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Volatile composition of red clover (*Trifolium pratense* L.) forages in Portugal: The influence of ripening stage and ensilage

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Abstract

The volatile organic compounds (VOCs) of three different red clover (*Trifolium pratense L.*) forages, fresh plant, hay and silage, were analyzed using GC and GC/MS. Comparing the volatile composition of hay and silage forages of red clover with the corresponding green plant, the effects of ripening and postharvest secondary metabolism can be noticed in hay and in ensilage. In hay, reductions of the percentages of alcohols, such as 3-methylbutanol and 1-hexanol, of aldehydes and of low boiling point ketones are observed. A sesquiterpene (β-farnesene; ca. 10%) and a phytol degradation product (6,10,14-trimethyl-2-pentadecanone; ca. 12%) were the most abundant compounds detected in hay. In silage, as a result of the fermentation of fresh red clover, esters (ca. 46%) are a more representative class of compounds.

Keywords: Red clover; *Trifolium pratense* L.; Volatile compounds; Hay; Silage

1. Introduction

Red clover (*Trifolium pratense* L.) is a perennial herb, native in Mediterranean and Red Seas countries. It is used in rotations for soil improvement and has also some medicinal applications, such as cancer, mastitis, joint disorders, jaundice, bronchitis, spasmodic coughing, asthma, and skin inflammations, e.g. psoriasis and eczema. Isoflavone products isolated from red clover have shown promising effects on conditions associated with menopause, such as hot flushes, cardiovascular health and the bone loss associated with osteoporosis (Atkinson, Compston, Day, Dowsett, & Bingham, 2004; Clifton-Bligh, Baber, Fulcher, Nery, & Moreton, 2001).

However, the main application of red clover is its use as grazing food for cattle and other livestock. In fact, red clover is a high quality forage that can be either grazed or used for hay. The use of this herb has some known advantages: in the form of hay it has a slightly higher net energy value and total digestible nutrients than has alfalfa hay (alfalfa being the most widely used forage in the USA), and two-thirds its digestible protein; protein in red clover has also been found to be degraded less extensively in the rumen than are proteins in other herbs that, like red clover do not contain condensed tannins (Broderick, Albrecht, Owens, & Smith, 2004; Owens, Albrecht, Muck, & Duke, 1999). A major disadvantage of preserving forage as hay is the risk of exposure to adverse weather conditions, since several days are usually required for drying (Owens et al., 1999).

Ensiling of crops is a popular method for preserving forage for animal feed, especially in humid regions (Sullivan, Hatfield, Thoma, & Samta, 2004). In the early 20th century, Hunter and Bushnell (1916) referred to the limited literature involving biological studies of silages at the time. Already then, the authors wrote that the chemical changes resulting from fermentation in silage, were caused by enzymes of the plant cells or microorganisms acting upon the cut forage plant. Two dependent processes were, therefore, involved

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in the silage formation: the biological action resulting from either enzymes or microorganisms acting on tissue cells, and the chemical action due to the by-products of direct enzyme action, acting on the siloed material.

Nowadays, it is known that, during harvesting and early stages of ensiling, plant membranes are ruptured, releasing proteolytic enzymes that rapidly degrade available substrates. Once fermentation by lactobacteria has progressed sufficiently to lower silage pH, to a value of about 5, proteolytic activity slows significantly (Sullivan et al., 2004). Most of the herbage protein is converted by plant proteinases and peptidases to free amino acids, ammonia and other forms of non-protein nitrogen (Elgersma et al., 2004).

Red clover, a forage with protein content similar to alfalfa, has up to 90% less proteolysis than has alfalfa during ensiling, which results in an economic added value of this plant, since the purchase of additional protein to supplement diets is avoided (Sullivan et al., 2004). The world is filled with flavors and scents, which are the result of volatile compounds produced and emitted by plants. These are specialized metabolites resulting from specific metabolic pathways (Gang, 2005).

The evolution of plant secondary metabolites is often considered to be closely associated with defence against herbivores and other parasites, but recently it has been proposed that plant chemical defence could primarily be aimed at abiotic stresses, such as photodamage and climate changes. The difference between constitutive and inducible volatile organic compounds (VOCs) is ambiguous, since most of the constitutive VOCs normally released from healthy intact plants, become inducible volatiles after foliage damage (Holopainen, 2004).

There is a broad diversity of known inducible VOCs, but the dominating compounds are C_6 green leaf volatiles, such as aldehydes, alcohols and esters that are derived via lipoxygenase cleavage of fatty acids, within seconds of injury, and immediately released, and terpenes, which are synthesized de novo several hours or even days after damage (Holopainen, 2004; Pichersky & Gershenzon, 2002). The lipoxygenase pathway is well described elsewhere (Salas, Sánchez, Garcia-González, & Aparício, 2005).

Many of the volatile compounds are also formed through transformation of the initial products by oxidation, dehydrogenation, acylation and other reaction types, involving P_{450} cytochrome and NADP/NAD-dependent enzymes, among others (Dudareva, Pichersky, & Gershenzon, 2004).

