Pulque production from fermented agave sap as a dietary supplement in Prehispanic Mesoamerica

Marisol Correa-Ascencio[a], Ian G. Robertson[b], Oralia Cabrera-Cortés[c], Rubén Cabrera-Castro[d], and Richard P. Evershed[a,1]

[a]Organic Geochemistry Unit, School of Chemistry, University of Bristol, Bristol BS8 1TS, United Kingdom; [b]Department of Anthropology, Stanford University, Stanford, CA 94305-2034; [c]School of Human Evolution and Social Change, Arizona State University, Tempe, AZ 85287-20402; and [d]Zona de Monumentos Arqueológicos de Teotihuacan, Instituto Nacional de Antropología e Historia, Teotihuacan, 55800 Mexico C.P., Mexico

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Although in modern societies fermented beverages are associated with socializing, celebration, and ritual, in ancient times they were also important sources of essential nutrients and potable water. In Mesoamerica, pulque, an alcoholic beverage produced from the fermented sap of several species of maguey plants (*Agavaceae*; Fig. 1) is hypothesized to have been used as a dietary supplement and risk-buffering food in ancient Teotihuacan (150 B.C. to A.D. 650). Although direct archaeological evidence of pulque production is lacking, organic residue analysis of pottery vessels offers a new avenue of investigation. However, the chemical components of alcoholic beverages are water-soluble, greatly limiting their survival over archaeological timescales compared with hydrophobic lipids widely preserved in food residues. Hence, we apply a novel lipid biomarker approach that considers detection of bacteriohopanoids derived from the ethanol-producing bacterium *Zymomonas mobilis* for identifying pulque production/consumption in pottery vessels. Gas chromatography–mass spectrometry selected ion monitoring (*m/z* 191) of lipid extracts of >300 potsherds revealed characteristic bacteriohopanoid distributions in a subset of 14 potsherds. This hopanoid biomarker approach offers a new means of identifying commonly occurring bacterially fermented alcoholic beverages worldwide, including palm wine, beer, cider, perry, and other plant sap- or fruit-derived beverages [Swings J, De Ley J (1977) *Bacteriol Rev* 41(1):1–46].

Mexico | pine resins | hopanes

Teotihuacan, located in the semiarid highlands of Central Mexico, is one of the largest urban centers of prehistory. Founded around 150 B.C., the city grew very rapidly, in part by absorbing a high proportion of the regional population. The city eventually covered around 20 km^2 and may have reached a population level of 100,000 inhabitants. Teotihuacan was the capital of a state that controlled the Basin of Mexico, and probably much of the adjacent parts of the Central Mexican Highlands, and maintained extensive trade relationships that in specific places and times involved military incursion. It survived as an important economic, political, and cultural power in Mesoamerica until roughly A.D. 650 (1, 2).

High altitude, low rainfall, and limited groundwater resources make most of the Teotihuacan Valley a high-risk area for cultivating maize, which was nevertheless a key crop for traditional farmers in the region and was thought to have been important in the diet of residents of ancient Teotihuacan (3–5). Although maize is a food of high caloric value, it contains only low concentrations of several important micronutrients and essential amino acids (e.g., B-complex vitamins, ascorbic acid, iron, calcium, lysine, tryptophan, methionine, and isoleucine) (2, 6). However, a maize-based diet supplemented with beans, which are especially rich in protein, can provide a reasonably balanced diet; although if the components are not consumed in the correct proportions, serious nutritional deficiencies occur (3, 6, 7). Indeed, human osteoarchaeological investigations provide clear evidence of nutritional stress within urban Teotihuacan, especially among lower-status households (8).

