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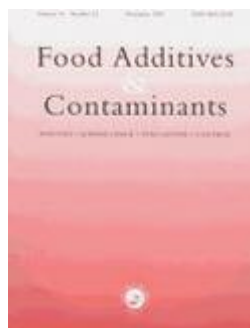
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Ochratoxin A in licorice as affected by processing methods

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1 Ochratoxin A in licorice as affected by processing methods

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17

18 **Running head:** *Effect of processing on ochratoxin A in licorice*

19

1
2
3 **1 Abstract**
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5
6 2 A study of the effect of several processing methods on the ochratoxin A (OTA)
7
8 3 concentration in licorice and derived products was carried out. The effect of sorting,
9
10 4 washing and peeling of fresh licorice roots was investigated, as well as the production
11
12 5 oat a laboratory scale of licorice extract and block licorice from dry roots. Finally, the
13
14 6 thermal stability of OTA was assessed. The OTA content was analyzed by HPLC-
15
16 7 fluorescence and confirmed by methyl ester formation. The OTA level in licorice
17
18 8 extract was stable to heat treatment at 150°C for 60 min. The OTA concentration was
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20 9 unaffected by sorting or washing, but it was much reduced by peeling (53.1%
21
22 10 reduction). A great reduction in the OTA level was found during the production of
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24 11 licorice extract (78.6%) and block licorice (91.8%).
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13 **Keywords:** Mycotoxins, ochratoxin A, HPLC, immunoaffinity, licorice root, licorice
14 extract, block licorice, licorice processing methods
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1 Introduction

2
3 Ochratoxin A (OTA) is a secondary metabolite of several species of fungi, notably
4 *Penicillium verrucosum* and *Aspergillus ochraceus*, but also *A. carbonarius* and *A.*
5 *niger* aggregate species (Esteban et al. 2006). OTA contaminates foods such as cereals,
6 coffee, dried fruit, grapes, cocoa and wine, and it can also occur in animal products.
7 Licorice and derived products may also contribute to human exposure to OTA,
8 particularly for high consumers of these foods, as it was noted in a recent international
9 conference (Fink-Gremmels 2005). Ochratoxin A is a mycotoxin with carcinogenic
10 (class 2B of IARC), nephrotoxic, teratogenic, and immunotoxic properties, and it has
11 been linked to nephropathy in humans (Creppy 2002).

12
13 The European Food Safety Authority considered that it would be prudent to reduce
14 exposure to ochratoxin A as much as possible, establishing a Tolerable Weekly Intake
15 (TWI) of 120 ng/kg b.w. (EFSA 2006). Recent analyses of the dietary exposure of adult
16 European consumers to OTA revealed that at present the weekly exposure ranges from
17 15 to 60 ng OTA per kg b.w., but more data would be needed to assess exposure rates
18 of infants and children, taking into account their dietary preferences (EFSA 2006).
19 Under Regulation No. 1881/2006 (EC 2006a), maximum limits for ochratoxin A were
20 set in cereal grains and derived products, dried vine fruits, coffee products, wine, grape
21 juice, musts and certain infant and dietary foods.

22
23 Licorice (*Glycyrrhiza glabra* L., Leguminosae) is a common dietary supplement used as
24 flavouring and sweetening agent in confectionery and other food products, such as
25 beverages and chewing gum (Gabriele et al. 2001), and its derivatives have been given

1 Generally Recognized as Safe (GRAS) status in the USA (Anonymous 1985). Licorice
2 is also a popular ingredient in herbal tea and in some botanicals, consumed to improve
3 overall health and to alleviate a wide range of diseases (Fiore et al. 2005). The natural
4 presence of ochratoxin A in licorice was first reported in Germany by Bresch et al.
5 (2000) and Majerus et al. (2000), who detected a high percentage of positive samples. A
6 recent survey conducted in our laboratory showed that all samples of licorice and
7 derived products contained ochratoxin A, and some of them had extremely high
8 concentrations up to 253 ng/g of OTA (Ariño et al. 2006). Therefore, every effort
9 should be made with regard to research and prevention measures to reduce the presence
10 of OTA in licorice as much as possible.