Acylation, most often with an acetyl moiety but also with larger acyls, such as butanoyl, benzoyl, hydroxycinnamyl, to make volatile compounds is also common. Such plant volatile esters are synthesized by alcohol acyltransferases which catalyse the transfer of an acyl group from an acyl CoA intermediate to the hydroxyl group of an alcohol (Dudareva et al., 2004; Gang, 2005; Salas et al., 2005).

Volatile compounds may also result from acid or enzyme hydrolysis of glycoconjugated compounds (Mastelić & Jerković, 2003; Sarry & Günata, 2004).

In the present work we have studied the volatile profile of three red clover forages: one of the green plant, another of the plant in its hay form and another resulting from the ensilage of the green material. We present here the results and proposals explaining the different volatile profiles observed, trying to relate them to the differential plant development stages/post-harvest metabolism (hay vs green red clover) and fermentation processes (silage vs starting material).

To the best of our knowledge, this is the first report featuring a study of red clover hay and silage volatile composition. Relatively to the green plant, we found two articles describing its volatile composition, one concerning its essential oil (Kami, 1978) and the other the fresh leaves, flowers and seed pods through the use of Tenax traps (Buttery, Kamm, & Ling, 1984). In the literature, the existing studies concerned with maturation stages are normally related to fruits (Almora et al., 2004) and not to plants.

2. Materials and methods

2.1. Samples

After ripening (green and hay-like) and silo opening (silage), samples of red clover (*Trifolium pratense* L.), were vacuum-packed and stored at −20 °C, until they were analyzed.

2.2. Isolation of volatile compounds

2.2.1. Sample preparation

After defrosting (about 2 h), the different forages (green, hay and silage) of *T. pratense* L. were chopped into approximately 2 cm lengths (scissors) and minced. They were previously subjected to a homogenizing process.

Aliquots of the minced material were weighed into 20 ml Agilent crimp-top headspace vial, so that the headspace volume in the vials was about one third of the total volume. The vials were immediately sealed with a teflon-lined silicone rubber septum and left overnight at 4 °C.

2.2.2. SPME experimental conditions

The vials containing the minced samples were placed in a water bath at 60 °C during 60 min. After this period, where the volatile compounds were allowed to equilibrate, the septum was pierced with the SPME holder (Supelco, Bellefonte, PA, USA) that was adjusted to #1, and the 2 cm SPME sampling fiber of 50/30 μm divinylbenzene–carboxen–polydimethylsiloxane extended into the sample vial headspace. The SPME holder was suspended above and held in position by a clamp. The extraction time for HS-SPME was 45 min at the same temperature.

The SPME apparatus was withdrawn, inserted into the injector port and the fiber exposed for 2 min in a gas chromatograph equipped flame ionization detector (FID).

2.3. GC/MS analyses

GC/MS analyses were done on a Thermoquest Trace MS (Thermofinigan) fitted with a DB-1 fused silica column (30 m × 0.32 mm × 0.25 μm). The oven temperature was held at 40 °C for 1 min. and then increased to 250 °C at 5 °C min^{-1} and held there for 10 min. Carrier gas (helium) flow rate was 2 ml min^{-1}. The source and interface temperatures were 200 °C and 275 °C. The detector operated in electron impact mode (70 eV), and the detection was performed in the scan mode between 30 and 400 Da.

All the peaks of the total ion chromatograms (TICs) were analyzed using the 3D feature of Xcalibur®, in order to discriminate the situations where co-elutions occur. Constituents were identified by comparison of their mass spectra with those of NIST, and other libraries available, and when possible confirmed with their relative retention indices from literature. Retention indices were determined externally with a series of *n*-alkanes (C_7–C_{20}), under the same chromatographic conditions, and calculated according to the formula given by Van Den Dool and Kratz (1963).

3. Results and discussion

The volatile components present in the three forages analyzed, and their percentages are given in Table 1.

Table 2 summarizes the results of Table 1, listing the components present in the different red clover forages grouped by classes. In this table, the intervals of percentages of the different classes of compounds are due to the co-elutions of those classes. The cases where there is no interval but only a value, mean that none of the compounds of that class co-elutes with another compound of any other class. The major value of the interval refers to all the co-elutions of compounds of each class; the percentage of the compounds of other classes that co-elute is null, while the minor value refers to the opposite situation. The percentage of each class, not considering the existence of co-elutions, is given between brackets in bold. The sum of all class percentages for a given forage is equal to the percentage of the total compounds C_7–C_{20} present in Table 2 for this forage. These were the values used in Charts 1 and 2.

As it can be seen in Table 2 and Chart 1, terpenes are the most abundant class of compounds in green and hay samples of red clover. Esters and fatty acids are the predominant compounds in silage.

