

## INADVERTENT INTRODUCTION OF SQUALENE, CHOLESTEROL, AND OTHER SKIN PRODUCTS INTO A SAMPLE

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**Abstract**—Recent developments in analytical techniques permit the chemical ecologist to achieve identification of naturally occurring compounds with relatively small amounts of the products of interest. However, the microanalytical techniques employed frequently require the handling of sample vials and other transferral instruments such as syringes and micropipets, where the analyst's hands come into close contact with the sample. Here we show how inadvertent contamination of a sample with skin lipids can occur simply by catching a 1-ml sample vial by the neck rather than the base or by activating a syringe by holding the plunger extension between the fingers rather than taking it by the head. Squalene, cholesterol, and, to a lesser extent, hydrocarbons and fatty acids from fingers are easily introduced into the sample in this manner. These findings are particularly relevant for a parasitology laboratory such as ours, investigating the function of vertebrate-derived products in hematophagous arthropods.

**Key Words**—Squalene, cholesterol, skin lipids, contamination, sample contamination.

### INTRODUCTION

Modern methods in analytical chemistry permit the chemical ecologist to work with very small amounts of extracts of biological origin for purposes of identification of naturally occurring products. The work is normally carried out with a minimum of solvent, as it is well known that the latter may contain some pollutants such as phthalates which, on concentration of an extract, may become so predominant as to obscure the product(s) of interest during analysis. Added

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to this is the obligation of keeping the use of solvents to a minimum in order to avoid environmental contamination and to reduce costs of elimination to a minimum. However, micro-methods require handling of syringes, micropipets, small sample vials, and other substrates. In the process, the investigator's fingers will frequently come near the solvent via the containers holding it. Here we show how some commonly occurring skin products from fingers can inadvertently find their way into a sample.

