Chapter 1

HPTLC: NEW APPLICATIONS IN THE FIELDS OF FOOD AND FOOD SUPPLEMENTS

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1. Introduction

The safety of consumers must be guaranteed by the quality control both at the production site and after distribution on the market. In the area of food and food supplements, the analytical approach requires special expertise, since the matrix is complex and data obtained could be altered by bias due to different factors.

Food supplements, and in particular those products containing botanical preparations, are widely consumed in Western diets and, unlike drugs, these products are generally considered by consumers positively, due to the misleading association ñnatural=safeò.

The popularity and the ease access in shops or via Internet have speeded up the diffusion of these products, with new concerns about their quality, composition, and safety [1-2]. The problem is particularly significant, when they are obtained from unregulated markets, where illicit activities are not infrequent. The so-called *parallel market* includes on-line distribution, gyms, smartshops and others. Among the illicit substances, those promoting weight loss, body-building, and

sexual performance are the most frequently detected. The use of performance-enhancing drugs in sport (so-called doping) is common, even though the practice is considered unethical by all international sports organizations and especially by the International Olympic Committee; moreover, it can be seriously detrimental to health. Among others, anabolic steroids affect cardiovascular and mental health and are associated with an increased risk of cancer [3].

Dietary supplements containing *Ephedra* alkaloids were associated with hypertension, tachycardia, stroke, seizures and death [4-6].

Also the presence of contaminants (mycotoxins, pesticides) is still an open problem and the development of fast and reliable methods for their detection and measure is highly recommended. On this basis, new applications of HPTLC method have been set up allowing a fast screening in food/food supplement analysis, even when the matrix shows a particular complexity.

Thin-Layer Chromatography (TLC) is a simple, flexible, relatively inexpensive and efficient separation technique for both qualitative and semi-quantitative/quantitative analysis, allowing a simultaneous separation of several samples. The recent evolution of TLC is the so-called HPTLC (High-Performance Thin-Layer Chromatography), which shows an optimized separation efficiency associated with the standardisation of all steps in the procedure. This approach allows precise sample deposition, reproducible chromatographic separation and computerised data analysis. Compared to most separation techniques, the costs for an HPTLC system (as well as its maintenance) is relatively low. Moreover, the possibility of image acquisition of separated samples on the plate is an interesting aspect of HPTLC.

The use of HPTLC is particularly useful, when a laboratory has to analyse a very high number of similar samples, searching for classes of molecules. In fact, HPTLC can be easily applied as a screening approach to define the presence/absence of certain analytes in a series of samples. After screening, only positive samples are included in further analyses, reducing significantly both costs and time spent for reaching the final results.

2. Objectives

The aim of this paper is the description of possible applications of HPTLC to quality control, with particular attention paid to food supplements for athletes.

3. Materials and Methods

Samples

The samples included in this study were food supplements for athletes taken by police in a gym and sent to our department to verify the presence/absence of dangerous ingredients.

3.1 HPTLC

Acids

- Sulphuric acid purity >95-97% (Merck KGaA, Darmstadt, D)

Solvents and Solutions

- Acetone for HPLC (Sigma-Aldrich Chemie, Schnnelldorf, D)
- Chloroform for HPLC (Sigma-Aldrich Chemie, Schnnelldorf, D)
- Metanol for HPLC (Merck KGaA, Darmstadt, D)
- Sulphuric acid in ethanol, 5% (v/v) - solution for detection

Purified steroid hormones

- Androstenedione, purity >99.1% (Sigma-Aldrich Chemie, St Louis, USA)
- Dehydroepiandrosterone, purity >99% (Sigma-Aldrich Chemie, St Louis, USA)
- Metandrostenolone (17ß-Hydroxy-17-methylandrosta-1,4-dien-3-one), purity >99% (Sigma-Aldrich Chemie, St Louis, USA)
- Nandrolone, purity >99.9% (Sigma-Aldrich Chemie, Schnnelldorf, D)
- Stanozolol, purity >99.71% (Steroid S.p.A, Cologno Monzese, MI)
- Testosterone, purity >99.9% (Sigma-Aldrich Chemie, Schnnelldorf, D)

- Testosterone enantate, purity >97% (Steroid S.p.A, Cologno Monzese, MI)

Standard solutions

The standards were stored according to the supplierôs instructions. Stock solutions were prepared in methanol at the final concentration of 1 mg/mL. Aliquots of 12 µL of each standard solution were applied onto HPTLC silica plates.

