HPTLC and Analysis of Residues in Biological Material

Hubert F. De Brabander*, **Felix Smets**, and **Gaspard Pottie**

Presented at the Symposium "The Rôle of Planar Chromatography (TLC) in Pharmaceutical and Biomedical Sciences", Ghent, Belgium, October 10 – 11, 1990.

Key Words:

HPTLC
Residue analysis
Biological material

Summary

The past, present and future rôle of (HP)TLC in residue analysis in biological material is discussed from the standpoint of harmonization in the EEC. Quality criteria for the use of HPTLC in residue analysis are reviewed and compared with those of other chromatographic methods. The future of (HP)TLC in residue analysis will depend strongly upon the possibilities for automation and specific detection at low detection limits.

1 Introduction

A residue may be defined as a trace of a component which is present in a matrix after some kind of administration. The matrix may be anything in which a residue may be present, trapped, or concentrated: examples are urine, soil, fatty tissue, *etc.* This paper focuses on residue analysis of biological material from animal origin, with special emphasis on growth promotors. There is no general agreement among scientists on the level which separates trace (residue) analysis from "minor components" analysis. The detection limit which can be achieved continually shifts to lower values as the detectors of commercially available apparatus are improved. Levels of 10 mg/kg (ppm) and lower can, however, certainly be considered as trace amounts. Ultra-trace analysis can be considered as the sub-ppm or even sub-ppb level. Unfortunately, analytical error increases as the detection limit is reduced [1].

Residue analysis should not be confused with microanalysis in which the amount of sample is small but the concentration of the analyte itself is high. Microanalysis is very valuable in residue analysis but also requires adequate clean-up and sample preconcentration.

A procedure for residue analysis consists of three distinct steps. First the analyte has to be extracted from the matrix. Then the extract is freed from as much interfering material as possible. The third step is the identification of the analyte (and eventually its quantification). The demands on methods used for residue analysis are high: the methods must be as simple and as cheap as possible; the recovery of each step in the procedure must be high (in order that the limit of quantification might be as low as possible); there must be a high sample throughput; and the results must be reliable. This last criterion includes a requirement that interlaboratory reproducibility should be reasonably high.

Chromatographic techniques play an important rôle in modern trace analysis, especially in residue analysis. The second and third steps of a residue analysis procedure consist mainly of chromatographic procedures: (HP)TLC could be used for clean-up as well as for the identification of the residue. In the past two decades several reliable (HP)TLC methods for residue analysis of growth promotors in biological material have been published. In this paper the past, present and future rôle of (HP)TLC in residue analysis in biological material is discussed from the standpoint of harmonization in the EEC.

2 Quality Criteria for the Use of (HP)TLC in Residue Analysis

In the EEC, minimum quality criteria for the identification of residues with different analytical techniques have been published [2]. For the use of (HP)TLC the following quality criteria have been decided by the Commission:

(i) the R_f value of the analyte should agree with the R_f value characteristic of the standard material; this requirement is fulfilled when the R_f value of the analyte is within 3 % of the R_f value of the standard material, under the same conditions;

(ii) the visual appearance of the analyte should be indistinguishable from that of the standard material;

(iii) the centre of the spot nearest to that of the analyte should be separated from it by at least half the sum of the spot diameters;

H. F. De Brabander, Laboratory of Chemical Analysis of Food from Animal Origin, Faculty of Veterinary Science of the University of Ghent, Casinoplein 24, B-9000 Gent, Belgium, and F. Smets and G. Pottie, IHE, J. Wytsmansstraat 14, B-1050 Brussels, Belgium.

(iv) for identification, additional cochromatography in the (HP)TLC step is mandatory; as a result only the spot presumed to arise from the analyte should be intensified; a new spot should not appear, and the visual appearance should not change;

(v) for confirmation two-dimensional (HP)TLC is mandatory.

