Assessment of Polycyclic Aromatic Hydrocarbon Content of Smoked Fish by Means of a Fast HPLC/HPLC Method

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Polycyclic aromatic hydrocarbon (PAH) concentrations in smoked food can reach levels hazardous for the human health, especially when the smoking procedure is carried out under uncontrolled conditions. In this work a simple and rapid method for the determination of PAHs in smoked fish samples is described. PAHs were extracted from the insoluble samples (together with fat substances) by homogenization with acidified chloroform. The fat extract was then submitted to a first LC sample preparation step (performed on a large silica column) to isolate PAHs from triglycerides. After reconcentration, the PAH fraction was finally injected into the reverse-phase analytical column. The proposed method, which presents good characteristics of recoveries and repeatability, was also used to analyze PAH content of some smoked trout samples packed under vacuum.

Keywords: *Polycyclic aromatic hydrocarbons (PAHs); smoked fish; HPLC; LC/LC chromatography*

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous in the environment as pyrolysis products of organic matter (Larsen and Poulsen, 1987). Food processing such as smoking, grilling, and toasting can lead to the formation of high amounts of PAHs (Larsson et al., 1983; Takatsuki et al., 1985; Joe et al., 1984; Speer et al., 1990; Yabiku et al., 1993). The high incidence of stomach cancer among some populations living in Nigeria (Alonge, 1988) or in Baltic countries (Dungel, 1961) has been related to the large consumption of fish smoked by artisanal wood combustion procedures generating high PAH levels.

Nonprocessed fish contains low PAH concentrations even when it comes from contaminated water because fish rapidly metabolize PAHs, resulting in low steady-state levels in the tissue (Lawrence and Weber, 1984).

Smoked fish normally contains <1 ppb of BaP (0.1−0.5 ppb), but this value can be exceeded in heavily smoked products (Larsen and Poulsen, 1987). In modern industrial ovens smoke is usually generated in a separate chamber, cleaned by using various techniques, such as electrostatic filters or smoke washing, and then led into the smoking chamber. This, together with the control of some important parameters such as temperature, humidity, smoke concentration, and circulation rate, can contribute to the minimization of PAH contamination (Larsen and Poulsen, 1987).

Various methods have been described in the literature for the determination of PAHs in smoked foodstuffs. Most of them consist of a saponification step and extraction of unsaponifiable matter followed by liquid−liquid partition, column chromatography, and analytical determination.

According to some authors, to isolate PAHs quantitatively from insoluble sample, saponification is an absolute necessity (Grimmer and Böhnke, 1975). Other experiences did not confirm this hypothesis and revealed that good results can be obtained simply by extracting PAHs, together with fat substance, with a suitable solvent (cold extraction) (Khran et al., 1988; Uthe, 1988; Moret and Conte, 1995).

In a previous work (Moret and Conte, 1998) we described a new, rapid method based on a bidimensional liquid chromatography (off-line LC/LC) for the determination of PAHs in vegetable oils. Sample preparation was performed on a large silica column able to retain fat and other interferents, allowing the polyaromatic fraction to elute. The PAH fraction was then collected, evaporated under a stream of nitrogen, and injected into a reverse-phase column for PAH quantitation by spectrofluorometric detection.

The purpose of this work was to test the possibility of applying this simple method also to lipidic extracts of smoked fish sample and to use it for a screening on PAH content of some industrially smoked and vacuum-packed trout samples.

EXPERIMENTAL PROCEDURES

Reagents. HPLC grade chloroform (Deselab, Piombino Dese, Padova, Italy), hydrochloric acid (37%) (Carlo Erba, Milan, Italy), anhydrous sodium sulfate (Baker, Deventer, The Netherlands), HPLC grade pentane, methylene chloride, acetonitrile, and 2-propanol (Baker), water purified with a Milli-Q system (Millipore, Bedford, MA), PAH standard mixture, 610 M (Supelco, Bellefonte, PA) consisting of acenaphthene (Ac), fluoranthene (Fl), naphthalene (Na), benz[*a*]anthracene (BaA), benzo[*b*]fluoranthene (BbF), benzo[*a*]pyrene (BaP), benzo[*k*]fluoranthene (BkF), chrysene (Ch), acenaphthylene (Ap), anthracene (A), benzo(*g*,*h*,*i*)perylene (BghiP), fluorene (F), phenanthrene (Pa), dibenz(*a*,*h*)anthracene (DBahA), indeno(1,2,3-*cd*)pyrene (IP), pyrene (P) were used.