At Teotihuacan, mural paintings (depicting maguey plants and scenes of possible pulque consumption) and artifactual remains (amphorae resembling vessels used to contain pulque during the later Aztec period and modern times, and types of stone end-scrapers thought to have been used in maguey sap extraction) have led to the suggestion that pulque might also have been important there (9). The maguey (Fig. 1*A*) withstands frost and drought that seriously affect less-hardy plants such as maize, with its sap (Fig. 1*B*) providing significant calories at times of shortfall in other crops (10). Because of its high viscosity (Fig. 1*C*), conferred by dextran-producing bacteria, consuming pulque at such times would have naturally helped satiate sensations of hunger. Pulque would also have helped alleviate more chronic nutritional deficiencies. Apart from the ethanol content of ca. 4.5% and pH of 3–4, pulque contains concentrations of most macro/micronutrients, which, together with lactic acid bacteria, confer probiotic properties (11–13). In addition, the presence of phytase mediates dephosphorylation of plant phytates (*myo*-inositol hexakisphosphate), an indigestible form of phosphorus occurring in high abundance in grains, thereby increasing the bioavailability of iron and zinc, the most deficient minerals in maize (14). Furthermore, the high concentration of ascorbic acid in pulque enhances the absorption of iron, and possibly zinc, into the digestive system (14).

Despite the significant challenges involved in identifying organic residues from alcoholic beverages in the archaeological record (15), the fermentation process in pulque production offers a potentially specific means of detecting this activity. The major bacterium involved in pulque fermentation is *Zymomonas mobilis*, which, together with yeast, produces alcohol (11, 12, 16). To be able to resist ethanol stress and low pH, *Z. mobilis* has evolved a membrane containing the highest concentration of hopanoids

Significance

This research provides the earliest direct chemical evidence for the production of alcoholic beverage pulque in Mesoamerica, based on organic residues recovered from pottery vessels from Teotihuacan. A novel bacterial lipid biomarker approach is reported, which provides a new means of documenting the consumption of bacterially fermented alcoholic beverages in antiquity worldwide. At Teotihuacan, we have evidence that pulque was stored in distinctive amphorae vessels sealed with pine resin, as well as in other, less specialized vessels. Direct evidence of pulque production provides new insights into how the nutritional requirements of Teotihuacanos were sustained in a region in which the diet was largely based on plants and crop failures, due to drought and frost damage, which resulted in frequent shortfalls in staples.

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[1]To whom correspondence should be addressed. Email: r.p.evershed@bristol.ac.uk.

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Fig. 1. (*A*) Maguey plant (*Agave salmiana*), (*B*) maguey sap (*aguamiel*) pooled within a maguey in the process of sap extraction, and (*C*) man transferring pulque to a 2.5-L container for sale in Apan, Mexico.

currently known in bacteria (i.e., ca. 30 mg g^{-1} dry cell mass), mainly as tetrahydroxybacteriohopane and its ether and glycosidic derivatives (16) (Fig. 2*A*). Hopanoids are extensively used as biomarkers in studies of extinct and extant bacteria in sediments (17–19); however, the use of hopanoids as biomarkers of alcoholic beverage production in archaeology is untested until now.

Materials and Methods

As part of broader paleodietary and cultural investigations at ancient Teotihuacan, organic residue analyses were performed on a wide range of systematically sampled archaeological potsherds from three localities within the city and immediate periphery: La Ventilla ($n = 148$), San José 520 ($n = 73$), and site 15:N1E6 ($n = 92$). Selected sherds dated to the Tlamimilolpa and Xolalpan phases (ca. A.D. 200–550). Absorbed lipids extractions were performed on 313 potsherds of various ware types and forms: ollas ($n = 144$), craters ($n = 83$), bowls ($n = 24$), and amphorae ($n = 62$), utilitarian cooking vessels that are more likely to have been used in the processing of food, and that thus have higher concentrations of organic residues. Briefly, ca. 1–3 g were sampled, and surfaces were cleaned with a modeling drill to remove any exogenous lipids. The potsherds were then ground to powder, an internal standard (*n*-tetratricontane, 2 μg g^{-1}) was added, and acidified methanol solution (5 mL H_2SO_4/MeOH, 2% vol/vol, 70 °C, 1 h) was added (20). The lipids were then extracted from the aqueous phase with hexane (4 × 3 mL). The solvent was evaporated under a gentle stream of nitrogen to obtain the total lipid extract (TLE). Aliquots of the TLE were derivatized using 20 μL *N,O*-bis (trimethylsilyl)trifluoroacetamide, at 70 °C, for 1 h and submitted to gas chromatography (GC) and GC–mass spectrometry (GC-MS) analyses.

