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Review

Immunoaffinity chromatography, its applicability and limitations in multi-residue analysis of anabolizing and doping agents

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ABSTRACT

The use of (multi-)immunoaffinity chromatography in residue analysis is discussed. After an introduction to the immunochemical background an overview of applications is given. A distinction is made between the following methods: (1) single-antibody, single-analyte procedures; (2) single-antibody, multi-analyte procedures; (3) multi-antibody, multi-analyte procedures. It is concluded that immunoaffinity chromatography is superior to most other techniques for sample preparation and extract clean-up. Its advantages in multi-residue procedures are most clear when compared with *e.g.* high-performance liquid chromatography. In combination with gas chromatography–low-resolution mass spectrometry, very effective multi-residue methods are possible. Most frequently they concern screening procedures which can fulfill the identification criteria for reference methods. It is concluded that the use of (multi-)immunoaffinity chromatography will proliferate further in the 1990s. However, its future viability is highly dependent on the interest of commercial firms and on the involvement of the EC Community Bureau of Reference in manufacturing and supplying the necessary materials.

CONTENTS

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1. INTRODUCTION

During the 1980s most of the analytical chemists involved in residue analysis were mainly concerned with the detection and identification aspects of analytical procedures. This state of affairs is explained by the fact that one of the major problems with residue analysis is the possibility of obtaining false-positive results [1]. Analytical strategies were developed, frequently based on screening with a procedure optimized to prevent false-negative results, followed by confirmation with a procedure optimized to prevent false-positive results. EC-guidelines now exist for both screening and confirming and they are mainly concerned with the identification criteria and general analytical quality control [2,3].

Notwithstanding this interest in identification, which was mainly based on the use of mass spectrometry but also included other spectrometric methods, other developments took place. Two of the most important ones are the introduction of solid-phase extraction (SPE) [4,5] and the further use of immunochemical procedures [6]. The main advantage of SPE is the fact that it is very easy to use in *e.g.* routine analysis. Immunochemical procedures are characterized by their specificity, sensitivity and by their ability to analyse a large number of samples within a single assay. However, the possibility of obtaining false-positive results limits their applicability to screening. In (clinical) biochemistry the use of antibodies in chromatography (immunoaffinity chromatography, IAC) has been known of since approximately 1960. Most applications have been in the field of protein chemistry, however, some analytical applications have been published for steroids [7,8]. During the second part of the 1980s some applications were also published in the field of residue analysis for anabolizing agents and veterinary drugs [9–15].

The aim of this contribution is to evaluate IAC as a technique for sample preparation (extract clean-up) within residue analysis. After giving an introduction to the immunochemical background of IAC, several types of methods will be discussed ranging from single-analyte to multi-residue, and from non-automated to automated procedures. As far as possible comparisons will be made between IAC procedures and alternative methods in order to give a clear view of the differences/advantages between the various approaches. The possible consequences of the use of IAC, combined with *e.g.* gas chromatography–mass spectrometry (GC–MS) detection and identification, with respect to analytical strategies, will be discussed.

2. IMMUNOCHEMICAL BACKGROUND

The use of immunological techniques in analytical chemistry started in the late 1950s with the work of Yalow and Berson [16] and Ekins [17] who worked on the quantification of low hormone concentrations in human serum. Since antibodies are used as an analytical tool in an *in vitro* system in these techniques the term immunochemical technique is perhaps more appropriate.

In present day terminology, an antibody is a member of the family of glycosylated proteins called immunoglobulins. However it was not before 1903 that scientists understood that "antibodies" could represent both phagocytic cells (cellular immunology) and individual molecules (immunochemistry).

The unique characteristic of antibodies is their ability to combine with an antigen. Simply explained, an antigen is merely the substance to which the antibody binds [29]. As shown *e.g.* by Landsteiner and Van der Scheer [18,19], antibodies can bind and have specificity for relatively small chemical groupings (haptens). In proteins such an haptenic site is typically three or four amino acids in size. The antibody–antigen combining reaction is called the primary interaction. Because antibodies are at least bivalent, their combination with multivalent antigens often leads to aggregates or clusters. The interactions which play a role in these reactions are called secondary interactions. However, since most veterinary drugs and anabolizing agents are relatively small molecules, they usually contain only one hapten, preventing the formation of aggregates by secondary interactions.

2.1. Primary antibody–hapten reactions

The antibody–hapten reaction can be described by the law of mass action for bimolecular reactions according to

$$P + Q \rightleftharpoons PQ$$

so that

$$K_a = \frac{[PQ]}{[P]\ [Q]} \tag{1}$$

in which [P] = equilibrium concentration of free ligand; [Q] = equilibrium concentration of free antibody binding sites; and [PQ] = equilibrium concentration of bound ligand.
Eqn. 1 can be rewritten as

$$K_a = \frac{B}{(p-B)\ (q-B)} \tag{2}$$

in which B = [PQ] = equilibrium concentration of bound ligand; p = total (bound + free) concentration of ligand; q = total concentration of binding sites. Since $(p-B)$ is the equilibrium concentration of free ligand, we can define a response variable:

$$R \equiv \frac{B}{F} \tag{3}$$

Substituting eqn. 3 into eqn. 2 results in

$$R = K_a q - K_a B \tag{4}$$

Eqn. 4 was first derived by Scatchard [20] and shows a linear relationship between R and B from which K_a and q can be calculated. Fig. 1 shows a Scatchard plot for testosterone as determined with a radioimmunochemical (RIA) procedure [21]. The resulting curve did not deviate significantly from linearity, the affinity constant $K_a = 5 \cdot 10^{10}$ and the binding capacity was 12 to 13 pg per incubation tube. A computer program developed by Rodbard and Lewald [22] was used for making calculations. The Scatchard model described above only holds for a single species of antibody combining sites. In some cases the resulting curve deviates significantly from linearity. In these cases a two-parameter model (K_a and q) is not able to describe the system properly.

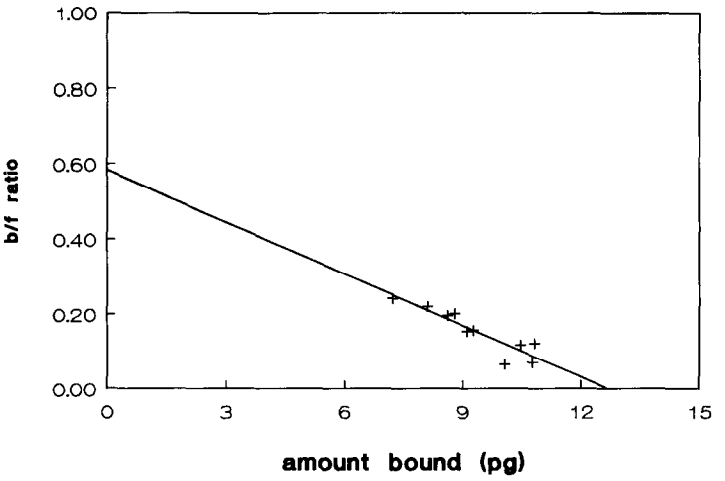

Fig. 1. Scatchard analysis (two-parameter model) for testosterone (RIA).

