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Review

Chromatographic methods for the determination of ochratoxin A in animal and human tissues and fluids

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Abstract

This paper gives a review of chromatographic methods used for the determination of ochratoxin A (OA) in animal and human tissues and fluids. These methods are needed for example for monitoring studies of OA occurrence in the food chain and for studies dealing with the OA carry-over. In this survey, emphasis was given to HPLC methods. The review includes sampling, sample storage, extraction, spiking procedures, clean-up, detection and determination, and confirmation procedures. Emphasis is laid on special problems associated with the analysis of animal tissues and fluids.

Keywords: Reviews; Sample preparation; Ochratoxins; Toxins; Mycotoxins

Contents

1. Introduction

Ochratoxin A (OA) is a mycotoxin with nephrotoxic, carcinogenic, teratogenic and immunosuppressive properties. It is produced by several *Aspergillus* and *Penicillium* species. The occurrence of OA in food and feed has been reported world-wide (for review see [1–4]). Cereals and derived products are assumed to be the major dietary source of OA. In addition, other products of vegetable origin, as nuts, beans, coffee, cocoa, spices, dried fruits and beer may contain OA. The intake of OA by contaminated

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feed may lead to residues in the blood, the kidney and the liver of pigs and of poultry and to a lesser extent in muscle tissue, adipose and eggs [4]. Thus, products of animal origin can contribute to the OA-intake of humans.

Investigations in a number of countries have shown that OA can be found in human blood; in Germany, 57% of 306 samples of human serum tested were positive for OA [5], in Sweden 13% of 297 samples [6], in Canada 40% of 159 samples [7], in Switzerland 100% of 368 samples [8]. The percentage of positive samples depends on the detection limit — in the Swiss investigation, a method with a very low detection limit of 0.01 ng/ml was used. OA was even detected in human milk [8–11].

A comparison of the OA values found in the literature is often problematic, as the methodology is not always specified and the quality of the methods used is frequently not known. Therefore, it is desirable that forthcoming studies should be carried out under analytical quality assurance regimes [1]. Of importance are methods validated at levels at which they are used and including methods for reliable confirmation of results [12]. Certified reference materials and inter-comparison studies are important parts of quality assurance, too. For the determination of OA in grain, official methods exist [13]. Certified reference material for OA determination in wheat is recently available [14,135]. In comparison, official methods or reference materials for the determination of OA in animal products still do not exist.

A review about OA analysis and several general reviews or books about mycotoxin analysis have been published in recent years [15–23]. In the Journal of the Association of Official Analytical Chemists, annual reports about mycotoxins, with emphasis on mycotoxin analysis, appear regularly in the first issue of every year [12,24].

This paper gives a review of chromatographic methods used for the determination of OA in animal and human tissues and fluids. Reliable methods for these matrices are important for monitoring the contamination of animal products and OA occurrence in humans, and for research studies dealing with the carry-over and metabolism of OA. In this survey, emphasis was given to HPLC methods. A great part of this paper deals with sampling, extraction and clean-up procedures for these matrices. Immunochemical methods are not part of this report. This increasing area of mycotoxin methodology was reviewed recently in two papers [25,26].

2. General remarks on the OA analysis

OA (M_r, 403.8) contains 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3R-methylisocoumarin that is linked through the 7-carboxy group to L-β-phenylalanine by an amide bond (Fig. 1). It is a colourless, crystalline compound, soluble in polar organic solvents, slightly soluble in water and soluble in diluted aqueous bicarbonate solutions. Physical data are published by IUPAC [27]. For the analytical procedures, the character of OA as a weak acid is important. The pK_a value for of the carboxyl group of the phenylalanine part is given as 4.4 [28], and the pK_a of the phenolic hydroxyl group as 7.3 [28] or 7.05 [29].

The concentration of OA standard solutions used for quantitative analysis must be determined spectrophotometrically, because generally only milligram amounts of OA are purchased for analytical purposes. The exact amount in the vial is not specified and exact weighing of such small amounts is hardly possible. Also the purity of the OA standard is questionable. Besides, handling of dry mycotoxins should be avoided because of the risk of dissemination due to electrostatic charge [13].

A survey from the literature of the molar absorption coefficient ϵ of OA in several solvents is given in Table 1. The dimension of ϵ was not mentioned in most papers. Obviously, the usual dimension $M^{-1}\,cm^{-1}$ (corresponding to $cm^2\,mmol^{-1}$ or 1000 $cm^2\,mol^{-1}$) was used in most cases. Generally, for reference purposes OA concentration is determined in benzene–acetic acid according to the AOAC

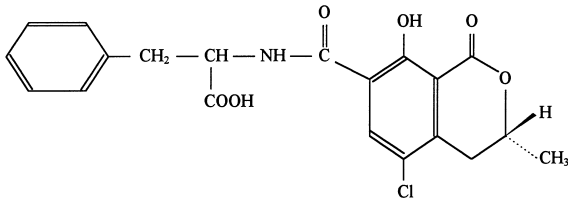

Fig. 1. Structure of ochratoxin A.

Table 1
Molar absorption coefficient ϵ of OA in different solvents[a]

Solvent	Concentration	λ (nm)	ϵ	Reference
Benzene–acetic acid (99:1, v/v)	~24 μg/ml	333	5550	[13]
Toluene–acetic acid (99:1, v/v)	~10 μg/ml	333 (?)	5440 $cm^2/\mu mol$[c]	[30]
Methanol	[b]	333	6400	[31]
Methanol	21.81 μ*M* (8.81 μg/ml)	332	633 m^2/mol (6330 $cm^2/mmol$)	[27]
Methanol	[b]	332	63 300	[2]
Methanol	10 μg/ml	333	6640	[5]
Ethanol	[b]	333	6100	[32]
Ethanol	[b]	333	5500	[33]
0.04 *M* Tris–H_2SO_4 buffer (pH 7.5)	10^{-4} *M* (40.38 μg/ml)	380	5680 $M^{-1}\ cm^{-1}$	[34]

[a] Specifications and dimensions as stated in the papers, converted values in parentheses.
[b] Not specified.
[c] Obviously, the dimension $cm^2/\mu mol$ is an error; $cm^2/mmol$ is correct.

procedure [13]. As benzene is highly toxic and its use is avoided in most laboratories, an ϵ coefficient in another solvent should be stated by the AOAC as an official value. The ϵ value in toluene–acetic acid, recently determined by an inter-laboratory study by direct comparison of the absorbance measurements in toluene–acetic acid and benzene–acetic acid, is a possible alternative [30].

OA is a stable compound, it is heat resistant, solutions in ethanol can be stored in the refrigerator for more than a year without loss [35]. Wood et al. reported problems with the stability of OA in methanol [30]. In the author's laboratory, no OA decomposition was observed in concentrated solutions in methanol over a period of some years when stored at −18°C. It is recommended to protect OA solutions from light since decomposition occurs on exposure to fluorescent light for several days [4]. However, indications of instability of OA in daylight was not found in more recent literature. An alternative to the storage of OA standard solutions is to evaporate the solvent, store the toxin as a film in the glass vial and reconstitute it only when needed.

