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Simultaneous determination of mycotoxins in biological fluids by LC-MS/MS

Alberto Ritieni[1], Antonello Santini[1], Michele Mussap[2], Rosalia Ferracane[1], Paolo Bosco[3], Diego Gazzolo[4,5], and Fabio Galvano[6]

[1]Department of Food Science, University of Naples Federico II, Portici, Naples, Italy, [2]Biochemical and Clinical Laboratory, "S. Martino" Hospital, Genoa, Italy, [3]IRCCS Associazione Oasi Maria S.S., Institute for Research on Mental Retardation and Brain Aging, Troina, Enna, Italy. [4]Neonatal Intensive Care Unit "Cesare Arrigo" Children Hospital, Alessandria, Italy, [5]Department of Pediatrics, "G. Gaslini Children's University" Hospital, Genoa, [6]Department of Biochemistry, Medical Chemistry and Molecular Biology, University of Catania, Italy

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1. ABSTRACT

We report a single, reliable, non-invasive analytical method for monitoring the fetal level of exposure to different mycotoxins. We assessed by tandem mass spectrometry levels up to 200 nanog/l for ochratoxin A and 500 nanog/l for aflatoxins in sample of serum (n= 71), urine (n= 18) and amniotic fluid (n= 21) of pregnant women. Aflatoxin G1 was present in one sample of serum (3.48 microg/l) and in four samples of urine (ranging from 14.0 to 18.8 microg/l), ochratoxin A was present in one sample of amniotic fluid (4.26 microg/l), whereas aflatoxin B1 (ranging from 0.4-2 microg/l) and B2 (ranging from 0.3-3 microg/l) were contextually present in two samples of urine. The very few contaminated samples did not allow statistical comparison between subjects grouped according to the frequency of consumption of commonly contaminated foods. Data confirm that mycotoxins can occur in fetal-maternal biological fluids. However, the incidence and the level of exposure to the investigated mycotoxins do not appear to pose risk for the mother and the fetus.

2. INTRODUCTION

Fungi belonging mainly to *Fusarium, Penicillium* and *Aspergillus* genera are able to produce variegate families of secondary metabolites well know as mycotoxins (1). Mycotoxins accumulation in food and feed can be a severe health risk both to humans and animals for their carcinogenic, mutagenic, teratogenic or toxic properties. In addition, mycotoxins are responsible for large economical losses worldwide in numerous commercial sectors, like crop production and food and feed processing (2) as well as for animal farming (3).

Till today, more than 300 mycotoxins have been isolated by several natural sources such as fruit, cereals, legumes, etc., and chemically characterized using a spectrometric approach. Less then 30 molecules are considered really dangerous for human and animals and, consequently, their surveillance and evaluation are primary targets for the European Community, Governmental Agencies and food control laboratories. Analytical protocols and quantitative analysis of mycotoxins in

complex matrixes are strongly correlated with experimental devices and technologies available for their identification and quantification. Moreover, the mode of action is partially clarified only for a few mycotoxins, and, on the other hand, their effects on cells, tissues, organs and organisms are widely reported in literature (4). Mycotoxins chronic exposure can produce different effects on humans, ranging from alterations of the response of the SNC to damages to the cardio vascular or respiratory apparatus and lead to carcinogenic, mutagenic or immunosuppressive effects (5-8).

Aflatoxins (AF) family (produced by *Aspergillus* spp.), Ochratoxin A (OTA), produced by *Aspergillus* and *Penicillium* spp., Zearalenone, Fumonisins and Thricothecenes, produced by *Fusarium* spp., are the most investigated mycotoxins because of the their well established toxicity confirmed by numerous animal and human studies (9-11).

OTA is a potent nephrotoxin. In animals OTA induces severe damages to liver and brain, being kidney the target organ where OTA induces an interstitial nephropathy that can lead to the develop of cancer of the kidney (12, 13). In humans OTA is also able to induce severe damage to kidney probably due to the unfavorable kinetic of elimination leading to a prolonged half-life (14-16). It has been observed that the main toxic effect of the OTA is the alteration of the protein synthesis and the onset of oxidative stress releasing free radicals and lipids peroxidation (17-20).

