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An improved method for the recovery of petroleum hydrocarbons from fish muscle tissue

A. Işiğigür, H. Heras[1] & R. G. Ackman*

Canadian Institute of Fisheries Technology, Technical University of Nova Scotia, P.O. Box 1000, Halifax, Nova Scotia, Canada B3J 2X4

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A method based on fish muscle hydrolysis with NaOH followed by addition of $CaCl_2$, and steam distillation, with direct analysis of a dichloromethane extract of the distillate by capillary gas chromatography (GC), was evaluated for the recovery of bioaccumulated water-soluble hydrocarbons from high-fat salmon muscle with the objectives of combining simplicity and minimal contamination by other materials. The 17 hydrocarbons tested as model compounds were primarily aromatics in the benzene–methylnaphthalene range, including several substituted benzenes and naphthalenes but as well included some shorter chain aliphatic compounds. The mixture is representative of the major hydrocarbons present in the muscle of salmon which have been exposed to the seawater-soluble fraction of crude oil. Recovery studies were conducted at three concentration levels. The standard solutions were spiked into 20 g of homogenized salmon fillets and the mixture hydrolysed in NaOH solution for 2 h in a stoppered distillation flask. This was followed by the addition of $CaCl_2$ in solution to reduce foaming and 2 ml of dichloromethane as a co-distillation solvent. The mixture was then steam distilled with the dichloromethane recovered in the distillate as the hydrocarbon carrier. Such distillates contained negligible amounts of non-hydrocarbon material, and were therefore suitable for direct GC analysis without the need of any clean-up step. Recoveries for all hydrocarbons analyzed were high and reproducibility acceptable in the higher spiking levels. The major advantage of the method is a reduced background level of non-hydrocarbon volatiles from control salmon flesh. This increases the sensitivity of determining bioaccumulated tainting hydrocarbons while maintaining the basic simplicity of steam distillation.

INTRODUCTION

The world's fishing industries have depended historically on the availability of various kinds of high quality and uncontaminated fish from offshore and inshore fisheries (Burt *et al.*, 1992). In addition to these traditional fisheries, salmon farming in Canada now has significant economic importance, the value being $245 million in 1993 (Department of Fisheries and Oceans, 1995). Growing salmon are kept in cages in seawater for almost 2 years and caged salmon do not have the ability to avoid polluted seawater. Salmon farms which are established near the oil tanker routes are particularly vulnerable to the adverse effects of petroleum contamination as demonstrated by the 1993 Braer wreck in the Shetland Islands. The resulting oil tainting from deposition of petrogenic hydrocarbons in salmon muscle necessitated destruction of an entire year's production, although depuration was in fact fairly rapid (Anon., 1993). Concern for the quality and marketability of this valuable commercial fish in such circumstances led us to reinvestigate an environmental approach to hydrocarbon recovery (Heras *et al.*, 1992) and reorient it to a food chemistry approach.

In our hands data were initially obtained for heavily contaminated samples by employing the simple steam distillation technique originated by Ackman & Noble (1973). However, with some less contaminated fish tissues, extra GC (gas chromatograph) peaks, obviously volatile non-hydrocarbons derived from unknown components of the fish tissue, were observed. There has recently been an increased use of direct alkali digestion of samples (Lebo *et al.*, 1991; Sasaki *et al.*, 1991; Shchekaturina *et al.*, 1995; Granby & Spliid, 1995; Snedaker *et al.*, 1995) as an initial step towards full recovery. Our evaluation of this approach led to the introduction of dichloromethane as the solvent of

[1]Present address: Instituto de Investigaciones Bioquímicas de La Plata, Fac. Medicina, Universidad Nacional de La Plata, 60 y 120 (1900) La Plata, Argentina.
*To whom correspondence should be addressed.