Apparatus. The equipment used was previously described (Moret and Conte, 1998).

Briefly, the first LC pump was an isocratic syringe one (Phoenix 30, Fison/CE Instruments, Milan, Italy) fitted with a 500 μL injection loop and a 10 mL backflush loop mounted on an additional valve and coupled to a Varian (Palo Alto, CA) UV−vis model 9050 detector, set at 254 nm. The second LC unit consisted of a Varian model 9010 liquid chromatograph

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with a 10 μL injection loop and a Varian spectrofluorometer model 9070 performing eight wavelength changes.

The preparation column was a 250 × 4.6 mm i.d. silica of 5 μm particle size (Spherisorb, Lab Service, Anzola dell'Emilia, Bo, Italy), whereas the analytical one was a reversed phase, Supelcosil LC-PAH, 15 × 4.6 mm i.d., and 5 μm particle size column.

Method. Ten grams of the ground sample was placed in a 300 mL flask with 150 mL of chloroform acidified with ∼2 mL of HCl (37%) and extracted with a Polytron homogenizer for 20 min at speed 3 (Kinematica, Luzern, Switzerland). The flask was maintained in an ice bath to avoid overheating. The extract so obtained was slowly filtered through 10 g of anhydrous sodium sulfate and concentrated under vacuum to a few milliliters of volume. The extract was then centrifuged and the supernatant taken to constant weight by using a rotavapor (Büchi, Switzerland).

The lipidic residue was diluted with pentane in a 5 mL volumetric flask, and 500 μL of this solution was used for sample injection into the silica column. The mobile phase was pentane and 5% of dichloromethane (CH_2Cl_2) with a flow rate of 0.8 mL/min. As soon as the PAH fraction had been eluted from the silica column, it was backflushed with 10 mL of CH_2Cl_2.

As already described (Moret and Conte, 1998), the purified PAH fraction was collected, concentrated, and injected into the analytical column. To reduce losses due to the evaporation step under nitrogen flow, the sample can be transferred from pentane/dichloromethane to acetonitrile without letting it go to dryness. In this case it is necessary to add a known amount of a standard (e.g., nitrobenzophenone) for volume control. The gradient elution program used consisted of 60% water and 40% acetonitrile for 5 min, programmed to 100% acetonitrile over 40 min at a flow rate of 1.5 mL/min.

RESULTS AND DISCUSSION

The capacity of a silica column to retain fat is proportional to its size (Grob et al., 1991) and depends on the mobile phase composition as well as the type and the byproducts (free acids and polymerized material) of the fat injected (Moret and Conte, 1998; Moret et al., 1996). For these reasons it was necessary to verify if the conditions used for vegetable oils (Moret and Conte, 1998) (maximum amount of injectable fat, mobile phase composition, fraction to collect) were also suitable for fat obtained from smoked fish samples.

The analyzed trout samples showed a fat content not higher than 10%. We diluted the fat obtained from 10 g of product in a 5 mL volumetric flask to inject, for each sample, a maximum of 100 mg of fat by using an injection loop of 500 μL.

In these conditions, using a mobile phase of pentane and 5% of dichoromethane, PAHs (from Na to BghiP) were eluted in a large fraction included between 5 and 15 min. As in the case of vegetable oils, we decided to collect a larger fraction ranging from 5 to 20 min.

In Figure 1 we can observe the spectrofluorometric chromatograms corresponding to two different aliquots of fat extracted from the same smoked trout sample, which underwent, respectively, (a) a traditional procedure of analysis involving liquid−liquid partition followed by SPE purification and RP-HPLC (Moret and Conte, 1995) and (b) the new off-line LC/LC procedure. Observing these traces, we can conclude that the two different methods are equivalent for what concerns sample purification.

The chromatographic "profile" of this sample appears to be very complicated for the presence of some interfering peaks (probably others PAHs not included in the standard mixture) that sometimes compromise a correct PAH quantitation.

