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ISOLATION AND IDENTIFICATION OF VITAMIN D_3, 25-HYDROXYVITAMIN D_3, 1,25-DIHYDROXYVITAMIN D_3 AND 1,24,25-TRIHYDROXYVITAMIN D_3 IN *SOLANUM MALACOXYLON* INCUBATED WITH RUMINAL FLUID

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Summary—It has been shown that *Solanum malacoxylon* contains 1α,25-dihydroxyvitamin D_3-glycoside. The presence of vitamin D_3 and 25-hydroxyvitamin D_3 has also been suggested. In the present study vitamin D_3 and three of its metabolites, including 1α,25-dihydroxyvitamin D_3, were detected in plant leaf extracts preincubated with ruminal fluid (SMRF). Extraction of SMRF with non-polar organic solvents and purification of the lipid extract by TLC followed by HPLC yielded nine ultraviolet-absorbing (264 nm) peaks. Four of them co-migrated on a Zorbax-Sil HPLC column with synthetic standards of vitamin D_3, 25-hydroxyvitamin D_3, 1α,25-dihydroxyvitamin D_3 and 1,24R,25-trihydroxyvitamin D_3, respectively. These compounds were unequivocally identified by means of mass spectrometry. The results confirm that *Solanum malacoxylon* contains, in addition to 1α,25-dihydroxyvitamin D_3, vitamin D_3, 25-hydroxyvitamin D_3 and possibly other as yet unidentified derivatives. As 1,24,25-trihydroxyvitamin D_3 is absent in plant extracts not incubated with ruminal fluid, the data also indicate that rumen microbes may convert 1α,25-dihydroxyvitamin D_3 into 1,24,25-trihydroxyvitamin D_3.

INTRODUCTION

It has been reported that leaves of *Solanum malacoxylon* (SM) contain a glycoside derivative of 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$-glycoside] [1–3]. Recent work has suggested that the plant also contains vitamin D_3 and 25-hydroxyvitamin D_3 [25$(OH)D_3$] as glycoside derivatives [4].

Other reports indicate that preincubation *in vitro* of SM leaf extracts with bovine or ovine ruminal fluid (SMRF) potentiates its biological activity on calcium and phosphate metabolism in animals [5–7]. It has been proposed that these effects could be related to the release of 1,25$(OH)_2D_3$ from the glycoside [5, 6]. The conversion of 1,25$(OH)_2D_3$-glycoside into free 1,25$(OH)_2D_3$ facilitates the interaction of the sterol with its receptor in target tissues [8].

However, data have been obtained indicating that modifications of the steroid moiety of 1,25$(OH)_2D_3$-glycoside may also occur by treating SM with ruminal fluid. Purification of SMRF by TLC and Sephadex LH-20 chromatography followed by HPLC has resulted in the isolation of two fractions with elution properties similar to 1,25$(OH)_2D_3$ and 1,24,25-trihydroxyvitamin D_3 [1,24,25$(OH)_3D_3$], respectively. Moreover, these fractions effectively competed with [^{3}H]1,25$(OH)_2D_3$ for binding to the chick intestinal 1,25$(OH)_2D_3$ receptor [9].

In the present work we have applied mass spectrometry analysis to unequivocally identify vitamin D_3 metabolites present in SM leaf extracts incubated with ruminal fluid.

EXPERIMENTAL

General

Solvents used for isolation and chromatographic procedures were HPLC grade. HPLC was performed with a Waters Model 600 E system controller equipped with a Model 490 multiwavelength programmable u.v. detector and a Model U6K automatic injector. Mass spectra (70 eV) were obtained on a Kratos MS-50 mass spectrometer.

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Preincubation in vitro of SM leaf extracts with ruminal fluid

Aqueous extracts from SM leaves were prepared and partially purified as previously described [10]. The ruminal fluid was obtained from a fistulated sheep. Three volumes of rumen fluid were mixed with 1 vol of aqueous extract of SM and bubbled with CO_2 for a few minutes until saturation. The mixture was incubated at 38°C for 72 h. After incubation, 4 vol of ethanol were added and the solution was allowed to stand at 4°C overnight. The precipitate formed was removed by centrifugation. The extracts were concentrated at 42°C under nitrogen in a rotary evaporator to give a final concentration equivalent to 2 g of original dry leaves per 0.5 ml.