Quality criteria (i) and (iii) are the same for any chromatographic procedure: in GC and HPLC also the retention times of the two peaks formed by the analyte and the standard should correspond, otherwise there is no doubt that the analyte differs from the standard. Criterion (iii) requires a resolution of at least unity between the analyte spot and any other. (In GC and LC this is equivalent to baseline separation of two peaks.) This quality criterion is, however, not precisely defined in the EEC document. The exact text should be: "separated from *the centre of* it by at least", otherwise the text could be interpretated as "the border" of the spot of the analyte which is clearly different.

Criterion (ii) requires that the visual appearance of the spots from the analyte and the standard should be indistinguishable. In this aspect (HP)TLC may offer more specificity than GC and HPLC (without MS as specific detector) for a particular analysis (*e.g.* residues of anabolics). Not only the R_f value but also the reaction of the analyte with certain reagents, the color of the spot when illuminated with different

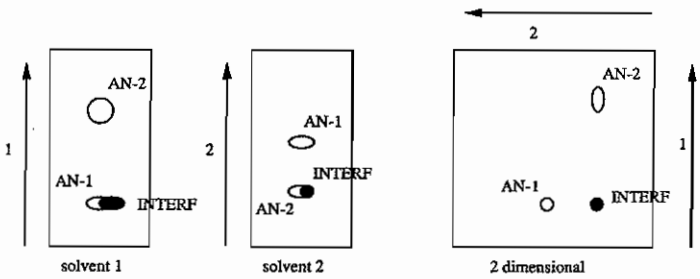

Figure 1

Comparison between 2D-(HP)TLC and two 1D-(HP)TLC separations: theoretical example.

sources (UV at λ = 254 and 366 nm, for example, and visible light) could be used. Moreover, (HP)TLC is not restricted to two R_f values only. The same sample could be chromatographed simultaneously on different stationary phases (*e.g.* silica 60, RP-18 and CN in the analysis of anabolics in injection sites [3]). The use of more than two R_f values could be considered as additional or specific quality criteria.

In criterion (v) 2D-(HP)TLC is mandatory for confirmation of the identity of an analyte. It should be emphasized that two dimensional thin layer chromatography is not the same as performing two TLC separations in different solvents. The resolving and identification power of 2D-(HP)TLC surpasses that of two 1D-(HP)TLC separations. This is illustrated with the theoretical example in **Figure 1.**

In this illustration it can be seen that an unknown product interferes with analyte 1 in the first development. In cases of bad luck, the same, or another, product will interfere with analyte 2 in the second development: using two monodimensional chromatograms neither of the two analytes will fulfil criterion (iii). By two-dimensional (HP)TLC both analytes are clearly separated from the interference.

Criterion (iv) states that cochromatography is necessary for proper identification of an analyte; this criterion is also mandatory for GC and HPLC (even for GC-MS). The application of cochromatography in (HP)TLC is, however, much easier than for the other chromatographic techniques because (HP)TLC is performed off-line.

In addition to these minimum quality criteria, specific criteria for specific groups of analytes should also be used. These will be discussed in the following sections.

3 Comparison of (HP) TLC with Other Methods Used in Routine Residue Analysis

Unfortunately, in a laboratory performing residue analysis scientific considerations are not always the overriding concern: the methods used must be affordable by the clients and, in many cases, the government. This comparison between (HP)TLC and other methods is, therefore, based on the practical use of the methods only. It is assumed that all the methods fulfil all the necessary quality criteria: the methods must be reliable and show a sufficient detection limit. The equipment used should also be in balance with the importance of the results: "it is not necessary to use a cannonshot to kill a fly."

R. Stephany [4, 5] published an interesting table which gives an indication of prices for the analysis of residues of anabolic agents and/or doping agents in the urine and meat of slaughtered animals. These prices should be in use by commercial laboratories in the Netherlands. The prices were converted from Dutch Guilders to a more international unit by using radioimmunoassay (RIA) units (the price of one RIA determination for one analyte being taken as unity. They are given in **Table 1.**

The prices given in Table 1 are applicable only in circumstances of analysis of a continuous series of samples.

