Fatty acid profile in two berseem clover (Trifolium alexandrinum L.) cultivars: effect of part of plant and phenological stage. Preliminary results

Cabiddu, A.[1], Wencelová, M.[2], Bomboi G.[3], Decandia, M.[1], Molle, G.[1], Salis, L.[1]

[1] Agris Sardegna, Olmedo, Italy

[2] Institute of Animal Physiology (IAP) of Slovak Academy of Sciences in Košice, Slovakia

[3] Facoltà di Medicina Veterinaria, Università di Sassari, Italy

Abstract

Grazing animals have diets rich in poly-unsaturated fatty acids (PUFA), in particular α-linolenic acid (C18:3n-3), so pasture is the richest and most environmentally sustainable source of unsaturated fatty acids for animal products. The concentration of PUFA in forages varies with species, cultivar, phenological stage, silage making technique, regrowth interval, nitrogen fertilization level and other management factors. An experiment was carried out, by a randomized block design with three replicates, to study in two widespread berseem clover cultivars (*Trifolium alexandrinum* L. Sacromonte and Marmilla) the influence of part of plant and phenological stage on fatty acid profile. In this study Sacromonte showed a higher proportion of leaves (94 vs 85% on DM basis; $P<0.05$) and lower proportion of stems (6 vs 15% on a DM basis; $P<0.05$) if compared with Marmilla during the vegetative stage. Leaves were characterized by higher levels of MUFA (0.50 vs 0.23 mg/g DM) and PUFA (10.33 vs 3.84 mg/g DM) if compared to stems ($P<0.01$) in both cultivars. Total unsaturated fatty acids represent 10.83 and 4.04 mg/g DM in leaves and stems respectively ($P<0.01$). No influence of phenological stage in different parts of plant was detected in terms of total unsaturated fatty acid and polyunsaturated fatty acids. These results firstly point out in berseem clover the change of FA profile in different parts of plant during vegetative and reproductive stage, but need to be confirmed in a long-term study.

Key words: berseem clover, fresh forage, grazing ruminants, unsaturated fatty acid

Introduction

In recent years, society's interest has been increasingly focused on foods with a positive effect on human health, reducing the seriousness of several chronic disease including cancer, atherosclerosis, obesity and diabetes (Elwood *et al*., 2010; Dewhurst, 2010). The concentrations of functional fatty acids (FA) are higher in products from grazing ruminants, reflecting the presence of FA in grass, such as alpha-linolenic acid; in fact, feeding with fresh herbage results in an increased content of beneficial poly-unsaturated FA, including conjugated linoleic acid (CLA) in milk and meat (Elgersma, 2015). Pasture is the richest and most environmentally sustainable source of CLA precursors. European dairy sheep production is mainly located in the Mediterranean basin (52% of the total dairy sheep stock in Europe is reared in Italy and Greece) and is based on semi-extensive farming systems. In particular, when we consider herbaceous pastures, grazing behavior is different between sheep and cows. In fact as reported recently, sheep on herbaceous pastures tend to graze only within the layer of green leaves and are reluctant to forage below this layer, where they find a prevalence of dead material and/or stems (e.g. Dumont *et al*., 1995). This leads to a higher selection ratio in grazing sheep than grazing cows. Lower leaf to stem ratios in the pasture resulted in a decrease in total dry matter intake and in vitro DM digestibility of total diet in grazing dairy ewes on annual ryegrass (Molle *et al*., 2004). These results demonstrate that understanding the variability of chemical composition of different plant parts (leaves, stems and flowers) is important to determine their contribution to the ingested diet of grazing ruminant. Several authors suggest that an increase in leafiness of the pasture could interfere with the level of polyunsaturated fatty acid (PUFA) content in sheep milk (Cabiddu *et al*., 2004; Buccioni *et al*., 2012). In addition, the concentration of PUFA in forages varies with species, cultivar, phenological stage, silage making technique, regrowth interval, nitrogen fertilization level and other management factors (Elgersma *et al*., 2003; Cabiddu *et al*., 2005; Elgersma, 2015). There is a low number of reports in literature about FA profile of different parts of plant; it can be assumed that plant leaves contain higher levels of PUFA (in particular C18:2 and C18:3) if compared with other plant parts (stems and flowers). Wyss (2012) observed that linoleic and linolenic acid contents is different between leaves and stems, with a higher level of linolenic acid observed in leaves than stems whereas linoleic acid content shown greater variability within parts of plant. Mediterranean climate is characterized by wet winters and hot dry summers with fast rise of temperatures and drop in soil moisture content during spring, which leads to a rapid transition from vegetative to reproductive stage in forage plants. This implies large changes in terms of plant chemical composition with a different development pattern if compared with continental climate grasslands.

Berseem clover is an annual forage legume widely grown in Mediterranean basin for its environmental adaptability and forage quality (Martiniello *et al*., 1996; Pecetti *et al.*, 2012). This variety has a good tolerance to drought, it is highly responsive to irrigation and shows a higher capacity to regrow after grazing than other species commonly used such as vetch, crimson clover (*Trifolium incarnatum* L.) and sulla (*Hedysarum coronarium* L.) (Giambalvo et.al, 2010). No data are currently available in literature, to evaluate the amplitude of FA variation in two forage cultivars of the same species in different parts of plant from growth to reproduction stage.

The aim of this research is to study in two widespread berseem clover cultivars the differences of chemical composition among part of plants and the influence of phenological stages.

Moreover, the research aims to investigate the relationship between macro chemical composition and fatty acid profile of berseem plants grown in Mediterranean basin.

