Evaluation of *n*-alkanes, long-chain alcohols, and carbon stable isotope enrichments of *n*-alkanes as diet composition markers in free-grazing animals

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Abstract

Context. Plant species exhibit different patterns of plant cuticular wax profiles, which can potentially be used as diet composition markers in free grazing herbivores.

Aims. Evaluate the suitability of the plant cuticular n-alkanes, long chain alcohols (LCOH) profiles and carbon stable isotope enrichment ($\delta^{13}C$) of n-alkanes for forage species to use as markers in the estimation of diet composition of grazing animals.

Methods. Forage samples were collected from 100 representative quadrats of 0.5 m x 0.5 m at 10 m transects and sorted by species and pooled from different quadrats to obtain enough quantities of representative individual species. A total of ten dominant forage species were identified and analyzed for n-alkanes and LCOH by gas chromatography (GC) and the isotopic ratio ($^{13}C/^{12}C$) by using Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS). Principal component analysis (PCA) was used to identify inter-species differences in the concentration patterns of plant wax components.

Key results. Odd-chain n-alkanes comprised the highest proportion of the total n-alkane concentration ranging from 79% in *Ischaemum afrum* to 95% in *Haplocarpha hastata*. N-alkanes C_{31}, C_{29} and C_{33} were the most abundant with an average 167, 80 and 61 mg/kg DM, in that order in all species. Even-chain LCOH comprised the highest proportion of the total LCOH concentration accounting for 92% in *Brachiaria scalaris* to 97% in *Ischaemum afrum*. The dominant even-chain LCOH were $C_{30}OH$, $C_{32}OH$, $C_{28}OH$ and $C_{26}OH$, with an average concentration of 362, 348, 266 and 237 mg/kg DM, respectively across species. The $\delta^{13}C$ of n-alkanes showed relatively large variations between forage species ranging from −19.7‰ in *Andropogon amethystinus* to −40.6‰ in *Trifolium mattirolianum*. The result of the PCA showed

that 81% of the variance in the pattern of concentrations of n-alkanes was explained by the first two principal components compared to 69.3 and 82.9% in the case of LCOH and $\delta^{13}C$ of n-alkanes, respectively.

Conclusions. Noticeable variations were observed for forage species studied in the patterns of plant wax components.

Implications. The differences in the patterns of concentrations of n-alkanes, LCOH and $\delta^{13}C$ of n-alkanes could be suitable as markers for diet composition estimation of grazing animals.

Keywords: assessment, diet estimation, forage species profile, location specific, marker concentrations, pasture, plant cuticular wax, principal component analysis.

Introduction

Ethiopian highlands are characterized by high species diversity and are considered a centre of species endemism due to large elevation range, heterogeneous landscape and climate (IBC 2005). These highlands, endowed with rich natural resources, have been cultivated agriculturally for millennia and now are heavily degraded due to mismanagement. This applies to grazing lands, and many studies have indicated that the grazing lands in Ethiopia are in poor to very poor condition and need immediate action (Mengistu *et al*. 2017). Improved management of grazing lands necessitates good knowledge of the nutritional qualities of different pasture species, biomass yields and botanical composition of grazing herbivores. Estimation of diet composition in freely grazing animals is challenging due to the invasiveness of the methods applied and associated in accuracies to simulate the natural grazing behaviour of animals. The use of plant cuticular wax hydrocarbons as diet composition markers has received increasing acceptance as it

is less invasive and allows diet composition estimation without restricting free movement of animals.

The basic precondition for estimating diet selection of ruminants using plant cuticular wax on a multispecies pasture is the presence of sufficient differentiation of marker profiles between plant species (Mayes and Dove 2000). Different plant species have different patterns of alkanes and other components in their cuticular wax (Dove and Mayes 2005) and this fact has successfully been used to estimate the diet composition of housed (Charmley and Dove 2007) and grazing animals (Piasentier *et al*. 2007). N-alkanes are saturated hydrocarbons present in the cuticular waxes of higher plants and their profile is specific to plant species and plant parts (Dove *et al*. 1996; Ferreira *et al*. 2005). When using plant cuticular wax markers as diet composition markers, the number of plant species consumed should be equal to or less than the number of markers used. In dealing with complex plant communities, the number of plant species available to the herbivore may well be high and necessitate use of other cuticular markers in addition to n-alkanes (Ali *et al* 2005; Bezabih *et al* 2011). This is due to the limited number of n-alkane markers available in high enough concentrations to be used for diet composition calculations (Brosh *et al*. 2003; Dove and Mayes 2005). One means of overcoming this constraint is to include more classes of plant wax components as markers such as stable isotope enrichment of carbon ($\delta^{13}C$) (Bezabih et al., 2011; Ferreira et al., 2014) and long-chain alcohols (LCOH) (Dove and Charmley, 2008; López et al., 2015; Heublein et al., 2017) for distinguishing between plant species.

Stable isotopes are used as tracers to determine the proportional contributions of several sources in a mixture (Phillips and Gregg 2003). The $\delta^{13}C$ for individual n-alkanes showed wide variation for some forage species at different locations (Bezabih *et al*. 2011; Ferreira *et al*. 2014). Orthogonal procurst rotation (OPR) results suggested that $\delta^{13}C$ values of alkanes provided different discriminatory information to that given by other markers. Similarly, LCOH of the plant wax components are potential diet composition markers that provide different or complementary information about plants to those provided by n-alkanes (Bugalho *et al*. 2004). Bugalho *et al*. (2004) reported that the proportional variation explained by using n-alkane, LCOH or their combination differed among plant mixtures, suggesting that marker choices are diet dependent. According to Samuels *et al*. (2008), environmental conditions and geographical locations could influence the pattern of the cuticular wax profile of plant species growing in different places. However, little information is available (Bezabih *et al*. 2011) regarding the patterns of plant wax components in forage species in the highlands of Ethiopia.

According to Ali *et al*. (2005), it is important to document location specific information on the n-alkane profiles of available herbage species to make effective use of these markers in nutritional studies. As a result, there is a need to extend the earlier work in the Mid Rift Valley rangelands (Bezabih *et al*. 2011) to pasture lands with different vegetation composition in the central highlands of Ethiopia and also characterize additional plant wax components. Therefore, the objectives of the present study were to quantify n-alkane, LCOH profiles and $\delta^{13}C$ of n-alkanes of forage species from the central highlands of Ethiopia, and to evaluate the potential of using these compounds as markers to estimate diet composition of grazing animals.