AF are the first mycotoxins to be detected and their discovery coincides with the birth of mycotoxicology in 1962. AF B_1 (AFB_1), G_1 (AFG_1), B_2 (AFB_2), and G_2 (AFG_2), which are the secondary metabolites of the molds *Aspergillus flavus*, *Aspergillus parasiticus* and *Aspergillus nomius* (21, 22), produce great risk by contaminating a wide variety of agricultural commodities and foodstuffs, especially those having high carbohydrate and/or fat contents, such as tree nuts and nut products, peanuts, corn, cereals, grains, oilseeds, dried figs and raisins, cottonseed, milk, feedstuffs, and dried spices (23). AF M_1 (AFM_1) and M_2 (AFM_2) are the hepatic metabolites of AFB_1 and AFB_2, respectively, excreted in milk by mammalians fed on contaminated food or feed. AFM_1 and AFM_2 are ten time less toxic of AFB_1 and AFB_2 and are also considered as biomarkers of the exposure to AFB_1 and AFB_2 (24). AF have been extensively studied with respect to their mechanisms of toxicity (25, 26). An understanding of metabolism, DNA adduct induction, mutagenicity and carcinogenicity has been paralleled by the development of biomarkers of AF exposure and biological effects, e.g. mutations, applied to human populations. The improvements in exposure assessment and their application in prospective epidemiological studies and the demonstration of a specific mutation in the TP53 gene in hepatocellular carcinomas from areas of high AF exposure have contributed significantly to the classification of AF as human carcinogens (27).

Basing on recent studies reporting that the assumption of OTA trough the diet correlates to the accumulation of the toxin in some biological fluids, e.g. blood and urine (28), one of the most interesting aspects to investigate in mycotoxin field, is to evaluate their early exposure during embryonic time.

In this study an optimized extraction and analytical procedure for biological fluids is proposed by its application to several biological samples to establish and evaluate the occurrence of the OTA and six AF in the serum, urine and amniotic fluid, considering that this last fluid is similar for quali-quantitative composition to the urine.

3. MATERIALS AND METHODS

3.1. Chemicals and equipments

OTA and AF standards were purchased from Sigma Chemical Company (St. Louis, Mo). All reagents used were on analytical grade and were purchased from Merck (Darmstadt, Germany). The mass spectrometer used was an Applied Biosystem (Toronto, Canada) Sciex API 3000, triple quadrupole, with a TurboIonSpray (TIS) source. Infusions were realized using a syringe pump from Harvard Apparatus (Massachusetts, USA). The HPLC connected to the mass spectrometer was formed by two Perkin Elmer (Toronto, Canada) series 200 micropumps. The chromatographic column used was a Phenomenex (USA) type Gemini 5microm C18 110A, 150x2 mm. For the data acquisition and elaboration the Analyst v. 1.4 software was used.

3.2. Subjects and sampling

From January 2005 to November 2006, at our 3rd level Centers for Fetal, Maternal and Neonatal Medicine Departments we collected, from a selected cohort of volunteers, samples from different biological fluids such as serum (n= 71), urine (n= 18) and amniotic fluid (n= 21) for mycotoxin analysis. The volunteers were selected on the basis of their medical history and exclusion criteria were: multiple pregnancies, gestational hypertension, diabetes and infections, fever, chromosomal abnormalities, metabolic diseases, diseases of the breast or central nervous system, malnutrition, maternal allergy, maternal addiction for tobacco, alcohol and cocaine. Informed consent was obtained from all pregnant women and from parents of the patients prior to inclusion in the study, for which local Human Investigations Committees approval was obtained.