11
12 A typical traditional process for licorice production, as outlined by Gabriele et al.
13 (2001), is essentially based on four different unit operations: grinding of dry licorice
14 roots, extraction with steam or hot water, filtering/centrifugation of the extract, and
15 concentration. The initial extraction and filtering yields the licorice extract, which is
16 further dehydrated to obtain a dark brown material called block licorice. In our previous
17 study of licorice derivatives and foods containing licorice, OTA was abundant in block
18 licorice (39.5 ng/g), as well as in licorice-confectionery (3.8 ng/g) (Ariño et al. 2006).
19 However, very little is known about the effects of licorice processing on the OTA
20 concentrations and the feasibility of decontamination methods for OTA.

21
22 The prevention of mycotoxin contamination should include all phases of food
23 production, because mycotoxins may be synthesized in the field, during storage, as well
24 as in transport. All methods of decontamination increase the cost of production and
25 should be used only in cases when preventive measures in the field have failed. With

1 regard to mycotoxin decontamination of foodstuffs, the European Community favours
2 the use of sorting procedures and physical decontamination processes, such as cleaning,
3 washing, peeling and heat processing. Chemical detoxification may be very effective,
4 but it does not meet the EC requirements, because some compounds leave behind their
5 toxic metabolites and others reduce the nutritional value of treated foodstuffs.
6 Therefore, foodstuffs containing mycotoxins shall not be deliberately detoxified by
7 chemical treatments (EC 2006a).

8
9 The objective of the present study was to obtain data on the effect of several processing
10 methods in the OTA concentration in licorice and derived products. First, the effect of
11 sorting, washing and peeling of fresh licorice roots was investigated. Then, licorice
12 extract and block licorice were produced at laboratory scale to assess the transfer rate of
13 OTA from the starting material (dry licorice roots). Finally, the thermal stability of
14 OTA in licorice extract was assayed.

16 **Materials and methods**

18 *Licorice samples*

19 The samples of fresh licorice root (for the study of sorting, washing and peeling) were
20 provided from local growers of Zaragoza, Spain. These fresh root samples were
21 classified into two types (healthy and damaged), distinguished by each other by the
22 presence of less uniformly shaped roots showing some brown coloration instead of the
23 typical bright yellow colour of the healthy roots. The samples of dry licorice root (for
24 the preparation of licorice extract and block licorice) were purchased from herbal stores

1 of Zaragoza, Spain. The licorice roots were ground in an IKA laboratory mill to a coarse
2 root powder, and kept refrigerated at 4°C until processing.

3 4 *Effect of washing and peeling of fresh licorice roots*

5 To assess the effect of first-stage processing of licorice roots on OTA contamination,
6 eight batches of freshly-collected roots were subjected to washing and peeling. Washing
7 was done in a suitable container using tap water with no detergent aid. Peeling was
8 carried out manually with a knife, removing the outer part of the licorice root. The peel:
9 root ratio (as a percentage of peel removed over total root weight) was around 16% in
10 all batches.

11 12 *Transfer of ochratoxin A into licorice extract and block licorice*

13 In order to evaluate the transfer rate of OTA from dry licorice roots to derived products,
14 experiments were carried out at laboratory scale to simulate the industrial processing of
15 licorice. First, licorice extract was obtained by hot water extraction in an Italian coffee
16 maker. For this, the container was filled with distilled water and the sample holder with
17 12 g of ground licorice root. The instrument was placed over a low flame and the
18 licorice extract leaching through the roots was collected and filtered through a Whatman
19 1 filter (volume ~ 120 ml). The extraction time was 1 minute and the temperature was
20 between 93-100°C. Then, the licorice extract was subjected to drying in a heater at
21 105°C during 12 h to obtain block licorice (~ 2.5 g). A total of 4 batches of dry licorice
22 roots were processed in this way into the corresponding derived products.

