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Automated On-line Solid-Phase Extraction−Liquid Chromatography−Electrospray Tandem Mass Spectrometry Method for the Determination of Ochratoxin A in Wine and Beer

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An automated on-line solid-phase extraction−liquid chromatography−electrospray tandem mass spectrometry (SPE-LC-ESI-MS/MS) method was developed for the determination of ochratoxin A (OTA) in alcoholic beverages. Mean recoveries for wine and beer were, respectively, 75 and 82%. Detection was achieved in negative ionization with a Q TRAP mass spectrometer operating in multiple-reaction monitoring (MRM) mode or enhanced product ion (EPI) mode, using the third quadrupole as linear ion trap. The MRM mode turned out to be more sensitive; the method allowed accurate determination of OTA in the range of 0.01−25 ng mL^{-1} using external calibration. Within-day and between-day relative standard deviation percentages were <6.2 and <9.1%, respectively. In EPI mode, fragmentation spectra at the limit of quantification (0.03 ng mL^{-1}) and good linearity could be obtained. Application of the method (MRM mode) to the analysis of several wine and beer samples purchased in local stores revealed OTA levels in the ranges of 0.03−1.44 ng mL^{-1} for wines and 0.02−0.14 ng mL^{-1} for beers.

KEYWORDS: Ochratoxin A; food analysis; automated method; liquid chromatography; mass spectrometry; linear ion trap

INTRODUCTION

Ochratoxin A, 7-(L-β-phenylalanylcarbonyl)carboxyl-5-chloro-8-hydroxy-3,4-dihydro-3*R*-methylisocumarin (**Figure 1**), is a widespread mycotoxin produced by the secondary metabolism of several *Aspergillus* and *Penicillium* species, mainly by *P. verrucosum*, *A. ochraceus* (*1*−*3*), and *A. carbonarius* together with a low percentage of the closely related *A. niger* (*4*, *5*). These fungi differ in their ecological niches, in the commodities affected, and in the frequency of their occurrence in different geographical regions. They can infect various plants and plant products such as cereals (wheat, barley, maize, and oats), legumes, groundnuts, spices, dried fruits, coffee, cocoa, and grapes. Consequently, contamination of ochratoxin A (OTA) can occur also in their derived products such as beer, wine, bread, and bakery products (*4*).

OTA has been shown to be nephrotoxic, hepatotoxic, teratogenic, and immunotoxic to several animal species and to cause kidney and liver tumors in mice and rats (*1*, *6*−*8*). For these reasons, in 1993 the International Agency for Research on Cancer (IARC) classified OTA as a possible carcinogen to humans (group 2B) (*9*, *10*).

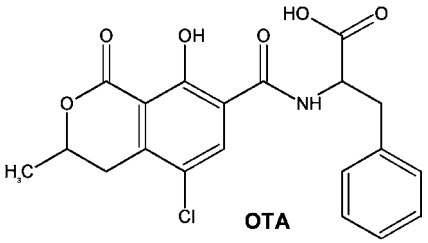

Figure 1. Structure and molecular weight of ochratoxin A (OTA).

With current scientific and technical knowledge, and despite improvements in production and storage techniques, it is not possible to prevent the development of these molds altogether. Consequently, OTA cannot be eliminated from food entirely. Limits should therefore be set as low as reasonably achievable. The World Health Organization (WHO) has set a provisional tolerable weekly intake level for ochratoxin A at 100 ng kg^{-1} of body weight (*11*), taking its potentially carcinogenic effect into account. Regulatory levels have been established within the European Union for products of wide consumption (*12*). Regulatory levels have been also discussed with regard to wine (*13*), for which a maximum residue limit (MRL) of 2.0 μg L^{-1} has been recently fixed (*14*). In 1999 a maximum guidance level

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of 0.2 μg L^{-1} for beer was set by the Italian Ministry of Health (*15*).

The occurrence of OTA in wine samples has been reported in several studies mainly dealing with European wines, showing a considerable level of contamination with high toxin concentration and incidence. Generally, red wines contain higher amounts of this mycotoxin than white and rosé wines (*16*), and southern European countries together with North Africa seem to be more affected by the OTA contamination (*8*). Differences are attributed to climatic factors, grape cultivation, winemaking techniques, and storage conditions.

Reversed-phase liquid chromatography coupled with fluorescence detection (LC-FLD), preceded by immunoaffinity column (IAC) cleanup, is currently the most applied method for OTA determination in wine and beer (*2*, *7*, *8*, *17*−*19*). An automated method using a robotic sample processor has been recently developed (*20*) to reduce analysis time. Immunochemical methods based on enzyme-linked immunosorbent assay (ELISA) are also used (*21*). However, problems arising from matrix complexity, such as cross-reaction, can lead to erroneous results. This problem may be overcome by employing mass spectrometry (MS) as a detection technique after LC (*6*, *7*, *22*).

