

Individual Comparisons of the Levels of (*E*)-3-Methyl-2-Hexenoic Acid, an Axillary Odor-Related Compound, in Japanese

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Abstract

The (*E*)-3-methyl-2-hexenoic acid (E3M2H), an axillary odor-related compound, is known to occur in Caucasians. The aims of this study were to clarify whether E3M2H contributes to axillary odor in Asians and to quantify and compare individual levels of E3M2H. Quantitative determination of E3M2H was performed by means of gas chromatography–mass spectrometry of sweat extracted from the axillary areas of T-shirts worn for 24 h by Japanese subjects. The amount of E3M2H was 15.9–34.6 nmol/ml in six of 30 subjects. Our method succeeded in quantitative analysis of E3M2H from axillary sweat collected individually; we also showed that E3M2H could be detected in Asians. This is the first report in which the amount of E3M2H in axillary sweat was quantified on an individual basis and compared to reveal individual differences. The results of this study indicate that E3M2H might contribute to axillary malodor in Asians as well as Caucasians.

Key words: apocrine gland, axillary odor, body odor, fatty acid, GC/MS, (*E*)-3-methyl-2-hexenoic acid

Introduction

The human axilla is a unique area that produces a characteristic odor, called “axillary odor.” In the axillary region of humans, various compounds are secreted by apocrine, eccrine, apoeccrine, and sebaceous glands. The characteristic odor is generated when microorganisms on the skin surface interact with apocrine secretions (Shehadeh and Kligman, 1963). It has been suggested that the characteristic odor in axillary secretion is due to the volatile steroids (androstenol, androstenone, and androstandienone), having musky and urinary odors, and isovaleric acid, having an acidic odor (Labows, 1982; Gower and Ruparelia, 1993). However, axillary odor is more distinct and pungent, suggesting the presence of other volatile compounds. In the 1990s, Zeng *et al.* (1991) demonstrated that the compounds contributing to the characteristic malodor in human axillary secretions were linear saturated, linear unsaturated, branched saturated, and branched unsaturated C6–C11 acids secreted by the apocrine gland. In particular, (*E*)-3-methyl-2-hexenoic acid (E3M2H), a C7 branched and unsaturated acid was identified as having a strong malodor. They also showed that E3M2H was released upon incubation of the aqueous frac-

tion of apocrine secretions with *Corynebacteria* or hydrolysis with NaOH, indicating that a water-soluble, covalent precursor must be present in apocrine secretions (Zeng *et al.*, 1992). Spielman *et al.* (1995) demonstrated that 3-methyl-2-hexenoic acid (3M2H) was liberated from apocrine secretion odor-binding proteins 1 and 2 (ASOB1 and 2) with apparent molecular masses of 26 and 45 kDa. Then, ASOB2 was identified as apolipoprotein D (apoD), whose glycosylation pattern differs from that of the apoD of serum (Zeng *et al.*, 1996b). On the other hand, a recent study revealed that *N*- α -3-methyl-2-hexenoyl-L-glutamine was contained in axillary secretions as the odorless precursor of E3M2H and that its N-terminal cleavage is mediated by *Corynebacteria* sp. (Natsch *et al.*, 2003). In recent studies, (*S*)-3-hydroxy-3-methylhexanoic acid (Natsch *et al.*, 2003; Hasegawa *et al.*, 2004) and (*S*)-3-methyl-3-sulfanylhexasan-1-ol (Hasegawa *et al.*, 2004) were also identified as contributing to axillary malodor.

E3M2H was first isolated and identified from whole-body sweat as a peculiar odor of Caucasian schizophrenic patients (Smith *et al.*, 1969). However, Gordon *et al.* (1973) showed

that E3M2H was detectable from the sweat of both normal and schizophrenic subjects, indicating there was no special relationship between E3M2H and schizophrenia. In a later study, as mentioned above, E3M2H was identified as an axillary odor-related compound in axillary secretions from Caucasian male subjects who have strong axillary odor.

