e-mail: yeboahso@mopipi.ub.bw

L. Wessjohann · J. Schmidt
Leibniz Institute of Plant Biochemistry, Halle, Germany

ion cyclotron resonance mass spectrometry with electrospray ionization (ESI-FTICR-MS) [2].

Characterization of oils and fats has thus far been focussed only on the principal components, which constitute the saponifiable fraction that comprises over 95% of oils and fats. However, it is now generally recognized that the minor components, which generally constitute the unsaponifiable matter, have important bioactive, nutritional, and characteristic compositional properties that affect the quality of individual oils and fats.

Phytosterols and the vitamin E compounds, tocopherols and tocotrienols (often called “tocols”), constitute, by far, the bulk of the unsaponifiable matter. Tocopherols and tocotrienols are natural antioxidants made by plants for protection against oxidative spoilage of plant materials such as oils and fats, and each plant species may have a characteristic composition of these tocols. Phytosterols often occur as a mixture of different but structurally similar compounds whose composition is now recognized to be characteristic of each plant species. The unique phytosterol composition in hazelnut oil, for example, has been used to detect adulteration of virgin olive oil by hazelnut oil [3]. Moreover phytosterols have now been shown to have important bioactive properties such as cancer prevention [4], lowering of plasma total cholesterol [5], and other physiological and nutritive properties. It has therefore become crucial to include profiling of their phytosterol, tocopherol and tocotrienol content in any detailed characterization of fats and oils.

In our general objective of comprehensive characterization of traditional seed oils from the sub-Saharan region of Africa, we here report the profiling of the phytosterol, tocopherol, and tocotrienol content of five seed oils from Botswana. This study is the natural extension of our earlier work on the profiling of the fatty acids and triacylglycerols in the seed oils of mkukubuyo, *Sterculia africana*, manketti, *Ricinodendron rautanenni*, mokolwane, *Hyphaene petersiana*, morama, *Tylosema esculentum*, and moretologa-kgomo, *Ximenia caffra* [6]. These seed oils have much economic importance in the areas where they are grown. Morama oil, for example is used widely for food preparations and is also turned into butter in the Kalahari region, and manketti oil, also known as mongongo oil, is a precious skin lotion and also used in food products. The ground manketti nut is used in preparing porridge or added to meat or vegetables to prepare delicious dishes. Moretologa oil is used by the Senn people in the Kalahari region as a skin lotion and also to soften leather and to treat bowstrings (Personal communications).

Tocopherols and tocotrienols have been successfully analysed as their acetates by GLC with packed columns.

They have also been analysed as their trimethylsilyl derivatives using capillary GC-FID [7–9]. However, in current practice analysis of tocopherols and tocotrienols is generally carried out by use of normal-phase HPLC techniques. In this study determination of the tocols was achieved by analysing the unsaponifiable matter of the test seed oils, using normal-phase HPLC with fluorescence detection (HPLC-FLD). Determination of plant sterols in oils and fats has traditionally been carried out by GC-FID or GC-MS analysis, after preliminary TLC fractionation and pre-concentration of the sterols from the total unsaponifiable matter. Other methods, for example solid-phase extraction (SPE) have, in recent times, been applied as a quicker and more efficient method for the fractionation step [10]. Indeed HPLC has been used for the fractionation step, and also as a direct method for the determination of phytosterols from the unsaponifiable matter [11]. In our study a preliminary determination of the relative percentage composition of the phytosterols in the test seed oils was carried out by GC-MS analysis of the acetylated total unsaponifiable matter. Then, in order to refine this analysis by determining the absolute amounts of the phytosterols in the seed oils, an SPE method was developed to fractionate the phytosterols into 4-desmethylsterols and 4-monomethylsterols, followed by acetylation of the fractions prior to GC-MS analysis.

Experimental Procedures

Materials

Manketti seeds, *R. rautanenii*, (500 g), mokolwane nuts *H. petersiana*, (150 g), morama beans, *T. esculentum*, (1.00 kg), mkukubuyo seeds, *S. africana* (200 g), and moretologa-kgomo seeds, *X. caffra*, (250 g), were obtained from Botswana Forestry Association, Kumakwane Village, Botswana.