For the bacteriohopanepolyols aging experiment, ∼20 g of alumina and crushed replica pot were saturated with ca. 30 mL pulque and then incubated at 100 °C for 2 wk. This experiment was performed in duplicate with experimental blanks of alumina and replica pot. After artificial aging, the lipid extraction was carried out as described earlier.

All TLEs were initially screened in a Hewlett-Packard 5890 Series II GC. Helium was used as carrier gas, and a flame ionization detector was used to monitor column effluent. The data were acquired and analyzed with Clarity software. The diluted TLE samples (1 μL) were injected into a fused silica capillary column (50 m × 0.32 mm i.d.) coated with a dimethyl polysiloxane stationary phase (J&W Scientific; CP-Sil 5 CB, 0.1-μm film thickness). The temperature program was as follows: initial temperature was held at 50 °C for 2 min, followed by an increase to 300 °C (10 min) at a rate of 10 °C min^{-1}. Peaks were identified by comparison of retention times with those of an external standard. Quantification was achieved by the internal standards method.

GC-MS analyses were performed using a ThermoFinnigan trace mass spectrometer. The sample (1 μL) was introduced using a programmed temperature vaporizing injector set to splitless mode onto a polydimethylsiloxane column (Phenomenex, ZB-1, 60 m × 0.32 mm i.d., 0-1 μm film thickness). The MS was operated in electron ionization mode at 70 eV with a GC interface temperature of 300 °C and a source temperature of 200 °C. The emission current was 150 μA, and data acquisition between *m/z* 50–650 at 1.3 scans per second. The temperature program was as follows: initial temperature held at 50 °C for 2 min, followed by an increase to 300 °C (10 min) at a rate of 10 °C min^{-1}. Lipid extracts were analyzed using the MS in total ion current and selected ion monitoring modes acquiring at *m/z* 105, 191, and 523.6 Daltons at 0.12 seconds per scan to check for the presence of ω-(*o*-alkylphenyl)alkanoic acids, hopanes, and *n*-dotriacontanol, respectively. To improve chromatographic peak resolution in resin and hopane-containing extracts and pulque aging, experiment samples were rerun using the following temperature program: initial temperature 50 °C for 2 min, followed by an increase to 270 °C (10 min) at a rate of 10 °C min^{-1}, and then from 270 °C to 300 °C at 6 °C min^{-1} (10 min). The MS was operated in total ion current and selected ion monitoring modes acquiring at *m/z* 191 Daltons at 0.12 seconds per scan. The acquisition and analysis of the data were carried out using XCalibur software.

Principal components analysis was used to investigate patterns in the variability of different biomarkers present in the lipid extracts recovered from the archaeological potsherds from Teotihuacan. The input variables are based on the presence and absence of peaks indicative of 75 biomarkers. XLSTAT 2014.3.01 was used for the analysis.

Results and Discussion

GC screening of the total lipid extracts showed that ca. 70% of the potsherds contained detectable lipids, although concentrations were low (mean, 16 μg g^{-1} for sherds containing lipids; maximum, 53 μg g^{-1}). The low lipid concentrations reflect the paucity of animal products and reliance on plants in the diet. Lipids were observed in 63% of the ollas, 78% of the craters, 85% of the bowls, and 65% of the amphorae.

GC-MS of lipid extracts revealed that these potsherds included mainly fatty acid, *n*-alkanes, and *n*-alkanols. The distributions of long-chain fatty acids ($C_{14:0}$–$C_{34:0}$), *n*-alkanes ($C_{23:0}$–$C_{33:0}$), and *n*-alkanols ($C_{16:0}$–$C_{34:0}$) confirmed an origin in plant oils or waxes (15) (Fig. 3*A*). In most extracts of this type, the biomarker of maize (C_{32} long-chain *n*-alkanol) was identified (21), which is consistent with the paleobotanical record of the region (3–5).