Sample preparation

Aliquots of sample (200 mg) finely grounded were precisely weighed and added to 10 mL of methanol. The solution was stirred for 15 minutes, and filtered on a 0.45 µm filter, dried with a nitrogen flow and finally solubilized in 1 mL methanol.

Plates

- HPTLC plates, silica gel 60 F254 (Merck KGaA, Darmstadt, D)

Equipment

The equipment for HPTLC was from CAMAG (Muttenz, Svizzera) and included a sample applicator (Camag Linomat 5), a TLC visualiser (Camag TLC visualizer) and the software for data elaboration (Camag VisionCats).

3.2 LC-MS

Acids

- Formic acid (Merck KGaA, Darmstadt, Germany)

Solvents

- Water for HPLC (Sigma-Aldrich Chemie, Schnnelldorf, D)
- Metanol for HPLC (Merck KGaA, Darmstadt, D)

Standard solutions

Standard solution of steroid hormones was prepared in methanol at the final concentration of 1 mg/mL. The solution was diluted 1:40 (v/v) with methanol in order to obtain the concentration of 25 µg/mL.

Sample preparation

Aliquots of sample (200 mg) finely grounded were precisely weighed and added to 10 mL methanol; the solution was stirred for 15 minutes. Solutions were filtered on a 0.45 μm filter, dried with a nitrogen flow and finally solubilized in 1 mL methanol. The solution was diluted 1:100 (v/v) with methanol before the injection in the LC-MS apparatus.

Equipment

Mass spectrometry used a linear trap (LTQ, ThermoElectron Co, San Jose, CA, USA) ESI ionization source coupled with a liquid chromatograph (LC) Surveyor Pump Plus autosampler and Surveyor Autosampler Plus (ThermoElectron Co., San Jose, CA, USA). For data acquisition and processing, a Dell workstation with Excalibur® software release 2.0 SR2 (ThermoElectron Co., San Jose, CA, USA) was used. Column was a Hypersil GOLD aQ, 2.1x100 mm, 3mm (Thermo Fischer, San Jose, CA, USA), maintained at 24°C.

The separation was performed in LC, with a gradient elution at 0.25 mL/min based on the following phases:

A. 0.1% formic acid in water

B. 0.1% formic acid in methanol

The gradient was set up as reported below and injection volume was 10 μL (Table 1).

Description of gradient Table 1

Time (min)	**Phase A (%)**	**Phase B (%)**
0	70	30
1.5	70	30
10	36	64
20	1	99
25	1	99
40	70	30

4. Results and discussion

Due to the large number of samples to control and the different classes of possible "dangerous" ingredients to search, a preliminary screening was necessary. HPTLC was considered the suitable tool and methods were developed for the detection of hormones and active amines, representing the classes of molecules, which are usually used in illicit activities, such as doping.

4.1 HPTLC

For the detection of steroid hormones, methanolic solutions of sample (4 and 8 μL) were applied on HPTLC silica plate in parallel to standard solutions (12 μL). The mobile phase used for the chromatographic separation was chlorophorm: acetone 85:15 (v/v) (10 mL). At the end of the chromatographic run, the plate was revealed at 254 nm and derivatised using a sulphuric acid solution (5% ethanol) and dried in extractor wood at 110°C for 5 minutes. The compounds were visualised at 366 nm and at visible light. The first step of the method development was the identification of Limit of Detection (LOD) for each hormone and to compare these values with the pharmacological activities of the molecules searched. Table 2 lists the LODs for the steroidal hormones included in the screening.

