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Characterization of three *Agave* species by gas chromatography and solid-phase microextraction–gas chromatography–mass spectrometry

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Abstract

Steam distillation (SD) extraction–solid-phase microextraction coupled to GC–MS was developed for the determination of terpenes and Bligh–Dyer extraction–derivatization coupled with GC for the determination of fatty acids such as ethyl esters were used. It was found that the three different *Agave* species have the same profile of fatty acids; the quantity of these compounds is different in each *Agave* variety. On the other hand, different terpenes were identified in the three *Agave* plants studied: nine in *A. salmiana*, eight in *A. angustifolia* and 32 in *A. tequilana* Weber var. azul.

Keywords: *Agave* spp.; Terpenes; Fatty acids; Volatile organic compounds

1. Introduction

Agave plants, which are often confused with cacti, belong to the family Agavaceae and are succulent plants with spirally arranged leaves forming a rosette. Some have definite trunks, but more often they are nearly stemless. The leaves are bluish green, over 1 m long in mature plants, and end in a sharp brown thorn. The widespread distribution of some 300 species, combined with the fact that the plants require approximately 8–12 years to mature and hybridize very easily, make the taxonomic and phylogenetic study of the genus *Agave* extremely complicated. The family Agavacea includes 20 genera and nearly 300 species. Of these, around 200 are found in México. Several species are important from an economic point of view. *Agave salmiana* and *A. atrovirensare* are valued for pulque production, *A. potatorum* and *A. angustifolia* for mezcal production and *A. tequilana* Weber var. azul for tequila production [1–4]. Tequila and mezcal are alcoholic beverages which manufacturing process involves harvesting the "piña", the stem of the *Agave* plant with leaves removed, followed by cooking in an oven to convert polysaccharides (inulin) to a mixture principally of fructose and glucose. Sugars are fermented with yeast, typically *Saccharomyces cerevisiae* and the fermented mash is then doubly distilled. The final product is diluted and alcohol content usually in the range of 40–50%. The final product, in both cases, is colorless with different characteristics of aroma and flavor. However, most mezcal use a rudimentary fermentation and distillation process. On the other hand, "pulque" is obtained by fermentation of the juice obtained from several species of *Agave*, by a complex succession of yeast and bacteria that produce ethanol, a diversity of chemical compounds and some polymers that give a sticky consistency to the final product. Pulque is sometimes mixed with fruits or vegetables, but has poor stability as it is neither distilled nor pasteurized.

During this process of production the raw material (e.g. *Agave*) undergoes many chemical and biochemical reactions which give a distilled product rich in different compounds. Their quality is a combination of different factors like *Agave* maturity, cooking, fermentation (yeast), distillation, etc. Therefore, their composition is very complex like in all kind of distilled products. There has been reported different compounds which are part of the aroma and flavor among them alcohols, fatty acids, esters, aldehydes, terpenes, phenols, lactones, sulfur compounds, etc. [5]. The primary

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aroma compounds come from the *Agave* which can undergo chemical transformations or can be modified during the process and the secondary aroma compounds which come from cooking, distillation and maturity process. Since the *Agave* plant is the raw material for the production of these alcoholic beverages which are important from a social and economic point of view, it is necessary to characterize and differentiate the varieties. The objective of this contribution is to characterize the terpenes and fatty acids as part of the chemical characterization of the three *Agave* plants.

2. Experimental

2.1. Reagents

Analytical-grade methylene chloride (99.8%), Methanol (99.9%), Ethanol absolute anhydrous (99.9%), hexane (100%), potassium hydroxide, hydrochloric acid 36.5–38% and sodium sulfate anhydrous were purchased from J.T. Baker, Xalostoc, Edo. de México, México.