Materials and Methods

Research site

The study was conducted in Kofele district, West Arsi Zone of Oromia Regional State, Ethiopia. It is situated at 7°07'N and 38°48'E with an altitude of 2660 masl with a predominantly loam soil type. The area has bi-modal rainfall distribution with short rains lasting from March to May and the main rainy season extending from June to September/October. The long term average annual rainfall is 1800 mm and the average daily temperature is 19.5°C.

Plant sampling and processing

Forage species samples were collected from 16 hectares of grazing land in October, 2017. Forage sampling was done from 100 representative quadrats of 0.5 m x 0.5 m at 10 m transects in the grazing land area. Forage sampling was done at 50% flowering stage when it was possible to easily identify the species, which coincided with the period when farmers start to use the pasture from enclosure areas known locally as "*kelo*". From a quadrat, whole plant species were mowed at 5 cm aboveground, sorted by species and weighed to determine the dominance of a species from a mixture. Forage species were pooled from different quadrats to obtain sufficient quantities of individual species in the sampling area for plant wax analysis. Forage species were identified by using guidebooks on the site and for those plant species that were difficult to identify on site, their local names were recorded and herbarium specimens were collected, pressed and dried properly by using a plant presser and identified and confirmed at the national herbarium, Addis Ababa University, Ethiopia. A total of 10 dominant available forage species as

they appeared naturally in the pasture (data not shown) consisting of grasses (4 species), legumes (3 species) and forbs (3 species) were selected for further analysis (Table 1). The individual forage species samples were oven-dried to a constant weight at 60 ^{0}C for 48 hours and then ground in a Willey mill to pass through a 1 mm sieve for subsequent laboratory analysis.

Table 1. Details of selected dominant forage species in the study area

Botanical name	Family	Plant type	Life form	To Ethiopia
Andropogon amethystinus	Poaceae	Grass	Perennial	Indigenous
Brachiaria scalaris	Poaceae	Grass	Annual	Indigenous
Ischaemum afrum	Poaceae	Grass	Perennial	Indigenous
Pennisetum thunbergii	Poaceae	Grass	Perennial	Indigenous
Trifolium cryptopodium	Fabaceae	Legume	Perennial	Indigenous
Trifolium mattirolianum	Fabaceae	Legume	Annual	Endemic
Trifolium tembense	Fabaceae	Legume	Annual	Indigenous
Centella asiatica	Apiaceae	Herb	Perennial	Indigenous
Haplocarpha hastata	Asteraceae	Herb	Perennial	Endemic
Uebelinia abyssinica	Caryophyllaceae	Herb	NA	Indigenous

Source: NDA (2011), NA=not available,

Extraction and analysis of plant wax markers

N-alkane and LCOH extraction and analysis were conducted at the isotope nutrition laboratory of James Hutton Institute, UK. Extraction and analysis of forage samples for n-alkanes was done

as described by Dove and Mayes (2006) by gas chromatography (GC), running analyses in duplicate, and for LCOH a modification of the method of Dove and Mayes (2006) was used. Long-chain fatty alcohols were extracted and analysed with 1-heptacosanol ($C_{27}OH$) being used as internal standard. Crude alcohol extracts were obtained using the method of Dove and Mayes (2006). Instead of using aminopropyl solid-phase extraction (SPE) columns to purify the crude alcohol extracts (Dove and Mayes 2006), a column-based urea adduction method (Mayes, unpublished) was adopted. To an empty SPE, fitted with polyethylene frits and closed at the bottom with a Luer syringe cap, a saturated solution of urea in ethanol was added followed by crude alcohol extract dissolved in n-heptane. After initial warming, the urea was allowed to crystallise and the solvents evaporated. The columns were placed in a positive-pressure SPE manifold and the Luer syringe caps removed. Sterols, stanols and any triterpenol impurities were removed by applying n-heptane to the columns (allowing the washings to run to waste). Water was then added in order to remove urea. The purified alcohol fraction was obtained using a second application of n-heptane. After removal of the solvent in the purified extract by evaporation, acetate derivatives of the LCOH were prepared by heating (50ºC) overnight with a mixture of acetic anhydride and pyridine. The pyridine and excess acetic anhydride were removed by evaporation and the derivatised extract was dissolved in n-dodecane prior to analysis by GC.

For GC analysis, the derivatised extract was injected (1 μL) into a Trace (Thermo Finnegan) gas chromatograph fitted with a split/splitless injector (running in splitless mode at 275ºC, with a splitless time of 5 min) and flame ionization detector (FID), using helium (flow rate 1 mL/min) as the carrier gas. The GC column was a non-polar bonded-phase capillary type Rtx-5 MS

(Restek) (30 m x 0.25 mm i.d. x 0.25 μm film thickness). The temperature programme used for the GC column oven was: 170ºC for 5 min; 30ºC/min to 210ºC; held at 210ºC for 1 min; 5.3ºC/min to 320ºC; held at 320ºC for 12 min. The fatty alcohol peaks from the detector were integrated and processed using EZChrom Elite software; the peak data was imported into an excel spreadsheet in order to calculate the fatty alcohol levels in the forage.

$$\text{Concentration alkane}_i\ (\text{mg/kg DM}) = \frac{[10 \times \text{area \% alkane}_i \times C_{34}\ \text{IS wt (mg)}]}{\text{sample wt (g)} \times \text{DM content} \times SRF_i \times FF_i}$$

$$\text{Concentration LCOH}_i(\text{mg/kg DM}) = \frac{[10 \times \text{area \% LCOH}_i \times C_{27}\text{OH IS wt (mg)}]}{\text{sample wt (g)} \times \text{DM content} \times SRF_i}$$

Where C_{34}ISwt and C_{27}OH ISwt is the weight of the solution containing the internal standard, and %Area is the area of n-alkane$_i$ or LCOH$_i$ calculated as the percent area of C_{34} or C_{27}OH, respectively. The DM content is the sample dry weight, SRF$_i$ is the average response factor, calculated as the percent area of n-alkane$_i$ or LCOH$_i$ in the mixed standard solution divided by the percent weight of n-alkane$_i$ or LCOH$_i$ in the mixed standard, and FF$_i$ is the fractionation factor.