Table 1. Concentrations of standard hydrocarbon mixtures spiked into salmon muscle homogenate (μg/g)

Hydrocarbon	High	Medium	Low
Benzene	15.822	7.915	0.009
Cyclohexane	6.794	3.401	0.008
Isooctane	0.581	0.294	0.007
n-Heptane	0.219	0.113	0.007
Methylcyclohexane	4.153	2.080	0.008
Toluene	62.424	31.216	0.009
Ethylbenzene	12.138	6.075	0.011
m+*p*-Xylene	17.260	8.647	0.035
o-Xylene	11.616	5.819	0.022
Propylbenzene	1.724	0.888	0.052
1,3,5-Trimethylbenzene	0.484	0.260	0.035
1,2,4-Trimethylbenzene	1.752	0.894	0.035
1,2,3-Trimethylbenzene	1.430	0.755	0.080
1,2,4,5-Tetramethylbenzene	0.144	0.085	0.026
Naphthalene	0.857	0.441	0.024
2-Methylnaphthalene	0.352	0.212	0.071
1-Methylnaphthalene	0.286	0.170	0.054

choice for both co-distillation and hydrocarbon recovery and for GC analysis (Zhou & Ackman, 1994). Further study was aimed at reducing non-hydrocarbon volatiles by improving on the steam distillation of Ackman and Noble with the alkali digestion technology. As a result we can now put forward a simplified and very efficient analytical technique that will allow high recovery of tainting compounds from salmon tissue exposed to the WSF of crude oil, with a minimal background of other volatiles occurring in the GC profile range for tainting hydrocarbons.

MATERIALS AND METHODS

Fish handling

Atlantic salmon (*Salmo salar*) used in the experiments were drawn from a stock kept in clean seawater at the Aquatron Laboratory, Dalhousie University (Halifax, NS). These fish were fed a commercial diet for several months and weighed approximately 1 kg when they were killed. Experimental fish were immediately transported to the Canadian Institute of Fisheries Technology at the Technical University of Nova Scotia, gutted, skinned and filleted in a cold room (4°C), and frozen in polyethylene bags at −35°C until required for experiments.

Preparation of the standard hydrocarbon mixture

The water-soluble fraction (WSF) of Flotta North Sea light crude oil served as the basis in selecting the experimental hydrocarbon mixtures (Zhou *et al.*, 1994). Hydrocarbon mixtures in proportions which reflected many of the important hydrocarbons present in salmon muscle tainted by WSF were prepared in dichloromethane at three different levels of concentration. Two concentrations were chosen to reflect the ranges of actual hydrocarbons likely to be encountered in the flesh of definitely tainted Atlantic salmon (Heras *et al.*, 1993). A much lower concentration level was chosen to match or to be lower than the minimum concentration that induces taint in salmon, as detected by sensory evaluation (Ackman & Heras, 1992; Heras *et al.*, 1993; Zhou & Ackman, 1994). The minimum is approximately 5 ppm of total hydrocarbons in salmon muscle, but depends on the mixture of hydrocarbons, and this changes with time of depuration (see below). The components of the mixtures and their concentrations are given in Table 1.

Recovery of hydrocarbons

The improved method for recovering the tainting compounds is based on alkaline digestion of salmon muscle followed by steam distillation to recover and isolate the hydrocarbons. The steam distillation apparatus consisted of 500 ml roundbottom glass flasks equipped with Barrett type distillation receivers (20 ml), with Teflon stopcocks, and Allihn glass water-cooled condensers. All apparatus was rinsed with distilled water, acetone and hexane and dried shortly before use. Heating and stirring were accomplished by rheostat-controlled heating mantles and magnetic stirrers respectively. Approximately 20 g of salmon muscle tissue from the laboratory-held fish, homogenized in a Sorvall OMNI Mixer, was weighed into the 500 ml flask; 100 ml of 1 M NaOH solution was then added. The mixture was then spiked with 100 μl of standard hydrocarbon solution in dichloromethane using a Hamilton microsyringe. The flask was stoppered and the contents were stirred with a bar magnet at room temperature for 2 h. At the end of this period 100 ml of calcium chloride solution (200 g/litre) and dichloromethane (2 ml) were added to the reaction mixture. The mixture was then distilled until 20 ml of condensate was collected. Distilling receivers were kept immersed in ice baths during the entire distillation. The 20 ml of condensate was transferred to 50 ml glass tubes with Teflon-lined screw caps, vortexed for 20 s and centrifuged for 15 min at 2000 ***g***. In all of the samples the dichloromethane layer separated cleanly after centrifuging. The bottom dichloromethane layer containing hydrocarbons was withdrawn using a glass syringe with a long needle and transferred to a screw-cap glass vial with a Teflon-lined cap for storage at −35°C until GC analysis. *n*-Heneicosane (C_{21}), used as the internal standard (IS), was added to the sample at this point as a solution in dichloromethane, the amount of IS being adjusted according to the expected concentration of the particular hydrocarbon mixture used. Spiked salmon tissues were analyzed in triplicate at each level of standard hydrocarbon mixture and unspiked salmon tissue samples from fish held in the same tank on the same diet were analyzed by the same technology as controls for laboratory blanks. The total method was also applied to fillet tissue from fish deliberately contaminated as part of other studies (Fig. 1A).

