

## Use of LC-MS/MS for xenobiotic metabolism studies in animals

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The use of LC-MS/MS in the identification of xenobiotic metabolism pathways in animals is described, on the basis of selected examples in the field of toxicologically relevant growth promoting agents. The structural elucidation of xenobiotic metabolites in complex biological matrices is achieved using various analytical methods and strategies using LC-MS with ESI or APCI ionisation techniques in combination with in-source CAD or MS/MS experiments. The use of some particular analytical tools (*e.g.* H/D exchange or MS<sup>n</sup> multisequential experiments) for solving specific structural ambiguities is also discussed.

### Introduction

Xenobiotics are defined as foreign compounds which are absorbed either intentionally or accidentally by animals or plants, and which lead to some physiological modifications. Thus, this term concerns a very wide range of chemical compound classes, including *e.g.* drugs, food additives, environmental contaminants or pesticides, each of which often requires the development of specific analytical strategies. Following their absorption, the biotransformation of xenobiotics is achieved *via* two principal classes of enzymatic systems (mainly localised in the liver for mammals), leading to the so-called phase I and phase II metabolites [1,2]. Phase I metabolites result from reactions of oxydation,

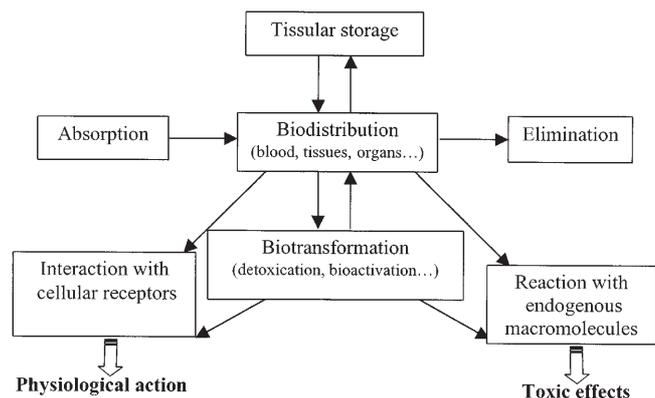


Figure 1. General description of xenobiotic fate in living organisms.

reduction or hydrolysis of the absorbed compound. Phase II metabolites are the result of the conjugation of an endogenous molecule (*i.e.* acetylation, conjugation to glucuronic acid (glucose for plants), glutathione, sulfate...) to the parent compound or to its phase I metabolites. These various processes often lead to the elimination of the formed metabolites, but they can also produce reactive metabolites, which may disrupt the cellular functioning [3] (see Fig. 1).

### The study of xenobiotic metabolism: an analytical challenge

These various metabolic steps often yield a mixture of metabolites, which can be very different from each other on a quantitative as well as on a qualitative point of view (lipophilicity, pKa, molecular weight...). Furthermore, the xenobiotic molecule and/or its metabolites may be biologically active at very low concentration levels in biological fluids. For this reason, the elucidation of xenobiotic metabolism necessitates highly sensitive and specific analytical tools. Modern mass spectrometry gathers these two features owing to the developments made in this field, more particularly over the past decade, as indicated by several review articles [4] or book chapters [5,6,7] covering this topic. This is principally due (i) to the introduction of soft ionisation techniques such as ESI, APCI or MALDI, which have rendered polar, unvolatile and labile compounds amenable to mass spectrometry and (ii) to the spectacular development of very sensitive and powerful mass analysers such as quadrupole ion traps or time of flight analysers. These technological developments have made the LC-MS coupling a robust routine technique, which has rapidly become a technique of choice in the biological and biochemical fields. In this context, the general analytical strategies for metabolite identification have moved from off-line strategies involving metabolite isolation and purification by peak collection in metabolic profiles towards on-line purification and

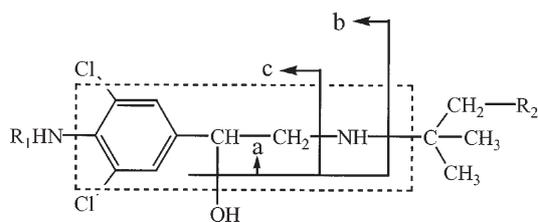
separation steps coupled to the mass spectrometric analysis of the xenobiotic related metabolites.