Table 1 shows that the most abundant identified compounds in the green sample of red clover are: 3-octanol (3.44%), 6,10,14-trimethyl-2-pentadecanone (3.46%), benzaldehyde (3.74%), (z)-β-caryophyllene (4.05%), β-farnesene (4.86%), 3-methyl-1-butanol (5.73%) and 3-octanone (co-eluting with 6-methyl-5-hepten-2-one) with 8.60%. In the case of hay, phenylethyl alcohol (2.28%), (z)-β-caryophyllene (2.65%), β-farnesene (10.4%) and 6,10,14-trimethyl-pentadecanone (11.8%) are among the most abundant identified compounds. In red clover silage, most abundant compounds are: ethyl hexanoate (co-eluting with 3-octanol) with 3.51%, phenylethyl alcohol (co-eluting with linalool) with 3.69%, 3-methylbutyl butanoate (3.77%), 3-methyl-1-butanol (5.06%), 3-methylbutanoic acid (co-eluting with 3-methyl-1-butanol acetate) with 6.73% and ethyl 2-methylpentanoate (co-eluting with benzaldehyde) with 6.83%.

The number of alkanes and alkenes present in Table 1 is reduced. No alkene was detected in silage and their concentration was higher in green forage than in hay. For alkanes the situation is rather different; the percentages of these compounds in hay are higher than that in the green plant and they are almost absent in the silage.

The percentage of aldehydes in the silage is almost nill and there are only a few representatives in hay, such as benzaldehyde and phenylacetaldehyde, which are in much smaller amounts compared with their percentages in green red clover. The presence of these compounds was reported in red clover essential oil (Kami, 1978). Benzaldehyde probably originated from oxidation reactions of cinnamic acid or phenylacetaldehyde, while phenylacetaldehyde must be the result of Strecker degradation of phenylalanine (Biehl & Ziegledir, 2003).

In almost all cases, there was a reduction of the percentage of alcohol components, from green to hay red clover forage. This reduction is more accentuated for 3-methyl-1-butanol and 1-hexanol. The first most likely results from the reduction of its corresponding aldehyde, which in turn is derived from leucine (metabolism of branched-chain amino acids), and 1-hexanol, like any green leaf volatile, is typically produced from polyunsaturated fatty acids (linoleic and α-linolenic acids) through the lipoxygenase pathway (Salas et al., 2005).

The decrease or complete disappearance of unsaturated alcohols and aldehydes is also reported in other studies (Pino, Sauri-Duch, & Marbot, 2006; Valette et al., 2003), that implicate the ripening and all changes, both chemical (resulting of changes of enzyme activities) and morphological, that occur during maturation, as the cause of these variations. The drying process of hay could also be responsible for the reduction of these compounds, which include the so-called green leaf volatiles. Since these are also the most volatile compounds, they are the most likely to disappear during the conversion (to hay) of the green material.

The 1-octen-3-ol is only detected in red clover hay and not in the fresh plant (Mastelić & Jerković, 2003), which may indicate that aliphatic alcohols like 1-octen-3-ol and *cis*-3-hexen-1-ol are aglycones of non-volatile glycoconjugates which accumulate in plant and fruit tissues and can originate from fatty acid catabolism.

In contrast to what happens in hay, where there is a drastic reduction of 3-methyl-1-butanol, the percentage of this volatile in the silage is just slightly inferior. Phenol and 4-ethyl-2-methoxyphenol are present only in red clover silage.

Table 1
Volatile compounds % of three different red clover forages: green, hay and silage