A more complicated Scatchard model is the four-parameter model. Here a second population of antibody combining sites is postulated with K_{a2} and q_2. These higher order Scatchard models were analysed by Feldman and Rodbard [23]. The four-parameter Scatchard model can be described with

$$R = K_{a1}\,(q_1 - [PQ_1]) + K_{a2}\,(q_2 - [PQ_2]) \tag{5}$$

The solution is a quadratic function of B with a hyperbole as the solution. In practice this model is seldom observed in the residue analysis of small molecules. However, an intermediate three-parameter model is sometimes observed. In this model the second population behaves like a population with $K_{a2} = 0$ but with an infinite binding capacity ($q_2 = \infty$). In a Scatchard plot this is observed as a horizontal asymptote.

2.2. Affinity constants in chromatography

The basic relation in chromatography which describes the retention of an analyte in a chromatographic system is [24]:

$$V_e = V_0 + V_0 \cdot K_D \tag{6}$$

in which V_e and V_0 are the elution volume of the substance and the void volume of the column respectively and K_D is the distribution coefficient between the chromatographic matrix and the analyte

$$K_D = \frac{\text{matrix-bound analyte}}{\text{free analyte}} = \frac{B}{F} = R \tag{7}$$

Eqn. 7 combines immunochemistry with chromatography under equilibrium. This combination can only be made in this way if we assume the fact that an antibody bound to a solid matrix does not influence the affinity of the analyte when compared to the affinity in solution (RIA incubation tube).

Combining eqn. 7 with eqn. 4 gives

$$V_e = V_0 + V_0\,(K_a q - K_a B) \tag{8}$$

$$\frac{V_e}{V_0} = 1 + k_a\,(q - B) \tag{9}$$

From eqn. 9 a set of basic requirements for the chromatographic system can be derived. (1) If the amount bound approaches the capacity of the system, retention

decreases, resulting in no retention: $V_e/V_0 = 1$, if $q = B$. (2) For high affinity systems, in which $B = p$ (all the analyte is bound) eqn. 9 can be simplified.

$$\frac{V_e}{V_0} = 1 + K_a (q-p) \tag{10}$$

From eqn. 10 it can be concluded that for a separation to be effective ($V_e/V_0 > 3$) $k_a(q-p)$ has to be 2 or higher. Table 1 shows the resulting V_e/V_0 for a column with a capacity of 20 ng testosterone and a volume of 1 ml ($q = 69.4$ nmol/l).

From Table 1 it can be concluded that as long as the amount applied to the column does not exceed the total capacity, retention is highly effective. The approximation $B = p$ is not of significant influence. For $p = 19$ ng ($6.597 \cdot 10^{-8}$ mol/l) the value of $B = 6.585 \cdot 10^{-8}$ mol/l, corresponding to 99.8% of p.

TABLE 1

RETENTION OF AN IAC COLUMN

$k_a = 5 \cdot 10^{10}$ l/mol, $q = 6.94 \cdot 10^{-8}$ mol/l.

p (ng)	$q-p$ (mol/l)	V_e/V_0
1	$6.59 \cdot 10^{-8}$	32987
2	$6.25 \cdot 10^{-8}$	31250
5	$5.21 \cdot 10^{-8}$	26042
10	$3.47 \cdot 10^{-8}$	17361
15	$1.74 \cdot 10^{-8}$	8681
18	$0.69 \cdot 10^{-8}$	3450
19	$0.35 \cdot 10^{-8}$	1736
19.9	$0.035 \cdot 10^{-8}$	174
19.99	$0.003 \cdot 10^{-8}$	17

The efficiency of IAC becomes even more apparent when we compare immunoaffinity with affinities corresponding to other interactions frequently used in chromatography, *i.e.* electrostatic interactions or hydrophobic interactions.

In Table 2 values for V_e/V_0 are given for different values of k_a at $p = q/2$ for $q = 10^{-4}$ and $q = 10^{-8}$, respectively. From Table 2 it can be concluded that most types of chromatography are not possible at $q = 10^{-8}$ mol/l, the capacity typically used in IAC. The lower affinity constant for other interactions has to be compensated for by increasing the capacity; this is also the case if only a relatively small amount of analyte has to be retained. In the case of residue analysis this increased capacity is not necessary since, in general, only nanogram amounts have to be isolated. However, another important characteristic of an interaction

TABLE 2

VALUES FOR V_e/V_0 AT HALF MAXIMUM CAPACITY ($p = q/2$) AS A FUNCTION OF k_a FOR $q = 10^{-4}$ AND $q = 10^{-8}$ mol/l

The values between parentheses are given for $q = 0.5 \cdot 10^{-8}$.

k_a (l/mol)	V_e/V_0	
	$q = 10^{-4}$	$q = 10^{-8}$
10^4	1.5 (2)	1
10^5	6 (11)	1
10^6	51 (101)	1
10^7	501 (1001)	1
10^8	$5 \cdot 10^3$ (10^4)	1.5
10^9	$5 \cdot 10^4$ (10^5)	6
10^{10}	$5 \cdot 10^5$ (10^6)	51
10^{11}	$5 \cdot 10^6$ (10^7)	501
10^{12}	$5 \cdot 10^7$ (10^8)	5.10^3

is the specificity. Immunochemical interactions are highly specific when compared to other interactions used in chromatography. A high capacity of non-IAC columns is therefore needed in order to obtain sufficient retention of the analyte and to prevent losses due to competition. From an analytical point of view this is not advantageous since under these conditions more cross-reacting compounds will be retained.