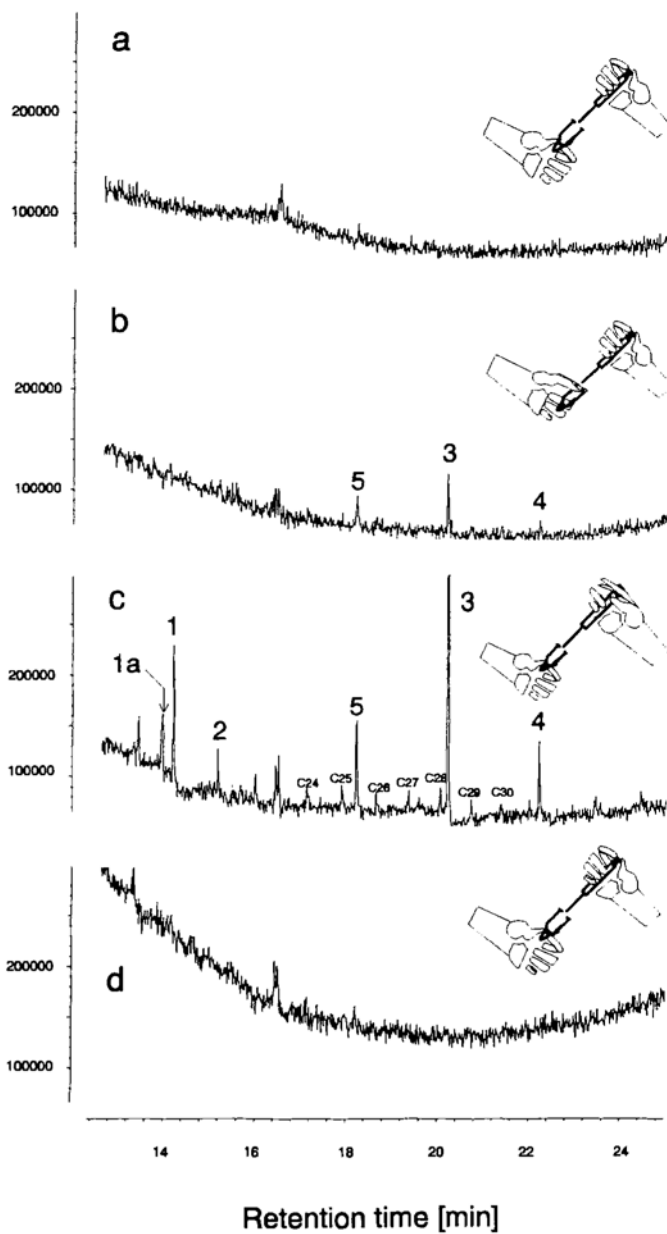
#### METHODS AND MATERIALS

*Contamination.* Contamination by fingers was investigated by manipulation of a small volume of solvent in a sample vial with a syringe in three different ways (Figure 1), and in addition, by purposely touching a piece of filter paper that was subsequently extracted and analyzed. One milliliter of chloroform (Merck, analytical grade) was introduced via a glass-PTFE dispenser (10 ml Repipet Dispenser, Labindustries, California), permanently mounted on the  $\text{CHCl}_3$  bottle, into a 1.1-ml tapered borosilicate screw-top vial (Chromacol, U.K.) held as specified below (a-d) and concentrated to dryness under a nitrogen stream. The Pasteur pipet, from which the nitrogen stream issued, was connected via stainless steel tubing and connections with PTFE ferrules to a fat-free metal bellows manometer on the nitrogen cylinder. The chloroform was remarkably pure, for even when it was concentrated 10,000 times, no trace of any of the products was detected. Then, 100  $\mu\text{l}$  of chloroform were added with a 500- $\mu\text{l}$  syringe (Hamilton 750 series microliter syringe, Bonaduz, Switzerland) to rinse down the walls of the vial and the solvent was again concentrated to dryness. The position of the fingers on the vial and syringe was varied systematically during four different manipulations to wash down the walls of the vial (Figure 1): (a), the vial was held by the bottom with the left hand, and the syringe plunger was activated by holding the head between the index finger and thumb

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FIG. 1. GC-MS analysis of a MSTFA-derivatized chloroform sample held in a 1.1-ml tapered vial and manipulated with a 500- $\mu\text{l}$  syringe. Dioctyl phthalate (5), squalene (3), and cholesterol (4) are introduced into the sample by holding the vial by the neck (b) rather than at the base (a). Activation of the syringe by holding the plunger extension between the index and middle fingers (c) leads to even more significant contamination of the sample with the skin products squalene and cholesterol, in addition to palmitoleic acid (1a), palmitic acid (1), stearyl alcohol (2), and the alkane series  $\text{C}_{24}$ - $\text{C}_{30}$ . Activation of the plunger by the head and holding the vial by the base avoids this inadvertent contamination of the sample (a and d). For details of the derivatization and analytical procedures see Methods and Materials. (b) Peak 3 represents ca. 1 ng, and (c) 10 ng.

Abundance



of the other hand (the remainder of the fingers being employed to hold the barrel of the syringe); (b) the same as in "a", but holding the vial by the neck; (c) the vial was held as in "a", but the syringe plunger was activated by holding it between the index and middle fingers just under the head, i.e., on the sleeve, designed for this purpose, 10 mm long on the plunger extension just under the head, which does not enter the barrel; (d) manipulations as in "a" (control).

The same sample vial was used in the four manipulations but washed between manipulations with  $\text{CHCl}_3$  and dried by heating in an oven at  $110^\circ\text{C}$ . The syringe was rigorously washed between successive manipulations by removing the plunger and washing it down with chloroform-soaked tissue and by evacuating several milliliters of chloroform through the syringe using a vacuum applied to the barrel. Each time, the dried-out content of the vial was redissolved in  $10\ \mu\text{l}$   $\text{CHCl}_3$  using the  $25\text{-}\mu\text{l}$  syringe employed for derivatization (below). This syringe, and the  $10\text{-}\mu\text{l}$  one used for injection of the sample for GC-MS analysis, were always activated holding the plunger by head.

The consequences of handling a piece of filter paper with the fingers was investigated by first washing a filter paper disk held by forceps (Schleicher & Schuell, 4.5-cm diam.) in some 10 ml of  $\text{CHCl}_3$ , and then drying at  $110^\circ\text{C}$  in the oven. Two small pieces of the disk (ca.  $4 \times 7$  mm) were cut with  $\text{CHCl}_3$ -cleaned scissors. One piece was held for about 3 sec between the index and thumb (which had been unwashed for 1 hr), then introduced with forceps into the sample vial described above and extracted for 10 min in  $200\ \mu\text{l}$   $\text{CHCl}_3$  by manipulating the syringe and vial as in "a" above. The piece of filter paper was then removed with forceps and the extract concentrated under nitrogen to dryness. Then  $10\ \mu\text{l}$  of  $\text{CHCl}_3$  was added with the  $25\text{-}\mu\text{l}$  syringe to rinse down the walls of the vial and to redissolve the extract for derivatization. The second piece of filter paper was treated as the first but without holding it between the fingers, thus serving as control for the extraction and derivatization procedures.