LOD of steroid hormones included in the HPTLC screening Table 2

Compound	**LOD (ng on plate)**	**LOD (mg/kg of product)**
Androstenedione	3.9	4.90
Dehydroepiandrostenedione	3.0	3.75
Metandrostenolone	3.0	3.75
Nandrolone	3.0	3.75
Stanozolol	3.9	4.90
Testosterone	3.0	3.75
Testosterone enanthate	3.0	3.75

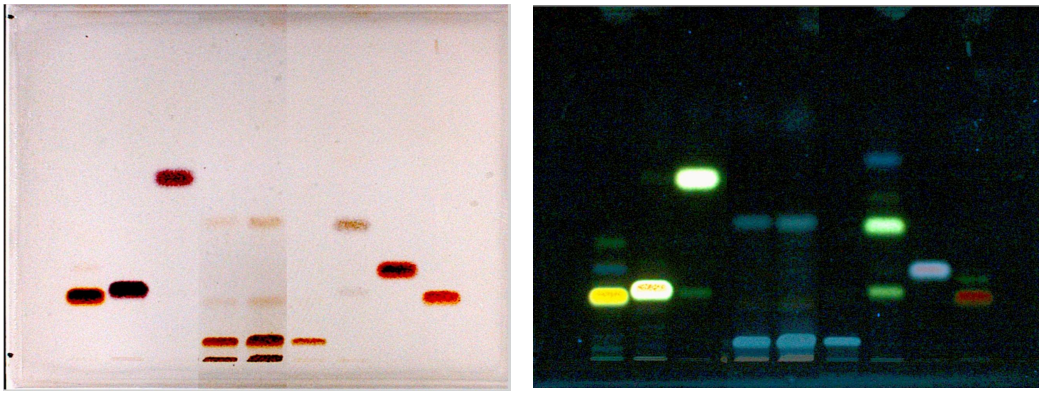
All LODs are highly below the level of pharmacological activity; so that if hormones are added for doping in a food supplement they can be easily identified by HPTLC.

Figure 1 illustrates the results obtained after chromatographic separation in HPTLC of standard solutions and food supplements.

The presence of stanozolol in the pattern of two samples separated in this plate is clearly evident, with both method of visualization.

The presence of a second hormone, androstenedione, could be hypothesised when HPTLC plate is visualised with visible light (Figure 1 left). This identification is not confirmed when the plate is exposed at 366 nm (Figure 1 right); at this wavelength the Rf of the spot in the sample correspond to androstenedione but the colour is different from the standard run in parallel.

A similar approach was used to screen the presence of active amines, and in particular of ephedrine and octopamine. Ephedrine is banned both in USA and Europe due to the severe adverse cardiovascular effects reported in the scientific literature [4-6]. Octopamine can be present in food supplements containing *Citrus aurantium* but its level is regulated.



N T TE FS_1 FS_2 S A D M N T TE FS_1 FS_2 S A D M

Fig. 1. *HPTLC of a PFS, revealed at visible light (left) and 366 nm (right)*

N = Nandrolone
T = Testosterone
TE = Testosterone enanthate
FS1/ FS2= Food Supplements
St = Stanozolol
A = Androstenedione
D = Dehydroepiandrostenedione
M = Metandrostenedione

For example in Italy, food supplements containing *C.aurantium* must contain with the daily dose $\leq$ 30 mg of sinephrine, while the sum of other amines (including octopamine) must be $\leq$ 1/8 (12.5%) of sinephrine. High amounts of octopamine are clearly associated to illicit addition.

4.2 LC-MS

In the second part of quality control of food supplements included in this study, LC-MS was use to confirm the positive results obtained by HPTLC. As an example, the LC-MS for the research of steroid hormones is here described in more detail.