2.3. Binding capacity from an experimental point of view

The above calculations were based on the results of a Scatchard analysis for testosterone. The data used were obtained in a RIA, a homogeneous incubation of antibody and analyte. The value of q which was obtained was much lower than the value used for calculating the retention on an IAC column with a capacity of 20 ng, since incubation vials in RIA usually have a binding capacity in the picogram range. In order to prepare IAC columns we must have an alternative way of controlling the capacity. Based on the results of a Scatchard analysis and an established need for capacity, *e.g.* 20 ng analyte per ml IAC material we can calculate the amount of antiserum equivalent which has to be coupled to 1 ml of gel. Earlier we reported on the preparation of IAC materials for nortestosterone (NT) and methyltestosterone (MT) [11]. Both antisera used had a capacity of 200 ng/ml of serum. The immunoglobulin G (IgG) fraction (see under Experimental) resulted in 15 mg of IgG from 1 ml of serum. With an estimated efficiency of 75% this was coupled to the activated matrix resulting in an expected capacity of 100 ng. The material for MT did indeed show this capacity, the capacity of the NT

material was 35 ng/ml of gel. Under the assumptions made during the capacity calculations and bearing in mind experimental uncertainties, both results could be regarded as being satisfactory, indicating the validity of eqn. 7.

2.4. Elution from an IAC matrix

In order to elute analytes from an IAC matrix we must dramatically change the conditions within the column. To elute 10 ng of analyte from a 1-ml column with a capacity of 20 ng we would need an elution volume of approximately 25 l. To elute this column with 5 ml of eluent we would need to change the affinity constant from $5 \cdot 10^{11}$ l/mol to approximately 10^8 l/mol. This type of elution can be classified as non-specific and can be achieved by *e.g.*:

— changing the pH
— changing the ionic strength
— changing the temperature
— changing the polarity (*e.g.* addition of methanol and ethanol)
— adding denaturating eluents such as urea, guanidine, detergents or chaotropic ions.

The elution conditions must be chosen in such a way that the adequate reduction of k_a is a reversible process so that a prolonged use of the columns is possible. It is common experience that IAC matrices are very stable as long as the conditions are not too extreme. The elution with water–ethanol has proved to be very useful resulting in an adequate elution and an instant reactivation of the IAC matrix after re-equilibration with water. Table 3 shows the experimentally determined values of V_0/V_e for NT by increasing the fraction of ethanol in the aqueous eluent. The affinity constant is calculated, based on these values.

TABLE 3

ELUTION OF NT BY MIXTURES OF ETHANOL AND WATER, VALUES FOR V_0/V_e AND CALCULATED AFFINITY CONSTANTS k_a AT $p = 0.5q$

Ethanol (%)	V_0/V_e	k_a (l/mol)
0	$\geqslant 20$	$0.5 \cdot 10^{10}$
10	>20	$5 \cdot 10^8$
16	12	$3 \cdot 10^8$
20	8	$2 \cdot 10^8$
24	3	$0.6 \cdot 10^8$
32	1	0

3. EXPERIMENTAL

The first requirement for preparing IAC matrices is an antiserum. The synthesis of immunogens and the subsequent immunization of *e.g.* rabbits, however, is not exclusively related to IAC but is an essential part of a variety of immunochemical techniques. Once a suitable antiserum is available a number of steps are necessary: (1) isolating the IgG-fraction; (2) estimating the protein concentration in this fraction; (3) coupling the IgG to an activated matrix; (4) characterizing the matrix.

(1) The two procedures most frequently used for the isolation of IgG are precipitation with ammonium sulphate and affinity chromatography with coupled protein A-Sepharose®. Most authors did not purify the IgG-fraction. However, in principle it is possible to further purify the IgG with *e.g.* IAC in which the analyte, against which the antibodies were raised, is coupled to the matrix. However, in view of the capacities which are obtained when the crude IgG fraction is used for coupling, further purification is not necessary for analytical applications.

(2) Estimating the protein concentration of the IgG fraction is not strictly necessary but it is used in order to estimate coupling efficiency, and from the point of view of quality assurance (QA). An alternative method which is possible if (radio)labelled materials are available, is to construct antibody dilution curves of the IgG fraction before and after coupling to the matrix (Fig. 2). When both the coupling efficiency for total IgG and the specific IgG are calculated and the binding capacity and affinity of the antibody are known, the percentage specific IgG can be calculated. Most antisera raised in our laboratory and used for RIA and IAC contain 1–2% specific IgG. This illustrates the possibility of increasing the capacity per ml column material by a factor of 50 to 100 and explains the higher capacity obtained by Van de Water and Haagsma [9] who used a mono-

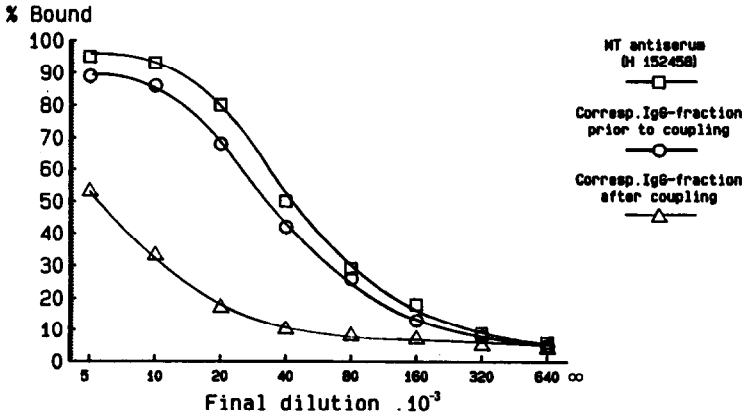

Fig. 2. Antibody dilution curves of the original antiserum and the IgG fraction prior to and after coupling to the activated matrix.

clonal antibody for the IAC of chloramphenicol. Monoclonal antibodies only contain one type of IgG and therefore the percentage specific IgG within the IgG fraction is 100.

(3) For many years cyanogen bromide (CNBr)-activated soft agarose gels were used, but also matrix material based on cellulose and polyacrylamide have been used. The disadvantage of using CNBr-activated matrices was the limited stability of protein binding. During the last few years a variety of activated matrices has become available, *e.g.* Tresyl-activated Sepharose® (Pharmacia, Uppsala, Sweden) and carbonyldiimidazole-activated Trisacryl (Pierce, Rotterdam, The Netherlands). Coupling procedures, including the deactivation (end-capping) of active sites which did not couple, are straightforward and are all clearly described by their respective manufacturers.

(4) The most important step in preparing IAC matrices is the characterization, especially when no preliminary data based on (R)IA are available. As discussed above, the most important parameters are affinity and capacity. The first step is to estimate the affinity. If (radio)labelled material is available the easiest method is to apply a small amount of labelled analyte and to elute stepwise (see Table 3). If suitable elution conditions are available the capacity is quickly and easy estimated by applying an aqueous solution containing *e.g.* 1 ng analyte per ml and a small amount of labelled analyte. The "break-through" point can be determined by stepwise application and monitoring the activity of the eluate. If no labelled material is available the quickest method is to apply a relatively large amount of analyte, wash the column extensively with water and preferably also with 5–10% ethanol and elute it with *e.g.* 80% ethanol in order to determine the amount of analyte in the eluate. Subsequently the affinity can be determined by testing the elution at intermediate ethanol concentrations.