Lauric acid (12:0) (99–100%), myristic acid (14:0) (99–100%), pentadecylic acid (15:0) (99%), palmitoleic acid (16:1) (99%), margaric acid (17:0) (99%), stearic acid (18:0) (99%) and oleic acid (18:1) (99%) from Sigma, St. Louis, MO, USA. Tridecylic acid (13:0) (99.5%), palmitic acid (16:0) (97.9%), linoleic acid (18:2) (99%) from Chemical Service, West Chester, PA, USA. Linolenic acid (18:3) (99%) from Alltech–Applied Science Labs., PA, USA. Stock standard solutions of each compound (2000–6000 mg/ml) were prepared in methylene chloride. A mixed standard solution was obtained diluting the stock solutions to a concentration of 200–250 μg/ml.

α-Phellandrene, α-terpinene, *p*-cimene, limonene, linalool, 4-terpineol, α-terpineol, nerol, geraniol, nerolidol and α-bisabolol were supplied by Fluka (Sigma–Aldrich, Buchs, Switzerland). Polydimethylsiloxane–divinylbenzene (PDMS–DVB, 65 μm) fiber was from Supelco (Bellefonte, PA, USA). The fiber was conditioned according to instructions provided by the supplier. Stock solution of selected terpenes was prepared by weighing and dissolving each compound in ethanol. These solutions were stored at 4 °C and used for the preparation of diluted standard solutions in methylene chloride for direct GC–MS injection.

2.2. Samples

Pines of *A. salmiana*, *A. angustifolia* and *A. tequilana* Weber var. azul were obtained from Tecamac, Edo. de México; Oaxaca, México and Tequila, Jalisco, México, respectively.

2.3. Instrumentation

A Hewlett-Packard Model 5890 Series II GC system equipped with a flame-ionization detection (FID) system and cool on-column injector was used for lipid analyses. An Omegawax (30 m × 0.32 mm i.d.) 0.25 μm film thickness (Supelco) column was used. The initial oven temperature was 40 °C for 1 min, then programmed from 40 to 280 °C at 10 °C/min with a final holding time of 17 min. A Hewlett-Packard Model 5890 with an HP 5971 mass selective detector and an HP ChemStation. A ZB-5M (30 m × 0.32 mm i.d.) 0.25 μm film thickness (Zebron Phenomenex, USA) column was used. The gas chromatographic conditions were as follow. The initial oven temperature was 40 °C for 1 min, then programmed from 40 to 210 °C at 5 °C/min, then programmed from 210 to 280 °C at 10 °C/min. The injector temperature was 280 °C, in splitless mode (1 min), and the desorption time for the SPME fiber was 10 min. The MS ionization potential was 70 eV; the ionization current 350 μA, and the ion source and transfer line temperature at 175 °C and 280 °C, respectively. Scan mode (50–550 *m*/*z*) was used for characterization of terpenes.

2.4. Lipid extraction

Lipids were extracted based on Bligh and Dyer's total lipid extraction method [6], with some modifications. The procedure applies to tissues that contain high percentage of water and low concentration of lipid (1% lipid or less). *Agave* tissue contains 83.4% water 50 g of fresh or frozen tissue of *Agave* were homogenized with 52 ml of methylene chloride, 104 ml of methanol (1:2:0.8) and 500 μl of internal standard (16.6 mg/ml) in a Waring Blender during 2 min. Then 52 ml of methylene chloride was added and blended for 30 s and finally 50 ml of water was added and blended again for another 30 s. The final proportion of methylene chloride, methanol and water was 2:2:1.8. These ratios represented

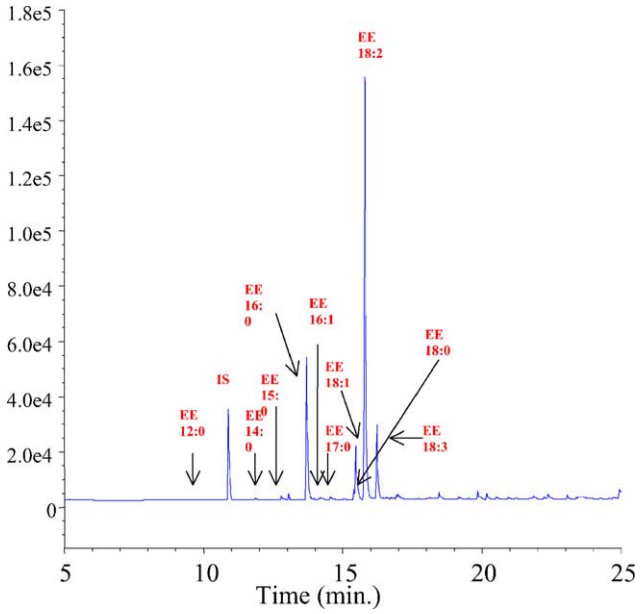

Fig. 1. Chromatograms of the fatty acids analysis of *Agave salmiana*. Chromatographic conditions in the text.