OA is stable under the acidic conditions used in most extraction procedures. Stability studies of OA in toluene–acetic acid (99:1, v/v) after storage at 25°C and 40°C for up to 8 weeks as well as at +4°C and −18°C for 6 months showed no evidence of decomposition [30]. On the other hand, it was reported that overnight exposure to 2 *M* HCl will change OA to ochratoxin α (Oα), a metabolite of OA, by hydrolysis of the amide bond [36]. In a recently published paper, hydrolysis of the lactone ring of OA after addition of 0.5 *M* NaOH to OA in DMSO for 2 h at 25°C was described. The lactone was reformed by acidification to a pH<1 for 6 h [37]. Several procedures for decontamination of OA contaminated feeds are based on an alkaline treatment by ammonia or sodium hydroxide [3,38]. As it has been shown that OA could be formed again in the feed after treatment with acid [39] and [H. Valenta, W. Richter (1997), unpublished results], formation of the ring-opened form of OA can be assumed as one possible reaction product by the treatment of contaminated feed by ammonia or NaOH.

All glassware used in OA analysis must be free of alkaline soap or detergent residues to avoid loss of the toxin from neutral solvents by salt formation, precipitation and/or adsorption onto glassware [13]. Hald et al. reported that in an inter-comparison study for the determination of OA in wheat poorer reproducibility of the results was observed when unsilanized glassware was used [40]. On the other hand, studies concerning adsorption effects of OA on glassware and their prevention by silanization were not found in the literature. In most papers, no indication of the use of silanized glassware is given.

In the author's laboratory, rinsing of the glassware with methanol prior to use in OA analysis proved valuable in order to desorb substances possibly adsorbed on the glassware. Methanol is used as it is one of the most potent desorbents [41].

As HPLC with fluorescence detection — the method which is used in most studies — is a very sensitive method and OA is generally analysed in trace levels, problems with interfering substances are common. These substances can originate not only from the matrix, but also from the chemicals, solvents and other materials, which are in contact with the sample during the analytical procedure. Therefore, it is important to use only solvents of HPLC quality and chemicals of analytical-reagent grade or better. Plastic, SPE (solid-phase extraction) cartridges or septa from vials are also possible sources of interfering substances. It is a good practice to make a complete analysis run (including extraction, clean-up etc.) without the sample, to detect entry of interfering substances during the preparation procedure.

Finally, as OA is a highly toxic substance, precautions must be observed when handling this substance according to the national rules. Safety instructions for working with mycotoxins are given in [13,42]. A method for destruction of OA in laboratory wastes and decontamination of glassware by a sodium hypochlorite solution has been described [43].

3. Survey of the methods

In Table 2, a survey of chromatographic methods for the determination of OA in animal and human tissues and fluids is given. In this table, only completely described methods published after 1980 are considered. Some older methods, especially TLC methods, were still used in recent years (AOAC method 973.37 [13]), [34,80,81]. Some of these methods, and methods which were developed for other matrices, but used also for animal or human tissues [82–84], are discussed below. This also covers methods with an incomplete description of the procedures [36,85–89].

The methods are arranged in chronological order. Each method is characterized by a brief description of the extraction, clean up and determination procedure. When indicated, a confirmation method is mentioned, too. In most papers, a detection limit was specified, often on the basis of a signal-to-noise ratio equal to 3. Additionally, some authors specified a quantitation limit which was defined in different ways. If only one value is given in Table 2, it is the detection limit in most cases. In the case of two values, the second value is the quantitation limit. Also included is the recovery, together with the standard deviation and the concentration range in which the recovery was determined, when stated in the paper.

3.1. Sampling and sample storage

General recommendations for sampling products for mycotoxin analyses have been published by Dickens and Whitaker [90] and Campbell et al. [91]. Sampling of meat and meat products, and eggs and egg products, suspected of containing aflatoxin residues is discussed in an older publication [92]. Sampling is regarded as one of the most serious problems associated with mycotoxin analysis. This concerns mainly particulate products (such as grain, peanuts and cottonseed) where mycotoxins are produced by growth of toxigenic molds. In these products the distribution is often very inhomogeneous. Most investigations relating to this problem are made on aflatoxins.

The distribution of mycotoxins in animal and human tissues and especially fluids after an ingestion of mycotoxins with contaminated foods and feeds is assumed to be rather homogeneous throughout the respective organ [90]. However, differences between the OA concentration in different regions of rat's kidney after an i.p. injection were found [79]. In case of small laboratory animals such as rats, generally the whole organs are taken for the analysis. Larger organs such as pig's kidney or pig's liver should be also homogenized in whole, prior to taking a smaller sample for the extraction [92]. Details about the original sample size in studies of OA levels in organs of pigs or other bigger animals are given only in few papers. One pig's kidney was blended, and an aliquot of 20 g was analysed in the study of Büchmann and Hald [52]. Canela et al. [93] blended and mixed both porcine kidneys and 500 g of chicken liver respectively before taking an analytical sample.

Table 2
Survey of chromatographic methods of analysis for ochratoxin A (OA) in animal and human tissues and fluids[a]