TLC fractionation of extracts incubated with ruminal fluid

SMRF was extracted twice with each of the following solvent mixtures: chloroform, chloroform–benzene (1:1, v/v), and benzene. The combined organic solvent phases were evaporated under a stream of nitrogen. Aliquots equivalent to 6 g of dried leaves were applied as a 15 cm band on a 500 μm thick silica gel G thin-layer plate. The plates were developed in chloroform–methanol (95:5, v/v). A portion of the chromatogram comprised by R_f values of 0.19 to 0.50, previously shown to contain most of the bioactivity [5], was scraped off and extracted with chloroform, chloroform–methanol (1:1, v/v), and methanol. The combined organic solvents were evaporated under nitrogen.

HPLC

The TLC fraction was analyzed by HPLC techniques on a Phenomenex Zorbax-Sil column (25 cm × 10 mm). A gradient elution of 4–60% isopropanol in hexane was carried out.

RESULTS

To identify the vitamin D metabolites produced by the action of rumen microbes on SM, the SM bioactive material isolated by TLC was further purified by HPLC in which runs were carried out on a straight phase HPLC system capable of resolving most of the known vitamin D metabolites. As shown in Fig. 1, nine

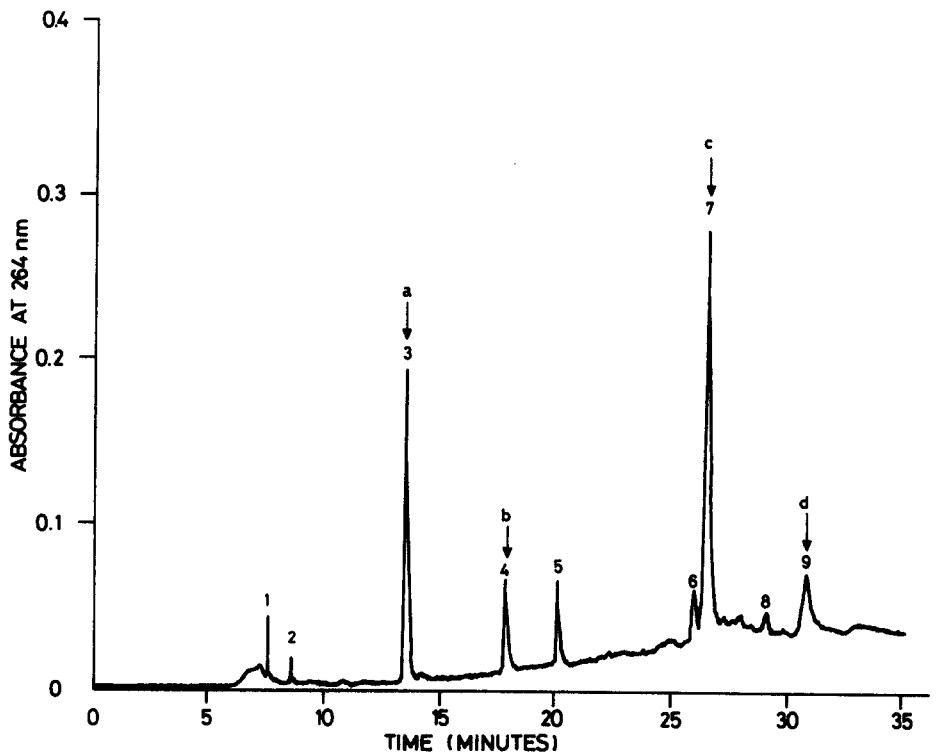

Fig. 1. HPLC profile of vitamin D compounds SMRF. SMRF was extracted with organic solvents and the vitamin D activity was isolated by TLC as described under Experimental. The bioactive material was applied to a Zorbax-Sil column (25 cm × 10 mm). The column was gradient-eluted with 4–60% isopropanol in hexane at a flow rate of 2 ml/min. Retention times of u.v.-peaks were: (1) 7.70, (2) 8.62, (3) 13.63, (4) 17.98, (5) 20.21, (6) 26.23, (7) 26.79, (8) 29.66 and (9) 30.56 min (average of 10 runs). Arrows indicate the elution of synthetic standards: a, vitamin D_3; b, 25(OH)D_3; c, 1α,25(OH)$_2D_3$; d, 1,24R,25(OH)$_3D_3$.

u.v.-absorbing (264 nm) peaks were obtained on a Zorbax-Sil column (25 cm × 10 mm) gradient eluted with 4–60% isopropanol in hexane. Peaks 3, 4, 7 and 9 emerged at 13.63, 17.98, 26.79 and 30.56 min, respectively, and exhibited similar retention times as authentic synthetic standards of vitamin D_3, 25(OH)D_3, 1α,25(OH)$_2D_3$ and 1,24R,25(OH)$_3D_3$, respectively.