For compound-specific isotope analysis, 90% of purified alkane extract obtained from each sample replicate was used and the remaining 10% was subjected to n-alkane analysis by GC. The carbon isotope composition of the alkanes was determined by fitting a GC with a split/splitless injector operated in split mode to a combustion interface which was connected to Gas Chromatography-Combustion-Isotope Ratio Mass Spectrometry (GC-C-IRMS) enabling the $\delta^{13}C$ values in individual n-alkanes to be determined. Full-base line separation of all individual

alkanes was achieved by fitting the Trace GC with a capillary column as described for n-alkane by Dove and Mayes (2006) and using helium as carrier gas. The temperature setting of the column was identical to that described for n-alkane. The isotope ratio of the alkanes was calculated in terms of conventional delta values ($\delta^{13}C$) as follows:

$$\delta^{13}C = \frac{1000\,(R_{sample} - R_{standard})}{R_{standard}}$$

Where, R_{sample} is the abundance ratio of ^{13}C to ^{12}C in the plant sample, and $R_{standard}$ is the abundance ratio of ^{13}C to ^{12}C in the standard sample.

Statistical analyses

Principal Component Analysis (PCA) was used to explore the pattern of n-alkane, LCOH and $\delta^{13}C$ of alkanes by grouping species along the principal component axes. The correlation matrix was used for the calculation, after the data was mean-centred and standardised. The first two principal components (PC1 and PC2) were plotted graphically where points on the graph represent plant species. The distance between species in the scatter plots is an indication of the difference in marker profile between the species. The species that are positioned close together in the scatter plots are the ones with a similar marker profile. On the other hand, the species that are placed far apart are expected to have large differences in their marker profiles according to Bezabih *et al*. (2011). Data were analyzed using Genstat (2008) for Windows (11th edition).

Results

Composition of n-alkanes in the whole plants

The n-alkane profiles of the forage species collected from grazing lands are presented in Table 2. The n-alkanes C_{22} and C_{34} are not shown as they were used as internal standards in the alkane analysis. Large variations in total C_{23} to C_{35} were observed between plant species, ranging from 58 mg/kg DM in *Centella asiatica* to 968 mg/kg DM in *Haplocarpha hastata*. The odd-chain n-alkanes comprised the highest proportion, being 79% of the total alkane concentration in *Ischaemum afrum* to 95% in *Haplocarpha hastata*. In all species, except *Brachiaria scalaris* and *Ischaemum afrum*, C_{31} was the most abundant, ranging from 13.2 mg/kg DM in *Centella asiatica* to 462.3 mg/kg DM in *Trifolium tembense* with an average concentration of 166.8 mg/kg DM. Next to C_{31} alkane, C_{29} was the most abundant alkane in most species, ranging from 9.3 mg/kg DM in *Centella asiatica* to 217.1 mg/kg DM in *Haplocarpha hastata* with an average concentration of 79.7 mg/kg DM, and the third dominant was C_{33} alkane. From the current study, *Centella asiatica* contained the lowest quantity in most alkane concentrations, with a total of 58 mg/kg DM.

Table 2. Concentration of n-alkanes for forage species collected from the central highlands of Ethiopia

Forage species	N-alkanes concentration (mg/kg DM)													
	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{35}	Total	TOC
Andropogon amethystinus	3.3	1.6	8.4	2.6	25.9	4.0	68.2	4.4	83.6	6.3	60.8	8.3	280	259
Brachiaria scalaris	1.5	1.0	6.4	1.5	18.3	4.9	53.4	8.6	97.3	14.6	184.8	42.8	437	405
Ischaemum afrum	6.7	6.4	34.0	8.4	52.8	8.6	28.2	9.9	36.8	15.1	24.2	6.1	240	189
Pennisetum thunbergii	2.3	1.9	4.7	2.8	19.4	4.4	51.8	5.4	93.2	8.1	81.0	10.6	288	263
Trifolium cryptopodium	11.1	11.3	17.6	11.2	45.7	14.2	143.2	14.0	292.6	12.9	31.7	3.6	613	546
Trifolium mattirolianum	2.2	1.8	6.9	2.9	27.0	5.9	54.7	8.6	107.9	7.0	13.5	2.4	243	215
Trifolium tembense	1.7	1.5	9.6	3.3	36.8	7.2	105.8	21.1	462.3	20.6	53.5	3.0	728	673
Centella asiatica	0.9	1.1	2.9	1.5	9.6	1.7	9.3	2.2	13.2	3.9	8.0	2.1	58	46
Haplocarpha hastata	1.0	1.4	3.6	1.4	52.0	6.5	217.1	21.7	419.4	16.4	145.0	79.8	968	918
Uebelinia abyssinica	1.2	1.3	5.1	2.3	29.3	4.4	65.0	5.5	61.9	4.5	8.7	1.9	193	173

TOC=total odd-chain alkanes

Composition of alcohol in the whole plants

The LCOH concentrations of forage species collected from the grazing lands are shown in Table 3. Large differences in the patterns of LCOH were observed among the plant species for $C_{22}OH$ to $C_{34}OH$, excluding $C_{27}OH$ which was used as internal standard. *Centella asiatica* forb showed the lowest total LCOH concentration (677 mg/kg DM) whereas *Trifolium mattirolianum* legume had the highest concentration (2228 mg/kg DM). Even-chain LCOH presented the highest proportion of the total LCOH concentration, ranging from 92% in *Brachiaria scalaris* to 97% in *Ischaemum afrum*. Most even-chain LCOH such as $C_{30}OH$, $C_{32}OH$, $C_{28}OH$ and $C_{26}OH$ were abundant with an average concentration of 362, 348, 266 and 237 mg/kg DM, respectively. *Trifolium mattirolianum, Trifolium tembense* and *Trifolium cryptopodium* legumes predominated in $C_{30}OH$ and *Andropogon amethystinus* and *Pennisetum thunbergii* grasses predominated in $C_{32}OH$.

