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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).