Mass spectrometry of the putative vitamin D_3, 25(OH)D_3, 1,25(OH)$_2D_3$ and 1,24,25(OH)$_3D_3$ was performed to elucidate their chemical structures. Mass spectra of u.v.-peaks pooled from 10 HPLC runs were obtained (Figs 2–5).

The electron impact mass spectrum of peak 3 showed a molecular ion at *m/z* 384 (Fig. 2). Loss of water and a methyl group from the parent peak gave *m/z* 351. The peak at *m/z* 325 was produced by loss of 59 atomic mass units (loss of water plus an isopropyl group) from the molecular ion. The peak at *m/z* 271 was caused by loss of the side chain from the parent ion. Loss of water from peak *m/z* 271 gave *m/z* 253. The peak at *m/z* 136 results from formal cleavage between carbons 7 and 8, whereas the base peak at *m/z* 118 results from dehydration of the peak at *m/z* 136.

Peak 4 of HPLC run displayed a parent molecular ion of *m/z* 400 (Fig. 3). Peak at *m/z* 382 represents loss of water from the molecular ion. Loss of water and a methyl group from the parent peak gave *m/z* 367. The peak at *m/z* 341 results from loss of water and an isopropyl group. The peaks at *m/z* 271, 253, 136 and 118 arise in the manner discussed above for Fig. 2.

Figure 4 shows the mass spectrum of HPLC peak 7, which displayed a molecular ion at *m/z* 416 as well as peaks at *m/z* 398 and 380 which are characteristic of the parent minus one and two molecules of water, respectively. The peak at *m/z* 287 is due to side chain cleavage from the main steroid molecule (C-17/C-20 cleavage). Loss of side chain plus one and two molecules of water of the parent ion gives the peaks at *m/z* 269 and 251, respectively. The peak at *m/z* 152 results from cleavage between carbons 7 and 8. From dehydration of the latter results the peak at *m/z* 134.

The mass spectrum of peak 9 (Fig. 5) has a molecular ion at *m/z* 432. Peaks at *m/z* 414 and 396 represent sequential losses of water molecules from the molecular ion. Peaks at *m/z* 287, 269, 251, 152 and 134 are discussed above for Fig. 4.

DISCUSSION

This paper reports the isolation and identification of vitamin D_3 and three of its metabolites

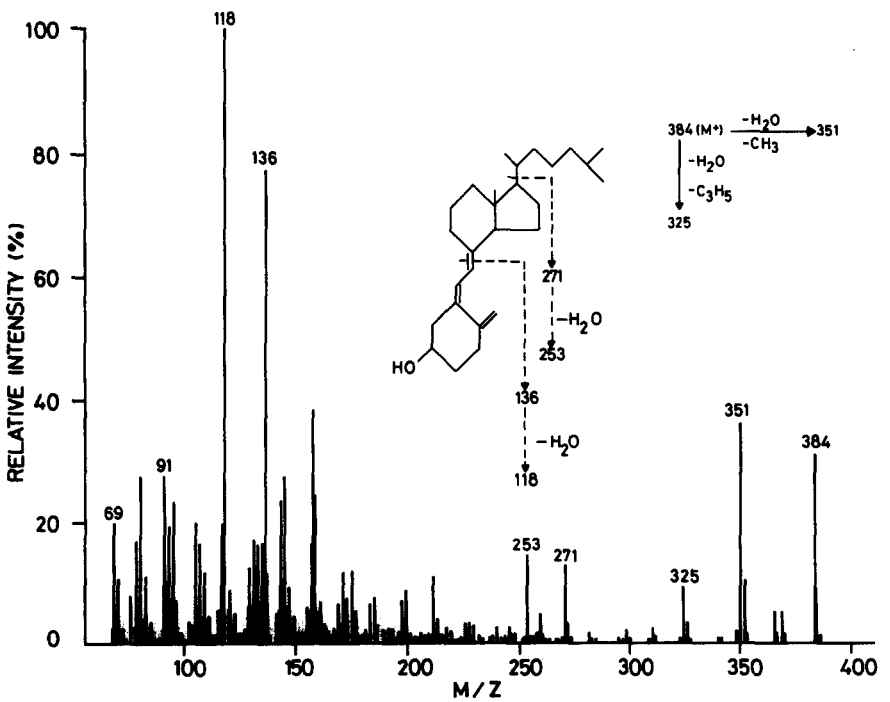

Fig. 2. Mass spectrum of u.v.-peak 3 from HPLC.

