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Characterisation of *in vitro* Gut Fermentation Products and their Metabolites from Corn Oil as Possible Source of Boldenone in Calves Nutrition

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Abstract: Phytosterols have been recently proposed as nutraceutics both in human and animal nutrition, because their influence on lipids metabolism. The reported possibility that phytosterols microbial fermentation could lead to the production of boldione as boldenone pro-hormone, pulsed us to set up two *in vitro* experiments for the characterisation of phytosterols gut fermentation products and their hepatic metabolites, by LC-MS/MS. A Todd-Hewitt broth (10 ml), enriched with corn oil as natural dietary source of phytosterols, was inoculated with 100 mg of calf faeces and aliquots drawn after 24, 48 and 72h of micro-aerobic incubation. After SPE extraction, LC-MS/MS analysis revealed the presence of Boldione in the 24h fraction at a concentration of 4 μg , while alpha and beta Boldenone were present in equimolar ratio in the 48h fraction at 12 ng each. Nor Boldione neither Boldenone were detected at 72h . The ether extract of the 48h fermented broth was further submitted to microsomial calf liver incubation. A not described major metabolite in a isomeric form, with a m/z 285 as protonated molecule has been found and its structure proposed . Such metabolite could be useful to trace in urine the origin of boldenone as metabolite from an alimentary source.

Key words: Characterisation, in vitro, fermentation, metabolites, com oil, possible source, boldenone and nutrition

Introduction

In human nutrition the supplementation with phytosterols, a group of biologically active phytochemicals present in the seeds oils and their by-products, in the diet has been proposed as a practice effective in lowering cholesterolemia, thereby reducing the risk of coronary heart disease (Lichtenstein and Deckelbaum, 2001). In the European Union (EU), plant sterol esters for use in margarines/spreads has been reviewed under the EU Novel Foods Regulation (Regulation EC No 25 8/97) and, in a recently published opinion, the Scientific Committee on Food concluded that the use of phytosterol-esters in yellow fat spreads (maximum level of 8% free phytosterols) is safe for human use (European Commission Health and Consumer Protection Directorate-General, 2000). Contemporary, in animal production, the use of a modified tall oil, a plant by-product with a high phytosterols content, has been proposed as feed additive to produce lean pig meat (O'Quinn *et al.*, 2002). Recently, the use of fat supplements have been suggested in beef in attempts to influence specific metabolic pathways and, ultimately, hormones that directly modulate ovarian cellular processes; the basis for this approach lies within an array of digestive, metabolic and reproductive sequelae that occur when cattle consume significant quantities of digestible fat originating from seeds oil and their partially hydrogenated by-products (Williams and Stanko, 2000). Due to the new refining technologies (Daguet and Coïc, 1999), it could be possible to use oil seeds by-products, phytosterols enriched, as feed material in farmed animals, use prompted in ruminant nutrition, as consequence of BSE crisis, to substitute tallow.

Despite such reported beneficial effects, phytosterols have been described as potential endocrine distrupting molecules non only in fish (Lehtinen *et al.*,1999) but also in mammalians (Nieminen *et al.*, 2003), where their dietary administration leads to increased levels of Testosterone ad 17 beta Estradiol. Moreover, there is the scientific evidence that boldione, considered as precursors of the anabolic steroid boldenone (Fig. 1) , can be produced by gut microflora in rats with a daily oral exposure to phytosterols of 500 mg kg^{-1}. b.w. (Yong *et al.*, 2000).

Because boldenone is ranked among anabolic steroids forbidden in meat production and due to an increased reporting about its positivities in urine of fattening steers and calves in the last two years, we aimed to verify if such analytical findings, considered as marker or proof of illegal treatment, could be related not only to illegal hormonal treatments but also to a possible dietary natural or nutraceutical source.

Previous research have already described the presence of large amounts of Boldione and Boldenone in its two isomeric forms, in faeces drawn for routine controls for veterinary drugs residue analysis and such findings were addressed as a consequence of illicit administration *via* feed of anabolics (Van Puymbroeck *et al.*, 1998a), even if the sterols fermentation by gut microflora was not excluded at all (Van Puymbroeck *et al.*, 1998b).