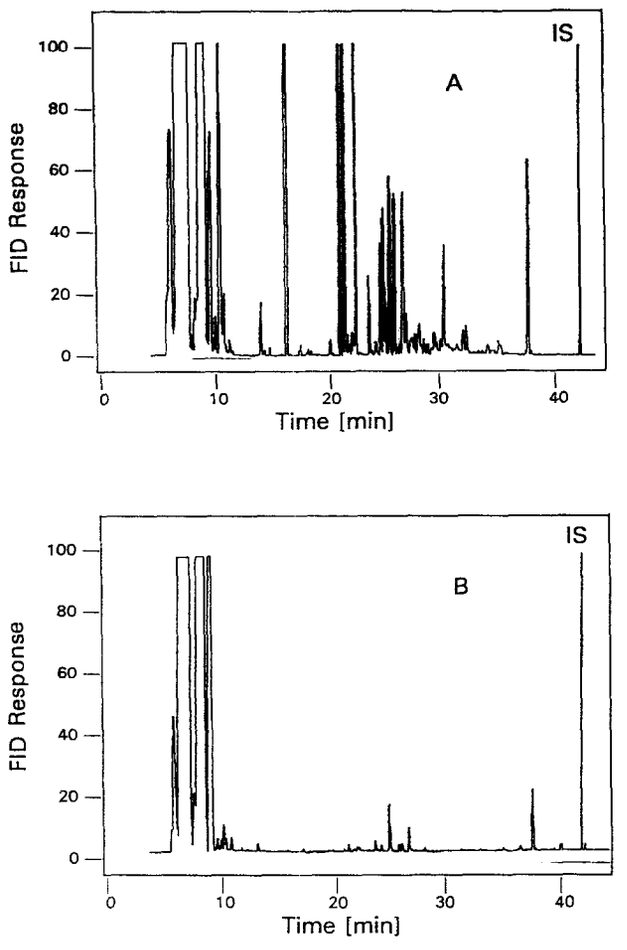

Fig. 1. A. Gas chromatogram of injection of 2 µl of dichloromethane distilled after saponification and $CaCl_2$ addition from 20 g of muscle of salmon exposed to 1 ppm water-soluble crude oil fraction for 36 h. IS = internal standard C_{21} hydrocarbon. The four offscale peaks from 17 to 22 min are, in elution order, toluene, ethylbenzene, *m*+*p*-xylene and *o*-xylene. These are followed by alkylated benzenes, etc. emerging prior to pristane at 38 min. B. Gas chromatogram of 2 µl of dichloromethane distilled from 20 g of muscle of control salmon treated as in A.

Gas chromatographic analyses

The hydrocarbon analyses were conducted with a Perkin Elmer 8420 capillary gas chromatograph equipped with a flame ionization detector (FID), a split injection system and a DB-1 methyl silicone fused silica capillary column (60 m, 0.25 mm ID, 0.25 µm film thickness). The operating conditions and temperatures were: flame ionization detector, 280°C; injector, 280°C. The column temperature was programmed as: initial temperature of 45°C held for 15 min, then increased at a rate of 13°C/min; final temperature 280°C for 30 min. The carrier gas was helium at a pressure of 140 kPa and detector hydrogen and air pressures were 91 and 161 kPa respectively. The split ratio was 1:20. Hydrocarbon concentrations in the samples were calculated with respect to the internal standard, and recoveries were corrected for GC response factors (Ernst *et al.*, 1989). All solvents used were glass distilled and blank readings were performed daily.