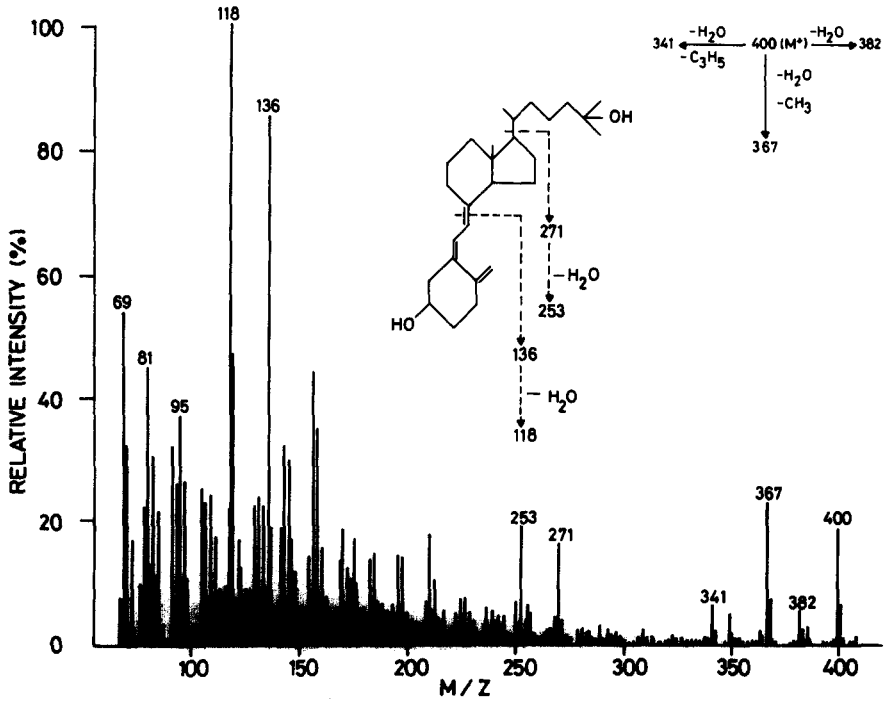

Fig. 3. Mass spectrum of u.v.-peak 4 from HPLC.

in SM leaf extracts incubated with ruminal fluid. The vitamin D_3 derivatives were unequivocally identified as $25(OH)D_3$, $1,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$.

The structural assignments as vitamin D_3, $25(OH)D_3$, $1,25(OH)_2D_3$ and $1,24,25(OH)_3D_3$ were determined by chromatographic and spectral means and by comparing the natural products with synthetic standards.

The synthetic compounds and the SMRF metabolites comigrated on HPLC.

The mass spectrum (Fig. 2) of u.v.-peak 3 (Fig. 1) indicated a molecular weight consistent with vitamin D_3 (peak at m/z 384). Peaks