To deepen the matter, this paper reports about *in vitro* possible formation of Boldione and Boldenone using a bacterial fermentation broth enriched with corn oil (1% phytosterols w/w) that was inoculated with mother-suckling veal calves faeces as source of microflora from the large intestine. Subsequently, the metabolic profile of such fermentation products was investigated on a calf liver microsomial preparation, to simulate a possible systematic absorption, with the aim to check for the presence of possible marker metabolites.
The LC-MS/MS approach was chosen, considering the opportunity both to have a good chromatographic resolution of alpha and beta boldenone isomers and to modulate the fragmentation of the analytes, thus achieving relevant structural informations.

Materials and Methods

Chemicals and Reagents: Standards of Boldione, beta boldenone, 6 beta OH boldenone 16 alpha OH boldenone, 16 beta OH boldenone, 6 beta 17 alpha boldenone and methenolone (Internal Standard), were purchased from Alltech (PA-USA); alfa Boldenone was kindly gifted by horseracing antidoping Centre of Milan (Italy). All solvents were of chemical pure grade from Carlo Erba, Milan- Italy. Isolute SPE MF C18 500 mg 6 ml columns (Stepbio, Bologna, Italy). Column X Terra MS C8 21x150 mm (5 micron) (Waters)
Todd-Hewitt Broth CM 189 B 10 ml vial (PBI, Milan, Italy) was enriched with 1 ml corn oil intended for human nutrition, available on the market.

Biological Materials: faeces were drawn from a holstein-fresian male veal calf aged 1 month and liver was from a 6 month calf, at slaughter

Equipments: Thermostatic bath (KW, Siena, Italy) and rotary evaporator, (Buchii, CH), bench centrifuge Megafuge 10.0 (Haereus Italia, Milan, Italy) SPE vacuum manifold (Supelco Italia, Milan, Italy)
Agilent 1100 series LC-MSD trap (Agilent Technology Italia, Milan, Italy)

Chemicals: Standards stock and working solution were prepared in methanol and stored at + 4°C. Calibration curves were built in the range 0.2 - 10.0 ng injected in the LC-MS/MS system. Recoveries were calculated according to the internal standard method.

Incubation of Phytosterols in Todd Hewitt Broth: A fresh fecal sample (2 g) was collected in steril 50 ml Falcon tube and diluted with 20 ml of sterile solution. After homogenisation by vortexing, 1 ml (equivalent to 100 mg of faeces) was added to 10 ml of the broth, previously enriched with 1 ml of corn oil containing 1% phytosterols and the mixture incubated at +37°C under 10% carbon oxide conditions for 72 hours. Negative controls (enriched fermentation broths not inoculated with faeces and not enriched with faecal inoculum, respectively) were inserted in the procedure. Fermentation was performed in duplicate and aliquots of 1 ml of broth drawn from each vial at 24h, 48h and 72h, respectively, to monitor the fermentation process.

***In Vitro* Metabolism:** *In vitro* metabolism of fermented phytosterols on microsomial calves liver preparations was performed according to Testai and Vittozzi (1986), to study only first phase metabolites. Sterols from 1 ml fermented broths (48 h) were extracted by two diethyl ether liquid liquid partition (2:1 v/v) under vortexing. Organic phases was recovered by centrifugation (800g 15 min +4° C), combined. and brought to dryness under a gentle nitrogen stream. Residue was redissolved in 50 μl methanol and added to 5 ml of the standard reaction mixture with 2 mg ml^{-1} microsomial proteins. Incubation was carried out at +37° C in stoppered vessels immersed in the thermostatic bath, under gentle shaking, overnight. As negative control, we used the correspondent 48h fermentation broth not phytosterols enriched.

SPE Purification of Fermentation Broths and Microsomial Incubates: Fermentation broths (1 ml) and microsomial incubates (5 ml), were spiked with 5 ng of Methenolone as internal standard and purified on a SPE MFC18 500 mg 6 ml columns previously conditioned with 6 ml MeOH and 3 ml of water. After the application (flow 2.0 ml min^{-1}) the column was rinsed with 4 ml water: acetone (5:1 v/v) solution and allowed to dry. After a further washing with 1 ml n-hexane, compounds of interest were eluted by 6 ml of diethyl ether, in a conic vial brought to dryness under nitrogen stream and dissolved in 50 μl of the LC-MS/MS mobile phase for the analysis.

