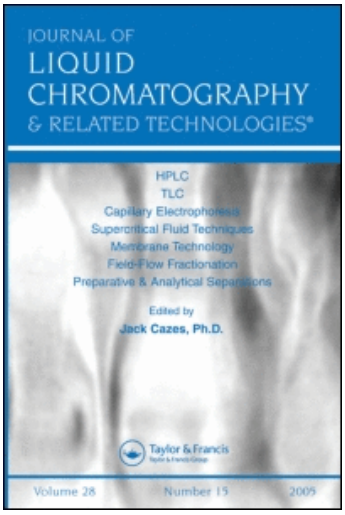

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REMOVAL OF TRITON X-100 AND REDUCTION OF TWEEN 20 CONCENTRATION IN EXTRACTS OF FATTY OXIDATION PRODUCTS

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ABSTRACT

A technique was developed using silica Sep-Paks to remove Triton X-100 and reduce the concentration of Tween 20 in fatty extracts. Tween 20 concentration was primarily reduced by using non polar solvents to lower solubility. Although each Sep-Pak was capable of adsorbing up to 75 mg of Triton X-100, separation of the detergent from sample extracts was most effective when using sample loads approximately one tenth that size.

INTRODUCTION

The detergents Triton X-100[3] (alkylaryl polyether alcohol) and Tween 20 (polyoxyethylene 20 sorbitan monolaurate) have many laboratory uses. Both detergents are used to disperse fats such as linoleic and linolenic acid for use as substrate in enzymic incubations involving lipoxygenase (1,2).

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2/ A laboratory cooperatively operated by the Agricultural Research Service, U.S. Department of Agriculture; the Minneosta Agricultural Experiment Station; the North Dakota Agricultural Experiment Station; and the Red River Valley Potato Growers' Association.

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Triton X-100 is especially useful in solubilizing membranes and membrane bound protein (3,4). Triton X-100 has been used to solubilize enzymes in the lipoxygenase oxidation system that include lipoxygenase (5), hydroperoxide isomerase (6), and hydroperoxide cyclase (7). Metabolite studies of this system are conducted by incubating extracts of the crude enzymes with linoleic acid substrate followed by extraction of the oxidation products. The organic (lipid) extracts of the oxidation products also contain portions of the Tween 20 originally used to disperse the substrate and Triton X-100 used to solubilize the enzymes. The presence of these detergents is not a serious problem when using TLC because they remain at the origin, thereby, causing little interference. However, when using reversed phase HPLC, Triton X-100 elutes with the oxidation products and it has strong UV absorption (8). Elution of Tween 20 is nearly undetectable because of its poor UV absorption.

The problem of detergents in the lipid extract is more troublesome when using normal phase HPLC. Polar compounds, such as Triton X-100, bind tightly to microparticulate silica columns. Each added injection coats more detergent onto the column. Typical non-polar gradient solvent systems will not elute the detergent. Detergent left bound to the silica could slowly alter the chromatographic properties of the column. Elution of the detergent with more polar solvents can also be difficult and it is troublesome to monitor clean-up of a column when changing from nonpolar to polar solvents because of drastic changes in baseline. A method is needed to remove these detergents from the organic extract before HPLC analysis.

Cheetham (9) developed a method using Amberlite XAD-2 to remove Triton X-100, however, this method was designed for use with aqueous extracts which were to be used for enzyme studies. We report here a quick and inexpensive technique using silica Sep-Paks to remove Triton X-100 from lipid extract and to minimize the amount of Tween 20 in the treated extract.

MATERIALS

Potatoes were macerated with a model 6001 Acme Supreme Juicerator, Acme Juicer MFG Co., Sierra Madre, CA. Membrane disruption was performed with a Sonic Dismembrator, Asteck Systems Corp., Farmingdale, NY. Triton X-100 and Tween 20 were purchased through J. T. Baker Chemical

Co, Phillipsburg, NJ. Linoleic acid was purchased from NuChek Prep Inc., Elysian, MN. Anhydrous diethyl ether and petroleum ether were purchased from Mallinkrodt, Inc., St. Louis, MO; all other solvents were purchased from Burdick and Jackson Laboratories, Inc., Muskegon, MI. TLC was conducted on Anasil HF plates obtained from Analabs, Inc., New Haven, CT.

Liquid chromatography was conducted using a 3.9 mm x 30 cm fatty acid analysis column, with dual M 6000A pumps, a model U6K injector, a model 730 Data Module and a model 720 System Controller, (Waters Assoc., Milford, MA). The UV detector was a variable wavelength model SF 770 (Schoeffel Instruments, Westwood, NJ) set at 214 nm. Detergent removal was accomplished with silica column cartridges, Sep-Paks, manufactured by Waters Assoc., Milford, MA.