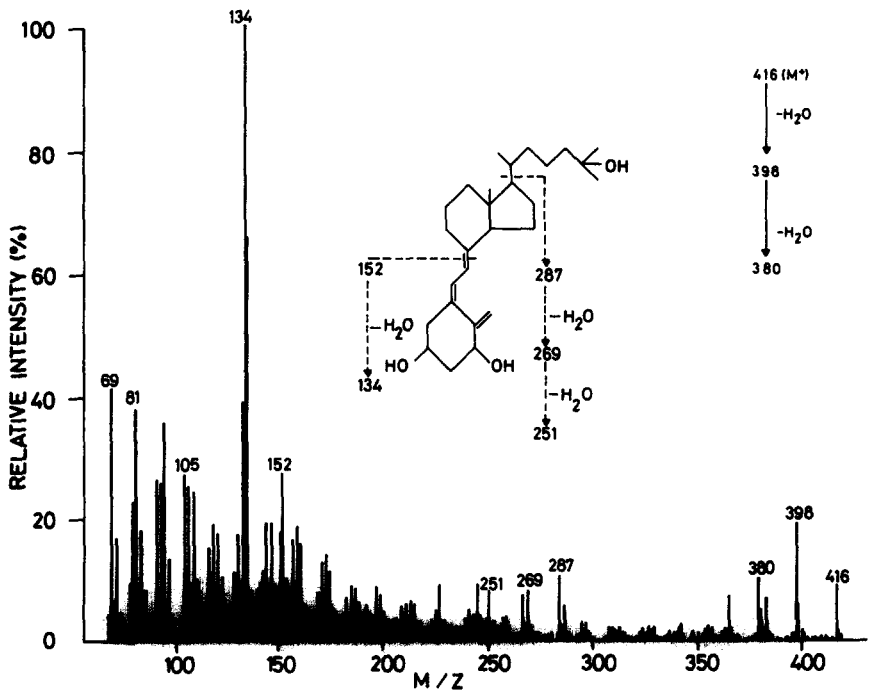

Fig. 4. Mass spectrum of u.v.-peak 7 from HPLC.

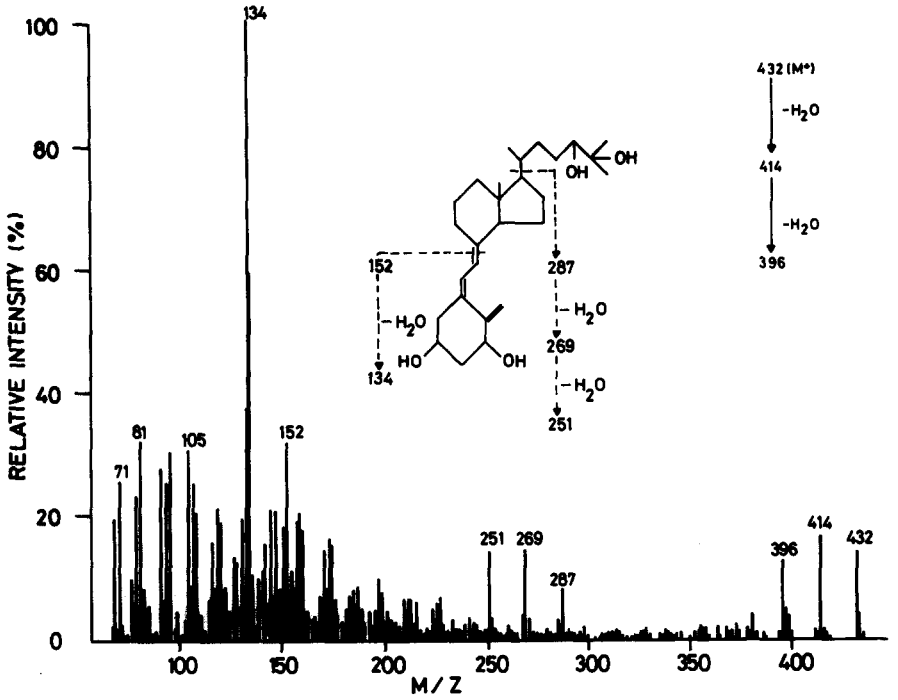

Fig. 5. Mass spectrum of u.v.-peak 9 from HPLC.

occurring at m/z 118 and 136 are characteristic of the vitamin D-triene system. These peaks and others of Fig. 2, demonstrated that this compound can be vitamin D_3.

Mass spectrum (Fig. 3) of u.v.-peak 4 in HPLC run exhibited a peak at m/z 400 in agreement with the molecular weight of 25(OH)D_3. Peaks at m/z 118 and 136 are discussed above for vitamin D_3. Peaks at m/z 271, 253, 136 and 118, which also appear in the mass spectrum of Fig. 2, collectively, indicated that the secoesteroid nucleus of their parent, vitamin D_3, has remained unchanged and that this metabolite is formed as a result of changes occurring only on their side chain. From the above data, this metabolite can be identified as 25(OH)D_3.