LC-MS/MS Analysis: To elucidate the strucure of possible metabolites, we selected those acquisition conditions, able to give an extensive fragmentation of the chosen precursor ion. To this purpose, Boldenone and Boldione standard solutions in acidified methanol were introduced by pump syringe at 8 μl/mL into the LC-MS/MS system and collision energy values varied while product ions were recorded in the range 75 - 400 amu.
The following chromatographic conditions were developed in order to basically resolve alpha and beta boldenone forms. Mobile Phase: water (1% acetic acid) and Methanol (1% acetic acid); Column X Terra MS C8 21x150 mm (5 micron) (Waters). Chromatographic run: a 18 min gradient, from 20% to 75% methanol in 12 min, then to 100% in 3 min. Such condition maintained

Table 1: LC-MS/MS conditions for the identifications of boldione, boldenone and its metabolites

Compound	RT (min)	Precursor Ion m/z	Main Product Ions m/z
6 beta OH Boldenone	10.2	303	285; 267
6 beta 17 alpha Boldenone	10.4	303	285; 267
16 alpha OH Boldenone	10.9	303	285; 267
16 beta OH Boldenone	11.4	303	285; 267
Boldione	12.6	285	267; 147
17 beta Boldenone	13.2	287	269; 135
17 beta microsomial Metabolite	13.3	285	267; 171
17 alpha microsomial Metabolite	13.8	285	267; 171
17 alpha Boldenone	14.0	287	269; 135
Methenolone	14.5	289	271; 253

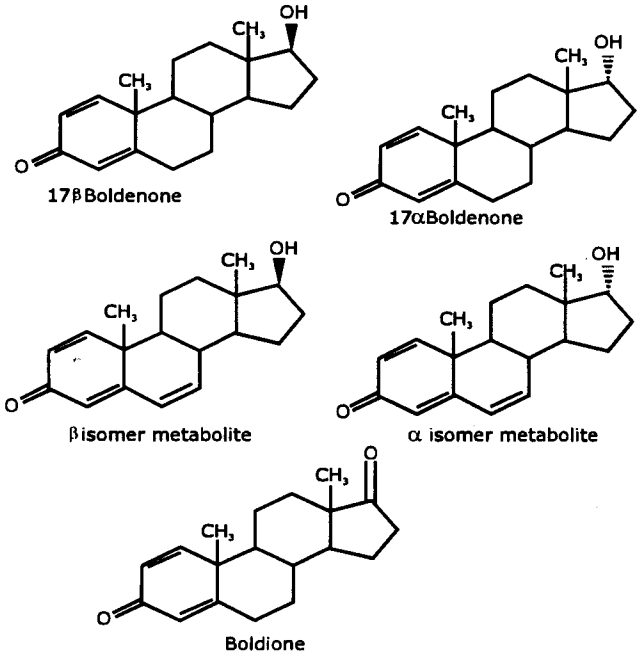

Fig. 1: Structures of Boldione, Boldenone and of the differential metabolite identified after microsomial incubation of the 48h phytosterols enriched fermented broth

for 3 min; flow: 0.300 ml min^{-1} ; nebulizer, 30 psi; Nitrogen as dry gas: 8 L min^{-1} ; Temperature: +350°C; collision energy: 0.9 eV. MS/MS acquisition was performed by building appropriate windows for the precursor ion of each compound considered, according to its Retention Time. Product ions were monitored in scan mode from 70 to 400 amu. From the 50 µl of the final extracts, 10 µl aliquots were injected into the system

Results

Calibration curves for Boldione, alpha and beta Boldenone and Methenolone in MRM mode were linear in the range 0.2 – 10.0 ng injected (r >0.9989). Recoveries, calculated by internal standard method, were above 85%

In Table 1 the LC-MS/MS elution profile of the molecules examined with the MRM products considered for the identification are reported

The analysis of fermentation broths at 24, 48 and 72 h revealed a kinetic in the formation of Boldione (at 24h) and Boldenone in both alpha and beta form (at 48h), with an almost complete disappearance at 72h of the analytes monitored. At 24h the Boldione mean concentration in the broths was estimated to be 4.46 µg ; at 48h, alpha and Beta boldenone were found at 24.6 ng, in equimolar ratio. LC-MS/MS chromatograms of the phytosterol -free and –enriched 48h broths are reported in Fig. 2.