Extraction: Solvents, and Reagents

All solvents and reagents used in this work, unless otherwise stated, were of analytical grade. Solvents used for HPLC were of HPLC grade. The solvents were obtained from Rochelle Chemicals (South Africa), BDH (Merck Chemicals, UK), Riedel-de Haën (Sigma Aldrich), or JT Baker Chemicals (Phillipsburg, NJ, USA). The seeds and nuts were manually dehulled and after thorough cleaning were macerated in a Waring commercial blender (Gateshead, UK). The powders were extracted with a 3:1 (v/v) mixture of *n*-hexane and 2-propanol in a Soxhlet apparatus for 6 h.

Analysis of Tocopherols and Tocotrienols

Saponification

Oil samples for analysis of tocopherols and tocotrienols were saponified according to the method reported by Panfili [12]. Oil sample (2.0 g) in an amber screw-capped bottle was flushed with nitrogen and 10 M potassium hydroxide (2.0 mL) was added. Absolute ethanol (2.0 mL) and 0.2 M sodium chloride solution (2.0 mL) were then added and the mixture flushed with nitrogen again. Ethanol pyrogallol (0.5 M, 5.0 mL) was finally added as antioxidant and the mixture was then flushed with nitrogen. The bottle was placed in a 70 °C water bath and mixed after every 5 min for 45 min, after which the bottle was cooled in ice and 0.2 M sodium chloride solution (15.0 mL) added. The suspension was extracted twice with 9:1 (v/v) *n*-hexane–ethyl acetate (15.0 mL). The combined organic layer was evaporated to dryness. The dry residue was dissolved in *n*-hexane–isopropanol (99:1) and passed through a Chromabond silica SPE cartridge. The filtrate was dried and re-dissolved in *n*-hexane (10.0 mL). This was then appropriately diluted prior to HPLC–FLD analysis.

HPLC–FLD Analysis

A Merck (Darmstadt, Germany) HPLC system that consisted of a Merck–Hitachi HPLC L-7100 Intelligent pump, a Rheodyne injector fitted with a 5- μ L loop, and a Merck L-7480 fluorescence detector was used for the tocopherol and tocotrienol analysis. The analysis was achieved by normal-phase HPLC on Nucleosil 100–5 column (5 μ m \times 4 mm \times 25 cm) from Macherey–Nagel (Düren, Germany). The mobile phase was *n*-hexane–isopropanol (99.7:0.3 v/v) at a flow rate of 1.0 mL/min. Fluorimetric detection of all peaks was performed at an excitation wavelength of 295 nm and an emission wavelength of 330 nm. Tocopherol peaks were identified and quantified against authentic tocopherols used as external standards. For each extract quantitative analysis was performed in triplicate. Tocotrienol peaks were confirmed by GC–MS and quantified against the corresponding tocopherols.

Analysis of Phytosterols

Saponification of Oil Samples

To the oil sample (2.5 g) in a 250-mL round-bottomed flask was added 0.5 M ethanolic potassium hydroxide solution (25.0 mL) and the mixture heated under reflux for 1 h. Water (100.0 mL) was then added down the condenser and the mixture was extracted with diethyl ether (100 mL \times 3)

in a 500-mL separatory funnel. The combined ether solution was washed with water (40.0 mL \times 3), and then washed successively with 0.5 M aqueous potassium hydroxide (40.0 mL), distilled water (40.0 mL), and repeatedly with aqueous 0.5 M KOH (40.0 mL) before finally washing with more 40.0 mL portions of distilled water until the washings were neutral to phenolphthalein indicator. The ether solution was then dried under anhydrous magnesium sulfate, and the solvent evaporated by distillation on a water bath to release the unsaponifiable matter.

Acetylation

The whole unsaponifiable matter (UM) was acetylated according to the method by Wilson et al. [11]. The UM (2.0 mg) was dissolved in 2:1 (v/v) pyridine–acetic anhydride solution (600 μ L) and the reaction was allowed to proceed at room temperature overnight. The excess reagents were then removed with a slight warming under a stream of nitrogen gas. The mixture was re-dissolved twice in dichloromethane and the solvent removed in a stream of nitrogen. The acetylated products were transferred into a sample vial and dissolved again in dichloromethane, flushed with nitrogen, and stored at 4 °C for GC–MS analysis.

Analysis by GC–MS

The acetylated lipids in dichloromethane were analysed in a ThermoQuest Voyager GC–MS coupled to ThermoQuest Trace GC 2000 SERIES (San Jose, CA, USA). Xcalibur version 1.3 software from Thermo Fischer Scientific (San Jose, CA, USA) was used to process the data. A DB-5MS capillary GC column (0.25 μ m \times 0.25 mm \times 30 m) from J&W Scientific (CA, USA) was used for separation and UHP helium was used as carrier gas at a flow rate of 1 mL/min. The injection temperature was 220 °C and the interface temperature was 300 °C. The initial temperature was 60 °C held for 1 min and then ramped to 200 °C at the rate of 15 °/min. It was then held for 1 min before the second ramp at the rate of 5 °/min to 300 °C. This was then held isothermally for 25 min.

