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Survey on the analysis of mycotoxins

Submitted by on behalf of the Analysis Committee of the European Brewery Convention

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1 Introduction

The Analysis Committee of the European Brewery Convention (EBC) decided in 2001 to survey among its members information relating to the analyses of mycotoxins. The aim of the survey was to collate information to help maltsters and brewers managing mycotoxins control.

A questionnaire was sent to members of the Analysis Committee of the European Brewery Convention (EBC) asking which mycotoxins were relevant to malting and brewing, which methods were used to analyse them and what were the detection limits of these methods. Information was gathered on the regulations and recommendations relating to mycotoxins in the different countries.

The questionnaire was sent to all EBC Analysis Committee members representing 19 countries. Answers were received from 12 countries.

2 Results

2.1 Which are the relevant mycotoxins to be analysed?

According to the survey, there is a consensus among members of the EBC Analysis Committee that the most important mycotoxins are the following: aflatoxins (AFB1, B2, G1, G2), ochratoxin A (OTA), tricothecenes, zearalenone and fumonisins (B1, B2). Of the tricothecenes, attention is paid mainly to deoxynivalenol (DON or vomitoxin), nivalenol and T2-toxin. Some laboratories also focused on HT2 toxin, 3-Ac-DON, DAS, Fusarenon-X. One laboratory also included MAS, Neosolaniol and 15-Ac-DON in the range of analyses. Other mycotoxins were also mentioned but only by a few members; these were patulin, sterigmatocystin and cytochalasin E.

Citrinin, which can be produced by different species of *Penicillium* (*expansum, verrucosum*) is indicated in the list of principal mycotoxins in the Manual of Good Practice on Malting Technology from the EBC but was not recorded in this survey (1). It is said to occur together with OTA but is not thermo-stable and is probably destroyed during mashing and boiling (1, 2).This could explain the current lack of interest among brewers and maltsters.

Surveys were published in the brewing world recently describing the most important mycotoxins, the producing agents and the toxicological effects and giving guidelines to avoid or reduce the level of mycotoxins in the barley beer chain (1, 3-6). Also, some studies on the detected levels in barley, malt and the fate of these compounds in the beer process were published recently (1, 7-12). More general surveys are also available (13, 14).

2.2 How to analyse them?

2.2.1 Sampling

The sampling method is critical. Contamination by mycotoxins can occur in the field, during storage or during the malting process. The production of mycotoxins is not homogeneous. For instance, Ochratoxin A contamination often occurs in “spots” during storage in silos. In consequence, a poor sampling procedure of the batch can miss the highly infected grains. Therefore, the European Commission has recommended a procedure for a sampling according to the size of the batch. This includes 10 to 100 incremental samples up to a total of 10 kilos that have to be milled before analyses (15). All results obtained from samples not taken by this method are generally underestimated (Dupire S., personal communication).

2.2.2 Method validation

The methods used should be validated and respond to the criteria for the selection of analysis methods for mycotoxins published in 1999 by the European Committee of Standardization (CEN). These include repeatability, reproducibility and minimum recovery levels (16). For instance, the recoveries for DON, zearalenone and fumonisins should fall 70 to 110 %. The validation of a method is often supported by international organisations such as CEN, AOAC (Association of Official Analytical Chemists), ISO (International Organisation for Standardisation), IUPAC (International Union of Pure and Applied Chemistry). The standardisation of a method is often required for commercial affairs and in the EC it is supervised by the CEN. When requesting a mycotoxin analysis to be carried out by an external laboratory, it is very important to ensure that this laboratory uses validated methods and is officially accredited for this analysis in a defined matrix (e.g. beer, malt, barley). The participation of the laboratories to international network and proficiency testing is an additional guarantee of the quality of their results (17).

2.2.3 Methods

There were two groups of methods reported in this survey; chromatographic ones and immunologic ones. The chromatographic methods measure the compound specifically after sample extraction and extract clean-up followed by a separation by gas or liquid chromatography and a specific detection. The extract clean-up by immunoaffinity (IA) column is more and more usual. According to this survey, the official thin layer chromatography (TLC) methods from the AOAC are almost not in use anymore. The immunologic assays are based on the ELISA principle (Enzyme Linked Immuno Sorbent Assay). Kits to perform the ELISA assays are available from different suppliers (R-biopharme, Rhone

Table 1 Detection limits reported for Aflatoxin B1 in barley, malt or beer

Laboratory	Method	Barley ppb	Malt ppb	Beer ppb
1	IA+ HPLC-Flu	0,35	0,35	0,2
2	IA+ HPLC-Flu	0,1		
3	IA+ HPLC-Flu	0,35		
4	IA+ HPLC-Flu	2		
5	IA+ HPLC-Flu	0,1		
6	IA+ HPLC-Flu	0,5	0,5	0,05
7	ELISA	1,7	1,7	