Significantly, an unusual lipid distribution (Fig. 3*B*) was observed in 14 potsherds from La Ventilla (a locality containing both high- and low-status residential contexts) (22), 10 of which were from amphorae and suspected for formal reasons to have been used in pulque production and/or transportation (Table 1). The main components of these potsherds displayed base peaks and molecular ions ($M^{+\cdot}$) at *m/z* 73, 374 ($C_{23}H_{38}O_2Si$); 241, 374 ($C_{23}H_{38}O_2Si$); 241, 374 ($C_{23}H_{38}O_2Si$); 239, 372 ($C_{23}H_{36}O_2Si$); 256, 374 ($C_{23}H_{38}O_2Si$); and 253, 386 ($C_{23}H_{34}O_3Si$), respectively. Comparisons with reference mass spectra indicated they were diterpenoid compounds, the major constituents and transformation products of coniferous resins (23): pimaric, isopiramric, palustric, dehydroabietic, abietic, and 7-oxodehyrdoabietic

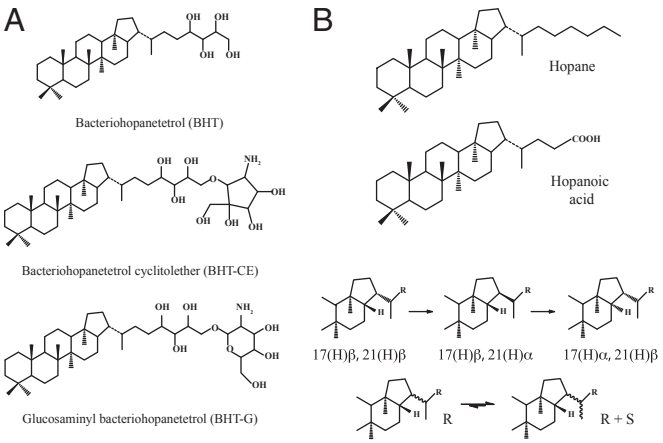

Fig. 2. (*A*) Major complex hopanoids in ethanol producer bacterium *Z. mobilis* (16). (*B*) Possible diagenetic products and transformations of complex hopanoids (17).