No.	Compounds	RI	Green	Hay	Silage	Id.
1	3-Methyl-1-butanol	702	5.73	0.29	5.06	RI/MS
2	Ethyl butanoate	779	–	–	1.34	RI/MS
3	1-Hexanol	844	1.27	0.47	–	RI/MS
4	3-Methylbutanoic acid	849	–	–	6.73	RI/MS
5	3-Methyl-1-butanol acetate	849	–	–	co.[a]	RI/MS
6	Dimethylpyridine	849	–	0.21	–	MS
7	Propyl butanoate	876	–	–	2.49	RI/MS
8	Methyl hexanoate	904	–	–	0.84	RI/MS
9	Benzaldehyde	917	3.74	0.44	co.[b]	RI/MS
10	Ethyl 2-methylpentanoate	920	–	–	6.83[a]	RI/MS
11	Isobutyl butanoate	935	–	–	0.69	RI/MS
12	Ethyl 3-methylpentanoate	944	–	–	0.15	RI/MS
13	3-Methylbutyl propanoate	949	–	–	0.39	RI/MS
14	1-Heptanol	951	0.42	0.46	–	RI/MS
15	1-Octen-3-ol	959	–	co.[b]	–	RI/MS
16	6-Methyl-5-hepten-2-one	959	8.60	1.55	0.46	RI/MS
17	3-Octanone	959	co.[a]	–	co.[a]	RI/MS
18	Phenol	971	–	–	0.24[b]	RI/MS
19	2-Pentylfurane	972	0.93	0.70	co.[a]	RI/MS
20	Butyl butanoate	975	–	–	0.81	RI/MS
21	3-Octanol	977	3.44	0.73	co.[b]	RI/MS
22	Ethyl hexanoate	980	–	–	3.51[a]	RI/MS
23	n.i. (1)	984	2.21	2.16	0.34	
24	3-Methylbutyl 2-methylpropanoate	995	–	–	0.27	RI/MS
25	Phenylacetaldehyde	998	1.02	0.16	0.71	RI/MS
26	Benzyl alcohol	1001	0.34	0.23	–	RI/MS
27	2-Ethyl-1-hexanol	1011	0.37	0.15	–	RI/MS
28	Hexanoic acid	1014	–	–	1.07[b]	MS
29	Limonene	1014	–	–	co.[a]	RI/MS
30	n.i. (2) – [MW = 139: 59(100)/81(80)/96(58)/43(21)/ 79(15)/41(14)/67(11)]	1017	–	1.77	–	
31	Isobutyl 3-methylbutanoate	1018	–	–	1.85	MS
32	Acetophenone	1023	0.36	0.11	–	RI/MS
33	3,5,5-Trimethylcyclohex-2-en-1-one	1026	0.48	0.12	–	MS
34	n.i. (3)	1030	0.57	0.34	0.10	
35	3,5-Octadien-2-one	1039	0.69	0.41	–	MS
36	Butyl 3-methylbutanoate	1040	–	–	3.77	RI/MS
37	3-Methylbutyl butanoate	1043	–	–	1.40	MS
38	n.i. (4)	1044	0.27	0.17	–	
39	(E)-2-Octen-1-ol	1051	0.43[b]	0.39[b]	–	RI/MS
40	n.i. (5)	1051	co.[a]	co.[a]	–	
41	n.i. (6)	1056	0.77	0.20	–	
42	Methyl benzoate	1062	0.61[b]	–	0.13	RI/MS
43	n.i. (7)	1064	co.[a]	0.49	–	
44	2-Nonanone	1068	0.50	0.57	–	RI/MS
45	n.i. (8)	1075	0.89	0.67	–	
46	Nonanal	1079	co.[b]	co.[b]	–	RI/MS
47	Phenylethyl alcohol	1079	2.65[a]	2.28[a]	3.69[b]	RI/MS
48	Linalool	1081	–	1.00	co.[a]	RI/MS
49	3-Methylbutyl 2-methylbutanoate	1088	–	–	0.46	RI/MS
50	3-Methylbutyl 3-methylbutanoate	1091	–	–	0.49	MS
51	2-Methylbutyl 3-methylbutanoate	1095	–	–	0.10	MS
52	Undecane	1103	0.60	0.51	–	RI/MS
53	Methyl octanoate	1113	–	–	0.23	RI/MS
54	1,2-Dimetoxybenzene	1114	–	0.24	–	RI/MS
55	n.i. (9)	1115	0.17	–	–	
56	1-(2-hydroxyphenyl)-ethanone	1118	–	0.74	–	MS
57	Octyl acetate	1121	–	–	2.10	RI/MS
58	Naphthalene	1123	3.82	0.15	–	MS
59	3-Methylbutyl pentanoate	1138	–	–	0.46[b]	MS
60	Isobutyl hexanoate	1138	–	–	co.[a]	MS
61	Ethyl benzoate	1140	–	–	0.47	RI/MS
62	Methyl 2-Phenylacetate	1142	–	–	0.10	MS
63	n.i. (10) – Phenol derivative	1150	–	–	0.57	

Table 1 (*continued*)

