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Review

Quantitative analysis of biomarkers, drugs and toxins in biological samples by immunoaffinity chromatography coupled to mass spectrometry or tandem mass spectrometry: A focused review of recent applications☆

Dimitrios Tsikas*

Institute of Clinical Pharmacology, Hannover Medical School, Carl-Neuberg-Str. 1, D-30625 Hannover, Germany

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ABSTRACT

Immunoaffinity chromatography (IAC), mass spectrometry and especially tandem mass spectrometry (MS/MS) represent the most efficient and reliable analytical techniques for specific isolation, unequivocal identification and accurate quantification of numerous natural and synthetic substances in biological samples. This review article focuses on the combined use of these outstanding methodologies in basic and clinical research and in life sciences for the quantitative analysis of low- and high-molecular mass biomarkers, drugs and toxins in urine, plasma or serum samples, in tissue and other biologicals systems published in the last decade. The analytes discussed in some detail include the biomarkers of oxidative stress 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ {15(*S*)-8-*iso*-$PGF_{2\alpha}$} and 3-nitrotyrosine, the major urinary metabolite of the lipid mediators cysteinyl leukotrienes, i.e., the leukotriene E_4 (LTE_4), melatonin, and the major collagen type II neoepitope peptide in human urine.

Contents

1. Introduction – mass spectrometry and immunoaffinity chromatography

Mass spectrometry (MS)-based techniques, e.g., GC–MS, GC–MS/MS, LC–MS and LC–MS/MS, are among the most efficient and versatile, currently available and increasingly used analytical techniques for qualitative and quantitative analysis of endogenous and exogenous substances in biological samples. The whole analytical process may comprise several pre-analytical and analytical procedures for sample generation, extraction and derivatization of analytes prior to their chromatographic and mass spectrometry separation and final detection. Scheme 1 summarizes the main steps commonly involved in MS-based analytical approaches.

In principle, LC–MS, LC–MS/MS and related techniques are applicable to both low-molecular-mass (LMM) and high-

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* Tel.: +49 511 532 3959; fax: +49 511 532 2750.
E-mail address: tsikas.dimitros@mh-hannover.de.

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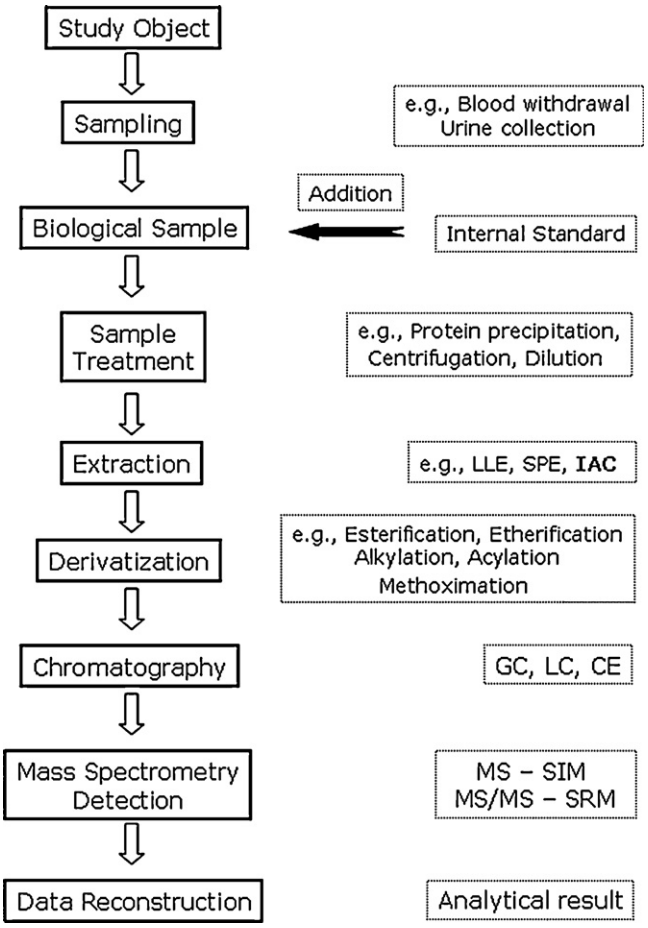
molecular-mass (HMM) analytes, mostly in their native, i.e., non-derivatized forms. By contrast, GC–MS and GC–MS/MS are used to analyze rather a very narrow spectrum of LMM analytes in their native form. This limitation can only partly be overcome by chemical conversion (derivatization) of the analyte. Nevertheless, even if not absolutely required, analyte derivatization may be advantageous in LC–MS and LC–MS/MS, for instance in terms of sensitivity enhancement [1]. Because of the very large number of analytes and fields of application and due to recent great instrumental progress, the LC–MS/MS technology becomes increasingly popular both in industry and in academia. However, the efficacy of the LC–MS/MS methodology, notably in quantitative analyses, is overestimated, not rarely in the author's opinion, and the matrix effects are frequently underestimated. Not without reason, matrix effects have been called the Achilles heel of quantitative LC–ESI-MS/MS [2]. Regrettably, the thought, that the specificity of the tandem mass spectrometry process and the use of stable-isotope labelled analogs of the analytes will entirely eliminate potential interferences and matrix effects, is widespread. As a consequence, frequently sample preparation is either not existent or minimal in LC–MS/MS-based methods. There are examples in the literature that minimization of sample preparation, for instance by simple matrix dilution, or renunciation of any sample preparation step or even the main LC separation step in quantitative analyses will definitely fail for numerous analytes [3]. Therefore, reliable quantitative analysis by LC–MS/MS regularly requires performance of adequate sample preparation procedures prior to quantification in addition to the LC step and the use of stable-isotope labelled analogs as internal standards (IS) at a proper concentration [4] (see Scheme 1). Indeed, these measures minimize ion suppression/enhancement effects.

By nature, in GC–MS/MS methods sample preparation and derivatization procedures are almost entirely indispensable for the majority of the analytes. It is worth mentioning, that, given the lower specificity of the single MS separation, in GC–MS and LC–MS methods the efficiency of the sample preparation steps may determine much more decisively the quality of the analytical result than in GC–MS/MS and LC–MS/MS. This issue is addressed in more detail in the next section for selected substances.

One of the most frequently used analytical procedures is the extraction of the target analyte and its internal standard from the biological matrix (Scheme 1). Extraction can be conducted by solvent extraction (i.e., liquid–liquid extraction, LLE), solid-phase extraction (SPE), and alternative techniques such as affinity chromatography and immunoaffinity chromatography (IAC). SPE of LMM analytes may be associated with considerable selectivity when special phases are used such as immobilized phenylboronic acid for 1,2- and 1,3-diols [5]. Certainly, the highest specificity is accomplished by IAC, because this particular extraction technique is based on the use of one or more chemically immobilized antibodies raised against the analyte or analytes to be extracted from a biological sample.

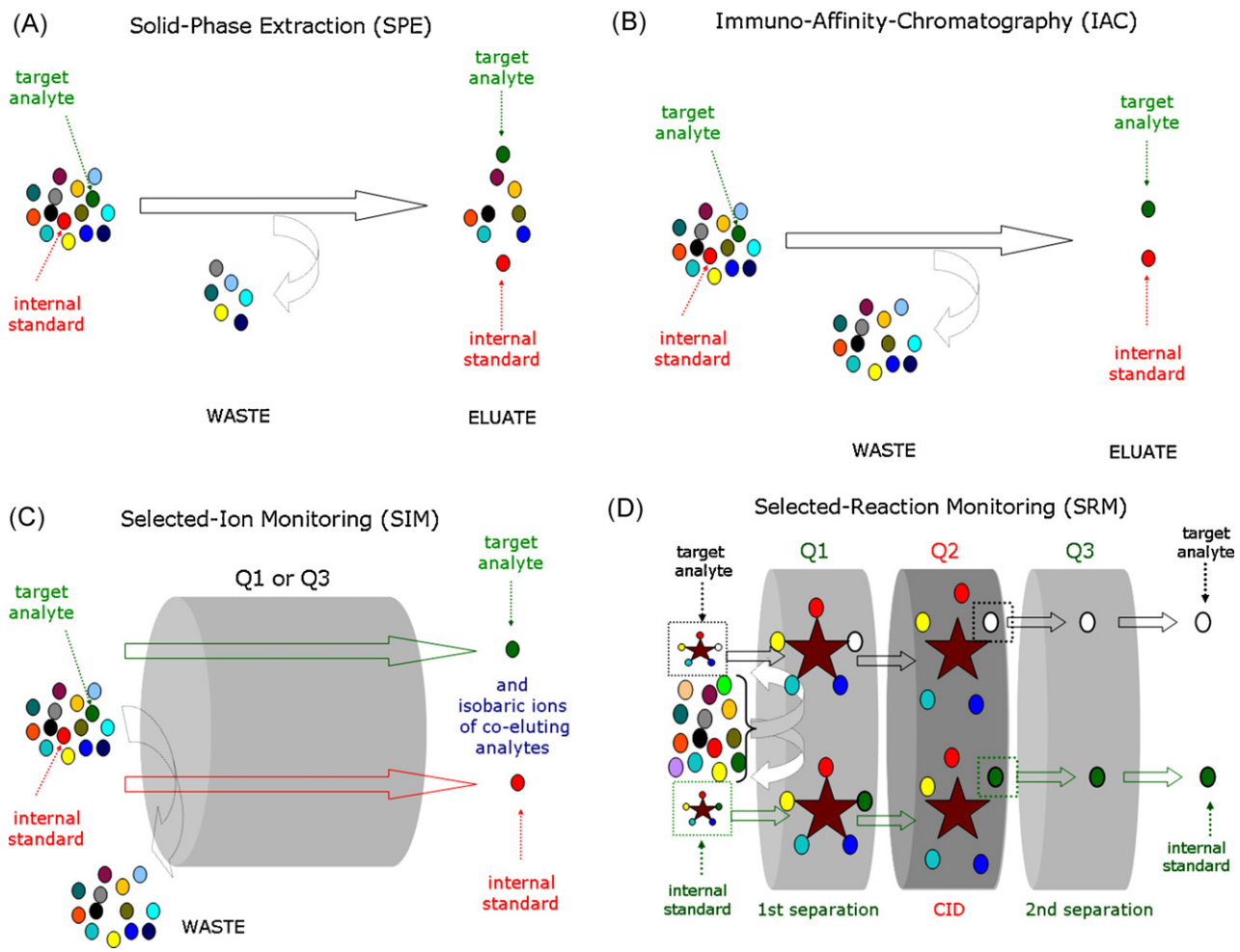
Formally, the processes that take place in IAC, on the one hand, and in LC–MS/MS and GC–MS/MS, on the other hand, are comparable in that IAC separates specifically a single compound among myriads of analytes and the MS/MS techniques separate specifically a single ion that can be unequivocally assigned to a single analyte (Scheme 2). Thus, it may reasonably be expected that the combination of IAC with MS-based techniques, notably tandem mass spectrometry, would provide the most reliable analytical result. Indeed, until the end of the 1990s, IAC has been used for the specific extraction of various endogenous and exogenous compounds from different biological fluids prior to quantitative determination by MS-based techniques [5]. Substance classes frequently analyzed in the past include arachidonic acid metabolites, i.e., the eicosanoids [6–11], steroids [12–17], DNA adducts of electrophiles [18–28], some drugs [29–35], and many toxins [36–43].