Table 1
Quantitative results of fatty acids analysis

	A. salmiana (μg/g)	R.S.D. (%)	*A. angustifolia* (μg/g)	R.S.D. (%)	*A. tequilana* (μg/g)	R.S.D. (%)
EE12:0	1.7	5.7	2.0	5.1	18.2	4.4
EE14:0	4.2	3.1	3.6	7.0	4.8	7.5
EE15:0	7.8	1.9	5.8	4.0	8.8	5.7
EE16:0	265.0	1.3	217.5	1.4	257.4	6.5
EE16:1	6.3	8.1	3.5	19.9	6.5	7.9
EE17:0	6.4	4.6	5.1	6.2	10.4	7.3
EE18:0	19.9	5.6	23.0	1.4	30.8	6.8
EE18:1	101.2	2.1	68.5	2.2	97.4	7.0
EE18:2	783.7	4.0	421.9	3.4	448.0	6.4
EE18:3	140.5	4.5	50.1	3.9	102.6	5.7
Total	1336.8	–	801.0	–	985.1	–

the total volumes in the ternary systems including the water present in the sample.

The homogenate was filtered through Whatman No. 5 filter paper on a Büchner funnel with slight suction. The lipid withheld in the tissue residue was recovered by blending the residue and filter paper with 50 ml of methylene chloride. The mixture was filtered again through the Büchner funnel and mixed with the original filtrate. The filtrate was transferred to a 500 ml separatory funnel allowing 10 min for complete separation and clarification. The methylene chloride layer was removed. This layer contained the purified lipid and was transferred to a rotating evaporation flask and concentrated at 60 °C to 10 ml final volume of lipid extract.

2.5. Saponification and esterification

A total of 250 μl of lipid extract was dried under N_2 and hydrolyzed with 1.5 ml 10% (in ethanol) KOH at 80 °C during 30 min. Then 1.5 ml HCl 10% (in ethanol) was added and heating at 80 °C during 60 min. After cooling 2 ml of water was added and the solution was extracted twice with 2 ml of hexane. The hexane was washed with 2 ml solution sodium hydrogencarbonate 4% (in water) and washed again with 2 ml of water. The final volume was adjusted with hexane to 5 ml and 1 μl was injected.

One milliliter from mixed standard solution (200–250 μg/ml) was evaporated under steam of nitrogen and esterificated and extracted in the same way as described above.