SPE Fractionation of Sterols

The SPE method of Damirchi et al. was modified for this determination [10]. A 500-mg, 3-mL silica solid-phase extraction (SPE) cartridge from Macherey–Nagel was used per sample. The cartridge was attached to a Macherey–Nagel vacuum manifold. Waste tubes were positioned to collect the conditioning solvent. The cartridges were conditioned by passing *n*-hexane (5.0 mL) through them. After conditioning, the *n*-hexane was discarded and a clean test tube was positioned to collect the phytosterol fraction. The

UM (2.0 mg) sample aliquots in 2 mL *n*-hexane were then loaded on to the cartridges. The UM was then washed with *n*-hexane–ethyl acetate (99:1v/v, 10.0 mL) to remove any non-sterol compounds. Vacuum was applied at 5 mmHg for each elution. This fraction was discarded after checking with analytical TLC that it did not contain compounds of interest. 4-Methylsterols were then eluted with *n*-hexane–ethyl acetate (99:1v/v, 10.0 mL), followed by 14.0 mL 97:3 (v/v) *n*-hexane–ethyl acetate. Prior to eluting pure 4-desmethylsterols, additional *n*-hexane–ethyl acetate (97:3 v/v, 2.0 mL) was used to wash the cartridges. 4-Desmethyl sterols were then eluted with *n*-hexane–ethyl acetate (97:3 v/v, 25.0 mL). The fractions were dried, spiked with 10 µg 5 α -cholestane as internal standard, and acetylated prior to GC–MS analysis. The components were quantified against 5 α -cholestane.

Data Analysis

Unless otherwise stated, experiments for determination of tocopherols, tocotrienols, and phytosterols by HPLC and GC–MS analyses were carried out in triplicate and results are expressed as mean values \pm SD.

Results and Discussion

Current awareness of the important bioactive properties of the minor constituents of oils and fats has expanded our notion of oil quality to include the nutritional and health-promoting benefits of phytosterols, tocopherols, and tocotrienols. Thus today the food industry actively seeks to identify plant sources rich in these minor constituents as nutraceuticals and for incorporation in functional foods.

In this study, tocopherol and tocotrienol composition was determined by normal phase-HPLC with fluorescence detection and quantified against external standards. Normal-phase HPLC was chosen for the separation of the tocols, because it is capable of resolving α and β -tocopherols and tocotrienols, unlike reversed-phase HPLC [13]. In this analysis the tocopherols and tocotrienols were eluted in order

of increasing polarity, starting with α and followed by the β , γ , and δ homologues, which corresponded to the decreasing order of methylation on the chromanol ring of the tocols. This order of elution was consistent with earlier reports [14]. The quantitative analysis was performed with calibration curves. A linearity test was carried out over the concentration ranges 1–5 ppm from which regression analysis of the plot of area response versus concentration for each isomer gave an excellent relationship with correlation coefficients of 0.9997 for α -tocopherol, and 1.0000 each for β , γ , and δ -tocopherols.

The tocopherol and tocotrienol composition of the test seed oils are given in Table 1, which also shows the combined tocopherols and tocotrienols as total tocol content for each of the oil samples. The most abundant single tocol detected in this study was γ -tocopherol (γ -T), 2232.99 µg/g for manketti seed oil, which also contained α -tocopherol (α -T) at 5.64 µg/g as the only other tocol in its oil. This high γ -tocopherol content of manketti seed oil agrees with an earlier report which gave the γ -tocopherol content in manketti oil as 565 mg/100 g, and attributed the long shelf-life of manketti oil in the hot African sun to its high γ -tocopherol content [15]. The other oil which contained γ -tocopherol was morama at 117.27 µg/g (Table 1). Morama had also α -tocopherol and β -tocopherol (β -T) at 81.43 and 0.36 µg/g, respectively, as its remaining tocols. Moretologa-kgomo seed oil contained no tocopherols but had α -tocotrienol (α -T3) and β -tocotrienol (β -T3) at 82.14 and 45.90 µg/g, respectively. Mkukubuyo seed oil, on the other hand, had only trace amounts of α -T and δ -T, but had 246.19 µg/g γ -tocotrienol (γ -T3), which was the highest amount of tocotrienol detected among all the test seed oils. The only tocopherol detected for mokolwane seed oil was 3.27 µg/g α -T, but it had 86.65 and 172.47 µg/g of α -T3 and β -T3, respectively. Thus mokolwane had the highest total amount of tocotrienols among all the test seed oils. This tocol profile for mokolwane oil seems to be consistent with other palm species as they are known to be one of the richest sources of tocotrienols [16].