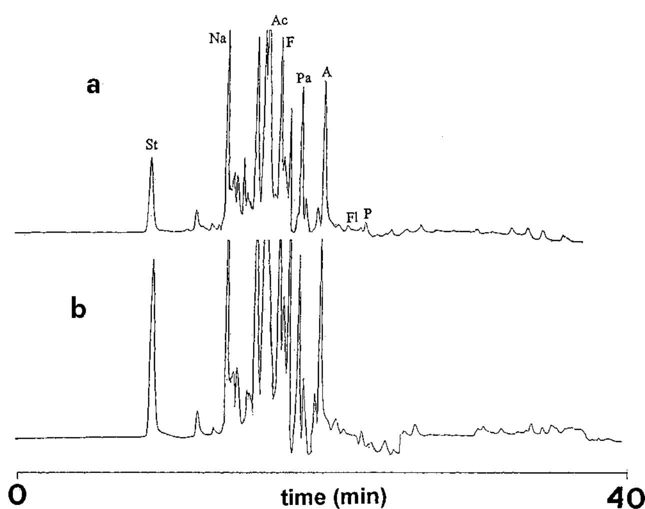

Figure 1. Spectrofluorometric traces of the same smoked trout samples that underwent, respectively, (a) the traditional analysis procedure involving a liquid−liquid partition followed by SPE purification and RP-HPLC and (b) the off- line LC/LC method.

Table 1. Recoveries of PAHs Obtained with the Proposed Method

PAH	recovery, %[a]	CV, %[b]	PAH	recovery, %[a]	CV, %[b]
Na	63	8.1	BaA	101	3.2
Ac	74	4.9	Ch	95	3.9
F	80	3.5	BbF	95	2.8
Pa	95	3.6	BkF	99	3.7
A	103	3.1	BaP	95	2.0
Fl	103	2.9	DBahA	94	2.9
P	97	4.2	BghiP	96	2.5

[a] Results are the means of three replicates. [b] CV, coefficient of variation.

Figure 2 shows the HPLC trace (always obtained with the new method proposed here) of another smoked trout sample purchased from a different supplier. In this case the profile appears to be clean. Differences in the chromatographic profiles of different samples are probably related to the different smoking technologies applied.

Recoveries were calculated on a trout sample whose fat (extracted from 10 g of fish sample) was fortified with a known amount of PAHs standard mixture, corresponding to a fish sample contamination ranging from 4 to 40 ppb for each compound. Results, given in Table 1, are the average of three replicates of the same fortified sample that underwent the entire procedure. Recoveries were practically quantitative starting from Pa, as already found for a vegetable oil sample (Moret and Conte, 1998). Figure 3a shows the UV trace (254 nm) obtained from the injection into the silica column of the fortified trout fat sample. Figure 3b shows the spectrofluorometric trace obtained after reconcentration and injection into the RP column of the corresponding PAH fraction eluted from the silica column between 5 and 20 min.

Repeatability data, resulting from seven replicate analyses of the same smoked sample, are reported in Table 2. Heavy PAHs were present only at trace level in the tested sample. Considering the complexity of the analyzed matrix, the results obtained appear good.