The number and the degree of the development of apocrine glands vary among ethnic groups as well as individuals (Ikeda *et al.*, 2001); hence, the strength and type of body odor may differ by ethnic group. For instance, the dry/wet cerumen type, which is decided by the development of ceruminous gland (a kind of apocrine gland), varies among several populations (Tomita *et al.*, 2002). So far, various investigations on the chemistry and microbiology related to the axillary odor have been performed, most of them on Caucasians (Russell, 1976; Doty *et al.*, 1978; Leyden *et al.*, 1981; Rennie *et al.*, 1991; Zeng *et al.*, 1991, 1992, 1996a). Asians also have an interest in controlling body odor, and several studies of the surgical treatment of axillary odor (osmidrosis) have been performed on them (Kao *et al.*, 2004; Nagasao *et al.*, 2004; Lee *et al.*, 2005). However, detection of E3M2H in axillary sweat of Asians has only been reported by Hasegawa *et al.* (2004). In addition, individual differences in the level of E3M2H in axillary sweat have not been determined, either for Asians or for Caucasians. In previous studies, Gordon *et al.* (1973) investigated the concentration of E3M2H not in axillary sweat but in whole-body sweat because E3M2H was thought to be a characteristic compound of whole-body sweat in schizophrenics. On the other hand, Zeng *et al.* (1992, 1996a) determined the concentration of E3M2H in the concentrated aqueous fraction of apocrine secretions collected from several donors by intradermal injection of adrenalin. In this study, we have succeeded at extracting lipophilic acidic compounds, such as E3M2H, selectively from axillary sweat using mixed-mode solid-phase extraction cartridges. Further, we have evaluated the concentration of E3M2H in axillary sweat collected individually by gas chromatography-mass spectrometric (GC/MS) analysis.

Materials and methods

Reagents

All reagents and solvents used in this study were research grade and were purchased from WAKO Pure Chemical Industries (Osaka, Japan) or Aldrich (Milwaukee, WI).

Synthesis of E3M2H

The 3M2H, a mixture of E- and Z-isomers, was synthesized as described in previous reports (Wadsworth and Emmons, 1961; Zeng *et al.*, 1991, 1992) with slight modifications. Sodium hydride (1.33 g) was placed in 80 ml of toluene. To this mixture, 12.4 g of triethyl phosphonoacetate was added

dropwise with stirring at room temperature for 30 min. The mixture was stirred at 40°C for 1 h. To the mixture, 4.76 g of 2-pentanone was added dropwise. The mixture was stirred at 40°C for 2 h. Then ice water (70 ml) was added to the reaction mixture. The reaction mixture was extracted with diethylether (3 × 40 ml). After being dried over anhydrous sodium sulfate, the solvent was removed from the extract by a rotary evaporator (Tokyo Rikakikai, Tokyo, Japan). To the residue, potassium hydroxide solution (5.0 g in 60 ml of MeOH/water at 50/50) was added. The mixture was refluxed for 2 h and cooled to room temperature. Then, the mixture was poured into ice water (100 ml) followed by extraction with diethylether (2 × 60 ml). The aqueous layer was acidified with dilute sulfuric acid until it became cloudy and was extracted with *n*-hexane (3 × 100 ml). The extract was dried over anhydrous sodium sulfate and concentrated by rotary evaporator to yield an oily compound.

Finally, E3M2H was isolated to above 99% purity by recrystallization of the (E/Z) mixture with petroleum ether (Costantini *et al.*, 2001). Isolation of the E-isomer of 3M2H was confirmed by the signals of 600-MHz [¹H] nuclear magnetic resonance (JEOL, Tokyo, Japan). The purity of the refined product was determined by GC/MS.

Preparation of stock solution of standard

To make up 50 mM of stock solution, 12.8 mg of E3M2H was dissolved in 2 ml of acetonitrile. To avoid esterification of free fatty acids, no alcohol was used for the preparation of the stock solution. The stock solution was stored at -20°C.