4. AN OVERVIEW OF APPLICATIONS

In order to evaluate the published applications of IAC we must recognize three different forms: (1) single-antibody, single-analyte (IAC); (2) single-antibody, multi-analyte ((M)IAC); (3) multi-antibody, multi-analyte (MIAC).

4.1. Single-antibody, single-analyte

The first applications of IAC in residue analysis were of this form [8,9]. A polyclonal or monoclonal antibody was coupled and a single analyte was isolated from an aqueous sample or aqueous extract. In our laboratory IAC was first used to determine the presence of trenbolone in urine and muscle. Strictly speaking, this is a pseudo-(M)IAC application since the antibody binds both 17α-trenbolone and 17β-trenbolone. Fig. 3a and b shows two chromatograms, one for a sample which does not contain trenbolone and one from a sample obtained from an animal treated with Trenbolonacetate®. In this method, in which detection is

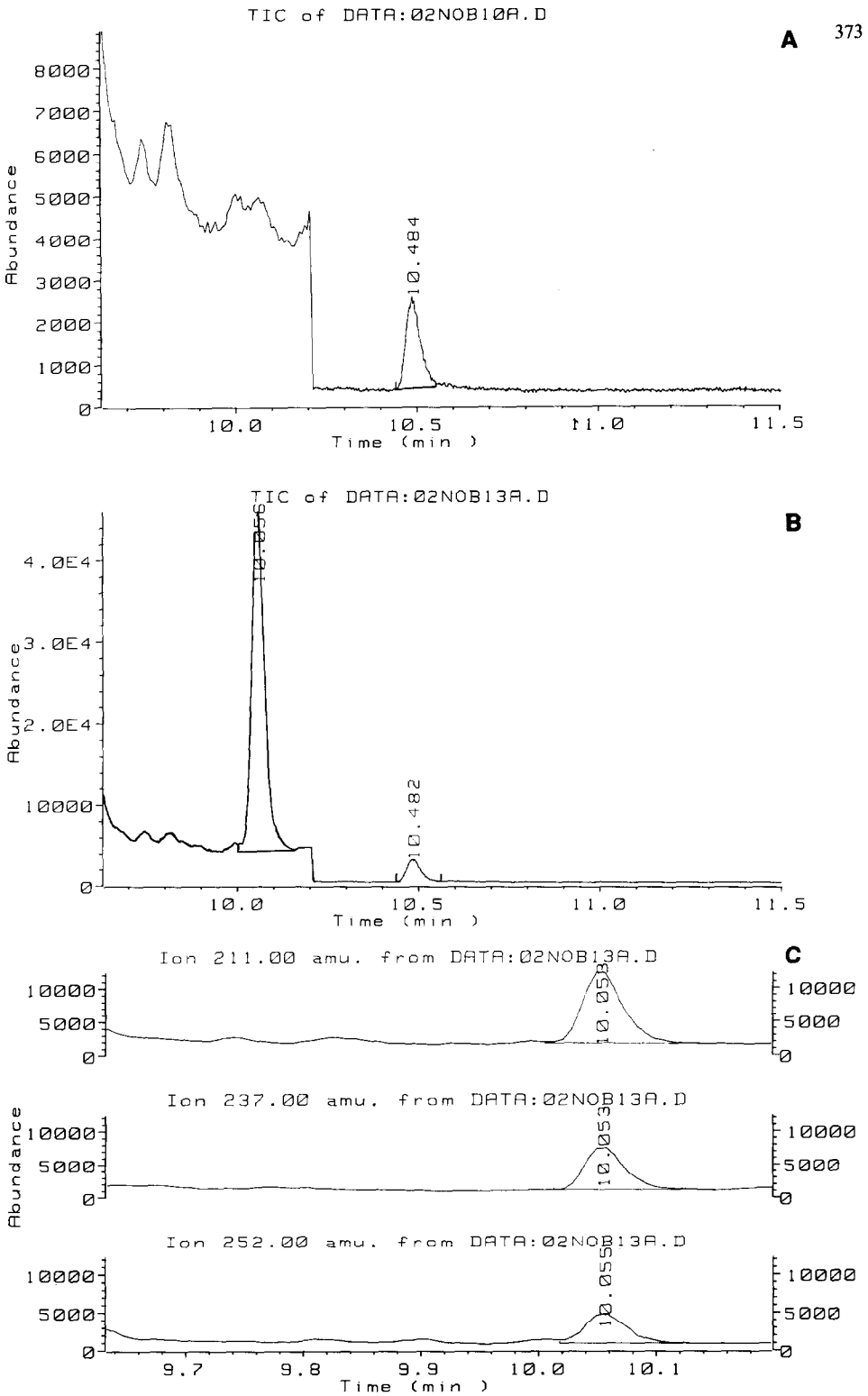

Fig. 3. GC–MS profiles for urine (TMS derivatives). (A) Sample spiked with 17β-Tb-d2 and containing no 17α- or 17β-Tb. (B) Sample spiked with 17β-Tb-d2 and containing 5 μg/l 17α-Tb. (C) Identification of 17α-Tb, monitoring of three additional fragment ions.