Table 2 Detection limits reported for Ochratoxin A in barley, malt or beer

Laboratory	Method	Barley ppb	Malt ppb	Beer ppb
1	TLC	10*		
2	C18+HPLC-FLD	2		
3	C18+HPLC-FLD	2		
4	IA+HPLC-FLD	0,7	0,7	0,1
5	IA+HPLC-FLD	1		
6	IA+HPLC-FLD	0,1		
7	IA+HPLC-FLD	0,3	0,3	0,05
8	IA+HPLC-FLD	0,1	0,1	
9	IA+HPLC-FLD	0,5	0,5	0,5
10	ELISA	1	1	

* in wheat

Table 3 Detection limits reported for Fumonisins B1 and B2 in barley, malt or beer

Laboratory	Method	Barley ppb	Malt ppb	Beer ppb
1	HPLC - FLD	100	100	100
2	IA+ HPLC -FLD	20	20	20

Table 4 Detection limits reported for DON in barley, malt or beer

Laboratory	Method	Barley ppb	Malt ppb	Beer ppb
1	GC-ECD	25	25	25
2	GC-ECD	10		
3	Myco-sep+ GC-ECD	20	20	10
4	Myco-sep+ GC-MS	30	30	10
5	GC-MS	5	5	5
6	GC-MS	5	5	
7	ELISA	100	100	

Diagnostix, International Diagnostic Systems Corp., Diffchamb, Neogen, Tepnel, Tome Laboratories and ELISA-technologies) and these methods are often used for screening purposes and on the field. Different versions exist that vary in the rapidity of the procedure and the quantification of the results. A very important step of validation is the detection of matrix effects (18), and since false positive can occur, positive results could require confirmation by more expensive chromatographic methods. Some authors have studied their correlation with the chromatographic methods (19-24). In this survey, the detection limits reported by the ELISA principle were always higher than the chromatographic methods (Tables 1 – 4).

New promising methods are in development; for instance LC-MS/MS seems to be very effective to achieve very low detection levels in beer (25). NIR, PCR, fluorescence polarization assays, biosensors and electronic nose technology are also currently under investigation for the detection of microbiological quality and mycotoxin potential in barley and malt (26-36).

2.2.4 Aflatoxins

There is to our knowledge very little evidence of the occurrence of aflatoxins in European cereals but a regulation exists in the EC and in some other countries (37). Therefore, the analysis of aflatoxins has to be included in mycotoxins management.

After IA extract clean up, aflatoxins (B1, B2, G1, G2) were reported to be measured in barley and malt by HPLC with fluorescence detection after a post column halogenic derivatization following the EN 12955 recommendations or the method published by Stroka *et al.* (38, 39).

Detection limits in barley and malt ranged from 0,1 to 0,5 ppb and 0,05 ppb in beer. An ELISA assay in barley and malt was also reported with a detection limit of 1,7 ppb (table 1).

2.2.5 Ochratoxin A

Ochratoxin A is mainly produced by *Penicillium verrucosum* and several species of *Aspergillus*. It is a storage mycotoxin, but it can be produced during the malting process (1).

A regulation for cereals (5 ppb) and malt (3 ppb) has been adopted in the European Community in 2002 (40). There is no European Regulation for beer at this moment. A directive concerning the sampling methods and the methods of analysis of ochratoxin A in foodstuffs has bee published by the European Community (41).

For the quantification of ochratoxin A, one lab reported the use of thin layer chromatography with a detection limit of 10 ppb in wheat (42). Two labs referred to an HPLC-FLD method for corn and barley using a clean-up of the extract on a C18 column published in 1992 and adopted in 1996 by AOAC (43, 44). The other laboratories measured OTA by HPLC-FLD and a clean up by IA. AOAC has recently adopted a method for OTA in barley (45, 46).

The detection limits reported by HPLC ranged from 0,1 to 2 ppb in barley and malt (Table 2). The highest detection limit was reported for method using the clean-up on C18. The ELISA test reported achieved 1 ppb in barley and malt. In beer, figures ranged from 0,05 to 0,5 ppb. A method following the one published by Visconti *et al.* has been adopted recently by AOAC and CEN (47-50).

2.2.6 Fumonisins

Fumonisins are mainly produced by *Fusarium moniliforme* in maize, current focus is on fumonisins B1 and B2 and little is known about the occurrence of fumonisins B3 and B4.