RESULTS AND DISCUSSION

The frequency of oil spills and tanker wrecks on a worldwide basis requires that analytical technology for petrogenic hydrocarbons be simple and suitable for implementation in any remote but well-equipped food laboratory. Environmental laboratories may have different perspectives and approaches to the hydrocarbon contamination problem (e.g. Payne *et al.*, 1988). Fortunately, except for the meats of whole shellfish, the food sample is usually edible fish muscle and is available in reasonable quantities. It is of interest that as long ago as 1977, Johansen *et al.* (1977) used alkali digestion for 20 g of animal tissues, or 2.5 g of fish fatty liver tissue, in a survey of hydrocarbons in marine organisms conducted off West Greenland. They extracted the distillate with pentane, concentrated this, and followed it with column chromatography on Al_2O_3 + SiO_2. They reported varying amounts of several hydrocarbons between pristane and their internal standard docosane (C_{22}) in fish lipids, but pristane ($C_{19}H_{40}$) was almost always around 100 times the total minor components. Squalene ($C_{30}H_{50}$) was also recognized as important in most organisms but is not included in our study because of the extended GLC retention time.

Monitoring of the hydrocarbon content of fish collected carefully from their natural environment (a ship is apparently designed to taint fish with hydrocarbons) has been almost non-existent, although in fact chronic exposure to petrogenic or refined hydrocarbons is also a problem in some areas (Lockhart & Danell, 1992).

Due to the complexity of the hydrocarbon mixtures involved in oil spills there is no one single method that can quantify all of the pollutants involved. Some of the analytical methods given in the literature by various investigators for recovering and quantifying the contaminating petrogenic hydrocarbons from fish or shellfish tissue include: vacuum sublimation–extraction (Shipton *et al.*, 1970), air trapping (Murray & Lockhart, 1988), direct steam distillation (Ackman & Noble, 1973), extraction–steam distillation (Connell, 1974), standard solvent extraction with chromatography on solids (McGill *et al.*, 1987; Williams *et al.*, 1989) and base digestion–extraction processes (Ogata *et al.*, 1979; Donkin & Evans, 1984; Lebo *et al.*, 1991; Sasaki *et al.*, 1991; Fowler *et al.*, 1992). These methods often refer to a particular set of hydrocarbons that were cited or recognized as taint-causing, e.g. diesel fuel (Ackman & Noble, 1973; Newton *et al.*, 1991). Gas chromatography (GC) is however almost universally available and applicable for the actual hydrocarbon analysis, although HPLC (high performance liquid chromatography) can be used, particularly for aromatic hydrocarbons, if GC is not available.

Figure 1A and B compare a typical chromatogram of (A) hydrocarbons recovered by this method from salmon muscle of approximately 10% lipid with the fish exposed to 1 ppm crude petroleum WSF for 36 h, and (B) the hydrocarbons recovered from the corresponding control fish tissue. The saponification step, followed by

$CaCl_2$ addition as suggested by Fowler *et al.* (1992), eliminates most of the non-hydrocarbon background peaks otherwise observed using only the simple steam distillation technique of Ackman & Noble (1973). Figure 2 is a part of a chromatogram from the muscle of a control fish treated by the original simple steam distillation process. The two obvious peaks between pristane and the IS are not hydrocarbons and must arise from the steam distillation of intact fish muscle. They are not observed in the chromatogram (Fig. 1A) from the modified method.

Table 2 shows the average recoveries with standard deviations of three replicate recoveries for each of the three spike levels. Recoveries for all surrogate hydrocarbons were reproducible and were generally similar at the high and medium concentration levels. Benzene, toluene, ethylbenzene, propylbenzene, 1,2,4,5-tetramethylbenzene and naphthalene had the highest recoveries, ranging from 53 to 90% of the amount spiked. Recoveries were consistently similar regardless of the concentration level spiked. In general, there was a trend of increasing recovery with increasing molecular weight up to naphthalene at all three levels. There were, however, exceptions such as benzene with a high recovery of 71–85%. The 2-methylnaphthalene and 1-methylnaphthalene, with a 23–35% recovery, are approaching the upper limit of molecular weight for solubility of aromatic hydrocarbons in water (Ernst *et al.*, 1987). Relatively low recoveries of naphthalene were noted by Murray & Lockhart (1988) and Granby & Spliid (1995). Recoveries for isooctane and *n*-heptane were low at high and medium concentration levels and erratic at the very low concentration level. Concentrations of individual hydrocarbons at the lowest spiked level were deliberately set (Table 1) at several hundred times lower than those of the higher spike levels, and some were thus close to the detection limits of the GC for an injection of 2 µl dichloromethane under our routine operating conditions. This borderline level can account for the erratic results and low reproducibility observed at this very low level of concentration. If samples contain such low amounts of hydrocarbons, a concentration step can be added to the procedure but this not only adds complexity but may require additional correction factors.