Matrix	Extraction	Clean-up[b]	Method[c]	Confirmation[c]	DL/QL[d] (ng/g)/(ng/ml)	Recovery[e] (%)	Remarks[f]	Ref.
Rat's blood serum, plasma, lymph, bile, urine, tissues	(a) HCl–$CHCl_3$ (b) direct	No	TLC	–	500	75–106		[44] [45]
Pig's kidney	H_3PO_4–$CHCl_3$	LLP	Two-dimensional TLC	OA methyl ester	2	82–94 (10)		[46]
Human blood serum	$MgCl_2$–HCl–$CHCl_3$	LLP	(a) Spectrofluorometric (b) HPLC–FL; C: RP18 E: CH_3OH–H_2O–HOAc (70:30:2, v/v)	HPLC of several derivatives	(a) 1–2	(a) 52±5 (2–75)		[47]
Chicken kidney, human blood plasma	H_3PO_4–hexane (hexane layer discarded) $CHCl_3$	No	Derivatised to O-methyl-methyl ester; HPLC–UV; C: RP18; E: CH_3CN–H_2O (60:40, v/v)	–	–	Kidney: 72–112 plasma: 78–109 (both 250–2000)		[48]
Pig's kidney	(a) Citric acid–CH_2Cl_2 (b) incubated with Subtilisin A; $CHCl_3$	LLP and SPE	TLC	MS; O-methyl-methyl ester	0.2	70 (2.6)		[49]
Pig's kidney and blood serum, human kidney and blood serum	$MgCl_2$–HCl–$CHCl_3$ (pH 2.5)	LLP	HPLC–FL; C: RP18; E: CH_3CN–H_2O–HOAc (570:410:20, v/v)	FL-spectrum; OA methyl ester; MS	0.1	Kidney: 76±14 (0.1–10.0) serum: 81±10 (0.1–10.0)	Applied for rat's urine, also	[50] [5] [79]
Pig's kidney	H_3PO_4–ethyl acetate	CC on Si	(a) TLC (b) HPLC–FL; C: RP 18; E: Propanol-2–CH_3CN–0.083 *M* H_3PO_4–H_2O (20:25:37:18, v/v)−0.370–94 (2–17) [51]	–	0.3	70–94 (2–17)		[51]
Pig's kidney	H_3PO_4–$CHCl_3$	Celite column [13]	TLC	OA methyl ester	10	87±9		[52]
Human urine	Incubation with β-glucuronidase $CHCl_3$	SPE on Si, CN and C_8	HPLC–FL; C: RP8; E: 0.083 *M* H_3PO_4–CH_3CN–propanol-2 (55:40:5, v/v)	Comparing ratio of peak heights with two detectors	0.7	72–93 (1–100)	Simultaneous determination of Cit and AFB1	[53]
Eggs, blood serum and kidney of layer hens albumin and yolk lyophilized	$MgCl_2$–HCl–$CHCl_3$ (pH 1.6) modified for yolk	LLP	HPLC–FL; C: RP18; E: CH_3CN–H_2O–HOAc (570:410:20, v/v)	FL-spectrum OA methyl ester	Yolk: 1.0 albumin: 0.3 serum, kidney: 0.1	Albumin, serum and kidney: 75 yolk: 14		[54]
Kidney, meat products	$MgCl_2$–HCl–$CHCl_3$	LLP on Extrelut column	HPLC–FL; C: RP18; E: 45% CH_3CN, 55% H_2O–HOAc (41:2)	OA methyl ester	<0.1	>80		[55]
Human milk	H_3PO_4–NaCl–$CHCl_3$ (pH 1.6)	LLP	HPLC–FL; C: RP18; E: CH_3CN–H_2O–HOAc (570:410:20,v/v)	MS; ELISA	0.1	83 (0.5–10.0)		[9]
Animal tissues	Citric acid–NaCl–acetone	LLP and CC on Si	TLC	–	10	80	Simultaneous determination of aflatoxins	[56]
Pig's blood serum	$MgCl_2$–HCl–$CHCl_3$ (pH<2.0)	SPE	HPLC–FL; C: RP18; E: Propanol-2–CH_3CN–0.083 *M* H_3PO_4–H_2O (20:25:37:18 v/v)	–	0.05/0.1	80–90 (0.5)		[57]
Human urine	HCl–$CHCl_3$–CH_3OH	CC on Si and HPLC	HPLC–FL; C: RP18; E: CH_3CN–0.005 *M* NaAc–HOAc (45:55:1.4, v/v)	OA methyl ester	0.005/0.01	65–75 (0.01–0.5)	Simultaneous determination of 4-OH-OA	[58]

(*Continued on p. 80*)

Table 2. Continued

Matrix	Extraction	Clean-up[b]	Method[c]	Confirmation[c]	DL/QL[d] (ng/g)/ (ng/ml)	Recovery[e] (%)	Remarks[f]	Ref.
Rumen fluid	H_3PO_4–$CHCl_3$	LLP	HPLC–FL; C: RP18; E: CH_3CN–Propanol-2–0.083 *M* H_3PO_4 (35:10:55, v/v)	OA methyl ester	0.1	72–88 (3–300)	Simultaneous determination of Oα, OB, OC	[59]
Blood serum	$MgCl_2$–HCl–$CHCl_3$	No	HPLC–FL; C: RP18; E: CH_3OH–H_2O–HOAc (70:30:1, v/v)	–	1.0	87–94 (5–50)		[60]
Pig's blood serum	Diluted by $MgCl_2$–HCl; extraction+clean-up on C_{18}-SPE		HPLC–FL; C: RP18; E: THF–CH_3CN–2% HOAc (30:15:55, v/v)	Ratio OA methyl ester/OA	0.1	–		[61]
Pig's kidney and liver	NaCl–H_3PO_4–$CHCl_3$	Si-SPE	HPLC–FL; C: RP18; E: H_2O–CH_3CN–HOAc (248:248:5, v/v)	OA methyl ester	0.5/1.0	93–106 (1–10)	Modified method for chicken liver	[62]
Human blood plasma	$MgCl_2$–HCl–$CHCl_3$	LLP	Ion-pair HPLC–FL; C: RP18; E: CH_3OH–phosphate buffer (pH 7.5) (63:37, v/v) with TBAB	OA methyl ester	0.02/0.05	83 (0.3–6.0)		[6]
Pig's kidney	H_3PO_4–$CHCl_3$	C_{18}-SPE	HPLC–FL; C: RP18; E: CH_3CN–H_2O–HOAc (99:99:2, v/v)	OA methyl ester	5	53–97 (average)[g] 24–224 (individual)[g]	AOAC-method for corn and barley	[63]
Pig's kidney and meat products	H_3PO_4–$CHCl_3$	IAC	HPLC-FL; C: RP18; E: CH_3CN–H_2O–HOAc (99:99:2, v/v)	–	0.2	Kidney: 79±3 (10), sausages: 74±6 (10)	For cereals mod. extraction procedure	[64]
Human blood serum	Diluted by $MgCl_2$–HCl; extraction+clean-up on C_{18}-SPE		HPTLC	OA methyl ester	0.5	95		[65]
Pig's blood serum, kidney, liver, muscle and fat	$MgCl_2$–HCl–$CHCl_3$ (pH 1.6–1.8)	LLP	HPLC–FL; C: RP18; E: CH_3CN–0.008 *M* H_3PO_4 (56:44,v/v), pH 2.8	ELISA	Serum, fat: 0.1; kidney, liver, muscle: 0.2	75–85 (1–20)		[66]
Pig's blood plasma	Protein precipitated by CH_3OH; dilution with H_3PO_4	No	HPLC–FL column switching; pre-C: PSDVB, C: RP18; E1: 0.01 *M* H_3PO_4; E2: CH_3CN–0.01 *M* H_3PO_4 (46:54, v/v)	Postcolumn pH shift	0.1	82±5 (0.5–12.0)		[67]
Pig's urine and faeces	Faeces lyophilized; $MgCl_2$–HCl–$CHCl_3$ (pH 1.6–2.0)	Si-SPE and LLP	HPLC–FL; C: RP18; E: CH_3CN–Propanol-2–0.083 *M* H_3PO_4 (47:10:43, v/v) (urine) CH_3CN–0.008 *M* H_3PO_4 (56:44) pH 2.8 (faeces)	ELISA	Urine: 0.3 faeces: 1.5	Urine: 93 (1–20) faeces: 60 (2–6)	Incubation with β-glucuronidase tested	[68]
Cow's milk human milk rat's milk	HCl–CH_3OH–$CHCl_3$	Self-packed Si-SPE and LLP	Ion-pair HPLC–FL; C: RP18; E: CH_3OH–phosphate buffer, pH 7.5 (51:49, v/v) with 10 m*M* TBAB	OA methyl ester	0.01/0.04, rat's milk: 0.1/0.3	Cow's milk: 85 human milk: 75 (both 0.01–0.5) rat's milk: 75		[10] [69]
Rat's blood serum and urine	$MgCl_2$–HCl–$CHCl_3$ (pH 2.5)	LLP	HPLC–FL; C: RP18; E: CH_3OH–CH_3CN–HOAc–NaAc (300:300:400:14, v/v)	–	–	–		[70]
Pig's kidney	Ethyl acetate–H_3PO_4	No	(a) ELISA (b) HPLC–FL; C: RP18; gradient mobile phase: A: H_3PO_4, pH 2.1 B: 90% CH_3OH–10% Propanol-2	Enzymatic hydrolysis to Oα	(b) 3.9	(b) 91–110 (3.9–15.6)		[71] [72]