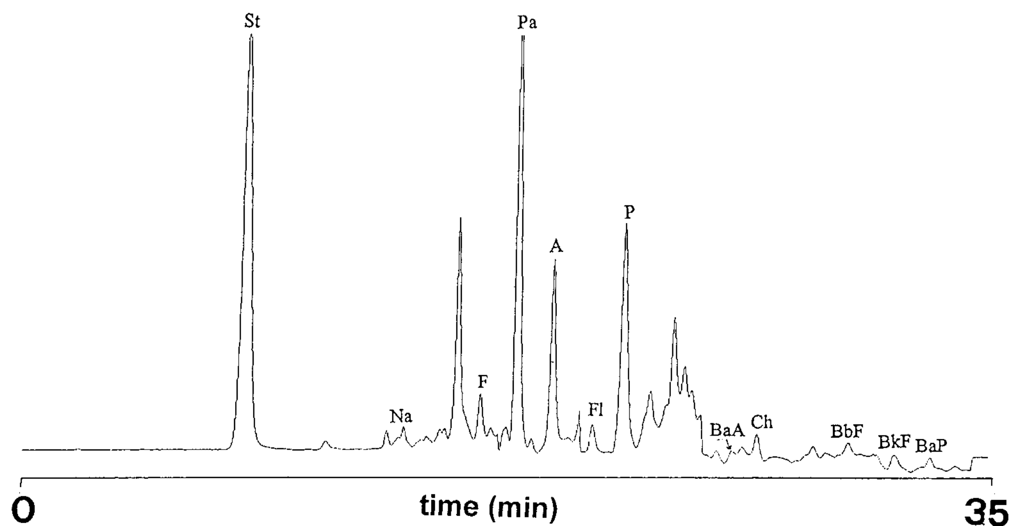

Figure 2. Spectrofluorometric chromatogram of a smoked fish sample.

Table 2. Analytical Repeatability of the Proposed Method

PAH	mean,[a] ppb	CV, %	PAH	mean,[a] ppb	CV,[b] %
Na	nq		BaA	0.1	10.0
Ac	nq		Ch	tr	
F	21.9	2.7	BbF	tr	
Pa	11.4	3.9	BkF	tr	
A	3.9	4.4	BaP	tr	
BghiP	1.5	7.4	DBahA	tr	
P	0.9	7.8			

[a] Results are the means of seven replicates of the same smoked trout sample that underwent the whole analytical procedure. nq, not quantified for the presence of interferring peaks; tr ≤ 0.05 ppb.

The new method developed here was then employed to investigate PAH content of some smoked fish samples stored under vacuum in plastic packaging.

At first, 11 samples of smoked, vacuum-packed trout, purchased from the same supplier and processed via different smoking technologies, were analyzed. Samples A were industrially smoked in an oven (using a traditional technology), samples B were processed by using a liquid smoke (sprayed on the products), and samples C were treated with "dehydrated liquid smoke".

We expected to find great differences in PAH content among different groups (A,−C), but as we can see from the results expressed as parts per billion (ppb) and reported in Table 3, PAH amounts of different samples appear not strictly related to the different smoking technologies applied. Often the differences between samples of the same group are bigger than those among samples of different groups. Only samples C seem to have always a slightly lower PAH content than samples A and B.

Some authors demonstrated the lowering of PAH concentration in a liquid smoke flavor by sorption into polyethylene packaging (Simko and Brucknova, 1993).

Smoked fish samples had been stored in their plastic packaging (in a freezer) for a long time prior to analysis. Therefore, we supposed that a partial adsorption of PAHs by the plastic packaging could have deleted differences in PAH concentration due to the different smoking procedures applied.

To verify this supposition, four smoked samples (from different suppliers), processed and vacuum packed with plastic film the same day, were stored at refrigeration temperature (4 °C) and analyzed, respectively, after 10, 20, 30, and 40 days. A smoked, not packed sample (control) was also tested for PAH content.

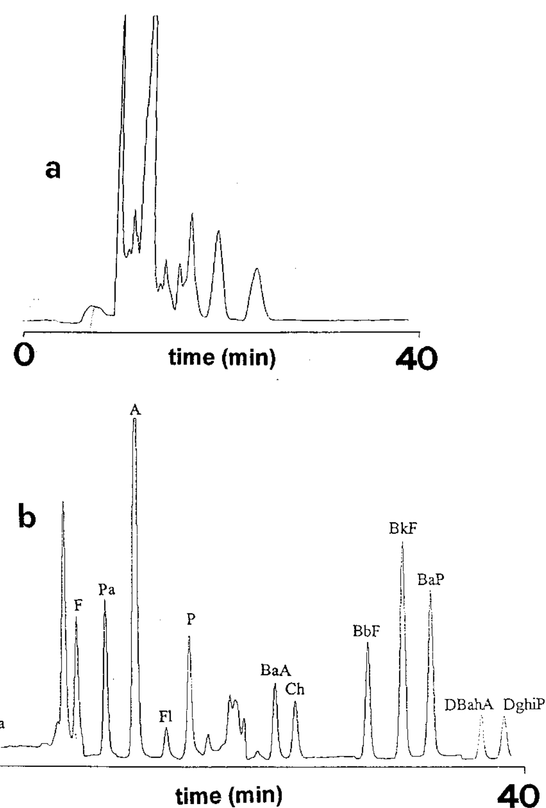

Figure 3. (a) UV trace (254 nm) of the injection of a fortified trout fat sample into the silica column; (b) spectrofluorometric trace obtained, after reconcentration, from the injection into the RP column of the corresponding PAH fraction eluted from the silica column between 5 and 20 min.

