Original

Investigation of Spectrophotometrically Determined Substances in Yucca Extract by GC/MS, TLC and On-column Injection GC

(Received December 25, 2003)

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Spectrophotometrically determined substances in Yucca extract, listed as "Yucca foam extract" in the "List of Existing Food Additives in Japan", were investigated by GC/MS, TLC and GC. A TLC method using an anisaldehyde color developing reagent similar to that employed in spectrophotometry was established for selective detection of saponin in Yucca extract. Several steroidal saponins were found by GC/MS in the fractions corresponding to spots on the TLC plate, and these were assumed to have contributed to the color development in spectrophotometry. Sarsasapogenin and smilagenin were the dominant saponins. An on-column injection GC method to determine these saponins in Yucca extract was also developed. The sum of these two saponins in Yucca extract was 0.9%. The total amount of saponin estimated by GC was approximately 2%, which was similar to that measured by spectrophotometry.

Key words: Yucca extract; Yucca foam extract; GC; GC/MS; TLC; sarsasapogenin; natural food additive; existing food additive; shelf-life extender

Introduction

Yucca extract (extract of whole plant of *Yucca arborescens* TREL. or *Yucca schidigera* ROEHL ex Ortgies) is listed as "Yucca foam extract" in the "List of Existing Food Additives in Japan", and its major use is as an emulsifier and processing agent¹⁾. Because steroidal saponins in Yucca were reported to exhibit antiyeast or antifungal activities²⁾, Yucca extract has been added to food particularly as a "shelf-life extender" (processing agent) in the Japanese market. Determination of the saponins is important for the quality control of products, because products of poor quality can easily lead to deterioration of food, when used as shelf-life extenders. However, the official method to ensure quality for food additive use ("food additive specification") has yet to be established.

Previously, we developed a spectrophotometric method to determine the total amount of saponins in Yucca extract³⁾. The saponins in Yucca extract obtained by separation with HP-20[®] were hydrolyzed, and the saponins generated were mixed with an anisaldehyde color developing reagent, heating developed a yellowish-green color with the maximum absorbance wavelength at around 430 nm, and the absorbance was measured with a spectrophotometer. One of the drawbacks of spectrophotometry is that the compounds contributing to the absorbance are not known. Hence, identification of substances contributing to the color development would make the spectrophotometric

method more reliable. The purpose of this work was to identify and quantitate the spectrophotometrically determined substances in Yucca extract by means of TLC and GC methods. Substances which contributed to the color development in spectrophotometry were separated and selectively detected on TLC with an anisaldehyde color developing reagent similar to that used in spectrophotometry. Fractions that corresponded to the detected spots were analyzed by GC/MS, and identified compounds were determined by GC.

Materials and Methods

1. Samples

Yucca extract was donated by Maruzen Pharmaceuticals Co., Ltd. (Hiroshima Japan).

2. Standard substances

Sarsasapogenin {(25S)-5 β -spirostan-3 β -ol} and smilagenin {(25R)-5 β -spirostan-3 β -ol}, (minimum, 98%) were from Sigma-Aldrich Japan (Tokyo, Japan).

3. Reagents

Diaion HP-20[®] was obtained from Mitsubishi Chemicals Co., Ltd. (Tokyo, Japan), *p*-anisaldehyde was from Wako Pure Chemical Industries Ltd. (Osaka, Japan), and the solid-phase extraction column Bond Elut[®] (SI, 500 mg) was from Varian Analytical Instruments (California, USA).

4. Instruments

Gas chromatography was performed on a GC-6890 (FID, Agilent Technologies, Palo Alto, USA). GC/MS was conducted with a mass spectrophotometer Auto-mass 50 (EI, JEOL Ltd., Tokyo, Japan), and a gas chromatograph (HP 5890 series II, Agilent Technologies), equipped with an on-column injector (OCI-5, SGE International Pty., Ltd., Ringwood, Australia).

5. TLC analysis

Samples were prepared as described previously³⁾, except that the sapogenin generated was dissolved in 2 mL of ethyl acetate instead of 20 mL. TLC was performed on silica gel 60 (High Performance Thin Layer Chromatography, HPTLC, 10×10 cm, layer thickness 0.2 mm, without fluorescent indicator, Merck KGaA, Darmstadt, Germany). A 1 μ L volume of sample solution was applied, and developed with a hexane–ethyl acetate (2 : 1) mixture.