*Derivatization.* Ten microliters of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA, Fluka puriss) were added with a  $25\text{-}\mu\text{l}$  syringe (Hamilton) to each of the six redissolved samples described above and the mixture held for 1 hr at room temperature under nitrogen. In this manner, all products with alcohol and acid functions are derivatized as trimethylsilyl esters. These less polar derivatives were more efficiently resolved by capillary gas chromatography (below). The sample was then brought to dryness under nitrogen and collected from the tapered vial in  $2\ \mu\text{l}$   $\text{CHCl}_3$  with a  $10\text{-}\mu\text{l}$  Hamilton syringe for GC-MS analysis.

*Gas Chromatography-Mass Spectrometry (GC-MS).* Derivatized samples were analyzed by GC-MS (Hewlett Packard 5890 series chromatograph linked to a HP 5971A mass selective detector), by injecting the  $2\ \mu\text{l}$  of derivatized sample on-column onto a DB-5HT nonpolar high-resolution fused-silica capillary column (15 m, 0.25 mm ID 0.10- $\mu\text{m}$  film thickness, J & W Scientific,

California) equipped with a precolumn (1 m deactivated fused-silica) and connected via a 1-m deactivated fused-silica capillary (0.25 mm ID) to the MS (ionization chamber temperature 300°C; ionization energy 70 eV). The detector, operating in the EI mode, scanned for masses of 30 to 650. Helium was used as carrier gas under constant flow (velocity 40 cm/sec at 60°C). The components of the sample were identified by comparing the mass spectra of unknowns with those of standards in the computer-based library of the GC-MS associated HP chemstation and by comparison of retention times of unknowns with those of standards, derivatized and underivatized, injected under the same conditions as the derivatized samples.

## RESULTS

Once the fingers were brought into contact with the mouth of the 1.1-ml vial containing the 1-ml solvent sample, both squalene and cholesterol were systematically found in the analysis of the concentrate, as well as increased levels of dioctyl phthalate (Figure 1b). Grasping the vial by the base while activating the plunger of the 500- $\mu$ l syringe by holding the sleeve on the plunger extension led to the greatest contamination of the sample (Figure 1c). Some 10 times more squalene and cholesterol was found in the concentrate in this case. In addition, other products were also detected in the concentrate such as palmitic acid, palmitoleic acid, stearic alcohol, dioctyl phthalate, the saturated hydrocarbon series  $C_{24}$ - $C_{30}$ , and wax esters, all at levels some 50% that of cholesterol. By contrast, simply holding the vial by the base and the syringe plunger by the head avoided all such pollution of the concentrate (Figure 1a and d).

Proof that the source of these commonly occurring chemicals is the fingers of the analyst was obtained by investigating the extract of the piece of filter paper that had been purposely handled. Here, palmitic acid, squalene, and cholesterol again predominated in the multicomponent extract (Figures 2a and 3). This extract also contained the alkane series  $C_{20}$ - $C_{34}$ , stearyl alcohol, the fatty acids palmitoleic, stearic and oleic acids, the wax esters palmityl and stearyl palmitate, palmityl, stearyl, arachidyl, behenyl, and lignoceryl palmitoleate, along with some steroids and other unidentified compounds, all at levels some 5-30% that of cholesterol. Overall, direct contact with the fingers yielded quantities of the products some 10 times higher than those obtained by manipulating a solvent sample with fingers on the plunger sleeve of the syringe ("b" above). By contrast, the extract of the untouched piece of filter paper (control) contained only a trace of the saturated  $C_{16}$  acid (Figure 2b).