Table 1

Indication of prices for routine residue analysis of anabolics (according to *R. Stephany* [4, 5])

Method	Analytes per sample	Price per: sample[a)]	analyte [%][a)]
RIA	1	1	100
(HP)TLC	10	6.3	63
GC-MS	100	8.8	8.8

a) RIA price was taken as a unit (1, or 100 %)

The idea of calculating the analysis cost by the price/analyte ratio is, of course, correct. Table 1 still, however, contains many unknowns. From the original table in *Stephany's* publications it could not be determined whether or not the price

included not only all the analysis steps, but also the necessary administration (which could take a lot of time), taxes, intra- and interlaboratory tests for quality control, some research for developing the methods adopted, *etc.* It is, moreover, our opinion that Table 1 is biased towards GC-MS.

In **Table 2** a recalculated scheme is given. There should, in our opinion, be a greater price difference between (HP)TLC and GC-MS. The difference in price between equipment for

Table 2

Indication of prices for residue analysis (reviewed)

Method	Analytes per sample	Price per: sample[a]	analyte [%][a]
RIA	1	1	100
(HP)TLC	20	8	40 %
GC-MS	30	12	40 %

a) RIA price was taken as a unit (1 or 100 %)

GC-MS and (HP)TLC is high and, moreover, prices and performances of GC-MS apparatus may vary widely: the price for an analysis with an Ion Trap 800 would not be the same as for more expensive apparatus. Also, the number of analytes which can be separated by (HP)TLC is larger than 10 (the figure mentioned by *Stephany* [4, 5]). For example, with HPLC clean-up and the "4 x 4" method, up to 20 anabolics may be screened simultaneously in urine by (HP)TLC [6, 7]. The simultaneous identification of 100 components of interest by GC-MS in one single run is imaginary: it is no problem to separate 100 and more components on a capillary column but they are rarely all components of interest. The real problem is finding the proper method for simultaneous clean-up for 100 analytes prior to GC-MS and separating those components in one run. We have, therefore, reduced this figure to 30, which is still high. The client is, moreover, not always interested in 100 components.

It is apparent from these figures that the price of GC-MS analysis is of the same magnitude as that for (HP)TLC. All depends on the exact number of analytes which has to be screened (the EEC, for example, stipulates only a limited number of anabolics). If that number is less than 30, GC-MS will be more expensive than (HP)TLC. It should also be emphasized that variations in the price of a single analysis are of minor importance if the number of analytes is very high.

4 Some Examples of the Use of (HP)TLC in Residue Analysis

Numerous (HP)TLC methods for the analysis of residues have been described. These examples are restricted to the analysis of growth-promotors in biological material (thyreostatic drugs, anabolics and beta-agonists).

4.1 Thyreostatic Drugs

Thyreostatic drugs inhibit the function of the thyroid gland. The use of these drugs in cattle results in a spectacular weight gain in a short time. This gain in weight consists, however, mainly of an increased filling of the gastro-intestinal tract and an augmented water retention in the carcasses; the quality of the meat is, moreover, reduced. Optimal detection of illegal treatment with thyreostatic drugs will be achieved through selection of the tissue or physiological fluid with the highest residue concentration. In most European countries the thyroid gland has been chosen as the target matrix. In our laboratory a specific (HP)TLC method for the determination of thiouracil and analogous compounds has been worked out [8, 9]. The method is based on fluorescence induction of the NBD-derivatives of the thyreostatic drugs with cysteine after (HP)TLC separation. By changing from the original to a more rapid and selective clean-up the quantitative determination of the thiouracils in various extracts of biological origin could also be achieved. These methods were adopted by the Benelux countries [10] and have been proposed by the EEC [11] for qualitative analysis of these drugs at the 50 ppb level.

4.2 Anabolic Agents

The use of estrogens, androgens, and gestagens as growth promotors in the fattening of animals is prohibited in all EEC member-states (EEC directives [12, 13]). In regulatory control at the farm, urine and/or faeces of the animals may be sampled. At the retail level or in cases of import/export, sampling is restricted to tissue only. At the slaughterhouse, tissue as well as excreta can be sampled.