Only in-house methods using HPLC-FLD with or without IA clean-up have been reported. According to the detection limits reported, the IA clean-up allows to lower it from 100 ppb to 20 ppb (Table 3). Both types of methods have been adopted by AOAC (51, 52). An official ELISA assay for total fumonisins has been approved by AOAC but was not reported to be used in this survey (53).

2.2.7 Tricothecenes

Tricothecenes are mycotoxins produced mainly in the field by different species of Fusarium. They are subdivided in two main groups, the tricothecenes type A (T2-toxin, HT2 toxin, DAS and neosolaniol) that do not display a carbonyl group at C8 and the tricothecenes type B (DON, nivalenol and others) displaying a carbonyl group at C8.

For DON, Nivalenol and T2-toxin, two main types of method are reported; both advise gas chromatography using either and electron capture detector (ECD) or a MS detector (54-57). A clean up (Romer Myco-sep) is mentioned by two labs. For DON, both techniques give the same range of detection limit from 5 to 30 ppb in barley and malt and from 5 to 25 ppb in beer (Table 4). For nivalenol, the detection limits vary from 5 to 50 ppb in beer, barley and malt. This variation is higher for T2 toxin (from 5 to 100 ppb). The immunologic test reported gave a limit of 100 ppb for DON.

For HT2-toxin, DAS, Fusarenon-X and Neosolaniol, only GC-MS is used. Variations from 5 to 40 ppb depending on the method were the laboratories reported for barley and malt. In beer the detection limits recorded range from 5 to 10 ppb.

2.2.8 Zearalenone

Zearalenone is also a field mycotoxin produced by some Fusarium species including *F. culmorum, F. graminearum* and *F. crookwellense*. These species are common on cereals and tend to develop particularly during cool, wet growing and harvest seasons. Zearalenone is measured by HPLC-FLD, generally after IA preparation (59-62). Typical limits of detection range from 1 to 50 ppb in barley and malt and 1 to 5 ppb in beer (Table 5). ELISA tests are also available with a reported detection limit of 50 ppb in cereals. An official ELISA test has been adopted by AOAC in 1994 (63). Zearalenone is converted largely to beta-zearalenol or alpha-zearalenol by brewing strains of *Saccharomyces cerevisae* (58). No labs reported the determination of zearalenol.

Table 5 Detection limits reported for Zearalenone in barley, malt or beer

Laboratory	Method	Barley ppb	Malt ppb	Beer ppb
1	HPLC-FLD	20		
2	HPLC-FLD	4	4	4
3	IA+ HPLC-FLD	1		
4	IA+ HPLC-FLD	5	5	5
5	IA+ HPLC-FLD	1,5	1,5	
6	IA+ HPLC-FLD	10	10	1
7	ELISA	50	50	

2.2.9 Regulation

Regulations for mycotoxins are currently under study by the European Commission. Consequently, the following data can be subject to modifications in the future. Regulations already exist for cereals and malt for aflatoxins and since 2002 for ochratoxin A (Table 6) (40). A distinction was made between unprocessed cereals (barley) and processed cereals for human consumption (malt).

Other levels mentioned in this table are guidelines maximum values in different countries received from the members of the Analysis Committee. It ranges from 500 to 2000 ppb for DON, 50 to 200 ppb for Zearalenone, 100 ppb for T2-toxin and 500 ppb for the sum of the fumonisins (FB1+FB2). Preliminary European proposals published by the Food Standard Agency and recommended in Germany for DON, zearalenone and fumonisins are similar to the lowest levels reported here (64-65). Working values from the EU for unprocessed cereals belong to the same range of value too (66).

In Czech Republic, values for sterigmatocystin (20 ppb) and patulin (100 ppb) were also received.

The data reported in this survey for beer were very limited. There are also no official regulations in the European Union and no

Table 6 Recommended maximum levels of mycotoxins reported in this survey for malt, unprocessed cereals (between brackets) and maize grits (only fumonisins) (in ppb)

Malt (unprocessed cereals)	Total afla	AF B1	OTA	DON	T2	Zea	FB1+FB2 (maize grits)	Patulin
EC	4*	2*	3 (5)*	(1000)**		(100)**	500**	
France***	4	2	3	500	100	50	500	
Bulgaria***	4	2	3	1000	100	200	1000	
Finland***				750				
Czech Rep.***				2000				100
Germany[64]				500		50	500	

* official maximum levels for European Commission; ** working maximum levels for European Commission (65);
*** guidelines information received from EBC members

maximum level for OTA has been established since the level in beer is indirectly controlled by the malt regulation (to be published). However, for OTA, a value of maximum 0,2 ppb in beer would be recommended in Bulgaria and in Italy [67].

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