The mass spectrometer operated in electrospray for positive ions (ESI+) using nitrogen as a sheath gas, auxiliary and sweep stream 15, 10 and 10 (arbitrary units) respectively. Other instrumental parameters were: vaporizer temperature 450 °C and collision energy (SID) 58%. The instrument was programmed to operate in MS^2 with helium as a collision gas. Table 3 illustrates the stanozolol parameters used in MS analysis by using the wide band activation.

Parameters used for the analysis of stanozolol in MS analysis Table 3

Compound	**Molecular Ion (M+)**	**Collision energy (%)**	**Transition m/z**
Stanozolol	329	58	121;229;203;147;189

Figure 2 shows the chromatograms of stanozolol in TIC (Total Ion Chromatography) and of the corresponding molecular ion m/z 329. Figures 3 illustrates MS spectrum of stanozolol (m/z 329); Figure 4 shows the corresponding MS^2 spectrum with two main ions m/z 311 and 329.

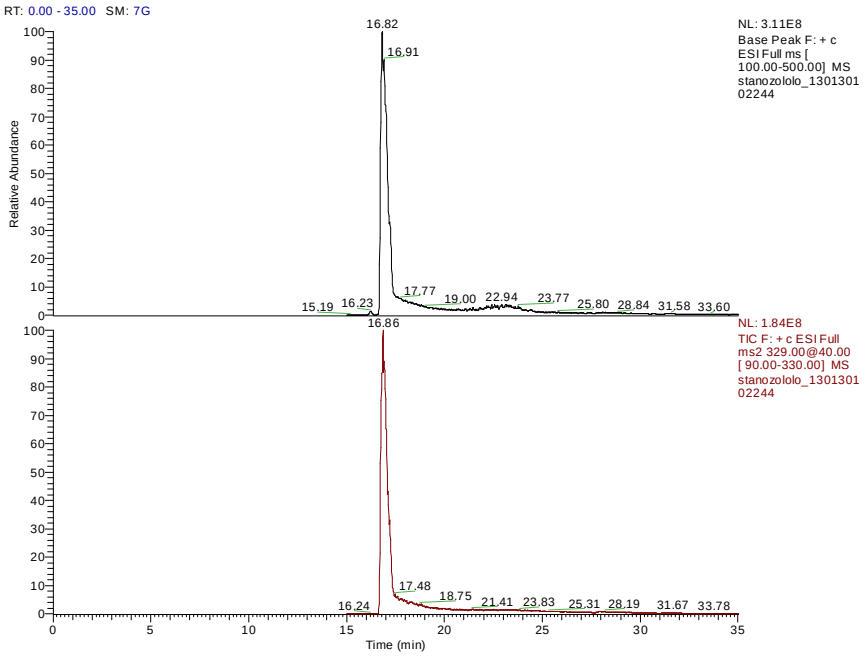


Fig. 2. *Total Ion Chromatography (TIC) of stanozolol (above) and the ion chromatogram of the ion m/z 329 $(M^{+}H)^{+}$ (below)*

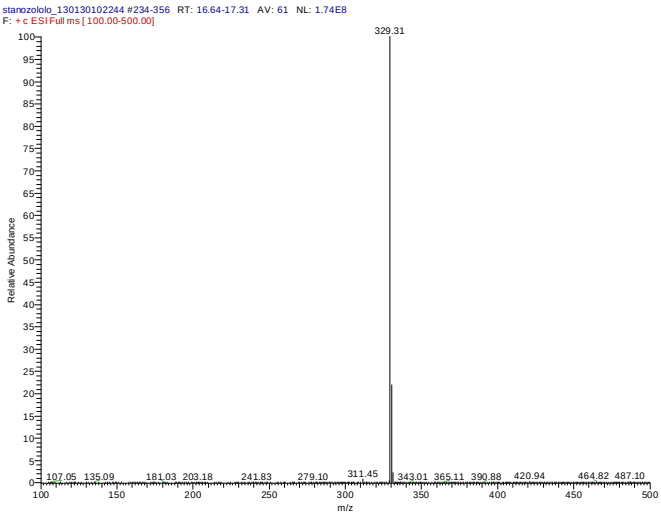


Fig. 3. *MS spectrum of the stanozolol. Ion at m/z 329 corresponds to $(M^{+}H)$*