No.	Compounds	RI	Green	Hay	Silage	Id.
64	1-Nonanol	1158	0.63	0.36	–	RI/MS
65	n.i. (11) – Terpenoid	1164	0.30	0.58	0.14	
66	(Z)-3-Hexenyl butanoate	1169	–	–	0.39	RI/MS
67	2-Decanone	1172	0.13	0.18	–	RI/MS
68	Hexyl butanoate	1178	–	–	0.70	RI/MS
69	Butyl hexanoate	1178	–	–	co.[a]	RI/MS
70	Decanal	1182	0.23	0.27[a]	–	RI/MS
71	n.i. (12) – Terpenoid	1182	–	co.[a]	–	
72	Ethyl octanoate	1183	–	–	0.29	RI/MS
73	n.i. (13) – Terpenoid [MW = 152; 137(100)/152(86)/123(74)/81(64)/67(61)/79(29)/91(29)/77(22)/39(20)]	1186	1.31	0.44	–	
74	1-Naphthalenol	1186	–	co.[a]	–	MS
75	Octanoic acid	1199	–	–	0.62	MS
76	Benzylacetone	1200	0.28	–	–	RI/MS
77	n.i. (14) – Terpenoid [MW = 152; 110(100)/109(89)/41(71)/43(60)/152(57)/95(53)/57(51)/81(46)/137(45)/71(40)]	1208	–	0.49	–	
78	n.i. (15)	1209	co.[b]	–	–	RI/MS
79	Methyl nonanoate	1209	0.20[a]	–	co.[b]	RI/MS
80	Ethyl benzoacetate	1210	–	–	0.46[a]	RI/MS
81	n.i. (16) – [(MW = 162; 91(100)/147(95)/105(68)/79(61)/162(52)/119(42)77(41)/93(36)/41(33)133(31)]	1213	0.53	–	–	
82	Phenylethyl acetate	1221	–	–	0.78	RI/MS
83	Cinnamaldehyde	1228	0.22[b]	–	–	RI/MS
84	n.i. (17)	1228	co.[a]	–	–	
85	3-Methylbutyl hexanoate	1235	–	–	1.03	RI/MS
86	Methyl 3-phenylpropanoate	1239	–	–	1.59	MS
87	4-Ethyl-2-methoxyphenol	1246	–	–	0.26	MS
88	1-Methylnaphthalene	1253	0.18	0.13	–	MS
89	n.i. (18) – [MW = 162: 147(100)/91(58)/105(48)/162(38)/119(23)/79(19)/77(18)/133(17)/41(17)/67(14)]	1258	0.68	0.14	–	
90	2-Undecanone	1272	0.26	0.56	–	RI/MS
91	n.i. (19) – Benzyl acid ester	1273	–	–	0.20	
92	Heptyl butanoate	1282	–	–	0.93	RI/MS
93	Tridecene	1289	0.13	–	–	RI/MS
94	Tridecane	1300	0.25	0.33	–	RI/MS
95	Methyl decanoate	1308	0.21	0.12	0.21	RI/MS
96	Ethyl 3-phenylpropanoate	1314	–	–	co.[b]	MS
97	Phenylethyl propanoate	1314	–	–	2.60[a,b]	RI/MS
98	n.i. (20) – [MW = 178: 85(100)/43(15)/91(15)/108(14)/71(12)]	1314	–	–	co.[a]	
99	Eugenol	1323	–	–	0.20	RI/MS
100	n.i. (21)	1325	–	0.94	–	
101	a-Cubebene	1337	0.11	0.26	–	RI/MS
102	n.i. (22)	1337	–	co.[a]	–	
103	Longicyclene	1350	0.22	–	–	RI/MS
104	1-Undecanol	1353	0.14	–	–	RI/MS
105	α-Copaene	1360	0.61	0.86	co.[b]	RI/MS
106	2-Phenylethyl 2-methylpropanoate	1361	–	–	0.42[a]	RI/MS
107	n.i. (23) – Terpenoid [MW = 204: 175(100)/119(92)/105(59)/133(43)/91(39)/41(33)/204(25)/189(24)/77(23)55(22)]	1363	0.33	–	–	
108	3,4-Dihydro-1(2H)-naphtalenone	1368	–	1.10[b]	–	MS
109	Decanoic acid	1368	–	co.[a]	co.[b]	RI/MS
110	Methyleugenol	1370	–	–	0.21[a]	RI/MS
111	b-Elemene	1375	0.92	1.04	0.74	RI/MS
112	n.i. (24)	1378	–	0.30	–	
113	Ethyl decanoate	1381			0.31	RI/MS
114	2-Dodecanone	1385	0.38	0.81	–	RI/MS
115	α-Cedrene	1389	0.18	0.46	0.13	RI/MS
116	(Z)-β-Caryophylene	1397	4.05	2.65	0.85	RI/MS
117	Tetradecane	1400	0.31	1.26	–	RI/MS
118	n.i. (25) – Terpenoid [MW = 204: 121(100)/161(98)/43(86)/93(52)/105(45)/119(44)/91(42)/81(39)/79(37)]	1407	0.43	0.83	–	
119	2-Phenylethyl butanoate	1409	–	–	3.19	RI/MS

(*continued on next page*)

Table 1 (*continued*)