Scheme 1. Schematic of regular analytical processes involved in the quantitative measurement of analytes in biological samples by mass spectrometry (MS)-based methods using stable-isotope labelled analogs as internal standards (IS). Used abbreviations are explained in the Nomenclature section.

In the first decade of the 21st century, IAC has been widely and increasingly used as a sample preparation step for reliable quantitative analysis of endogenous and exogenous biomarkers, drugs and toxins in various biological matrices including human plasma and urine by LC–MS, LC–MS/MS, GC–MS or GC–MS/MS. The present review focuses on recent applications in this area and discusses selected representatives of the substance classes analyzed by IAC-involving MS approaches.

Table 1 summarizes in alphabetical order (and chronological order within the same substance class) papers published from 2000 to 2009. Scheme 3 shows the chemical structures of some of the analytes reviewed and discussed here in more detail. For the sake of simplicity, analytes are discussed in groups which include eicosanoids, 3-nitrotyrosine, toxins, drugs including steroids and other anabolics, DNA and proteins adducts of LMM substances such as malondialdehyde (MDA), and peptide/protein biomarkers. With the exception of the review article on IAC techniques in food analysis by Şenyuva and Gilbert [115], other articles included in this special issue of the journal, as well as those being based on other kinds of affinity chromatography including molecularly imprinted methodology, were not considered in the current review.



Scheme 2. Schematics for the comparison of two extraction procedures, i.e., (A) solid-phase extraction (SPE) and (B) immunoaffinity chromatography (IAC) extraction, and of two quantification modes as can be performed for instance in single-stage quadrupole (SSQ) and triple-stage quadrupole (TSQ) instruments using the stable-isotope dilution technique, i.e., by the use of stable-isotope labelled analogs as internal standards (IS). (C) Quantitative analysis by selected-ion monitoring (SIM) of two ions specific for an analyte and for the IS, respectively. (D) Quantitative analysis by selected-reaction monitoring (SRM) of two specific product ions generated by collision-induced dissociation (CID) of the corresponding parent ions. Q means quadrupole. Regarding selectivity, formally SPE is comparable with SIM, and IAC is comparable with SRM. In other words, IAC and SRM are considerably more specific than SPE and SIM. Also, multi-analyte IAC is comparable with SRM of many analytes and their corresponding stable-isotope labelled analogs.

2. Review of applications in the decade 2000–2009

2.1. Eicosanoids – 15(S)-8-iso-PGF$_{2\alpha}$ and LTE$_4$

The eicosanoids are an outstanding example of analytes, the reliable determination of which is difficult in biological fluids and requires several purification steps and sophisticated methodologies (reviewed in Ref. [116]). The major difficulty in quantifying arachidonic acid metabolites, such as prostaglandins, thromboxane, leukotrienes, and their metabolites, is the occurrence of a very large number of structurally closely related compounds and their extremely low concentrations in plasma and urine (pM to nM). The specificity both of GC–MS methods and immunoassays, initially being the most frequently used approaches in eicosanoid analysis, was greatly improved by incorporating IAC for analyte extraction. In the past, several members of the eicosanoid family have been in the focus of many groups who developed and used IAC methods for their quantification by GC–MS [6–11]. Regrettably, this effort has not resulted in commercially available IAC sorbents or columns for all of these eicosanoids. At least, for some prominent biomarker-eicosanoids, such as 15(*S*)-8-*iso*-prostaglandin F$_{2\alpha}$ {15(*S*)-8-*iso*-PGF$_{2\alpha}$}, prostaglandin E$_2$ (PGE$_2$), and the cysteinyl leukotriene E$_4$ (LTE$_4$), IAC columns and sorbents have been commercially available for several years. However, only very few groups have used this IAC material for quantitative purposes thus far (Table 1).

2.1.1. 15(S)-8-iso-prostaglandin F$_{2\alpha}$ – 15(S)-8-iso-PGF$_{2\alpha}$

We found that use of commercially available IAC columns with immobilized antibodies raised against 15(*S*)-8-*iso*-PGF$_{2\alpha}$ (Scheme 3), one of the 64 theoretically possible F$_2$-isoprostanes which are considered lipid peroxidation biomarkers [102], allows for highly specific extraction of 15(*S*)-8-*iso*-PGF$_{2\alpha}$ from human urine, plasma and serum and quantification by GC–MS/MS or GC–MS with comparable accuracy in the higher concentration range [99] (Fig. 1; see also Fig. 2). It is worthy to mention, that performance of quality control (QC) samples for urine and plasma 15(*S*)-8-*iso*-PGF$_{2\alpha}$ of humans, rats and mice in our group revealed that the commercially available IAC columns for 15(*S*)-8-*iso*-PGF$_{2\alpha}$, which were purchased from the same manufacturer, are of consistent quality over several years (data not shown).

Despite considerable primary costs of IAC columns for 15(*S*)-8-*iso*-PGF$_{2\alpha}$, we have calculated that IAC columns are finally cheaper than regular SPE cartridges, because these IAC columns are reusable

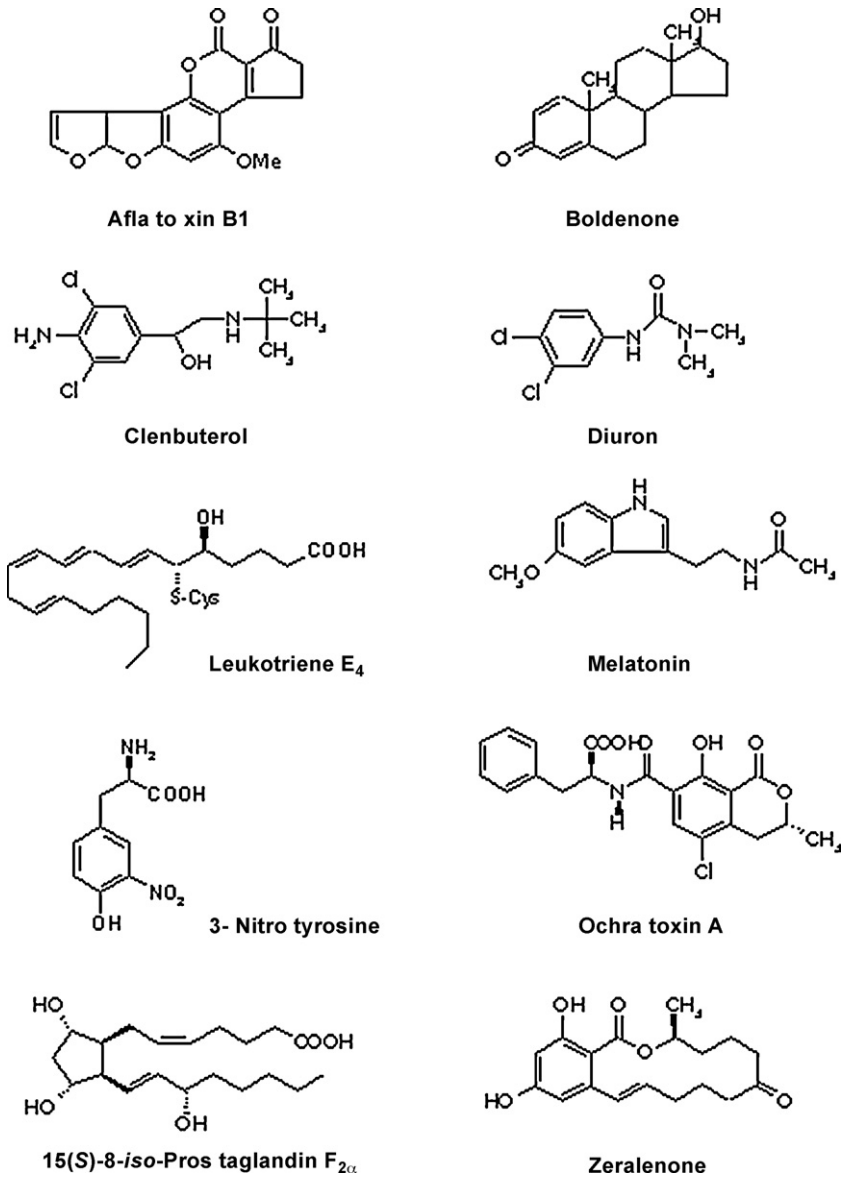
Table 1
Summary of published mass spectrometry-based methods for qualitative and quantitative of endogenous and exogenous analytes in biological samples using immunoaffinity chromatography for sample purification.