23 24 *Thermal treatments*

1 The thermal stability of ochratoxin A was carried out in a thermo-resistometer TR-SC
2 as previously described (Raso et al. 1998). The corresponding treatment medium was
3 licorice extract (350 ml) with an OTA concentration of 7.7 ng/ml. The heat treatment
4 was conducted isothermally at 150°C, once the temperature had attained stability. At
5 preset intervals (15, 30 and 60 min), samples for each treatment time were collected and
6 analyzed for ochratoxin A by LC-FLD. Experiments were performed in duplicate and
7 results were expressed as the average value.

9 *Apparatus and reagents for ochratoxin analysis*

10 HPLC grade acetonitrile and methanol were purchased from Lab-Scan (Dublin, Ireland)
11 and HPLC grade acetic acid and hydrochloric acid (37%) from Merck (Darmstadt,
12 Germany). Ultrapure water was obtained from a Milli-Q Plus apparatus from Millipore
13 (Milford, MA). The immunoaffinity columns RIDA Ochratoxin A were supplied by R-
14 Biopharm AG (Darmstadt, Germany). Ochratoxin A standard was provided by Sigma
15 (St. Louis, MO) and stock solutions (0.01 mg/ml) were prepared in methanol and stored
16 at -21°C. Reagents for sodium bicarbonate buffer pH 8.1 (0.13M) and phosphate buffer
17 saline pH 7.4 (20 mM) were provided by Panreac (Barcelona, Spain).

18
19 The LC system consisted of an Agilent Technologies 1100 high-performance liquid
20 chromatogram coupled to an Agilent fluorescence detector at 333 nm (excitation) and
21 460 nm (emission). The LC column was Ace 5 C₁₈, 250 x 4.6 mm, 5 µm particle size
22 (Advanced Chromatography Technologies, Aberdeen, Scotland). The isocratic LC
23 mobile phase was distilled water, acetonitrile and acetic acid (51:48:1 v/v); the flow
24 rate, 1 ml/min.

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3 1 *Analysis of ochratoxin in licorice and derived products*
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5
6 2 The analytical method for ochratoxin has been previously described elsewhere (Ariño et
7
8 3 al. 2006), and it is based on immunoaffinity column/HPLC method EN 14132:2003 for
9
10 4 ochratoxin A in cereal grains and roasted coffee of the European Committee for
11
12 5 Standardization (CEN, 2003). Briefly, 3 g of licorice roots (12 ml for licorice extract, 1
13
14 6 g for block licorice) was extracted with 30 ml sodium bicarbonate 0.13 M and methanol
15
16 7 (9:1 v/v). The extract was centrifuged at 3500 rpm during 15 min, and 10 ml of it was
17
18 8 purified through the RIDA ochratoxin A immunoaffinity columns. The ochratoxin-
19
20 9 containing eluate was evaporated to dryness in a heating block under a gentle stream of
21
22 10 nitrogen, and re-dissolved in the injection solvent, a mixture of methanol, distilled water
23
24 11 and acetic acid (30:69:1 v/v). Aliquots of 20 µl were injected into the LC-FLD system.
25
26 12 The ochratoxin residues were confirmed by production of their methyl-ester derivatives,
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28 13 as described later.
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36 15 The analytical method was validated in-house with respect to precision and recovery.
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38 16 The average recovery and relative standard deviation (RSD_r, repeatability) obtained by
39
40 17 the described method for ochratoxin A were 90.6% (RSD 3.8%). The performance
41
42 18 characteristics were within the acceptable margins indicated in the Commission
43
44 19 Regulation No 401/2006 (EC 2006b). The study of sensitivity indicated that the limit of
45
46 20 quantification (LOQ) for ochratoxin A was 0.5 ng/g.
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53 22 *Confirmation procedure*
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55 23 The identity of OTA was confirmed by methyl ester formation according to Li et al.
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57 24 (1998). To an amber vial containing 25 µl of OTA residue, 75 µl of hydrochloric acid
58
59 25 (6N) and 400 µl of methanol were added. The vial was shaken, closed and left for 24 h
60

1 in an incubator at 25°C. A 20 µl aliquot was analyzed using liquid chromatography
2 coupled with fluorescence detection, and compared with blanks and standards prepared
3 in the same manner.