After illicit administration the identity of the anabolics is not known. From the analysis of injection sites it is known that cocktails of different anabolics are used [14]. In order to protect the consumer, screening of the samples by a multiresidue method should be carried out. (HP)TLC is a valuable multiresidue method for the determination of residues of steroids. In our laboratory an (HP)TLC method for the analysis of anabolic agents at the 1 – 10 ppb level was developped by *R. Verbeke* [15]. The method is based on fluorescence induction by reaction of the steroids with sulfuric acid. As the original clean-up described [15] was too time-consuming for routine analysis, modifications of *Verbeke's* method have since been described [16 – 18].

These procedures could also be combined with an additional clean-up by HPLC with column switching, as described by *F. Smets* [6, 19]. By fraction collection very "clean" extracts are obtained. The reduction of matrix components considerably improves the interpretation of the two-dimensional (HP)TLC analysis. The use of HPLC also increases the cost of analysis, however. In Belgium the use of HPLC with column switching prior to (HP)TLC is mandatory for regulatory analysis, in order to fulfil an extra quality criterion.

4.3 Beta-agonists

A new line of drugs, the beta-agonists, has recently found illegal application in animal breeding. The compounds are similar in structure to epinephrine and nor-epinephrine and at doses higher than those applied therapeutically have been found to produce extra weight gain in the animals together

with weight redistribution from fatty to muscular tissue. For this reason the compounds are also known as repartitioning agents.

An (HP)TLC method for the determination of clenbuterol and cimaterol has been described by *D. Courtheyn* [20, 21]. Urine (18 ml) or an extract of meat was made alkaline (pH 12) and extracted through a Chem Elut column with a mixture of toluene and dichloromethane; the beta-agonists are re-extracted from the organic phase with a minimum amount of acid (100 µl). This concentrate was then analyzed by 2D-(HP)TLC on silica gel 60 and the beta-agonists revealed by spraying with a modified Ehrlich's reagent. The detection limit is *ca.* 1 ng, corresponding to a detection limit in urine of 1 ppb. This method was ring tested in Belgium, together with other methods (RIA, ELISA, HPLC, GC-MS, MS-MS), with excellent results.

5 Wishes and Dreams for the Future of (HP)TLC

The future of (HP)TLC in residue analysis will be strongly dependent on two factors: firstly, (HP)TLC has to be automated, and secondly, the detection limit and specificity of the method has to be improved in order to enhance qualitative accuracy and precision.

5.1 Automation of (HP)TLC

Thin layer chromatography is essentially an off-line technique and so not easily automated. The use of two-dimensional development, which is a necessity in residue analysis, complicates automation. This disadvantage could, however, be compensated by carrying out several chromatographic runs at the same time.

In comparison with the other chromatographic techniques, automation in (HP)TLC is poor. Most of the manipulations are still made by hand, requiring high personnel costs. Sample application is a very time-consuming step in residue (HP)TLC (up to one third of the total analysis time). Using the "4x4" application mode [7] and the semiautomated "4x4" spotter [22] four samples can be simultaneously applied to an (HP)TLC plate in *ca.* 7 minutes. This apparatus has been in use in our laboratory for two years and saves a lot of time. Recently, automatic spotters have appeared on the market. The Camag Automatic TLC sampler III, is, for example, capable of the unattended application of 16 samples to any point on four (HP)TLC plates. For really automated application, this is still not sufficient. We can only dream of a (HP)TLC spotter capable of applying 200 samples (and 600 standard mixtures) to any point on 50 (HP)TLC plates. Building such an apparatus is technically possible: it would include a bigger sample rack and an automated (HP)TLC plate loader.

The development and visualization of (HP)TLC plates could and should be automated. By using a laboratory robot, automatic development with different solvent pairs, drying, spraying or dipping could become less laborious. The boring and time-consuming tasks of the analyst could be replaced by insertion of samples vials in the sample rack, filling of solvent bottles, and keying commands into the computer which controls the whole event. Not only would more time become available for the study of new techniques, and for quality control but the automation of the procedures would also enhance the reproducibility and possibilities of quality control.

