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QUANTITATIVE HPLC ANALYSIS OF THE LEVEL OF FECAPENTAENES AND THEIR PRECURSORS IN HUMAN FECES BY A CHEMICAL CONVERSION METHOD

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ABSTRACT.—A fast and reliable hplc method for the quantitative analysis of total fecapentaene-12 (FP-12 and its precursors) and total fecapentaene-14 (FP-14 and its precursors) in human feces is described. The analysis is based on the rapid chemical conversion of fecapentaenes and their precursors to more stable methoxytetraenols and the use of synthetic, not naturally occuring, fecapentaene-13 (FP-13) as an internal standard. The synthesis and physical properties of this internal standard are described.

The convenience and reproducibility of the method were illustrated by applying the procedure to stool samples obtained from twelve individuals on 3 consecutive days. Levels of total FP's were in the range of 0.1–25.4 μg for total FP-12 and 0.1-8.5 μg for total FP-14 per g of wet feces. Appreciable fluctuations were observed between levels in samples from the same individual on different days. Reproducibility and recovery were shown to be good.

Quantitative analysis of fecapentaene-12 (FP-12) [**1**] and fecapentaene-14 (FP-14) [**2**], [two major representatives of a class of strongly mutagenic compounds present in human feces (1–8)], is hampered by the minute quantities present in the feces (ppb level) and by the extreme sensitivity of these polyunsaturated enol ethers to light and oxygen. We observed variable losses of fecapentaenes during isolation, purification, and hplc-analysis, even when taking all the precautions described (4). Several hplc methods for the detection and separation of fecapentaenes have been described (9–11). In the bowel, the fecapentaenes are produced by common anaerobic bacteria of the genus *Bacteroides* from precursors of as yet unknown origin that are present in much larger amounts (up to fiftyfold) (12). Although the exact nature of these precursors remains to be determined, there are strong indications that they are phospholipids of type **3**, already containing the pentaenol ether structure (12). In order to quantify the level of fecapentaenes and their precursors (total FP), several papers have described methods in which this level is analyzed after incubation of feces for 96 h at 37° (6, 13–17) in the presence of a fecapentaene-producing strain of bacteria.

We have developed a rapid and efficient method to convert the unstable fecapentaenes as well as their precursors into stable 10-methoxy-2,4,6,8-tetraenols **5** (Scheme 1). Treatment of a sample of human feces with HCl in MeOH caused conversion of the enol ether compounds (fecapentaenes and precursors) into methoxy-substituted tetraenals **4**. These conjugated aldehydes were subsequently reduced by $NaBH_4$ to the corresponding alcohols **5**. The first step in this conversion involves the addition of MeOH to the ω-carbon atom of the polyene chain, accompanied by a flow of electrons towards the enol ether oxygen atom and loss of the (protonated) glyceryl moiety as allyl alcohol and H_2O. More information about this transformation, which is unique for this class of compounds, is described in a separate article (18). Chromatographic purification can now be effected with minor or no losses prior to hplc analysis. The methoxy substituted tetraenols were analyzed with a diode-array detector because of their characteristic uv absorptions and high extinction coefficients.