For each woman a questionnaire was completed, reporting diet and eating habits focused on foods more likely to be sources of AFM_1 and OTA according to the European Commission Food Science and Techniques indications (29). Based on questionnaire results, study population was sub-grouped according to the frequency of consumption, in moderate consumers (up to seven times a week), or habitual consumers (more than seven times a week) of foods demonstrated to be likely sources of AFM_1 and OTA (2). The focused foods were: cereals foods (pasta, bread, breakfast cereals, cookies, bakery products), corn, rice, legumes, poultry, bovine meat, pork meat (fresh and cured), milk products (milk, yoghurt, cheese),

beverages (white and red wine, beer, black tea, coffee, juices), fruits, dried fruits (peanuts, chestnuts, walnuts) and chocolate.

Samples of fetal-maternal fluids were stored after collection at -40°C before the mycotoxin analysis. All tests were performed against a blank sample and run in triplicate.

3.3. Recovery tests

Recoveries from the three fluids of each investigated mycotoxin were obtained by artificially contaminating samples of urine, amniotic fluid and human serum with standard solutions of 20 microg/l of each mycotoxin. All extraction was repeated at least three times in two separate days with different operators. Data were evaluated statistically and the average recovery values of each experiment reported.

3.4. Procedure of extraction of OTA and AF from sera

An aliquot of serum (500 microl) was added of 2.5 ml of a 0.1 M $MgCl_2$ solution and the mixture stirred; subsequently 2.5 ml of chloroform were added and the mixture was stirred again for 10 minutes and then centrifuged at 4°C for 10 min at 2000 rpm. The organic phase was separated and the organic solvent evaporated using a rotating evaporator. Before the LC-MS/MS analysis the samples were dissolved in 200 microl of a solution of CH_3OH/H_2O 70:30 *v/v*.

3.5. Procedure of extraction of OTA and AF from urine and amniotic liquid

An aliquot of 500 microl of each sample (urine or amniotic liquid) was added of 2.5 ml of a 0.1 M $MgCl_2$ solution and the mixture was stirred; subsequently, 2.5 ml of chloroform were added, the solution was acidified with HCl 6N, and the mixture was stirred again for 2 minutes. The samples were then ice cooled for 20 minutes and then centrifuged at 4°C for 10 min at 3000 rpm. The organic phase was separated and the organic solvent evaporated using a centrifugal evaporator ThermoSavant (Savant Instruments Inc., Farmingdale, NY, USA). Before the LC-MS/MS analysis the samples were dissolved in 200 microl of a solution of CH_3OH/H_2O 70:30 *v/v*.

3.6. LC-MS/MS Analysis

The quantitative method for the simultaneous evaluation of OTA and AF (B_1, B_2, G_1, G_2, M_1 and M_2) was obtained by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). A volume of 20 microl of each sample was injected using the full loop technique. The flow rate was set to 0.2 ml/min. A mobile phase a 0.2 % (*v/v*) formic acid solution in water was used, while the B mobile phase was a mixture CH_3OH/CH_3CN (90:10 *v/v*). The elution was performed using a linear gradient. The gradient program was as follows: 20-80% B (12 min), 80-100% B (2 min), 100% B isocratic conditions (6 min), 100-20% B (2 min).

The calibration curves for each mycotoxin were obtained by injecting different solutions at known concentrations and the obtained areas from the peaks integration were linearly correlated as a function of the concentrations. All the experiments were performed in triplicate. The linearity for the analyzed mycotoxins is in the range 1-50 microg/l ensuring a good sensitivity and reproducibility. The limit of detection (LOD) was 200 nanog/l for the OTA and of 500 nanog/l for the six AF investigated. The limit of quantification (LOQ) was 500 nanog/l for the OTA and 1 microg/l for the other mycotoxins analyzed. The experimental acquisition was performed in the positive ion mode for the AF and the negative ion mode for the OTA. The optimal parameters used were determined as: ionization potential (IS) +5500V and -4500V in the positive and negative ion mode, respectively, drying gas (air) was heated to 350°C. The acquisition was performed in MRM (Multiple Reaction Monitoring). The Declustering Potential (DP) and Collision Energy (CE) were optimized for each compound infusing directly into the mass spectrometer standard solution of each compound at a concentration of 10 microg/mL at a constant flow rate of 8 microl/min, using a Model 11 Syringe pump (Harvard Apparatus, Holliston, MA, USA). Table 1 reports the MS/MS characteristic parameters for the analyzed toxins.