5.2 Enhancing Specificity in (HP)TLC

In 2D-(HP)TLC the identity of an unknown analyte is determined on the basis of two R_f values corresponding within 3% (according to the minimum quality levels). In addition, the specific reaction of the analyte with a reagent (*e.g.* sulfuric acid with anabolic steroids with the formation of typical colors (illumination at, *e.g.*, λ = 366 nm and with visible light) gives an extra confirmation. There is, however, still the possibility that an unknown product (an interference) will survive the clean-up, and show the same R_f value and produce the same specific reaction as the analyte. (In such an eventuality the probability is high that the interfering compound has a structure very similar to that of the analyte).

5.2.1 Nortestosterone

An example of the limitations of (HP)TLC specificity is provided by the analysis for residues of nortestosterone (NorT) in urine. NorT is a very widely used anabolic agent which differs from the natural male hormone, testosterone (T) only by the absence of a methylgroup in the 19 position. Originally, the illegal treatment of cattle with NorT derivatives was controlled by searching for residues of the "parent" product (*beta*-NorT) only. Later it was found [23] that *beta*-NorT was metabolized in cattle to *alpha*-NorT and estranediol and that in treated cattle *alpha*-NorT should be the "target" metabolite in urine. As soon as standards of these metabolites became available, control was focused on the presence of *alpha*-NorT in urine. The problem was, however, complicated by two discoveries: firstly, it was found that nortestosterone was a naturally occurring steroid in some animal species, such as the stallion [24] and the boar [25, 26], and, secondly, it became apparent during tests on veal calves [27] that a metabolite of testosterone, *alpha*-testosterone, was interfering with *alpha*-nortestosterone analysis performed using (HP)TLC with the classical clean-up as described by *Verbeke* [15]. The specificity of this clean-up was not sufficient for the control of these particular drugs: especially with veal calves treated with natural hormones (*e.g.* testosterone – in the EEC the administration of T is also inhibited) there was a high risk of false positive results for nortestosterone.

The specificity of the analysis could be enhanced in three ways:

(i) initial screening of the urine samples with an RIA method (highly selective RIA for nortestosterone) this procedure was used by the Institute of Veterinary Control for the screening of bovine urine in slaughterhouses;

(ii) using HPLC as clean-up prior to (HP)TLC; *alpha*-NorT and *alpha*-testosterone elute in different fractions and interference between the two anabolics is excluded; this

clean-up was described by the National Reference Laboratory, the IHE [6], and is mandatory in Belgium for analysis of urine for anabolics;

(iii) using MS as additional confirmation; positive (HP)TLC results (after HPLC clean-up) for most anabolics could be confirmed by GC-MS.

The identity of the analyte follows from two R_f values, a characteristic reaction, a retention time and a mass spectrum. A combination of these three strategies is in use for routine inspection in Belgium.

5.2.2 The Future of (HP)TLC in Residue Analysis

From the example given above it should be clear that the future of (HP)TLC in residue analysis is highly dependent upon the possibility of enhancing the specificity. The enhancement of the detection limit is also important: if lower amounts of analyte could be detected the amount of matrix used for analysis could be reduced.

Detection limits in fluorescence (now *ca.* 1 ng) could be enhanced by using strong excitation sources of the correct excitation wavelength. For visible inspection of (HP)TLC plates there is a need for strong (trans)illumination sources of variable wavelength. The coupling of (HP)TLC to spectrometric methods (UV-visible and fluorescence TLC scanners) is commercially available. Taking of spectra of the suspect spots and comparing them with standard spectra could also enhance specificity. UV-VIS spectra are, however, more valuable for quantitative than for qualitative analysis. The coupling of (HP)TLC to FT-IR could contribute considerably to the specificity since IR spectra could produce a characteristic fingerprint of a molecule. However FT-IR usually needs more analyte than is available on a thin layer plate.

5.2.3 (HP)TLC-MS

A real step forward will be the direct coupling of (HP)TLC with MS (or (MS-MS). Several systems for attaining this goal have been described. Off-line mass spectra of TLC spots has been described by several authors [28] using FAB (Fast Atom Bombardment) probes. A FAB probe for the automatic scanning of a small TLC plate (10 mm x 65 mm) is commercially available (Jeol). Unfortunately the mass spectrometer involved is so expensive that the probe is only of theoretical value for routine residue analysis; there is, moreover, little information on the sensitivity of the probe.