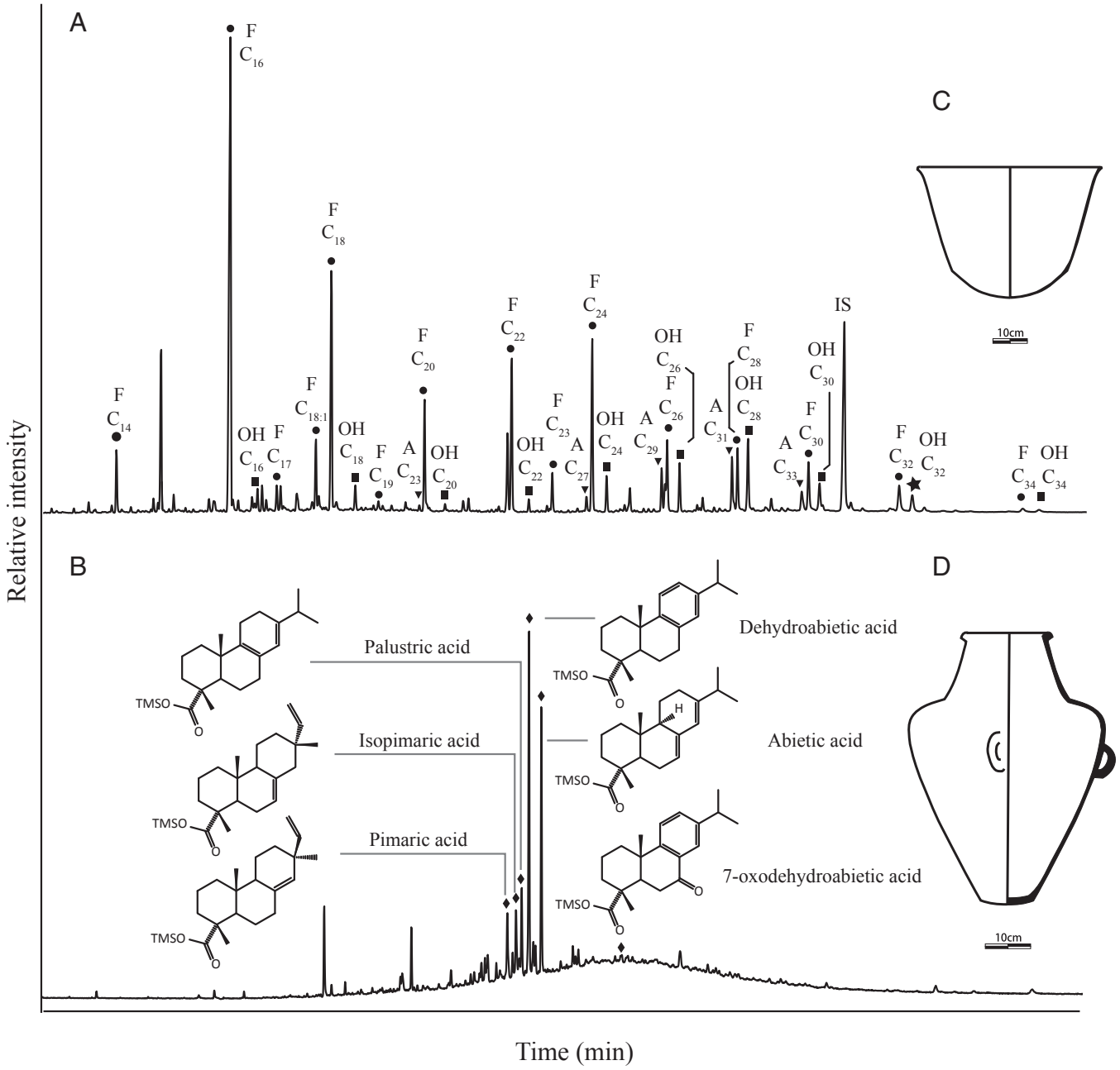

Fig. 3. Partial gas chromatograms of total lipid extracts from pottery from the locality of La Ventilla. (*A*) Crater: characteristic distribution of plant waxes (LV-058). (*B*) Amphora: abietic acid derivatives (LV-014). Drawings of representative (*C*) crater and (*D*) amphora vessels. A, *n*-alkane; F, fatty acid methyl ester; OH, *n*-alkanol; OH C_{32}, biomarker of maize (21); IS, internal standard. Lipid extracts were run in different GC temperature programs to improve chromatographic peak resolution.

acids [as trimethylsilyl (TMS) derivatives; Fig. 3*B*]. Archaeological and ethnographic examples of postfiring treatment of pottery vessels with tree resins have been associated with waterproofing unglazed ceramic vessels to improve storage of liquids (24). The finding of coniferous resin only in these potsherds (despite intensive searching of the extracts in the entire assemblage) pointed to a specialized function. The low abundance of aromatic diterpenoid components suggests the resin was applied in a relatively fresh form, rather than being a pyrolytic product. The latter also rules out an origin for the diterpenoids in wood smoke condensate from heating over a fire (23, 25, 26).

Further inspection of the full-scan GC-MS data from the lipid extracts of potsherds containing the coniferous resin biomarker derivatives revealed a further series of compounds characterized by a base peak at *m/z* 191 and molecular ions ($M^{+\cdot}$): 370 ($C_{27}H_{46}$), 398 ($C_{29}H_{50}$), 412 ($C_{30}H_{52}$), 426 ($C_{31}H_{54}$), 440 ($C_{32}H_{56}$), 454 ($C_{33}H_{58}$), and 468 ($C_{34}H_{60}$) corresponding to hopanes 17(H)α,21(H)β C_{27}, C_{29}, C_{30}, C_{31}(S,R), C_{32}(S,R), C_{33}(S,R), and C_{34}(S,R), respectively (17) (Fig. 4*A*). Remarkably, these hopanes were only detectable in potsherds from resin-containing vessels, despite extensive searches, including using GC-MS-selected ion monitoring (*m/z* 191), which increases detection limits by several orders of magnitude. The absence of these characteristic hopanoids biomarkers from the vast majority of the assemblage, and their co-occurrence specifically in the resin-treated potsherds, excludes exogenous, pre- or post-excavation, contamination as a possible source.