In this paper, we will illustrate the use of LC-MS methods for structural elucidation in the field of xenobiotic metabolism studies conducted on animals in the context of food safety. Examples will be treated from several selected applications focused on growth promoting agents used in farm animals (*i.e.* beta-agonists and estrogens), which may exert a toxicological impact on the meat consumer at the end of the food chain.

### Beta-agonists

Beta-agonists are used as bronchodilators in the treatment of pulmonary diseases in human and animal therapeutics, and they also act as strong repartitioning agents, as a result of reduced protein catabolism and enhanced lipolysis [8]. For this reason, they are considered as doping agents in humans and their use as growth promoting agents in farm animals is severely regulated, if not prohibited. However, several cases of human intoxication have been related to the consumption of liver from beta-agonist illegally treated animals [9,10]. Many analytical methods have been developed for the detection of beta-agonists in biological fluids, using formerly GC-MS [11] or GC-MS/MS [12] as the most powerful characterisation techniques. In the beginning of the 90's, the emerging technology of atmospheric pressure ionisation techniques like electrospray coupled to liquid chromatography [13,14] and to capillary electrophoresis [15] showed very promising results both for the detection and the structural characterisation of beta-agonists. Under collisional activation carried out either in the collision cell of a triple quadrupole or in the skimmer region of an atmospheric pressure ionisation source [16], the beta-agonists molecular species produced by electrospray [16] or APCI [17,18] dissociate according to common and specific decomposition pathways, which can be used for the LC-MS detection of these compounds in biological fluids by selected decomposition reaction monitoring [19]. These characteristic decomposition pathways are presented in figure 2 for clenbuterol, the most widely used beta-agonist in animals. They mainly concern an initial dehydration of the protonated molecule, and consecutive eliminations of the alkyl side chain (*tert*.butyl for clenbuterol) and ammonia. These fragmentation pathways also apply to beta-agonist metabolites and were used for their structural identification in metabolism studies conducted *in vitro* on rat [19], pig [20] or bovine [19,21] liver preparations or *in vivo* in rat [22,23] and bovine [24] treated with clenbuterol.

In these studies, the starting material consisted of either the incubation media or the biological matrices (urine, tissues...) from *in vitro* or *in vivo* experiments, respectively. In all cases, the sample was in general simply submitted to centrifugation, and evaporation to dryness before the reversed phase HPLC separation of the various metabolites, carried out either on-line [19,21] (LC-MS) or off-line [20,22] (peak



	MH <sup>+</sup> , <i>m/z</i>	a, <i>m/z</i>	b, <i>m/z</i>	c, <i>m/z</i>
<b>Clenbuterol</b>	277 (282)	259 (262)	203 (206)	186 (188)
<b>1</b> R <sub>1</sub> = H, R <sub>2</sub> = OH	293 (299)	275 (279)	203 (206)	186 (188)
<b>2</b> R <sub>1</sub> = OH, R <sub>2</sub> = H	293 (298)	275 (278)	219 (222)	202 (204)

**Figure 2.** Chemical structure and characteristic fragmentation patterns of clenbuterol metabolites (dashed line : general structure of beta-agonists). In parenthesis, the *m/z* values obtained from H/D exchange experiments carried out in MeOD/D<sub>2</sub>O.

collection) with their mass spectrometric characterisation (mainly achieved using electrospray ionisation). An additional solid phase extraction step was generally carried out before the HPLC analysis for samples obtained from *in vivo* experiments [22,23,24].

Hence, the main metabolic pathways of clenbuterol could be established on the basis of the collisional-activated dissociation (CAD) spectra obtained from the metabolites. They involved the N-dealkylation of the parent compound and further oxidative cleavages of the clenbuterol side chain, the formation of glucuronic acid [21,22] as well as mercapturic acid [25] conjugates, and enzymatic hydroxylations occurring at different sites of the molecule [20,21,22]. This latter feature was particularly studied because positional isomers could be generated, which toxicological relevance could be very different. Indeed, a new and major metabolic pathway of clenbuterol involving the *in vivo* formation of a N-hydroxy-arylamine [23] could be identified. The unambiguous identification of this metabolite imposed to solve some structural features concerning the precise hydroxylation site. Hence, H/D exchange experiments were designed for the determination of the number of exchangeable hydrogen atoms. These experiments also provided valuable information on fragmentation mechanisms, which were very informative in the structural elucidation of clenbuterol metabolites [26].