It is worth noting that although no δ -tocotrienol was found in any of the test seed oils, a trace of δ -tocopherol

Table 1 Tocopherol and tocotrienol content (µg/g) in selected test seed oils obtained by HPLC–FLD

Oil sample	α -T	β -T	γ -T	δ -T	α -T3	β -T3	γ -T3	Total tocol
Mokolwane	3.27 \pm 0.72	–	–	–	86.65 \pm 3.52	172.47 \pm 11.00	–	262.40 \pm 9.8
Morama	81.43 \pm 0.74	0.36 \pm 0.19	117.27 \pm 0.50	–	–	–	–	199.1 \pm 1.0
Moroteloga-kgomo	–	–	–	–	82.14 \pm 2.63	45.90 \pm 1.59	–	128.0 \pm 3.7
Mukukubuyo	Trace	–	–	Trace	–	–	246.19 \pm 6.03	246.2 \pm 6.0
Manketti	5.64 \pm 0.45	–	2232.99 \pm 42.62	–	–	–	–	2238.6 \pm 42.3

Values are averages \pm SD from three replicate analyses

“Trace” denotes levels less than 0.10 µg/g of oil

(δ -T) was detected in the seed oil of mkukubuyo. The average total tocol content for mokolwane, mkukubuyo, and manketti seed oils was 262.40, 246.20 and 2,238.60 $\mu\text{g/g}$, respectively, all of which fell within the typical total tocol range of 200–8,000 $\mu\text{g/g}$ for vegetable oils [17]. The average total tocol content for morama (199.10 $\mu\text{g/g}$) and moretologa-kgomo (128.0 $\mu\text{g/g}$) fell below the typical range for vegetable oils. It should be noted that the results of this analysis did not show any obvious correlation between degree of unsaturation and total tocol content. Indeed it has previously been shown that total vitamin E content of a seed oil sample may not be a foolproof indicator of oil stability, because the stability of vegetable oils can be affected by natural anti-oxidants and synergists other than vitamin E [18]. To the best of our knowledge this may be the first report of the tocopherol and tocotrienol composition of the seed oils of mokolwane, morama, moretologa-kgomo, and mkukubuyo.

In our study analysis of the phytosterols in the test seed oils was performed at two levels. The first level involved GC–MS determination of the relative percentage composition of the sterols in the acetylated total unsaponifiable fraction of each of the seed oils. The results from this analysis were contrasted with the results of the second level, which involved determination of the absolute amounts of 4-desmethylsterols obtained from SPE fractionation of the unsaponifiable matter from each of the seed oils before GC–MS analysis. At both levels of analysis acetate derivatives of the sterols were preferred to

trimethylsilyl (TMS) derivatives, because acetylated derivatives are more stable and enable easier detection of side-chain unsaturation in the sterol molecules, and also enable easier distinction between Δ^5 and Δ^7 phytosterols [19].

In the GC–MS determination of the relative composition of the phytosterols, the components in the region where phytosterols eluted were selected and the identification of the phytosterols was based on comparison of retention times and mass spectra with those of authentic standards. The partial total ion currents (TIC) for the acetylated unsaponifiable matter from morama seed oil is shown in Fig. 1. The relative percentage composition of each sterol was calculated as a ratio of its peak area to the total area of all identifiable sterol peaks in the oil sample. Table 2 shows the results of the relative percentage composition of the phytosterols in the test seed oils. Phytosterols are generally classified into three subclasses based on the degree of methyl substitution at carbon-4 in the sterol molecule; these are 4-desmethylsterols, which are often the most abundant, and the minor components 4-monomethylsterols and 4,4-dimethylsterols.