Table 2. Continued

Matrix	Extraction	Clean-up[b]	Method[c]	Confirmation[c]	DL/QL[d] (ng/g)/ (ng/ml)	Recovery[e] (%)	Remarks[f]	Ref.
Animal tissues	Citric acid–NaCl–methanol	CC on Si	TLC	AOAC-procedure [13]	–	50 (150–200)	Multi myco-toxin method; qualitative OA-analysis	[73]
Pig's bile	$MgCl_2$–HCl–$CHCl_3$ (pH 1.6–2.0)	Si-SPE and LLP	HPLC–FL; C: RP18; E: CH_3CN–0.008 *M* H_3PO_4 (56:44, v/v), pH 2.8	ELISA	0.3	84 (1–17)	Incubation with β-glucuronidase tested	[74]
Human milk	$MgCl_2$–HCl–ethyl acetate	No	HPLC–FL; C: RP18; E: 2% HOAc–CH_3CN (43:57. v/v)	OA methyl ester ammoniation	0.1	80–87 (0.5–10.0)		[11]
Pig's liver	CH_3OH–PBS	IAC	HPLC–FL; C: RP18; E: CH_3CN–H_2O–HOAc (51:47:2, v/v)	–	–	40 (10)	In wheat 87% recovery	[75]
Human blood, serum and milk	H_3PO_4–NaCl–$CHCl_3$	IAC, milk: LLP and IAC	HPLC–FL; C: RP18; E: CH_3OH–9% HOAc pH 2.3 (18:7, v/v); postcolumn addition of NH_4OH	OA methyl ester and Oα methyl ester	0.005–0.01	85±10 (0.01–5.0)	Reuse of IAC studied	[8]
Cow's milk	HCl–CH_3OH–$CHCl_3$ (pH 1.6–2.0)	Si-SPE	HPLC–FL; C: RP18; E: CH_3CN–0.008 *M* H_3PO_4 (60:40, v/v) pH 2.8	ELISA; OA methyl ester	0.01/0.03	84±7 (0.03–0.5)	Incubation with β-glucuronidase tested	[76]
Pig's blood serum	[34] ($MgCl_2$–HCl–$CHCl_3$)	No (?)	HPLC–FL; C: RP18; gradient mobile phase: A: H_3PO_4 (pH 2.1) B: CH_3OH–Propanol-2 (90:10, v/v)	Enzymatic hydrolysis to Oα; LC–MS	0.3	87 (0–24)		[77]
Meat and meat products	(a) Citric acid–CH_2Cl_2; (b) $MgCl_2$–HCl–$CHCl_3$, (pH<1.6)	(a) LLP and Si-SPE (b)IAC	HPLC–FL; C: RP18; E: CH_3CN–H_2O–HOAc (570:410:20, v/v)	–	(a) 0.002–0.005 (b) 0.02–0.05	Both methods: 50–90		[78]

– Not specified.

[a] Specifications as stated in the papers.

[b] LLP, liquid–liquid partitioning; CC, column chromatography; SPE, solid-phase extraction; IAC, immunoaffinity column; Si, silica gel.

[c] TLC, thin-layer chromatography; HPTLC, high-performance thin-layer chromatography; HPLC, high-performance liquid chromatography. FL, fluorescence; MS, mass spectrometry; ELISA, enzyme-linked immunosorbent assay; C, column; E, mobile phase; THF, tetrahydrofuran; HOAc, acetic acid; NaAc, sodium acetate; TBAB, tetrabutyl ammonium bromide; PSDVB, polystyrene–divinylbenzene.

[d] DL, detection limit; QL, quantitation limit.

[e] In parentheses, concentration range in ng/g.

[f] Oα, ochratoxin α; OB, ochratoxin B; OC, ochratoxin C; 4-OH-OA, 4-OH-ochratoxin A; Cit, citrinin; AFB1, aflatoxin B1.

[g] From an inter-laboratory study.

As problems with homogeneity are not expected in most tissues and fluids, smaller sample sizes can be taken for the extraction compared with grains, for example. The size of an analytical sample depends on the total sample size available, on the detection limit which is demanded, on the determination method and on the instrumentation. Generally, only restricted sample sizes of human fluids and of animal blood are available. In most cases, less than 1–5 ml of blood or human milk and 5– 50 g of pig's kidney, respectively, were analysed. The progress in instrumentation led to an improved sensitivity of new fluorescence detectors for HPLC in comparison with the older types. For example, it was necessary to start with 50 ml cow's milk to reach a detection limit of 0.01 ng/ml [76]; after a new fluorescence detector has been installed, only 5 ml milk was required to reach the same detection limit [H. Valenta, unpublished results]. Low sample sizes are recommended to minimize the amount of solvents needed for extraction. This diminishes the costs of the solvents and of waste disposal.

For an inter-comparison study, OA was shown to be stable in naturally contaminated pig kidney

material which was frozen, freeze-dried, ground, bottled in brown glass vials under nitrogen, irradiated and stored over a period of 6 months at −18°C to +40°C [94]. This treatment was necessary to ensure that no deterioration of the material occurred when sending it to the participants of the inter-comparison study. However, in general, samples of tissues and fluids are stored without special pretreatment, so that the enzyme activity is not stopped completely. In most cases, they are stored at −18°C. No study was found in the literature on the influence of the storage conditions on the OA amount in naturally contaminated animal or human tissues or fluids. The stability of OA in spiked cow's milk at very low concentration levels of 0.01–0.05 ng/ml was tested during storage at −18°C over a period of 6 weeks; no decrease of the OA concentration was observed [76]. However, it must be considered that results of storage experiments with spiked material cannot be applied to storage of naturally contaminated material without restrictions. Binding of OA to the matrix is not the same in both cases, and, when spiking, generally a solvent is added to the sample which can have effects on the natural biological activity. Therefore, in case of liquids and aqueous samples, in which the spiking solvent cannot be allowed to evaporate, it is recommended to add OA in a small volume of solvent. Solvents which are miscible with water as methanol or acetonitrile are preferred to ensure good distribution throughout the sample.