The 1,3,5-trimethylbenzene results reveal an example of the need for a reduction in background volatiles natural to fish muscle. Despite the use of a high resolution GC column one of some components originating in the muscle sample must coincide exactly with this hydrocarbon peak in order to increase the apparent recovery with less spiking as shown. Lebo *et al.* (1991) had a similar problem with 2-methylnapthalene and a C_4-alkylbenzene.

The aliphatic fatty acids up to C_8–C_{10} are commonly called the VFA (volatile fatty acids) because they can be concentrated by steam distillation. These and higher fatty acids up to C_{16} are volatile enough to be readily passed through GC columns (Ackman *et al.*, 1963; Hrivnak & Palo, 1967) and have been observed in such steam distillates from acidified aqueous mixtures (Ackman, unpublished results). It seems likely the simple steam distillation of fish muscle could include such high

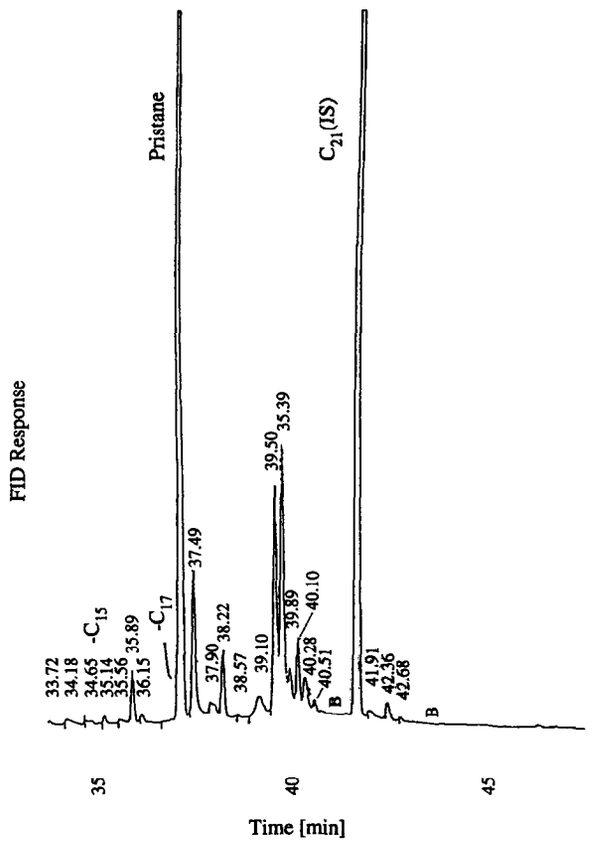

Fig. 2. Part of a gas chromatogram of a dichloromethane extract of a steam distillate of a fish muscle sample treated as described by Ackman & Noble (1973).

Table 2. Average percent recovery of standard hydrocarbons from spiked salmon muscle homogenate

Hydrocarbon	Spike level		
	High	Medium	Low
Benzene	84.7 ± 3.5	71.9 ± 4.0	83.3 ± 10.9
Cyclohexane	40.7 ± 7.9	23.9 ± 3.7	11.0 ± 9.4
Isooctane	21.8 ± 4.5	25.7 ± 1.1	[a]
n-Heptane	21.6 ± 3.0	20.2 ± 9.8	0.0
Methylcyclohexane	28.7 ± 2.9	25.7 ± 0.2	15.1 ± 12.8
Toluene	76.3 ± 7.2	72.9 ± 4.1	90.3 ± 4.5
Ethylbenzene	62.8 ± 7.4	59.3 ± 4.4	63.7 ± 45.7
m + *p*-Xylene	36.8 ± 1.4	36.4 ± 2.9	102.3 ± 6.8
o-Xylene	60.5 ± 6.1	59.4 ± 4.4	145.3 ± 9.0
Propylbenzene	55.0 ± 4.4	53.0 ± 2.2	60.2 ± 1.5
1,3,5-Trimethylbenzene	68.5 ± 21.6	105.4 ± 3.6	152.3 ± 15.6
1,2,4-Trimethylbenzene	44.8 ± 4.2	40.1 ± 7.4	97.7 ± 21.7
1,2,3-Trimethylbenzene	44.7 ± 3.4	42.6 ± 3.6	47.0 ± 1.6
1,2,4,5-Tetramethylbenzene	85.1 ± 26.4	78.5 ± 26.0	74.7 ± 10.9
Naphthalene	82.6 ± 4.5	72.3 ± 7.2	80.5 ± 7.8
2-Methylnaphthalene	24.3 ± 3.1	26.4 ± 2.8	33.0 ± 1.8
1-Methylnaphthalene	34.7 ± 1.9	23.4 ± 9.0	34.3 ± 2.0