Water for the HPLC solvent system was purified by passage through Reverse Osmosis and Super Q water systems followed by final treatment with an Organex Cartridge-Filtration apparatus (Millipore Corp., Bedford, MA).

METHODS

Enzyme extracts from Norchip Potatoes (Solanum tuberosum L.) were prepared by freezing cubes (100 g) of potato flesh in liquid nitrogen, macerating the frozen cubes and extracting with K-Phosphate buffer (100 ml, 0.1 M, PH 6.0) containing 0.3% Triton X-100. The enzyme extract combined with the pulp was then sonicated 45 sec at a setting of 60 (80 watts, 22.2 kcycles/sec) with a Sonic Dismembrator. Linoleic acid substrate was prepared using Tween 20 as a dispersing agent (1). Enzyme extract (120 ml) and substrate (60 ml) were incubated in K-phosphate buffer (1500 ml, 0.05 M, pH 6.0). After the appropriate incubation period (30 to 45 min) the enzyme activity was stopped by adding enough citric acid to lower the pH to 3.5. The fatty acid oxidation products were extracted (3X) with chloroform-methanol (2:1, V/V). The organic extract of lipid and unwanted detergent was dried with anhydrous Na-sulfate and the solvent was evaporated. The concentrated extract of fat and detergent was esterified with diazomethane.

The detergent concentration in the esterified lipid extract was initially reduced by evaporating the ether solvent with nitrogen and dissolving the lipid in 5 ml of hexane-ether (1:1, V/V). The hexane-

ether solvent combination also provided the proper polarity for adsorption onto silica Sep-Paks.

A silica Sep-Pak cartridge was fitted to a 10 ml gas tight syringe and purged with 10 ml of water at approximately 12 ml/min. The cartridge was then similarly activated with 10 ml of anhydrous methanol, 15 ml of absolute ethanol and 8 ml of anhydrous diethyl ether, followed by equilibration with 20 ml of hexane-ether (1:1, V/V). A small portion (0.5 ml) of the lipid extract, containing esterified fat and detergent, was diluted to 1 ml with hexane-ether and applied by syringe to the activated Sep-Pak. The methyl esters of the fatty acid oxidation products were eluted with 4 to 12 ml of hexane-ether, followed by 4 to 8 ml of ether. Adsorbed (or bound) detergent could then be eluted with 4 ml of methanol.

Thin layer chromatography of the concentrated, eluted fractions was accomplished by three developments with petroleum ether-anhydrous diethyl ether-acetic acid (60:40:1, V/V/V) and visualized by charring.

The average binding capacity of a Sep-Pak cartridge for Triton X-100 was determined by passing successive 4 ml volumes of 0.5% Triton X-100 (5.33 mg/ml in hexane-ether, 1:1, V/V) through the Sep-Pak and monitoring the eluate for non-bound Triton X-100 at 277 nm (9,10).

High performance liquid chromatography was conducted with an initial 5 min isocratic period (40% acetonitrile - 60% water) followed by a linear gradient (curve 6 on the System Controller) to 25 min (85% acetonitrile - 15% water) and a subsequent isocratic period to 40 min (85% acetonitrile - 15% water). The flow rate was 3 ml/min.

RESULTS

Solubility Of Products and Detergent

After methylation, the crude organic extract containing enzymically oxidized lipid and detergent was taken up in hexane-ether (1:1, V/V). This solvent solubilized the reaction products but dissolved little of the Tween 20. Gravimetric experiments indicated that the hexane-ether solvent was capable of dissolving 1.8 mg of Tween 20 per ml at saturation at 23^{0}C. It was not practical to spectrophotometrically monitor the presence of Tween 20 in experiments or subsequent HPLC because of its poor UV absorption. The residue, created after taking the lipid extract

up in hexane-ether, was redissolved in methanol and analyzed chromatographically. Results from HPLC indicated the redissolved residue contained no lipid oxidation products or Triton X-100. On TLC, no oxidation products were visible and the residue remained at the origin and was therefore presumed to be Tween 20.

Adsorption Capacity of Silica Sep-Paks for Triton X-100

The average capacity of a Sep-Pak to adsorb Triton X-100 was evaluated by cummulative additions of the detergent (0.5% V/V in hexane-ether) and monitoring the eluate for non-bound Triton X-100 at 277 nm (Fig. 1) (8,10). The 0.5% Triton X-100 stock solution would have an equivalent absorbance of 12.5 (10). The same binding or adsorption curve was obtained in experiments using 1.0% Triton X-100 solutions.