The positive ESI-MS spectra obtained from the two different hydroxylated clenbuterol metabolites **1** and **2** (Fig. 2) in a MeOH-H<sub>2</sub>O mixture exhibited MH<sup>+</sup> species at *m/z* 293 for both derivatives. Their fragmentation pattern was characteristic of those described for beta-agonistic drugs [16,17] and allowed to identify metabolite **1** as the result of a hydroxylation on the *tert.*butyl side chain of the parent compound (Fig. 2). On the other hand, the fragment ions obtained from the collisionally activated dissociation of the isomeric metabolite **2** did not allow to determine the exact hydroxylated site of the molecule (*i.e.* the nitrogen atom of

the aromatic amine or one of the aromatic carbons), since the **c** (*m/z* 202) fragment ion (Fig. 2) was not discriminant in this case. The use of H/D exchange experiments in MeOD-D<sub>2</sub>O mixtures showed that the molecular ion was shifted from a MH<sup>+</sup> species (*m/z* 293) to a M<sub>0.5</sub>D<sup>+</sup> species (*m/z* 299) for metabolite **1**, or to a M<sub>0.4</sub>D<sup>+</sup> species (*m/z* 298) for metabolite **2**. In the latter case, the number of exchangeable hydrogen atoms was consistent with an aromatic hydroxylamine whereas the formation of a phenolic site by direct hydroxylation of the aromatic ring should have give rise to the presence of one more exchangeable hydrogen. Collisional activation of the protonated and deuterated molecular species of each derivative also produced characteristic fragments and were used as an additional tool for their differentiation. Metabolite **2** was of particular toxicological interest because of the involvement of N-oxidation pathways in various toxicological mechanisms, which result from the binding of reactive metabolites to DNA or proteins. In addition, from an analytical point of view, the chemical instability of this compound prevented from making extensive sample clean-up prior to the mass spectrometric analysis. Therefore, the use of LC-MS afforded an efficient way for the identification of such an unstable metabolite by using minimum sample preparation.

## Estrogens

Estrogens constitute an important family of endogenous steroid hormones, which play a major role in the regulation of various physiological processes. Besides, when administered as xenobiotics to meat producing animals, they act as growth promoting agents and 17β-estradiol is widely used for this purpose in several countries. However, this molecule is also known to generate genotoxic compounds [27,28], and thus, the assessment of the toxicological impact of this molecule on the meat consumer is of particular interest. We will present here some results illustrating the role of liquid chromatography coupled to ion trap tandem mass spectrometry in the understanding of estrogen metabolic pathways in the bovine. Because of their potential toxicological relevance, two metabolic pathways will be emphasised, *i.e.* (i) the esterification of the C17 hydroxyl function of estradiol by long chain fatty acids [29] giving rise to estradiol esters which can be stored in fat and act as pro-estrogenic species, and (ii) the formation of catechol estrogens, which further quinoid oxidation products are known to induce DNA alteration processes [27,28,30].

### Estradiol fatty acid esters

Estradiol fatty acid esters extracted from the bovine fat were analysed by LC coupled to negative ESI-MS/MS. The chromatographic separation was carried out by reversed-phase HPLC using pure methanol as the mobile phase, and a post-column addition of an isopropanol-ammonia mixture was made in order to enhance the negative ionisation efficiency [31]. In these conditions, the CAD mass spectra of the