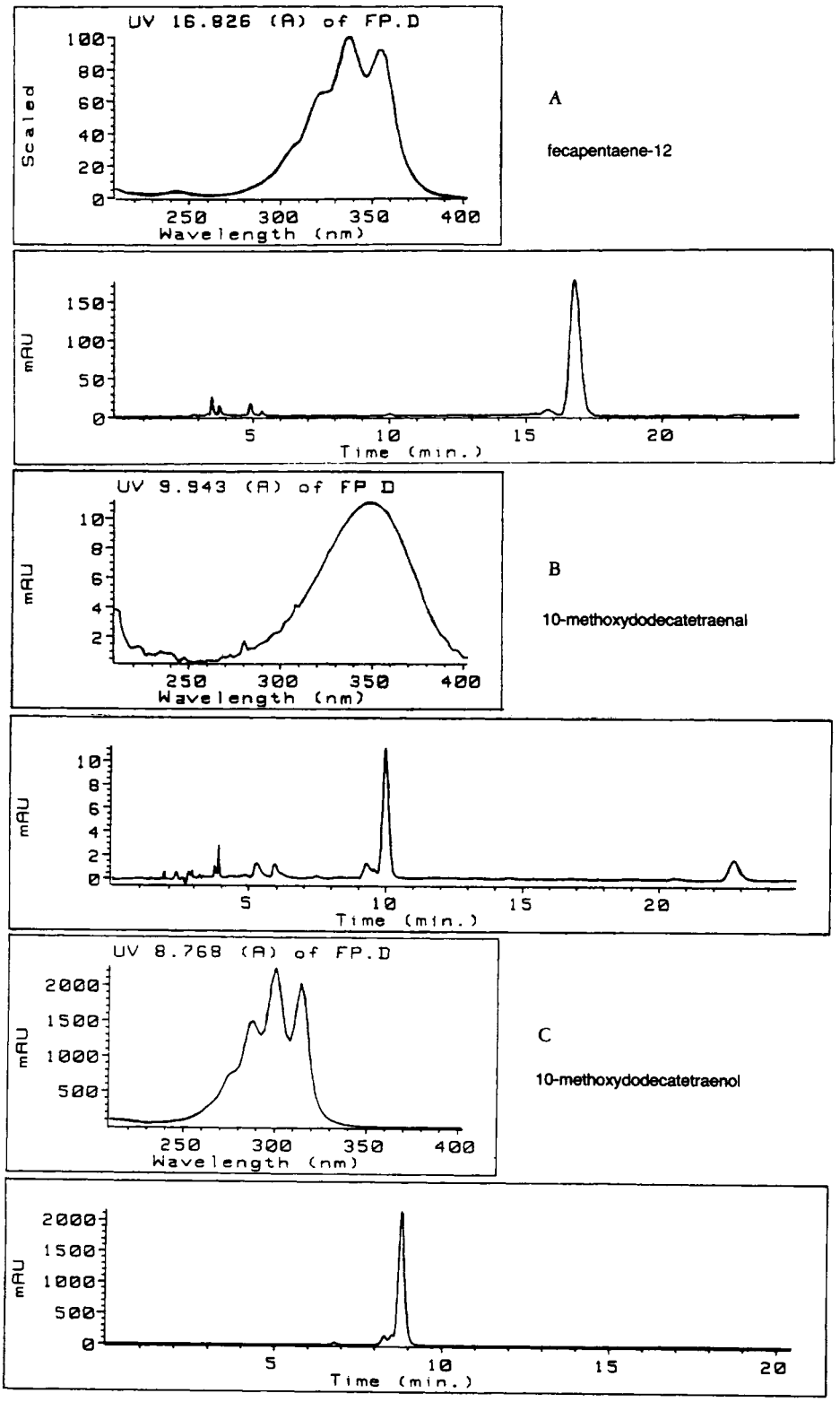
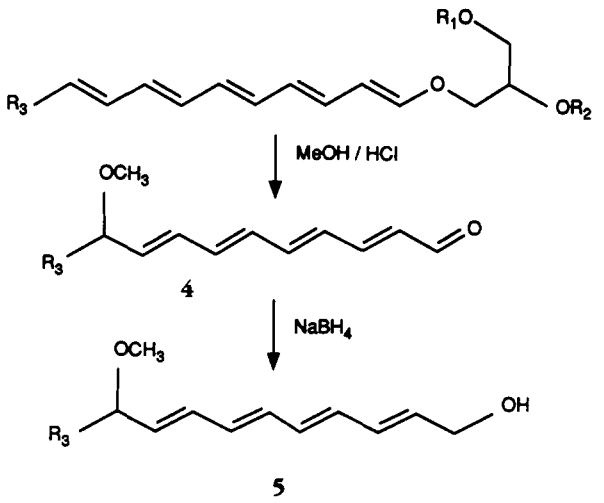


FIGURE 1. Hplc chromatograms and uv spectra of products outlined in Scheme 1: (A) fecapentaene-12, (B) 10-methoxydodecatetraenal, (C) 10-methoxydodecatetraenol; au = absorption units.