The relative composition of the phytosterols shown in Table 2 indicates that 4-desmethylsterols were always the most abundant of the three subclasses of sterols. Indeed in the seed oils of moretologa-kgomo, mkukubuyo, and manketti the 4-desmethylsterols constituted about 90% of the total sterol content, with β -sitosterol being the single most abundant sterol, an observation that is consistent with

Fig. 1 Partial TIC profiles of the acetylated unsaponifiable matter fraction in morama bean oil by GC–MS in the full-mode scan at 70 eV. 1, campesterol; 2, stigmasterol; 3, sitosterol; 4, Δ^5 -avenasterol; 5, unidentified; 6, unidentified; 7, cycloartenol; 8, 22-dihydrospinasterol; 9, Δ^7 -avenasterol; 10, 24-methylenecycloartanol; 11, citrostadienol

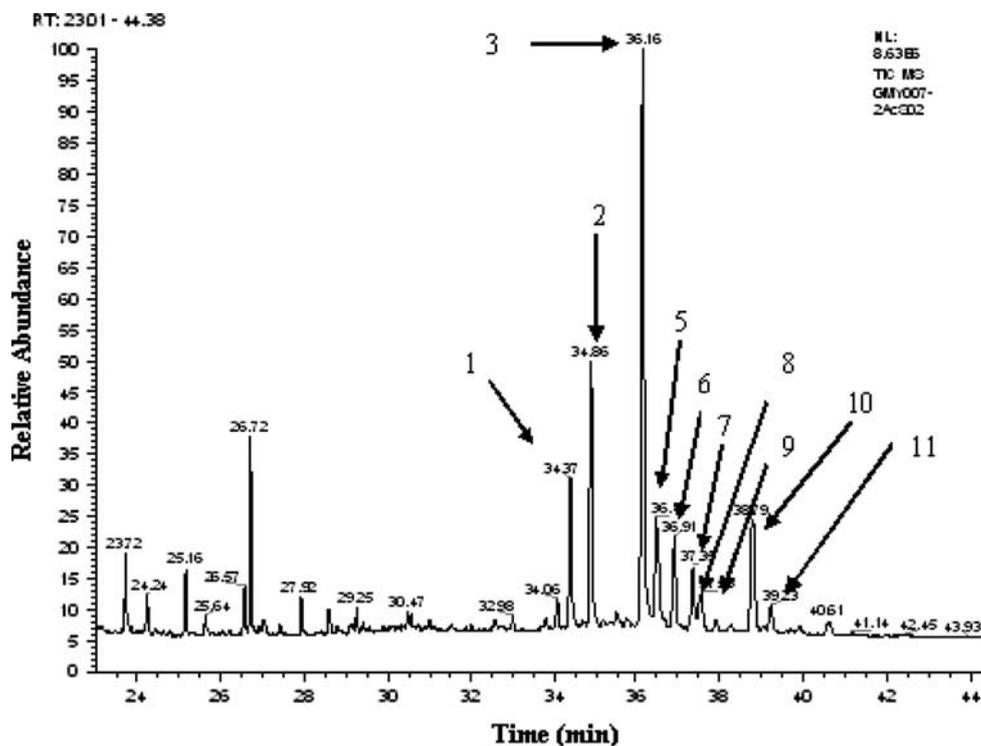


Table 2 Relative percentage composition of phytosterols in the test oil samples, determined by capillary GC–MS

Phytosterol	Mokolwane	Morama	Moretologa-kgomo	Mkukubuyo	Manketti
Cholesterol	0.91 ± 0.02	–	–	–	–
Campesterol	3.59 ± 0.12	9.64 ± 0.66	9.14 ± 0.10	10.00 ± 0.10	5.86 ± 0.72
Campestanol	–	–	–	–	–
Stigmasterol	3.87 ± 0.03	15.55 ± 0.19	–	23.67 ± 0.12	3.33 ± 0.22
Stigmastanol	0.51 ± 0.01	–	–	–	–
Sitosterol	37.08 ± 0.1	39.74 ± 0.52	46.49 ± 0.91	63.28 ± 0.06	78.46 ± 0.71
β -Amyrin	–	–	25.41 ± 1.12	–	–
Δ^5 -Avenasterol	–	8.76 ± 0.31	4.41 ± 0.23	3.04 ± 0.12	7.65 ± 0.12
Sitostanol/ Δ^5 -avenasterol	6.31 ± 0.04	–	–	–	–
Cycloartenol	36.9 ± 0.21	4.61 ± 0.13	–	–	–
Lupeol	–	–	5.21 ± 0.16	–	–
22-Dihydrospinasterol	–	2.95 ± 0.07	–	–	2.19 ± 0.12
Δ^7 -Avenasterol	–	0.78 ± 0.03	–	–	1.43 ± 0.02
24-Methylene-cycloartanol	9.03 ± 0.04	8.63 ± 0.09	5.98 ± 0.16	–	–
Citrostadienol	1.05 ± 0.05	2.48 ± 0.03	–	–	1.06 ± 0.17
Others	0.55 ± 0.02	6.86 ± 0.22	1.87 ± 0.08	–	–