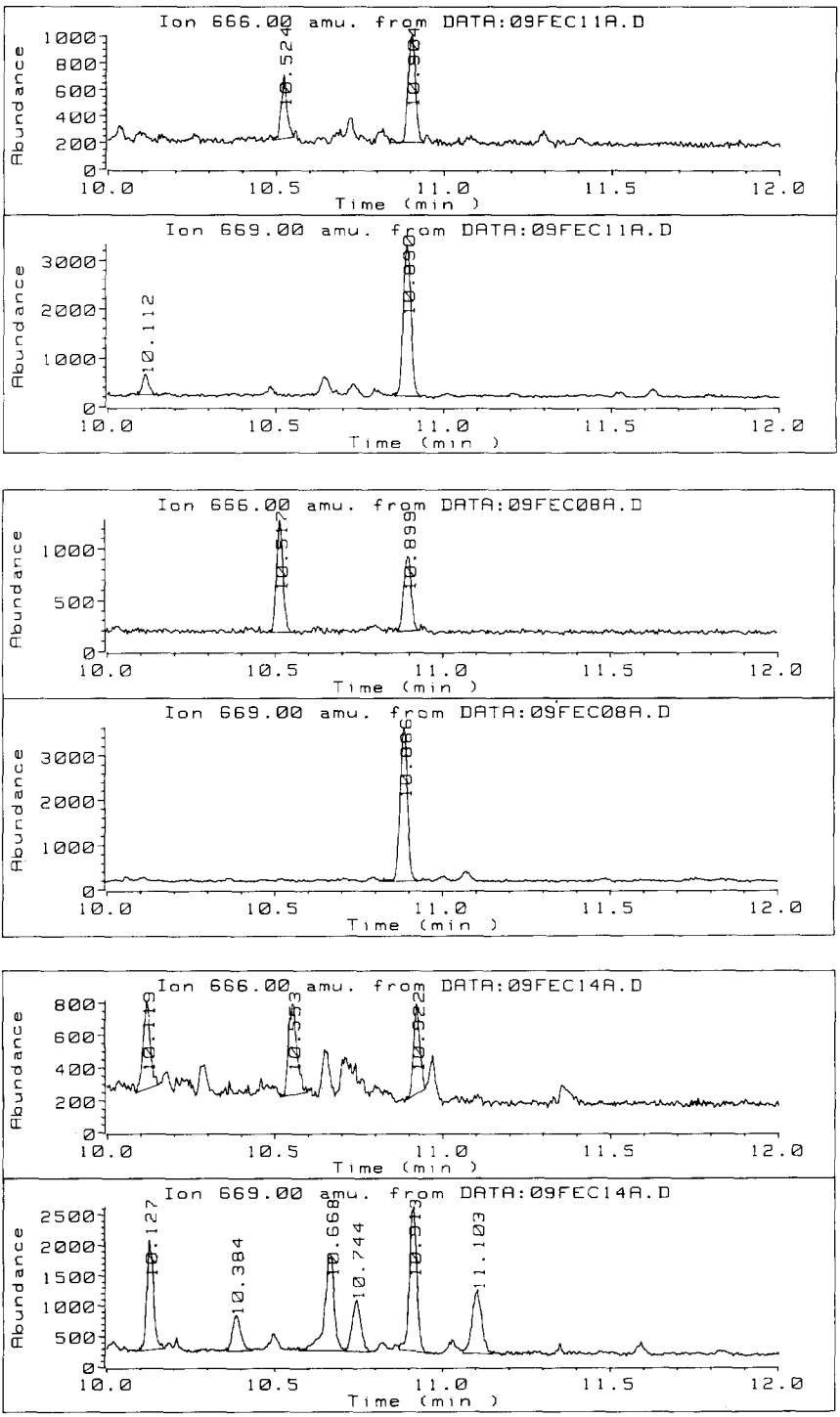

Fig. 4. GC–MS profiles for NT in urine (HFB derivatives), samples spiked with 2 μg/l 17β-NT-d_3. Each time m/z = 666 (NT-di-HFB) and m/z = 669 (NT-d3-di-HFB) are shown. Upper two panels after IAC, middle two after HPLC and lower two after SPE.

based on GC–MS, we use 17β-trenbolone-d2 as the internal standard. The chromatogram in Fig. 3b, represents a sample containing 5 μg/l 17α-trenbolone, of which 2 ml were analysed. In order to confirm the identity three ions are monitored in addition to the molecular ion (Fig. 3c). This procedure, which has now been in use for over three years, has proved to be a reliable method for controlling the residues of trenbolone. However, during the last decade a large number of methods with similar limits of detection and even identification [2] have been published, indicating that IAC is not the only possible approach. The object of this discussion is to evaluate the use of IAC and compare it with other procedures, especially that of extract clean-up. Fig. 4 demonstrates the differences between the use of IAC, high-performance liquid chromatography (HPLC) and solid-phase extraction (SPE) in determining the presence of NT and its metabolite 17α-NT in urine. A test portion of 5 ml was hydrolysed enzymatically and extracted with *tert.*-butyl methyl ether (TBME). After removing the solvent the residue was dissolved in water and purified with IAC, HPLC or SPE. The purified extracts were analysed by GC–MS. From Fig. 4 it can be concluded that the results after IAC and HPLC are very similar. However, with HPLC the recovery of 17α-NT (t_R = 10.5 min) is slightly less. The recovery of the internal standard, spiked at a level of 2 μg/l, was over 90%. After SPE the results are clearly inferior. The chromatogram clearly shows a number of additional compounds. The high capacity but relative low selectivity of this type of sample clean-up is clearly demonstrated in this experiment. The procedure, however, is suitable for screening purposes at this level.

The test procedure used in the experiment described above contained a solvent extraction step with TBME. For IAC, the dry residue was dissolved in distilled water prior to its application in the column. However, this extraction step is not strictly necessary. For most samples no differences are observed between a test portion which was extracted prior to IAC and one which was not. Some samples, however, especially those obtained from older cattle, show lower recovery after direct application. In these cases the sample matrix components do not allow the immunochemical reaction to be optimal. The recovery rate can be improved by extracting the sample, diluting it with distilled water or by passing it more slowly through the column. The affinity of most of the antibodies is constant over the pH range 4.5–8.

4.2. Single-antibody, multi-analyte

Specificity, the ability of *e.g.* an antibody to discriminate between different molecules, has always been a key feature of immunochemical methods. The reasons are obvious, identification is based to a large extent on the affinity of compounds in a purified extract of the antibody used in a particular assay. The use of immunochemistry in IAC does not impose such strict demands on the antibodies. The identification of analytes isolated by IAC is based on an entirely different

process, *e.g.* UV spectrum (diode array) detection, GC–MS or other procedures which are frequently based on spectroscopy and yield direct information about the molecular structure of the analyte.