Coloring reagent was prepared as follows: 0.5 mL of *p*-anisaldehyde was dissolved in 9 mL of ethanol, to which 0.5 mL of sulfuric acid and 0.1 mL of acetic acid were added (anisaldehyde reagent). After drying, the plate was sprayed with the coloring reagent, and heated at 110°C for 10 min.

6. Investigation of color-developing substances

(1) Preparation of sample solutions corresponding to spots on TLC

In order to obtain substances corresponding to each spot on the TLC plate, chromatography was performed on a solid-phase extraction column packed with silica gel (Bond Elut[®] SI, 500 mg). Hydrolyzate of *Yucca* extract prepared by a previously reported method³⁾ was dissolved in a small amount of hexane–ethyl acetate (2 : 1) mixture, and applied to the column. Fractionation was performed with 20 mL of the hexane–ethyl acetate (2 : 1) mixture. Fractions collected every 1 mL were subjected to TLC together with total sample solution (t) (hydrolyzate of *Yucca* extract prepared in a separate experiment), and analyzed by the TLC method described above. Fractions corresponding to the three major spots were combined (fractions a–c). Fractions a–c were divided into two parts, and evaporated to dryness under a stream of nitrogen.

(2) Sample preparation for GC/MS analysis

One part of fractions a–c was dissolved in 1 mL of ethyl acetate (sample solutions a–c). The other was acetylated with 200 μ L of pyridine and acetic anhydride at 40°C for 30 minutes. After evaporation of the reagents under a stream of nitrogen, the residue was dissolved in 1 mL of ethyl acetate (sample solutions a'–c'). Half of the total sample solution (t) was acetylated as well.

(3) GC/MS conditions

A 30 m×0.32 mm i.d. column (BPX 5, film thickness 0.25 μ m, SGE International Pty., Ltd.) was employed for GC/MS. The column oven temperature program was as follows: 40°C (2 min)→(10°C/min)→320°C (30 min). The

column inlet pressure was 30 kPa (helium), the interface temperature was 300°C, the ion source temperature was 210°C, and the scanned mass range was from 40 to 600 amu. Aliquots (1 μ L) of the sample solutions before (a–c and t) and after (a'–c' and t') acetylation were applied to the GC column by on-column injection.

7. GC analysis

(1) Sample preparation for GC analysis

Samples were prepared as described previously³⁾. A portion of the sample solution was taken in a flask, and the ethyl acetate in the solution was removed under reduced pressure. The residue was acetylated in the same manner as the sample solutions used for GC/MS analysis.

(2) Preparation of standard solution for GC

Stock standard solutions of sarsasapogenin and smilagenin were prepared by dissolving 5 mg of the standard substances in 50 mL of ethyl acetate. The stock solutions were diluted and mixed to prepare mixed standard solutions at a concentration of 1–400 μ g/mL. The standard solutions were acetylated in the same manner as sample solutions.

(3) GC conditions

The separation column and the column oven temperature program were the same as for the GC/MS analysis. The column flow was 2.6 mL/min (helium). The detection temperature was 350°C. Aliquots (1 μ L) of the acetylated sample solutions were injected into the GC column by on-column injection.

(4) Determination of sapogenins by GC

The amounts of sarsasapogenin and smilagenin were calculated by using calibration curves obtained with the corresponding standard substances.

Results and Discussion

1. Detection of sapogenin by TLC

Saponins in *Yucca* extract appeared blue on the TLC plate following the treatment with anisaldehyde reagent, probably due to the sugar moiety of the glycoside. However, sarsasapogenin exhibited a yellowish-green color, characteristic of steroidal sapogenins. Hence, TLC of sapogenin was preferred over TLC of saponin for better selectivity to detect effective compounds in *Yucca* extract. Separation of sapogenins with various hexane–ethyl acetate mixtures⁴⁾ as a developing solvent was attempted, and the 2 : 1 mixture was chosen because of the relatively better separation of the three yellowish-green spots. HP-20 treatment was preferable to remove other color-developing substances on the TLC plate, *i.e.*, sterols, and two blue spots (spots e and d in Fig. 1), which were later found to be hydroxyl-methylfurfural and its ethyl adduct, respectively, that may have been formed from sugars during hydrolysis of the *Yucca* extract (data not shown). The detection limit by TLC for standard sarsasapogenin was 50 ng (1 μ L of 50 μ g/mL), corresponding to 0.025% in *Yucca* extract.