1 $R_1=R_2=H$, $R_3=C_2H_5$ (FP-12)
2 $R_1=R_2=H$, $R_3=C_4H_9$ (FP-14)
3 R_1=phosphate ester,
$R_2=CO(CH_2)_nMe$
$R_3=C_2H_5$ or C_4H_9 (hypothetical precursor)

SCHEME 1. *Conversion of fecapentaenes and precursors into 10-methoxytetraenols.*

3-(1,3,5,7,9-Tridecapentaenyloxy)-1,2-propanediol (FP-13) [**6**] was chosen as an internal standard because of the retention characteristics of its alcohol derivative (as expected it elutes between the alcohols derived from FP-12 and FP-14), its absence in natural feces samples (confirmed by analysis without addition of FP-13), and its structural analogy to the compounds to be determined.

In accordance with our previous work on the synthesis of naturally occurring fecapentaenes (19–21), our approach to the synthesis of homologue **6** (Figure 2) was based on the construction of the enol ether double bond by Horner-Wittig coupling of an appropriate polyunsaturated aldehyde with phosphine oxide **7**, containing the glyceryl moiety in a suitably protected form (Scheme 2). The requisite aldehyde **8** [(2*E*,4*E*,6*E*,8*E*)-dodecatetraenal] was prepared from (2*E*,4*E*)-octadienal (22–24) by a reaction sequence described by Wollenberg (25).

EXPERIMENTAL

EXPERIMENTAL PROCEDURES.—^1H and ^{13}C nmr spectra were recorded on a Jeol JNM FX-200 spectrometer. The chemical shifts are given in ppm (δ) relative to TMS as internal reference. Coupling constants (*J*) are given in Hz. FP-13 was measured in DMSO-d_6 as solvent and the other compounds in $CDCl_3$. Hplc analysis was performed on a Hewlett Packard 1040 A liquid chromatograph, equipped with a Lichrospher 100 RP-18 column (5 μm, 250 × 6.4 mm). For glc analysis a Packard 437 A gas chromatograph was used, equipped with a Chrompack capillary CP-sil 5 CB column. N_2 was used as carrier gas. Mass spectral data were obtained with an AEI MS 902 and a Kratos MS 9/50 apparatus. Uv absorptions were recorded on a Varian DMS 200 spectrophotometer, using 96% EtOH as solvent. Flash chromatography was performed with Si gel (230–400 mesh, Merck). Solvents and reagents were used as high grade commercial products. All syntheses of poly-unsaturated aldehydes and 3-(1-alkenyloxy)-1,2-propanediols were carried out in oxygen-free solvents in the dark in an oxygen free atmosphere.

(2*E*,4*E*)-OCTADIENAL.—This compound was prepared from butanal using the method of Pippen and Nonaka (22). It is also commercially available (Ventron-Alfa Produkte, FRG).

(2*E*,4*E*,6*E*,8*E*)-DODECATETRAENAL [**8**].—*n*-Butyllithium (1.6 M solution in hexane, 108 mmol) was slowly added at −80° to the stannane obtained by radical addition of tributyltinhydride to distilled commercially available methoxybutenyne (Fluka AG, Switzerland) (37.8 g, 101.3 mmol) dissolved in dry THF (125 ml). After stirring for 90 min at −80°, (2*E*,4*E*)-octadienal (101.3 mmol) in 20 ml of THF was added in 60 min and stirring was continued for an additional 90 min at −80°. After quenching with H_2O (200 ml), the organic layer was separated and the H_2O layer was extracted with Et_2O (3 × 100 ml). The combined organic layers were washed with saturated brine (2 × 50 ml), dried with anhydrous K_2CO_3, and evaporated in vacuo. The crude adduct was purified by flash chromatography [Et_2O-petroleum ether 40–