4. RESULTS AND DISCUSSION

This experimental work focuses on the set up of an efficient fast and inexpensive procedure of extraction and simultaneous analysis on seven mycotoxins from biological fluids. The nature of the analyzed samples required a special care in the samples preparation and manipulation and the use of different protocol as reported by previous studies (30, 31). On the basis of methods successfully used for analysis of OTA and AF in biological fluids, e.g. blood (32, 33) and urine (34, 35), two different extraction protocols have been set up: one used for the serum and the other used for the urine and for the amniotic liquid since the last two matrices show analogies with respect to the extraction and analysis protocols. Different tests have been performed before identifying the final procedure with the aim of evaluating the efficiency of the extraction procedure applying it on mixtures containing the above mentioned toxins in a known quantity. The use of a solution of acetonitrile (36) gave a recovery from sera less then 10%, whereas the use of a mixture of chloroform and n-hexane was more effective, giving an higher recovery for some mycotoxins from sera. More in detail, the best recovery results (87.5% and 61%) where obtained in sera added with 20 microg/l of AFB_1 and AFM_1, respectively, whereas a recovery of 21% was obtained for OTA, probably due to the use of the *n*-hexane that could extract a part of in the first phase of extraction procedure. The third used method, suggested by Becker *et al.* in 1991 (33), was then applied and results are reported in Table 2. Recovery was obtained from sera contaminated with a mixture of the investigated toxins at a concentration of 20 microg/l. This procedure is simpler then the other two tested and uses chloroform in HCl acid solution. In this work we adopted the method proposed by Becker with a slight but important modification consisting in the absence of the extract purification step. The elimination of prepurification step is an important improvement because

Table 1. MS/MS parameters for each analyzed mycotoxin

Mycotoxin	Retention times (min)	Precursor Ion *m/z*	Product ions *m/z*
Aflatoxin B_1	11.2	$[M+H]^+$ 313.1	269 285
Aflatoxin B_2	10.8	$[M+H]^+$ 315	259 287
Aflatoxin G_1	10.3	$[M+H]^+$ 329	243 283.1
Aflatoxin G_2	9.9	$[M+H]^+$ 331	312,2 245 257.2
Aflatoxin M_1	9.9	$[M+H]^+$ 329	273.1 258.9 301.1
Aflatoxin M_2	9.2	$[M+H]^+$ 331	273 285.1 259.1
Ochratoxin A	15.0	$[M-H]^-$ 402	251.2 211.2

Table 2. Recovery from sera, urine and amniotic fluids each contaminated with toxins at a quantity of 20 microg/Kg

Sample	AFB_1	AFB_2	AFG_1	AFG_2	AFM_1	AFM_2	OTA
Urine and amniotic fluids	50.2	65.4	37.0	52.6	61.1	53.2	57.5
Sample	69.1	67.4	72.1	78.1	67.2	68.2	70.3