State-of-the-art, on-line solid-phase extraction (SPE)-LC-MS provides high precision, sensitivity, and a higher sample throughput as compared to off-line SPE (*24*). Several technical devices for coupling on-line SPE with LC are commercially available, but also simpler homemade apparatuses may offer good performances (*24*). To the best of our knowledge there is only one published paper, dealing with the determination of fumonisins (*25*), that makes use of an on-line SPE-LC-MS method for determining these mycotoxins in corn-based feed in which the SPE is realized by coupling an immunoaffinity capture column to a polymeric resin-filled trap.

SPE-LC-MS/MS methodologies have been developed especially for drug monitoring (*26*), pharmacokinetic application (*24*, *27*, *28*), and analysis of pesticides in water (*29*). To achieve very high throughput methods, short (20−50 mm) columns working at low-resolution condition are often adopted for biological samples. SPE systems coupled with these stressed minicolumns are equally very fast operating, and a total time of 2.5 min or less could be achieved (*24*, *26*, *27*). Such a considerable shortening in time of analysis is, at least in part, obtained at the expense of selectivity and limit of detection (only very low enrichment factors are possible), relying on MS/MS for accurate quantitation at a very low level.

The fact that in the electrospray ionization (ESI) process coextracted and coeluted matrix components can decrease the yield of analyte ion production by competition is now a well-recognized effect. The use of an internal standard (IS) that undergoes the same signal suppression as the analyte surely eliminates the inaccuracy problem, but the signal is still weakened. Narrowing the peak by very fast elution may compensate for ion production decrease only as long as the extra analytical column contribution to peak width becomes the limiting factor. Limits of detection reported in a recent work for compounds well amenable to positive ESI were ~0.1 ng/mL in blood (*26*). Moreover, when a more complex matrix such as urine was evaluated, good performances were obtained with run times of >10 min, although deuterated IS were used (*30*).

The aim of this work was to develop and evaluate an "on-line" SPE-LC method coupled to ESI-MS/MS for confirmation analysis of OTA in wine and beer. The pursued characteristics of this method were (1) high performances, (2) total automation, (3) simple apparatus, and (4) high throughput.

MATERIALS AND METHODS

Chemicals and Materials. Standard of ochratoxin A was purchased from Sigma-Aldrich (Milan, Italy).

Acetonitrile and methanol, both of LC grade, as well as ethanol and formic and acetic acids were supplied by Carlo Erba (Milan, Italy). Deionized water was further purified using a Milli-Q apparatus (Millipore, Billerica, MA).

A standard stock solution (200 ng μL^{-1}) was prepared by dissolving OTA in CH_3CN. Working standard solutions were prepared at different concentrations immediately before use by diluting the stock solution with suitable volumes of H_2O/EtOH (85:15, v/v) and 10 mmol L^{-1} HCOOH. All solutions were kept at −20 °C and allowed to equilibrate at room temperature before use.

The cartridge employed for on-line SPE extraction was a 4.0 × 4.0 mm i.d. LiChroCART 4-4 containing 100 mg of C_{18} (5 μm average particle size), purchased from Merck (Darmstadt, Germany). For sample filtration PTFE syringe filters (0.45 μm; 15 mm diameter) were employed (Chemtek Analytica, Bologna, Italy).

Wine and Beer Samples. Bottled and boxed Italian wine samples (glass or Tetrapak packaging) were purchased from local stores. A total of 66 wine samples were analyzed: 43 red, 16 white, and 7 rosé. Bottled and canned beer samples were also purchased from local stores. A total of 18 beer samples (of Italian and foreign origin) were analyzed.

Instrumentation. The liquid chromatography system used was a series 200 apparatus from Perkin-Elmer (Norwalk, CT). The system consisted of a binary LC micropump, a binary LC pump, two vacuum degassers, and an autosampler equipped with a 200 μL loop. The chromatographic column was a 150 mm × 1 mm i.d. Alltima (Alltech, Deerfield, IL) filled with C_{18} reversed-phase packing, 5 μm average particle size, equipped with a 4 × 2.1 mm i.d. guard column (Phenomenex, Torrance, CA).

Electrospray mass spectrometry (ESI-MS) was carried out on a Q TRAP quadrupole-linear ion trap instrument equipped with a TurboIonSpray (TISP) interface and with a built-in eight-port valve (Applied Biosystems/MDS Sciex, Concord, ON, Canada).

The LC-MS system was controlled by Analyst software (1.3.2 version, Applied Biosystems/MDS Sciex).