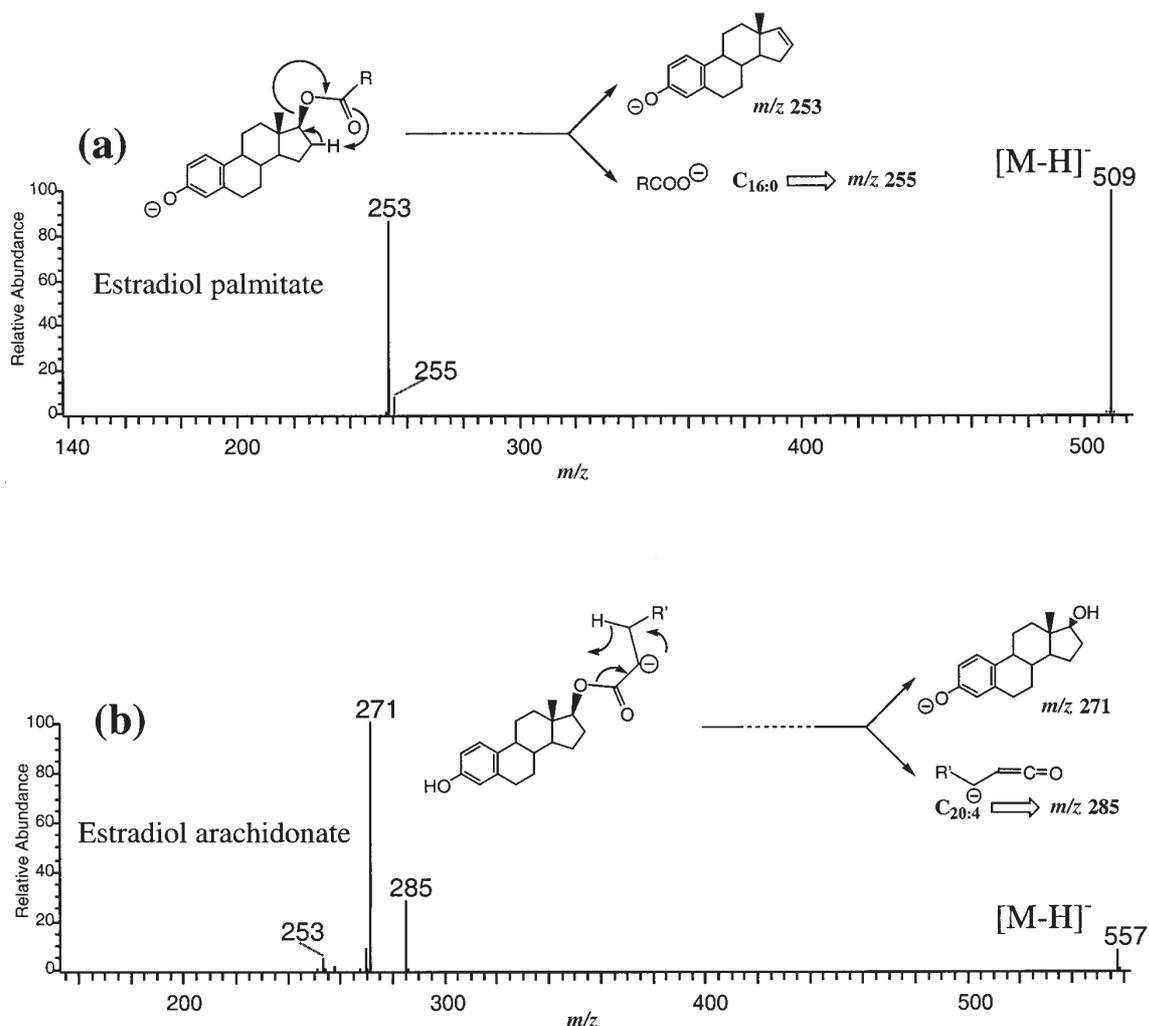


Figure 3. CAD spectra and decomposition mechanisms of ESI-produced  $[M-H]^-$  ions of estradiol fatty acid esters<sup>31,34</sup>.

various fatty acid ester  $[M-H]^-$  ions displayed diagnostic fragment ions for both the steroid moiety and the fatty acid part of the molecule. For example, as shown in figure 3a, the decomposition of estradiol palmitate led to a  $m/z$  253 daughter ion corresponding to the steroid, whereas the fatty acid side chain appeared as its carboxylate ion at  $m/z$  255. In the case of estradiol arachidonate, the situation was different and led to the phenoxy ion of estradiol at  $m/z$  271 and to a ketene anion form of the side chain at  $m/z$  285 (Fig. 3b), meaning that these derivatives decomposed according to different pathways. Indeed, for such bifunctional compounds, deprotonation competitively took place at both acidic sites of the molecule, *i.e.* the phenolic function of the steroid and the enolisable position of the fatty acid ester side chain (Fig. 3) [32,33]. The regioselectivity of the deprotonation depended on the chain length and the degree of unsaturation of the esterifying fatty acid, and gave rise to different pairs of complementary fragment ions (Fig. 3) which were