Collection of axillary sweat

Axillary sweat was collected by a simple and noninvasive method as follows: New cotton T-shirts were washed with water in order to remove detergent and starch before use. Healthy donors (*n* = 30, 21 males and 9 females) wore the cotton T-shirt for 24 h without using any deodorant sprays, deodorant soaps, or perfumes. Soaps made from animal fats were also avoided. Sampling took place from 17 February to 29 March 2005 in Chiba, Japan. The averages of the highest and lowest temperatures for these days were 12.9°C and 5.7°C, respectively. An approximately 10 × 10-cm area was cut from each of the two underarm areas of a cotton T-shirt worn by a healthy Japanese donor for 24 h, and an extraction procedure was performed on each using 20 ml of 10% EtOH for 2 h on a rotary shaker (TAITEC, Saitama, Japan). The extracts of both right and left sides were combined, frozen at -80°C, and concentrated by lyophilization. The sample was redissolved in 0.8 ml of distilled water. To check the background presence of E3M2H, the underarm area of a washed clean T-shirt was used as a control and was confirmed as being under the limit of detection (LOD). Written informed consent was obtained from each donor. All procedures involving human subjects were approved by

the Institutional Review Board of the National Research Institute of Police Science.

Collection of whole-body sweat for verification of the analytical methods

For verification of the recovery rate of E3M2H from sweat by solid-phase extraction, whole-body sweat was collected as blank sweat from two healthy donors (one male and one female) as follows: 15 ml polypropylene tubes were applied to the surface of the face, neck, or chest after induction of sweating by physical exercise or sauna. Before sampling, no deodorant sprays, deodorant soaps, or perfumes were used. The collected sweat samples were pooled and stored at -20°C until use.

Hydrolysis and solid-phase extraction of collected sweat

To liberate the bounded E3M2H, 0.7 ml of axillary sweat stain extract was hydrolyzed by 5 M of NaOH at 100°C for 20 min. The sample was reacidified by 5 M of HCl and applied to a mixed-mode solid-phase extraction cartridge (Oasis MAX cartridge, Waters, MA). The cartridge was conditioned with 1 ml each of MeOH and distilled water before use. The cartridge was washed with 50 mM of sodium acetate (pH 7.0) and eluted with 1 ml of MeOH (Elute 1) followed by 0.5 ml of MeOH with 2% formic acid (Elute 2). In this solid-phase extraction procedure, the lipophilic acidic compounds were selectively eluted in Elute 2, which was then subjected to GC/MS.

GC/MS analysis of E3M2H and other compounds extracted from axillary sweat

A model GCMS-QP2010 GC/MS system (Shimadzu, Kyoto, Japan) and DB-5ms column (film thickness $0.25 = \mu\text{m}$, 0.25-mm inner diameter $\times 30\text{ m}$, Agilent Technologies, Tokyo, Japan) was used for GC/MS analysis of E3M2H and other compounds extracted from axillary sweat. The column temperature was programmed as follows: initial hold at 50°C for 1 min, $7^{\circ}\text{C}/\text{min}$ to 190°C , then $3^{\circ}\text{C}/\text{min}$ to 250°C , and a 20-min hold. The electron ionization mode was used for analysis. The ion source temperature was 250°C , and the ion source energy was 70 eV. Detector voltage was 1.0 kV. A prepared sample ($1.0\ \mu\text{l}$) was injected by a splitless injection mode. The total-ion chromatogram was monitored, and the mass spectrum was scanned from m/z 20 to m/z 500. For quantitation, single-ion monitoring was also performed at m/z 128 for the molecular ion of E3M2H. E3M2H in the axillary sweat stain extract was identified by matching the retention time and mass spectrum with those of authentic material.

Recovery rates of E3M2H from sweat by solid-phase extraction

The recovery rates of E3M2H extracted from the whole-body sweat by solid-phase extraction were determined.