Analyte	Matrix	Species	Approach	Remark	1st author	Ref. no.
Abscisic acid	Tobacco	N.A.	LC–MS	IAC; home-made	Hradecká	[44]
Aflatoxin B_1	Smoke	N.A.	LC–MS; LC–MS/MS; ID	IAC	Edinboro	[45]
Aflatoxin B_1-N^7-G.	Urine	Human	LC–MS/MS; ID	IAC	Egner	[46]
Aflatoxin B_1	Spices	N.A.	ELISA	Tandem IAC	Goryacheva	[47]
Aflatoxin	Urine	Human	LC–MS/MS	Automated IAC	Everley	[48]
Aflatoxins	Maize	N.A.	LC–MS/MS	Multi-toxin IAC (11 toxins)	Lattanzio	[49]
Aflatoxin M_1	Milk	Human	LC–MS/MS	IAC	Chen	[50]
Amanitin	Urine	Human	LC–MS	IAC; reusable (50×)	Maurer	[51]
Aminoflunitrazepam	Urine	Not reported	LC–MS/MS	IAC; SPME; home-made	Lord	[52]
Anabolics	Urine	Human	GC–MS; LC–MS/MS	IAC	Saugy	[53]
Avermectins	Liver	Swine	LC–TOF-MS	IAC; home-made	Wu	[54]
Benzopyrene-DNA	Urine	Human	LC–IT-MS	IAC; home-made	Bhattacharya	[55]
Bisphenol A	Water	N.A.	LC–MS/MS	IAC monolith; home-made	Li	[56]
Boldenone	Urine	Horse	LC–TOF-MS/MS; ID	IAC; home-made	Ho	[57]
Clenbuterol	Retina	Bovine	LC–MS/MS; ID	IAC	Lau	[58]
Cytokinins	Tissue	Plants	UPLC–MS/MS; ID	IAC	Novák	[59]
Dioxins/furans	Serum	Bovine	GC–HR-MS; ID	IAC; home-made	Huwe	[60]
Diuron	Wastewater		LC–MS/MS	IAC; on-line; home-made	Zhang	[61]
rhErythropoietin	Plasma	Equine	LC–MS/MS	IAC; magnetic-beads-based	Guan	[62]
rhErythropoietin	Plasma	Equine	LC–MS/MS	IAC	Guan	[63]
Fumonisins	Cornflakes		LC–MS/MS	IAC	Paepens	[64]
Gonadotropin	Urine	Human	LC–MS/MS	IAC; home-made	Gam	[65]
Herbicides	Food; water		LC–MS/MS	IAC, sol–gel glass-based	Degelmann	[66]
GnR hormone	Serum; urine	Human	CE–MS	IAC; on-line; home-made	Guzman	[67]
GnR hormone	Urine	Human	LC–MS/MS	IAC; magnetic-beads-based	Thomas	[68]
Insulin analogs	Urine	Human	LC–MS/MS	IAC	Thevis	[69]
Insulins	Urine	Equine	LC–MS/MS	IAC	Kuuranne	[70]
Leukotriene E_4	Urine	Human	LC–MS/MS; ID	IAC; 37 pg/mg	Armstrong	[71]
MDA adducts	Model peptides	N.A.	LC–MS/MS	IAC; home-made	Fenaille	[72]
MDA–DNA adducts	Urine	N.A.	LC–MS/MS; ID	IAC; home-made	Otteneder	[73]
Melatonin	Serum	Human	LC–MS; ID	IAC; home-made; Ser: <305 pM	Rolcík	[74]
Methamphetamine	Urine	N.A.	LC–MS; ID	IAC; home-made	Lua	[75]
Microcystins	Lake water	N.A.	LC–MS	IAC; home-made; reusable (3×)	Kondo	[76]
Microcystins	Surface water	N.A.	HPLC; LC–MS	IAC; sepharose- or silica-based	Aranda-Rodriguez	[77]
Microcystins	Water, green algae		HPLC	IAC; home-made; polyclonal	Mhadhbi	[78]
Mycotoxins	Foods	N.A.	LC–MS/MS	Acetonitrile–water; 33 mycotoxins	Spanjer	[79]
Mycotoxins	Foods, feeds	N.A.	LC–MS/MS	Review	Krska	[80]
3-Nitrotyrosine	Various	N.A.	LC–MS/MS	IAC; PL: 0.5–3 nM; U: 0.25–3 nM	Radabaugh	[81]
Ochratoxin A	Foods, plasma		LC–MS/MS	IAC; SPE	Scott	[82]
Ochratoxin A	Foods	N.A.	LC–MS/MS	IAC	Monaci	[83]
Ochratoxin A	Grape	N.A.	Nano-LC–MS	IAC	Timperio	[84]
Ochratoxin A	Maize	N.A.	LC–MS/MS	See Aflatoxins above	Lattanzio	[49]
Phthalic anhydride	Plasma	Human	GC–MS; LC–MS/MS	IAC; home-made	Johannesson	[85]
Peptide	Plasma	Rat	LC–MS	IAC; on-line; home-made	Zheng	[86]
Peptide	Plasma, urine	Human	LC–MS	IAC; home-made	Junot	[87]
Peptides/proteins	Cancer cells	Human	LC–MS/MS	IAC; beads-based; home-made	N.A.	[88–96]
8-*iso*-$PGF_{2\alpha}$	Plasma, urine	Human	GC–MS	Various methods	Gopaul	[97,98]
15(*S*)-8-*iso*-$PGF_{2\alpha}$	Plasma, urine	Human	GC–MS/MS; ID	IAC; reusable (7×) PL: 3 pg/ml; U: 78 nmol/mmol	Tsikas	[99]
15(*S*)-8-*iso*-$PGF_{2\alpha}$	Urine	Human	GC–MS; ID	IAC; U: 286 nmol/mmol	Donovan	[100]
15(*S*)-8-*iso*-$PGF_{2\alpha}$	Urine	Human	LC–MS/MS; ID	IAC; PL: 106 pg/ml; U: 115 nmol/mmol	Sircar	[101]
8-*iso*-$PGF_{2\alpha}$	Plasma, urine	Human	GC–MS/MS, LC–MS/MS; ID	IAC; SPE; TLC, HPLC	Schwedhelm	[102]
Quinolone antibiotics	Muscle	Animal	LC–MS/MS	IAC; mixed-bed; home-made	Li	[103]
Solanine, chaconine	Serum		MALDI-TOF-MS	IAC; home-made	Driedger	[104]
Steroids	Various		LC–MS, LC–MS/MS	IAC	Stolker	[105]
Steroids	Microsomes	Human	LC–MS, LC–MS/MS	IAC	Onorato	[106]
Steroid estrogens	Wastewater		LC–MS; ID	IAC; home-made	Ferguson	[107]
Steroids	Urine		GC–C-IR-MS	IAC; home-made	Desroches	[108]
Synacthen	Plasma	Human	LC–MS/MS	IAC	Thevis	[109]
Tetrodotoxin	Serum, urine	Human	HPLC-FL	SPE	O'Leary	[110]
THC-delta-9	Plasma, urine		GC–MS	IAC	Feng	[111]
Zearalenol analogs	River water		LC–MS/MS; ID	IAC; cross-reactivity	Erbs	[112]
Zearalenone	Plasma/urine	Horse	LC–APCI-MS; ID	IAC; cross-reactivity	Songsermsakul	[113]
Zeranol	Muscle	Bovine	GC–MS	IAC; home-made; reusable (20×)	Zhang	[114]

Note: N.A., not applicable; PL, plasma, Ser, serum; U, urine; data in urine are corrected for creatinine excretion.

(about 5 times for plasma and 7 times for urine) without loss of specificity and recovery [99]. As can be seen in Table 1, the reusability of IAC material has been explicitly reported by some authors for other analytes. For instance, reusable IAC material has been used up to 50 times for amanitin [51] and up to 20 times for zeranol [114].

When using IAC columns specific for 15(*S*)-8-*iso*-$PGF_{2\alpha}$, and more generally IAC material specific for one of the two or more possible forms of a compound, it is important to note that in comparison studies including those in which non-immunological methods have been applied, the issue of specificity demands special consideration, otherwise wrong conclusions would be drawn [99,101] (see below). For instance, the 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC column/sorbent is highly specific for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ but does not retain the (*R*)-isomer of this isoprostane, i.e., 15(*R*)-8-*iso*-$PGF_{2\alpha}$.



Scheme 3. Chemical structures of some of the compounds that have been analyzed by the combination of IAC with GC–MS, GC–MS/MS, LC–MS or LC–MS/MS and are discussed in this article.

Thus, in non-chiral GC, LC and TLC, separation of native as well as derivatized 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and 15(*R*)-8-*iso*-$PGF_{2\alpha}$ would be incomplete, unlike in IAC [99]. Difference in specificity is most likely the reason why GC–MS/MS quantification of 8-*iso*-$PGF_{2\alpha}$ in the same human urine samples provided about 2 times higher concentrations than sample cleanup by non-chiral TLC [99] (see also below).

By using IAC and GC–MS/MS we measured a mean urinary excretion rate of 78 nmol/mmol creatinine, a mean serum concentration of 73 pg/ml for free + esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and a mean serum concentration of 7 pg/ml for free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in healthy non-smoking subjects [99] (Table 1). We measured by the same method considerably lower concentrations of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in bronchoalveolar liquid (BAL) samples of healthy subjects as compared to serum. Interestingly, BAL concentrations of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ were statistically significantly lower in smokers than in non-smokers (Fig. 3), unlike in serum and urine samples of the same volunteers [99]. It is worthy to mention that the combination of TLC and GC–MS/MS would have most likely not allowed to quantify free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in human plasma and in BAL samples of healthy smoking and non-smoking subjects because of the considerably higher LOQ value (about 25-fold) as compared to the combination of IAC and GC–MS/MS, i.e., 5 pg/ml versus 0.2 pg/ml (see Fig. 3).

The combination of IAC with GC–MS/MS allows reliable quantification of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in only 100-μl aliquots of urine, plasma and serum samples, and in 1-ml aliquots of BAL. However, in case of plasma and serum, use of 1-ml sample aliquots is recommended for accurate quantification because of the very low 15(*S*)-8-*iso*-$PGF_{2\alpha}$ concentrations in these matrices [99,101,102].

Sircar and Subbaiah [101] have used IAC columns from the same manufacturer for the quantitative analysis of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in human plasma and urine by LC–MS (Fig. 4). These authors compared LC–MS with LC–MS/MS, found a very good correlation and recommend use of LC–MS if IAC columns are used for the extraction of urinary 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [101]. The combination of IAC with LC–MS provided a mean concentration of 106 pg/ml in plasma and a mean urinary excretion rate of 115 nmol/mmol creatinine for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [101]. These concentrations are close to those we measured for this analyte in plasma (for free + esterified forms) and

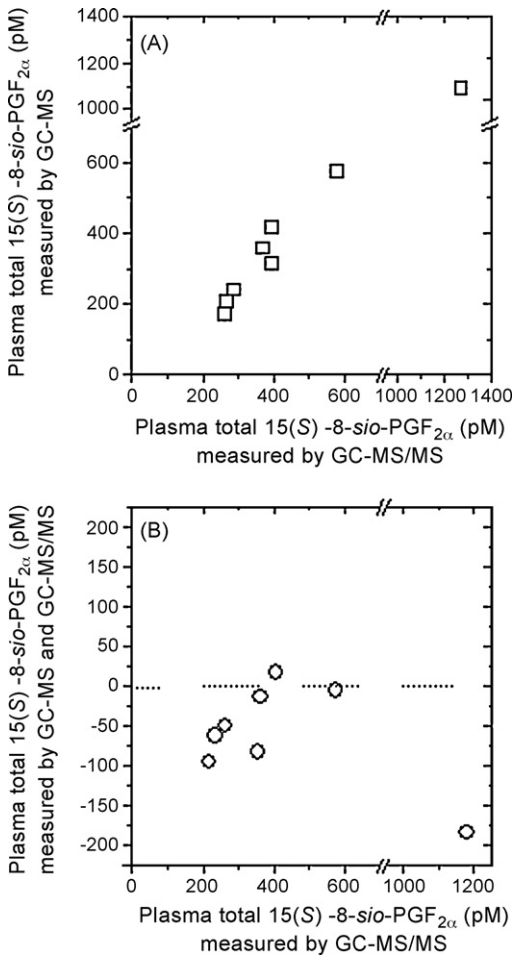


Fig. 1. Quantitative determination of free + esterified 15(*S*)-8-*iso*-prostaglandin $F_{2\alpha}$ {15(*S*)-8-*iso*-$PGF_{2\alpha}$} in eight human plasma samples by GC–MS and GC–MS/MS after IAC extraction as described elsewhere [99]. Same samples were analyzed by GC–MS and GC–MS/MS. Data comparison by (A) linear regression analysis and by (B) the Bland–Altman method.

urine (for the free form) by combined use of IAC and GC–MS/MS [99] (Table 1).