Fig. 1. TLC of hydrolyzed Yucca extract

(i): hydrolyzate of Yucca extract without HP-20 purification

(ii): hydrolyzate of Yucca extract purified with HP-20

(iii): standard sarsasapogenin

○: yellowish green, ●: blue

2. Investigation of spectrophotometrically determined substances

Three yellowish-green spots (spots a, b, c in Fig. 1) were observed on the TLC plate of the hydrolyzate of Yucca extract. They were assumed to represent the substances which exhibited a yellowish-green color with a maximum wavelength at around 430 nm in spectrophotometry. Figure 2 shows total and selected ion mass chromatograms for sample solution t' and a'-c' (acetylated fractions corresponding to total hydrolyzate, and spots a-c), obtained as described in the Experimental section. Peaks 1 and 2 corresponded to spot a, 5 and 6 to spot b, and 3 and 4 to spot c. The fragment ion observed at $m/z = 139$ was the base peak in the mass spectra of 6 major and several minor GC peaks for these solutions. Because it is a typical fragment ion of steroidal sapogenin⁵⁾, these peaks were assumed to represent steroidal sapogenins.

Peak 2 was the largest, and was identified as sarsasapogenin. Peak 1 followed, and was identified as smilagenin by comparing the retention times and mass spectra with those of standard substances. Mass spectra of

Fig. 2. Total ion and selected ion chromatograms of acetylated sapogenins in Yucca extract

(t): correspond to total hydrolyzate

(a)-(c): selected ion chromatograms correspond to spots a-c

Peaks 1 and 2: correspond to spot a in Fig. 1.

Peaks 3 and 4: correspond to spot c in Fig. 1.

Peaks 5 and 6: correspond to spot b in Fig. 1.

peak 2 (before and after acetylation) are shown in Fig. 3.

The mass spectra of peaks 5 and 6, and the corresponding peaks before acetylation were similar to those

Fig. 3. Mass spectra of sarsasapogenins in Yucca extract before and after acetylation

(a): before acetylation

(a'): after acetylation

of hecogenin [{(25*R*)-12-oxo-5 α -spirostan-3 β -ol}; molecular ion at m/z =430 before acetylation, and m/z =472 after acetylation), but the retention times were different. Hence, these peaks were assumed to represent substances with structures similar to that of hecogenin. The presence of carbonyl groups in fraction b was confirmed by Fourier transform infrared (FT-IR) spectroscopy (data not shown).

Peaks 3 and 4 have ion peaks at m/z =516. Peaks corresponding to these peaks before acetylation have ion peaks at m/z =432. Steroidal sapogenins with two hydroxyl groups such as markogenin (25*S*)-5 β -spirostan-2 β ,3 β -diol} and samogenin {(25*R*)-5 β -spirostan-2 β ,3 β -diol}, gitogenin {(25*R*)-5 α -spirostan-2 α ,3 β -diol} and neogitogenin {(25*S*)-5 α -spirostan-2 α ,3 β -diol} that were found in the acid-hydrolyzed glycoside fraction of *Yucca schidigera*⁶⁾ would give these ion peaks. Steroidal saponins which have these aglycones were isolated and identified from *Yucca*, as well⁷⁾. Hence, peaks 3 and 4 might represent these sapogenins.

Although further investigation will be required to identify these compounds, these peaks were assumed to represent steroidal sapogenins from the base peak at m/z =139, the similarities of the mass spectra to those of known steroidal sapogenins, and the specific color developed on TLC.

3. Determination of major sapogenins by GC

A GC method was established for the determination of two major sapogenins, sarsasapogenin and smilagenin, which are the aglycones of major antimicrobial saponins⁸⁾.

Although a GC peak of underivatized standard sarsasapogenin was observed, calibration curves were not linear, probably because polar hydroxyl groups in the compound would result in strong adsorption on the inner wall of the glass insert liner, or the surface of the separation column. Hence, derivatization with various reagents was investigated. Acetylation was chosen because the peak of the trifluoroacetate was broad, whereas that of the acetate was sharp.

Injection techniques (splitless injection and on-column injection) and GC columns (0.25, 0.32 and 0.53 mm diameter of BPX5) were next investigated. GC peak areas obtained by on-column injection into a 0.32 and 0.53 mm column were 5 times larger than those obtained by splitless injection into a 0.25 or 0.32 diameter capillary column. However, the separation between neighboring sapogenin peaks with the 0.53 mm column was insufficient. GC peaks with on-column injection into the 0.32 mm column were twice as large as those into the 0.25 mm column. Thus, we chose on-column injection and a 0.32 diameter column.