Thin Layer Chromatographic Profiling of Sep-Pak Fractionated Lipid and Detergent

The extracted products and detergent were applied to a Sep-Pak, and eluted fractions were collected and chromatographed by TLC (Fig 2). Lane one of the TLC is the crude non-fractionated extract and shows the detergent remaining at the origin. Lipid can be eluted from the Sep-Pak with hexane-ether (1:1 and 1:2.3) without desorption of detergent (lanes 3, 4, and 5). Elution with ether desorbed remaining polar lipid products and presumably some Tween 20 (lane 6). With our sample loads (6 - 8 mg) and range of product polarities, final elution with methanol (lane 7) desorbed the detergent and showed that no polar lipid remained.

HPLC Profiling of Sep-Pak Fractionated Lipid and Detergent Extract

HPLC was conducted on the crude extract and fractions prepared by Sep-Pak treatment (Fig 3). Figure 3E shows the peak at 11.5 min, Triton X-100, to be the single largest absorbing component in the extract. Although Triton X-100 was spectrophotometrically quantitated at 277 nm (8,10), the UV spectrum for this compound has its largest absorption maxima at 216 nm, very close to the 214 nm wavelength used in our HPLC monitoring. After applying the crude sample to the Sep-Pak, lipid could quickly be eluted for HPLC with 4 ml of hexane-ether (1:1) followed by 8 ml of ether. HPLC of the combined hexane-ether and ether eluates shows removal of Triton X-100 (Fig 3D). HPLC of the final Sep-

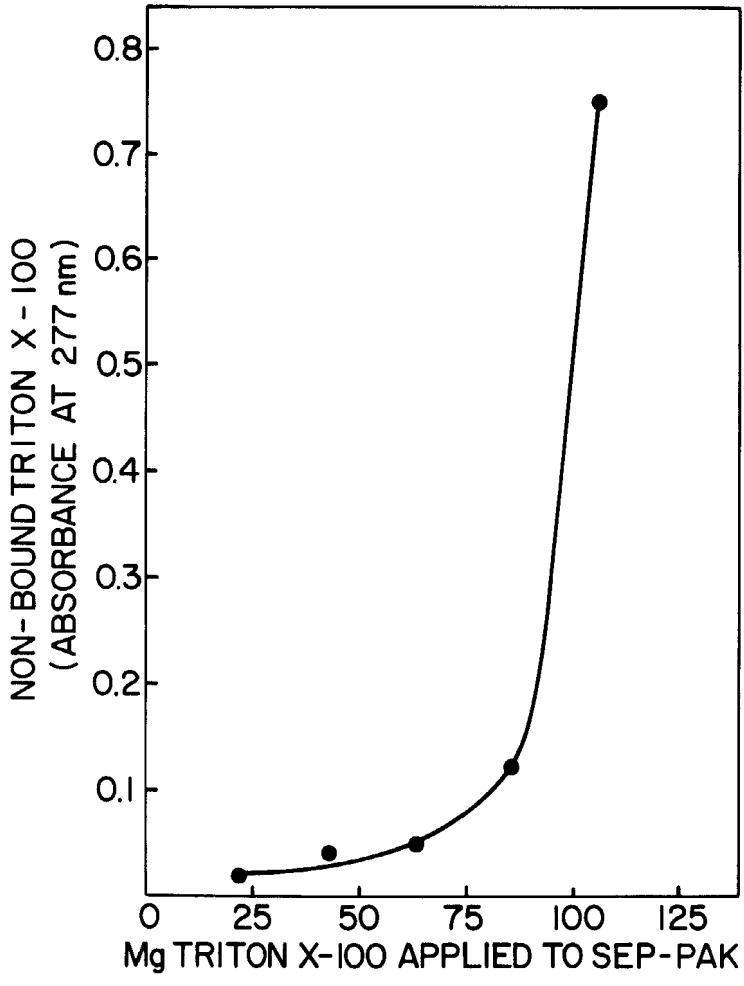

FIGURE 1. Silica Sep-Pak binding capacity for Triton X-100. Cummulative 4 ml aliquots of Triton X-100 (0.5%, 5.33 mg/ml) were applied to a silica cartridge and the non-bound Triton X-100 was measured in the eluate.