Materials and Methods

Experimental site and experimental design

The experiment was carried out at the Bonassai research station (NW Sardinia, Italy, 40° 39’46’’N, 8° 21’ 46’’E, 33 m a.s.l) from autumn 2006 to spring 2007 on a flat terrain with calcareous clay soil. The average annual rainfall and min and max temperatures are 573 mm, 11.1 °C and 20.8 °C, respectively (climatological normals "CLINO" for the period 1961-1990). During October 6 plots of 12 m^2 were sown with *Trifolium alexandrinum* L. in order to compare 2 commercial cultivars (CV), Marmilla and Sacromonte. The first one derives from Sardinian native genotypes, the latter is an old widespread variety that provides in Sardinia high forage yield over the years (Pecetti *et al.*, 2012). The experimental design was a randomized block with three replicates. The plot size was 1.5 x 8m. The sowing rate was 30 kg ha^{-1} and 92 kg ha^{-1} of P_2O_5 were applied before seeding. Two different 0.5 m^2 areas per plot, were cut at ground level by a mower and sampled in March during vegetative (GW) stage and in May during reproductive (REP) stage. A standardized procedure was selected for definition of different phenological stages, according to Moore *et al.* (1991). In particular, vegetative stage was assigned when no flower buds (0%) were visible on plants, and reproductive stage was assigned when more than 50% of plants had at least 1 open flower, given that the onset of flowering in berseem clover occurred over an extended period. Samples from each plot were collected separately to evaluate forage dry matter yield (DM, g m^{-2}). A sub sample of fresh material, coming from both CV (150 g), was partitioned into leaves, stems and flowers to determine plant structure for flowering plants sampled at the REP stage. Leaf to stem ratios were also calculated. The material was frozen (−20 °C) immediately after separation and then freeze-dried, ground and stored at -20 °C until the chemical analysis.

Chemical analysis

All samples (including Marmilla or Sacromonte cultivars, collected separately) were freeze dried and then ground to pass through a 1-mm sieve. The following parameters have been then determined: dry matter (DM), ash, ether extract (EE), crude protein (CP) and protein fractions (AOAC, 1990), neutral detergent fiber on an ash-free basis (NDFom), acid detergent fiber on an ash-free basis (ADFom), acid detergent lignin (ADL; Van Soest *et al.*, 1991) and water soluble carbohydrates (WSC, Deriaz, 1961).

Fatty acid analysis

1 g DM of the freeze-dried sample was used for lipid extraction. Extraction was carried out with chloroform: methanol (2/1; v/v) based on the method described by Christie (1989). Fatty acids were determined according to the method of Stanton *et al.*, (1997), using trans-methylation catalyzed by acid. Separation and quantification of methyl esters was carried out with a gas chromatography system (Varian

3900; Varian, Harbor City, CA), equipped with a split/splitless injector and a flame ionization detector. Methyl ester separation was carried out on a capillary column SP2560 (100 m × 0.25 mm i.d., 0.25 µm of phase; Supelco Inc., Bellefonte, PA, USA) using helium as the carrier gas (constant flow, 1 ml/min). The injector and detector temperatures were set at 290 °C. The injection was undertaken in split mode with a 1:100 split-ratio. The temperature of the column was initially 75 °C for 1.5 min, and then increased to 190 °C at 8 °C/min, kept at this temperature for 25 min, then increased again to 230 °C at 15 °C/min, and kept for a further 4.5 min at 230 °C. The results were analyzed using Star system 6.0 Varian software. Individual FAMEs were identified by comparison with a standard mixture of 37 components (Matreya Inc., Pleasant Gap PA, USA). Calibration curves with internal standards were performed to quantify fatty methyl esters (FAMEs). In particular, the internal standard Me-C9:0 was used to quantify short chain FA methyl esters from Me-C8:0 to Me-C10:0, the internal standard Me-C13:0 was used to quantify medium chain FA methyl esters from Me-C11:0 to Me-C17:0 and the internal standard Me-C19:0 to quantify long chain FA methyl esters from Me-C18:0 to Me-C26:0. The concentration of each internal standard added to the sample was 10 mg/g of DM.

Statistical analysis

Each cultivar gave raise to 15 samples, including 6 leaf samples (3 from GW and 3 from REP stage), 6 stem samples (3 from GW and 3 from REP stage) and 3 flower samples. Data on plant structure were analyzed by a GLM with CV, part of plant (PP) and phenological stage (PS) as fixed effects. Means were compared by Tukey *t-tests*. Since no Cv effect was detected, we decided to split the results separately by Cv and report in tables only the effect of PP and PS and their first order interaction. The probability level for evaluation of the significance of effects was set at $P < 0.05$ and trends were assumed with P ranging between 0.05 and 0.10. Comparison of the chemical composition of flowers from the two CVs was carried out separately from analysis of composition of other plant parts because flowers were present only in REP stage. Therefore, for this variable the GLM included only CV as fixed effect.

Results

DM production and plant morphology

During the experimental period, maximum temperatures were slightly higher, whereas rainfall and minimum temperatures were lower than average seasonal values. The two CVs showed the same seasonal DM production, 580±4 g m^{-2}, with 70% of production observed at the REP stage for both varieties. During the vegetative stage, Marmilla showed a lower quantity (% of plant DM) of leaf and higher quantity of stem material if compared to Sacromonte (P<0.05) (Figure 1), with a lower leaves/stems ratio (P<0.01) (Figure 2). No effect of the CV was detected during the REP stage on leaves, stems, flowers (expressed as % DM) and flower/leave and flower/stem ratios (Figure 1 and 2). Macro chemical and fatty acid composition was not significantly affected by CV, however, for the sake of precision, results for PP and PS factors are shown separately for both cultivars.

Macro chemical composition

Both CV leaves (tables 1, 2) had a higher content of EE, CP and TP (P<0.01), while stems were characterized by higher levels of WSC and NPN (P<0.01). The level of ADF was higher (P<0.05) in stems if compared to leaves, at both phenological stages, of CV Sacromonte. Levels of DM, Ash, NDF, ADF, ADL and BSP increased in leaves and stems as plants transitioned from vegetative to reproductive stage. A decreasing effect was detected for insoluble protein (IP) in leaves and stems as plants matured from vegetative to reproductive stage (P<0.01). Overall, a strong interaction between PP and PS was observed for both CV (tables 1 and 2).