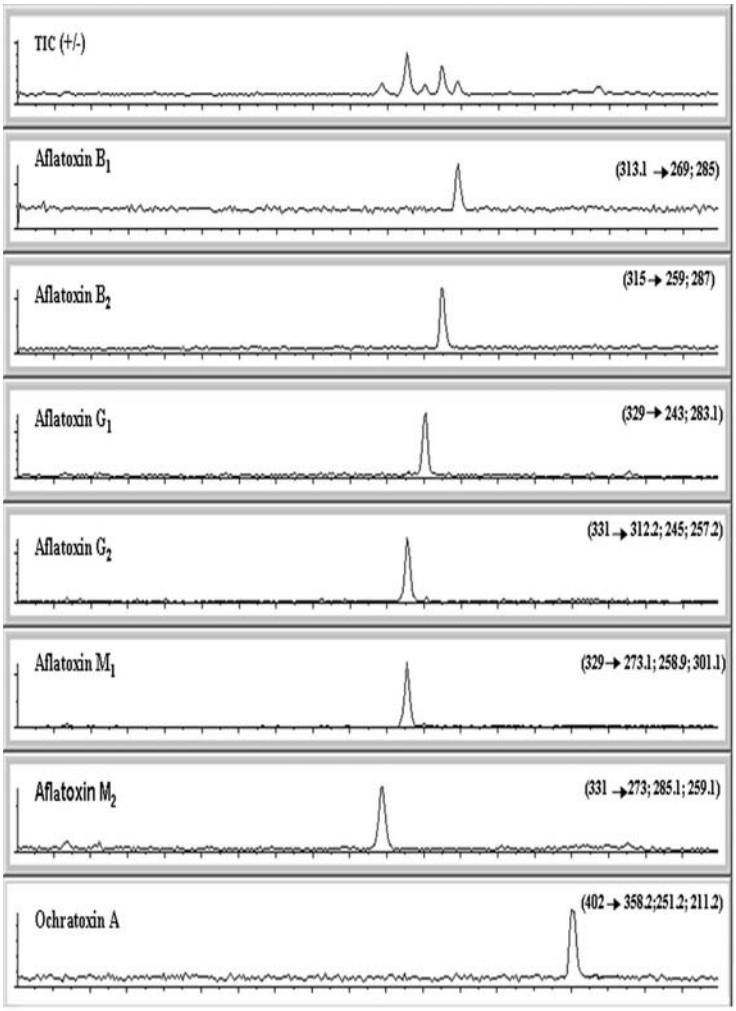

Figure 1. Total Ion Chromatogram (TIC) and Extracted Ion Chromatograms (XICs) of a standard mixture containing AFB_1, AFB_2, AFG_1, AFG_2, AFM_1, AFM_2 and OTA at a concentration of 50 microg/l.

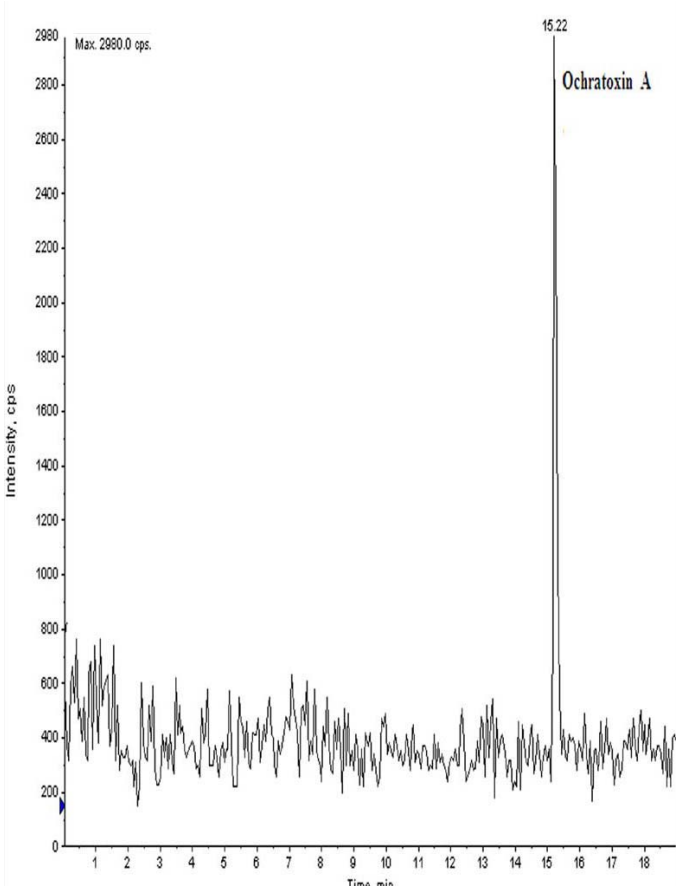

Figure 2. Extracted Ion Chromatogram (XIC) of the sample of amniotic liquid positive to the Ochratoxin A.