Sample Preparation and Analytical Conditions. Ten milliliter aliquots of beer samples were previously degassed by sonication for 30 min. After this step, the analytical procedure was the same for both alcoholic drinks. Before analysis, wine and beer samples were filtered on 0.45 μm PTFE filters, and without any further processing a 200 μL aliquot was injected directly into the apparatus by the autosampler.

The two alternate positions of the software-controlled eight-port valve allowed flow switching in the SPE extraction cell. The LC binary pump (pump 1) was used to deliver a high flow (1 mL min^{-1} flow rate) through the extraction column to load the sample and wash out very polar matrix compounds and subsequently to flush and equilibrate the extraction column. Instead, the LC binary micropump (pump 2) was used to deliver a low flow (50 μL min^{-1} flow rate) to elute the analyte from the extraction column in back-flushing mode and from the chromatographic column and subsequently to flush and equilibrate the latter one. Mobile phases used for sample loading and washing were ethanol (B) and water (A). Mobile phases for analyte elution were methanol (C) and water (A). All of the above-cited mobile phases contained 10 mmol L^{-1} HCOOH. Gradient profiles and valve position are reported in **Table 1**. For more details see the schematic of the on-line system available as Supporting Information.

The column effluent was directly transferred into the mass spectrometer. Taking advantage of the two different analyzers available on the Q TRAP, data acquisition and quantitation were performed working either in multiple-reaction monitoring (MRM) mode or in enhanced product ion (EPI) mode. Instrument tuning parameters were suitably adjusted by infusing at 10 μL min^{-1} flow rate an OTA standard solution, 10 ng μL^{-1}, prepared in MeOH/H_2O (1:1, v/v) containing 10 mmol L^{-1} HCOOH.

Mass calibration and resolution adjustments on the resolving quadrupoles were performed automatically by using a 10^{-5} mol L^{-1} solution of polypropyleneglycol (PPG) introduced via a model 11 Harvard infusion pump. The peak width was set on both resolving

Table 1. Gradient Profiles of the Two LC Binary Pumps and Valve Positions

time[b] (min)	pump 1, Φ[a] = 1 mL min^{-1}, % B[c,d]	valve position	pump 2, Φ[a] = 50 μL min^{-1}, % C[c,e]
0–1	15	1[f]	60
1–3	15	2[g]	60
3–3.5	15	2[g]	60→63
3.5–8	15→85	1[f]	63→90
8–10	85	1[f]	90
10–24	15	1[f]	60

[a] Flow rate. [b] Time starts after autosampler injection. [c] Solvent A was H_2O containing 10 mmol L^{-1} formic acid. [d] Ethanol containing 10 mmol L^{-1} formic acid. [e] Methanol containing 10 mmol L^{-1} formic acid. [f] Loading, extraction, and equilibration position. [g] Elution position.

quadrupoles at 0.7 Th (Thomson) measured at half-height for all MS and MS/MS experiments. Mass spectra recorded in full-scan mode were obtained by scanning over the range *m*/*z* 100−450 in 2.6 s. TISP interface was operated in the negative ionization mode, by applying to the capillary a voltage (IS) of −4.2 kV. Nitrogen was used as curtain gas (CUR), whereas air was used as nebulizing (GS1) and turbo spray gas (GS2, heated at 350 °C), with the optimum values set, respectively, at 20, 20, and 40 (arbitrary values). Collisionally activated dissociation (CAD) MS/MS was performed in the collision cell (Q2), operating at medium pressure (arbitrary value) of nitrogen as collision gas. Declustering potential (DP) was set at −30 V, whereas entrance potential (EP) was set at −10 V; collision energy (CE) was optimized at 25 eV.

Operating in MRM mode, Q1 (first quadrupole) and Q3 (third quadrupole) resolutions were set at unit *m*/*z* resolution power and the collision cell exit potential (CXP) was set at −2 V. Two transitions were monitored for the deprotonated molecular ion $[M - H]^-$ of OTA: *m*/*z* 402→358 and 402→211. However, only the former was used for quantitation, whereas the latter was used for confirmation purpose. Operating in EPI mode, linear ion trap (LIT) fill time was optimized at 50 ms and Q3 entry barrier at 8 V, whereas the scan rate was set at 4000 amu/s. For quantitation purpose the *m*/*z* 358 product ion (0.5 Th width) was selected.

Calibration Curve, Quantification, and Evaluation of Method Performance. Calibration solutions were prepared daily by dissolving a suitable amount of OTA standard in H_2O/EtOH (85:15, v/v), containing 10 mmol L^{-1} HCOOH, at 15.0, 5.0, 1.0, 0.1, and 0.025 ng mL^{-1} concentration levels. For quantification, a calibration curve was constructed by plotting the observed peak area against the OTA concentration. The linear regression equation was obtained in duplicate by least-squares analysis from the five-point calibration curves. The calibration solutions were injected and then the curves plotted, at the beginning and end of each analytical batch.