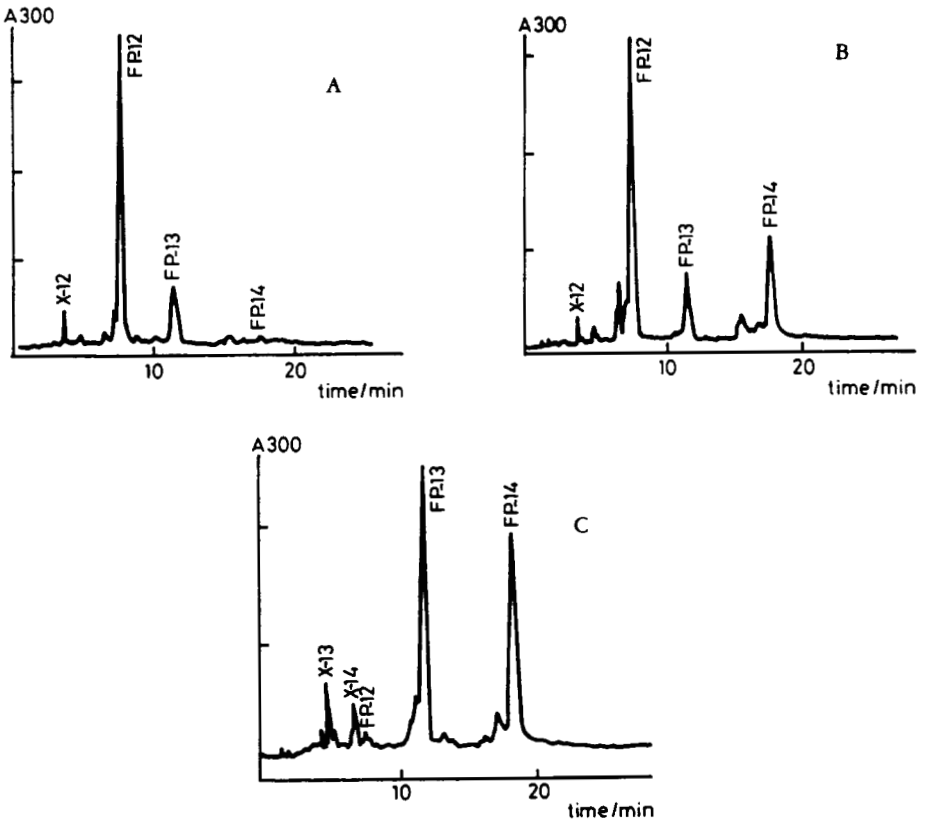
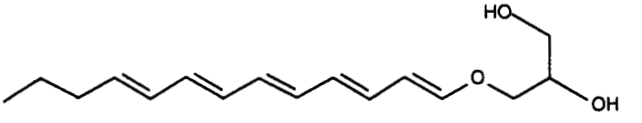


FIGURE 2. Examples of hplc tracings of methoxytetraenols prepared from human feces: (A) Subject E: FP-12 producer; (B) Subject F: FP-12 + FP-14 producer; (C) Subject G: FP-14 producer. The 10-hydroxytetraenols derived from FP-12, FP-13, and FP-14 are marked X-12, X-13, and X-14, respectively.

60°-triethylamine (5:3:1)]. The purified adduct was dissolved in 5% aqueous THF (80 ml), and a catalytic amount of *p*-toluenesulfonic acid monohydrate (*p*-TsOH·H_2O) was added. After stirring for 30 min, the reaction mixture was filtered through a column with magnesium oxide and Florisil (1:4). Evaporation of the solvent yielded pure **8**. Yield 11.5 g (65%) yellow solid: uv λ max (EtOH) 355 nm (ε = 47000 l mol^{-1} cm^{-1}); ms *m/z* $[M]^+$ 176 (86), 147 (22), 133 (40), 105 (48), 91 (100), 79 (42), 77 (35); exact mass 176.1199 ($C_{12}H_{16}O$ requires 176.1201); ^{1}H nmr δ 0.92 (3H, t, *J* = 7.3, Me), 1.44 (2H, tq, *J* = 7.3 and 7.1, MeCH_2), 2.12 (2H, dt, *J* = 7.1 and 7.3, CH_2CH), 5.90 (1H, dt, *J* = 14.8 and 7.3, CH_2CH), 6.12 (1H, dd, *J* = 15.0 and 8.0, *CH*CHO), 6.15 (1H, dd, *J* = 11.5 and 14.3, H-8), 6.22 (1H, dd, *J* = 14.5 and 10.8, H-6), 6.40 (1H, dd, *J* = 11.5 and 14.3, H-4), 6.46 (1H, dd, *J* = 14.8 and 10.8, H-7), 6.69 (1H, dd, *J* = 14.3 and 11.0, H-5), 7.13 (1H, dd, *J* = 11.5 and 15.0, H-3), 9.59 (1H, d, *J* = 8.0, CHO); ^{13}C nmr δ 13.46 (C-12), 21.93 (C-11), 34.81 (C-10), 128.74 (C-4), 129.09 (C-6), 130.06 (C-8), 130.20 (C-2), 139.11 (C-7), 139.49 (C-9), 142.88 (C-5), 151.81 (C-3), 193.04 (C-1) ppm.