Fatty acid profile

Overall α-linolenic and palmitic fatty acids were the dominant FAs in berseem clover (tables 3 and 4). For both CVs, leaves showed the highest content of α-linolenic, C16:0, C18:0, PUFA and total FA, as reported in tables 3-4. Moreover, leaves showed higher levels of MCSFA, MUFA, UFA, total UFA and total FA if compared to stems (P<0.01). Phenological stage affected the level of C20:2 in leaves and stems with an increasing content as plants matured from vegetative to reproductive stage (P<0.01). Several interactions were observed with both CV, between PP and PS, that originated not only from the magnitude of effects, but also from the effects caused by phenological stage on the different parts of plant.

As reported in figure 1, flowers represent 20% of plants mass during reproductive stage. In terms of macro composition, the main representative constituents (on a % DM basis) are: NDF (36.93), ADF (26.99), CP (21.86), ADL (8.72), ash (8.01), WSC (5.81), and EE (3.75). The FA profile of flowers is characterized by 5.45 mg/g DM of UFA (57% as total FA) where the main component (95%) are PUFA with a higher ($P< 0.5$) SAT/UNSAT FA ratio (0.75) when compared to leaves (0.33) and stems (0.57) as results of higher level of saturated fatty acid in comparison with other parts of plant.

Discussion

DM production and plant morphology

The production of berseem clover cultivars under study were similar to those of cultivars of crimson clover, (650±70 g m^{-2}), and Balansa clover (T. *michelianum* Savi) (600±20 g m-2) grown in the same area, whereas persian clover (T. *resupinatum* L.) showed lower DM production (470±80 g m^{-2}). Over recent decades, papers have been published on the effect of CV, phenological stage and grazing management on FA content of fresh herbage (Dewhurst *et al*., 2001; Elgersma *et al*., 2003; Boufaied *et al*., 2003; Clapham *et al.*, 2005; Dewhurst *et al.*, 2006; Kalac and Samkova, 2010; Buccioni *et al.*, 2012; Elgersma, 2015). On the other side, at the moment there are few references on the effect of different plant parts on fatty acid profile in forage species, which take into consideration different phenological stages (Rebolé *et al.*, 2004; Wyss, 2012). In this study (figure 2) we found that Sacromonte showed a higher proportion of leaves (94 vs 85% on a DM basis; $P<0.05$) and lower proportion of stems (15 vs 6% on a DM basis; $P<0.05$) if compared with Marmilla during the GW stage. No differences were observed in leave, stem and flower ratios between CVs during the REP stage. Plants showed a decrease in proportion of leaves and an increase in proportion of stems during transition from GW to REP stage, in agreement with Rebolé *et al.* (2004) and Suzuki *et al.* (2010). The ratio between leaves and stems appeared very high in our study if compared to the findings of Suzuki *et al.* (2010) and Rakić *et al.* (2013) as probable consequence of differences in characteristics of the forage species under investigation in the different studies. In fact, our results are consistent with the work of De Santis *et al.* (2004) on the same forage species in a Mediterranean environment. Leafiness was double in Sacromonte during the GW stage if compared to Marmilla (leave/stem ratio = 16 vs 7; P<0.01) (figure 2). This information is very useful to support the strategy previously proposed by Cabiddu *et al.* (2004) for achieving higher levels of PUFA in grazing animal products by the choice of leafy CV and appropriate grazing management.

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Macro composition

In both CV, EE and CP content were higher in leaves if compared with stems and flowers (P<0.01) while stems contained higher levels of WSC in agreement with Molle *et al.* (2003) and Chaves *et al.* (2006), who found similar results in sulla. As expected, the DM, NDF, ADF and ADL of leaves and stems increased at the REP stage if compared with GW stage in agreement with the findings of Rebolé *et al.* (2004) probably because the weather was warmer and drier as also observed by Molle *et al.* (2003). On the other side, CP, EE and WSC showed higher levels during GW if compared with REP stage partly in agreement with Hakl *et al.* (2015) in their study on alfalfa chemical composition. Soluble protein (NPN + BSP) were higher (P<0.05) in REP than in GW stage, also in agreement with Halkl *et al.* (2015). The magnitude of change in macro chemical composition was much higher for WSC in leaves whereas stems showed greater changes in DM, NDF, ADF, Ash and CP contents. The large significant effect for the interaction between PP and PS is probably due to the magnitude of change in composition detected in leaves if compared with stems during transition from GW to REP stage. In fact, for both CV only WSC content showed a different pattern in leaves and stems with change in phenological stage.