Recently methods which are based on the use of a non-specific antibody have been developed for the isolation of β-agonists from aqueous samples and extracts [13]. Fig. 5 shows the structure of five N-*tert.*-butyl group-containing β-agonists. The immunogen which was used was prepared, as described earlier, by coupling diazonium-clenbuterol to bovine serum albumin (BSA) [25]. The antibodies obtained were all directed against the N-*tert.*-butyl group of the molecule. Detection and identification was based on GC–MS. Fig. 6 shows a chromatogram of the five β-agonists tested and a number of additional compounds [trimethylsilyl (TMS) derivatives]. Baseline separation was achieved between all the compounds with the exception of carbuterol and clenbuterol which were impossible to separate on the column used. Sotalol and the β-blocker carazolol formed two different derivatives. The β-agonists included in this assay are partly very different molecules, *e.g.* phenolic *versus* non-phenolic. This makes multi-analyte extraction

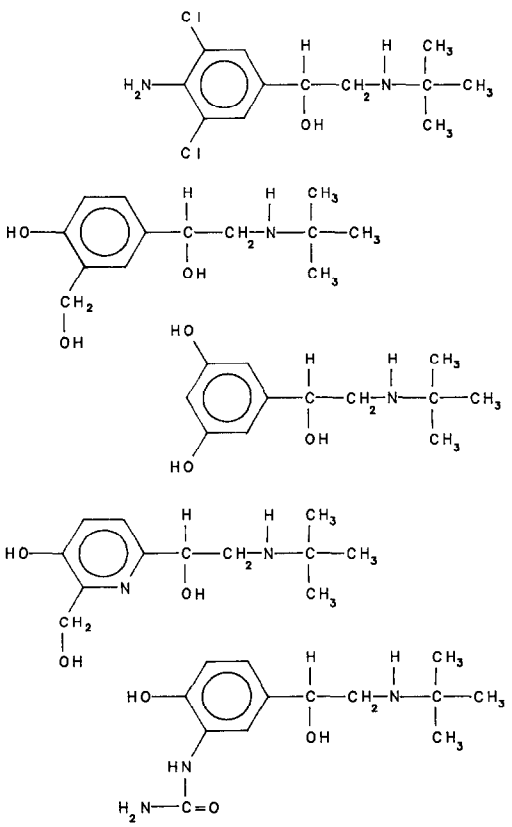

Fig. 5. Structures of five different N-*tert.*-butyl β-agonists.

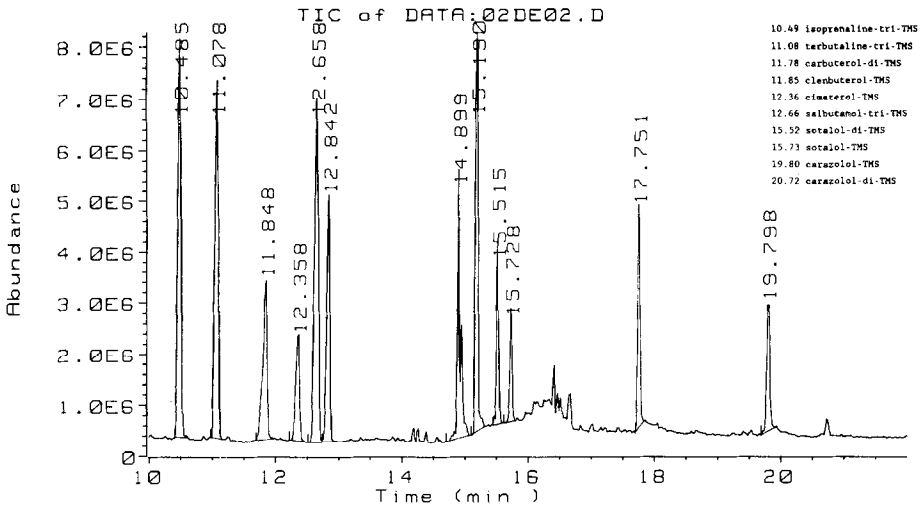

Fig. 6. GC–MS profile for some β-agonists and related compounds.

from an aqueous sample a rather complicated procedure. To obtain good recovery rates it is necessary to use ion-pair extraction. We used sodium dodecyl sulfate since this counter ion proved to be suitable in the HPLC analysis of clenbuterol [27]. From pH optimization experiments with clenbuterol, salbutamol and the N-isopropyl group containing cimaterol it was concluded that the best results are obtained at pH 7.8. The latter compound, however, clearly shows lower affinity with the IAC matrix since it does not contain the N-*tert.*-butyl group.

In our experience (M)IAC is the most suitable technique for extract clean-up for multi-residue analysis of β-agonists. Currently a new IAC column containing antibodies directed against N-*tert.*-butyl and N-isopropyl β-agonists is being evaluated in order to extend the number of analytes within a single analysis.

4.3. Multi-antibody, multi-analyte

Nowadays most laboratories are involved in the development of multi-residue methods — multi in the sense of multi-analyte as well as multimatrix. In our laboratory several multi-residue methods were also developed, partly based on MIAC.

The first method is an almost completely automated procedure for the detection of NT/17α-NT, MT, Tb/17αTb, zeranol (Z) and its metabolites taleranol and zearalanon, testosterone (T), estradiol (E2) and diethylstilbestrol (DES) in urine. To prepare the MIAC matrix seven individual IAC matrices were combined in a single column. The procedure used testosterone-d2 (T-d2) as the internal standard. Urine samples (5 ml) are enzymatically hydrolysed, centrifuged and placed in an auto injector (Gilson, Villiers-le-Bel, France). The MIAC column is placed

in the injection port of the auto injector. The sample is applied to this column and the column is automatically washed with water to remove all urine and with 10% ethanol–water to elute low affinity absorbed compounds. Subsequently the analytes are eluted with 50% ethanol–water. The eluate is diluted with water to lower the ethanol content to 10% and the analytes are trapped on a reversed-phase pre-contration column (Chrompack, Middelburg, The Netherlands). In the next step the analytes are eluted from the pre-column into the HPLC system (ODS-Hypersil, Shandon). The analytes are detected by UV absorbance (λ = 254 nm and λ = 350 nm) and most of the eluate is collected as a single fraction. Fig. 7 shows two chromatograms obtained for a sample of urine (veal calve) obtained from a non-treated animal. Fig. 8 shows the same sample spiked at the 2 μg/l