Table 3. Concentration of LCOH for selected forage species collected from the central highlands of Ethiopia

Forage species	LCOH concentrations (mg/kg DM)													
	C_{22}	C_{23}	C_{24}	C_{25}	C_{26}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{34}	Total	TEC
Andropogon amethystinus	89.7	6.7	59.2	7.7	152.1	370.0	21.7	194.5	32.3	757.2	9.0	31.2	1731	1654
Brachiaria scalaris	36.3	4.4	43.8	4.4	138.4	75.7	10.5	53.9	26.1	327.1	13.0	25.5	759	701
Ischaemum afrum	23.2	2.8	51.0	4.8	297.2	454.5	10.4	78.9	9.6	119.1	2.9	3.1	1058	1027
Pennisetum thunbergii	76.4	5.9	49.9	4.9	170.5	191.8	15.6	116.6	32.2	605.8	10.1	41.2	1321	1252
Trifolium cryptopodium	68.4	10.4	87.3	10.0	155.6	129.7	30.5	572.1	50.8	457.7	18.6	44.2	1635	1515
Trifolium mattirolianum	41.4	5.8	55.4	8.6	210.9	511.0	54.7	1072.0	24.8	196.7	7.6	38.8	2228	2126
Trifolium tembense	71.6	6.7	59.6	9.7	176.6	140.6	48.7	956.5	67.0	306.7	11.9	35.2	1891	1747
Centella asiatica	17.6	2.5	40.1	8.2	178.8	208.8	14.9	113.2	12.4	74.9	1.9	3.2	677	637
Haplocarpha hastata	51.5	3.4	129.2	12.4	632.4	467.5	23.8	336.5	99.9	381.1	7.2	20.9	2166	2019
Uebelinia abyssinica	44.9	5.9	38.6	5.1	256.0	107.7	13.0	128.7	25.0	251.0	7.2	16.2	899	843

LCOH=long chain alcohol; TEC=total even-chain LCOH

Compositions of $\delta^{13}C$ of n-alkanes in the whole plants

The $\delta^{13}C$ values of the n-alkanes are given in Table 4. The isotope enrichment of C_{34} was not shown as it was used as internal standard in GC-IRMS analysis. Patterns of $\delta^{13}C$ of the n-alkanes for all n-alkanes were between −19.7‰ in *Andropogon amethystinus* and −40.6‰ in *Trifolium mattirolianum*, which showed relatively large variations between forage species. The $\delta^{13}C$ of n-alkanes for the grass species ranged from −19.7‰ for C_{29} alkane in *Andropogon amethystinus* to −38.1‰ for C_{32} alkane in *Brachiaria scalaris,* whereas it ranged from −29.2‰ for C_{24} alkane in *Trifolium tembense* to −40.6‰ for C_{32} alkane in *Trifolium mattirolianum* legume species. On average, the odd-chain alkanes tended to be greater in magnitude compared to the subsequent even-chain with 1.0 delta unit in *Brachiaria scalaris* to 7.2 delta units in *Trifolium mattirolianum* except in *Uebelinia abyssinica* in which there was no data for even-chain alkanes C_{24} and C_{32} due to insufficient alkane present during analysis. The enrichment of $\delta^{13}C$ for C_{35} alkane was only presented for half of the plant species studied in the current study and insufficient alkane was observed for the other plant species during GC-IRMS analysis. *Andropogon amethystinus* comprised the highest concentration of $\delta^{13}C$ for alkanes C_{23} to C_{29}. *Trifolium mattirolianum* contained the lowest concentration as carbon length increase from C_{25} to C_{33}.

Table 4. Concentration of $\delta^{13}C$ for selected forage species collected from the central highlands of Ethiopia.

Forage species	$\delta^{13}C$ values (‰) of n-alkanes											
	C_{23}	C_{24}	C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}	C_{35}
Andropogon amethystinus	−23.9	−25.9	−20.9	−22.8	−20.0	−22.9	−19.7	−25.4	−21.3	−29.1	−29.8	−24.0
Brachiaria scalaris	−29.8	−28.8	−29.7	−27.5	−30.9	−34.7	−33.0	−36.4	−34.6	−38.1	−29.9	−36.8
Ischaemum afrum	−26.8	−28.4	−22.0	−26.3	−21.1	−24.9	−22.0	−20.2	−22.7	−21.9	−29.0	ND
Pennisetum thunbergii	−27.2	−28.5	−25.4	−25.8	−21.9	−24.7	−21.1	−25.3	−21.9	−28.1	−29.7	−23.8
Trifolium cryptopodium	−30.7	−30.1	−30.4	−30.5	−33.0	−34.3	−34.6	−36.9	−35.2	−37.0	−29.9	ND
Trifolium mattirolianum	−30.4	−29.8	−31.5	−31.3	−35.0	−38.4	−37.1	−39.9	−37.8	−40.6	−30.0	ND
Trifolium tembense	−30.4	−29.2	−31.4	−33.2	−34.7	−35.5	−36.6	−37.9	−37.0	−38.3	−29.8	ND
Centella asiatica	−29.1	−31.4	−27.4	−33.1	−30.1	−32.6	−28.5	−30.3	−26.9	−30.1	−29.5	ND
Haplocarpha hastata	−29.9	−28.6	−28.8	−30.7	−33.2	−36.1	−34.9	−37.7	−35.8	−37.6	−29.9	−36.0
Uebelinia abyssinica	−30.8	ND	−27.6	−29.7	−31.6	−34.3	−35.4	−36.8	−35.6	ND	−29.7	ND

ND=not detected

Principal Component analysis

The result of the PCA showed that 81.1% of the variance in the profile of n-alkanes was explained by the first two principal components (PC1 and PC2), whereas 69.3 and 82.9% was explained in the case of LCOH and $\delta^{13}C$ of n-alkanes, respectively (Table 5). The two principal component scores were used to present the position of forage species in a two-dimensional space as shown in Fig. 1. For n-alkanes, PCA showed that some of the forage species showed scattering along PC1 and PC2. *Haplocarpha hastata, Brachiaria scalaris* and the two legumes (*Trifolium cryptopodium and Trifolium tembense)* were separated from other species. On the other hand, the two grasses (*Andropogon amethystinus* and *Pennisetum thunbergii*), *Trifolium mattirolianum* and *Uebelinia abyssinica* clustered close to each other. When the PCA analysis was based on LCOH, a distinct pattern of cluster was observed with groupings observed between *Ischaemum afrum*, *Centella asiatica, Brachiaria scalaris*, and *Uebelinia abyssinica*. When the PCA analysis was based on $\delta^{13}C$ of n-alkanes, most of the forage species studied were separated except for the three legumes which clustered close to each other. When the data from markers were combined (n-alkanes and $\delta^{13}C$ of n-alkanes, n-alkanes and LCOH, LCOH and $\delta^{13}C$ of n-alkanes and the three markers), better scattering of forage species was observed, except when n-alkanes and LCOH combined in which *Andropogon amethystinus, Pennisetum thunbergii* and *Trifolium mattirolianum* clustered close to each other (Fig. 2).