3.2. Extraction

OA can be extracted from a water phase into a less polar solvent not miscible with water such as chloroform only at pH<7.0, as under neutral and alkaline conditions it is present in the dissociated form. In a protein matrix such as blood, the extraction is made additionally difficult by the binding of OA to protein. In blood, more than 99% of OA is bound, mainly to serum albumin (for review to pharmacokinetics of OA see [29,95]). Uchiyama et al. [28] showed that the binding ability of OA to bovine serum albumin was high in the range of pH 4.0 to 6.0. The major binding type was presumed to be ionic. The binding was weakened with increasing ionic strength of the solution and with increasing content of methanol. The authors recommended that the pH of the sample solution should be adjusted to less than 2.0 to ensure complete extraction of OA from matrices rich in protein.

In recently published papers adducts and conjugates of OA were found in fungal and plant cell cultures. Xiao et al. [96] found macromolecule conjugates of OA and of two OA metabolites in fungal cultures; the binding was assumed to be covalent. In studies of Ruhland et al. [97] and Bokern et al. [98] with [^{14}C]OA in plant cell cultures of wheat, maize and barley 7–58% of the radioactivity was found in nonextractable residues. Besides that, glucosides of two hydroxy-OA-metabolites were detected [97]. Therefore, further adducts and conjugates can be expected in animal tissues. DNA-adducts of OA were detected in monkey kidney cells and in three mouse tissues [99,100]. Two older methods for OA determination in kidney include an enzymatic digestion with Subtilising A or papain prior to extraction [81,49]. Higher concentrations of OA were measured in the samples after enzymatic digestion with Subtilising A [49]. Nevertheless, it seems that these procedures were not applied in later studies.

As glucuronide conjugates with other mycotoxins, e.g. aflatoxins and zearalenone, were found in urine and milk [101–104], incubation with β-glucuronidase prior to the extraction was studied in a few papers. In bile of mice, OA glucuronide conjugates were detected [105]; in three naturally contaminated bile samples of swine, higher levels of OA after enzymatic incubation were also found [74]. No glucuronide conjugates could be detected in two samples of naturally OA contaminated pig's urine [68], and in cow's milk [76]. In cow's milk, a peak occurred at the retention time of OA in HPLC chromatogram after enzymatic incubation with β-glucuronidase, but OA could not be confirmed by enzyme-linked immunosorbent assay (ELISA). Orti et al. [53] included incubation with ß-glucuronidase in their method for OA determination in human urine. However, as naturally contaminated samples were not analysed in this study, OA conjugation with glucuronic acid in human urine could not be proven.

Transformation of OA is known to occur in microorganisms, in fungal systems, in plant cell cultures, in cells of animals and in animals in vivo

[96,97,106,107]. Several metabolites have been identified, e.g. in animals and animal cells ochratoxin α, 4-*R*- hydroxyochratoxin A, 4-*S*-hydroxyochratoxin A, 10-hydroxyochratoxin A and, recently, lactone-opened ochratoxin A [37]. Besides, analogues of OA were isolated from cultures of *Aspergillus ochraceus*, in which the phenylalanine moiety was replaced by other amino acids [108]. Tyrosine-OA, an analogue of OA, was found in the liver of OA poisoned animals [109]. In a recently published study [72], much higher levels of Oα relative to the amount of OA were detected in naturally contaminated swine kidney samples after enzymatic hydrolysis of OA to Oα by carboxypeptidase A. Two possibilities were discussed that the free Oα could be derived from the cleavage of either the present conjugated forms of OA or of present OA analogues by carboxypeptidase. The toxicity of OA metabolites is in part as high as of OA. Thus, an additional health hazard may derive from the occurrence of OA conjugates, adducts, metabolites and analogues in animal-derived food products which are generally not extracted and/or not determined by the analytical procedures for OA. Therefore, further research in this area is necessary [110].

As can be seen in Table 2, in most studies OA was extracted from blood or animal tissues by chloroform after acidification with a solution of hydrochloric acid and magnesium chloride to pH 2.5 or less than 2.0 [6,47,50,55,57,60,66,78]. Addition of magnesium chloride should not only increase the ionic strength but also inhibit the extraction of interfering substances [34]. In some studies, OA was extracted by chloroform after addition of a solution of phosphoric acid and sodium chloride [8,62]. Zimmerli and Dick [8] compared the two procedures: They extracted 2 ml of a naturally contaminated sample of pig plasma using 5 ml of chloroform after addition of (1) 10 ml of 0.1 *M* $MgCl_2$ and 0.05 *M* HCl (pH 2.5), or (2) 10 ml solution of 0.5 *M* H_3PO_4 (pH 1.6) and 2 *M* NaCl. The first procedure gave a mean concentration of 0.20 ± 0.02 ng/g ($n=4$), the second procedure gave a 40% higher value of 0.29 ± 0.03 ng/g ($n=4$). The authors concluded that the addition of the 2 *M* NaCl solution was the reason for the better extraction result using the second extraction procedure. However, both procedures differed also in the pH value. In several studies, higher concentrated solutions of $MgCl_2$ and HCl with a higher ionic strength and pH of less than 2.0 were used [50,57,74,78]. It is therefore not sure, whether the extraction in presence of NaCl and H_3PO_4 generally leads to better results than the extraction in presence of $MgCl_2$ and HCl. As extraction technique, shaking for up to 30 min is used in some methods [5,6,52,68], blending or intensive mixing for some minutes in other methods [46,62–64]. At the end of extraction, the two phases must be separated by centrifugation. When emulsion occurs, centrifugation in a refrigerated centrifuge is recommended.

In some studies, especially those dealing with the OA determination in kidney, OA was extracted by chloroform after acidification with phosphoric acid without an addition of a salt solution [46,52,63,64], or by dichloromethane and citric acid [49]. Milk and urine were extracted with chloroform using similar procedures to those described above, partly with an addition of methanol [9,10,53,58,68,76]. It is remarkable that, generally, chlorinated solvents are used for the extraction in the vast majority of the studies. Even recently developed methods with a clean-up using IAC (immunoaffinity column) include extraction by chloroform [8,64,78]. For reasons of environmental conservation, halogenated solvents should be replaced where possible. This is not easy, because they have very good solvent properties, and more polar solvents such as ethyl acetate, extract to a greater extent polar interfering substances from the matrix. Wilkens et al. [51] and Clarke et al. [71] extracted kidney with ethyl acetate and phosphoric acid, Miraglia et al. [11] used ethyl acetate together with magnesium chloride and hydrochloric acid solution for the extraction of human milk. In the method of Langseth et al. [67], plasma is extracted using methanol only, without acidification. In [61,88,111] extraction and clean up of serum or urine were combined on a C_{18} cartridge after acidification. Marley et al. [75] extracted pig's kidney with methanol and phosphate buffered saline (PBS) (1:1, v/v) prior to clean-up on an IAC. Obviously, this extraction procedure was not suitable for kidney, as the recovery of 40% was very low.