Sircar and Subbaiah [101] have also compared the combined method of IAC extraction and LC–MS analysis for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ (*x*) with a GC–MS method (*y*) that involves non-chiral TLC separation and provides the sum of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and additional mostly unknown F_2-isoprostanes in urine. This comparison revealed a weak correlation ($r = 0.81$) with the regression equation $y = 0.996 + 3.2x$, i.e., high *y*-axis intercept and a high slope values which differ from the theoretical values of 0 and 1, respectively. Furthermore, in the Bland–Altman comparison method the difference between the two methods was very high (2.8 ± 1.1 ng/mg creatinine) and, importantly, it increased with the concentration of the mean of the methods, suggesting a considerable proportional error. As mentioned above, this kind of discrepancy is expectable and explainable by the use of separation approaches of distinctly different specificity regarding F_2-isoprostanes.

Sicilia et al. reported that IAC is required for the quantitative determination of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in rat urine by LC–MS/MS [117]. This conclusion is greatly supported by the results and chromatograms reported by this group [117] (see also Ref. [3]). Indeed, Sicilia et al. [117] obtained completely different results from the LC–MS/MS quantification of 8-*iso*-$PGF_{2\alpha}$ in rat urine after direct injection of the diluted urine sample, i.e., without any sample treatment, as compared to the use of IAC.

IAC columns are also commercially available for PGE_2. We found that these IAC columns are very useful for the quantitative determination of PGE_2 in various matrices including human and mice urine (unpublished data), cultured airway epithelial cells (Fig. 5), and in incubation mixtures of arachidonic acid and isolated cyclooxygenase in aqueous buffer [99].

In summary, use of (commercially available) IAC columns for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ and PGE_2 allows for specific and accurate quantitative determination by GC–MS/MS and LC–MS/MS in plasma, serum and urine samples of humans and animals. IAC makes superfluous sample purification by other chromatographic procedures like SPE or TLC. Also, incorporation of IAC may allow quantification by using the less expensive GC–MS and LC–MS variants. In LC–MS, IAC extraction of 15(*S*)-8-*iso*-$PGF_{2\alpha}$ is indispensable for accurate analysis and superior to SPE.

2.1.2. Leukotriene E_4 – LTE_4

Urinary LTE_4 is the major metabolite of the cysteinyl leukotrienes which are potent lipid mediators of bronchoconstriction and inflammation in asthma. LTE_4 in urine is a suitable biomarker to assess cysteinyl leukotriene synthesis in various conditions including asthma. Armstrong et al. [71] reported a LC–MS/MS method for urinary LTE_4 after IAC extraction on a commercially available sorbent with immobilized antibodies raised against various cysteinyl leukotrienes including LTE_4. The combination of IAC with LC–MS/MS provided LTE_4 values in urine of 29–143 pg/mg creatinine. This order of magnitude agrees with that reported by GC–MS/MS and other techniques including RIA several years ago [116]. By contrast, EIA without preceding IAC separation was by far less precise and, more importantly, provided LTE_4 values which were 30–40 times higher, i.e., 639–5685 pg/mg creatinine, than those measured by LC–MS/MS [71]. This study clearly and convincingly demonstrated that the combination of IAC with LC–MS/MS is a reliable method for the measurement of urinary LTE_4. Furthermore, Armstrong et al. [71] showed that IAC-coupled LC–MS/MS is by far less time-consuming than GC–MS/MS which requires HPLC separation of LTE_4, its catalytical desulphurization/reduction and two derivatization steps (reviewed in Ref. [116]), whereas EIA without preceding IAC purification is obviously doomed to failure.

2.2. 3-Nitrotyrosine

Considerable attention has been paid to 3-nitro-L-tyrosine (Scheme 3) because of its potential to function as a biomarker of oxidative/nitrative stress. 3-Nitrotyrosine occurs both as a soluble amino acid (NO_2Tyr) and as a residue in proteins ($NO_2TyrProt$). Many different methods have been reported for NO_2Tyr and $NO_2TyrProt$ so far, with the most reliable being GC–MS/MS and LC–MS/MS (reviewed in Refs. [118–120]). It is worth mentioning that $NO_2TyrProt$ is usually measured as NO_2Tyr after enzymatic proteolysis or chemical hydrolysis (acid- or base-catalyzed).

LC–MS/MS quantification of NO_2Tyr and $NO_2TyrProt$ in various biological samples, including plasma, serum, urine, synovial fluid and cerebrospinal fluid (CSF) after IAC extraction of 3-nitrotyrosine on home-made IAC column has been recently reported by Radabaugh and colleagues [81,121]. Regrettably, this group has not reported basal NO_2Tyr concentrations in human plasma or serum. The basal concentration of NO_2Tyr measured in rat plasma of 0.5–4 nM [81] is comparable to that we measured in rat plasma by GC–MS/MS following HPLC separation [122]. The basal concentration of $NO_2TyrProt$ in human plasma ranged between 1 and 5 nM in the study of Radabaugh et al. [81]. This order of magnitude is comparable to that published by most of the reported GC–MS/MS and LC–MS/MS methods that do not include any IAC purification step [119,120]. Nemirovskiy and colleagues have used this IAC-coupled

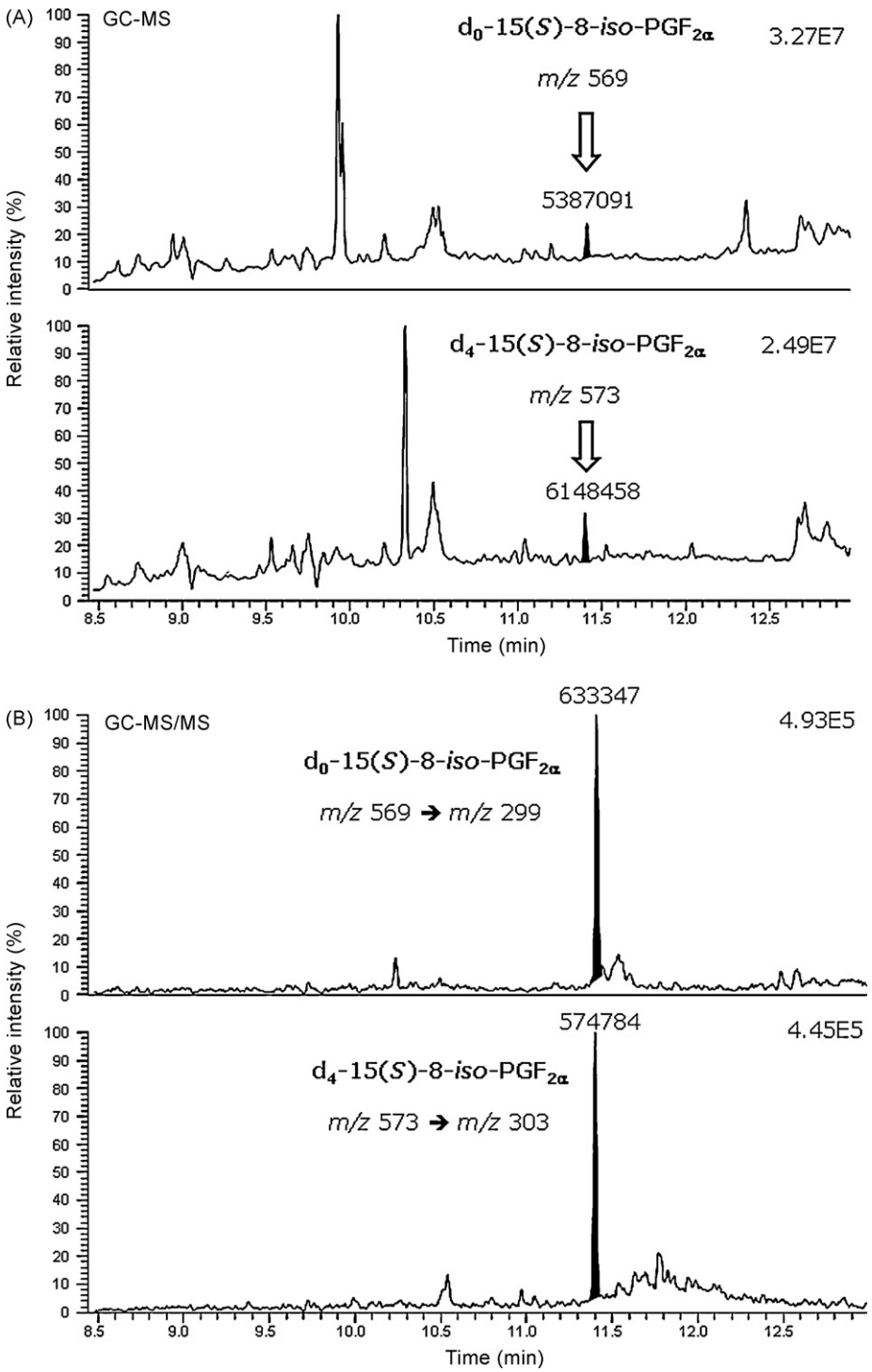


Fig. 2. Partial chromatograms from the GC–MS (A) and GC–MS/MS (B) analysis of free + esterified 15(*S*)-8-*iso*-$PGF_{2\alpha}$ in a human plasma sample after IAC extraction as described elsewhere [99]. See also Fig. 1. 15(*S*)-8-*iso*-$PGF_{2\alpha}$-specific IAC columns were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

LC–MS/MS method to quantify NO_2Tyr in urine and measured concentrations of 0.25–3 nM in healthy humans [121]. Similar NO_2Tyr concentrations, i.e., 1.6–33 nM, have also been measured by us in urine of healthy subjects by GC–MS/MS coupled to preceding NO_2Tyr separation by HPLC [123]. These collaborating findings suggest that urinary NO_2Tyr excretion varies considerably in humans and other species [81,123]. Yet, the reason for this variation remains to be investigated.