The metabolic pattern of the 48h fermentation broth microsomial incubation revealed the extensive metabolisation of the beta boldenone, leading to the formation of the correspondent hydroxy metabolites (Fig. 3). In addition, we recorded the presence of two differential peaks with almost the same mass spectra, sharing the same precursor ion of Boldione (m/z 285), but at two different retention times (Fig. 3). The slightly differences in their fragmentation pattern, with respect to that of Boldenone (m/z 287) and Boldione, suggest the presence of an hydroxyl group on C17 position, responsible of the isomers and a dehydrogenation on the B ring at C6-C7 level (Fig. 1) (Fig. 4) as consequence of bacteria dehydrogenase activity.

Discussion

In vitro fermentations of phytosterols with selected Mycobacterium strains under strictly controlled aerobic, temperature and pH conditions, have been already studied in applied microbiology and some of these processes patented by the pharmaceutical industry; with high yields (up to 40%) in the production of steroids, such as Boldione and Testosterone, due to the activity of dehydrogenase steroid by *Mycobacterium* (Lo *et al.*, 2002) (Seidel and Horhold 1992). Moreover, a recent paper reported about Boldione as fermentation

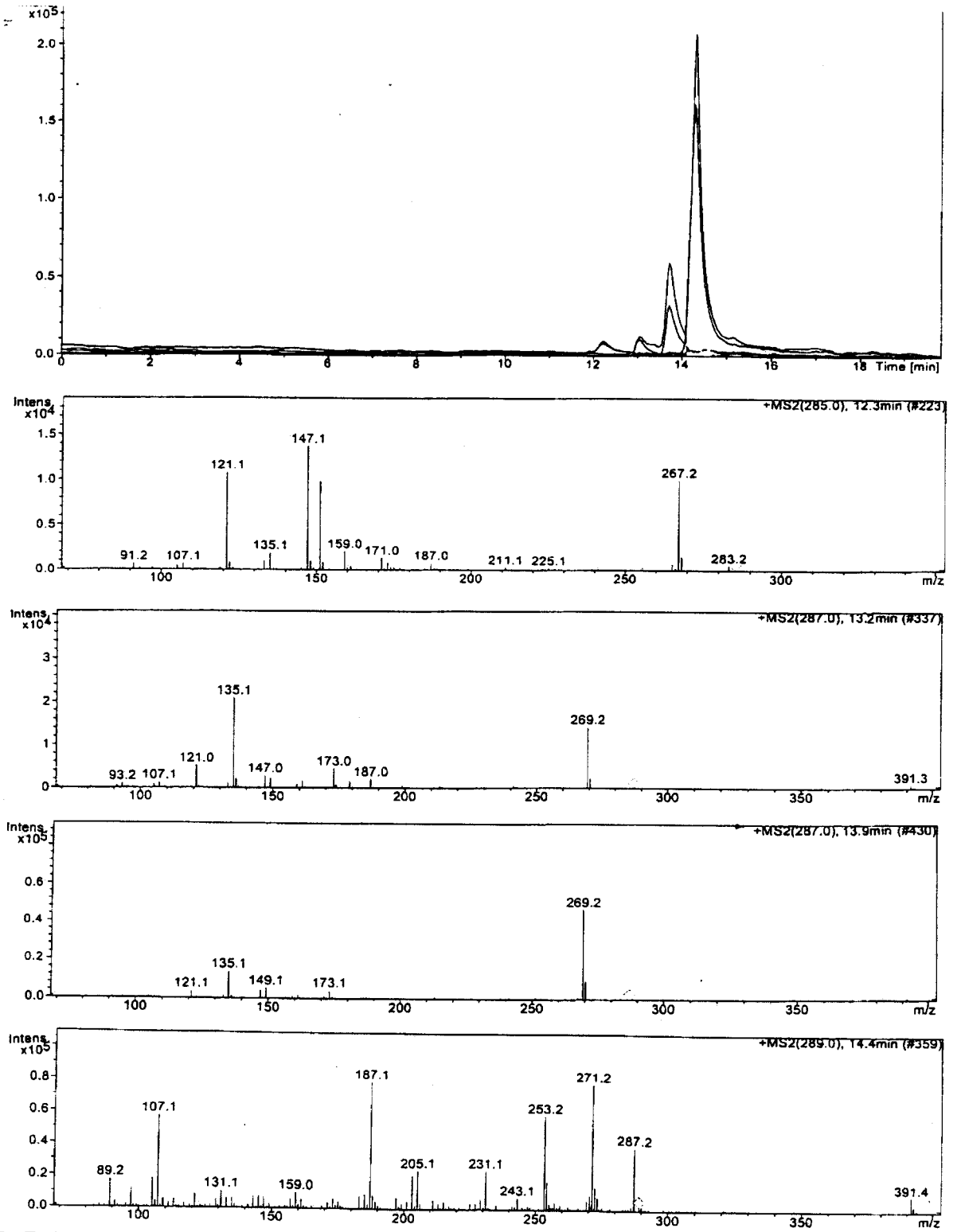

Fig.2a To be continued

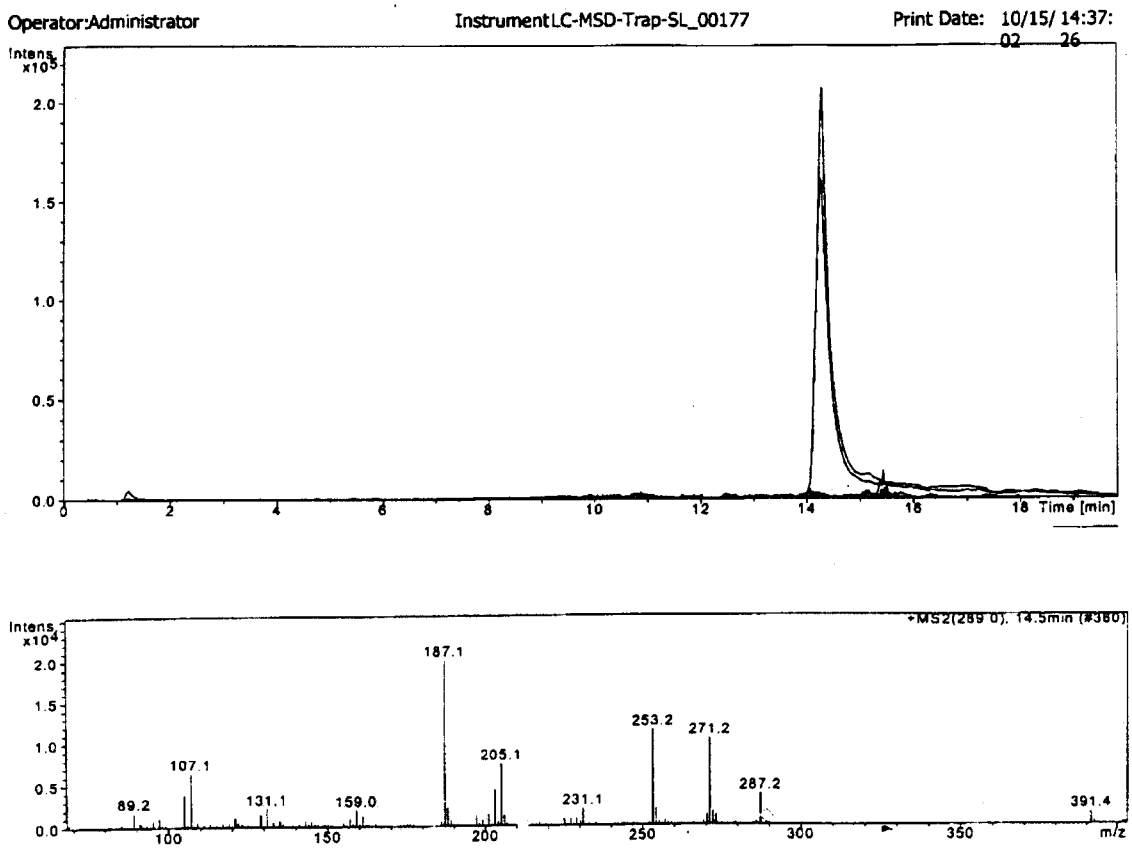

Fig. 2: LC-MS/MS identification of Boldione and beta and alpha Boldenone in the 48h enriched fermentation broth (a). Not enriched fermentation broth as control, Methenolone (RT 14.4) as Internal standard (b).

product from bamboo phytosterols, due to different *Bacillus* strains (Sarangthem and Singh 2003). *Mycobacteria* both as slow- and rapid grow strains are ubiquitous in cattle, at faecal level. Some *Bacillus* strains are licensed as probiotics in animal nutrition, to improve feed conversion. Because at farm such standardised fermentation conditions are not reproducible at all, we chose the faeces from mother suckling veal calves, to minimise as much as possible the influences of different dietary regimens on the gut microflora. Corn oil was selected as source of phytosterols, because corn and its by-products are among the most important feed materials of the diet of meat producing animals.

We could confirm the possibility that both Boldione and Boldenone (alpha and beta) could be sequentially produced by veal gut microflora drawn from the large intestine, just after a 24h - 48h micro-aerobic fermentation, using a commercial corn oil as phytosterols enrichment. The presence of beta Boldenone, with no traces of its metabolites (Fig. 2 a) indicates at least faecal bacteria did not show any an appreciable 17 beta steroids dehydrogenases and isomerases activity under such conditions. These enzymes are described as the main detoxification mechanisms for the biological active beta hormones, such as Estradiol, among mammalians and cattle.

The overall observed very low bio-fermentation efficiency from phytosterols to Boldione ($25x10^3$: 1 w/w) and to both alpha and beta boldenone ($4x10^6$:1 w/w), nevertheless, was still able to give positive findings to LC-MS/MS analysis settled around a target Minimum Required Performance Limit of 1.0 ppb, currently required for reliable residue analysis in urine. The observed bio-transformation kinetics, with the target analytes detectable only at 24h (Boldione) and 48h (Boldione and both Boldenone isomers in equimolar ratio), (Fig. 2) and no more after 72h, constitutes a valuable information to understand the possible temporary and irregular presence of boldenone in farmed calves. To this respect, other key factors, such as the phytosterols concentration in the diet, the