4 *Statistical Analyses*

5 The results from OTA analyses obtained by the LC-FLD method were subjected to
6 descriptive and comparative statistics according to Sachs (1978). Calculations were
7 performed on StatView™ SE+Graphics (Abacus Concepts, Inc. 1988, Berkeley, CA)
8 for Macintosh personal computers.

9 **Results and discussion**

10 *Occurrence of OTA in healthy and damaged fresh licorice roots*

11 Healthy fresh licorice roots were uniformly shaped and bright-yellow coloured while
12 damaged roots were unevenly shaped with some brownish parts. However, no samples
13 were moldy in appearance. All samples tested positive for OTA, with mean levels
14 ranging from 10.4 ng/g in healthy roots to 10.0 ng/g in damaged roots. The subsequent
15 statistical analysis revealed no significant differences between samples ($p>0.05$),
16 indicating that first-stage sorting of licorice based on appearance may not be effective in
17 separating out contaminated samples. However, Bresch et al (2000) reported that visual
18 analysis of licorice indicated that homogeneous bright yellow samples were less
19 contaminated by OTA than those containing brownish material and being less
20 homogeneous in shape. The natural occurrence of OTA in botanical roots has been also
21 reported by Trucksess et al. (2006), who found OTA concentrations in kava-kava (6
22 ng/g) and licorice (20 ng/g).

1 *Effect of washing and peeling of licorice roots on OTA contamination*

2 Eight batches of fresh licorice roots were subjected to water washing and manual
3 peeling to assess for OTA removal (Table I). The mean OTA level in the starting
4 material was 10.2 ng/g, and remained essentially unchanged after washing (11.0 ng/g,
5 $p>0.05$). However, in all of the samples, the peeling process caused a reduction of the
6 OTA level. Thus, peeling significantly removed the 53.1% of the OTA present in fresh
7 roots ($p<0.05$), as initial levels dropped from 10.2 to 4.9 ng/g after peeling. This shows
8 that on average, nearly half of the OTA contamination in licorice roots is found in the
9 outer root layer. However, the distribution of OTA in the roots is not homogeneous, as
10 peeling caused varying reductions from 12 to 85% even though the peel: root ratio was
11 around 16% in all batches. Majerus et al. (2000) analyzed commercial samples and
12 reported that peeled licorice roots were much less contaminated by OTA than unpeeled
13 roots. Similarly, in the processing of cocoa beans, shelling has been reported to cause
14 more than 50% reduction in OTA levels (Amezqueta et al. 2005).

15
16 “[Insert Table I about here]”

17 *Transfer of OTA from dry licorice roots to derived products*

18 Licorice derivatives are largely used in different areas of food industry, such as
19 confectionery and beverages. Licorice trade is usually made with licorice extract
20 (liquid) or crude dried block licorice (solid). The few reports available on the OTA
21 contamination of licorice derivatives indicate a high degree of contamination, as the
22 mycotoxin was detected in almost all analyzed samples by LC-FLD and LC-MS
23 (Majerus et al. 2000) and by LC-FLD plus methyl-ester confirmation (Ariño et al.
24

1 2006). However, very little is known about the effects of licorice processing into its
2 derivatives.