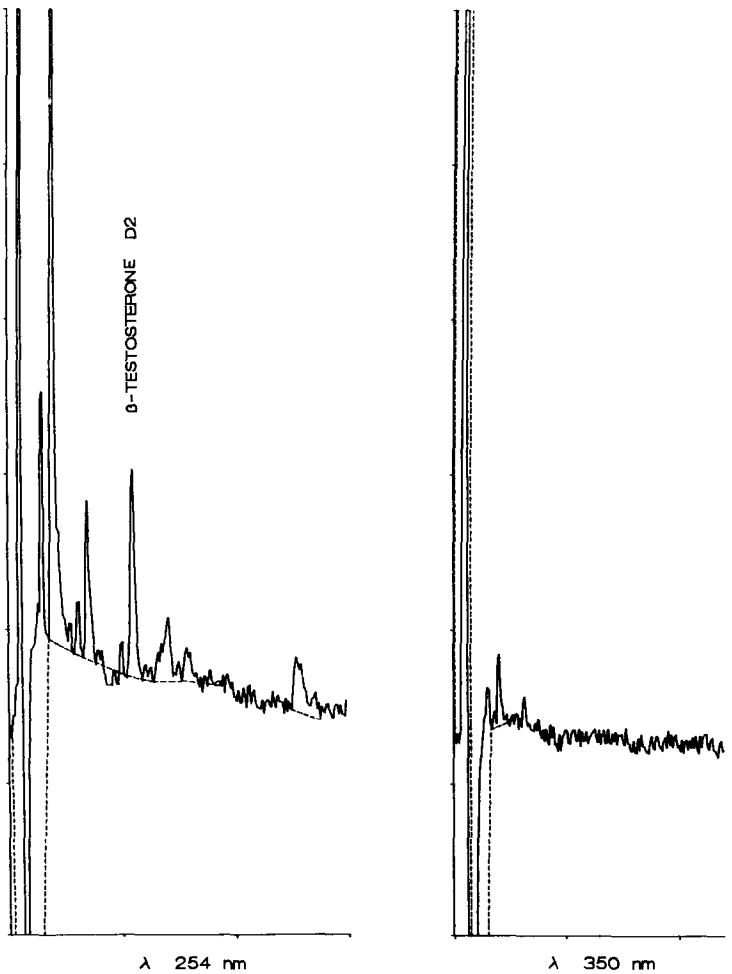

Fig. 7. HPLC of a urine sample spiked with 2 μg/l T-d2 and containing no analytes, as obtained with on-line MIAC–HPLC–UV detection.

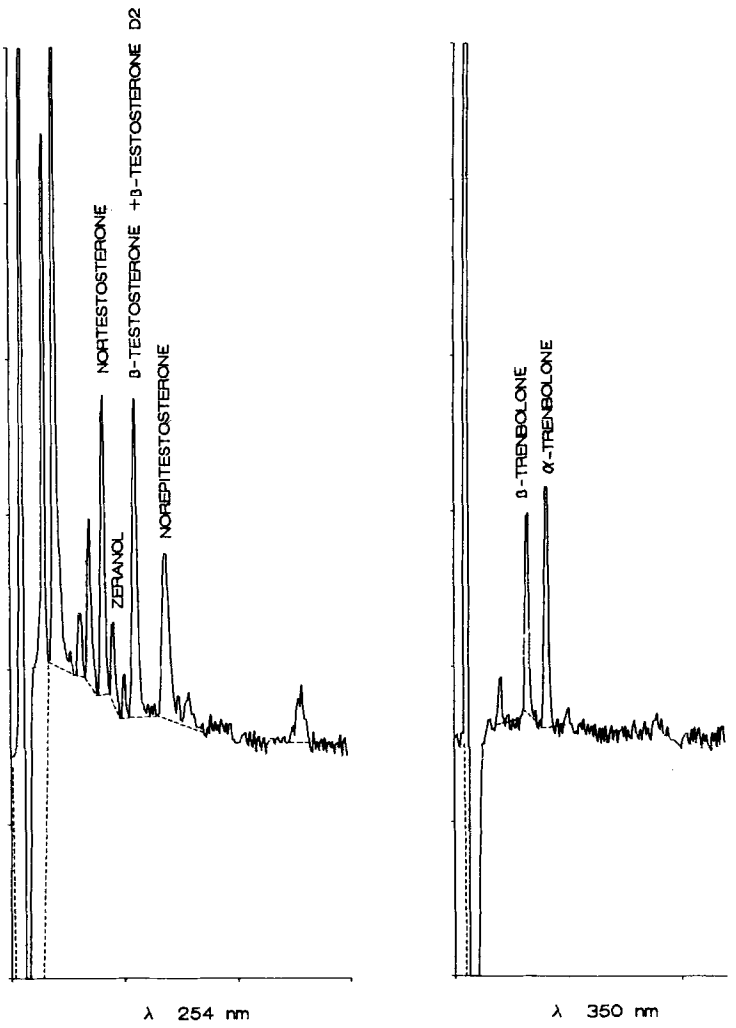

Fig. 8. HPLC of a urine sample spiked with 2 μg/l T-d2 and all the analytes included in the method, as obtained with on-line MIAC–HPLC–UV detection.

level. If the chromatogram of a sample shows compounds at a retention time corresponding to one of the analytes or if the chromatogram does not permit unambiguous detection at the 1 μg/l level, the total fraction is extracted and subjected to GC–MS analysis. With this procedure it is, in principle, possible to analyse over one hundred samples a week, including standards and control samples, with a work-load of approximately 5–10 h (not including GC–MS confirmation).

We developed two multi-residue methods for the detection of anabolic compounds in meat, including minced meat; one based on MIAC (method I) and one based on HPLC (method II).

Method I	*Method II*
Nortestosterone (NT)	Boldenone (Bol)
Methyltestosterone (MT)	Ethinylestradiol (EEZ)
Trenbolone (Tb)	Chlormadinoneacetate (CMA)
Zeranol (Z)	Medroxyprogesteroneacetate (MPA)
Diethylstilbestrol (DES)	Megesterolacetate (MGA)
Testosterone (T)	
Estradiol (E2)	

Both procedures are based on preparing a primary extract with Subtilisin A enzymatic digestion and defatting with petroleum ether. In method II an additional basic hydrolysis is included to free chlormadinone, medroxyprogesterone, and megesterol from their corresponding acetates. The TBME extracts are subsequently purified with MIAC, resulting in a single fraction, or with HPLC, resulting in three different fractions. Both procedures are suitable for the detection of anabolics at the 1–2 μg/kg level. The advantages of MIAC over HPLC are: the higher recovery at the 2 μg/kg level, approximately 90% *versus* approximately 60% with HPLC; the need to analyse only a single fraction with GC–MS instead of three after fractionation with HPLC. After the above mentioned methods had been developed within our laboratory approximately 300 samples of meat, including minced meat, were analysed by both procedures. One technician can analyse 50 samples with method I (producing 350 results) or 30 samples with method II (producing 150 results), in one week, respectively. In addition it can be concluded that the purified extracts contain less interfering compounds after MIAC than those obtained after HPLC. A direct comparison of the limits of detection is not possible since more parameters than just extract clean-up exert their influence. However, a detailed comparison was made for 17β-NT which was analysed with method I and included in Method II by slightly modifying it. When detected as a heptafluorobutyryl (HFB) derivative, the limit of detection with method I was approximately 0.2 μg/kg and 0.8–1 μg/kg with method II. The limits of identification were 1.0 and 1.5 μg/kg, respectively. In practice, GC–MS with negative-ion chemical ionisation is used for the confirmation of results less than 1 μg/kg [11].