Fatty acids

Several authors refer to the effects of CV on FA profile (Elgersma, 2003; Elgersma *et al.*, 2003; Bouafied *et al.*, 2003;) detected in whole plant. In the present study, no effect of CV was detected, probably because the objective was to evaluate the FA profile in different plant parts and not in the whole plant as reported in other research. A study carried out in Italy to evaluate nutritive value of berseem clover (De Santis *et al.*, 2004) showed no effect of CV on CP content and *in vitro* digestion. As reported in tables 3 and 4, leaves are characterized by higher levels of MUFA and PUFA if compared to stems (P<0.01). Total UFAs represent 75 and 64% of total FA content in leaves and stems respectively (P<0.01), partially in agreement with Clapham *et al.* (2005) who found similar results for total UFA content in the whole plant. In particular, the proportion of linolenic acid and linoleic acid (as % of total FA) appears lower in this study if compared to the results reported by Elgersma *et al.* (2003) with perennial ryegrass. In addition, the total FA and UFA content was higher as leaf content increased (Elgersma *et al.*, 2003), in agreement with results reported by Cabiddu *et al.* (2004), who found a negative relationship between pasture leafiness and sheep milk Δ^9 desaturase activity, probably due to the higher level of UFA in leaves. In fact, linolenic acid is the main FA observed in leaves followed by palmitic acid and linoleic acid, in agreement with findings reported by several authors (Crombie, 1958; Hawake, 1973; Elgersma *et al.*, 2015). Lettuce leaves represent the main site for linolenic acid synthesis as proposed by Nichols (1963); in this study a decrease in C18:3 content during transition from GW to REP stage in leaves was only observed with CV Marmilla (P<0.01). Overall, stems are characterized by lower levels of LSCFA and UFA than leaves (P<0.01). Total fatty acid content was highest in leaves and lowest in stems, in agreement with Gray *et al.* (1967). Leaves are the main site of lipid synthesis in the plant probably due to a higher content of chloroplasts (Hawke, 1973; Sasikala *et al.*, 2013), if compared to stems. In fact, CP content is positively correlated with total FA content (R^2 =0.78; P<0.001 data not shown), and with total UFA content (R^2= 0.75; P<0.01) (figure 3) probably as result of the higher efficiency of lipid synthesis in the leaves than in stems, as reported by Mayland (1976). In fact a positive relationship has been found between chlorophyll and total fatty acid, included PUFA in plant (Dierking *et al.*, 2010), and it is probable that chlorophyll will be an important criterion in plant breeding for manipulation of fatty acids levels. A negative relationship was observed between WSC and EE (R^2=- 0.47, P<0.01; data not shown) in agreement with Cabiddu *et al.* (2000), Witkowska *et al.* (2008) and Gregorini *et al.* (2008), probably because during plant lipid metabolism some of the carbohydrate pool, generated by the photosynthetic cycle, is complexed with FA to produce polar lipids, in particular phospholipids and glycolipids (Hawke 1973). Moreover, we need to consider that following synthesis, WSC accumulates in stems, as reported by Pollock and Cairns, (1991). In our study, WSC content was strongly influenced by plant part, in particular during the REP stage where we observed that the sugar content of stems was twice higher than in leaves in both CV (+135%, P<0.01), as reported by Hawke (1973). These results agree with Takahashi *et al.* (2001) who showed in wheat that WSC decreased in leaves after anthesis when plant turns from vegetative to reproductive stage, whereas it increased in plant stems. Chlorophyll (pigment-protein complex) content in leaves is positively related to crude protein and EE (Bojović and Marković 2009); whereas CP correlates negatively with WSC (R^2=0.68; P<0.01, data not shown) in agreement with Delagarde *et al.* (2000); Cabiddu *et al.* (2000) and Witkowska *et al.* (2008). The positive relationship between CP vs linolenic acid content (R^2=0.76; P<0.01), and TP vs linolenic acid content (R^2=0.57, P<0.01 data not shown) is due to both CP and true protein (TP) as part of the pigment complex (chlorophyll) of chloroplast located in thylakoid membranes, which are the main site of unsaturated fatty acid synthesis (Hawke 1973). In fact, the NPN fraction (peptides of 3 amino acids or less) which is not included in the pigment complex of chloroplast, showed a negative relationship with C18:3 c9c12c15 (R^2=0.60, P<0.001, figure 4). A similar relationship was observed between NPN and total FA (R^2=0.59; P<0.05, data not shown) content in both CV and in all parts of plant. In general, a strong interaction was observed between PP and PS for both CVs (tables 3 and 4). All these interactions can be accounted by differences in the extent of change and trends in composition occurring in PP at different PS. In both CVs, LCSFA and the ratios between saturated and unsaturated FA (Sat/Unsat) increased more in leaves than in stems during the REP stage. On the other side, PUFA content increased in stems from vegetative to reproductive stage. Leaves usually contain higher level of unsaturated fatty acids during vegetative than reproductive stage. These fatty acids confer fluidity to membranes and they are more concentrated in young plants than in the old ones (Heldt and Piechulla, 2011). The higher level of UFA in leaves during vegetative stage is also linked to a better tolerance of the cold during winter (Heldt and Piechulla, 2011). In our study, these results are consistent in both CVs, only when we consider the FA profile as a percentage of total FA (P<0.01). In stems we observed an increasing effect in both forage species on PUFA content from GW to REP stage, not in line with Wyss (2012). Grassland with a lower ratio Sat/Unsat should be suitable to increase the transfer ratio of beneficial fatty acid on animal products as reported by Buccioni *et al*., 2012. The chemical composition of flowers must be considered separately, independently from leaves and stems during the vegetative stage.

Conclusion

Chemical composition of fresh herbage is influenced by part of plant with less influence of phenological stage. Stems represent the great storage site for WSC in both CV and phenological stages. Leaves are richer in PUFA, MUFA and total UFA than stems in both phenological stages. In this study, any effect of CV was detected except for the plant structure. Nevertheless, the different leave/stem ratio could imply a different chemical composition of the herbage offer as a whole during vegetative stage. This character could be considered in breeding programs among the selection criteria such as flowering time. The use of cultivars with higher leave/stem ratio and late reproductive stage could give pastures with better UFA content, making it possible to produce high quality cheese even in late spring. Overall, these results firstly point out the changes of FA profile in different part of forage plant during vegetative and reproductive stage, but they must be confirmed and better investigated under grazing conditions in a long-term study.

Acknowledgements

Monica Wencelova is grateful to Slovak Research and Development Agency for providing a scholarship at Agris Research institute, Bonassai Italy (APVV 14/0169).

Table 1. Chemical composition of different parts of plant during growth (GW) and reproductive stage (REP) of Marmilla cultivar

		GW		REP		SEM[§]	*Significance level*		
		Leaves	Stems	Leaves	Stems		PP[†]	PS[‡]	PP*PS
DM	%	14.61	7.39	20.22	20.02	1.3328	ns	**	**
Ash	% DM	12.31	20.22	12.03	8.12	0.4934	ns	**	**
EE	% DM	5.97	2.62	5.24	2.33	2.6975	**	ns	**
NDF	% DM	31.74	26.17	33.38	49.68	2.6073	ns	*	**
ADF	% DM	18.21	18.91	21.22	39.14	0.4817	ns	*	**
ADL	% DM	2.39	1.94	4.06	5.95	0.6515	ns	**	**
WSC[¶]	% DM	6.43	7.80	5.04	10.40	1.9273	**	ns	**
CP	% DM	25.51	16.49	22.48	9.04	2.1307	**	ns	**
Protein fraction									
TP[††]	% CP	86.16	78.15	89.89	74.00	2.3321	**	ns	**
IP[‡‡]	% CP	78.10	69.82	62.10	58.62	2.1376	ns	**	**
NPN[§§]	% CP	13.84	21.88	10.09	26.08	2.8059	**	ns	**
BSP[¶¶]	% CP	8.06	8.33	27.79	15.38	0.0011	ns	**	**