Table 5. The variance (%) in the pattern of cuticular wax marker concentration explained by the first two principal component scores (PC1 and PC2) for each data set

	% Variance explained by:		
	PC1	PC2	Total
n-alkanes	47.6	33.5	81.1
LCOH	41.4	27.9	69.3
$\delta^{13}C$ of n-alkanes	65.0	17.9	82.9

LCOH=long chain alcohol; PC=principal components

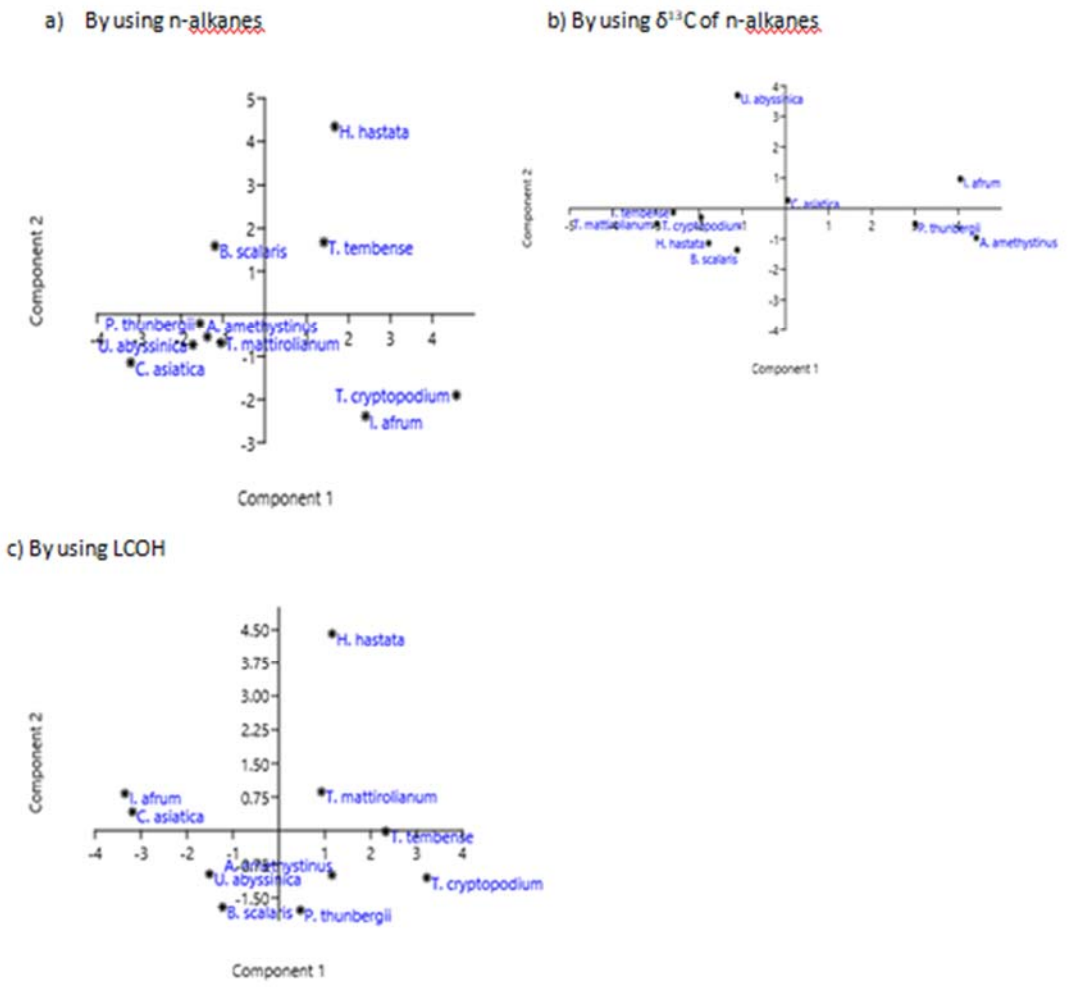

Fig. 1: Two-dimensional scatter plots of pasture species from principal component scores (PC1 and PC2) based on individual markers.

Grasses: *A. amethystinus= Andropogon amethystinus*; *B. scalaris= Brachiaria scalaris*; *I. afrum= Ischaemum afrum*; *P. thunbergii=Pennisetum thunbergii*; legumes: *T. cryptopodium= Trifolium cryptopodium*; *T. mattirolianum= Trifolium mattirolianum*; *T. tembense= Trifolium tembense*; forbs: *C. asiatica= Centella asiatica*; *H. hastata= Haplocarpha hastata; U. abyssinica= Uebelinia abyssinica*