The whole-body sweat was used as blank sweat to determine extraction recovery. The level of endogenous 3M2H in blank sweat was confirmed as being under the LOD. The test group was spiked with E3M2H stock solution in 0.5 ml of blank sweat to reach final concentrations of 10 and 100 nmol/ml. On the other hand, the control group was spiked with acetonitrile, a solvent of the stock solution. Then, hydrolysis and solid-phase extraction were performed in both groups as above. For the control group, E3M2H stock solution was spiked in Elute 2 to reach final concentrations of 10 and 100 nmol/ml. Elute 2 of both groups was applied to GC/MS. The recovery rate was calculated as the ratio of the peak area of E3M2H of the sample group to that of the control group using single-ion monitoring at m/z 128.

Quantitation of E3M2H in axillary sweat

Concentration of E3M2H in axillary sweat was calculated with a calibration curve constructed from E3M2H at concentrations of 5, 10, 20, 50, 100, 200, 500, and 1000 μM . A stock solution of E3M2H was diluted and spiked into MeOH with 2% formic acid, an eluate of Elute 2. GC/MS analysis was performed as above. The calibration curve was constructed within a linear range of concentrations of E3M2H. The LOD and limit of quantitation (LOQ) were calculated as having signal-to-noise ratios of above 3 and 10 by five replicate analyses.

Results

GC/MS analysis of E3M2H

The total-ion chromatogram and single-ion monitoring at m/z 128 are shown in Figure 1. The retention time of spiked E3M2H in blank sweat was 7.88 min. Isomers of 3M2H were completely separated by the analytical method established in this study (data not shown). In the single-ion monitoring at m/z 128, the E3M2H peak was independent from peaks of other compounds extracted from sweat samples (data not shown). As shown in Figure 2, the mass spectrum of synthesized E3M2H following electron ionization showed a molecular ion (m/z 128) and a main fragment ion (m/z 113, 100, and 95). This corresponds with results of previous reports (Smith *et al.*, 1969; Zeng *et al.*, 1991). LOD and LOQ were 2 and 5 nmol/ml, respectively, calculated as signal-to-noise ratios of more than 3 and 10 by five replicate analyses.

Solid-phase extraction

As shown in Table 1, the recovery rates of spiked E3M2H in blank sweat by mixed-mode solid-phase extraction with Oasis MAX were $93.6 \pm 7.0\%$ at 10 nmol/ml and $82.4 \pm 4.0\%$ at 100 nmol/ml. The reproducibility of this solid-phase extraction method calculated as a coefficient variation (CV) value was 7.5% at 10 nmol/ml and 4.9% at 100 nmol/ml. The extraction recoveries and the reproducibilities were calculated by five replicate analyses.

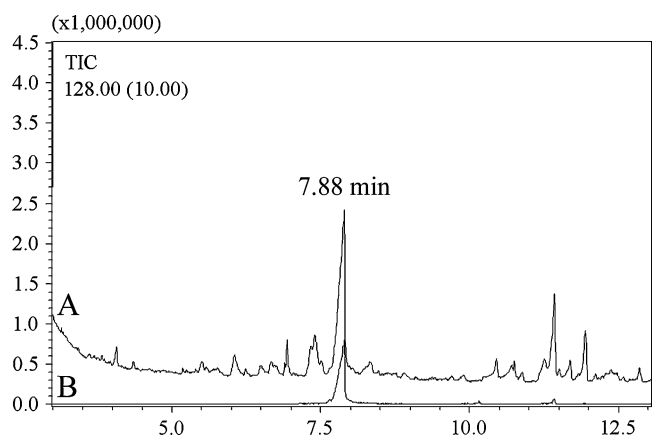


Figure 1 GC/MS analysis of E3M2H-spiked blank sweat. **(A)** Total-ion chromatogram and **(B)** single-ion monitoring at m/z 128 for the molecular ion of E3M2H ($\times 10$ intensity). E3M2H-spiked blank sweat was hydrolyzed and extracted with an Oasis MAX mixed-mode solid-phase extraction cartridge. The retention time of E3M2H was 7.88 min in this analytical method.