3
4
5 Four batches of dry licorice roots were processed into licorice extract and block licorice
6 at laboratory scale, and the effects on OTA concentrations are shown in Table II. The
7 level of OTA contamination in dry roots varied from 53 to 253 ng/g, in licorice extract
8 ranged from 1.1 to 5.8 ng/ml, and concentrations in block licorice were between 20.4 to
9 98.4 ng/g. On average, 21.4% of the OTA present in dry roots was transferred to the
10 corresponding licorice extract, what is to say that 78.6% of OTA was retained in the
11 brewed dry licorice root powder during extraction. The subsequent concentration of the
12 licorice extract further reduced OTA level by 91.8%, as only 8.2% of the initial amount
13 was carried over into block licorice. In comparison, a 32.1% reduction of the OTA level
14 was attained by brewing roasted coffee in an Italian coffee maker (Perez de Obanos et
15 al. 2005). The different percentage of OTA transfer in licorice extract (21.4%) and
16 coffee brew (67.9%) could be explained in terms of the higher fibre content of licorice
17 roots (contain root bark) and the fact that coffee is roasted, which may have facilitated
18 the transfer of OTA into the corresponding brew.

19 “[Insert Table II about here]”

20
21 In summary, the net effect of licorice processing showed a 91.8% reduction of the OTA
22 concentrations found in the starting material. However, due to the high OTA
23 concentrations detected in the roots, the mycotoxin was still present in its derivatives.
24 This is a cause for concern, as for example licorice sweets may contain between 5 to
25 10% of licorice derivatives. Majerus et al. (2000) detected OTA in 24 out of 26 samples

1 of licorice confectionery (candies and other sweets) at a mean level of 1.1 ng/g, and
2 Ariño et al. (2006) found OTA in 4 samples of licorice sweets averaging 3.8 ng/g.

3

4 *Effect of thermal treatment on OTA levels in licorice extract*

5 Where they are legally permitted, detoxification or decontamination processes will
6 involve degradation, destruction and/or inactivation of the mycotoxins. These processes,
7 while reducing the concentration of the mycotoxins to safe levels, should not produce
8 toxic degradation products or reduce the nutritional or palatability properties of the
9 commodities. In our study, licorice extract was subjected to heating to assess the effect
10 on OTA concentrations. A thermoresistometer instrument was loaded with licorice
11 extract containing OTA at 7.7 ng/ml, and tested at 150°C at preset time intervals. The
12 results showed the extreme stability of the ochratoxin to heat, as the levels remained
13 constant during the process (Figure 1). Ochratoxin is a thermostable compound; in a
14 study of the stability in cereals during heating, the calculated half-lives (time to 50%
15 destruction of ochratoxin A) were 707 and 201 min, respectively, at 100 and 150°C for
16 dry wheat, and 145 and 60 min, respectively, at 100 and 150°C for wheat heated under
17 wet conditions (Boudra et al. 1995). The presence of water (50%) increased the
18 decomposition of OTA at 100 and 150°C.

19

20 “[Insert Figure 1 about here]”

21

22 Licorice roots and derived products are frequently contaminated with OTA, and
23 licorice derivatives are largely used in different areas of food industry. OTA level in
24 licorice extract was stable to heat treatment at 150°C during 60 m. Sorting of damaged
25 roots based on visual appearance has not proved useful to separate out contaminated

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1 roots. Washing the roots was also ineffective in removing OTA, whereas peeling the
2 roots significantly reduced OTA contents by more than 50%. A great reduction in the
3 OTA level was attained during the production of licorice extract (21.4% remaining) and
4 block licorice (only 8.2% of the original OTA remains). Thus, the combined unit
5 operations of peeling, processing into extract and dehydration considerably reduced the
6 OTA concentrations in finished products. However, due to the high OTA concentrations
7 detected in the roots, the mycotoxin was still present in its derivatives. Further research
8 is needed for the prevention of the contamination by ochratoxin-producing molds in the
9 fields where the licorice is grown, during sun-drying and storage of the roots, and for
10 methods of selection of the raw material to effectively separate out contaminated roots.