The LC–MS/MS chromatograms shown in Fig. 6 suggest that measurement of NO_2Tyr and NO_2TyrProt in biological samples is a difficult undertaking even by the combination of IAC extraction of NO_2Tyr and LC–MS/MS quantification. By contrast, in GC–MS/MS combined with HPLC separation of NO_2Tyr usually only the peaks of NO_2Tyr and its internal standard appear in the chromatogram (see Fig. 7). In the particular case of 3-nitrotyrosine, HPLC seems to be as effective and specific as IAC, but, without any doubt, considerably more time-consuming. Fig. 7 shows representative chromatograms from the GC–MS/MS analysis of HPLC-isolated NO_2Tyr from plasma of rats before and 2 h after milk protein supplementation (see Ref. [122]). It is worthy to mention that the remarkable increase in

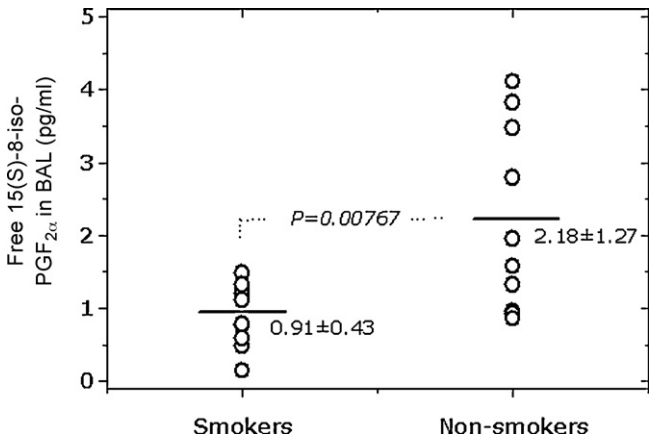


Fig. 3. Concentration of free 15(*S*)-8-*iso*-$PGF_{2\alpha}$ measured in 1-ml aliquots of bronchoalveolar liquid (BAL) obtained from healthy male smokers ($n = 10$) and non-smokers ($n = 11$) by GC–MS/MS after IAC column extraction previously reported for serum and urine samples [99]. Horizontal bars indicate the mean values. 15(*S*)-8-*iso*-$PGF_{2\alpha}$-specific IAC columns were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

plasma NO_2Tyr concentration seen in that study [122] is quite rare when GC–MS/MS and LC–MS/MS methods are applied [119,120], and it can not be excluded that this increase is due to dietary 3-nitrotyrosine.

Tyrosine-nitrated proteins (NO_2TyrProt) are currently in the focus of proteomic studies. Recently, Bischoff and coworkers have reviewed this interesting and challenging topic from a physico-chemical, biological and analytical point of view [124]. Approaches based on mass spectrometry, and sporadically on IAC, have also been used in proteomic studies on tyrosine-nitrated proteins and peptides. Thus far, however, the focus of these investigations has been the identification rather than the quantification of macro-biomolecules [124].

2.3. Toxins

Toxins such as the mycotoxins are natural contaminants in foods and feeds. Major mycotoxins include aflatoxins, ochratoxins, fumonisins, deoxynivalenol and zearalenone (see Scheme 3 and Table 1). These toxins are of particular interest because they can have serious effects on health and have been recognized as a significant source of food-borne illnesses [49,125,126]. From the analytical point of view, the interest in mycotoxins and other toxins has increased considerably during the last decade in comparison to the past 20 years [5,36–43]. For recent reviews of analytical methods of toxins including the mycotoxins see Refs. [80,82,83,115].

Interestingly, single- and multi-toxin IAC has been increasingly used as a cleanup step for the quantitative determination of numerous toxins and their metabolites in foods, feeds, surface waters and other matrices including smoke [45] and human urine [46] by LC–MS or LC–MS/MS at the expense of GC–MS and GC–MS/MS. Furthermore, for reasons of higher compatibility, IAC has been coupled on-line to LC–MS and LC–MS/MS techniques for various analytes including toxins such as ochratoxin A [84], herbicides such as diuron [61], hormones [67], and peptides [86,94].

Substantial efforts have been made for the identification and simultaneous determination of different classes of mycotoxins by LC–MS/MS. Until recently, about 87 mycotoxins have been identified by LC–MS/MS [80]. An interesting method for the simultaneous determination of 11 toxins, i.e., various aflatoxins, ochratoxin A, fumonisins B1 and B2, deoxynivalenol, zearalenone, T-2 and HT-2 toxins, in maize has been reported by Lattanzio et al. [49] who used a commercially available multi-toxin IAC column. Because of differences in chemical structure and polarity, mycotoxins were first extracted by a double extraction procedure using phosphate buffer and methanol, then isolated from the extract by the multi-toxin IAC column, separated by LC and quantified by tandem mass spectrometry with ESI in the negative mode for deoxynivalenol and zearalenone, and in the positive mode for the remaining toxins (Fig. 8). In that study no stable-isotope labelled analogs of the investigated mycotoxins were used as internal standards. Quantification was performed by measuring peak areas in the SRM mode and by using a calibration curve. On the other hand, Spanjer et al. have reported that 33 mycotoxins can be analyzed simultaneously by LC–MS/MS in a 30-min run after a single extraction with acetonitrile–water from various foods [79]. Nevertheless, IAC, being a sophisticated purification technique, increasingly replaces the conventional and by far more less specific SPE technique in routine mycotoxin analysis (see Table 1). It is worth mentioning, that in the past, IAC has been applied almost exclusively by using laboratory-prepared IAC columns, whereas in recent years IAC columns are commercially available for most known mycotoxins [115].

One important issue in IAC is the cross-reactivity which has been recently exemplified and utilized analytically for zearalenone (see Scheme 3) and its metabolites α-zearalenol, β-zearalenol,

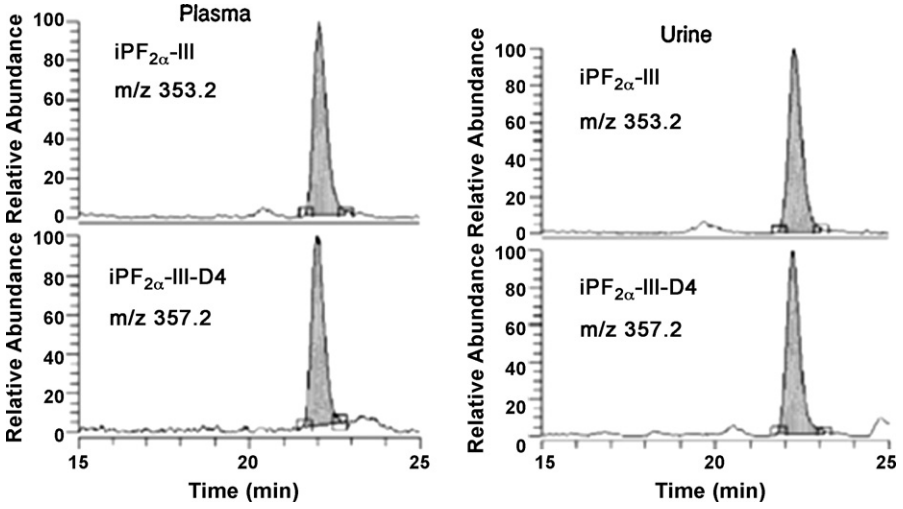


Fig. 4. LC–ESI-MS chromatograms from the analysis of 15(*S*)-8-*iso*-$PGF_{2\alpha}$, also known as $iPF_{2\alpha}$-III [102], in human plasma and urine after IAC column extraction. Fig. 2 from Sircar and Subbaiah [101] with permission.

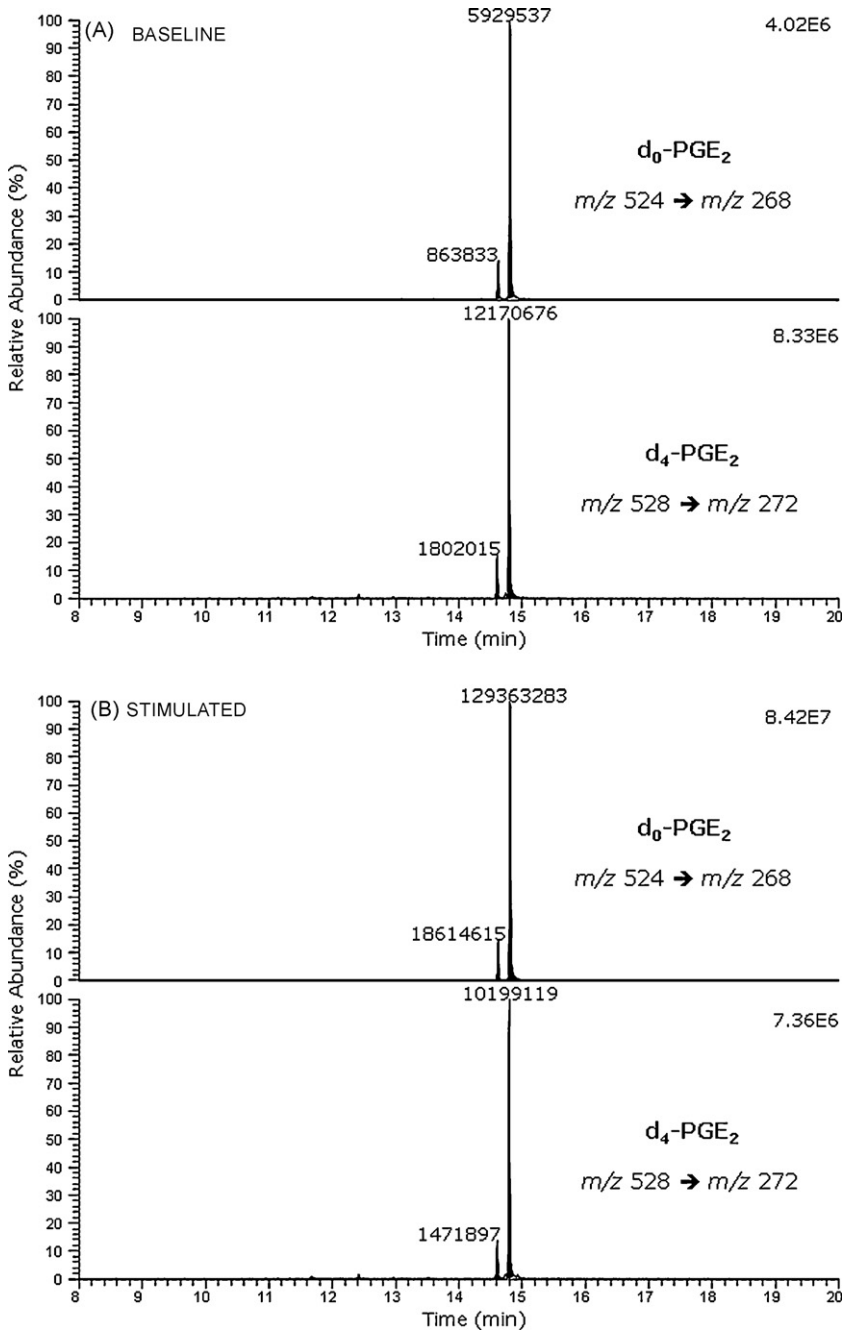


Fig. 5. Partial chromatograms from the GC–MS/MS analysis of PGE_2 in supernatants of airway epithelial cells before (A) and after (B) stimulation. PGE_2 was extracted from a 1-ml sample aliquot by using a commercially available IAC column (Cayman Chemicals, USA) with immobilized antibodies raised towards PGE_2. IAC extraction of PGE_2 was performed as described for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [99]. The IAC eluate (95 vol.% ethanol) was evaporated to dryness, and unlabelled (d_0-PGE_2) and deuterium-labelled PGE_2 (d_4-PGE_2) were converted to their pentafluorobenzyl ester methoxyamine trimethylsilyl ether derivatives [116]. The double peak is due to the syn- and anti-methoxime isomers. PGE_2-specific IAC columns were obtained from Cayman Chemicals (Ann Arbor, MI, USA).

zearalanone, α-zearalanol and β-zearalanol [112–114]. The original antigen zearalenone and its above mentioned metabolites act as cross-reactants in both commercially available [112,113] and home-made [114] IAC columns designed for zearalenone. Despite differences in the extent of cross-reactivity and irrespective of the source, zearalenone IAC columns were found to be useful for the quantitative determination of this estradiol-like compound and its metabolites in various biological samples by LC–MS, LC–MS/MS and GC–MS techniques. However, accurate quantitative analysis of zearalanone and its metabolites requires use of the respective stable-isotope labelled analogs for IAC extraction to compensate for cross-reactivity differences [113] (Fig. 9).