Pak eluate (Fig 3C) shows that only Triton X-100 remained adsorbed until elution with methanol. The presence of Tween 20 could not be determined for HPLC by UV monitoring. The solvent gradient (Fig 3B) changed the refractive index and caused the baseline rise depicted by the blank run in Figure 3A and found in the chromatograms of Figures 3C, 3D and 3E. The small peaks found in the first 5 min of Figure 3A are from the injection of 10 ul of hexane-ether control. The peaks within the remaining portion of Figure 3A are from trace enrichment of the organic material in the water component of the solvent system.

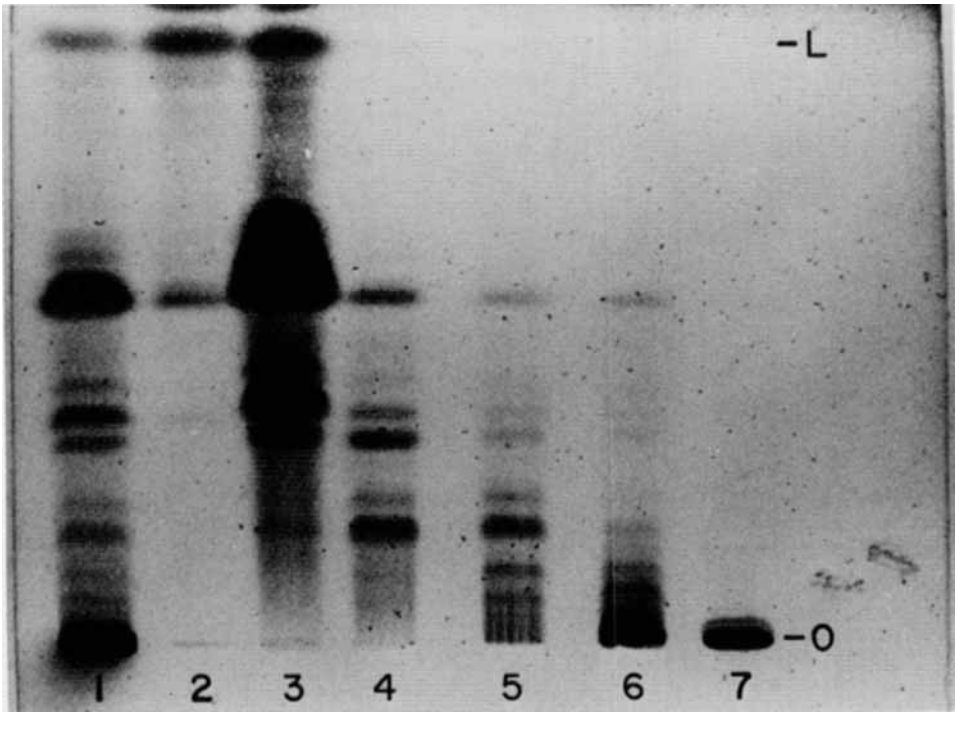

FIGURE 2. Thin layer chromatographic profile (3 developments, petroleum ether-anhydrous diethyl ether-acetic acid, 60:40:1, V/V/V) of (1) the crude extract containing lipid and detergent, followed by profiles of fractions from silica Sep-Pak separation, (2) eluate from sample application, (3) eluate from 4 ml hexane-ether (1:1), (4) eluate from 4 ml hexane-ether (1:1), (5) eluate from 4 ml hexane-ether (1:2.3), (6) eluate from 4 ml anhydrous diethyl ether, and (7) eluate (Triton X-100) from 4 ml methanol. Detergent remained at origin (-0) and linoleic acid (-L) migrated to near the top of the TLC plate.