SIMS (Secondary Ion Mass Spectrometry) with a home-designed apparatus is described by *Busch et al.* [29, 30]. With this technique a two-dimensional mass map of a (HP)TLC plate could be produced. Unfortunately the apparatus is not commercially available and there is also little information upon the detection limits of this technique.

In our laboratory an Ion Trap detector (ITD 800) has been coupled to (HP)TLC. The goal of this research was the use of a relatively inexpensive apparatus for taking a full spectrum of a particular (suspect) spot on a (HP)TLC plate in order that highly specific information of the component(s) present in the spot could be obtained. The interface built in our laboratory is shown in **Figure 2.**

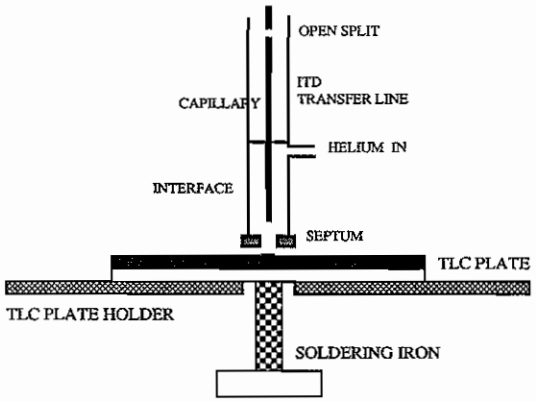

Figure 2

Schematic diagram of (HP)TLC interface as constructed in our laboratory.

The ITD was interfaced with the open air by means of an helium curtain. By positioning the interface close up the (HP)TLC plate a small vaporization chamber containing the spot of interest was formed. By heating the (HP)TLC plate at the back of the spot (*e.g.* with a specially adapted soldering iron) the spot is vaporized and transferred into the ITD. By programming the heating procedure and using a small column (1.5 m) in the long transfer line of the ITD, a separation of different products present at the place of the spot is possible. In this case the transfer line acts as a small GC. In **Figure 3** is shown an "evaporatogram" of naphthalene, (the substance used to test the ITD) from an (HP)TLC plate.

As can be seen, 10 ng of naphthalene gives a high response on the ITD and a full spectrum could be recorded.

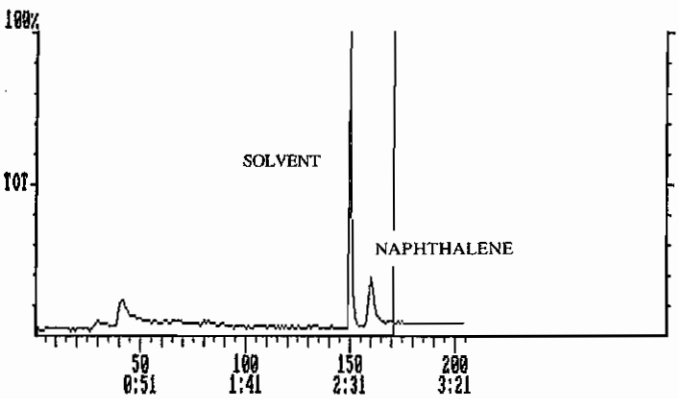

Figure 3

Evaporatogram of naphthalene (10 ng) from an (HP)TLC plate

6 Conclusion

In the years 1975 – 1985 (HP)TLC was the method of choice for the analysis of residues of veterinary drugs in food from animal origin. The main reason for the choice of this technique was the relatively low detection limits which could be reached by the use of fluorescence induction (*ca.* 1 ng). The relatively low price of (HP)TLC in comparison with GC-MS was also important. Nowadays, GC-MS is more and more af-

fordable. As the demands on specificity increase, the competition of (HP)TLC with these hyphenated techniques is also increasing. The future of (HP)TLC in residue analysis will, therefore, be highly dependent upon the possibilities both of automation and of specific detection at low detection limits.