it is time and cost effective, due to elimination of immunoaffinity column, besides avoiding sample manipulation. If applied to the analysis of food extracts or raw material this procedure could likely lead to reduced performances, however, biological samples require a different approach based on high sensitivity, univocal structural identification and the minimum manipulation of the samples. The proposed procedure simplifies the method and has been confirmed by the absence of any interference peak in the analyzed "blank" samples and by the mass spectrometer that does not require any previous purification step of the samples before the analysis. The same approach to the set up of the analytical procedure for the amniotic fluid was also adopted for urine, in consideration of the analogue composition of the two biological fluids. Initially the method proposed by Pascale and Visconti (35) was used but the observed recoveries were not satisfactory, being 22%, 34% and 25% for OTA, AFB_1 and AFG_1, respectively. The method here proposed provided better recovery results for all the tested mycotoxins. Indeed, in samples contaminated with 20 microg/l of a mixture of the investigated mycotoxins the recovery was always above the 50% (Table 2). The seven analyzed mycotoxins were simultaneously evaluated using a tandem mass spectrometer, LC-MS/MS, that confirmed the efficiency of the applied extraction procedure. Figure 1 reports the TIC (Total Ion Chromatogram) and the XIC (Extracted Ion Chromatogram) in which the selected transitions for each compound of a standard toxins mixture containing the seven analyzed toxins, at a concentration of 50 microg/l, are monitored. Figures 2, 3 and 4, report the XIC (Extracted Ion Chromatogram) for positive samples of amniotic liquid, urine and serum, respectively. The advantages of the proposed method are the employment of a reduced sample aliquot (500 microl), the speed of the extraction step and the absence of any clean-up step as required by literature methods. This last circumstance is highly desirable considering the delicate nature of the analyzed samples.

With regard to the analytical results very few samples of fluids resulted contaminated. More in detail, AFG_1 was present in one sample of serum (3.48 microg/l) and in four samples of urine (ranging from 14,0 to 18.8 microg/l); OTA was present in one sample of amniotic fluid (4.26 microg/l), whereas AFB_1 (ranging from 0.4 to 2 microg/l) and AFB_2 (ranging from 0.3 to 3 microg/l) were contextually present in two samples of urine. It can be observed that the most frequent contamination from AF was found in urine, that is also the biological fluid richest in toxic metabolites eliminated from the human body. OTA was found only in one sample and only in the

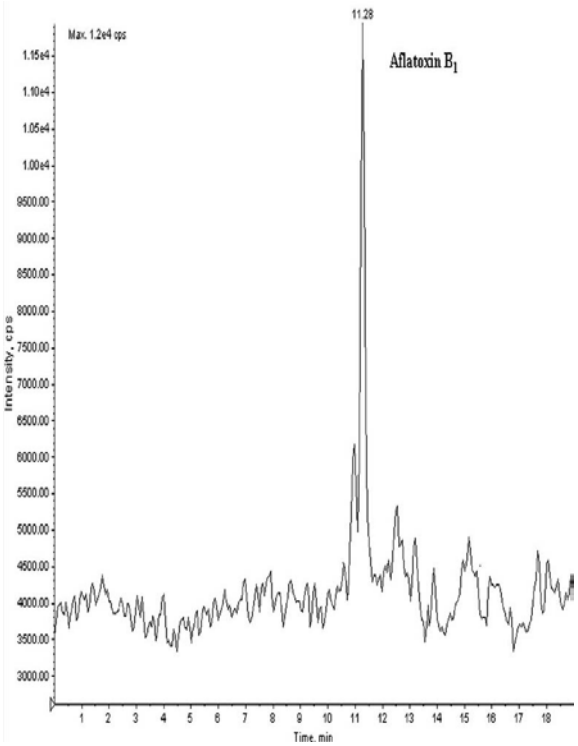

Figure 3. Extracted Ion Chromatogram (XIC) of the urine sample positive to the AFB_1.