Abundance

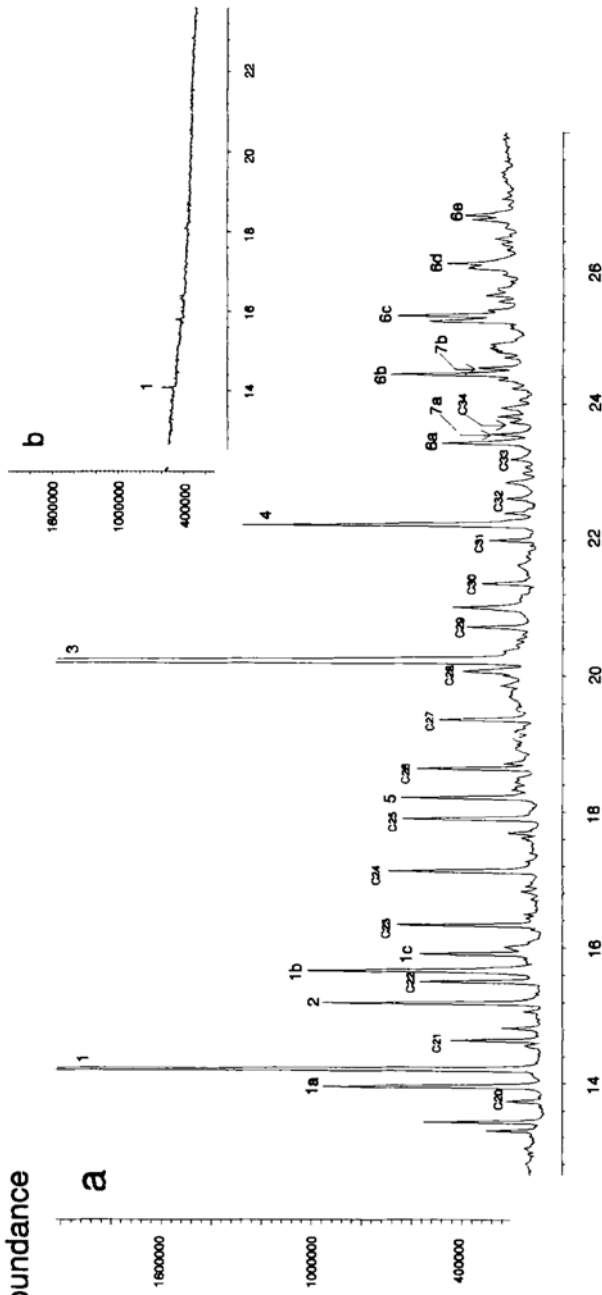


Fig. 2. GC-MS analysis of a MSTFA-derivatized chloroform extract of two 28-mm<sup>2</sup> pieces of filter paper. One was held between index and thumb for 3 sec (a) and the other left untouched (b). The major skin components that impregnated the filter paper in "a" are squalene (3), palmitic acid (1), and cholesterol (2). Other skin products detected by the mass selective detector include palmitoleic acid (1a), oleic acid (1b), stearic acid (1c), stearyl alcohol (2), the alkane series C<sub>20</sub>-C<sub>34</sub>, dioctyl phthalate (5), and the wax esters palmityl palmitate (7a), stearyl palmitate (7b), palmityl palmitoleate (6a), stearyl palmitoleate (6b), arachidyl palmitoleate (6c), behenyl palmitoleate (6d), and lignoceryl palmitoleate (6e). For details of the derivatization and analytical procedures see Methods and Materials. Peak 3 represents ca. 100 ng.