Problems with the extraction of freeze-dried pig kidney material were recently reported from an intercomparison of methods for the determination of OA in pig kidney, carried out within the European

Commission, Measurements and Testing Programme [95]. Freeze-dried samples of naturally contaminated and blank kidney material were analysed by 20 European laboratories. Only four of the laboratories obtained a recovery greater than 70% in the preliminary study; a further eight laboratories achieved a recovery greater than 70% after adapting their methods. The choice of solvent used for the extraction (chloroform, dichloromethane, ethyl acetate or acetonitrile under acidic conditions) did not greatly influence the results obtained. Specific difficulties in extracting freeze-dried pig kidney material were assumed to be the cause for the low recoveries. Four laboratories reported that better recoveries of OA were obtained from fresh pig kidney material although the same method was used in both cases.

As recovery experiments use blank samples spiked with a standard solution of the toxin prior to extraction, spiking will be briefly discussed in this section. It was demonstrated in an inter-comparison study for the determination of OA in wheat that the spiking procedure used by each laboratory had a great influence on the results [14]. When the mixture toluene–acetic acid (99:1, v/v) was used as the spiking solvent, it was essential to leave the samples for sufficient time (at least 18 h) to allow evaporation of the solvent. Otherwise, low recoveries could be obtained which led to abnormally high values of the results when corrected for spike recovery. Use of methanol as a spiking solvent did not have such a profound effect. In the inter-comparison study of methods for kidney which was discussed above [94], the freeze-dried kidney material was spiked by an OA solution in toluene–acetic acid and the solvent was allowed to evaporate prior to a reconstitution of the samples with water. However, when analysing fluids or water containing matrices such as fresh kidney, an evaporation of the spiking solvent is hardly possible. As mentioned in Section 3.1, in this case it is recommended to spike with a low volume of a standard solution in a solvent which is miscible with water, and to mix the sample to ensure good distribution of OA in the sample. One must still consider that the results of recovery experiments cannot be applied to naturally contaminated samples without restrictions. Therefore, it is necessary to test extraction procedures on naturally contaminated samples, especially with regard to the extraction time [16].

3.3. Clean-up

Clean-up of the sample extract is generally necessary, when low detection limit is required. Also, clean-up is recommended to protect the HPLC column, when using this technique. Some methods mentioned in Table 2 contain no clean-up step, especially those for blood serum or plasma which are the ‘cleanest’ matrices [11,60,71,77]. Usually, clean-up is simple or omitted in methods used in animal experiments with high OA doses, because, in these cases, a low detection limit is not necessary. For the detection of OA metabolites, clean-up of the extract is problematic because in most cases the suitability of the clean-up procedures for the metabolites is not proven.

Classic methods for clean-up are liquid–liquid partition (LLP) and column chromatography (CC). The LLP method is based on the solubility of OA in a solution of sodium hydrogen carbonate, as OA is dissociated in slightly alkaline medium. The chloroform extract obtained by the extraction of the sample or a reconstituted extract in chloroform after evaporation of the extraction solvent is partitioned into a solution of sodium hydrogen carbonate. Subsequently, the solution is acidified, and OA is extracted back into chloroform. This procedure is simple, effective in removing interfering substances, and of low costs, when using low amounts of solvents like in the method of Bauer and Gareis [5]. Because of small volumes, the mixing can be done directly in centrifuge tubes on a high speed shaker; it is not necessary to use separating funnels. This method, some times with modifications, is till now widely used for analysing serum, plasma, kidney or liver [112–114]. This procedure could also be applied to some metabolites of OA, e.g. Oα [59]. Tris-buffer (pH 7.5), was also used instead of sodium hydrogen carbonate [6,47]. The disadvantage of these clean-up methods is that they cannot be automated, and the cleaning effect is not sufficient for more complicated matrices such as urine or faeces.

The second classic procedure — CC on large self-packed columns — requires high amounts of solvents and is time consuming. This technique, used

by Wilken et al. [51], for example, has been replaced by solid-phase extraction (SPE) in the last 10 years in most cases. In the latter technique, small prepacked disposable cartridges are used which are filled with silica gel or with bonded phases like C_{18} of smaller and more uniform particles than those used previously in CC. Because of the high capacity, only 500 mg of the packing material and a few millilitres of solvent for elution of OA are sufficient in most cases. Generally, sample solutions and elution solvents are applied to the cartridges under light vacuum. Special apparatus allow simultaneous processing of several samples.

Although a variety of packing materials is commercially available, only silica gel and C_{18} cartridges are widely used for OA analysis of tissues and fluids [10,57,62,63,76,82]. A combination of LLP and SPE was used for the more complicated matrices urine, faeces, bile, human and rat's milk [10,68,69,74]. Orti et al. [53] used a combination of three cartridges — Si, CN and C_8 — for clean-up of human urine. When using silica-gel cartridges, the sample extract in chloroform was applied directly onto the column, or the sample extract was evaporated, redissolved in a rather unpolar solvent such as chloroform or toluene, and then applied. OA was eluted with a mixture of chloroform, methylene chloride, toluene or methanol containing an acid. For clean-up on C_{18} cartridges, the sample extract in chloroform was partitioned into a sodium hydrogen carbonate solution which was then applied onto the column. OA was eluted by a mixture of ethyl acetate, methanol and acetic acid [63]. As mentioned in Section 3.2, in a few studies serum or urine were directly applied to a C_{18} cartridge after acidification, and eluted with methanol or with a mixture of methanol and sodium hydrogen carbonate [61,65,89,111]. In [88,115], TLC was used for clean-up of urine, faeces and rumen fluid. This method was also suitable for determining Oα.

The most important development in the field of clean-up methods during the last few years are the IACs. The principle of this method is the same as in ELISA: the packing of the column consists of immobilised antibodies against the specific mycotoxin. The sample extract is applied onto the column, extraneous material is washed off by water or aqueous buffer, and the toxin is eluted by acetonitrile, methanol or methanol–buffer. In case of other mycotoxins, e.g. aflatoxins, biological fluids such as milk can be applied directly onto the column.

This is not possible in case of OA, because OA binds to protein, as discussed above. This is why some methods for determination of OA in animal fluids and tissues with IAC clean-up included a conventional extraction by acidified chloroform. The chloroform extract was then partitioned with sodium hydrogen carbonate solution and an aliquot of the aqueous phase was applied onto the affinity column [64,78]. In [8], in the case of blood and serum, the chloroform extract was evaporated to dryness and the residue was dissolved in PBS–methanol before application to the column. In the case of human milk, a clean-up step by LLP was included before the clean-up on an affinity column. The recovery of these methods (determination by HPLC with fluorescence detection) was in the range 79% (pig's kidney, [64]), 50–90% (meat and meat products, [78]), and 85% (human serum and milk, [8]). As already mentioned, extraction of pig's liver by methanol–PBS (1:1) and clean-up of the extract on an affinity column after dilution with PBS led to a recovery of only 40% [75]. An acidification of the extraction solvent was not possible, because the presence of an acid in the extract could be toxic to the antibody on the immunoaffinity column [75]. Dietrich et al. [116] treated cow's milk with protease–PBS in order to digest the protein, and applied the supernatant onto an affinity column. This procedure proved to be successful for spiked milk samples, recovery was 74–85% (determination by ELISA).