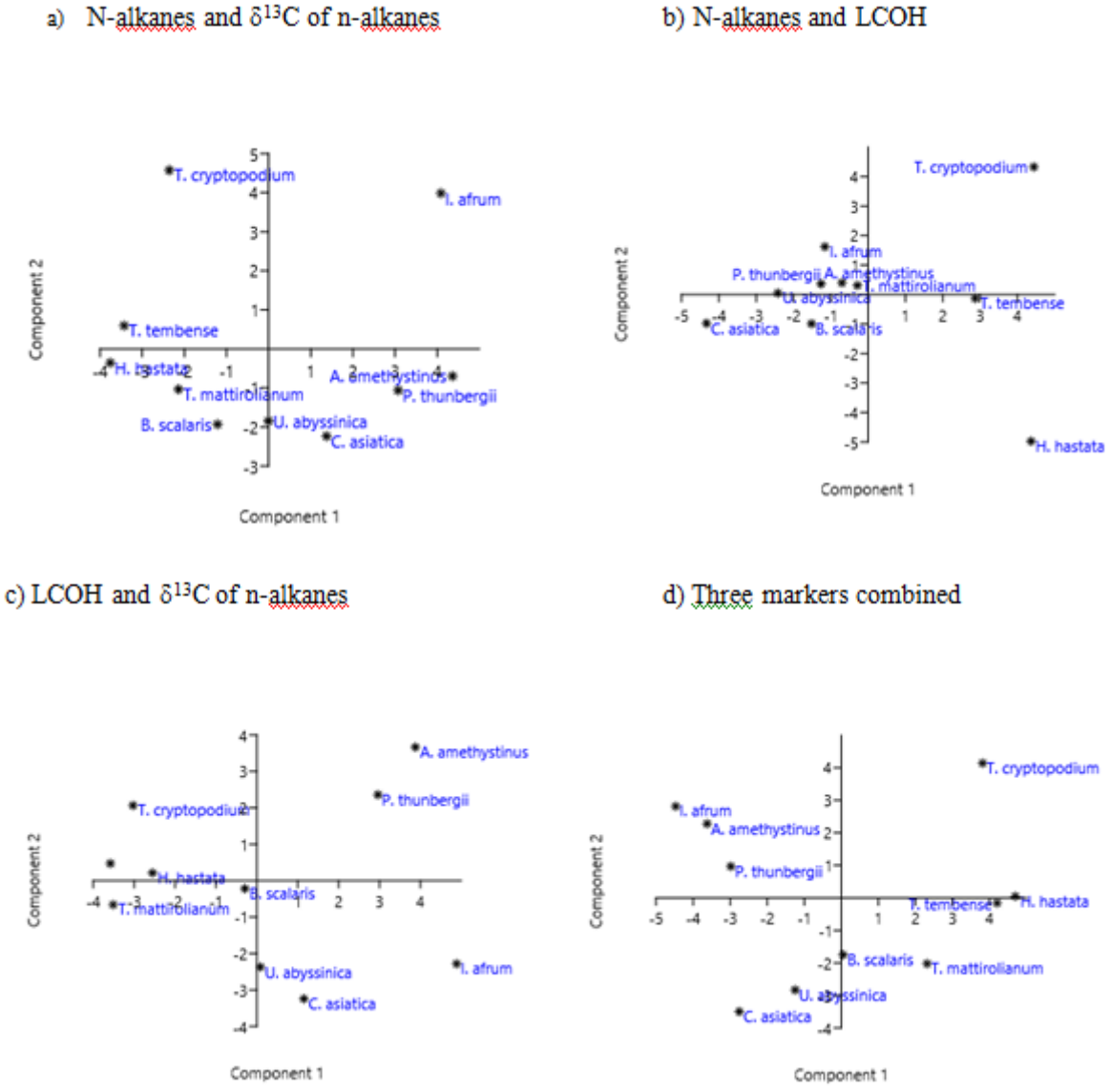

Fig. 2: Two-dimensional scatter plots of pasture species from principal component scores (PC1 and PC2) based on marker combinations.

Grasses: *A. amethystinus= Andropogon amethystinus*; *B. scalaris= Brachiaria scalaris*; *I. afrum= Ischaemum afrum*; *P. thunbergii=Pennisetum thunbergii*; legumes: *T. cryptopodium= Trifolium cryptopodium*; *T. mattirolianum= Trifolium mattirolianum*; *T. tembense= Trifolium tembense*; forbs: *C. asiatica= Centella asiatica*; *H. hastata= Haplocarpha hastata; U. abyssinica= Uebelinia abyssinica*

Discussion

Composition of n-alkanes, LCOH and δ^{13}C of n-alkanes *in the whole plants*

The plant wax concentrations in the majority of the species analyzed in this study have not been reported in the literature as most of them are endemic and indigenous to Ethiopia (Table 1). The n-alkane profile of the plant species has been documented in the literature (Ferreira *et al*. 2013; López *et al*. 2015; Heublein *et al*. 2017). Also Bezabih *et al*. (2011) evaluated the n-alkane and δ^{13}C of n-alkanes of forage species collected from the Mid Rift Valley rangelands of Ethiopia. In all cases considerable variability was observed in the n-alkane profile as well as the isotopic enrichment of n-alkanes among the studied species. As Ali *et al*. (2005) observed, it is necessary to document location specific information on the marker profiles of herbage species as environmental conditions and geographical locations could influence the pattern of the cuticular wax profile of plant species growing in different places (Samuels *et al*. 2008; Bezabih *et al*. 2011).

In agreement with the previous findings (Ali *et al*. 2005; Dove and Charmley 2008; Bezabih *et al*. 2011), the odd-chain n-alkanes were presented in a greater concentration than even-chain alkanes in the current study. Laredo *et al*. (1991) suggested 50 mg/kg DM to be the minimum concentration that any n-alkane must have to be used as a marker. The alkanes C_{29} and C_{31} except for *Ischaemum afrum* and *Centella asiatica* fulfilled this minimum requirement for evaluated forage species. Following the two alkanes, C_{33} alkanes recorded more than 50 mg/kg DM for three grasses (*Pennisetum thunbergii*, *Andropogon amethystinus* and *Brachiaria*

scalaris), *Trifolium tembense* legume and *Haplocarpha hastate* forb. The dominances of alkanes C_{29}, C_{31} and C_{33} for most species in the forage species conforms the earlier findings (Ferreira *et al*. 2007; Bezabih *et al*. 2011), and these alkanes tended to be suitable for diet composition, feed intake and digestibility estimation. The enrichment levels of $\delta^{13}C$ of alkanes for forage legume species in the current study are in agreement with the values obtained for C3 plant species as observed by Bezabih *et al*. (2011) and Ferreira *et al*. (2014). The lower enrichment level of $\delta^{13}C$ of alkanes for forage grasses, except *Brachiaria scalaris*, in the current study supports the earlier findings by Schweizer *et al*. (1999), and is typical of C4 plants.

Large differences between forage species in their LCOH concentration were observed in the current study in agreement with the earlier findings (Ferreira et al., 2013; López et al., 2015), which indicates the usefulness of these compounds as plant wax markers in diet composition estimation. Similarly, predominant even-chain compared to odd-chain LCOH reported by others (Dove and Mayes 2006; Ferreira *et al*. 2013) were observed from the present study. The observed higher concentration of total LCOH compared to n-alkane (Table 2 and 4) is in agreement with previous reports (Ferreira *et al*. 2005; Oliv´an *et al*. 2007).