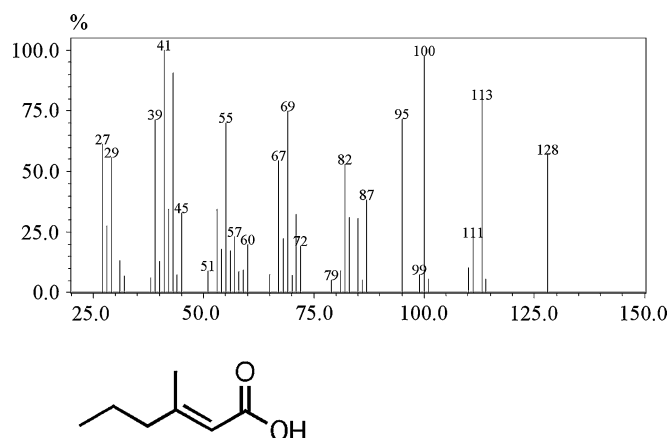


Figure 2 Mass spectrum of synthesized E3M2H-spiked blank sweat.

Quantitative determination of E3M2H in axillary sweat of Japanese subjects

The results of quantitative determination of E3M2H in axillary sweat of Japanese subjects collected for 24 h are summarized in Table 2. Six of 30 samples contained E3M2H at quantitative levels of 15.9–34.6 nmol/ml in redissolved solution after lyophilization. The total-ion chromatogram and single-ion monitoring at m/z 128 of the axillary sweat of a representative of these six subjects (number 30) are shown in Figure 3. Figure 4 shows the mass spectrum of E3M2H, which was identified by matching the retention time and mass spectrum with those of authentic material (Figures 1 and 2). Another seven of 30 samples contained E3M2H at levels that were detectable but below the LOQ (5 nmol/ml). Consequently, the detection rate of E3M2H in axillary

Table 1 Recovery rates and reproducibilities of E3M2H from blank sweat by mix-mode solid-phase extraction

E3M2H (nmol/ml)	Mean (%)	SD (%)	CV (%)
10	93.6	7.0	7.5
100	82.4	4.0	4.9

Data calculated by five replicate analyses.

sweat stains of Japanese subjects was 43.3% (13 of 30 samples) in the analytical method established in this study.

Discussion

Most of the various studies on axillary odor—its chemistry, gender differences, intensity, and related microorganisms—have been performed on Caucasians (Russell, 1976; Doty *et al.*, 1978; Leyden *et al.*, 1981; Rennie *et al.*, 1991; Zeng *et al.*, 1991, 1992, 1996a). In these studies, E3M2H, which is secreted by the apocrine gland, has been identified as an axillary odor-related compound (Zeng *et al.*, 1991, 1992, 1996a). The number and the degree of development of apocrine glands vary among ethnic groups as well as individuals (Ikeda *et al.*, 2001); hence, the strength and type of body odor may differ by ethnic group. So far, detection of E3M2H in axillary sweat of Asians has only been reported by Hasegawa *et al.* (2004). Previous research has also left it unclear whether and to what degree there are individual differences in the levels of E3M2H in axillary sweat, for either Asians or Caucasians. Therefore, in order to clarify whether E3M2H also contributes to axillary malodor in Asians, quantitative determination by means of GC/MS was performed on axillary sweat extracts from the underarm area of T-shirts worn by Japanese subjects for 24 h. The result of this study showed that about half of the axillary sweat samples contained E3M2H at detectable levels. In these samples, six samples (numbers 1, 2, 3, 10, 16, and 30) contained E3M2H at quantifiable levels at high concentrations (15.9–34.6 nmol/ml, Table 2). Thus, it was shown that levels of E3M2H using our methodology differ among individuals from under the LOD to high concentrations.