5. DISCUSSION

New techniques, applications and alternative approaches regularly enter the field of residue analysis and frequently claim to be superior to techniques which have been developed in the past. Sometimes these techniques find their way into the residue laboratory. However, only a limited few find applications outside the laboratory which originally developed them.

The suitability of a technique is not solely determined by its analytical parameters such as specificity and reproducibility but also by non-analytical parameters such as costs and the availability of critical reagents and equipment.

IAC is a technique which can only be used in extract clean-up and has to be combined with other procedures for detection purposes and, if it is to be applied to the preparation of a primary extract. Its applicability therefore also depends on the possibility of combining it to a variety of other analytical steps.

We can conclude that IAC is at least equivalent to but in most cases superior to other analytical procedures with respect to the analytical parameters. The selectivity of the interaction surpasses all other selectivities used for extract clean-up in residue analysis. Only HPLC which isolates the fraction containing the analyte of interest, can compete with IAC. The use of HPLC in extract clean-up, however, can not be regarded as a multi-residue technique because of the differences in retention time between the different analytes which results in the need to collect several fractions for further, *e.g.* GC–MS, analysis.

The reproducibility of IAC columns must be regarded at three different levels: the reproducibility between columns of a single batch; the reproducibility between batches; the reprodubility of a single column during prolonged use.

The way in which columns are prepared, usually 10–20 ml of IAC material in a single batch, is such that no inhomogeneity problems occur between the columns. However, each batch has to be tested for both capacity and affinity. Based on the same IgG solution, the preparation of IAC material is highly reproducible providing that the protein content is monitored accurately. IAC colums are regenerated after use and therefore their reproducibility during prolonged use is also important. All the columns which have been prepared by us sofar have been shown to last at least 100 runs, provided that the sample is properly defatted and does not contain solid particles.

IAC is easily combined with other steps in an analytical procedure, *e.g.* thin-layer chromatography (TLC), HPLC and GC–MS. For our applications GC–MS proved to be the most useful. After IAC the eluate is extracted and the dry residue is derivatized. The gas chromatograph–mass spectrometer is programmed in such a way that around the retention time of a particular analyte, one or two diagnostic ions, and if necessary, one ion of the deuterated internal standard, are monitored.

All the methods used in our laboratory are multi-residue procedures. The first analysis is always regarded as screening. Therefore, no attempt is made to detect more than one or two diagnostic ions which are needed for reference analysis [3]. Frequently, when deuterated internal standards are used the detection of more diagnostic (fragment) ions is not relevant since the fragments of the analyte and the internal standard become identical due to the loss of deuterium from the internal standard. When a positive result is obtained with one of the multi-residue methods the sample is reanalysed in duplicate, once spiked with the internal standard (quantification) and once without spiking (identification). In this confirmatory analysis IAC is used with a matrix containing only the relevant antibody. If the concentration is above 2 ppb, HPLC is used instead of IAC to optimize the independence of the methods used. Using MIAC as the method for extract clean-

up and GC–MS as the technique for detection and identification (MIAC/GC–MS) a highly reliable analytical strategy is available which is suitable for use in all possible situations, even in the case of international conflicts and it can be combined with relative "simple" low-resolution MS using a bench-top machine. The reliability of this approach was demonstrated during an "experimental chemometrics" session organised during an EC workshop on the use of GC–MS in residue analysis.

When using MIAC/GC–MS the line between screening and confirmation techniques becomes very thin. The major advantage of screening methods has always been the high sample throughput. Indeed, most (immunochemical) methods can analyse up to 30 samples every day. However, since most methods are single analyte procedures, the maximum number of test results obtained each week is 150, which is less than the number obtained with MIAC/GC–MS. On the other hand, the selectivity of MIAC/GC–MS is such that it can be used for confirmation and even reference analysis. In view of the low detection limits and identification limits which are obtainable with MIAC/GC–MS during initial screening it becomes a problem to confirm results by a different (independent) procedure, especially at levels below 1–2 ppb. Suitable alternatives, however, can be found within the GC–MS region by using a different derivative or ionisation procedure.

Overall, it can be concluded that MIAC can claim a prominent place in residue analysis, based on its analytical qualities. However, to become a truly popular technique, more is required. The major problem, of course, is the availability of materials. The use of antibody is far greater in IAC than in, for example, RIA procedures. One IAC column contains roughly the same amount of antibodies as 1000 RIA tubes. However, this difference is compensated to a large extent by the prolonged use (over 100 times) of the columns.

Based on this use of antibody, the price of a single column is estimated to be in the range Dfl. 100–500 (US$ 50–250) depending on the number of columns to be prepared. In comparison with an average HPLC column the price of a (M)IAC column is economically advantageous. When columns are available, the introduction of MIAC in a laboratory is relatively easy, comparable to SPE but with an additional need for quality control since the columns are to be used for a long time. When only antiserum is available the use of (M)IAC is still economically advantageous but greater immunochemical expertise is needed. The preparation of the materials, however, is straight-forward and is based on commercially available materials. The only real problem is the synthesis of an immunogen and the production of antibodies. To circumvent these problems the possibility of supplying material through *e.g.* the EC Bureau of Reference (BCR) is currently being studied. The general need for relevant, and if possible certified, reference materials (RMs) has currently resulted in a number of candidate RMs, some of which are presently being certified. Up to now all activities have been in the direction of reference samples. However, a laboratory actively involved in the preparation of materials could supply, Under BCR contract, columns to other laboratories.

Recently an Italian firm involved in biotechnology also announced the sale of MIAC columns, three to six analytes per column, which were suitable for the isolation of anabolic agents.

In the near future new developments can be expected. One interesting possibility is the development of quick "field-like" tests. Currently we are working on an assay based on very small IAC columns with a capacity of 10–100 pg suitable for extracting NT from urine and subsequent detection with an immunochemical procedure (IACIA). The use of different antibodies, raised against different immunogens, provides adequate selectivity for a screening procedure. The possibility of homogeneous RIAs, based on the scintillation proximity counting of low tritium activities, provides the necessary sensitivity.

IAC is not a new technique. Its applicability in residue analysis has, however, been sofar limited. Nevertheless, the technique deserves a prominent place in residue analysis during the coming years when taking into account its analytical qualities and the cost-effectiveness of MIAC combined with *e.g.* GC–low-resolution MS. The availability of MIAC materials is the major factor which will determine its success or not.

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