The on-line SPE-LC-ESI-MS/MS method was evaluated for the following parameters: linearity, within-day and between-day accuracies and precisions, matrix effect, specificity, limit of identification (see later for definition), limit of quantification, and performance stability.

Linearity in the working range was evaluated from a calibration curve on five points, each in triplicate (concentration range of 0.1−25 ng mL^{-1}). Accuracy and precision were measured using, as quality check samples, simulated wine samples (SWSs) at three different known concentration levels (1.0, 3.0, and 15.0 ng mL^{-1}) prepared in H_2O/EtOH (85:15, v/v) containing 10 mmol L^{-1} HCOOH from a separate dilution of the standard stock solution. Each SWS was analyzed in triplicate per single batch on five different days. Specificity was assessed by analyzing several different wine or beer samples in MS/MS EPI mode and comparing the obtained spectra with that of a standard solution.

Matrix effect on recovery was assessed by comparing the slope of the calibration curve obtained from the hydroalcoholic standard solution with the slope of calibration curves obtained by injecting wine (red or white) or degassed beer spiked at the same concentration level of the standard solution. The calculated mean recoveries were utilized for quantitative analysis of naturally contaminated samples.

To assess performance stability, red wine samples spiked at five different concentration levels, covering the entire working range, plus a blank were processed by the on-line system. This set of samples was re-injected up to a total of 60 processed samples (10 cycles).

RESULTS AND DISCUSSION

General Remarks. This method has been specifically developed for confirmation analysis purposes in compliance with the European Union laws in force (*31*). On this basis, a substance can be identified using LC-MS/MS, in MRM mode, by at least two transitions. For this reason, we prefer to indicate a method identification limit (MIL), estimated as the minimum concentration giving a signal-to-noise (S/N) ratio = 3 for the second most intense MRM transition instead of a limit of detection. Moreover, this implied achieving a limit of quantification (MQL), S/N = 10, of at least 0.1 ng mL^{-1} for wine. To implement this objective we devised a nonlaborious, reasonably low cost, high-throughput, robust system suitable for routine use in a specialized laboratory.

To assess the best conditions for the on-line extraction and analysis of OTA, each part of the analytical setup was optimized off-line first; then, after the whole system was set up, the mutual influence of each parameter was evaluated and reoptimized. In preliminary experiments conducted with SWSs we selected some fast conditions that failed when applied to actual wine samples. Irreproducibility, signal weakening, and frequent clogging were the main problems. This was probably due to the fact that, like some foods, wine is a complex matrix and requires selective extraction and efficient chromatographic separation to minimize ion suppression (*32*, *33*); therefore, a new more selective setup was planned and tested.

One advantage of automated on-line methodologies, if compared with off-line ones, is that sample manipulation is reduced to a minimum, and then more reproducible results are obtained. Another advantage is that sensitivity is usually enhanced because the whole sample, instead of an aliquot of the final extract such as in off-line systems, is transferred to the chromatographic system. In the methodology developed only filtration of the sample and loading of the autosampler were manually performed. A small volume of the sample (200 μL) was injected into the SPE trap and transferred to a 1 mm chromatographic column, with virtually no loss. Considering that in an analytical column like the one used in this study normally no more than 5 μL of sample can be injected without peak broadening, a 40-fold enrichment factor was achieved.

Method Optimization: ESI-MS/MS Conditions. The presence of a carboxylic group in OTA structure, with regard to chromatographic separations, implies acid or buffer (*6*) addition to mobile phases to avoid peak broadening and tailing. It is known that in these conditions OTA can be detected in positive ESI-MS (*3*), because it forms either proton adduct at *m*/*z* 404 or sodium adduct at *m*/*z* 426, ions that can be monitored. However, using MeOH and H_2O as mobile phases and a relatively low concentration of HCOOH as mobile phase modifier, we noticed that negative ionization gave a more intense $[M - H]^-$ ion at *m*/*z* 402.

Generally, in negative ESI the responses of compounds containing a carboxylic acid are decreased as formic acid concentration increases. On the other hand, the presence of acid helps to retain analytes on the column and gives a better chromatographic resolution and lower matrix effect. We tried to find the best compromise between these two opposite