†= part of plant; ‡= phenological stage; PP*PS= interaction between PP and PS; Significant differences between means *= $P < 0.05$; **= $P < 0.01$; ns= not significant; [§]= standard error of mean; [¶]= water soluble carbohydrates; [††]= true protein; [‡‡]= insoluble protein; [§§] = non-protein nitrogen; [¶¶]= buffer soluble protein

Table 2. Chemical composition of different parts of plant during growth (GW) and reproductive stage (REP) of Sacromonte cultivar

		GW		REP			*Significance level*		
		Leaves	Stems	Leaves	Stems	SEM[§]	PP[†]	PS[‡]	PP*PS
DM	%	15.53	6.62	19.03	17.93	1.51	ns	**	**
Ash	% DM	11.23	22.99	11.57	9.25	1.69	ns	*	**
EE	% DM	5.82	2.73	5.09	2.42	0.45	**	ns	**
NDF	% DM	27.40	26.41	28.46	47.09	2.64	ns	*	**
ADF	% DM	15.23	18.38	18.25	36.86	2.61	*	*	**
ADL	% DM	2.20	1.85	3.38	5.50	0.44	ns	**	**
WSC	% DM	8.20	9.22	4.41	11.99	0.91	**	ns	**
CP	% DM	22.76	17.79	24.68	9.75	1.81	**	ns	**
Protein fraction									
TP[††]	% CP	88.36	79.34	92.01	74.79	2.81	*	ns	ns
IP[‡‡]	% CP	77.72	72.62	63.68	60.57	2.55	ns	**	*
NPN[§§]	% CP	11.66	21.97	7.99	25.24	2.67	**	ns	*
BSP[¶¶]	% CP	10.64	7.80	28.33	14.22	2.60	ns	**	**

†= part of plant; ‡= phenological stage; PP*PS= interaction between PP and PS; Significant differences between means *= $P < 0.05$; **= $P < 0.01$; ns= not significant; [§]= standard error of mean; [¶]= water soluble carbohydrates; [††]= true protein; [‡‡]= insoluble protein; [§§] = non-protein nitrogen; [¶¶]= buffer soluble protein

Table 3. Fatty acid profile of different parts of plant during growth (GW) and reproductive stage (REP) of Marmilla cultivar

		GW		REP		SEM[§]	*Significance level*		
		Leaves	Stems	Leaves	Stems		PP[†]	PS[‡]	PP*PS
C10:0	mg/g DM	0.003	0.008	0.005	0.002	0.003	ns	ns	ns
C12:0	“	0.007	0.006	0.029	0.006	0.0076	*	ns	**
C14:0	“	0.034	0.015	0.078	0.019	0.0023	*	ns	**
C15:0	“	0.024	0.030	0.020	0.018	0.1563	ns	ns	ns
C16:0	“	2.316	1.523	2.080	1.025	0.032	**	ns	**
C16:1c7	“	0.276	0.035	0.197	0.035	0.0017	**	ns	**
C16:1	“	0.015	0.010	0.018	0.007	0.0018	*	ns	ns
C17:0	“	0.025	0.018	0.033	0.024	0.0464	*	*	**
C18:0	“	0.409	0.308	0.631	0.257	0.0142	**	ns	**
C18:1c9	“	0.166	0.115	0.182	0.074	0.0036	**	ns	**
C18:1c11	“	0.045	0.040	0.048	0.020	0.0006	*	ns	**
C18:1c12	“	0.011	0.012	0.012	0.014	0.0126	ns	ns	ns
C18:2t9t12	“	0.136	0.063	0.093	0.026	0.073	**	ns	**
C18:2c9c12	“	1.585	1.369	1.696	1.156	0.0032	**	ns	*
C18:3c6c9c12	“	0.007	0.021	0.019	0.010	0.9594	ns	ns	ns
C18:3c9c12c15	“	8.651	1.554	6.949	1.675	0.0741	**	ns	**
C20:0	“	0.046	0.047	0.413	0.049	0.0602	ns	ns	ns
C20:2	“	0.254	0.228	0.666	0.571	0.0141	ns	**	**
C22:0	“	0.136	0.093	0.189	0.071	0.0049	**	ns	**
C23:0	“	0.061	0.065	0.076	0.062	0.0078	ns	ns	ns
C24:0	“	0.150	0.144	0.166	0.105	0.0044	*	ns	**

C26:0	"	0.030	0.023	0.047	0.017	0.004	*	ns	ns
MCSFA[¶]	"	2.409	1.601	2.331	1.093	0.16	**	ns	**
LCSFA[††]	"	0.832	0.679	1.522	0.561	0.14	*	ns	*
MUFA[‡‡]	"	0.513	0.196	0.457	0.149	0.048	**	ns	**
PUFA[§§]	"	10.632	3.235	9.422	3.438	1.03	**	ns	**
Tot UFA[¶¶]	"	11.145	3.134	9.880	3.587	1.08	**	ns	**
Sat/Unsat[†††]	ratio	0.291	0.692	0.388	0.461	0.043	**	ns	**
Tot FA[‡‡‡]	mg/g DM	14.386	5.308	14.35	5.241	1.32	**	**	**

[†]= part of plant; [‡]= phenological stage; PP*PS= interaction between PP and PS; Significant differences between means *= $P < 0.05$; **= $P < 0.01$; ns= not significant; [§]= standard error of mean; [¶]= medium chain saturated FA; [††]= long chain saturated FA; [‡‡]= mono unsaturated FA; [§§]= poly unsaturated FA; [¶¶]= total unsaturated FA;[†††]= saturated FA/unsaturated FA; [‡‡‡]= total FA