generated *via* the formation of ion dipole complex intermediates [34]. Results obtained from the analysis of a series of estradiol long chain fatty acid esters showed that for saturated short chain fatty acid esters like palmitate or stearate, the first mechanism involving the phenoxide parent ion was favoured, whereas in the case of polyunsaturated long chain fatty acids such as arachidonate, the initial deprotonation took place preferentially on the fatty acid side chain. For intermediate species like estradiol linolenate, both competitive processes could be observed. These two decomposition pathways were used for the identification of different estradiol fatty acid esters in bovine fat, by monitoring the appropriate selected decomposition reaction, allowing to evidence the occurrence of estradiol stearate, oleate, palmitate, linoleate, arachidonate and linolenate in bovine fat [31]. Thus, LC-MS/MS provided product ion data allowing the structural identification of the analysed metabolites, according to a classical analytical approach in xenobiotic LC-MS

studies, *i.e.* obtaining and interpreting CAD spectra in a first step in order to perform the most efficient targeted product ion experiments for the identification of the metabolites in biological matrices.

### Catechol estrogen derivatives

In xenobiotic metabolism studies, *in vitro* experiments using incubations of the parent molecule with hepatocytes, microsomes or tissue slices represent a powerful tool, which often accelerates the identification of metabolites because they offer the ability to rapidly generate metabolites in a relatively clean matrix [6]. For the assessment of catechol estrogen formation at the cellular level, isolated rat hepatocyte incubations were used. The formation of such derivatives can be evidenced either by their direct detection or by scrutinising their subsequent biotransformation products, *i.e.* glucuronide or glutathione conjugates as well as O-methoxylated derivatives.

After centrifugation of the incubation media, the supernatant was directly injected onto a C18 reversed phase HPLC column operated under a classical water (0.5 % acetic acid) – acetonitrile gradient elution. When radio-labelled molecules were concerned, a post-column splitting could be operated in order to achieve the radioactive detection of the metabolites together with their mass spectrometric characterisation. In this study, both ESI and APCI ionisation techniques were used for the determination of polar as well as apolar metabolites [35]. Using ESI, the catechol estrogen derivatives of estradiol and estrone could be detected as their  $[M-H]^-$  ions at  $m/z$  287 and 285, respectively. Their corresponding CAD spectra were identical to those of standard 2-hydroxylated compounds, whereas no metabolite resulting from a hydroxylation at position C4 of the steroid was observed. In addition, the glucuronide ( $m/z$  463 and 461) and glutathione ( $m/z$  592 and 590) conjugates of these derivatives were detected and were clearly identified on the basis