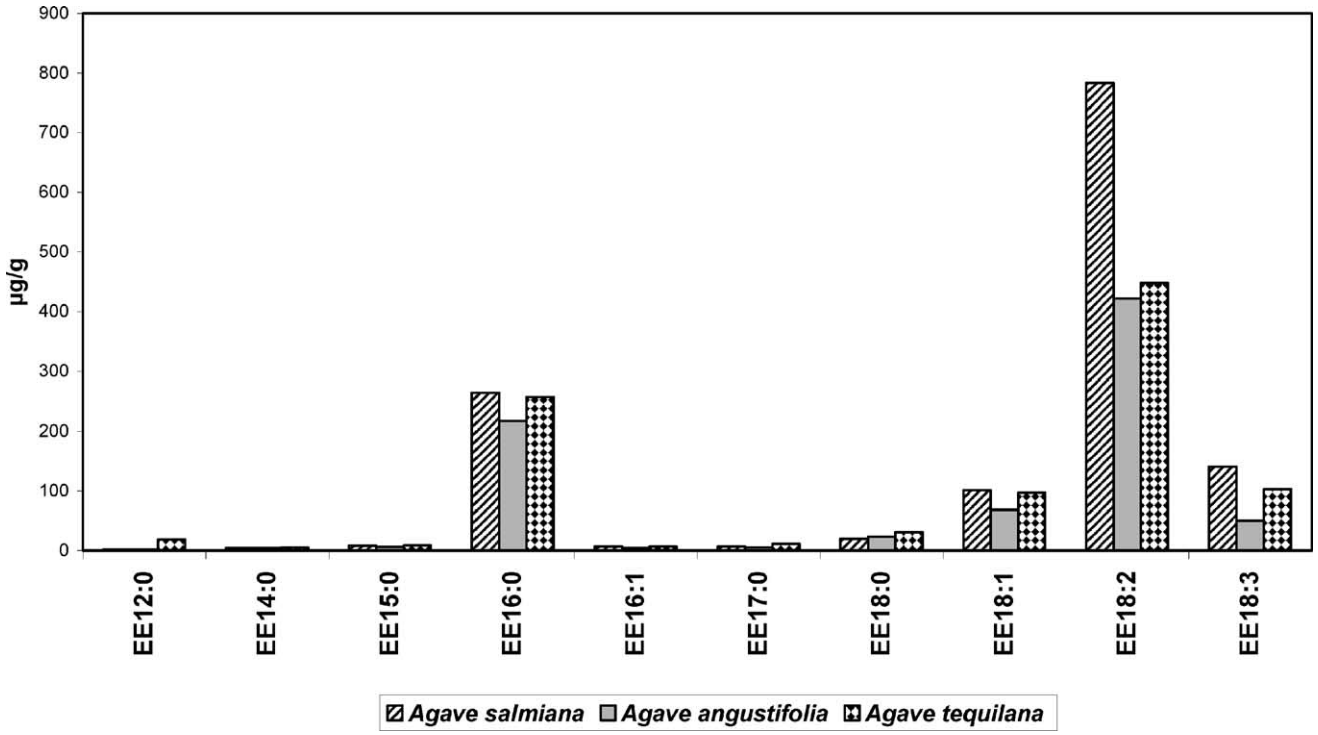

Fig. 2. Distribution of fatty acids ethyl esters of the *Agave* plants.

2.6. Steam distillation (SD)–solid-phase microextraction (SPME) of terpenes

The main parameters that affect the SPME process, such as extraction time profile, desorption time, stirring and salt addition were optimized in a previous work in our laboratory [7]. Before the analysis, 160 g of frozen or fresh *Agave* was transferred to stem distillation equipment and 400 ml were collected. Samples were prepared by placing 5 ml from distilled in a 7 ml vial which was sealed with a PTFE septum; a 10 mm × 3 mm magnetic stirring bar at 1100 rpm was used. Headspace (HS) SPME was performed.

3. Results and discussion

Fatty acids and terpenes are important since normally contribute to characteristic flavor but excessive concentrations of any can lead to off-flavors. For example high concentrations of butyric acid and 3-methyl butyric acid will give cheesy or sickly off-flavor and caproic, caprylic and capric acids all have rancid or goaty flavor characteristics however their esters give fruity notes. Off-flavors due to these acids normally arise from excess formation during fermentation [8]. On the other hand, terpenes possess particularly desirable flavor notes. For instance monoterpenes like β-myrcene, limonene, cymene or some sesquiterpenes as β-murolene and cardinene.

3.1. Lipid analysis

The analysis of lipids had shown the same profile for the *Agave* plants studied as is illustrated in Fig. 1. Ten fatty acids as ethyl esters were identified using external standards and was confirmed by GC–MS: lauric acid ethyl ester (EE12:0), myristic acid ethyl ester (EE14:0), pentadecylic acid ethyl ester (EE15:0), palmitic acid ethyl ester (EE16:0), palmitoleic acid ethyl ester (EE16:1), margaric acid ethyl