Table 4. Fatty acid profile of different parts of plant during growth (GW) and reproductive stage (REP) of Sacromonte cultivar

		GW		REP			*Significance level*		
		Leaves	Stems	Leaves	Stems	SEM[§]	PP[†]	PS[‡]	PP*PS
C10:0	mg/g DM	0.002	0.018	0.025	0.002	0.005	ns	ns	ns
C12:0	“	0.006	0.005	0.039	0.004	0.006	ns	ns	ns
C14:0	“	0.033	0.014	0.109	0.015	0.017	ns	ns	ns
C15:0	“	0.021	0.025	0.024	0.020	0.001	ns	ns	ns
C16:0	“	2.273	1.734	2.198	1.213	0.129	**	ns	**
C16:1c7	“	0.274	0.038	0.230	0.036	0.035	**	ns	**
C16:1	“	0.015	0.008	0.020	0.005	0.002	**	ns	**
C17:0	“	0.025	0.023	0.032	0.021	0.002	ns	ns	ns
C18:0	“	0.421	0.412	0.625	0.266	0.044	*	ns	**
C18:1c9	“	0.144	0.138	0.198	0.075	0.018	ns	ns	ns
C18:1c11	“	0.044	0.051	0.050	0.019	0.004	ns	ns	**
C18:1c12	“	0.010	0.025	0.012	0.004	0.003	*	ns	*
C18:2t9t12	“	0.124	0.070	0.093	0.024	0.011	**	ns	**
C18:2c9c12	“	1.544	1.464	1.703	1.388	0.043	*	ns	*
C18:3c6c9c12	“	0.007	0.011	0.017	0.011	0.001	ns	ns	ns
C18:3c9c12c15	“	8.306	1.929	8.008	1.919	0.994	**	ns	**
C20:0	“	0.050	0.062	0.167	0.044	0.019	ns	ns	*
C20:2	“	0.250	0.180	0.612	0.765	0.078	ns	**	**
C22:0	“	0.144	0.128	0.168	0.072	0.011	*	ns	**
C23:0	“	0.060	0.052	0.070	0.059	0.003	*	ns	ns
C24:0	“	0.156	0.170	0.156	0.118	0.007	ns	ns	ns
C26:0	“	0.032	0.027	0.035	0.014	0.003	*	ns	*

MCSFA[¶]	"	2.361	1.818	2.428	1.274	0.216	**	ns	**
LCSFA[††]	"	0.864	0.851	1.221	0.573	0.105	ns	ns	*
MUFA [‡‡]	"	0.486	0.259	0.510	0.142	0.048	**	ns	**
PUFA [§§]	"	10.231	3.654	10.433	4.107	1.033	**	ns	**
Tot UFA[¶¶]	"	10.716	3.913	10.942	4.410	0.051	**	ns	**
Sat/Unsat[†††]	Ratio	0.301	0.687	0.337	0.451	1.157	**	ns	**
Total FA[‡‡‡]	mg/g DM	13.941	6.583	14.591	6.398	1.251	**	ns	**

[†]= part of plant; [‡]= phenological stage; PP*PS= interaction between PP and PS; Significant differences between means *= $P < 0.05$; **= $P < 0.01$; ns= not significant; [§]= standard error of mean; [¶]= medium chain saturated FA; [††]= long chain saturated FA; [‡‡]= mono unsaturated FA; [§§]= poly unsaturated FA; [¶¶]= total unsaturated FA;[†††]= saturated FA/unsaturated FA; [‡‡‡]= total FA