In the present study, no female samples contained E3M2H at detectable levels, although Zeng *et al.* (1996a) reported that E3M2H was contained in pooled axillary extracts and hydrolyzed apocrine secretions of females as well as males. On the other hand, Leyden *et al.* (1981) demonstrated that the prevalence of lipophilic diphtheroids, which could be related to the typical pungent “apocrine” odor, was lower in female axilla than in male axilla. Although the presence of microorganisms was not determined in this study, the results may support the aforementioned paper.

Although it is difficult to compare our results with concentrations of E3M2H found in previous studies because of differences in sampling and analytical procedures, Gordon *et al.* (1973) determined the concentrations of E3M2H in

Table 2 Quantitative determination of E3M2H in axillary sweat of Japanese subjects

Sample numbers	Sex	E3M2H in Elute 2 (nmol/ml)	E3M2H in redissolved solution (nmol/ml)	E3M2H in axillary sweat stain (nmol)
1	M	22.3	15.9	12.7
2	M	22.4	16.0	12.8
3	M	22.4	16.0	12.8
4	M	<5	<3.57	<2.86
5	M	<5	<3.57	<2.86
6	M	<5	<3.57	<2.86
7	F	ND	ND	ND
8	M	<5	<3.57	<2.86
9	M	ND	ND	ND
10	M	28.8	20.6	16.4
11	M	ND	ND	ND
12	M	ND	ND	ND
13	M	ND	ND	ND
14	M	ND	ND	ND
15	F	ND	ND	ND
16	M	26.5	19.0	15.1
17	M	<5	<3.57	<2.86
18	M	ND	ND	ND
19	M	<5	<3.57	<2.86
20	F	ND	ND	ND
21	F	ND	ND	ND
22	F	ND	ND	ND
23	M	<5	<3.57	<2.86
24	F	ND	ND	ND
25	F	ND	ND	ND
26	F	ND	ND	ND
27	M	ND	ND	ND
28	F	ND	ND	ND
29	M	ND	ND	ND
30	M	48.4	34.6	27.7

M, male; F, female; ND, not detected. LOD was 5 nmol/ml at elute of solid-phase extraction in the analytical method established in this study.

whole-body sweat collected by placing normal or schizophrenic subjects in large plastic bags for 80 min to be <2 to 198 ng/ml. On the other hand, Zeng *et al.* (1992, 1996a) determined the concentrations of E3M2H in the concentrated aqueous fraction of apocrine secretions collected from

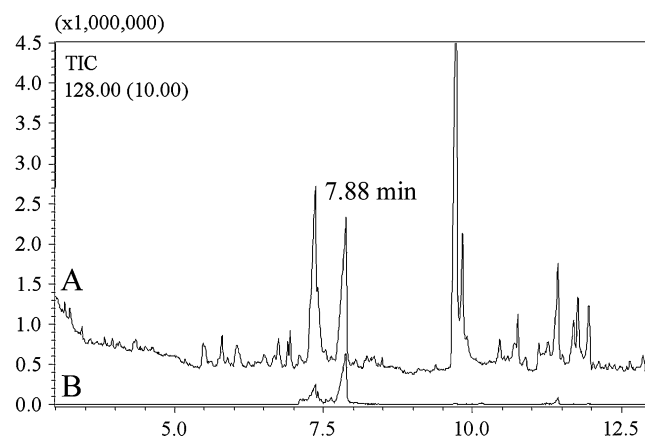


Figure 3 GC/MS analysis of axillary sweat of a Japanese subject (number 30). (A) Total-ion chromatogram and (B) single-ion monitoring at m/z 128 for the molecular ion of E3M2H ($\times 10$ intensity). An approximately 10×10 -cm area was cut from each of the two underarm areas of a cotton T-shirt worn by a healthy Japanese donor for 24 h, and an extraction procedure was performed using 20 ml of 10% EtOH for 2 h on a rotary shaker and concentrated by lyophilization. The sample was redissolved in 0.8 ml of distilled water and extracted with an Oasis MAX mixed-mode solid-phase extraction cartridge. Elute 2 of Oasis MAX containing acidic lipophilic compounds was injected to GC/MS. The analytical conditions of GC/MS are shown in Materials and Methods. E3M2H in axillary sweat was identified by matching the retention time and mass spectrum with those of authentic material.