No.	Compounds	RI	Green	Hay	Silage	Id.
120	n.i. (26) – Terpenoid [MW = 204: 121(100)/43(88)/161(62)/93(47)/41(39)/119(35)/81(33)/79(32)/136(32)/123(31)]	1415	0.40	0.31	–	
121	(E)-α-Bergamotene	1421	0.20	0.21	–	RI/MS
122	Geranyl acetone	1425	0.51	0.86	–	RI/MS
123	α-Humulene	1430	3.35	1.22	0.21[b]	RI/MS
124	3-Methylbutyl octanoate	1430	–	–	co.[a]	MS
125	β-Farnesene	1445	4.86	10.35	2.98	RI/MS
126	3-(Hexyloxy)phenol	1449	0.17	0.45	0.55[b]	MS
127	Phenylpropionic acid	1449	–	–	co.[a]	MS
128	β-Ionone	1453	2.43[b]	1.49	1.48[b]	RI/MS
129	n.i. (27) – Terpenoid [MW = 204: 161(100)/105(60)/119(53)/91(37)/93(35)/79(32)/133(28)/81(26)/204(21)]	1456	co.[a]	0.71	co.[a]	
130	n.i. (28) – Terpenoid [MW = 204: 105(100)/119(79)/93(72)/79(71)/107(69)/91(68)/41(66)/67(58)/81(56)/132(46)]	1461	1.07	–	0.95	
131	n.i. (29) – Terpenoid [MW = 202: 119(100)/132(83)/105(65)/91(41)/41(38)/131(29)/145(26)/55(26)/120(25)/93(24)]	1461	–	2.30[b]	–	
132	n.i. (30) – Terpenoid [MW = 204: 119(100)/121(64)/93(59)/111(49)/105(42)/91(39)/77(27)/79(24)/204(23)/109 (22)]	1461	–	co.[a]	–	
133	n.i. (31)	1465	0.84	1.80	0.37	
134	n.i. (32) – Terpenoid (MW = 204: 93(100)/189(86)/107(82)/91(78)/105(77)/81(73)/133(72)/161(49)]	1472	1.30	1.45	0.73	
135	2-Tridecanone	1476	–	1.93[b]	–	RI/MS
136	n.i. (33)	1476	–	co.[a]	–	
137	n.i. (34) – Terpenoid [MW = 204: 93(100)/119(87)/41(70)/69(58)/55(57)/79(44)/91(41)/107(34)/105(30)/77(30)]	1480	1.10	2.89[b]	2.54	
138	n.i. (35) – Terpenoid [MW = 204: 119(100)/93(77)/105(68)/91(44)/161(40)/77(24)/94(23)/204(17)]	1480	–	co.[a]	–	
139	1-Pentadecene	1489	0.99	0.66	–	RI/MS
140	α-Farnesene	1493	0.75	0.99	1.15	RI/MS
141	Pentadecane	1500	1.50[b]	2.37[b]	0.32[b]	RI/MS
142	δ-Cadinene	1500	co.[a]	co.[a]	co.[a]	RI/MS
143	Methyl dodecanoate	1508	0.18[b]	–	0.31	RI/MS
144	n.i. (36)	1509	co.[a]	0.13	co.[b]	
145	n.i. (37)	1512	–	–	0.18[a]	
146	α-Calacorene	1528	0.10	–	–	RI/MS
147	Spathulenol	1542	0.18	–	–	MS
148	n.i. (38)	1542	–	0.45	–	
149	Caryophyllene oxide	1545	0.18	0.26	–	RI/MS
150	Dodecanoic acid	1558	–	0.19	–	RI/MS
151	Ethyl dodecanoate	1580	–	–	0.25	RI/MS
152	n.i. (39) – Terpenoid [MW = 204: 107(100)/135(80)/93(51)/41(48)/91(44)/43(35)/105(28)/79(23)/77(22)/55(21)]	1582	0.13	0.30	0.59	
153	Hexadecane	1597	0.84	3.39	0.12	RI/MS
154	2-Phenylethyl pentanoate	1603	–	–	0.13	RI/MS
155	β-Eudesmol	1610	–	0.23	–	RI/MS
156	n.i. (40) – Terpenoid [MW = 222: 43(100)/81(93)/135(70)/71(68)/67(53)/41(46)/93(44)/95(43)/109(41)/55(40)]	1613	–	–	0.18	
157	α-Eudesmol	1615	–	0.20	–	RI/MS
158	1-Tetradecanol	1640	–	0.21	–	MS
159	Methyl tetradecanoate	1700	–	–	0.26	RI/MS
160	Tetradecanoic acid	1747	–	0.25	–	RI/MS
161	Ethyl tetradecanoate	1777	–	–	0.25	RI/MS
162	Octadecane	1798	0.20	0.68	–	RI/MS
163	6,10,14-Trimethyl-2-pentadecanone	1829	3.46	11.79	0.63	
164	n.i. (41)	1837	0.37	3.85	0.40	
165	Methyl hexadecanoate	1916	–	–	1.09	RI/MS
166	Hexadecanoic acid	1956	–	–	0.22	RI/MS
167	Ethyl 9-hexadecenoate	1976	–	–	0.10	MS
168	Ethyl hexadecanoate	1987	–	–	1.05	RI/MS

Compounds present in percentages below 0.10% and/or outside the work range: C_7–C_{20}, were not considered.
n.i: not identified.
co.: Co-eluting.
[a] Co-eluting with previous compound.
[b] Co-eluting with next compound.

Table 2
Distribution of compounds present in red clover forages by classes

	Red Clover		
	Green	Hay	Silage
Total compounds (C_7–C_{20})	79.2%	83.7%	81.1%
By classes			
Alkanes (%)	2.20–3.70 (**3.70**)	6.17–8.54 (**8.54**)	0.12–0.44 (**0.44**)
Alkenes (%)	1.12 (**1.12**)	0.66 (**0.66**)	0.00 (**0.00**)
Aldehydes (%)	5.16–8.03 (**5.38**)	0.60–1.54 (**1.54**)	0.71–7.54 (**0.71**)
Alcohols (%)	12.5–15.6 (**15.6**)	3.06–7.72 (**5.73**)	5.89–13.3 (**9.82**)
Ketones (%)	15.7 (**15.7**)	15.3–19.9 (**19.9**)	1.09 (**1.09**)
Esters (%)	0.21–1.20 (**1.20**)	0.12 (**0.12**)	31.8–52.9 (**46.0**)
Acids (%)	0.00 (**0.00**)	0.44–1.54 (**0.44**)	0.84–9.40 (**8.64**)
Terpenes (%)	24.5–26.0 (**24.5**)	31.3–34.6 (**32.0**)	11.2–18.6 (**13.1**)
Others	7.13–8.12 (**7.13**)	13.4–16.0 (**13.4**)	1.39–3.99 (**1.39**)
n.i. (%)	4.93 (**4.93**)	1.43 (**1.43**)	0.00–0.24 (**0.00**)

n.i – not identified.