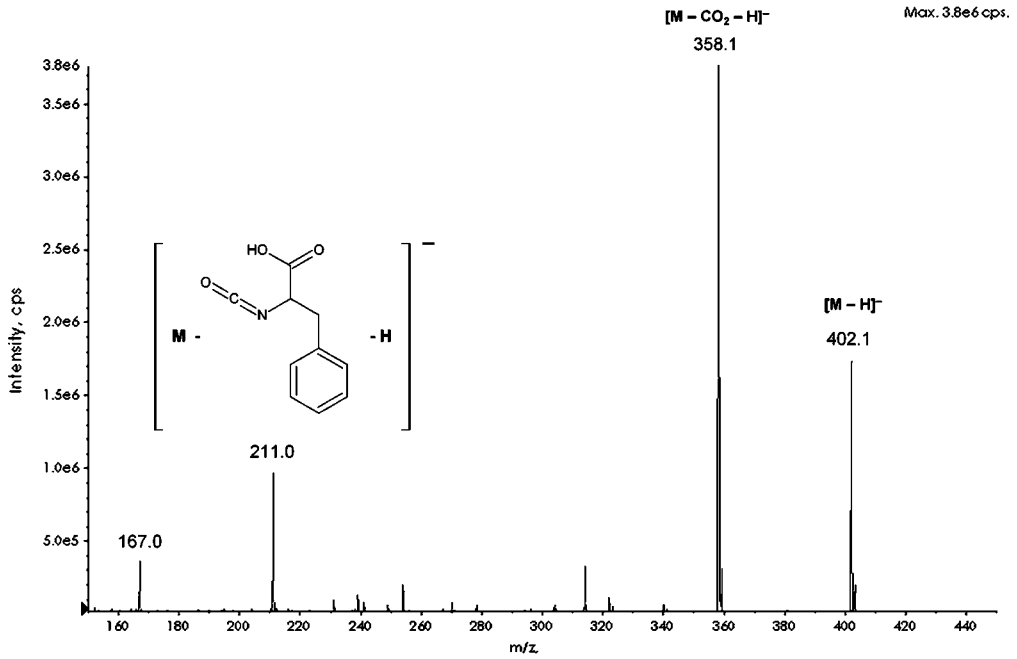

Figure 2. Enhanced product ion (EPI) spectrum of OTA $[M - H]^-$ ion (CE = 25 eV).

tendencies. We noticed that the addition of 10 mmol L^{-1} HCOOH to both mobile phases did not suppress the signal dramatically and gave symmetrical peaks. Moreover, by performing flow injection analysis in MeOH/H_2O (70:30, v/v) we surprisingly noticed that acid addition up to 1 mmol L^{-1} to mobile phases instead of suppressing the signal increased it slightly (data not shown). This is in agreement with recent studies performed by Wu et al. (*34*) that described how ionization efficiency in negative ESI is influenced by mobile phase pH. In particular, it was observed that the intensity of the deprotonated ion signal of acidic substances sometimes is enhanced by acidification. The formation of charged droplets in electrospray, indeed, is achieved mainly through ion reduction on capillary surface. Positive ions, produced by protonation in an acidic environment, increase the reduction process rate and enable the spray to carry more easily a negative charge excess, which then is transferred to the analyte. In **Figure 2** the fragmentation spectrum of the $[M - H]^-$ ion of OTA is shown. As can be seen at a CE of 25 eV the two major fragments formed are *m*/*z* 358, $[M - CO_2 - H]^-$, followed by the less intense *m*/*z* 211. For quantitative analysis only the transition *m*/*z* 402→358 was used, whereas both were selected for identification purposes.

At present, chromatographic separation followed by MRM mass spectrometric detection of two fragment ions is judged to be exhaustive to confirm the identity of a molecule. However, in view of a more stringent regulatory framework in which the identity of the molecule might have to be confirmed with greater confidence, it would be preferable to have the entire fragmentation spectrum of a molecule.

Traditional triple-quadrupole instruments can perform product ion scans, but when this acquisition mode is utilized, the sensitivity is greatly diminished. To overcome this, we investigated the capability of the hybrid quadrupole ion trap MS/MS system to address both qualitative and quantitative issues by taking advantage of the two available configurations of Q3. The system employed is a modified triple-quadrupole where the Q3 region can be operated either as a conventional quadrupole mass filter or as a linear ion trap with axial ion ejection. The instrument encompasses the functionality of an ion trap mass spectrometer, with its associated high sensitivity for product ion scanning, and that of a triple-quadrupole mass spectrometer with the capabilities to perform MRM mode. An exhaustive description of the system and of its capabilities can be found in the work of Hager et al. (*35*, *36*). Then acquisition of EPI chromatograms of *m*/*z* 402 was made. The extracted current of the *m*/*z* 402→358 transition (using a width of *m*/*z* 0.5) was used for quantitative analysis. In **Figure 3** the MRM and EPI (*m*/*z* 402→358) chromatograms of a red wine spiked at 0.05 ng mL^{-1} are shown. As can be seen, the sensitivity reached in EPI mode was lower than the sensitivity reached in MRM mode (S/N in MRM mode = 3.5× S/N in EPI mode), but still sufficient to comply with current regulation requirements. Also in the EPI mode the instrumental response was found to be linear in the range of 0.05−25.0 ng mL^{-1}, and no space-charge effects, typical of a traditional quadrupole ion trap analyzer, were observed.