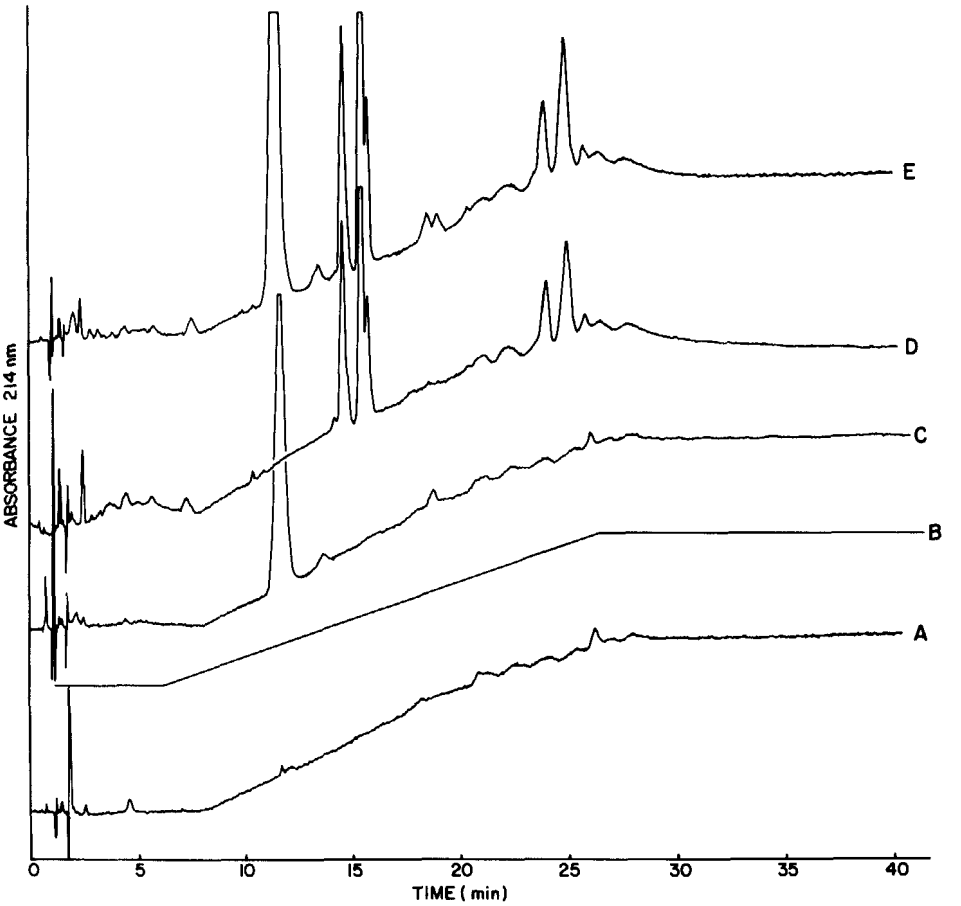

Figure 3. HPLC profiles of: (A) a blank chromatogram of the solvent gradient with injection of 10 ul of hexane-ether; (B) the solvent gradient starting with a 5 min isocratic period (40% acetonitrile-60% water), then a 25 min linear gradient, (85% acetonitrile-15% water) followed by a 15 min isocratic period; (C) a chromatogram of the final Sep-Pak eluate using 4 ml of methanol to desorb bound Triton X-100; (D) a chromatogram of the combined Sep-Pak eluates containing fatty acid methyl esters eluted with 4 ml of hexane-ether (1:1, V/V) and 8 ml of ether; (E) a chromatogram of the crude fatty extract prior to removal of Triton X-100 by fractionation with silica Sep-Pak. The flow rate through the 3.9 mm x 30 cm fatty acid analysis column was 3 ml/min.

DISCUSSION

In addition to the successfull removal of Triton X-100 from extracts of oxidized linoleic acid, we found that the level of Tween 20 could also be reduced to approximately 1.8 mg/ml of solvent by dissolving the final 5 ml concentrate of methyl esters in hexane-ether (1:1, V/V).

Experiments showed that silica Sep-Paks would adsorb Triton X-100 (Fig 1) and easily bind up to approximately 75 mg before appreciable Triton X-100 appeared in the eluate. However, separation of Triton X-100 from the lipid extract was most efficient when smaller amounts of total crude extract, 6 to 8 mg, were applied. Application of larger sample loads caused some lipid to adhere with the detergent to the silica even after elution with ether. Furthermore, oxidation products of higher polarity, if present, may also remain bound with the detergent and later appear in the methanol eluate.

Although elution with ether appeared to desorb some Tween 20 from the Sep-Pak, ether appeared necessary to desorb products which were "less" non-polar (Fig 2).

Ether did not elute Triton X-100, as indicated by the chromatogram of the combined eluates (Fig 3D). However, a skewed peak with a retention time similar to that of Triton X-100 was observed when the water component of the solvent system was purified at the final stage with two 7.8 mm x 61 cm columns packed with Bondapak C_{18}/Porasil B. The Organex-Cartridge Filtration system eliminated the skewed peak (Fig 3A).

Several other solvent systems employing $CHCl_3$, THF and water were tried with silica Sep-Paks and with C_{18} Sep-Paks, all with less success. An older batch of Sep-Paks which were yellowish in color required additional clean-up to leach soluble antioxidants from the casing.

The use of a small silica column, such as the Sep-Pak, provided a quick, inexpensive method to effectively remove Triton X-100 from fatty acid extracts for HPLC analysis. The elimination of Triton X-100 simplified the chromatogram when using C_{18} reversed phase HPLC and, more importantly, prevented detergent build-up on analytical silica HPLC columns. Our experience has shown that Triton X-100 will bind very tightly to an analytical silica HPLC column and it was not easily removed with normal phase solvent systems.

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