Some species which have very low concentrations of n-alkane had higher concentrations in LCOH. For example, *Brachiaria scalaris* grass which was the second lowest in total LCOH become higher in total n-alkane concentration compared to others. On the other side, *Trifolium mattirolianum,* which was the highest in magnitude in total LCOH concentration, had lower total n-alkane concentration. This is an important observation, as it suggests the value of using a combination of markers to improve the discriminatory power of the wax profiles in diet

composition estimation (Charmley and Dove 2007). As a result, it was possible to select marker types (alkanes or LCOH) for diet estimation depending on their concentration in the available plant species (Ferreira *et al*. 2013). *Centella asiatica*, *Uebelinia abyssinica* and *Ischaemum afrum* had lower concentrations of both total n-alkane and LCOH, which may necessitate evaluation of another marker to avoid lower accuracy in diet estimation due to their lower concentrations. The predominance of $C_{30}OH$ concentrations for *Trifolium mattirolianum, Trifolium tembense* and *Trifolium cryptopodium* agrees with Body (1974) who observed prevalent $C_{30}OH$ alcohol in white clover. Oliv´an *et al*. (1999) found higher concentrations of $C_{30}OH$ in dicotyledons compared to monocotyledons. This indicates the usefulness of $C_{30}OH$ alcohol in distinguishing between the dicotyledons and monocotyledons in forage legumes.

Principal Component analysis

The majority of the forage species showed distinct positions along PC1 and PC2, which showed the level of separation between species based on the respective marker profiles. Even though the ultimate test could only be achieved by actually employing these markers to estimate diet composition, such multivariate statistics can provide an indication of which species are likely to be distinguishable from each other (Mayes and Dove 2000; Ali *et al*. 2005)**.** From the present study, $\delta^{13}C$ of n-alkanes showed relatively larger variation as explained by the first two principal components and this indicated that $\delta^{13}C$ of n-alkanes had the greatest variability among the studied forage species. According to Mayes (1998), for any compound to qualify as a diet composition marker, a wide variation in the pattern of marker concentration should exist among plants, and in many instances plant wax hydrocarbons have proved to be suitable.

The three wax markers (n-alkanes, LCOH and $\delta^{13}C$ of n-alkanes) scattered and clustered forage species differently which favours multiple markers for diet composition estimations. For instance, the three legumes (*Trifolium cryptopodium*, *Trifolium mattirolianum* and *Trifolium tembense*) clustered close to each other when $\delta^{13}C$ of n-alkanes was used as a marker, but much wider when either n-alkanes or LCOH were used. Similarly, the two grasses (*Andropogon amethystinus* and *Pennisetum thunbergii*), and *Trifolium mattirolianum* and *Uebelinia abyssinica* showed resemblance when n-alkanes were used, but scattered when either LCOH or $\delta^{13}C$ of n-alkanes were used. This trend is in agreement with Kelman *et al*. (2003) and Ali *et al.* (2005) for alkanes and alcohols and Bezabih *et al*. (2011) for alkanes and $\delta^{13}C$ of n-alkanes, who observed different plant wax markers clustering and scattering of species differently. Dove and Mayes (1996) postulated that using a single marker type for estimating the composition of the diet of animals found in complex environments, such as forests or rangeland, is likely to produce less reliable diet composition estimates than using multiple marker types**.** Similar trends were observed in the current study as combining markers resulted in more scattering of forage species compared to individual markers. When more species are present in a mixture, the possibility of existence of species with similar patterns of markers would increase.

Conclusions

The results from the present study showed huge variability in the patterns of concentration of n-alkanes, LCOH and $\delta^{13}C$ of n-alkanes among forage species studied from the central highlands of Ethiopia. Odd-chain n-alkanes were found to be greater when compared to the subsequent even-chain alkanes, even though the reverse was observed for LCOH. Most of the inter species

variances studied in forage species were explained by the first two principal components, and the studied plant wax marker studied scattered and clustered forage species differently, which favoured multiple markers for diet composition estimations. This will allow a better discrimination between plant species than using one marker alone, which shows the potential use of multiple plant wax markers for diet composition estimations. It was concluded that the differences in the patterns of concentrations of n-alkanes, LCOH and $\delta^{13}C$ of n-alkanes in the present study could make them suitable as markers for diet composition estimation of grazing animals.

Conflicts of interest

The authors declare no conflicts of interest.

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Data Availability Statement

The data that support this study will be shared upon reasonable request to the corresponding author.

References

Ali HAM, Mayes RW, Hector BL, Ørskov ER (2005) Assessment of n-alkanes, long-chain fatty alcohols and long-chain fatty acids as diet composition markers: the concentrations of these compounds in rangeland species from Sudan. *Animal Feed Science and Technology* **121**, 257–271. https://doi.org/10.1016/j.anifeedsci.2005.02.026.

Bezabih M, Pellikaan WF, Tolera A, Hendriks WH (2011) Evaluation of n-alkanes and their carbon isotope enrichments ($\delta^{13}C$) as diet composition markers. *Animal* **5**, 57–66. https://doi.org/10.1017/S1751731110001515.

Body DR (1974) Neutral lipids of leaves and stems of *Trifolium repens*. *Phytochemistry* **13**, 1527–1530.

Brosh A, Henkin S, Rothman .J, Aharoni Y, Orlov A, Arieli A (2003) Effects of faecal n-alkane recovery in estimates of diet composition. *Journal of Agricultural Science* **140**, 93–100.

Bugalho MN, Dove H, Kelman W, Wood JT, Mayes RW (2004) Plant wax alkanes and alcohols as herbivore diet composition markers. *Journal of Range Management* **57**, 259−268.

Charmley E, Dove H (2007) Using plant wax markers to estimate diet composition and intakes of mixed forages in sheep by feeding a known amount of alkane labelled supplement. *Australian Journal of Agricultural Research* **58**, 1215–1225. https://doi.org/10.1071/AR7187.