FECAPENTAENE-13 [**6**], [3-(1,3,5,7,9-TRIDECAPENTAENYLOXY)-1,2-PROPANEDIOL].—In an atmosphere of dry N_2, phosphine oxide **7** (10.7 g, 20.0 mmol) dissolved in dry THF (100 ml) reacted smoothly with lithium diisopropylamide (22.0 mmol) at −50° to give a solution of the deeply red colored anion. After stirring for 15 min, the solution was cooled to −80° and aldehyde **8** (20.0 mmol) dissolved in



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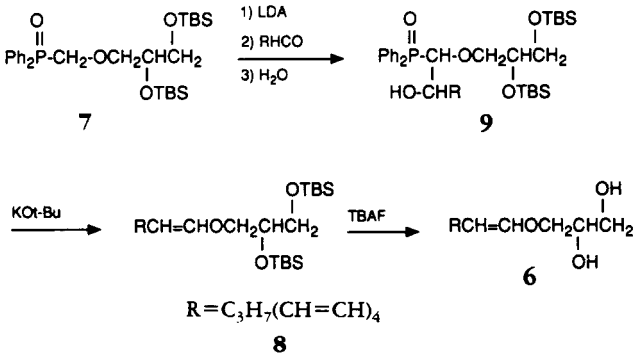

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SCHEME 2. Synthesis of FP-13.

dry THF (20 ml) was added dropwise. After stirring for 1 h at −80°, the reaction mixture was quenched with saturated aqueous NH_4Cl (150 ml). The THF layer was separated and the H_2O layer was extracted with Et_2O (3 × 150 ml). The combined organic layers were washed with saturated brine (50 ml), dried with anhydrous $MgSO_4$, and evaporated in vacuo to yield the silyl-protected adduct **9**. The crude adduct was subsequently treated with potassium *t*-butoxide (KO*t*-Bu) (2.5 g, 22.2 mmol) in dry THF (100 ml) at −50°. The reaction mixture was stirred overnight at −17°. After addition of saturated brine (200 ml), the THF layer was separated and the H_2O layer extracted with Et_2O (3 × 50 ml), dried with Na_2CO_3, and evaporated in vacuo to afford the crude silyl-protected fecapentaene-13, which was purified by flash chromatography (10% triethylamine in petroleum ether 40–60°). The purified silyl-protected fecapentaene-13 was dissolved in THF (80 ml), and 2.2 equivalents tetrabutylammonium fluoride trihydrate in THF (80 ml) were added at 0°. After stirring for 1 h at 0°, the reaction mixture was concentrated in vacuo at temperatures not exceeding 10° and the resulting syrup was subjected to flash chromatography (5% MeOH and 10% triethylamine in Et_2O). Fecapentaene-13 [**6**] was thus obtained as a mixture of geometric isomers around the enol ether double bond; *E*/*Z* ratio 3:1 (^{13}C and glc; oven temp. 150–250°, rise 7°/min.). The nmr spectra of the isomers were assigned by analogy with the spectra of two separated isomers of fecapentaene-12 (20). No impurities were detected by either nmr or glc. Yield: 2.23 g (42%) of pale yellow solid. Uv λ max (EtOH) 323, 338, 356 nm (ε = 45000, 66000, 59000 l mol^{-1} cm^{-1}); ms *m*/*z* 265 (18), $[M]^+$ 264 (100), 190 (10), 147 (12), 117 (33), 91 (50), 75 (12), 61 (15); exact mass 264.1704 ($C_{16}H_{24}O_3$ requires 264.1725); ^1H nmr *E*-isomer: δ 0.85 (3H, t, *J* = 7.3, Me), 1.36 (2H, tq, *J* = 7.3 and 7.3, MeCH_2), 2.04 (2H, dt, *J* = 7.3 and 7.3, CH_2CH), 3.27–3.38 (2H, m, CH_2OH), 3.63 (1H, m, *CH*OH), 3.59–3.79 (2H, m, OCH_2), 4.63 (1H, d, *J* = 5.7, CHO*H*), 4.87 (1H, t, *J* = 5.2, CH_2OH , 5.60 (1H, dd, *J* = 12.0 and 10.3, H-2), 5.66 (2H, dt, *J* = 7.3 and 14.0, H-10), 6.00–6.28 (7H, m, H-3–H-9), 6.75 (1H, d, *J* = 12.0, H-1); *Z*-isomer: δ 3.68-3.91 (2H, m, OCH_2), 5.07 (1H, dd, *J* = 6.0 and 11.3, H-2), 5.68 (1H, dt, *J* = 14.0 and 7.0, H-10), 6.00–6.28 (6H, m, H-4–H-9), 6.20 (1H, d, *J* = 6.0, H-1), 6.47 (1H, dd, *J* = 11.3 and 14.8, H-3) ppm; other signals identical to *E*-isomer.