References

[1] *W. Horwitz, L. R. Kamps,* and *K. W. Boyer,* J.A.O.A.C. **63** (1980) 1344 – 54.

[2] EEC directive 89/610 (1989) Nr L 351/39.

[3] *F. Smets, H. F. De Brabander, P. J. Bloom,* and *G. Pottie,* J. Planar Chromatogr, in preparation.

[4] *R. W. Stephany,* Belg. J. Food Chem. Biotechn. **44** (1989) 4, 139 – 153.

[5] *R. W. Stephany,* J. Chromatogr. **489** (1989) 3 – 9.

[6] *F. Smets* and *G. Vandewalle,* Z. Lebensm.-Unters. Forsch. **178** (1984) 38 – 45.

[7] *H. F. De Brabander, F. Smets,* and *G. Pottie,* J. Planar Chromatogr. 1 (1988) 33 – 38.

[8] *H. F. De Brabander* and *R. Verbeke,* J. Chromatogr. **108** (1975) 141 – 151.

[9] *H. F. De Brabander* and *R. Verbeke,* Proc 30th Europ. Meeting Meat Res. Workers, Bristol, UK (1984) 387 – 388.

[10] *R. Verbeke* and *H. F. De Brabander,* Doc. Benelux Econ. Unie SP/LAB/h 75-1.

[11] *R. Verbeke* and *H. F. De Brabander,* Commission of the European Communities (1984) Doc 3186/VI/84 File 6.21 II-4.

[12] EEC Directive 81/602 (1981) Nr L222/32.

[13] EEC Directive 86/469 (1985) Nr L275/36.

[14] *H. F. De Brabander, F. Smets,* and *G. Pottie,* unpublished results.

[15] *R. Verbeke,* J. Chromatogr. **177** (1979) 69 – 84.

[16] *H. F. De Brabander, P. Vanhee, S. Vanhoye,* and *R. Verbeke,* J. Planar Chromatogr. **2** (1989) 33 – 38.

[17] *L. Van Look, Ph. Deschuytere,* and *C. Van Peteghem,* J. Chromatogr. **489** (1989) 213 – 18.

[18] *H. F. De Brabander* and *J. Van Hoof,* J. Planar Chromatogr. **3** (1989) 237 – 41.

[19] *F. Smets,* Doc Benelux Econ Unie SP/LAB/h (88) 33.

[20] *D. Courtheyn, R. Schilt,* and *A. Winne,* Doc. Benelux Econ Unie SP/LAB/h (88) 41.

[21] *D. Courtheyn, I. Boonen,* and *V. Bakeroot,* Doc. Benelux Econ Unie SP/LAB/h (88) 42.

[22] *H. F. De Brabander, G. Rasschaert,* and *W. Vanneste,* J. Planar Chromatogr. **2** (1989) 484 – 485.

[23] *L. M. G. Tuinstra, P. G. M. Kienhuis, R. J. Van Mazijk,* and *W. A. Traag,* Proc. IV Int. Symp. Vet. Lab. Diagnosticans, Amsterdam (1986) 765 – 767.

[24] *E. Houghton, J. Copsy, M. C. Damasia, P. E. Haywood, P. E. Moss,* and *P. Taele,* Biomed. Mass Spectrom. **11** (1984) 96 – 99.

[25] *G. Debruykere, C. Van Peteghem, H. F. De Brabander,* and *M. Debackere,* Vet. Quaterly **12** (1990) 246 – 51.

[26] *G. Maghuin-Rogister, A. Bosseloire, P. Gaspar, P. Dasnois,* and *G. Pelzer,* Ann. Med. Vet. **132** (1988) 437 – 40.

[27] *M. Debackere* and *H. F. De Brabander,* unpublished results.

[28] *T. T. Chang, J. O. Lay,* and *R. J. Francel,* Anal. Chem. **56** (1984) 111 – 113.

[29] *K. L. Busch,* Trends Anal. Chem. **6** (1987) 95 – 100.

[30] *M. S. Stanley* and *K. L. Busch,* Anal. Chem. Acta **194** (1987) 199 – 209.

MS received: December 12, 1990
Accepted by SN: December 17, 1990