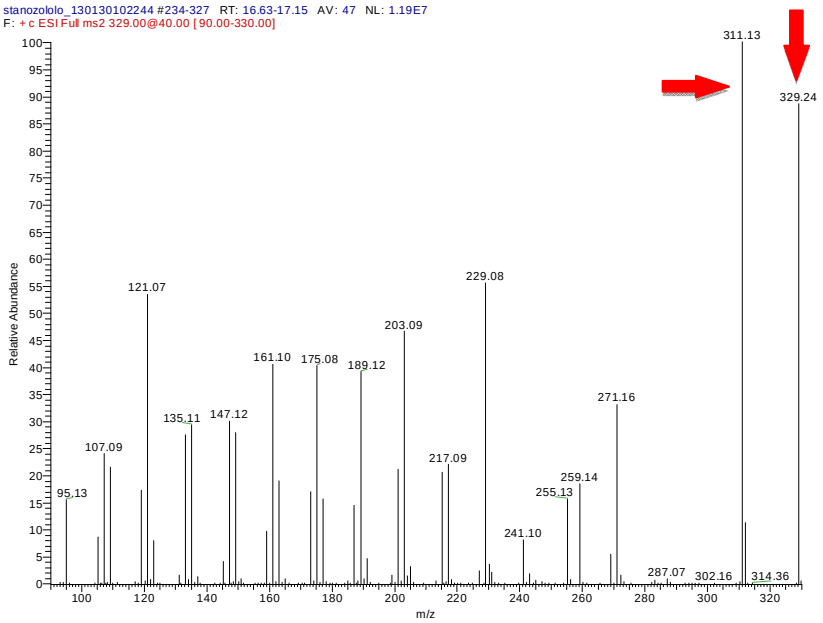


Fig. 4. *MS^2 spectrum of standard stanozolol.*
Ions at m/z 329 $(M^+H)^+$; m/z 311[$(M^+H)^+$ - H_2O]

Figures 5 show the chromatogram of one of positive food supplements in TIC and of the corresponding molecular ion m/z 329, respectively.