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8 3 Table I. Effect of washing and peeling of fresh licorice roots on ochratoxin A
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10 4 concentrations (ng/g).
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Sample	Fresh root (ng/g)	After washing (ng/g)	After peeling (ng/g)	^a Percentage of reduction
1	10.7	17.1	5.7	47.0 %
2	8.1	10.4	7.1	12.0 %
3	12.2	13.2	7.0	42.8 %
4	14.7	11.5	8.6	41.3 %
5	10.3	10.7	3.3	67.9 %
6	9.9	8.5	3.1	68.2 %
7	8.6	8.1	3.4	60.4 %
8	7.3	8.7	1.1	84.7 %
^b Mean	10.2 A	11.0 A	4.9 B	53.1 %
Standard error	± 0.9	± 3.0	± 0.9	± 7.9 %

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7 ^aPercentage reduction of OTA from the starting material (fresh root) to the finished
8 product (peeled root); ^bMeans with different letter in a row are significantly different by
9 ANOVA test ($p < 0.05$).
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8 3 Table II. Transfer of ochratoxin A into licorice extract and block licorice at laboratory
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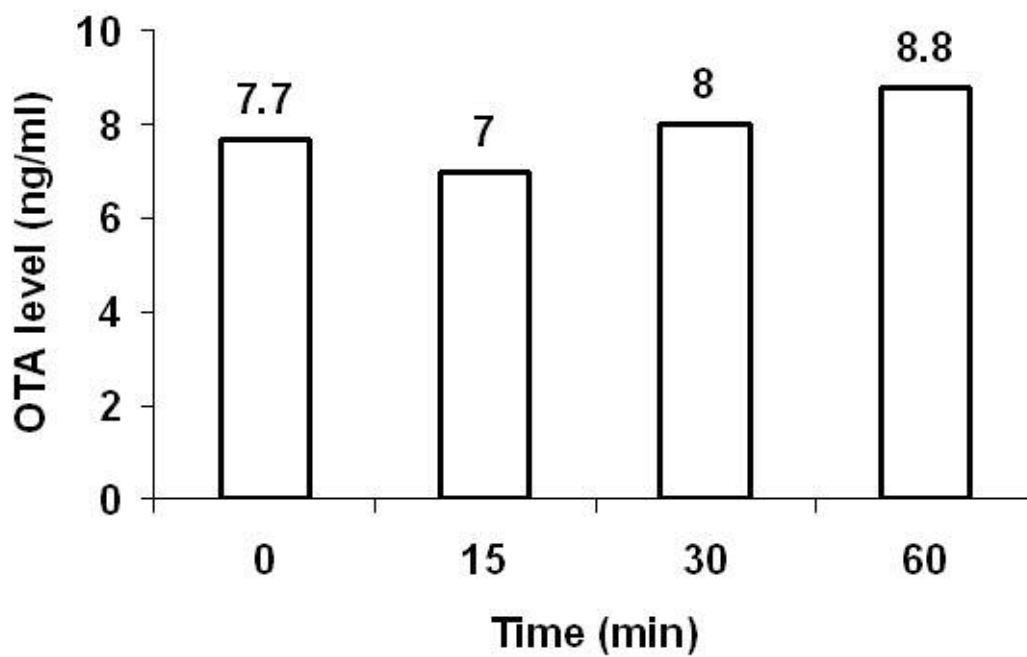
Dry root (~ 12 g)	<u>Licorice extract (~ 120 ml)</u>		<u>Block licorice (~ 2.5 g)</u>	
OTA ng/g	OTA ng/ml	^a Transfer (%)	OTA ng/g	^b Transfer (%)
253	5.8	22.9	98.4	8.1
112	2.1	19.1	39.2	7.3
88	2.0	22.6	39.7	9.4
53	1.1	21.0	20.4	8.0
Mean ± SE		21.4 ± 0.9		8.2 ± 0.4

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38 7 ^aTransfer rate of ochratoxin A from the dry root to the licorice extract; ^bTransfer rate of
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40 8 ochratoxin A from the dry root to the block licorice.
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Figure 1.
Effect of thermal treatment on ochratoxin A heated at 150°C in licorice extract during 60 min. Initial OTA concentration was 7.7 ng/ml.



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view Only