2.4. Steroids, anabolics, hormones, and drugs

In certain analogy to the potential hazard of the food and feed toxins discussed above, the need for doping control in human and animal sports has initiated much scientific work and led to the development of simple, rapid and highly specific analytical methods for steroids and other anabolics (Table 1). This concerns both the unequivocal identification and the accurate quantitative determination of prohibited and misused steroids, anabolics, hormones and drugs, for instance as regulated and prescribed by international agencies such as the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA). The use of IAC for sam-

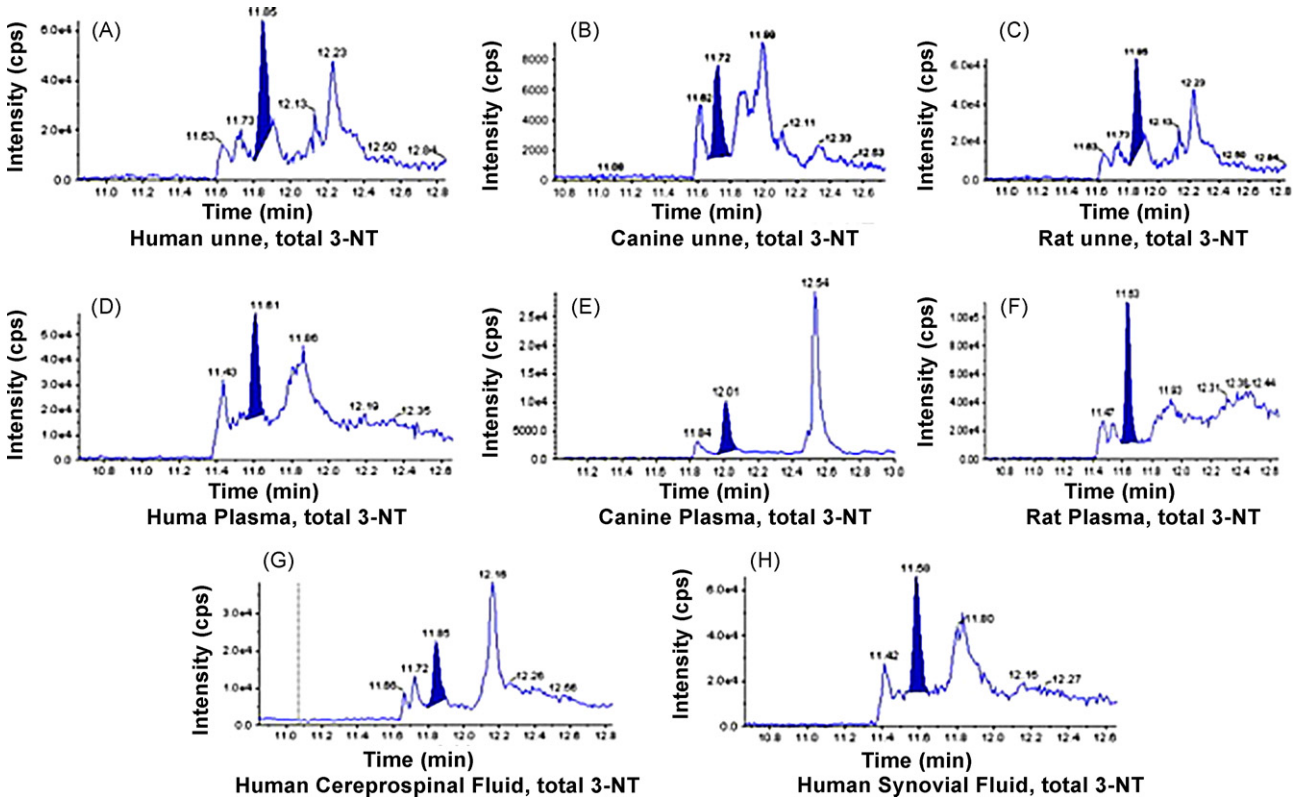


Fig. 6. LC–MS/MS chromatograms from the analysis of 3-nitrotyrosine in various matrices after IAC extraction of free soluble NO_2Tyr or NO_2Tyr obtained from pronase-catalyzed proteolysis. Fig. 4 from Radabaugh et al. [81] with permission.

ple purification of these analytes has constantly increased, with the LC–MS and LC–MS/MS techniques being increasingly and massively replacing other techniques, notably HPLC-UV, GC–MS and immunoassays, which have been frequently used until the 1990s. Nevertheless, the spectrum of analytes for which IAC columns or sorbents are commercially available is still narrow, and IAC columns must still be prepared in the laboratory. Below a few representative examples are discussed.

Boldenone (see Scheme 3) is a common veterinary anabolic agent. Interestingly, by the combination of IAC with LC–MS/MS and GC–MS, Ho et al. [57] provided direct evidence that both boldenone and its sulphate conjugation metabolite occur endogenously in the urine of non-castrated male horses. GC–MS quantification revealed that free boldenone and boldenone sulphate were detectable in post-race urine samples of entire horses (range 0.1–4.4 ng/ml), but they were not detectable in post-race urine samples of geldings [57]. The authors recommended use of GC–MS for reliable quantification of low concentrations of boldenone.

β-Receptor agonists such as clenbuterol (see Scheme 3), salbutamol and cimaterol are used in human and veterinary medicine for the treatment of pulmonary disorders. At supra-therapeutic doses these substances can act as anabolic steroids. This tempts to misuse, notably in farm animals, to increase meat production. Lau et al. [58] reported on the simultaneous quantitative determination of clenbuterol, salbutamol and cimaterol in bovine retina by LC–ESI-MS/MS following commercially available clenbuterol β-receptor agonist IAC column and by using stable-isotope labelled analogs of clenbuterol and salbutamol [58]. Interestingly, salbutamol and cimaterol are extracted by the clenbuterol-designed IAC column due to cross-reactivity (not explicitly reported by the authors) [58].

The phytohormones cytokinins [59], recombinant human erythropoietin (rhErythropoietin) [62,63], insulin and insulin analogs [69,70], gonadotropin-releasing (GnR) hormone [67,68], and melatonin [74] (see Scheme 3) have been analyzed qualitatively and in part quantitatively by IAC and LC–MS/MS or CE–MS/MS. Fig. 10 shows impressively the high specificity and analytical superiority of the IAC extraction to the conventional SPE as a purification step in the LC–MS analysis of melatonin in a human serum sample [74]. The concentration of melatonin in serum samples of five male volunteers (27–34 years of age) 1 h before the end of the dark period was determined to range between 136 and 305 pM by this IAC-coupled LC–MS method using [*O-methyl*-$^2H_3$]melatonin as the internal standard. Various melatonin-related compounds including the melatonin metabolite 6-hydroxymelatonin showed minimal (<0.02%) or very low (2.6%) cross-reactivity against the home-made anti-melatonin antibody [74].

2.5. DNA and protein adducts – protein biomarkers

Exogenous and endogenous alkylating agents, such as various aldehydes including 4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA), attack nucleophilic functionalities of DNA, peptides, proteins and other biomolecules to yield a variety of reaction products. DNA- and protein-adducted aldehydes are commonly used as biomarkers of oxidative stress. Analogous to 3-nitrotyrosine (see above), the concentration of MDA and other electrophiles adducted to DNA, peptides and proteins is very low, for instance one adduct in 10^7 to 10^9 parent DNA bases, which represents a formidable analytical challenge. IAC of adducted biomolecules prior to analysis turned out to be a very effective separation method for highly specific and sensitive quantitation, primarily by GC–MS until the end of the 1990s [5,23], and increasingly by LC–MS/MS in the last decade (Table 1). However, preparation of specific/selective IAC material requires knowledge of the structures of the target analytes. This represents a serious obstacle, given their large number.