Results are the mean of triplicate analysis ± S.D.
[a]Results were too variable to report.

molecular weight materials unless the distillation is from an alkaline medium. Foaming during distillation is sometimes a problem but is prevented to a certain extent by the additional step of adding $CaCl_2$, presumably by the formation of inert calcium soaps of the saturated (C_{14}, C_{16}) fatty acids. The muscle fat content of fish ranges from 0.5 to 20% (Ackman, 1989) and there is a rough correlation between uptake of hydrocarbon and fat level as well as in the persistence of 'tainting' (Heras *et al.*, 1993). In our work with Atlantic salmon *Salmo salar* we have determined that the hydrocarbons of the WSF of crude oil are unlikely to be recognized by sensory panels in depurated fish at totals of less than 5 ppm (Heras *et al.*, 1993; Ackman *et al.*, 1994; Zhou & Ackman, 1994). This is because the monoaromatic hydrocarbons (Fig. 1A, region 10–24 min) of very objectionable flavour are more rapidly depurated than the later-eluting polycyclic aromatic hydrocarbons.

There is now limited information in the literature about hydrocarbon bioaccumulation in fish muscle due to exposure to the WSF (water-soluble fraction) of crude oil (Brandal *et al.*, 1976; Ackman & Heras, 1992; Heras *et al.*, 1992, 1993; Martinsen *et al.*, 1992; Zhou & Ackman, 1994). Basically, *n*-alkanes above C_{10} are essentially not soluble in seawater, compared to aromatic hydrocarbons (Zhou *et al.*, 1994) and are not present in WSF, and hence are not accumulated.

The objective of investigating hydrocarbon contamination of edible fish muscle can be met by selecting or combining a number of equally important but often mutually conflicting requirements such as

- maximizing recovery of a specific range of tainting hydrocarbons,
- minimizing contamination of such hydrocarbons by carryover of non-hydrocarbon materials,
- avoiding concentration steps, or extensive preliminary chromatography and/or partitioning steps requiring large volumes of solvents,
- retaining analytical sensitivity adequate to recognize hydrocarbon 'tainting' as distinct from any natural (biogenic) hydrocarbons in the sample, and
- simplifying the technology for the isolation and recovery of hydrocarbons since there are usually very large numbers of samples.

The salmon used had been raised on commercial fish feeds including herring or similar fish oils, and imported fish meal. Some natural hydrocarbons from these sources could have accumulated in the salmon adipocytes and contributed to the minor peaks for the control fish of Fig. 1B. Hydrocarbons are also to be found in fats of wild fish of all types, for example in North Sea dabs *Limanda limanda* (McGill *et al.*, 1987) and in several species of Pacific salmon taken on the high seas (Sasaki *et al.*, 1991, 1993), but qualitatively and quantitatively this would depend on food, location etc. The hydrocarbon pristane, which can be derived from chlorophyll, was found to be nearly half the hydrocarbon total in samples of Pacific salmon flesh and is the major background hydrocarbon shown in Fig. 2. It was also present, but was relatively less important, in hydrocarbons of the fish *Sardina pilchardus* taken in the Adriatic Sea (Serrazanetti *et al.*, 1991) The proposed method requires only 2 ml of solvent per sample and allows a cleaner recovery of the hydrocarbons actually present in the tissue when compared to the results obtained using the original simple steam distillation technique (Ackman & Noble, 1973; Ernst *et al.*, 1989). Compared to many other published methods this procedure does not require a prior lipid extraction, usually time-consuming, and the 2 ml of dichloromethane does not require a solvent concentration step, always a risky technique if the 'tainting' by low-boiling monoaromatic hydrocarbons is a key requirement of matching hydrocarbon burden and sensory panel results.

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