a portion of dimethyl yellow (R_F = 0.68); the R_F of benzyl orange was 0.80, according to the control, and the lines c and d were marked at positions corresponding to R_F 0.81 and 0.71, respectively, and this band also included bromothymol blue, Congo red and brilliant yellow. In the second development with acetone bromothymol blue (R_F = 0.50) and the remnants of dimethyl yellow were separated; the R_F of benzyl orange was 0.05. In the third run with acetone–ethanol (3:1) the latter product was completely separated (R_F = 0.48) from Congo red (R_F = 0.10) and brilliant yellow (R_F = 0.18). The three steps of the process were carried out in glass cuvettes 22 × 6 × 22 cm lined with saturated paper.

The separated colours were identified by means of their R_F values determined independently. After the third development brilliant yellow and benzyl orange can be characterized if necessary by exposing the plate to the vapours from aqueous solutions of NH_4OH and HCl. The former will have a reddish colour with NH_4OH and is violet with HCl and the latter reacts only with HCl to show up as a red spot.

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The separation of fatty acids and their derivatives on urea-impregnated paper

Urea forms insoluble adducts with aliphatic compounds possessing a straight chain with at least eight methylene groups. The adducts of unsaturated compounds are usually unstable and branched derivatives are mostly non-adduct-forming. Various types of fatty acids have been separated on urea–Celite columns[1] on the basis of the different stabilities of their urea adducts. By using relatively non-polar solvent systems, fatty acids have been separated, on paper or thin layers impregnated with urea[2,3], into three fractions: (1) saturated fatty acids, remaining on the start; (2) branched fatty acids, with an R_F value of about 0.4–0.6, and (3) short-chain and unsaturated fatty acids, moving with the front. Waxes have also been separated by chromatography on thin layers impregnated with urea[4].

A separation using a polar solvent system and an excess of urea is reported in this paper. The mobility of various groups of fatty acids in this scheme renders it very suitable for the prefractionation of some complicated mixtures.

Procedure

Whatman No. 3 paper was impregnated with a saturated methanolic solution of urea. Amounts of sample up to 1.0 – 1.5 mg/cm were developed by ascending chromatography using methanol saturated with urea as solvent. The running distance was 14 cm, the developing time 90 min, and the optimum temperature 18 – 25°. Free fatty acids were detected by 5% aqueous cupric acetate solution followed by 3% aqueous potassium ferrocyanide solution. Neutral lipids were detected by 0.001% Rhodamine B dissolved in 0.25 *M* dipotassium phosphate[5] after previous exposure to iodine vapour[6]. Polyhydroxylic acids were detected by cutting up the chromatogram, eluting the strips, acetylating the isolated fatty acids, and determining the amount of ester groups by the hydroxamic acid procedure[6]. Peroxides were detected with 3% aqueous ammonium ferrous sulphate followed by 5% aqueous ammonium thiocyanate solution. Carbonyl derivatives were detected with 1% benzidine solution in acetic acid, with a saturated solution of 2-thiobarbituric acid in acetic acid, or by spraying with a 2,4-dinitrophenylhydrazine solution followed by 0.5 *N* ethanolic potassium hydroxide solution. The iodine value of the eluted fractions was determined by the Hanuś method the peroxide value iodometrically (reaction time 20 min, at room temperature). The purity of the substances was checked by gas–liquid chromatography (Chrom III, EGA columns).

Results

When compared with separations using less-polar solvent systems[2,3] the mobility of all adduct-forming substances was much lower when urea-saturated methanol was used as solvent. Caprylic and lower acids had R_F = 0.8–0.9, decanoic 0.5–0.6, lauric 0.1–0.2, while myristic and higher saturated acids were left on the start. Unsaturated fatty acids, if the first double bond was on the 9th or a more distant carbon atom from the carboxyl group (oleic, elaidic, linoleic, linolenic, 11-eicosenoic, erucic, nervonic) also remained on the start. Impregnation of the paper with mercuric acetate and saturation of the solvent with mercuric acetate did not affect the results.

Ricinoleic and chaulmoogroic acids remained on the start but acetylated or ethylene oxide-treated[8] ricinoleic acid moved with the front. Fatty acids with the first double bond on the 4th carbon atom (arachidonic, clupanodonic) also proceeded with the front. The cod liver oil fatty acids were separated into a fraction on the start (iodine value = 117.6) and a fraction at the front (iodine value = 267.3), the latter having the typical fish oil odour. The R_F values of the polyhydroxylic fatty acids produced from oleic, linoleic and linolenic acids by potassium permanganate oxidation (9,10-dihydroxystearic, 9,10,12,13-tetrahydroxystearic, and 9,10,12,13,15,16-hexahydroxystearic acid) were 0.8–0.9, 0.6–0.9, and 0.3–0.9, respectively. 9,10,12,13-Tetrabromostearic acid (prepared by bromination of linoleic acid) advanced with the front.

Methyl, isopropyl and butyl esters of saturated and unsaturated fatty acids had R_F values similar to those of free fatty acids. Stearamide, stearonitrile and stearaldehyde remained on the start.

Methyl esters of olive oil fatty acids are oxidized on thin layers at 60% to a peroxide value of 1,200 – 1,500 mequiv./kg. After the separation on a urea-impregnated paper, the fraction on the start gave a negative peroxide reaction and only slight

reaction for carbonyl groups while the fraction proceeding near the front had a peroxide value of 3,100 – 3,300 mequiv./kg and gave an intense reaction with benzidine, 2-thiobarbituric acid and 2,4-dinitrophenylhydrazine.

Discussion

According to the previous procedures[2,3] loosely bound fatty acids are extracted by non-polar solvents from the adducts so that *cis*-unsaturated acids, for instance, are eluted with the solvent front. These methods are, therefore, inconvenient in those cases when the non-adduct-forming substances need to be separated from both saturated and unsaturated straight-chain fatty acids. For this reason, the sample was separated under conditions favouring the formation of adducts, *i.e.* in methanol saturated with urea. The medium chain saturated fatty acids then have lower R_F values than those obtained by the previous procedures[2,3], and all monoenoic acids remain on the start. Even the substances forming adducts with difficulty[8], such as linolenic, ricinoleic[9] and chaulmoogroic acids, do not advance from the start. Only compounds unable to form adducts at all can proceed with the front, in particular derivatives with a voluminous side chain or some polysubstituted compounds *e.g.* tetrabromostearic acid[10]. In case of polyhydroxylic acids, the steric configuration may play an important role[11]. The fatty hydroperoxides and dimers are separated from the adduct-forming non-oxidized material. The present procedure thus enables the non-adduct-forming substances to be separated from both the saturated and the unsaturated fatty acids and their esters.

Another advantage of the use of urea-saturated methanol as a solvent is the increased capacity of the chromatographic paper so that the separation may be used for preparative purposes as well. The procedure is particularly useful for the removal of oxidized products from unsaturated fatty acids and their derivatives.

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