IAC has the great advantage that OA is bound specifically to the antibody, so that the matrix can be removed nearly completely. This is particularly important, where complex matrices are analysed. Relating to the clean-up procedures LLP (with low solvent amounts) and SPE, the saving of solvent and analytical time is not so evident, when chloroform is used for the extraction further on, and when a liquid–liquid extraction step is involved. This would change, when a suitable extraction procedure would be developed allowing the application of the extract directly onto the column. The results of an intercomparison of methods for the determination of OA in pig kidney [94] showed that improvements in the methodology are necessary, as low recoveries in the

range 43–68% were obtained using an immunoaffinity column clean-up. The relatively high costs of the columns are a disadvantage of the method. This problem could be solved, if reuse of the columns were possible, as tested by Zimmerli and Dick [8].

3.4. Detection and determination

3.4.1. Thin-layer chromatography

Many reviews on TLC analysis of mycotoxins, including OA, were published (for recent reviews see [117–119]). As only few papers about new applications or improvements of the methods for OA analysis were published in the last years, this subject will be discussed here only briefly.

Despite few recent references in publications to the technique, TLC continues to be used routinely, particularly outside Europe and North America, because this method can be applied without expensive analytical instrumentation. However, TLC methods have generally higher detection limits and higher relative standard deviations in quantitative analysis, compared to HPLC. Usually, one-dimensional TLC on silica-gel plates is used. Paulsch et al. [46] used two-dimensional TLC for OA determination in pig's kidney which provided better separation. Later investigations led to the use of small-particle-size silica-gel, i.e., high-performance TLC (HPTLC). Using HPTLC, a detection limit of 0.5 μg/l was reached for OA analysis in human blood serum [65]. Abramson et al. [120] studied chromatography of mycotoxins, OA included, on precoated reversed-phase thin-layer plates, and recommended this technique as a confirmation method for mycotoxins appearing in normal-phase (silica) thin-layer chromatographic screening procedures.

In the most widely used TLC method (AOAC method for OA in barley [121]) which is also recommended for animal tissues, benzene–methanol–acetic acid (18:1:1, v/v) is used as developing solvent. Büchman and Hald [52] used toluene–ethylacetate–formic acid (5:5:1, v/v) as developing solvent for OA analysis in kidney. The blue–green fluorescence of the OA spot which is brought out by UV light is used for detection. The detection can be done by visual observation or instrumentally by a densitometer. OA fluorescence is enhanced approximately 10-fold after exposure to methanol–ammonia; the excitation maximum is shifted from 333 to 370 nm [118]. This procedure is recommended in a modified method of Nesheim et al. [122]; the detection limit is given as 5 μg/kg tissue.

3.4.2. High–performance liquid chromatography

HPLC with fluorescence detection (FL) as the final separation and determination method of OA has become the widely used method in the last 10 years. As can be seen from Table 2, most methods for OA determination in animal and human tissues, published in the last years, are HPLC methods. In an inter-comparison of methods for the determination of OA in pig kidney, carried out within the European Commission, Measurements and Testing Programm, all 20 laboratories used reversed-phase (RP) HPLC as the determinative step [94]. One reason for the preferential use of HPLC is the very low detection limit that can be reached by HPLC–FL, as OA is a substance with high natural fluorescence. Enhancement of the efficiency of fluorescence detectors in the last few years further improved the sensitivity of OA measurements. Low detection limits of OA in foods and animal and human tissues are important for carrying out a health risk assessment. Another reason for the popularity of HPLC for OA detection is that, in general, OA separation on a RP18 column is rather nonproblematic when considering the conditions described below. The possibility to automate the analysis is a further advantage.

General conditions for the HPLC analysis have not changed in the last years. RP-HPLC on C_{18} columns was used in nearly all methods mentioned in Table 2, Orti et al. [53] used a C_8 column for OA determination in human urine. With the exception of some older methods, in which 10 μm material was employed, 5 μm was the standard particle size. Breitholtz-Emanuelsson et al. [10] and Langseth et al. [67] used columns filled with 3-μm material. Columns with the standard size 250×4 or 4.6 mm were most often employed.

As OA is a weak acid, the mobile phase in RP-HPLC must be acidic to avoid strong tailing and unspecific adsorption to the column. As can be seen in Table 2, mixtures of acetonitrile or methanol with diluted acetic or phosphoric acid were used as the mobile phase in most cases. Acetonitrile was preferred, as acetonitrile–water mixtures have a lower

viscosity and better separation efficiency than methanol–water mixtures [123]. A disadvantage of acetonitrile is the higher price and higher toxicity. Mixtures of two solvents (with an acid) were used in special cases, e.g. in [51,53,57,68]. An addition of a second solvent can — in some cases — improve the separation of the OA peak from interfering matrix peaks. Isocratic elution was used in most cases. Gradient elution was applied in the methods of Clarke et al. [71] and Ominski et al. [77] for pig's kidney and serum which have no clean-up step in the sample preparation. In general, gradient elution is necessary, when other metabolites should also be detected [115,124]. Usually, the fluorescence detector is set to λ_{exc}=330 nm and λ_{em}=460 nm.

Ion-pair chromatography was used in the methods of Breitholtz-Emanuelsson et al. for the determination of OA in blood plasma and in human, cow's and rat's milk [6,10,69]. This technique which was used previously for OA determination in coffee [125] allows HPLC determination of OA at pH of 7.5, because a counter-ion is added to the mobile phase which forms a complex with the dissociated OA. Under alkaline conditions, a shift of the absorption maximum of OA occurs from 330 nm to 380 nm, associated with an increased signal. With this technique, very low detection limits of 0.01 ng/ml in human and cow's milk and 0.02 ng/ml in blood plasma were achieved [6,10]. A disadvantage of the method is that small changes in the composition of the mobile phase cause considerable variations in the OA retention time. Another approach for increasing the sensitivity of OA determination is the post-column addition of ammonia [8,67,81] — a method which was derived from TLC (see Section 3.4.1). Zimmerli and Dick [8] reported a six-fold increase of the response for OA after ammoniation and a quantitation limit of 0.005–0.01 ng/g for human milk and serum. Langseth et al. [67] used a HPLC column-switching method for the determination of OA in pig blood plasma. The method consisted of a precipitation of protein by methanol, dilution of the supernatant with phosphoric acid and direct injection into the HPLC apparatus. The extract was cleaned up and preconcentrated on a polystyrene–divinylbenzene precolumn, and, after column-switching, chromatographed on a C_{18} analytical column.