Method Optimization: On-line SPE-LC System Conditions. The most critical parameters influencing method performance were valve switching time and binary pump flow rate. These parameters were interdependent, because their combination influenced both recovery and chromatographic peak shape and, consequently, the MQL. To optimize these parameters, the signal-to-noise ratio of the OTA peak in the obtained chromatogram versus the two parameters, for a given amount injected, was monitored. A loading flow rate of 1 mL min^{-1} and a 1.0 min loading time were found as optima (data not shown). A higher loading time gave analyte losses, whereas slower flow rates caused chromatographic peak broadening. Back-flushing elution of the extraction cartridge turned out to be better because it gave sharper chromatographic peaks. With regard to the sample volume injected, 200 μL was estimated to be sufficient to achieve a proper sensitivity; greater volumes were still amenable by the system with higher sensitivity (and lower MQL), but, on the other hand, the extraction cartridge duty cycle was diminished.

To avoid cartridge clogging after a few analysis cycles when wine samples were injected, acidification of pump 1 mobile phases was necessary. Without acid addition the extraction

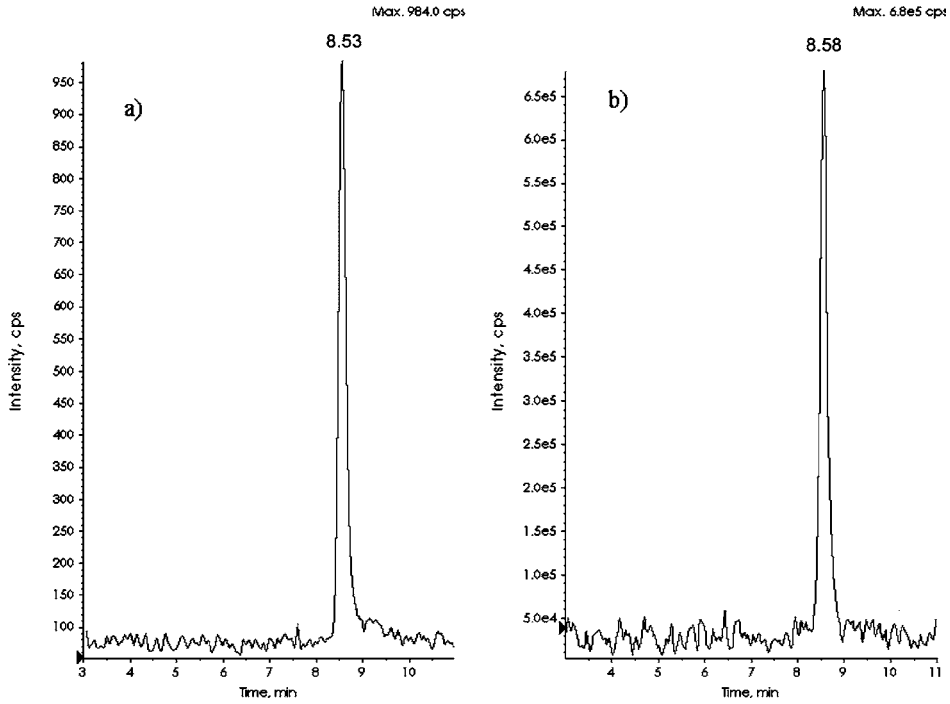

Figure 3. (**a**) 402→358 *m*/*z* MRM and (**b**) EPI LC-MS/MS chromatograms obtained by analyzing a red wine spiked at 0.05 ng mL^{-1} OTA.

Table 2. Calibration Curve Parameters and Recoveries Relative to SWS and MILs[e] for OTA Determination in Beer and Wine Samples Using the Developed On-line Automated System

calibration matrix[a]	regression equation[b,c]	coefficient of determination (R^2)	recovery[d] ± RSD % (%)	MIL[c,e] (ng mL^{-1})	OTA unspiked concn (ng mL^{-1})
SWS[f]	PA × 10^{-4} = 0.36 + 1.27C	0.999		0.007	
	PA × 10^{-7} = *0.29* + *1.03C*	0.998		*0.02*	
beer	PA × 10^{-4} = 0.45 + 1.04C	0.999	82 ± 3	0.008	0.09
	PA × 10^{-7} = *0.37* + *0.85C*	0.999		*0.02*	
white wine	PA × 10^{-4} = 0.44 + 0.96C	0.999	76 ± 4	0.01	0.09
	PA × 10^{-7} = *0.36* + *0.79C*	0.999		*0.03*	
red wine	PA × 10^{-4} = 0.63 + 0.94C	0.998	74 ± 4	0.01	0.31
	PA × 10^{-7} = *0.52* + *0.77C*	0.998		*0.03*	

[a] Calibration from five points plus a blank, each in triplicate, in the range of 0.05–25 ng mL^{-1}. [b] PA = *a* + *bC* (PA, peak area; *a*, intercept; *b*, slope; *C*, concentration). [c] Values in italic type refer to EPI chromatograms, others to MRM chromatograms. [d] Percent signal with respect to SWS. Means calculated from the ratio of the slopes of regression equation in both MRM and EPI modes. [e] Method identification limit (see text for definition). [f] Simulated wine sample.

cartridge underwent rapid clogging after a few injections and, in addition, reproducibility was affected.