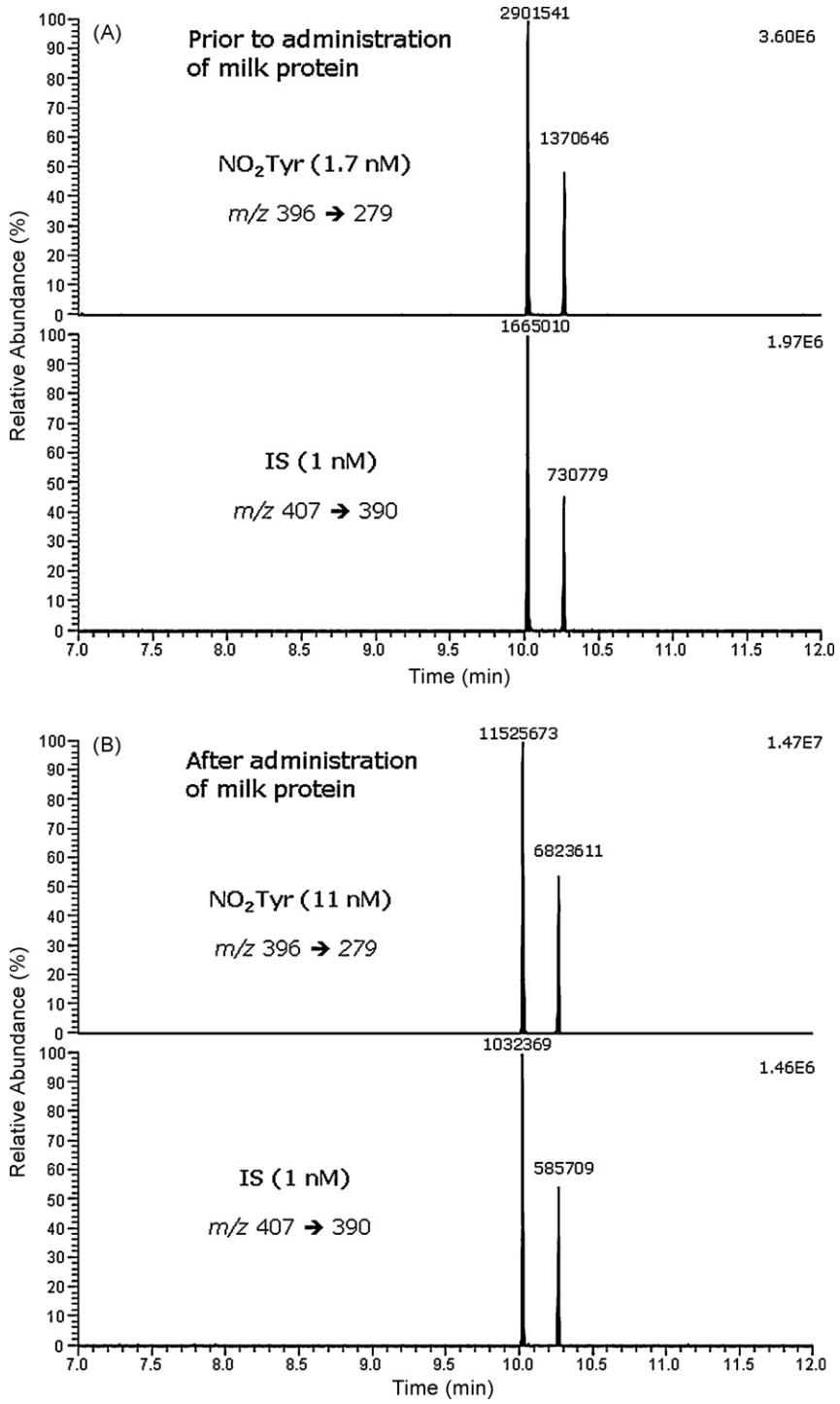


Fig. 7. Chromatograms from the GC–MS/MS analysis of free soluble 3-nitrotyrosine (NO_2Tyr) in plasma of a rat prior to (A) and after (B) administration of milk protein. The internal standard (IS) was [$^{15}N_2$,$^{13}C_9$]-3-nitrotyrosine. The whole animal study and the GC–MS/MS method have been reported in Refs. [122,123], respectively. Note that the GC–MS/MS method involves a HPLC step but not an IAC step for sample purification. The *n*-propyl-pentafluoropropionyltrimethylsilyl ether derivatives elutes as a double peak due to thermally induced isomerization of the derivatives of unlabelled and stable-isotope labelled NO_2Tyr.

Therefore, it is not surprising that most of the reported applications are based on in-house prepared IAC materials for structurally elucidated adducted DNA, peptides and proteins (Table 1). Below, some representatives are discussed.

Fenaille et al. [72] reported that IAC sorbent extraction using anti-HNE or anti-dinitrophenyl antibodies is useful for the specific isolation and enrichment of HNE and MDA adducts of peptides and proteins prior to LC–MS/MS characterization, i.e., for qualitative purposes.

Otteneder et al. [73] reported on the development and application of a stable-isotope dilution LC–MS/MS technique suitable for the quantitative determination of MDA–deoxyguanosine adduct in rat urine after SPE and IAC extraction. Fig. 11 shows an LC–ESI-MS/MS chromatogram obtained from the analysis of deoxyguanosine-adducted MDA added to a rat urine sample at a final concentration of 50 pM, with the [7,9-^{15}N,^{13}C]-labelled internal standard of MDA–deoxyguanosine being added at 200 pM. Otteneder et al. [73] have applied their method to measure the

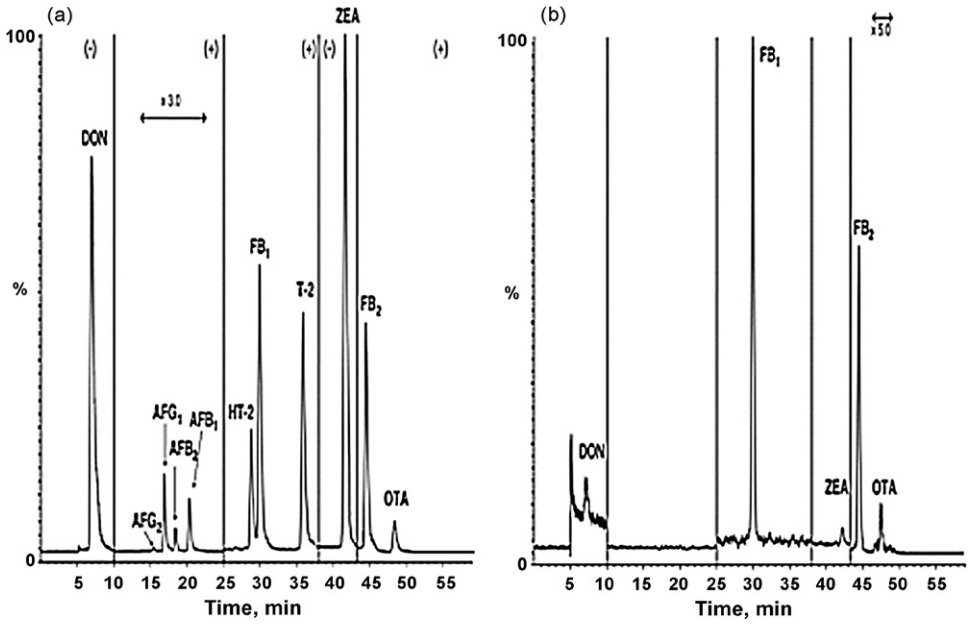


Fig. 8. LC–MS/MS chromatograms from the simultaneous determination of 11 mycotoxins in unspiked and spiked maize after double extraction and multi-toxin IAC extraction. DON, deoxynivalenol; AFB1, AFB2, AFG1, AFG2 means aflatoxin B1, B2, B3 and B4, respectively; FB1, fumonisin B1; FB2, fumonisin B2; ZEA, zearalenone; OTA, ochratoxin. (−) and (+) means positive and negative ESI. Fig. 1 from Ref. [49] with permission.

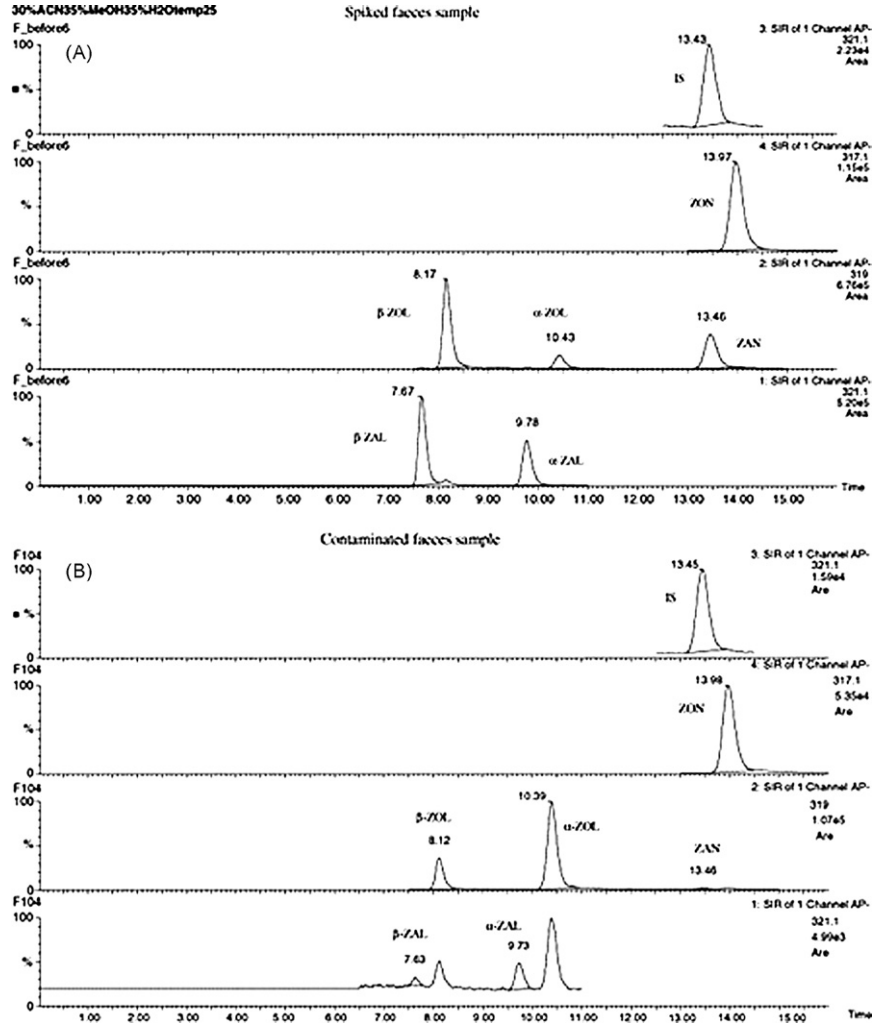


Fig. 9. LC–APCI-MS chromatogram from the analysis of unspiked and spiked horse faeces for zearalenone and its metabolites. Fig. 5 from Ref. [113] with permission.

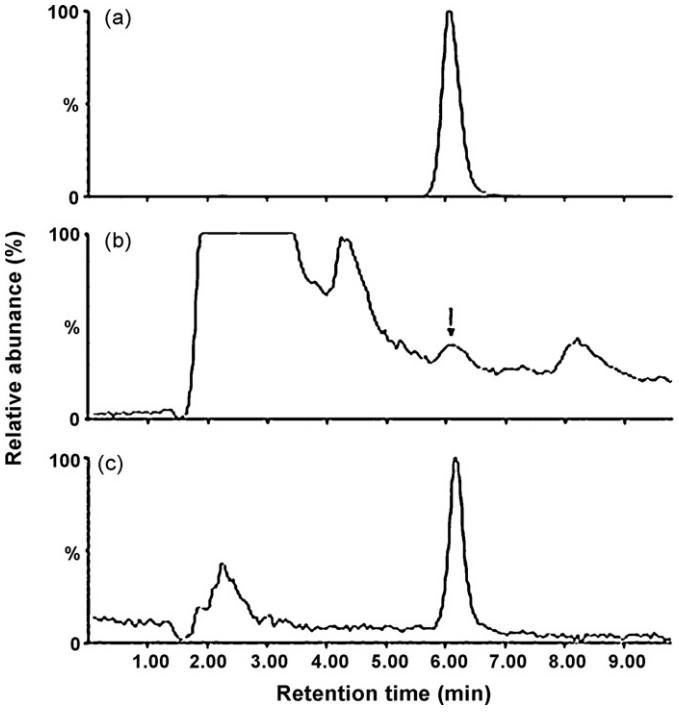


Fig. 10. LC–ESI-MS chromatograms from the analysis of synthetic melatonin (a) and of a human serum sample after SPE (b) or melatonin-IAC extraction (c). Fig. 2 from Ref. [74] with permission.