The suitability of mass spectrometry (MS) as LC detection method for OA was proved by Abramson [126] and Rajakylae et al. [127]. Thermospray (TS) was used as LC–MS interface in [127], direct liquid introduction (DLI) was applied in [126]. In the latter study, the use of negative-ion chemical ionisation (NCI) gave a 40-fold sensitivity increase compared to positive-ion chemical ionisation (PCI). Applying NCI and selective ion monitoring (SIM), OA concentrations at approximately 3 μg/kg were readily detectable in barley. As other more simple and sensitive methods are available, the use of LC–MS for routine analysis of OA is not expected. However, an increasing importance of LC–MS for the detection of OA metabolites can be expected, because, compared to GC–MS, no derivatisation is needed, and more polar metabolites can be detected. In [96], LC–MS was used for the identification of OA metabolites in rat urine and in a culture of *Aspergillus ochraceus*. Besides, fast atom bombardment (FAB)-MS was used for the identification of OA metabolites in cell cultures of wheat and maize [97].

3.4.3. Other methods

OA cannot be analysed by gas chromatography (GC) directly, as it is not volatile. Identification of OA in food samples by chemical derivatisation and GC–MS was reported for the first time in 1992 [128]. OA was converted into its O-methylochratoxin A methyl ester derivative which was identified by capillary GC–MS with negative-ion chemical ionisation (NCI). The detection limit was 0.1 μg/kg. A disadvantage of this method is that the highly toxic diazomethane is required for derivatisation. As mentioned above for LC–MS, the use of GC–MS in routine analysis of OA is not expected. However, the method is suitable for confirmation of positive findings.

Immunoassays, especially ELISA, have become important in mycotoxin analysis. These methods are used for screening purposes and, in recent years, also for determination. RIA (radioimmunoassay) and ELISA were used for example for OA determination in blood [111,129–131], kidney or milk [116,132,133]. As these are not chromatographic methods, they are not further discussed in this review. A spectrofluorometric method for the determination of OA was used earlier [34,47]. The toxin was cleaved into Oα and phenylalanine, using

the enzyme carboxypeptidase. For quantitation, the loss of fluorescence intensity at 380 nm after enzymatic hydrolysis was measured.

3.5. Confirmation

The confirmation of positive findings is now considered an essential quality assurance requirement [17]. As can be seen in Table 2, OA is confirmed in most methods by derivatisation to the OA-methylester. The derivative is then determined by TLC or HPLC. A boron trifluoride (BF_3)–methanol mixture is used for the preparation of the methyl ester in most methods. This procedure is also a part of the official AOAC method for OA determination in corn and barley by HPLC [13]. Zimmerli and Dick [8] reported problems in finding a commercial BF_3–methanol reagent that gave an acceptable blank value. Finally, they used a HCl–methanol mixture which yielded in 82% of the methyl ester. Takeda et al. [61] used H_2SO_4 and methanol for a controlled derivatisation procedure at 15°C for 30 min which led to a conversion rate of 46%. The peak height was used for the quantitation of OA and the ratio (OA-Me/OA) was employed for the confirmation of OA identity in pig's blood serum. Several authors reported problems in confirming low concentrations near the detection limit by derivatisation to OA methyl ester, e.g. in blood plasma and kidney [6,50], human milk [11] or in cow's milk [76].

In a few papers, derivatisation to Oα and partly to Oα methyl ester was described as a confirmation method [8,47,72,77]. However, the chromatographic separation of Oα on a RP18 column is more difficult than that of OA, because Oα elutes prior to OA as a more polar substance. In this part of chromatogram, interfering matrix peaks are common. Ominski et al. [77] and Frohlich et al. [72] used gradient elution to separate the peaks. Miraglia et al. [11] used ammoniation by postcolumn derivatization as a confirmation method for OA in human milk. Recording of the fluorescent spectrum of OA during a HPLC measurement by stop flow was also employed as a confirmation method [5,50,54]. This is only possible, when the OA peak is not too small.

Another possibility to confirm positive results is to apply an independent method based on other principles. ELISA, as a very sensitive method, was found to be suitable for confirming OA levels down to the detection limit in human milk, in swine tissues, serum, bile, urine and faeces and in cow's milk [9,66,68,74,76]. MS techniques are highly specific confirmation procedures. Formerly, direct probe (DIP) MS was used for qualitative confirmation of OA. The samples had to be cleaned up extensively by TLC or HPLC prior to the introduction into the MS [9,49,50], and several sample extracts had to be combined to reach an acceptable detection limit. The LC–MS and GC–MS methods, developed in the last few years, which are discussed in Section 3.4 now offer better possibilities of confirming positive results; sample clean-up as required for HPLC–FL is sufficient. A GC–MS method, similar to that described by Jiao et al. [128], was used for confirmation of OA in green and roasted coffee [134]. The LC–MS method of Abramson [126], also described in Section 3.4, was recently used for confirmation of OA in pig's blood serum [77].

4. Conclusions

Reliable and sensitive methods for the determination of OA in animal and human tissues and fluids are needed for monitoring studies on the OA occurrence in the food chain and in humans, for studies dealing with the carry-over from feed to animal tissues, and for studies on the elucidation of the mode of action of OA in animals and humans. In recent years, improvements in analytical methodology of OA have been made mainly in the fields of clean-up methods and instrumentation resulting in lower detection limits, easier clean-up procedures and automation of the chromatographic determination.

HPLC with fluorimetric detection has now become the most popular method for OA determination, at least in Europe and North America. The method is very sensitive and reproducible, and can be easily automated. TLC remains a reliable routine method which can be applied without expensive instrumentation. With further progress in the instrument development it can be expected that LC–MS will become more important, especially for the detection

of OA metabolites. As an alternative to the chromatographic methods, ELISA methods which are not discussed in this review were also used for screening and determination in recent years. The confirmation of positive findings is now considered as an important part of each method. The derivatisation to OA methylester, followed by the determination with HPLC or TLC is used in most cases. In addition, ELISA, GC–MS and LC–MS are also suitable as confirmation methods

Extraction and clean-up are important parts of OA determination methods of animal or human tissues and fluids. In general, for the properties of OA as a weak acid and its protein binding, the extraction is performed at a low pH. Results of recent studies indicate the possibility that further OA adducts and conjugates could be present in tissues and fluids. Additionally, new metabolites and compounds similar to OA were isolated in the last few years whose toxicity is not always known. This underlines the necessity of further research in this field, as the standard extraction procedures and analytical methods possibly lead to an underestimation of OA contents in the organs and of the toxic potential. At present, most methods used for OA determination in tissues and fluids include SPE or LLP as clean-up procedures. The most important development of the last years in the field of clean-up procedures are IACs. The advantages of this approach are the possibility to obtain very clean extracts and to simplify the extraction and clean-up procedures. However, in case of OA analysis in tissues and fluids, further improvements in the methodology are required.

The subjects of sampling, sample storage and spiking of tissues and fluids were until now neglected, nevertheless investigations in this area are also needed. There is a trend in recent years towards minimizing the amounts of solvents used in the analytical procedures, and to replace toxic and ecotoxic solvents such as chloroform with less problematic solvents. However, as halogenated solvents are used at present in most methods for OA determination in tissues and fluids, further research is needed to find alternatives. Finally, the efforts for the development of a certified reference material for determination of OA in kidney should be continued, as reference materials play a key role in the quality assurance of analytical laboratories.

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