Values are averages ± SD from three replicate analyses

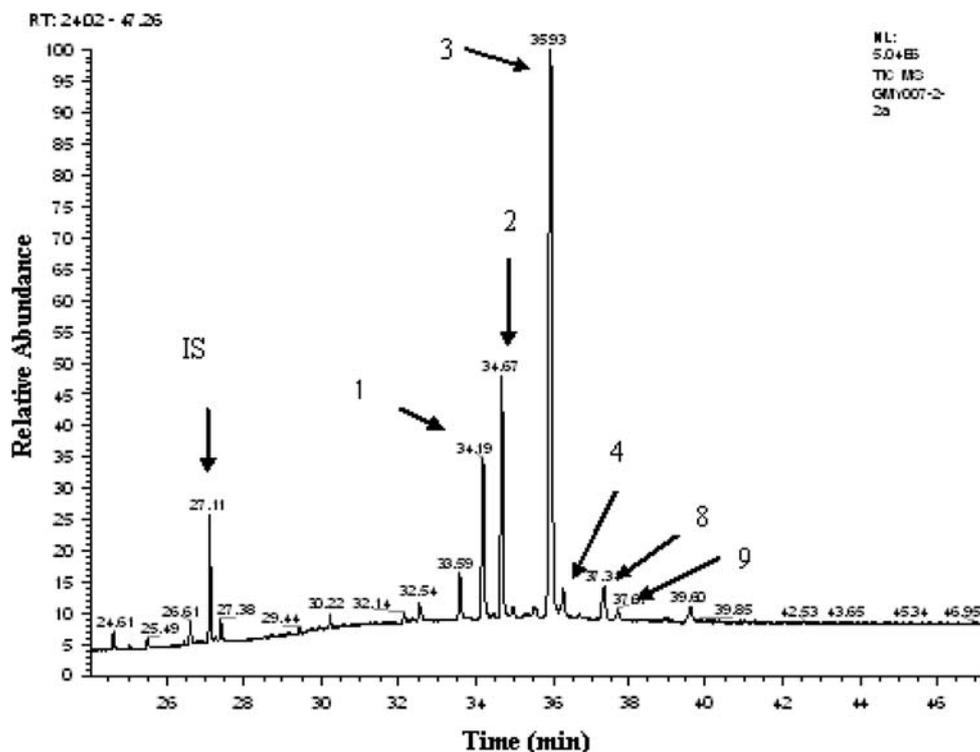
literature reports [20]. Other 4-desmethylsterols present in significant amounts were campesterol and stigmasterol. The phytosterol profile for mokolwane was quite different from those of the other test seed oils by having the 4-desmethylsterols constituting only about 50% of its total sterol content, which included 37.08% of β -sitosterol. In contrast, mokolwane seed oil contained the largest percentage composition of 4,4-dimethylsterols with 36.9% cycloartenol and 9.03% 24-methylenecycloartanol. Citrostadienol, 1.05%, was the only 4-monomethylsterol detected in the mokolwane seed oil. Morama seed oil contained 4-desmethylsterols to the extent of about 77%, chief among which were β -sitosterol (39.74%), stigmasterol (15.55%), campesterol (9.64%), Δ^5 -avenasterol (8.76%), and 22-dihydrospinasterol (2.95%). The 4,4-dimethylsterols which occurred in morama were 24-methylenecycloartanol (8.63%) and cycloartenol (4.61%). Morama was one of three of the test seed oils that contained the 4-monomethylsterol, citrostadienol (2.48%). Morama also contained significant amounts of unidentified sterols (6.86%).