^{13}C nmr *E*-isomer: δ 13.54 (C-13), 21.98 (C-12), 34.36 (C-11), 62.45 (C-3′), 69.98 (C-2′), 71.97 (C-1′), 107.04 (C-2), 127.33, 129.90, 130.28, 130.89, 131.24, 131.65, 133.20 (C-3–C-9), 134.11 (C-10), 152.30 (C-1); *Z*-isomer: δ 62.39 (C-3′), 70.48 (C-2′), 74.34 (C-1′), 105.81 (C-2), 126.34, 128.65, 130.90, 131.16, 131.25, 132.24, 133.20 (C-3–C-9), 134.55 (C-10), 148.33 (C-1) ppm; other signals identical to *E*-isomer.

10-METHOXY-2,4,6,8-DODECATETRAENOL [**5**, R_3 = Et].—This product was synthesized on a 0.8 mmol scale from synthetic FP-12, following the same reaction sequence as described in the analytical procedure. Detailed reaction conditions have been described in a separate paper (18). This synthetic methoxytetraenol was used to confirm the structure of the products obtained from FP-12 and FP-14 and their precursors by the two-step procedure described and also to determine the efficiency of this chemical conversion. Uv λ max (EtOH) 314, 300, 288 nm (ε = 20000, 23000, 17000 l mol^{-1} cm^{-1}); ms *m*/*z* 209 (28), $[M]^+$ 208 (74), 179 (57), 161 (27), 117 (67), 91 (91), 71 (84), 57 (100); exact mass 208.1476 ($C_{13}H_{20}O_2$ requires 208.1464); ^1H nmr δ 0.87 (3H, t, *J* = 7.5, Me), 1.56 (2H, dq, *J* = 7.5 and 6.4, MeCH_2), 1.88 (1H, s, OH), 3.25 (3H, s, OMe), 3.49 (1H, dt, *J* = 6.4 and 7.9, *CH*OMe), 4.18 (2H, d, *J* = 5.8, CH_2OH), 5.50 (1H, dd, *J* = 14.8 and 7.9, *CH*CHOMe), 5.82 (1H, dt, *J* = 5.8 and *J* = 14.4, *CH*CHO), 6.1-6.3 (6H, m, H-3–H-8); ^{13}C nmr δ 9.67 (C-12), 28.35 (C-11), 56.15 (C-13), 63.22 (C-1), 83.51 (C-10), 134.41, 132.89, 132.63 (2×), 132.48, 132.42, 132.28, 131.31 (C-2–C-9) ppm.