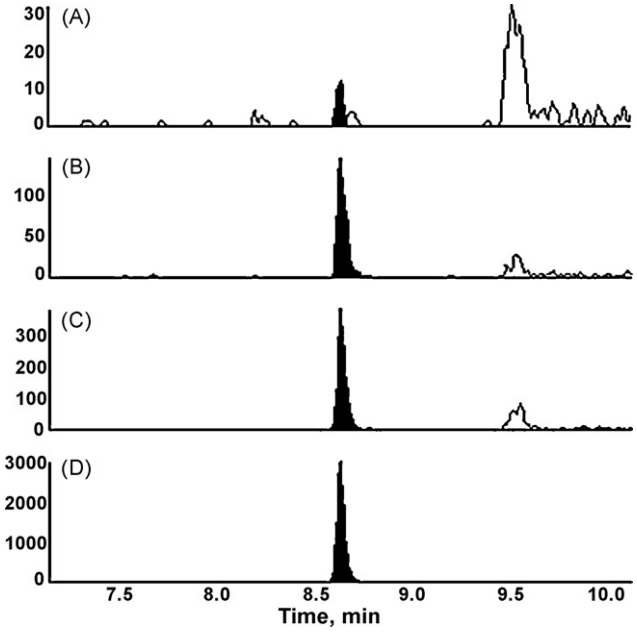


Fig. 12. Chromatograms from the quantitative determination of the 45-mer peptide uTIINE in human urine by using a stable-isotope dilution LC–ESI-MS/MS technique following IAC separation. Trace (A): blank elution buffer; trace (B): uTIINE standard at 156 pg/ml; trace (C): unspiked urine sample containing 338 pg/ml; trace (D): the custom-synthesized stable-isotope labelled internal standard peptide. Fig. 3 from Ref. [93] with permission.

major MDA–deoxyguanosine adduct (M_1GdR) in urine of untreated rats and of rats orally administered with a single dose of CCl_4 (100 or 1000 mg/kg body weight). Despite an LOQ value of the IAC-coupled LC–MS method of about 100 pM of M_1GdR (analyzed as 5,6-dihydro derivative) in urine, these authors did not find this metabolite in urine of control or CCl_4-intoxicated rats.

As has been rightly concluded by Ackermann and Berna in their review article on coupling IAC with MS techniques for low-abundance protein biomarkers [94], the field of proteomics is rapidly turning towards targeted MS method, i.e., to quantitative determination of structurally known proteins using stable-isotope labelled proteins as internal standards. Yet, quantitative IAC proteomics seems to be still in its infancy (see Table 1).

It is worth mentioning a thoroughly validated IAC-coupled LC–MS/MS method reported recently by Li et al. [93] for the quantification of the endogenous major collagen type II neoepitope (uTIINE) peptide in human urine. uTIINE is considered a biomarker of matrix metalloproteinase activity and osteoarthritis in human urine. This method was applied to measure basal concen-

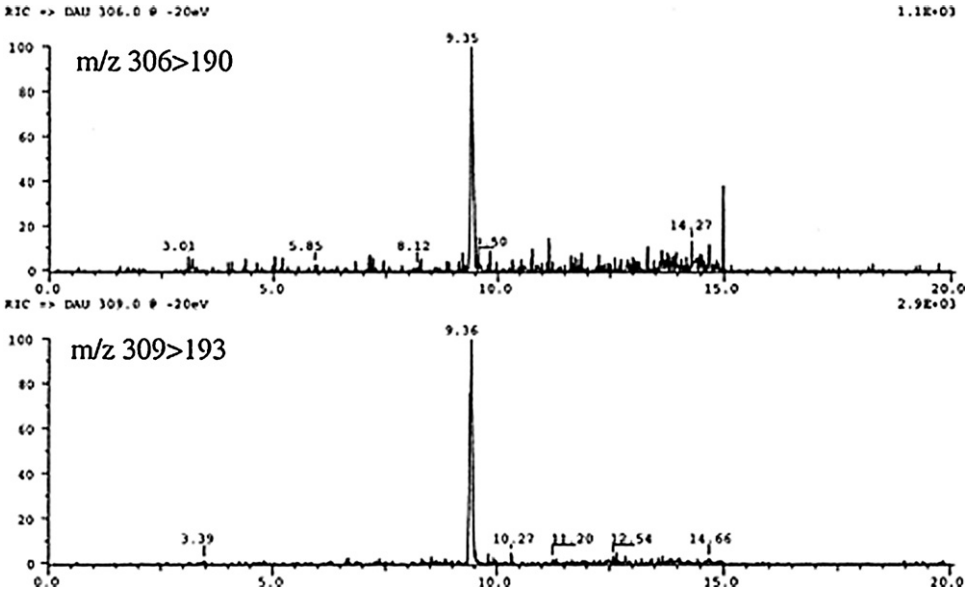


Fig. 11. LC–ESI-MS/MS chromatogram obtained from the analysis of deoxyguanosine-adducted MDA added to a rat urine sample at a final concentration of 50 pM. Fig. 4 from Ref. [73] with permission.

trations of uTIINE in normal subjects and osteoarthritic patients (Fig. 12), as well as to evaluate the clinical utility of uTIINE as a biomarker of osteoarthritis [93]. By means of this reportedly accurate and precise IAC-coupled LC–MS/MS method, Li et al. [93] found considerable intersubject variability of the creatinine-corrected urine concentration of uTIINE (0.05–0.8 ng uTIINE per μmol creatinine).

3. Conclusions and outlook

Analytical methods based on MS in combination with on-line GC or LC separation, i.e., GC–MS, GC–MS/MS, LC–MS and LC–MS/MS, are highly efficient and reliable analytical techniques for qualitative and quantitative analyses of numerous natural and synthetic LMM and HMM substances in biological systems. However, despite the inherent sensitivity and selectivity especially of the GC–MS/MS and LC–MS/MS variants, the quality of the analytical result is greatly dependent upon the applied sample preparation procedures (Scheme 1). These approaches aim at eliminating potential interferences, isolating selectively structurally closely related analytes, or preferably at specifically isolating the target analyte and its stable-isotope labelled analog that serves as internal standard, and eventually at minimizing matrix effects. Expectedly, sample preparation procedures that include IAC extraction of a single target analyte (i.e., use of a single antibody immobilized on the IAC material) or of a group of target analytes (e.g., use of multiple antibodies immobilized on the IAC material or of antibodies with cross-reactivity) turned out to be the most appropriate separation steps for MS-based analysis. Numerous published analytical papers in the past 30 years convincingly demonstrated the overwhelming superiority of the combined use of IAC extraction and MS/MS separation for accurate quantitative measurement of various substances in biological systems.

IAC extraction of endogenous and exogenous analytes even from very complex biological samples such as plasma is commonly performed in a routine fashion very easily and safely. IAC is very compatible with LC–MS/MS and can be automated to a high degree. So far, there are no reports that target analytes and their stable-isotope labelled analogs behave differently on IAC sorbents, unlike in HPLC and GC where part or even complete separation of analyte and internal standard may occur. The user needs only to take in consideration the potential cross-reactivity of the immobilized antibody for related substances and the capacity of the IAC column for the analyte and its internal standard. This information is available for IAC material from commercial sources. Regarding cross-reactivity and binding-capacity, homemade IAC material needs to be characterized experimentally prior to use.

When comparing two different methodologies that involve chromatographic and immunologic separation techniques, discrepancies in analyte concentrations are highly expected and need to be properly considered in study evaluation. Due to the high specificity of the immobilized antibody, for instance 15(*S*)-8-*iso*-$PGF_{2\alpha}$-IAC columns do not possess analytically relevant affinity to 15(*R*)-8-*iso*-$PGF_{2\alpha}$ and other possible isomers that may co-elute and thus interfere in GC–MS/MS and LC–MS/MS, it is important to recognize that the combination of IAC and MS would provide distinctly different results than a combination of regular SPE and/or TLC and MS approaches. This has been demonstrated for 15(*S*)-8-*iso*-$PGF_{2\alpha}$ [99] and is important in method comparison as well as for generating reference values and intervals for biomarkers.

Perhaps with the exception of toxins [115], until the present day, the major limitation to IAC is still the absence of commercial sources for immunoaffinity resins for the majority of requested analytes. In consideration of the high specificity/selectivity of the IAC technique, commercialization of immunoaffinity resins and IAC columns for a much larger number of analytes is still a task for industrial firms. On the other hand, analysts are encouraged to make much more use of the currently commercially available IAC material despite the clearly higher primary costs in comparison to conventional SPE cartridges such as octadecyl silica (ODS). IAC columns designed for different analytes are reusable for many times without real loss of analytical quality (see Table 1), so that finally the use of IAC columns or immunosorbents may be even less expensive than the use of regular SPE cartridges. In any case, the analytical profit from using IAC materials in quantitative analysis of LMM and HMM biomarkers, drugs and toxins by MS-based approaches would be greatest. Especially in LC–MS/MS, renunciation of any sample preparation step is very tempting. However, literature reports advice us not to save at the wrong end.

Nomenclature

APCI	atmospheric pressure chemical ionization
API	atmospheric pressure ionization
BAL	bronchoalveolar liquid
CE	capillary electrophoresis
CID	collision-induced dissociation
CSF	cerebrospinal fluid
EIA	enzyme immunoassay
ESI	electrospray ionization
GC–C-IR-MS	gas chromatography-combustion-isotope ratio mass spectrometry
HMM	high-molecular mass
HNE	4-hydroxy-2-nonenal
HRMS	high-resolution mass spectrometry
IAC	immunoaffinity chromatography
ID	isotope dilution
IS	internal standard
LC–IT-MS	liquid chromatography-ion trap-mass spectrometry
LLE	liquid–liquid extraction
LMM	low-molecular mass
LT	leukotriene
MDA	malondialdehyde
MS	mass spectrometry
NO_2Tyr	soluble free 3-nitrotyrosine
NO_2TyrProt	3-nitrotyrosine residue in proteins
PG	prostaglandin
QC	quality control
RIA	radioimmunoassay
SIM	selected-ion monitoring
SPE	solid-phase extraction
SPME	solid-phase microextraction
SRM	selected-reaction monitoring
SSQ	singe-stage quadrupole
TLC	thin-layer chromatography
TSQ	triple-stage quadrupole

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work of many other scientists could not be considered. The author deeply apologizes for this inconvenience.

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