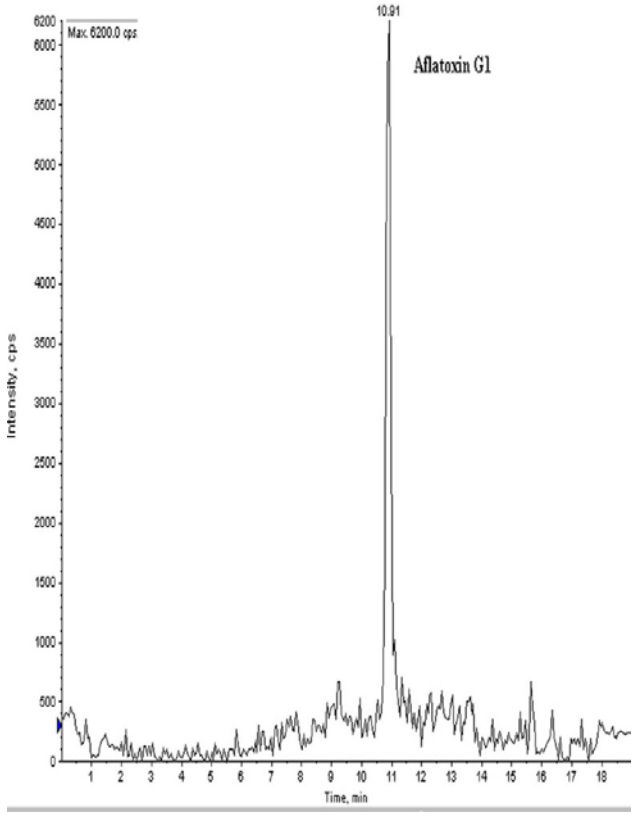

Figure 4. Extracted Ion Chromatogram (XIC) of the serum sample positive to the AFG_1.

amniotic fluid. This observation agrees with reported literature data where the carry-over of the OTA trough the placenta is reported (37, 38).

We grouped the subjects according to the frequency of consumption, recorded by the dietary questionnaire, in moderate consumers (up to seven times a week), or habitual consumers (more than seven times a week) of foods demonstrated to be likely sources of AF and OTA. Regrettably, the very few contaminated samples of fetal-maternal fluids did not allow statistical comparison between groups. However, the donor whose amniotic fluid was contaminated by OTA (4.26 microg/l) was an habitual consumer (more than seven times a week) of foods demonstrated to be likely sources of OTA such as bread, bakery products and cured pork meat. No significant seasonal differences were observed for OTA and AF when comparing samples collected in the period November–April with those collected in the period May–October.

According to these observations, it was not possible to correlate the presence of the mycotoxins and the exposition for the individuals. Indeed, it has been observed that only a long term significant exposures to these toxins originates chronic simptomathology due to the accumulation of the compounds in the body (39).

In conclusion, our data confirm that mycotoxins can occur in fetal-maternal biological fluids. However, at least in the present survey, the frequency and the level of exposure to the investigated mycotoxins do not appear to pose risk for the mother and the fetus. At the same time, the satisfactory analytical results of the simultaneous quantification of OTA and AF in biological fluids confirmed the robustness of the extraction, chromatographic and spectrometric steps also allowing time and cost effective manipulation of the samples.

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Send correspondence to: Fabio Galvano, Department of Biological Chemistry, Medical Chemistry and Molecular Biology, University of Catania, V.le A. Doria 6, 95125 Catania, Italy, Tel: 39-095-7384081, Fax: 39-095-7384220, E-mail: fgalvano@unict.it

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