Molecular ion at m/z 416 in the mass spectrum of the u.v.-peak 7 (Fig. 4) indicates that this metabolite contains one additional hydroxyl group when compared to 25(OH)D_3. This peak at m/z 416 is in accordance with the molecular weight of 1,25(OH)$_2D_3$. The cleavage between carbons 7 and 8 results in a peak at m/z 152, which represents the A ring plus the carbon 6 and 7 fragment. Loss of water from peak at m/z 152 results in the base peak at m/z 134. Collectively, these peaks and others exhibited in the mass spectrum of Fig. 4, indicate that this metabolite can be 1,25(OH)$_2D_3$.

The electron impact mass spectrum of the u.v.-peak 9 (Fig. 5) has a molecular ion at m/z 432 [molecular weight of 1,24,25(OH)$_3D_3$]. This parent peak suggests that this metabolite is a trihydroxy metabolite of vitamin D_3. Therefore, it can be concluded that there is an additional hydroxyl group on the side chain of this metabolite when compared with its parent 1,25(OH)$_2D_3$. Peaks at m/z 134 and 152 were discussed above for 1,25(OH)$_2D_3$. Peaks at m/z 287, 269, 251, 152 and 134 indicate that the secoesteroid nucleus of their parent 1,25(OH)$_2D_3$ has remained unchanged. This finding indicates that this metabolite is formed as a result of changes occurring only on the side chain of their parent 1,25(OH)$_2D_3$. Collectively, from the above data, a definitive structure assignment for this metabolite could be 1,24,25(OH)$_3D_3$.

The relative distribution of vitamin D compounds in SMRF as determined by measurements of areas of HPLC peaks was 28.6% for vitamin D_3, whereas its derivatives 25(OH)D_3, 1,25(OH)$_2D_3$ and 1,24,25(OH)$_3D_3$ represented 8.4, 53.2 and 9.8%, respectively. However, the possibility that additional vitamin D_3 metabolites are present in other HPLC fractions which were not analyzed by mass spectrometry cannot be excluded.

It is likely that the plant material used for preincubation with ruminal fluid may be the

source of vitamin D_3, 25(OH)D_3 and 1,25(OH)$_2D_3$ found in SMRF. 1,25(OH)$_2D_3$-glycoside has been detected in SM leaf extracts [1–3]. In addition, chromatographic, u.v. and biological data consistent with the presence of vitamin D_3 and 25(OH)D_3 as glycoside derivatives in SM extracts have been obtained [4]. Vitamin D_3 appears to be synthesized in plants by the photolytic activation of a precursor similar to that which occurs in the skin of vertebrates [11]. Moreover, the presence of enzymes in SM involved in the conversion of vitamin D_3 to 25(OH)D_3 and in turn of 25(OH)D_3 to 1α,25(OH)$_2D_3$ has been shown [4]. On the other hand, the 1,24,25(OH)$_3D_3$ detected in SMRF may be the result of the action of rumen microbes on vitamin D compounds from SM extracts. The presence of 1,24,25(OH)$_3D_3$ in SM has been previously excluded on the basis of HPLC and 1,25(OH)$_2D_3$ receptor binding assays [9]. The occurrence of this metabolite in rumen samples was also ruled out (data not shown). Moreover, previous work has shown that incubation of [^{3}H]1,25(OH)$_2D_3$ with ruminal fluid results in the formation of a labeled metabolite with chromatographic properties similar to 1,24,25(OH)$_3D_3$ [9]. This and previous investigations [9] therefore, provide evidence on the production of 1,24,25(OH)$_3D_3$ in ruminal fluid. Synthesis of this metabolite in kidney and to a lesser extent in intestine and cartilage is known to occur [12]. Other examples of metabolism of vitamin D compounds in rumen are known. The formation of 19-nor-10-ketovitamin D derivatives upon incubation of vitamin D_3/D_2 and 25(OH)D_3 with bovine rumen contents has been reported [13]. These compounds could have been present in HPLC peaks (Fig. 1) not identified in the present work. At the present time studies are underway in our laboratory to achieve their identification.

A calcinotic disease in cattle which ingest SM in grazing areas of Argentina has been reported [14]. The intoxication has been attributed to the presence of 1,25(OH)$_2D_3$ in the plant. The conversion of 1,25(OH)$_2D_3$ into 1,24,25(OH)$_3D_3$ in rumen of animals which become intoxicated with SM may represent a first step to deactivation into metabolites of less activity than the hormone or into inactive derivatives.

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