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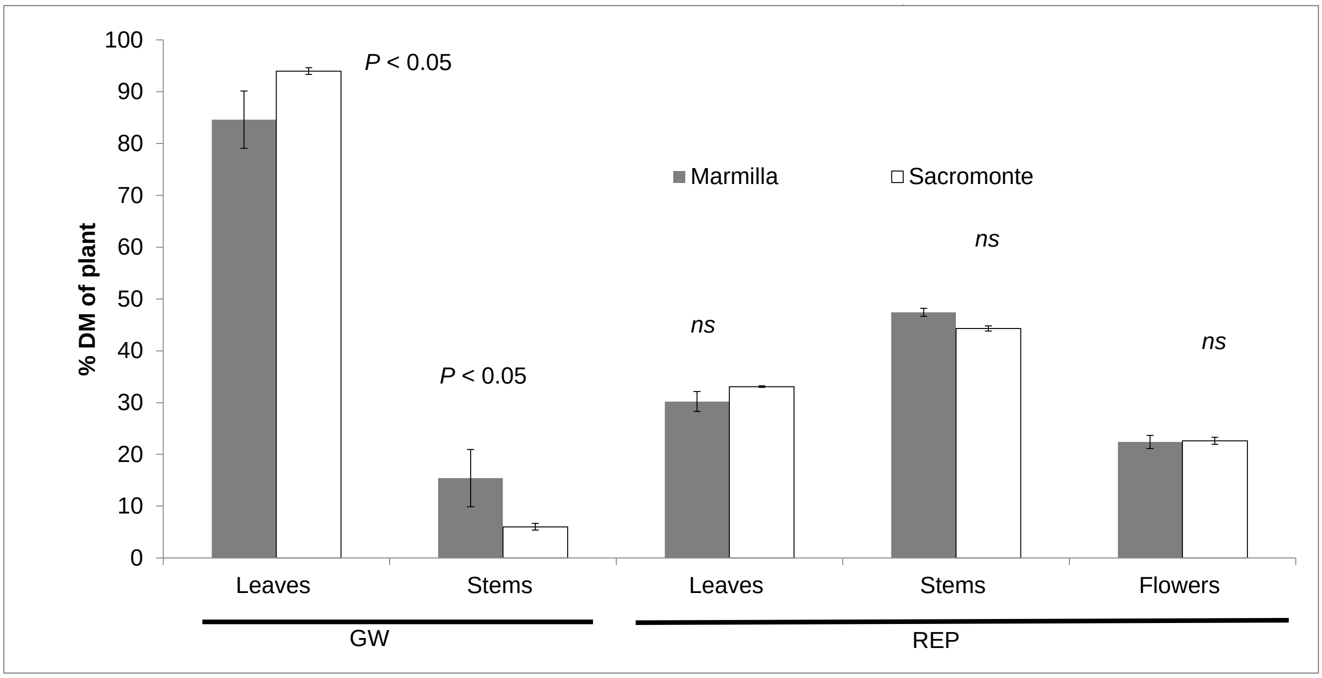
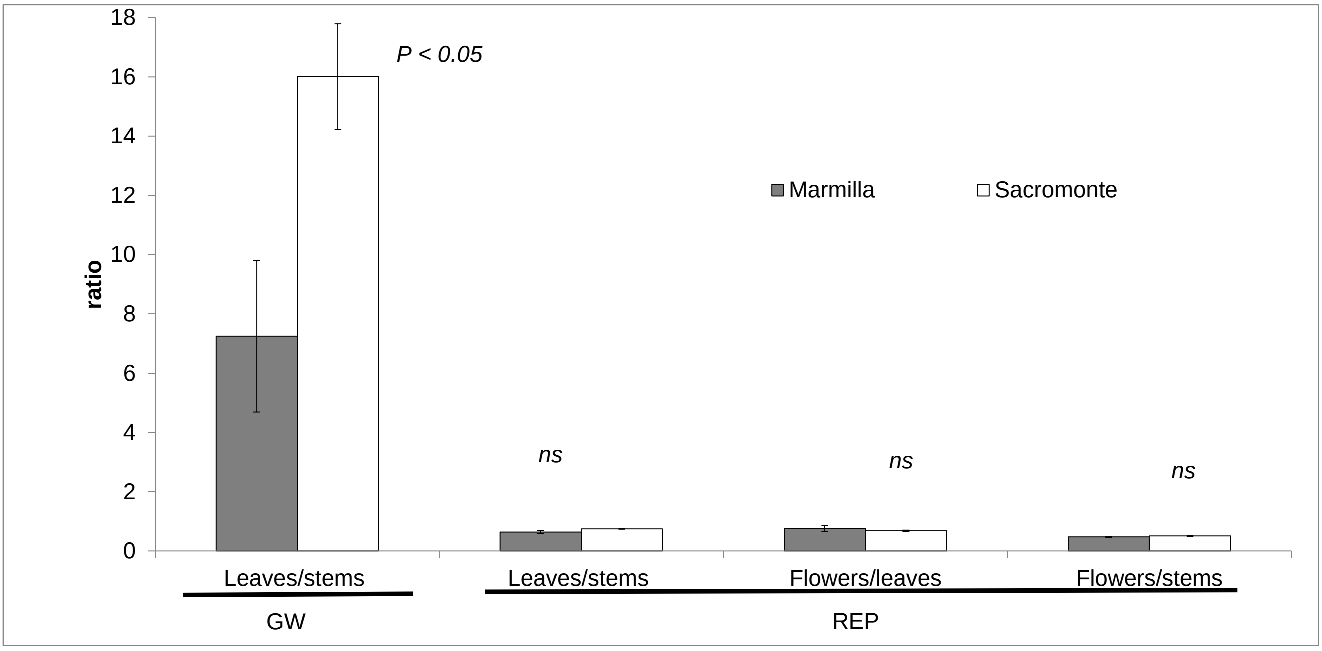
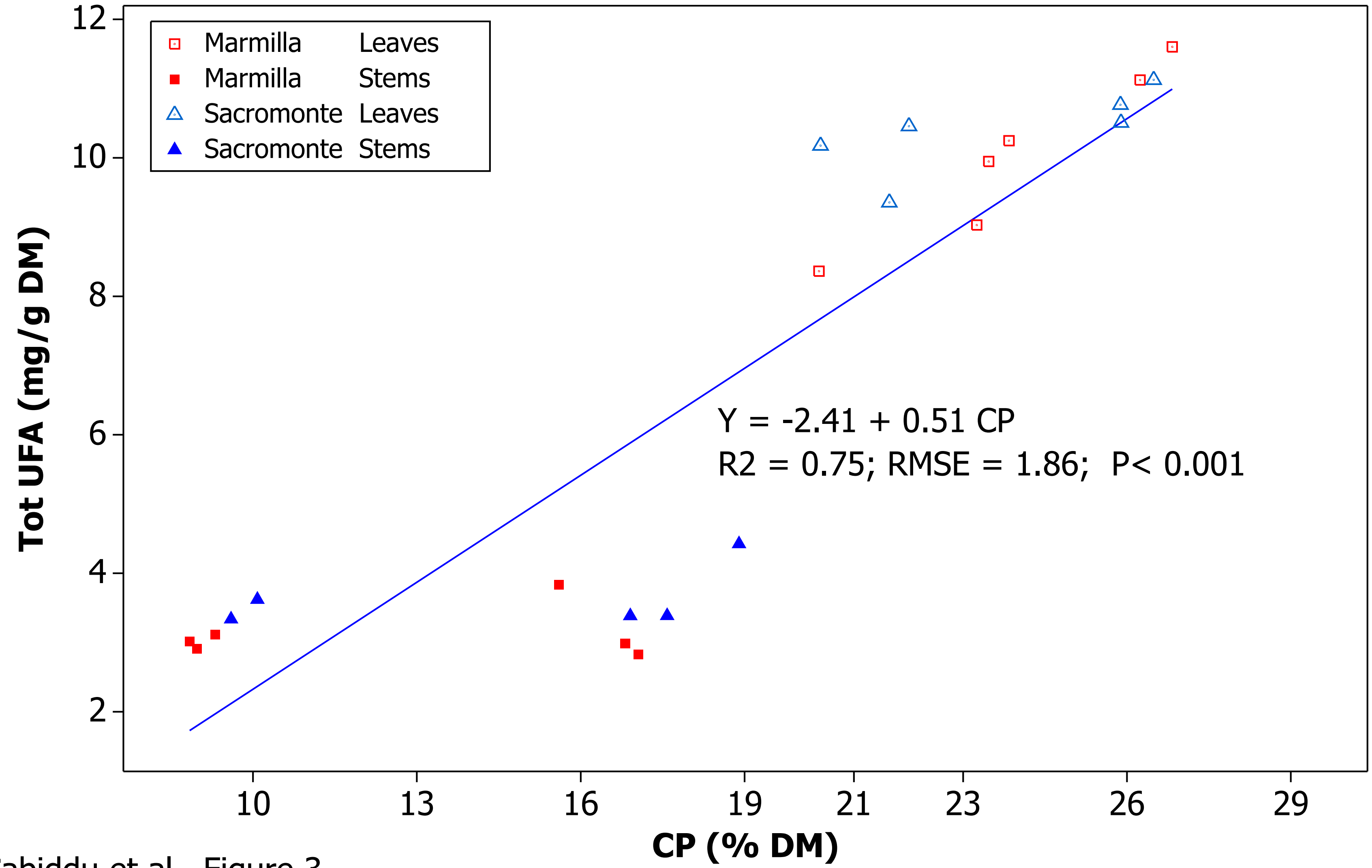