For each sample skin and flesh were analyzed separately. PAH contents (ppb) are given in Table 4.

We can observe a great difference between PAH distribution in skin and flesh samples. The skin presents higher PAH concentration, especially for the light components, demonstrating that skin is an effective barrier against PAH penetration in the inner tissue. This is not always true for heavy PAHs that probably migrate easily toward the inner tissue. A similar

Table 3. PAH Content (Parts per Billion) of Trout Samples (Vacuum Packed) Smoked via Different Technologies

PAH	A1	A2	A3	A4	B1	B2	B3	B4	C1	C2	C3
F	21.6	38.7	21.9	33.4	33.1	25.0	23.9	49.5	15.5	20.8	34.8
Pa	17.6	27.3	11.4	25.7	14.2	16.5	14.6	36.7	10.0	13.6	21.4
A	3.6	6.8	3.9	5.8	4.4	5.6	3.4	8.0	2.5	2.9	6.1
Fl	3.3	2.3	1.5	1.9	3.3	2.0	1.8	2.9	3.6	1.2	1.9
P	1.1	1.6	0.9	2.3	3.8	5.0	0.9	3.2	1.4	0.9	1.7
BaA	0.1		0.1	0.1	0.1	tr	tr	tr			0.1
Ch	0.1	tr	tr	0.1	0.1	0.1	tr	tr		0.1	tr
BbF	0.1	0.2	tr	0.1	0.1	tr	0.1	0.2	0.1	0.1	0.1
BkF	0.5	tr	tr	tr	tr	tr	0.1	0.2	tr	tr	tr
BaP		0.1	tr	0.1	tr	tr	0.3	tr	tr	tr	0.1
DBahA		tr	tr	tr	tr	tr	tr	tr	tr	tr	0.1
BghiP		tr	tr	0.1	tr	tr	tr	0.2	0.1	0.1	0.1

Table 4. PAH Content (Parts per Billion) of Vacuum-Packed Smoked Trout Samples during Storage in Their Plastic Packaging (at 4 °C)

	control[a]		after 10 days		after 20 days		after 30 days		after 40 days	
PAH	flesh	skin	flesh	skin	flesh	skin	flesh	skin	flesh	skin
F	0.9	4.1	2.2	3.4	2.7	15.0	1.9	2.5	1.7	4.2
Pa	3.7	32.2	7.2	20.3	5.6	44.0	6.5	15.2	3.5	1.8
A	0.3	2.7	0.5	1.6	0.8	3.6	0.4	0.9	0.4	2.2
Fl	1.0	12.9	1.4	8.4	1.7	9.3	1.4	3.0	1.0	7.9
P		18.0	0.7	9.2	0.8	8.9	1.0	2.7	0.6	9.8
BaA	0.2	0.3	0.2	0.3	0.3	0.3	0.1	0.1	0.1	0.4
Ch	0.3	2.6	0.3	1.0	0.6	1.0	0.1	0.4	0.2	1.2
BbF	0.6	2.0	0.4	1.1	0.9	0.8	0.1	0.3	0.3	1.4
BkF	0.3	0.7	0.3	0.4	0.5	0.2	0.1	0.1	0.1	0.2
BaP	0.2	0.4	0.1	0.2	0.3	0.1	0.1	0.1	0.1	0.3
DBahA	0.6	0.4	0.5	tr	0.9		tr	tr	tr	tr
BghiP	0.5	1.0	0.2	0.5	0.7		tr	tr	0.2	0.1

[a] control = smoked, not packed sample.