The phytosterol profile for moretologa-kgomo seed oil consisted of about 60% 4-desmethylsterols and about 40% 4,4-dimethylsterols. The 4-desmethylsterols included β -sitosterol (46.49%), the most abundant; campesterol (9.14%), and Δ^5 -avenasterol (4.41%). The 4,4-dimethylsterol content included substantial amounts of β -amyrin (25.41%), 24-methylenecycloartanol (5.98%), and lupeol (5.21%), which was detected only in moretologa-kgomo seed oil. The phytosterol profile of mkukubuyo seed oil consisted exclusively of 4-desmethylsterols (100%), made

up of β -sitosterol (63.28%), stigmasterol (23.67%), campesterol (10.00%), and Δ^5 -avenasterol (3.04%). Manketti seed oil, on the other hand, had a sterol profile that consisted of about 98.92% 4-desmethylsterols, which included 78.46% β -sitosterol as the dominant sterol, followed by Δ^5 -avenasterol (7.65%), campesterol (5.86%), stigmasterol (3.33%), 22-dihydrospinasterol (2.19%), and Δ^7 -avenasterol (1.43%). In addition, manketti seed oil also contained the 4-monomethylsterol, citrostadienol (1.06%), as the only other sterol. It is worth noting that among the test seed oils cholesterol was detected in trace amount (0.91%) in mokolwane seed oil only. This observation tends to uphold the general view that cholesterol is generally prominent mainly in animal sources.

Determination of the relative composition of the phytosterols is a quick and useful technique to reveal the overall sterol profile of oils, but it does not determine the absolute amount of each phytosterol in the seed oil. Thus, in order to refine the data, an SPE method was developed to fractionate the sterols into two main fractions—4-desmethylsterols and 4-monomethylsterols. As described in the [experimental](#) section, Damirchi's SPE method was modified by using *n*-hexane–ethyl acetate mixtures for gradient elution and employing a vacuum pressure of 5 mmHg to elute the fractions [10]. However, because of a lack of authentic standards for the 4-monomethylsterols, only the 4-desmethylsterol fraction was acetylated and analysed by GC–MS, using 5 α -cholestane as the internal standard. The partial total-ion current (chromatogram) for the acetylated 4-desmethylsterols in the morama seed oil is shown in Fig. 2, which looks cleaner than that for the full unsaponifiable

Fig. 2 Total-ion chromatogram of the acetylated 4-desmethylsterols in morama bean oil. IS, internal standard (5 α -cholestane); 1, campesterol; 2, stigmasterol; 3, sitosterol; 4, Δ^5 -avenasterol; 8, 22-dihydrospinasterol; 9, Δ^7 -avenasterol



matter shown in Fig. 1. The absolute amount of each identifiable 4-desmethylsterol was calculated as $\mu\text{g/g}$ of the seed oil by using the relationship, 4-desmethylsterol = $(A_z \times m_{is}) / (A_{is} \times m)$, where A_z = start peak area, A_{is} = internal standard peak area, m_{is} = mass (μg) of internal standard, and m = mass of oil in g weighed for the analysis [19]. The results obtained from the analysis are shown in Table 3, and agree very closely with the results shown in Table 2 for the general phytosterol profiles of the test seed oils. Both Tables 2 and 3 agree that β -sitosterol was the most abundant phytosterol in all the test seed oils.

Table 3 shows that only Δ^5 -phytosterols were present in the seed oil of mokolwane, with β -sitosterol (987.08 $\mu\text{g/g}$) as the dominant sterol followed by Δ^5 -avenasterol (129.95 $\mu\text{g/g}$) and campesterol (81.43 $\mu\text{g/g}$), making a total of 1291.88 $\mu\text{g/g}$ 4-desmethylsterols. This level of phytosterol content of mokolwane seed oil is higher than that reported for palm kernel but is in the range for groundnut oil [21]. Manketti seed oil showed the presence of both Δ^5 and Δ^7 -4-desmethylsterols, as was also detected earlier (Table 2). As expected β -sitosterol (1326.74 $\mu\text{g/g}$) was the dominant sterol, followed by Δ^5 -avenasterol (111.89 $\mu\text{g/g}$), campesterol (93.53 $\mu\text{g/g}$), stigmasterol (36.24 $\mu\text{g/g}$), 22-dihydrospinasterol (28.31 $\mu\text{g/g}$), and Δ^7 -avenasterol (20.70 $\mu\text{g/g}$), making a total of 1617.41 $\mu\text{g/g}$ 4-desmethylsterols in the manketti seed oil, which compares very favourably with the phytosterol content of groundnut oil (900–2884 $\mu\text{g/g}$) [22].