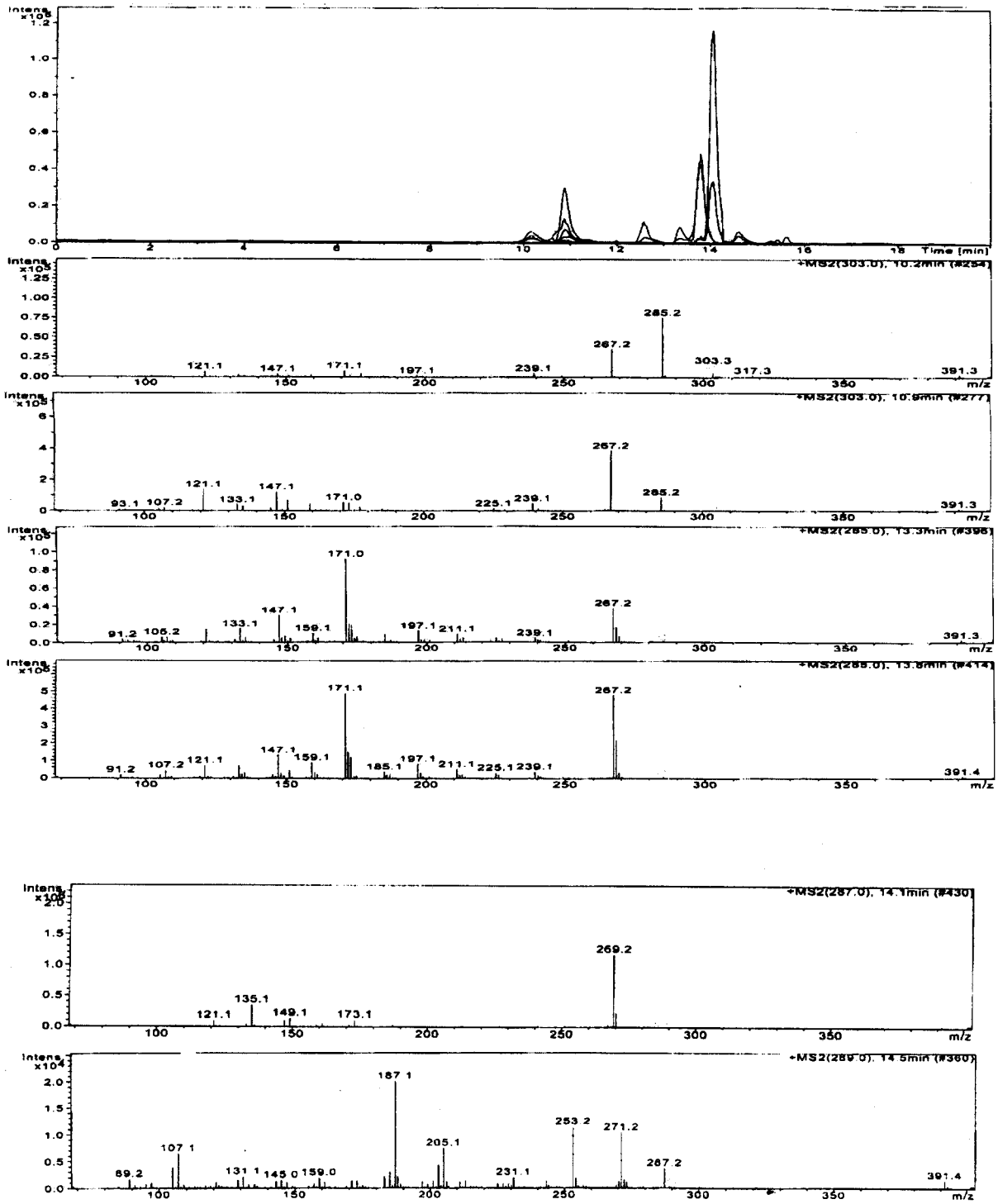

Fig.3 To be continued

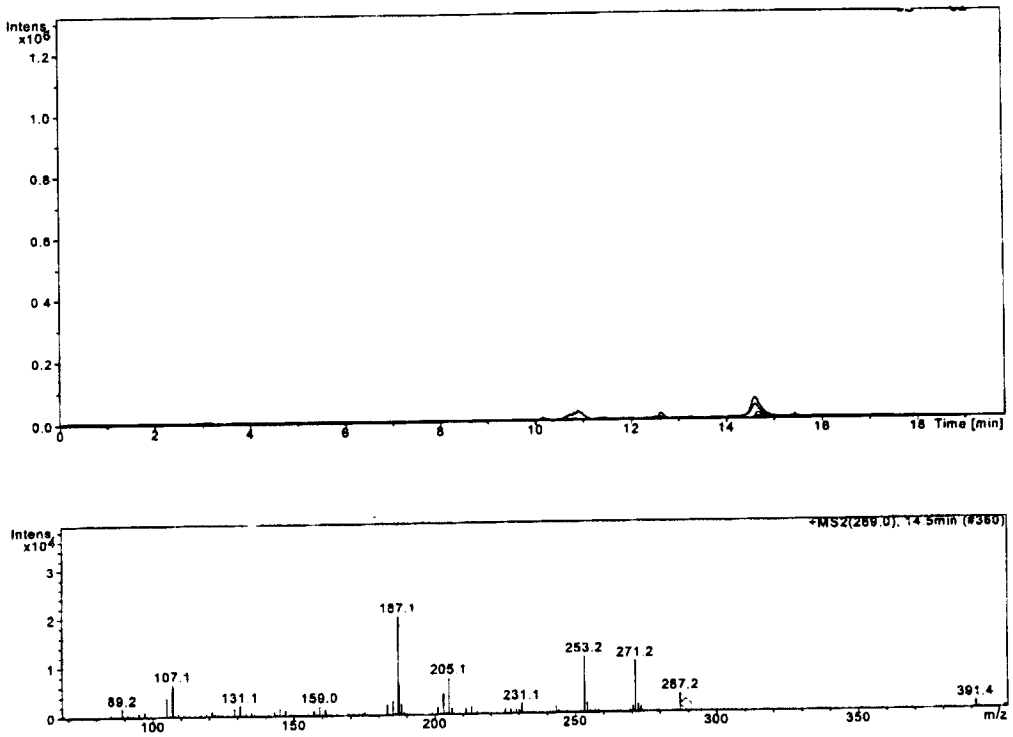

Fig. 3: Metabolic pattern of 48h fermented broths extracts after calf liver microsomial incubation: a) non phytosterols enriched broth extract; b) phytosterols enriched broth extract, with, from the top to the bottom: 6 beta OH Boldenone (RT 10.2), 16 alpha OH Boldenone (RT 10.9), the new metabolite in the two isomeric forms (RT 13.3 and 13.8, respectively) and alpha Boldenone (RT 14.1);. Methenolone as Internal Standard (RT 14.5)

environment, the ecology of gut microflora, with special emphasis devoted to the presence of Mycobacteria and the contemporary administration of probiotics, should be also taken into account as key factors in the fermentation process. The use of micro aerobic condition could not exclude that the observed formation of Boldione and Boldenone could be only limited to faeces and manure fermentation and not likely to occur also in the large intestine.

Until now, there is open debate how to consider the presence and the amount of alpha and beta Boldenone residues in calves and steers urine for the compliance/ not compliance of the meat, as proof of illegal anabolic treatment. In national residue plans, until now no specific attention has been paid to a possible dietary origin, nor dependent by the activity of the endogenous endocrine system, neither consequence of xenobiotics administration.

To this respect, our *in vitro* metabolic studies indicate the beta Boldenone, eventually absorbed by gut, immediately undergoes to extensive metabolism, leading to the formation and probable elimination in the urine of its hydroxy metabolites, with alpha Boldenone and Boldione still detectable (Fig. 3a). In addition, the identification of a major metabolite, recorded as microsomial differential isomeric peaks in the 48h fermented broth, could help to deepen the matter about the possible source of Boldenone. No differential