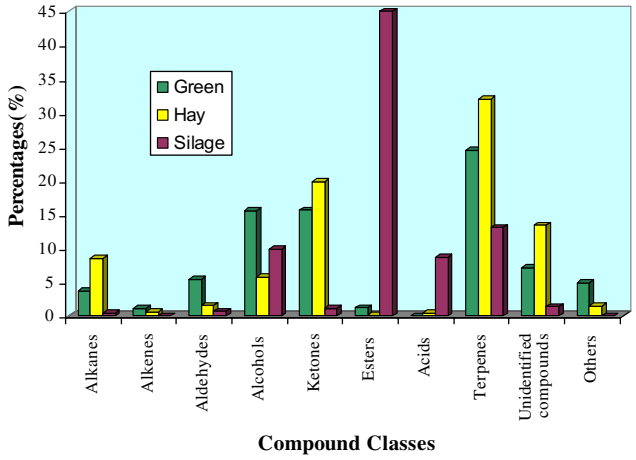

Chart 1. Red clover volatile components variation by chemical classes.

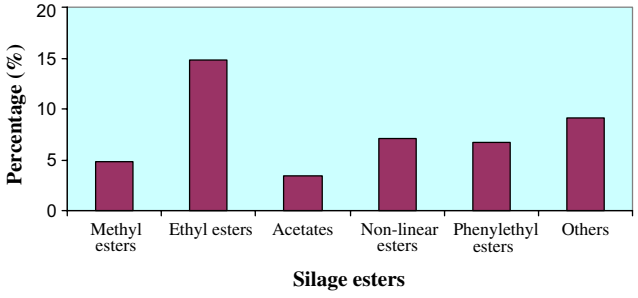

Chart 2. Ester subclasses present in silage.

Besides 3-methyl-1-butanol, phenylethyl alcohol is the only alcohol identified in red clover forages. Phenylethyl alcohol is thought to result from Strecker degradation of phenylalanine (Nogueira, Lubachevsky, & Rankin, 2005); some authors (Xu, Yan, & Zhu, 2005) indicate that it can also result from glycoconjugate hydrolysis.

Benzyl alcohol, another of the alcohols identified, is most likely derived from benzaldehyde in a reversible reaction catalyzed by a NADP/NAD-dependent oxidoreductase (Dudareva et al., 2004).

The presence of 3-methyl-1-butanol, hexanol, 1-octen-3-ol, phenol and benzyl alcohol, among other alcohols, in red clover, has been described elsewhere (Kami, 1978). Hexanol and 1-octen-3-ol were also identified (Buttery et al., 1984).

Evidently only 6-methyl-5-hepten-2-one and 6,10,14-trimethyl-2-pentadecanone are present in all the red clover forages, these being the only ketones identified, in red clover silage. In general, the low molecular weight ketones are more abundant in green red clover than in the hay and others, such as 2-decanone, 2-undecanone and 2-dodecanone, are present in higher amounts in hay. These methyl ketones can be synthesized either from fatty acids or by direct oxidation of hydrocarbons (Forney & Markovetz, 1971).

The 6,10,14-trimethyl-2-pentadecanone is the most abundant compound detected in red clover hay and one of the most abundant detected in the green sample. This is a phytol degradation biomarker in chlorophyll (Alves, Pio, & Duarte, 2001). Phytone, as it is also named, was, along with 3-octanone, the only ketone identified in red clover essential oil (Kami, 1978).

Benzylacetone was only detected in the green sample, while a few compounds, such as 3,4-dihydro-1(2H)-naphthalenone, were only observed in the hay.

Acetophenone, present in small amounts in the green and hay forages, was also identified in red clover (Buttery et al., 1984), being the major component of red clover flowers.

No free fatty acids were observed in red clover green forage. According to the previous work (Elgersma et al., 2004), almost all of the total fat in fresh grass is present as esterified fatty acids while, in silage, a large proportion is present as free fatty acids. Kami (1978) identified a considerable number of free fatty acids in a methylated acid fraction of red clover essential oil.

The hexadecanoic acid present in the silage could result from enzymatic hydrolysis of esters (namely the ethyl and methyl hexadecanoate), a process that occurs during fermentation via esterases (Vianna & Ebeler, 2001). With this exception, there is a tendency for the lower molecular

weight acids to be present in the silage and absent in the hay, while higher acids, e.g. decanoic, dodecanoic and tetradecanoic, are present in the hay forage and are not found in the silage. The low molecular weight fatty acids are common in the last stage of fermentation, which is characterized by the development and dominance of aerophilic bacteria that metabolize them (Biehl & Ziegledir, 2003).

Except for methyl decanoate, which is also present in green and hay forages, and methyl nonanoate and methyl benzoate, that appear in the green plant, we can conclude that the esters are the most abundant class of volatile compounds present in red clover silage.