several male or female donors by intradermal injection of adrenalin to be 50 or 244.7 ng/ μ l, respectively. In this study, we determined the concentration of E3M2H in axillary sweat collected individually from Japanese subjects using a simple and noninvasive method. The amount of E3M2H of axillary sweat was <5 to 34.6 nmol/ml in redissolved solution after lyophilization (Table 2). Total E3M2H in axillary sweat stain was <2.5 to 24.4 nmol. This is the first report in which the amount of E3M2H in axillary sweat from different individuals was compared.

A different result might have been obtained if sampling took place during a different season, such as one accompanied by heat and high humidity, because sweating rates are affected by environmental conditions. While apocrine secretion is thought to be accelerated more by emotional stimulation than by thermal stimulation (Shelley and Hurley, 1953; Ikeda *et al.*, 2001), the release of free E3M2H by microorganisms is believed to be less in cool and dry conditions than in hot and high-humidity conditions. Consequently, the loss of E3M2H by volatilizing caused by microorganisms was suppressed in the cool and dry conditions at which sampling took place in this study.

It is known that short-chain fatty acids, such as E3M2H, are hard to detect because of their high volatility. Iverson and Sheppard (1977) reported that it was difficult to collect the methyl esters of short-chain fatty acids. We also failed to collect the methyl ester of E3M2H standard solution in spite of high concentrations (data not shown). Therefore, methyl

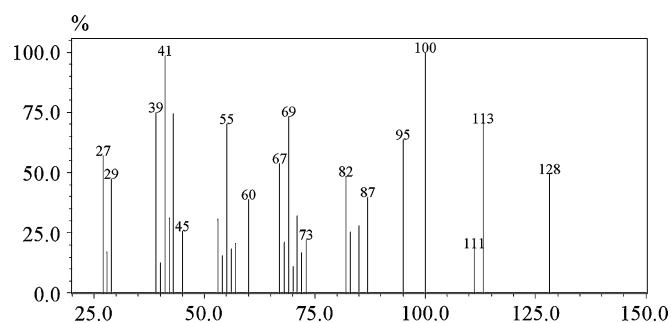


Figure 4 Mass spectrum of E3M2H detected in axillary sweat of a Japanese subject (number 30).

esterification is not suitable for those acids even though methyl esterification is generally used in the analysis of free fatty acids by GC/MS. On the other hand, Gordon *et al.* (1973) applied butyl esterification by BF_3 -butanol for the derivatization of E3M2H. Butyl esters of short-chain fatty acids are less volatile than their methyl esters (Lambert and Moss, 1972; Iverson and Sheppard, 1977). However, it might be difficult to recover the trace amount of the butyl ester of E3M2H from sweat samples because of the complicated method of recovery for the derivatives of BF_3 -butanol. Zeng *et al.* (1991, 1992, 1996a) succeeded in separating and detecting the volatile acid components of axillary secretion without derivatization. Therefore, we also tried to analyze E3M2H without derivatization to avoid the loss of trace amounts of E3M2H.

The results showed that the spiked E3M2H in sweat was extracted selectively at a high recovery rate by Oasis MAX mix-mode solid-phase extraction cartridges. We succeeded in the quantitative analysis of E3M2H from axillary sweat collected individually. E3M2H could be detected in nano-mole quantities in axillary sweat.

In the present study, it has been demonstrated that E3M2H, which is known to be an axillary odor-related compound in Caucasians, was also detected in the axillary sweat of Asians. In addition, our method succeeded in the quantitative analysis of E3M2H from axillary sweat collected individually. E3M2H might contribute to axillary malodor in Asians as well as Caucasians.

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