The calibration curves were linear at 1–400 μ g/mL (R^2 =0.9991), when 1 μ L was injected. Hence the detection limit (referring to the sample) was 10 μ g/g. Recoveries from *Yucca* extract were 95.1–99.8% (n =3).

A typical gas chromatogram of *Yucca* extract is shown in Fig. 4.

Fig. 4. Gas chromatogram of acetylated sapogenins in *Yucca* extract
Peaks: 1, smilagenin; 2, sarsasapogenin

Table 1. Concentrations of Sapogenins in *Yucca* Extract Used as Food Additive

Sample	Sarsasapogenin	Smilagenin	Total sapogenins ^{a)}
1	0.66	0.26	1.89
2	0.58	0.25	1.82
3	0.63	0.19	1.94
4	0.66	0.22	2.03

^{a)} Calculated from the sum of the GC areas of peaks 1–6 in Fig. 4

4. Contents of major sapogenins

Contents of sarsasapogenin and smilagenin in commercial *Yucca* extracts (n =4) were 0.58–0.66% and 0.19–0.26%, respectively (Table 1). The sum of the two compounds was approximately 0.9%. Total steroidal sapogenin amounts in *Yucca* extract were 1.8–2.0%, as estimated by summing the areas of the six GC peaks for which m/z =139 was observed in mass spectra, using a calibration curve for standard sarsasapogenin, on the assumption that the FID response of these compounds was the same as that of sarsasapogenin. The total amounts of steroidal sapogenin estimated by GC in this way (Table 1) were similar to those obtained by spectrophotometry³⁾. The sum of sarsasapogenin (peak 2 in Fig. 2) and its stereoisomer smilagenin (peak 1 in Fig. 2) accounted for nearly half of the steroidal sapogenins, and these two sapogenins were considered to be major sapogenins in *Yucca* extract.

Conclusion

The major spectrophotometrically determined substances in *Yucca* extract were found to be steroidal sapogenins. Sarsasapogenin and smilagenin were the dominant sapogenins, the sum of which in *Yucca* extract was 0.9%. The total amount of sapogenins estimated by GC was approximately 2%, which is similar to that measured by spectrophotometry.

Acknowledgments

We thank Professor Ichiro Kudo and Professor Yoshiteru Ida from Showa University for helpful advice during this work, and Maruzen Pharmaceuticals Co., Ltd. for donating *Yucca* extract.

References

- 1) List of Existing Food Additives (List of food additives from natural origin compiled and published by the Ministry of Health and Welfare on April 16, 1996): The Ministry of Health and Welfare Announcement, No. 120, Tokyo, Japan.
- 2) Tanaka, O., Tamura, Y., Masuda, H., Mizutani, K., Application of saponins in foods and cosmetics: saponins of Mohave Yucca and *Sapindus mukurossi*. Adv. Exp. Med. Biol., 1–11 (1996).
- 3) Uematsu, Y., Hirata, K., Saito, K., Kudo, I., Spectrophotometric determination of saponin in Yucca extract for food additive use. J. AOAC Int., **83**, 1,451–1,454 (2000).
- 4) Blunden, G., Hardman, R., Quantitative estimation of monohydroxy saturated steroidal sapogenins in plant materials by densitometric thin-layer chromatography. J. Chromatogr., **34**, 507–514 (1968).
- 5) Budzikiewicz, H., Djerassi, C., Williams, D. H., Structure elucidation of natural products by mass spectrometry, Volume II: Steroids, terpenoids, sugars, and miscellaneous classes. Holden-Day, Inc., 1964, p. 112–113.
- 6) Kaneda, N., Nakanishi, H., Staba, E. J., Steroidal constituents of *Yucca schidigera* plants and tissue cultures. Phytochemistry, **26**, 1,425–1,429 (1987).
- 7) Miyakoshi, M., Tamura, Y., Masuda, H., Mizutani, K., Tanaka, O., Ikeda, T., Ohtani, K., Kasai, R., Yamasaki, K., Antiyeast steroidal saponins from *Yucca schidigera* (Mohave Yucca), a new anti-food-deteriorating agent. J. Nat. Prod., **63**, 332–338 (2000).
- 8) Killeen, G. F., Madigan, C. A., Connolly, C. R., Walsh, G. A., Clark, C., Hynes, M. J., Timmins, B. F., James, P., Headon, D. R., Power, R. F., Antimicrobial saponins of *Yucca schidigera* and the implications of their *in vitro* properties for their *in vivo* impact. J. Agric. Food Chem., **46**, 3,178–3,186 (1998).