metabolite could be present in the case of faecal and manure contamination of urine, in absence of a the systematic absorption of fermented sterols from gut , their subsequent liver metabolism and urinary excretion. It is worth noting the proposed metabolite formula, according to the acquired fragmentation pattern (Fig. 1), shows a dehydrogenation on the B steroid ring at C6-C7 position. The evaluation of the presence of such new metabolite also in urine of animals under exogenous treatment with boldenone would be helpful to deepen the matter .

For regulatory purposes, because the possible natural origin of boldenone from dietary phytosterols, the contemporary sampling and analysis of faeces and urine of the same animal in the cleanest way could be suggested, in order to evaluate both analytes

concentrations and their first and second phase metabolites patterns. On the basis of such data it could be possible to calculate metabolites ratio, thus tracing back the probable origins of Boldenone, exogenous, natural or eventually endogenous, in farmed animals.

To conclude, the use of nutraceutics in animal production should be evaluated within a risk analysis frame, according to animal welfare, food safety and environmental impact items (Lange *et al.*, 2003). To this respect, it is in our aim to deepen this study, by reproducing this experiment using various feed materials as substrate, thus predicting possible situations that *in vivo* could determine or inhibit the natural production of Boldione and Boldenone from gut flora and, eventually, that could influence animal welfare, by interfering with the production of the endogenous hormones Testosterone and 17 beta Estradiol. Moreover, the possibility that the phytosterols enriched dietary regimen could enhance the selection and microbial load of potentially pathogenic fast growing Mycobacteria strains will be also investigated, at farm.

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