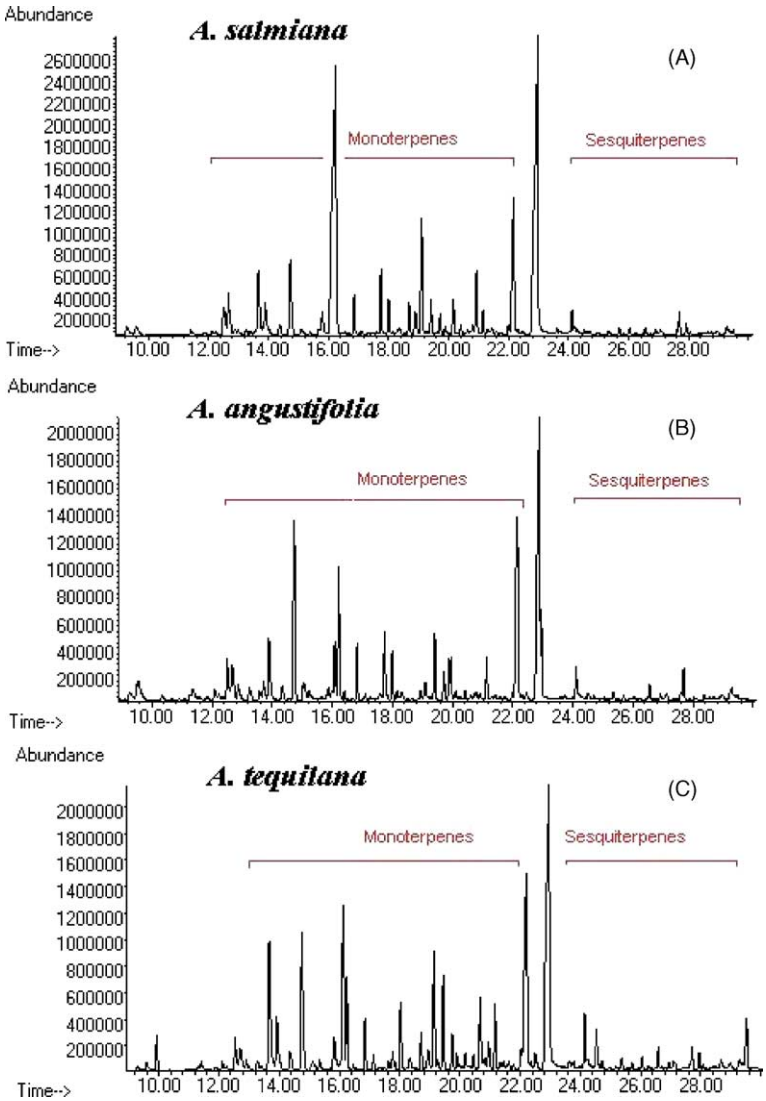

Fig. 3. SD–SPME–GC–MS analysis of terpenes. (A) *A. salmiana*, (B) *A. angustifolia* and (C) *A. tequilana* Weber var.azul.

Table 2
Terpenes identification of *A. salmiana*, *A. angustifolia* and *A. tequilana* Weber var. azul

Compound	*Agave*[a]	t_R (min)	Compound	*Agave*[a]	t_R (min)
α-Phellandrene	1, 3	13.129	Antrastreptene	3	25.090
α-Terpinene	1,3	13.527	Bergamotene	3	26.374
p-Cimene	1, 2, 3	13.786	β-Farnesene	3	26.793
Limonene	1, 2, 3	13.945	Naphtalene 1,2,3,4	3	27.572
β-*trans*-Ocimene	1, 2, 3	14.156	Germacrene	3	27.661
β-*cis*-Ocimene	3	14.508	α-Curcumene	3	27.941
Sabinene (H_2O)	3	14.840	α-Muurolene	3	28.167
Linalool	1, 2, 3	16.236	α-Bisabolene	3	28.279
2,4,6-Octatriene	3	17.571	Cadinene	3	28.770
4-Terpineol	1, 3	18.812	α-Pirovetivene	3	28.863
α-Terpineol	2,3	19.216	Cedrol	3	29.050
Nerol	2, 3	20.282	*trans*-Nerolidol	1, 2, 3	29.624
Bornil formate	3	20.438	Cardelene	3	31.503
Geraniol	1, 2, 3	21.031	Cadinol	3	31.861
α-Cubebene	3	24.644	Patchouli alcohol	3	32.518
Copaene	3	24.827	α-Bisabolol	3	32.745