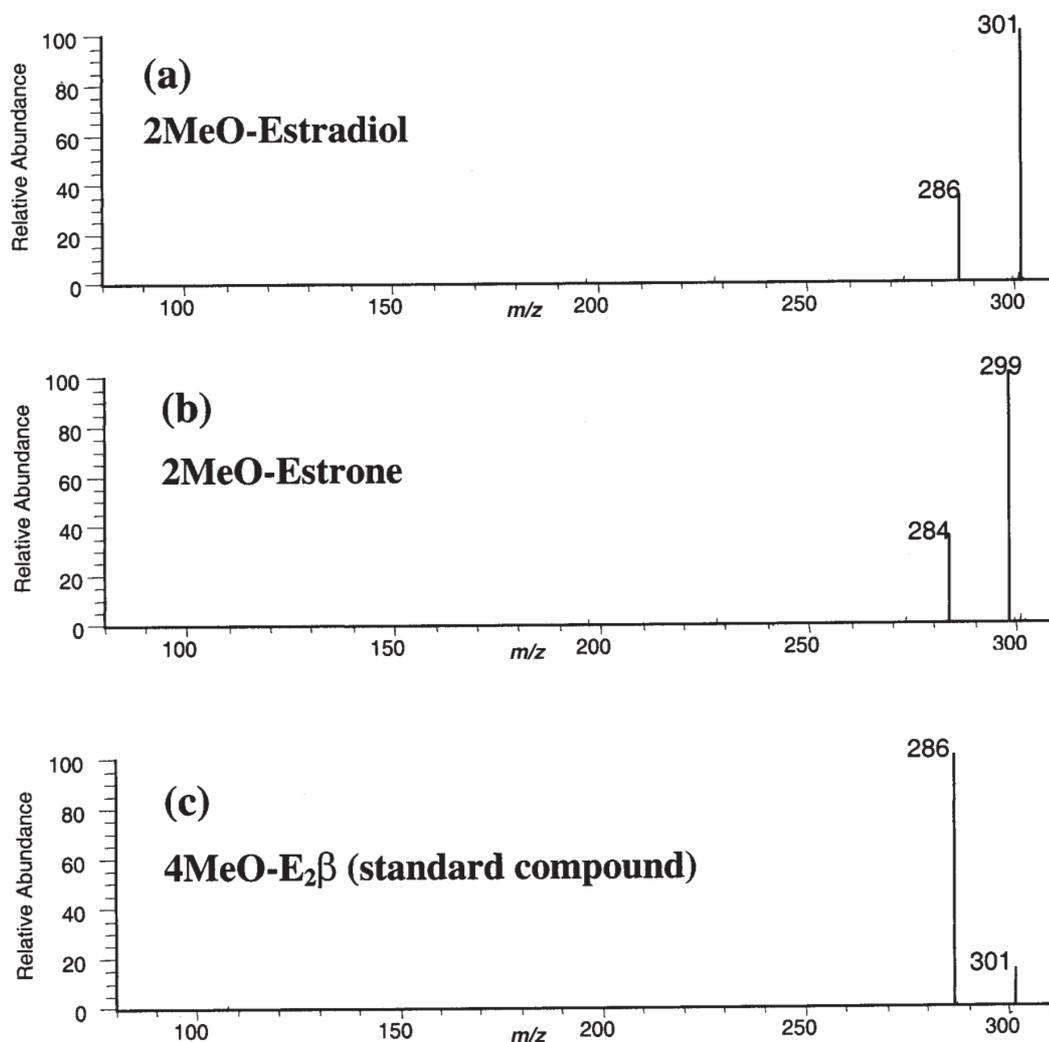


Figure 4. CAD spectra of APCI-produced  $[M-H]^-$  ions from (a) 2-methoxy estradiol, (b) 2-methoxy estrone and (c) 4-methoxy estradiol.

of their CAD spectra obtained by MS/MS experiments [35,36,37].

Negative APCI proved to be more suited than ESI for the detection of less polar metabolites like methoxylated catechol estrogens. The CAD mass spectra obtained from the selected  $[M-H]^-$  ions of the methoxylated catechol derivatives of estradiol and estrone ( $m/z$  301 and 299) are presented in figure 4a and 4b, respectively. Here again, tandem mass spectrometry allowed to identify these metabolites as the 2-methoxy derivatives of estradiol and estrone, respectively, on the basis of their fragmentation pattern which differed from the 4-methoxy isomers in the same collision conditions, concerning in particular the stability of the molecular species (Fig. 4a, 4c). The LC-MS methods developed were subsequently used for the identification of estradiol metabolites in edible tissue extracts (liver, kidney, muscle) prepared from  $^{14}C$  estradiol treated steers. In these *in vivo* experiments,  $17\alpha$ -estradiol and  $17\beta$ -estradiol-3-glucuronide were identified as the main metabolites of  $17\beta$ -estradiol in bovine liver also. In some particular cases, the high-resolution power and mass accuracy attainable with time of flight instruments can also be of great importance in solving structural problems [38]. Concerning estradiol metabolism, exact mass measurements performed on the  $MH^+$  ions generated from  $17\beta$ -estradiol-3-methylether ( $C_{19}H_{27}O_2$ ; calculated mass: 287.2011, measured mass: 287.2013) and hydroxyestrone ( $C_{18}H_{23}O_3$ ; calculated mass: 287.1647, measured mass: 287.1656), allowed to easily distinguish estradiol metabolites exhibiting the same nominal mass ( $MW = 286$ ) but different elementary formula.