Mkukubuyo seed oil showed the third highest total content of 4-desmethylsterols (861.47 $\mu\text{g/g}$), which fell in

the range reported for coconut oil (470–1140 $\mu\text{g/g}$). The 4-desmethylsterols present were the same phytosterols found in the earlier analysis in about the same order of abundance: β -sitosterol (560.80 $\mu\text{g/g}$), stigmasterol (177.32 $\mu\text{g/g}$), campesterol (92.92 $\mu\text{g/g}$), and Δ^5 -avenasterol (30.54 $\mu\text{g/g}$) (Table 3). Morama bean oil contained the second lowest total content of 4-desmethylsterols among the test seed oils, at 149.15 $\mu\text{g/g}$, which is comparable with the phytosterol content of olive oil (100 $\mu\text{g/g}$). It is interesting to note that morama seed oil and olive oil have similar fatty acid compositions, with oleic acid being the dominant fatty acid [6]. Morama seed oil phytosterols consisted of β -sitosterol (84.50 $\mu\text{g/g}$), stigmasterol (32.07 $\mu\text{g/g}$), campesterol (21.92 $\mu\text{g/g}$), 22-dihydrospinasterol (5.26 $\mu\text{g/g}$), Δ^5 -avenasterol (4.45 $\mu\text{g/g}$), and Δ^7 -avenasterol (1.58 $\mu\text{g/g}$). This phytosterol profile very distinctly distinguishes morama oil from olive oil, whose phytosterol profile is reported as: β -sitosterol (75–80 $\mu\text{g/g}$), Δ^5 -avenasterol (4–14 $\mu\text{g/g}$), stigmasterol (0–4.0 $\mu\text{g/g}$), campesterol (0–4.0 $\mu\text{g/g}$), Δ^7 -stigmasterol (0–0.5 $\mu\text{g/g}$), cholesterol (0–0.5 $\mu\text{g/g}$), and brassicasterol (0–0.1 $\mu\text{g/g}$) [23].

The lowest total content of 4-desmethylsterols was found in the moretologa-kgomo seed oil, at a level of 109.11 $\mu\text{g/g}$, which was made up of β -sitosterol (61.27 $\mu\text{g/g}$), campesterol (38.76 $\mu\text{g/g}$), Δ^5 -avenasterol (4.96 $\mu\text{g/g}$), stigmasterol (2.41 $\mu\text{g/g}$), and cholesterol (1.71 $\mu\text{g/g}$), all of which, except cholesterol, were found in the earlier analysis (Table 2). As far as we are aware this may be the first report of the phytosterol profiles for the seed oils of mkukubuyo, *Sterculia*

Table 3 4-Desmethylsterols ($\mu\text{g/g}$ of oil) content in test oil samples determined by GC–MS after SPE fractionation^a

Phytosterol	Mokolwane	Morama	Moretologa-kgomo	Mkukubuyo	Manketti
Cholesterol	–	–	1.71	–	–
Campesterol	81.43	21.29	38.76	92.92	93.53
Stigmasterol	92.23	32.07	2.41	177.33	36.24
Sitosterol	987.08	84.5	61.27	560.68	1326.74
Δ^5 -Avenasterol	129.95 ^b	4.45	4.96	30.54	111.89
22-Dihydrospinasterol	–	5.26	–	–	28.31
Δ^7 -Avenasterol	–	1.58	–	–	20.70
Total 4-desmethylsterol	1291.88	149.15	109.11	861.47	1617.41

^a Values are for one run of SPE fractionation prior to GC–MS

^b Sitostanol/ Δ^5 -avenasterol mixture

africana, mokolwane, *Hyphaene petersiana*, morama, *Tylosema esculentum*, and moretologa-kgomo, *Ximenia caffra*.

This study has clearly demonstrated how analysis of the constituents of the unsaponifiable matter, i.e. the minor components of seed oils, is an essential part of detailed characterisation of oils and fats. Manketti seed oil has been shown to be a rich source of γ -tocopherol and β -sitosterol, ingredients which must justify its reported use as a nutritious and protective skin-care and hair-care oil [24]. Mkukubuyo, with oil yield of 31.7% (w/w), can serve as a source of 4-desmethylsterols (861.47 $\mu\text{g/g}$) and γ -tocotrienol (246.19 $\mu\text{g/g}$) for the nutraceutical industry. The study has revealed qualitatively and quantitatively the particular compounds that make up the bulk of the unsaponifiable matter in the test seed oils. The study has further demonstrated the uniqueness of the composition of these compounds as being characteristic of the oil content of a particular plant species. As shown above, the phytosterol profiles for morama and olive oils clearly distinguish between the two oils, indeed much more so than their fatty acid compositions. Thus in many cases the composition of the minor fractions, i.e. the profiles of phytosterols, tocopherols, and tocotrienols more distinctly define the genuineness of individual oils and fats than their respective fatty acid compositions.

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