[a] (1) *A.* salmiana; (2) *A. angustifolia*; (3) *A. tequilana* Weber var. azul.

ester (EE17:0), stearic acid ethyl ester (EE18:0), oleic acid ethyl ester (EE18:1), linoleic acid ethyl ester (EE18:2) and linolenic acid ethyl ester (EE18:3). The quantification was made using response factors of each fatty acid and tridecylic acid as internal standard (IS). The repeatability of the method was <8.0% R.S.D. ($n = 3$) for all the fatty acids (Table 1). As is shown in Fig. 2 and Table 1 Linoleic acid ethyl ester (EE18:2) has the highest concentration in the three *Agave* plants: *A. salmiana* 783.7 μg/g, *A. angustifolia* 421.9 μg/g and *A. tequilana* 405.4 μg/g. However, the highest total concentration of these fatty acids was observed in the *A. salmiana* (1336.8 μg/g). As expected the concentration of unsaturated fatty acids is higher than saturated ones. On the other hand, although the concentration of EE15:0 and EE17:0 found in *Agave* was low they has been reported in tequila [9].

3.2. SD–SPME procedure

The concentration of terpenes in the plant is low therefore it was necessary to select a method for their extraction. Different procedures for the extraction of terpenes from the *Agave* were applied for instance direct extraction, liquid extraction and SD combined with SPME. The SD extraction showed higher content of terpenes hence it was the extraction method selected. The conditions for the SPME analysis were: Headspace analysis, 5 ml sample volume, 20 min equilibrium time (60 °C), 30 min extraction time, 60 °C extraction temperature, 1100 rpm. Salting out did not improve the analyte extraction under optimum conditions.

The chromatogram of terpenes analysis is shown in Fig. 3, as it is observed, many compounds are present, some of them are mainly aldehydes, esters, acids and terpenes. Some terpenes were not well resolved and their concentration is very low which made it difficult to identify them. Therefore the identification was obtained comparing the retention time of standards, Kovats Index and the NIST library of the MS. In the preliminary identification, nine terpenes were identified in *A. salmiana*, eight in *A. angustifolia* and 32 in *A. tequilana* (Table 2) observing that the linalool is the main terpene in the three *Agave* plants. The concentration of some terpenes in *A. salmiana* and *A. angustifolia* is little, therefore, it is not possible to identify them with certainty. More monoterpenes than sesquiterpenes were found in *A. Salmiana* and *A. angustifolia* conversely, more sesquiterpenes than monoterpens in *A. tequilana* were found. On the other hand, the monoterpenes and sesquiterpenes have particularly desirable notes of flavor and pleasant odor that probably contribute to the flavor and aroma of tequila, mezcal and pulque and can be tracers characteristic of the different *Agave* plants under study.

4. Conclusion

The methodology applied for the analysis of fatty acids had <8.0% R.S.D. ($n = 3$). Ten fatty acids were identified and quantified in the three *Agave* plants. Although the profile was the same, Linoleic acid has the higher concentration followed by palmitic acid. Probably some of these fatty acids found for instance in tequila can come from the *Agave* and did not undergo any modification during the cooking, fermentation and distillation process.

The profile of terpenes of the *Agave*s studied was different. In preliminary results, nine terpenes were identified in *A. salmiana*, eight in *A. angustifolia* and 32 in A. *tequilana*. The concentration of terpenes is low which made difficult their identification however, some of them can be tracers characteristic of the several varieties under study. Obviously, not only the content of fatty acids and terpenes in *Agave* is important for the chemical characterization of *Agave* plants nor for the production of tequila, mezcal and pulque but it

is important to know their characterization because they are part of the different compounds groups in the *Agave* plants. This project is not finished yet, we are still characterizing the other classes of compounds (aldehydes, alcohols, furans, etc.) in order to understand the use of the different *Agave* plants in the manufacturing of alcoholic beverages.

Acknowledgements

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