### DNA adducts

Limited in the past few years to the study of circulating or excreted metabolites, the recent developments of the LC-MS coupling technologies now allows the study of metabolic pathways leading to the so called bound metabolites resulting from the bioactivation of the parent xenobiotic compound and the subsequent covalent modification of macromolecules by reactive metabolic species, which may affect the enzymatic or genetic equipment of the cell. For example, the metabolic activation of catechol estrogens yield highly electrophilic quinoid forms [27], which are known to covalently bind to DNA bases [27,39,40,41].

From model reactions conducted on estrogen-quinones and deoxyribonucleosides, several adducts could be isolated and identified using NMR and tandem mass spectrometry [39]. However, by direct analysis of the crude reaction mixtures, liquid chromatography coupled to ion trap tandem mass spectrometry proved to be very efficient for the characterisation of additional unstable adducts which underwent degradation during their purification [41]. In several cases, multisequential  $MS^n$  mass spectrometry was successfully used when MS/MS failed for the differentiation of regioisomeric adducts [41,42,43]. Some additional information on the regio- as well as the stereochemistry of the addition of the DNA base on the steroid could also be obtained using various deuterium labelling experiments [44].

The major adduct characterised in these works has subsequently been characterised *in vivo* [40], showing that the chemical model reactions accurately predicted the *in vivo* formation of DNA adducts. In this field, the coupling of micro or nano separative techniques (capillary electrophoresis, micro and nano HPLC) to mass spectrometry is able to achieve (after extraction and enzymatic digestion of the DNA material) the detection of covalent adducts at sensitivity levels corresponding to a few modified bases for  $10^6$ - $10^8$  bases (*i.e.* ca. 150 fmol of adducts per mg DNA [45,46]). This allows considering in the near future the detection and structural characterisation of covalent DNA adducts in physiological situations, thus allowing the extension of our knowledge on the critical initiation phases of several cancers.

### Conclusion and perspectives

The spectacular development of atmospheric pressure ionisation techniques (ESI, APCI) as well as the technological evolution of modern mass analysers have greatly contributed to impose LC-MS/MS as one of the most powerful analytical tools in the field of xenobiotic metabolism studies. LC-MS/MS analyses are able to provide both qualitative (metabolite structural identification) and quantitative (pharmacokinetics) information with sensitivity already compatible with the detection of minor cellular disruptions. Concentration techniques such as solid phase extraction, switching-column or immunoaffinity clean-up procedures also greatly contribute to the improvement of the sensitivity and selectivity of analytical processes ending with mass spectrometric measurements. The new possibilities offered by recent ionisation sources more tolerant towards salts (biological extracts, buffered mobile phases...) and powerful mass analysers (ion traps, time of flight) have made analytical strategies evolving towards simplification, automation and direct coupling of sample preparation steps with the LC-MS analysis. Moreover, the analysis of the less polar xenobiotic molecules, which remained rather difficult using the classical ESI- or APCI-LC-MS interfaces, may be rendered much easier with the development of new atmospheric pressure ionisation sources such as atmospheric pressure photoionisation [47] or atmospheric pressure electronic capture [48], which may give access to high sensitivity normal phase LC-MS analyses. On the other hand, the development of fast LC coupled to tandem mass spectrometry, task automation as well as the apparition of multiple parallel ionisation sources or mass analysers will give access to higher and higher throughput LC-MS analyses, allowing extended activities in fields such as metabolite screening or rapid metabolite profiling.

In conclusion, mass spectrometry now offers a wide range of equipment for the LC-MS/MS studies of xenobiotic metabolism, including in particular hybrid quadrupole-time of flight mass spectrometers which provide great sensitivity, speed of operation and high resolution power in screening steps, and ion trap mass analysers which  $MS^n$  capabilities are very useful for structural analysis purposes. Moreover,

mass spectrometry companies now also propose software packages for accelerating metabolism studies, which allow automated metabolite identification from information such as the parent compound formula, and several decision criteria (exact mass criteria for TOF analysers, peak detection or spectrum quality thresholds), which are naturally applied to xenobiotic metabolism.

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