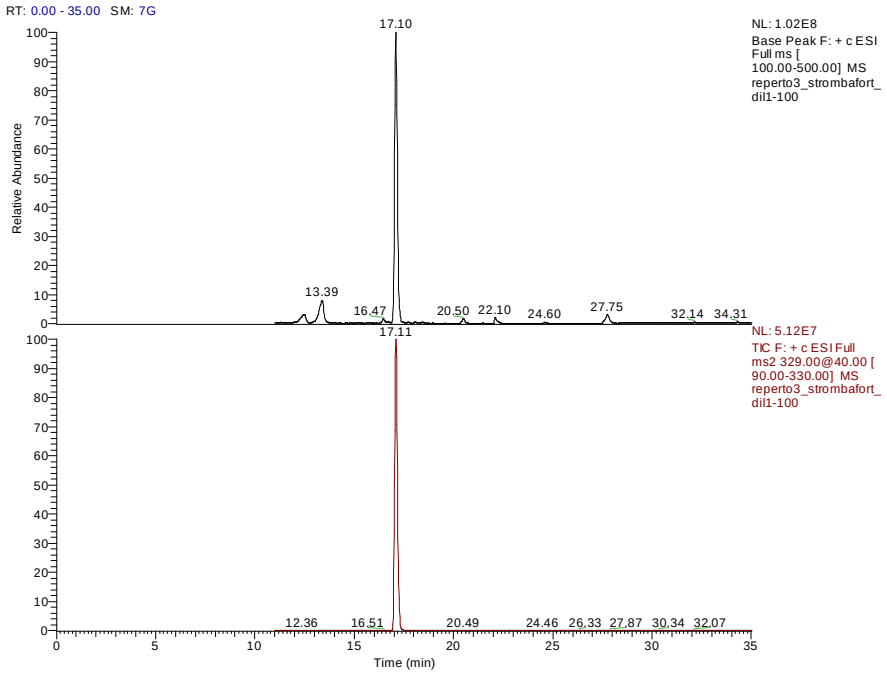


Fig 5. *Chromatogram of the food supplement in TIC (above) and of the corresponding molecular ion at m/z 329 (below)*

The peak retention time was 17.10 min, close to the retention time of the reference compound (16.82 min). Figures 6 and 7 show MS and MS^2 spectrum of the molecular ion m/z 329. Also in this case, ions at m/z 311 and 329 were the most abundant. The presence of stanozolol was then confirmed; on the contrary, as already supposed by HPTLC, androstenedione was not present in the two products.

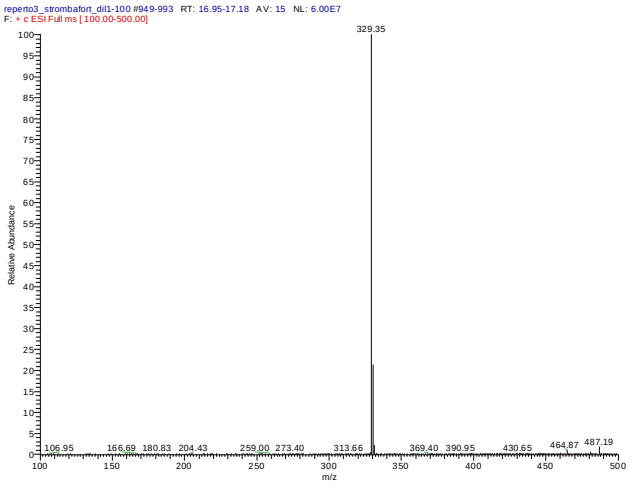


Fig. 6. *MS spectrum of the molecular ion at m/z 329 of stanozolol present in the PFS*

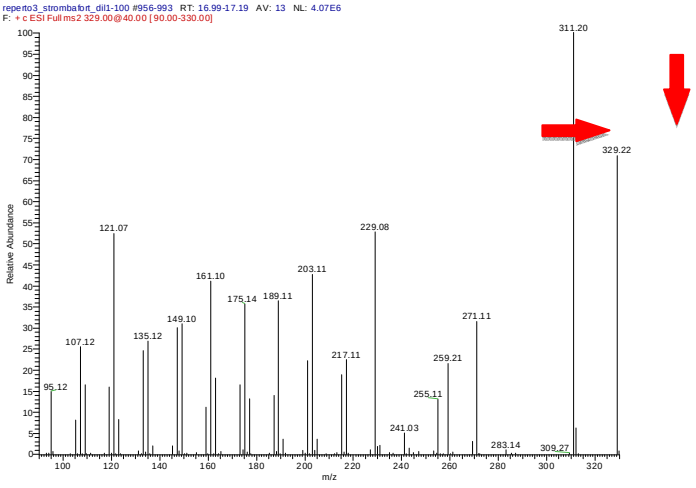


Fig. 7. *MS^2 spectrum of molecular ion at m/z 329 of stanozolol present in the PFS*

5. Conclusions

The quality control of food supplements is particularly critical for certain categories of food supplements, such as those products aimed to improve athletes' performances.

To optimise the consumers' protections, quality control should be carried out throughout the various steps of production, but this does not protect from illicit additions.

The possibility to use a fast screening of products present on the market or in parallel distributors (gyms, smartshops and internet) could allow a larger control and a more effective struggle against illicit activities.

In this context, HPLC represents a very useful and relatively inexpensive tool to identify irregular products.

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This paper does not necessarily reflect the Commissionôs views or future policy in these areas.

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