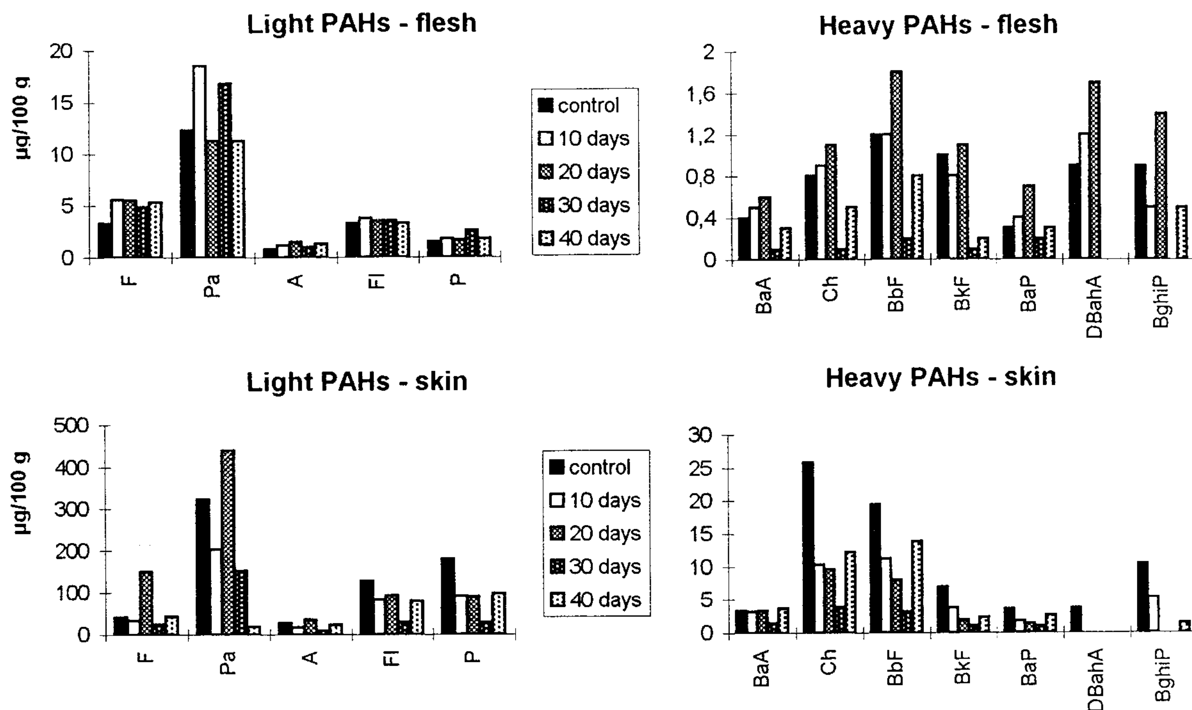

Figure 4. PAH content (expressed as micrograms per 100 g of fat) of vacuum-packed smoked trout samples after different storage times.

behavior was already observed in a smoked "ricotta" sample, in which light PAHs were mostly concentrated in the superficial layer, whereas some of the heavier ones (DBahA, BghiP) had about the same concentration in the superficial and inner parts (Moret, 1997).

As PAHs are lipophilic substances and the analyzed samples presented different fat contents, we decided to express PAH quantities on fat amount (micrograms per 100 g). Data are visualized in Figure 4.

PAH concentrations of flesh samples appear generally rather homogeneous, whereas those of skin samples are more variable. Even if in this situation it is not possible to identify a clear trend depending on storage time, we can see that, with some exceptions, PAH contents of packed skin samples are lower than those of the control upon long-term storage in a plastic film.

Considering the results of this screening, it is difficult to say if the plastic film really interacts with the

products, adsorbing PAHs and determining a reduction of their concentration. The high variability existing among the different samples, due, for example, to uncontrolled parameters such as a different position in the smoking chamber, could mask the real trend.

CONCLUSIONS

The method here proposed is suitable for rapid screenings on PAH content of smoked products. Except for the losses of the more volatile components, due to the evaporation step, PAH recoveries are practically quantitative.

The analyzed samples presented BaP levels ranging from trace to 0.5 ppb. F was the most represented among PAHs, followed by Pa, A, Fl, and P. Further studies on smoked and vacuum-packed trout samples are required to confirm the hypothesis concerning a possible reduction of PAH content due to sorption by the packaging.

The possibility to realize a completely on-line LC/LC method for PAH analysis of oils and lipidic extracts, by using an on-line evaporator able to couple a normal-phase silica column with a reversed-phase one, will be the object of further investigations. The off-line LC/LC method remains a good alternative to the traditional methods (both for analysis time and for volume of solvents employed), and it is easy to realize with the normal instrumentation available in a modern laboratory.

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