During method development, for quantification purposes, to achieve a better precision the use of ochratoxin B (OTB) as internal standard was tested, but no improvements were achieved and, on the contrary, a decrease of performance was noticed. This was, probably, due to the fact that the optimal SPE-LC conditions for the internal standard were different from those for OTA. An external calibration procedure was therefore preferred.

Method Performance. In the studied range (0.05−25 ng mL^{-1}), instrumental response was found to be linear, showing a coefficient of determination (R^2) of 0.999 (**Table 2**). The response was found to be linear in the same range also for wine and beer samples but, as can be noted, total mean recoveries ranged from 82 to 74%. Signal suppression or analytical recovery related problems could be responsible for this matrix effect, and individual contribution was not investigated. The analysis of several different types of wine and beer samples allowed a reproducible matrix effect to be noted within each alcoholic beverage type; the relative standard deviation (RSD) of the signal reduction coefficient found for each class of beverage was <4%. Thus, by using these coefficients, as correction factor, a good accuracy can be achieved. Specificity and high confidence in analyte identification are given by the OTA fragmentation spectrum obtained in EPI mode. In **Figure 4** (top) the ion current profile for the *m*/*z* 358 fragment ion from the LC-EPI data set of a beer sample naturally contaminated at 0.03 ng mL^{-1} is shown. In the bottom panel of **Figure 4** the background-subtracted EPI spectrum obtained at OTA retention time is also reported. As can be seen, the characteristic fragment ions (namely, *m*/*z* 358, 314, 211, and 167) are all present. This demonstrates that with the new hybrid quadrupole-linear ion trap instrument, more stringent confirmatory data can be achieved without the linearity range narrowing in instrumental response and with only a 3.5-fold decrease in S/N.

Within-day precision expressed as relative standard deviation percentage (RSD %) evaluated from SWSs, as quality control samples, ranged from 4.8 to 6.1%. Between-day precision evaluated at the same concentration level ranged from 7.8 to 9.0%. The recovery percentage expressing the within-day and

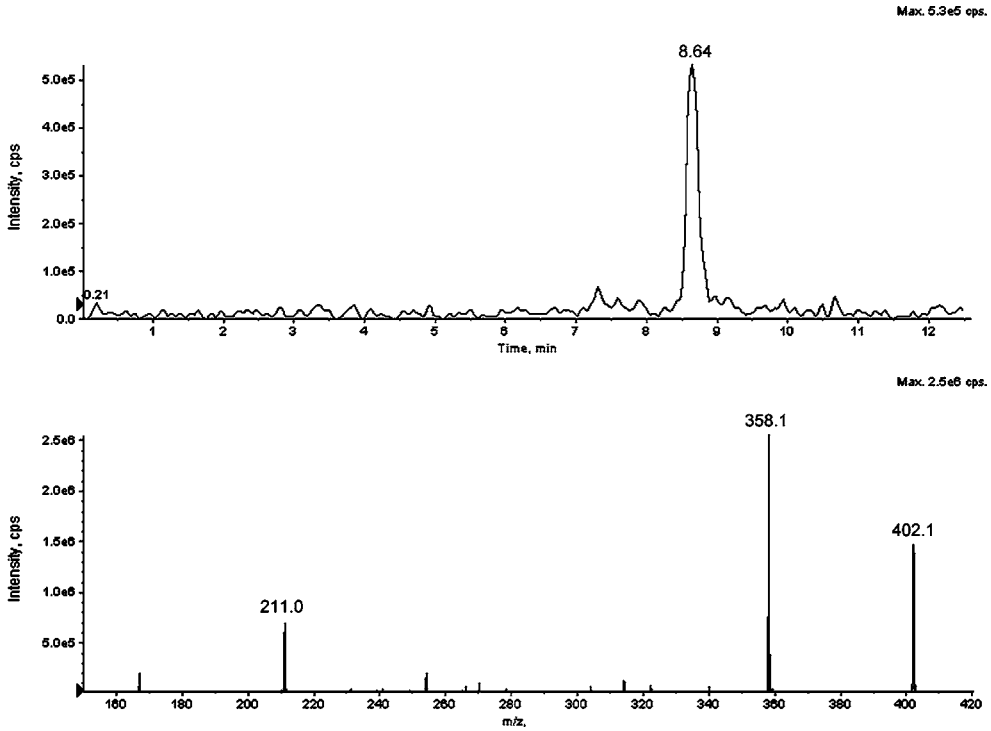

Figure 4. EPI scan of *m*/*z* 402→358 extracted current chromatogram (top) and EPI spectrum obtained by analyzing a beer sample naturally contaminated at 0.03 ng mL^{-1} OTA (bottom).