Dove H, Charmley E (2008) Using the alkanes and long-chain alcohols of plant cuticular wax to estimate diet composition and the intakes of mixed forages in sheep consuming a known amount of alkane-labelled supplement. *Animal* **2**, 1474–1485. https://doi.org/10.1017/S1751731108002735.

Dove H, Mayes RW (1996) Plant wax components: A new approach to estimating intake and diet composition in herbivores. *Journal of Nutrition* **126**, 13–26.

Dove H, Mayes RW (2005) Using n-alkanes and other plant wax components to estimate intake, digestibility and diet composition of grazing/browsing sheep and goats. *Small Ruminant Research* **59**, 123–139. https://doi.org/10.1016/j.smallrumres.2005.05.016.

Dove H, Mayes RW (2006) Protocol for the analysis of n-alkanes and other plant-wax compounds and for their use as markers for quantifying the nutrient supply of large mammalian herbivores. *Nature Protocols* **1**, 1680–1697. https://doi.org/10.1038/nprot.2006.225.

Dove H, Mayes RW, Freer M (1996) Effects of species, plant part, and plant age on the n-alkane concentrations in the cuticular wax of pasture plants. *Australian Journal of Agricultural Research* **47**, 1333–1347.

Ferreira LMM, Celaya R, Santos AS, Mayes RW, Rodrigues MAM, Osoro K (2013) Application of long-chain alcohols as diet composition markers in sheep fed on grass–white clover and

heather–gorse plant species. *Grass and Forage Science* **70**, 30–43. https://doi.org/10.1111/gfs.12083.

Ferreira LMM, Daniela JB, Celaya R, Santos AS, Osoro K, Rodrigues MAM, Pellikaan WF (2014) Utilization of carbon isotope enrichments ($\delta^{13}C$) of alkanes as faecal markers to estimate diet composition of goats fed with heath land vegetation. *Animal Feed Science and Technology* **191**, 26–38. http://dx.doi.org/10.1016/j.anifeedsci.2014.02.004.

Ferreira LMM, Garcia U, Rodrigues MAM, Celaya R, Dias-da-Silva A, Osoro K (2007) The application of the n-alkane technique for estimating the composition of diets consumed by equines and cattle feeding on upland vegetation communities. *Animal Feed Science and Technology* **138**, 47–60. https://doi.org/10.1016/j.anifeedsci.2006.11.007.

Ferreira LMM, Oliv´an M, Garcia U, Rodrigues MAM, Osoro K (2005) Validation of the alkane technique to estimate diet selection of goats grazing heather–gorse vegetation communities. *Journal of the Science of Food and Agriculture* **85**, 1636–1646. https://doi.org/10.1002/jsfa.2162.

Genstat (2008). Genstat for windows, 11th edition, VSN International Ltd., Oxford.

Heublein C, Südekum KH, Gill FL, Dohme-Meier F, Schori F (2017) Using plant wax markers to estimate the diet composition of grazing Holstein dairy cows. *Journal of Dairy Science* **100**, 1019–1036. https://doi.org/10.3168/jds.2016-11494.

IBC (2005) Institute of Biodiversity Conservation, national biodiversity strategy and action plan, Government of the Federal Democratic Republic of Ethiopia, vol. 115. https://doi.org/10.1016/S0006-3495(96)79498-5.

Kelman W, Bugalho MN, Dove H (2003) Cuticular wax alkanes and alcohols used as markers to estimate diet composition of sheep (*Ovis aries*). *Biochemical Systematics and Ecology* **31**, 919–927. https://doi.org/10.1016/S0305-1978(03)00081-4.

Laredo MA, Simpson GD, Minson DJ, Orpin CG (1991) The potential for using n-alkanes in tropical forages as a marker for determination of dry matter intake by grazing ruminants. *Journal of Agricultural Science* **117**, 355–361.

López López C, Celaya R, Santos AS, Rodrigues MAM, Osoro K, Ferreira LMM (2015) Application of long-chain alcohols as faecal markers to estimate diet composition of horses and cattle fed with herbaceous and woody species. *Animal* **9**, 1786–1794. https://doi.org/10.1017/S1751731115001196.

Mayes RW (1998) New potential markers for determining diet composition. In 'Proceeding of the IXth European intake workshop'. pp. 63–66. (Institute of Grassland and Environmental Research North Wyke, UK).

Mayes RW, Dove H (2000) Measurement of dietary nutrient intake in free-ranging mammalian herbivores. *Nutrition Research Reviews* **13**, 107–138.

Mengistu A, Kebede G, Feyissa F, Assefa G (2017) Review on major feed resources in Ethiopia: Conditions, challenges and opportunities. *Academic Research Journal of Agricultural Science and Research* **5**, 176-185. https://doi.org/10.14662/ARJASR2017.013.

NDA (2011). Natural database for Africa, version 2.0, Addis Ababa, Ethiopia.

Oliv´an M, Dove H, Mayes RW, Hoebee SE (1999) Recent developments in the use of alkanes and other plant wax components to estimate intake and diet composition in herbivores. *Revista Portuguesa de Zootecnia* VI: 1–25.

Oliv´an M, Ferreira LMM, Garcı´a U, Celaya R, Osoro K (2007) Application of n-alkanes as diet composition markers in grazing/browsing goats and sheep: effect of using different faecal recovery corrections and plant species grouping approaches. *Australian Journal of Agricultural Research* **58**, 1013–1022.

Phillips DL, Gregg JW (2003) Source partitioning using stable isotopes: coping with too many sources. *Oecologia* **136**, 261–269.

Piasentier E, Sacca, E, Bovolenta S (2007) Dietary selection and ingestive behaviour of fallow deer and sheep grazing on adjacent monocultures of white clover and tall fescue. *Small Ruminant Research* **71**, 222–233. https://doi.org/10.1016/J.SMALLRUMRES.2006.07005.

Samuels L, Kunst L, Jetter R (2008) Sealing plant surfaces: cuticular wax formation by epidermal cells. *Annual Review of Plant Biology* **59**, 683–707. https://doi.org/10.1146/annrev.arplant.59.103006.093219.

Schweizer M, Fear J, Cadisch G (1999) Isotopic (^{13}C) fractionation during plant residue decomposition and its implications for soil organic matter studies. *Rapid Communications in Mass Spectrometry* **13**, 1284–1290.