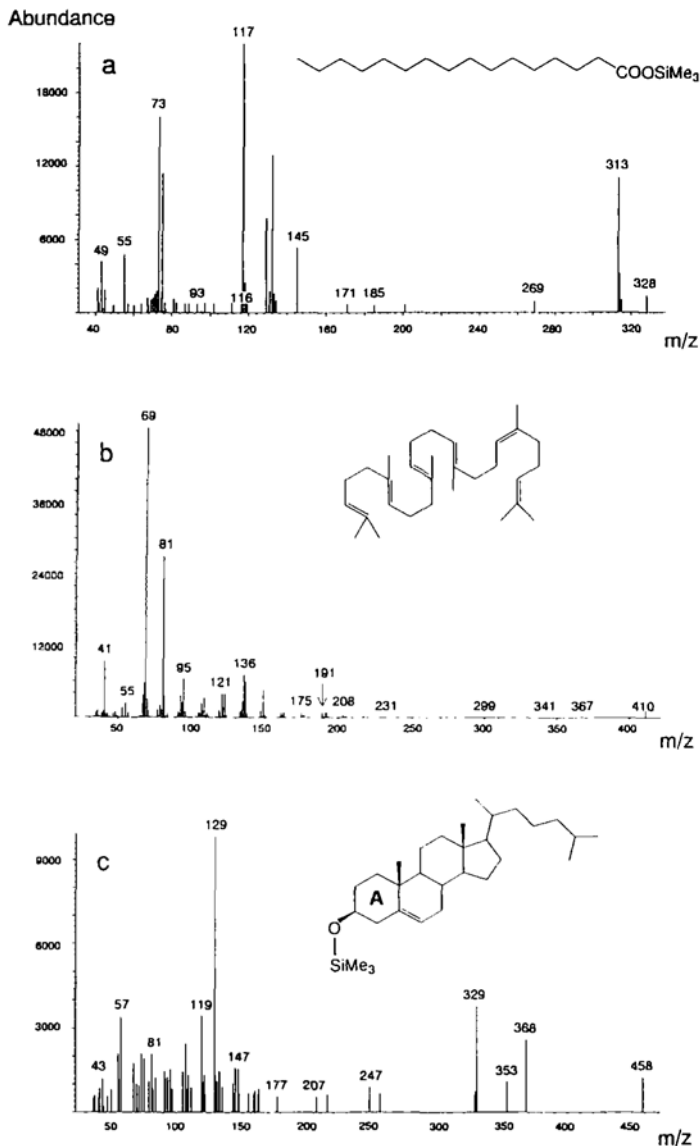


FIG. 3. Mass spectra of the major constituents of the MSTFA-derivatized chloroform samples. (a) Trimethyl silyl derivative of palmitic acid with  $M^+$  328, intense key fragment ion  $m/z$  313 ( $M^+ - 15$ ) and base peak  $m/z$  117 of  $(CO_2SiMe_3)^+$ ; (b) squalene with a weak  $M^+$  410 and key fragment base peak  $m/z$  69 of  $(C_3H_9)^+$ ; and (c) the trimethyl silyl derivative of cholesterol with  $M^+$  458, the key fragment  $m/z$  368 ( $M^+ - 90$ ) due to loss of  $(OSiMe_3)^+$ ,  $m/z$  329 ( $M^+ - 129$ ) and base peak  $m/z$  129 of  $(C_3H_4OSiMe_3)^+$  after cleavage of the A ring.

## DISCUSSION

The possibility that a sample could be contaminated by material from the analyst's fingers was brought to our attention by the presence of squalene, and other products of vertebrate origin, in quite diverse samples. All of the products detected in the samples analyzed in this study are well-known constituents of skin (Biedermann and Grob, 1991; Downing et al., 1981, 1987; Liu et al., 1976). Squalene (2,6,10,15,19,23-hexamethyl-2,6,10,14,18,22-tetracosahexaene) is well known to analytical chemists as a contaminant in handled samples. It is a precursor of cholesterol synthesis in vertebrates. However, one would never have thought that levels up to 1 ng of this product could be transferred to the pure solvent sample simply by placing one's fingers at the mouth of the vial, at some 9 mm from the solvent surface. Admitting that the solvent underwent a ca. 1000-fold concentration step, levels of squalene at over 10 ng/ $\mu$ l in the concentrated sample where the plunger was held by the sleeve are such as to be visible in most routine analyses. Evidently, evaporation and recondensation of chloroform within the vial (as with the volatiles along the walls of a glass of brandy) suffices to recover the skin materials, but what happens by holding the syringe plunger by the sleeve is even more dramatic. Here the solvent creeps up within the barrel on repeated movements of the plunger of the 500- $\mu$ l syringe, and this solvent evidently comes into close enough contact with the sleeve to pick up skin products every time the plunger is pushed home. Mixing of this solvent around the plunger with that in the barrel of the syringe is apparently sufficient to contaminate the sample being manipulated.

The levels of squalene on the fingers are indeed very high, as indicated by the amounts of about 100 ng recovered from the piece of filter paper that was purposely handled for just 3 sec. The same experiment made with fingers that had been unwashed for several hours showed an amount of more than 1  $\mu$ g. Levels of 60  $\mu$ g were recovered by Biedermann and Grob (1991) from one finger tip extracted with hexane. These authors demonstrate solvent contamination with the same type of products described here via inadvertent touching of glassware. The high levels of squalene detected are not very surprising. A maximum of about 475  $\mu$ g/g dry weight has been measured in human skin, but the amounts of squalene contained in the blood or secreted by the skin of humans will vary enormously with diet (Liu et al., 1976). Wearing latex gloves will hinder the inadvertent introduction of skin products, but the consequence is the introduction of phthalates from the latex into the sample. Other substances reported from the skin surface of humans include branched fatty acids, phospholipids, cerebroside, ceramides, sterol esters, and triglycerides (Downing et al., 1987; Melnik et al., 1989).

Inadvertent pollution of a sample is a bothersome occurrence for any analyst. However, the contamination described here is particularly acute for labo-



ratories investigating the origin and function of natural products of mammalian origin. In this laboratory, for example, we investigate the role of semiochemicals for hematophagous arthropods. These organisms do not synthesize products such as squalene and cholesterol, but may sequester these products or their derivatives for a number of essential functions. For example, cholesterol esters are employed as pheromones in ticks (Hamilton et al., 1989). Host-derived arachidonic acid (20:4) is concentrated in the salivary glands of ticks, presumably as a precursor of prostaglandins, which are employed by ticks to overcome hemostasis, inflammatory responses, and host immunity (Shipley et al., 1993; Bowman et al., 1994). Ascribing particular amounts of products associated with human skin to the parasite, or to one of its organs, can at most only be tentative if the possibility of inadvertent contamination of the sample by the investigator has not been eliminated, i.e., with an adequate control.

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