Table 3. Precision and Accuracy of the Method for OTA Determination on Simulated Wine Samples (SWS) Using the Automated On-line SPE-LC-ESI-MS/MS System[a]

OTA concn in SWS (ng mL^{-1})	within-day precision (RSD %)	between-day precision (RSD %)	within-day accuracy (recovery %)	between-day accuracy (recovery %)
1.0	6.1	8.1	104.2	107.6
3.0	4.8	7.8	96.3	95.3
15.0	5.3	9.0	105.5	109.4

[a] Three replicates of each SWS were done per single batch on five different days.

between-day accuracy ranged from 96.3 to 105.5% and from 95.3 to 109.4%, respectively. Values are reported in **Table 3**.

MIL was estimated as the minimum concentration giving a S/N ratio = 3 for the second most intense MRM transition. The noise (N) used in the calculation is based on 3σ of the baseline signal and was directly provided by the instrument software. Because there is no meaning in quantifying an unidentified substance, although at MIL the S/N ratio of the most intense transition was 30, we set the MQL = MIL. The MILs were 0.008 ng mL^{-1} for beer and 0.01 ng mL^{-1} for red or white wines. The second value is 200 times lower with respect to the maximum residue limit imposed by the current EU regulation for wine (*14*).

The performance stability test made with 10 replicates of a red wine sample (alcohol 12% v/v, dried extract ~18 g L^{-1}) spiked at five concentration levels plus blank showed no carry-over effect in blank samples during analysis, whereas values of relative standard deviation of signal intensities (peak area counts) at the various concentration levels were in the range of 4.7−7.7%. Thus, it can be assumed the apparatus is able to process up to 60 samples without performance deterioration. Consequently, this performance with minimum attendance (only daily routine instrument maintenance, sample filtration, and autosampler loading are required) makes this method suitable to be employed in private and public laboratories involved in food safety controls.

Table 4. Occurrence (Incidence, Mean of Positives, and Range) of OTA in Different Wine and Beer Samples Purchased in Local Stores during April–June 2004

sample type	incidence (pos/tot)	incidence (%)	mean (ng mL^{-1})	range (ng mL^{-1})
red wine				
bottled	31/38	82	0.29	0.04–1.44
boxed	5/5	100	0.33	0.17–0.87
white wine				
bottled	8/13	62	0.16	0.03–0.42
boxed	2/3	66	0.14	0.11–0.17
rosé wine				
bottled	5/7	71	0.32	0.10–0.82
beer				
bottled	5/12	42	0.07	0.02–0.14
canned	3/6	50	0.09	0.04–0.12

[a] Concentration levels were obtained using MS/MS acquisition in MRM mode.

Naturally Contaminated Wine and Beer Sample Analysis. The method was tested, using MS/MS acquisition in MRM mode, in a survey of some wine and beer samples purchased in local stores. Sampling was made during April−June 2004. The purpose of the study was (other than to test the methodology) to have an indication of the possible average intake of OTA associated with wine consumption. Therefore, the wines chosen were those commonly on sale in supermarkets in a medium price range. Also, for beers the most common Italian and foreign brands that can be found on sale were chosen.

In **Table 4** the results of the survey are summarized. These are not surprising and in agreement with the literature (*1*, *7*, *17*, *37*, *38*). We planned this work, bearing in mind the WHO-estimated tolerable weekly intake level for OTA of 100 ng kg^{-1} of body weight (*11*), when a lower MRL for wine in the EU [0.5 μg L^{-1}, as originally proposed (*13*)] was expected. A MRL

of 2 μg L^{-1} means that a person of 70 kg body weight, drinking daily 250 mL of wine at a still legal level of OTA, ingests with this beverage almost 50% of the tolerable intake. Among the wine samples analyzed, only 5 of the 38 of red wines and 1 of the 7 of rosé wine have an OTA concentration between 0.5 and 2.0 μg L^{-1}, in agreement with other authors (*39*). This finding means that a reasonable effort to improve winemaking practices may allow in a short time the revision of high MRL without serious economical consequences.

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Supporting Information Available: Schematic of the on-line system. This material is available free of charge via the Internet at http://pubs.acs.org.

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