Ethyl esters, among the total ester components, constitute in the most representative subgroup as can be seen in Chart 2. Comparing ethyl with methyl esters we notice that, with the exception of methyl hexanoate and methyl benzoate, probably due to their volatility, there is a close resemblance between the proportion of the ethyl esters and homologous methyl esters. Fatty acid ethyl esters are obtained from ethanolysis of acyl CoA, that is formed during fatty acid synthesis or degradation (Vianna & Ebeler, 2001). The greater diversity of that class of compounds must be related to the amount of ethanol produced during anaerobic fermentation of siloed material.

Methyleugenol, found in silage and also reported in red clover essential oil (Kami, 1978), is a typical example of a compound resulting from the methylation of a hydroxyl group, in this case eugenol (also present in the silage), as a result of a reaction catalyzed by a methyltransferase (MT) in which s-adenosyl-L-methionine (SAM) acts as the methyl donor (Dudareva et al., 2004). Methyl benzoate can also result from benzyl alcohol by this reaction.

In the group of methyl and ethyl esters, methyl nonanoate is the only one of all the identified non-aromatic esters, that does not correspond to a fatty acid with an even number of carbon atoms, as they normally occur in nature. This was nevertheless one of the methyl esters identified Kami (1978).

Considering the non-linear esters, those that derive from 3-methyl-1-butanol (3-methylbutyl esters) are the most important. Notable also is the number of 2-phenylethyl esters, which must be correlated, as previously, with the presence and abundance of the converting material, in this case phenylethyl alcohol.

Acetate esters, such as 3-methyl-1-butanol acetate, are the result of the reaction of acetyl CoA with alcohols that are formed from the degradation of amino acids or carbohydrates (Vianna & Ebeler, 2001). These have also been reported in other red clover volatile studies (Buttery et al., 1984; Kami, 1978).

Terpenes are the most representative class of compounds in green and hay forages, and one of the more important in silage (see Chart 1).

Linalol, found in red clovers hay and silage, is one of the few identified monoterpenes listed in Table 1. An explanation for its appearance might be the increase of glycosidase activities in the processes that lead to these forages, considering that glycosides of tertiary alcohols, such as linalool, are more readily hydrolyzed (Sarry & Günata, 2004). According to other authors (Degen, Dillmann, Marion-Poll, & Turlings, 2004), compounds like linalol seem to be quite common or ubiquitous components of herbivore-induced odor emissions.

The absence of monoterpenes might be a result of plant metabolism and/or a result of mechanical damage to the plant. In this respect, a study involving white cabbage plants reports a decrease of about 50% in monoterpenes from intact to herbivore-damaged plants (Vuorinen, Reddy, Nerg, & Holopainen, 2004).

Irregular monoterpenes, such as 6-methyl-5-hepten-2-one and β-ionone are reported by some authors, in studies involving carrots (Kjedsen, Christensen, & Edelenbos, 2003) as most likely to be formed from carotenoids. A recent study shows that red clover is particularly rich is these plant metabolites, containing for instance lutein, epilutein, (*E*)-β-carotene and 13-(*Z*)-β-carotene (Cardinault, Doreau, Poncet, & Noziere, 2006).

The terpenes are the most diverse class of plant-specialized metabolites, with sesquiterpenes being the most diverse group of these.

Analyzing the variations of sesquiterpenes in all the red clover forages studied, it appears that, while in the silage there is generally a decrease of the percentage of sesquiterpenes, in hay, (compared once again to the fresh plant), there is a different situation: there are same sesquiterpenes not detected in the green plant, such as the eudesmol isomers that are observed; some sesquiterpenes, e.g. (*Z*)-β-caryophylene and α-humulene, are present in smaller amounts; some sesquiterpenes, of which β-farnesene is the most illustrative example, have their percentage raised considerably from the green plant to the red clover hay.

The decline of sesquiterpenes that occurs in silage, results most likely from bioconversion by microorganisms involved in the ensilage fermentation. The few exceptions noticed, could be due to the acid hydrolysis of terpene glyconjugates.

Of the sesquiterpenes present in all the red clover forages, β-caryophyllene and α-farnesene, are among the most typical inducible sesquiterpenes emitted by herbivore-damaged plants (Holopainen, 2004). The drop of the percentage of β-caryophyllene, could be due to less ability to generate inducible volatiles in hay than in the green plant. β-Caryophyllene and β-farnesene, two of the most predominant sesquiterpenes found in all the studied forages, have already been mentioned elsewhere (Buttery et al., 1984).

The presence of large amounts of sesquiterpene oxides in hay is correlated with the oxidizing reactions that take place during drying.

In conclusion, the volatile composition and its variation were explored in three different forages of commonly used red clover in Portugal.

Concerns about the quality of food products and their origin have increased dramatically in the past few years. Red clover is one of the most diffuse grassland herbs, and

this kind of work may prove useful, for determining volatile components that provide discriminant markers for ruminant PDO products, such as milk and meat.

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