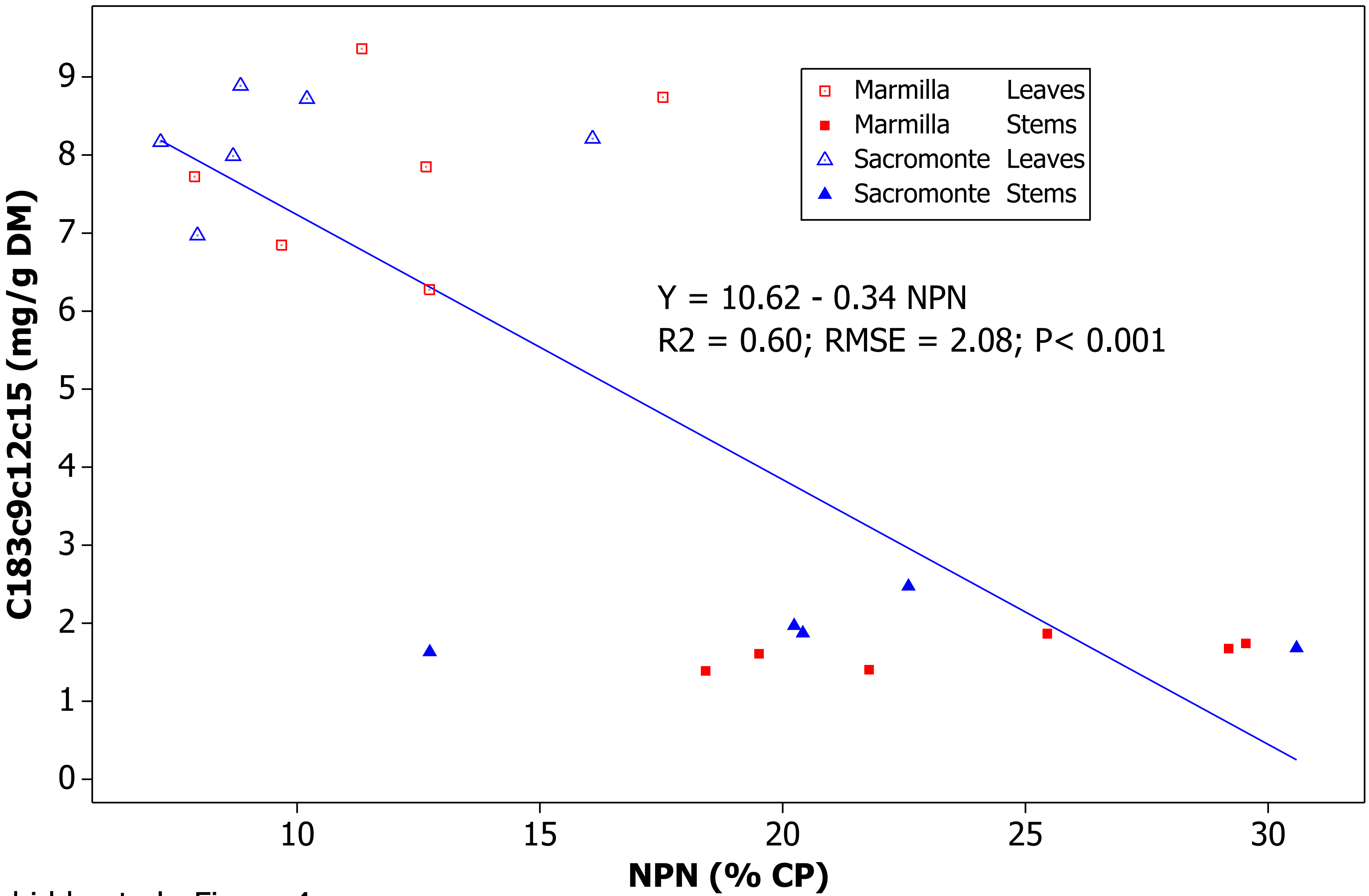
Cabiddu et al., Figure 1



Cabiddu et al., Figure 2



Cabiddu et al., Figure 3



Cabiddu et al., Figure 4

Figure 1. Proportion of leaves stems and flowers during vegetative (GW) and reproductive (REP) stage of Marmilla and Sacromonte cvs.

Figure 2. Ratio of leaves/stems flowers/leaves and flowers/stems during vegetative (GW) and reproductive (REP) stage of Marmilla and Sacromonte cvs.

Figure 3. Relationship between crude protein (CP) content and total unsaturated fatty acid (UFA) in different part of plants of Marmilla and Sacromonte cvs.

Figure 4. Relationship between non protein nitrogen (NPN) content and linolenic acid (C18: 3 c9c12c15) in different part of plants of Marmilla and Sacromonte cvs.