DETERMINATION OF FECAPENTAENES AND PRECURSORS IN HUMAN FECES.—In order to avoid possible losses of active material during sample treatment, analyses have been directly performed on wet

feces. From a homogenized sample of feces an amount of about 2.5 g was accurately weighed. To this sample, 3 ml of 0.5 M aqueous Na_2CO_3 solution (argonized before use) and 3 ml of MeOH were added. The ingredients were slurried with a spatula and the final mixture was shaken with 7 ml Et_2O (containing 0.02% BHT) for about 30 sec. Subsequently an appropriate amount (usually 10 μg of FP-13) of the internal standard solution was added. This latter solution was prepared by dissolving ca. 2 mg of FP-13 in a mixture of 10 ml of EtOAc-toluene (1:3), containing 0.2% (w/v) of BHT; the concentration was determined spectrophotometrically prior to use. The extraction mixture was shaken for an additional 10 sec. The two phases were separated by low-speed centrifugation. The supernatant was pipetted off and rapidly concentrated with a stream of N_2 at room temperature to an end volume of about 1 ml.

To the turbid, brownish suspension was added rapidly in one portion 3 ml of MeOH containing 2% v/v of 37% aqueous HCl. This mixture was allowed to react for 15 min at 20°; most of the MeOH was removed with a stream of N_2 until a volume of 1 ml was reached. Subsequently Et_2O (2 ml) and H_2O (2 ml) were added. The mixture was shaken and the Et_2O layer, containing the unsaturated aldehydes, was treated with an excess of $NaBH_4$ (about 50 mg), freshly suspended in MeOH (1 ml), for 10 min at 20°.

Successively, Et_2O (2 ml) and H_2O (2 ml) were added, the mixture was vortexed, and the organic layer, containg the tetraenols, was taken to dryness under N_2. The procedure as outlined above has to be performed without delay, with minimal exposure to air and heat; both the enol ethers and the unsaturated aldehydes are unstable and should never be allowed to come to dryness. The methoxytetraenols are appreciably easier to manipulate; they can be chromatographed and stored at −20° for longer periods of time (several months) without decomposition. The preparation obtained contained not only 0.5–50 μg of methoxytetraenols but also a relatively large amount of other lipids, including coprostanol, coprostanone, cholesterol, and free fatty acids. Direct hplc of this mixture is possible, but results in rapid contamination and deterioration of the hplc column. For this reason, a purification step on a small Si gel column was included in the procedure. The crude methoxytetraenol mixture was dissolved in 1 ml of *n*-hexane-Et_2O (4:1), and the solution was brought onto a 1 ml Si gel (Kieselgel 60, Merck) column, prepared in a Pasteur pipette. The column was washed with 6 ml of hexane-Et_2O (4:1) to remove less polar compounds. Subsequently the methoxytetraenols were eluted with 6 ml of Et_2O. The eluate was taken to dryness, and the residue was dissolved in 50 μl of MeOH. Reversed phase hplc analysis was performed with THF-MeCN-MeOH-H_2O (6:30:24:40) with a flow rate of 1.0 ml/min; detection at 300 nm; injection volume 5-10 μl. The methoxytetraenols from FP-12 and FP-14 were quantified by comparing their peak areas with that of the methoxytetraenol derived from a known amount of internal standard.

Hplc chromatograms and uv-spectra of FP-12 and its reaction products are shown in Figure 1. The uv spectra of the other fecapentaenes and their reaction products are, as expected, identical. Identification of the peaks was facilitated by using synthetic samples and a photodiode array detector, showing the characteristic uv absorptions of compounds shown in Figures 1A–1C.

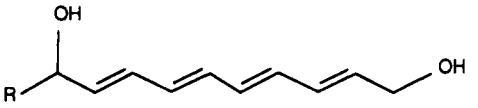
RESULTS AND DISCUSSION

Samples, collected during 3 successive days from twelve healthy volunteers, were worked up and analyzed according to the procedure described; the results are shown in Table 1. Levels of total FP's were in the range of 0.1–25.4 μg for total FP-12 and 0.1–8.5 μg for total FP-14 per g of wet feces. When comparing the data shown in the table with those obtained by others using freeze-dried samples, it should be realized that freeze-drying reduces the sample weight by about 70%. Bearing this in mind, the results presented here agree remarkably well with those recently reported by Peters *et al.* (15) who applied an analytical procedure based on the addition of ^{3}H-labelled fecapentaenes to the feces samples. The results presented here confirm the earlier finding by several groups (6, 9, 13–16) that large differences occur, both in total amount and in distribution pattern of the FP's and their precursors, between different individuals. The observation that large fluctuations may occur in FP excretion in the feces of a single individual on consecutive days has, to the best of our knowledge, not been made before. Our results show that by the reaction procedure described not only the fecapentaenes themselves but also their precursors are rapidly converted into the corresponding methoxytetraenols **5**. This method thereby allows direct determination of total FP-12 and total FP-14 without going through a lengthy incubation procedure. Representative hplc recordings of typical excreters are shown in Figure 2.

When mixtures of FP-12, FP-13, and FP-14 (individual concentrations exactly known by spectrophotometrical analyses) were worked up and analyzed by hplc, the

same relative composition of the corresponding methoxytetraenols was found, showing that, as expected, these FP homologues are converted in corresponding amounts. The yield of the chemical conversion of the FP homologues to their methoxytetraenols was over 90%. The overall recovery was about 70%.

The only side-products that can be observed after reaction of the FP's in the two-step sequence described are small amounts of 10-hydroxytetraenols **10**. The identity of these compounds was confirmed by comparison with synthetic samples. Their formation, which does not affect the outcome of the analysis, can be kept to a minimum by keeping the H_2O content of the solvolysis mixture low.



10 R = C_2H_5, C_3H_7, or C_4H_9

Samples of feces analyzed without addition of FP-13 did not show any peak in the chromatogram of the methoxytetraenols at the expected retention volume of 10-methoxy-2,4,6,8-tridecatetraenol-1, which demonstrates that FP-13 is not produced endogenously and confirms its suitability as an internal standard.

When FP-13 is directly added to the feces sample, it is not protected by the matrix to the same extent as its naturally occuring homologues (4). To avoid unequal degradation, we therefore prefer to add the internal standard after shaking the feces slurry with the extraction solvent. The lowest level of detection by the method described here was shown to be about 20 ng of total fecapentaenes per g of wet feces. The results of duplicate analyses of a number of samples (values between brackets, Table 1) show the reproducibility of the method to be good.

TABLE 1. Content of Total FP's (free FP + precursors) in Feces of Healthy Male Subjects (aged 25–60 years) with a General Type of Diet.

Subject	FP-12 (μg/g wet feces) day			FP-14 (μg/g wet feces) day		
	1	2	3	1	2	3
A	7.5	3.9 (3.86)[a] (3.94)	5.7	0.5	0.2 (0.21)[a] (0.19)	0.3
B	1.8	1.8	—[b]	0.6	0.6	—[b]
C	1.9	0.8	2.5	0.9	0.7	1.1
D	0.1	0.1	0.1	0.8	0.9	0.8
E	11.0	9.6 (9.19) (10.05)	15.9	1.0	0.5 (0.47) (0.52)	0.6
F	7.2	9.0	11.9	5.3	6.7	8.5
G	—[b]	0.1	0.1	—[b]	4.3	2.1
H	9.4	25.4	8.2	0.7	0.9	0.1
I	5.0	4.2 (4.43) (3.95)	5.8	1.0	0.7 (0.74) (0.68)	1.9
J	0.1	0.5	0.7	0.2	1.0	1.4
K	16.2	7.6	8.6	2.5	1.4	0.9
L	2.9	3.0 (2.78) (3.26)	—[b]	1.9	3.0 (2.89) (3.11)	—[b]

[a]Duplicate assays are shown in parentheses.
[b]—No feces produced.

With this newly developed method a sensitive, fast, and reproducible routine determination of the fecapentaenes and their precursors is possible. The technique is now being used to study the influence of dietary factors on the presence of these compounds in the bowel.

ACKNOWLEDGMENTS

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