Table 1. Subset of potsherds shown by GC-MS and GC-MS selected ion monitoring to contain diterpenoids diagnostic of pine resin and bacteriohopane proxies of *Z. mobilis*

Sample	Phase	Ware	Form	Vessel part	Provenience	Lipid concentration, μg g^{-1}
LV-001	Tlamimilolpa	Granular	Amphora	Neck	Frente 5	21
LV-002	Tlamimilolpa	Granular	Amphora	Neck	Frente 5	17
LV-003	Tlamimilolpa	Granular	Amphora	Body	Frente 5	12
LV-009	Tlamimilolpa	Granular	Amphora	Body	Frente 5	9
LV-010	Tlamimilolpa	Granular	Amphora	Body	Frente 5	6
LV-011	Tlamimilolpa	Granular	Amphora	Body	Frente 5	17
LV-012	Tlamimilolpa	Granular	Amphora	Body	Frente 5	11
LV-013	Tlamimilolpa	Burnished	Olla	Body	Frente 5	14
LV-014	Tlamimilolpa	Granular	Amphora	Neck	Frente 5	22
LV-020	Tlamimilolpa	Burnished	Olla	Body	Frente 5	30
LV-082	Xolalpan	SMO*	Amphora	Body	Frente 5	10
LV-100	Xolalpan	SMO	Amphora	Body/base	Frente 5	14
LV-138	Tlamimilolpa	Burnished	Olla	Neck/body	Southern Area, PGC†	7
LV-150	Tlamimilolpa	Burnished	Olla	—	Southern Area, PGC	17

All 14 cases come from La Ventilla excavation materials.
*San Martín Orange.
†Patio of the Glyphs Compound.

Principal components analysis was carried out to characterize major patterning in the occurrence of biomarkers within the entire pottery assemblage. Fig. 4 shows the projection of the data onto the first two principal components, which capture just over 40% of the variance in the original data. Two distinct sets of scores on principal component 2 (PC2) divide the pottery into well-separated groups. Group 4 (in the upper part of the plot) is composed entirely of the 14 sherds containing abietic acid derivatives and hopanes. The rest of the samples show roughly the same range of scores on PC2 but can be subdivided into three less salient groups by scores on PC1. Group 1 (right) corresponds to potsherds showing the most complex lipid distributions, dominated by fatty acids, *n*-alkanols, *n*-alkanes, hydroxyacids, campestanol, and/or sitostanol. Group 2 (middle) is formed by samples showing fatty acids, *n*-alkanols, hydroxyacids, plant sterols, plant triterpenoids, and/or ω-(*o*-alkylphenyl)alkanoic acids; the lipid distributions of these potsherds are similar to, but simpler than, those in Group 1. Finally, Group 3 (left) corresponds to pottery samples with no appreciable lipids. In summary, PC1 is a dimension that reflects variation in the complexity of lipid composition profiles, whereas PC2 simply isolates cases associated with residues of tree resin and fermentation-derived hopanes from all others.

The detection of the hopanoid biomarker proxies of *Z. mobilis* in vessels likely used to contain pulque is consistent with the complex biohopanoids it produces, having been transformed during vessel use and burial via microbial action, or possibly abiologically, via reactions catalyzed by clay minerals and possibly involving heating in the past (27). The principal transformations of the complex biohopanoids include defunctionalization and isomerization (17, 18, 27). Such reactions give rise to C_{32} hopanoic acid or the parent hydrocarbons, hopanes (up to C_{35}; Fig. 2*B*) (17, 27). In early diagenesis, the biological conformation 17(H)β,21(H)β generally remains, subsequently isomerizing to the thermodynamically more stable 17(H)α,21(H)β configuration; changes in stereochemistry at C-22(R) can also occur, producing a mixture of C-22(R) and 22(S) isomers (17, 18, 27) (Fig. 2*B*). In general, these processes are thought to occur over extended periods; however, laboratory experiments on steroids and studies involving recent sediments suggest the latter transformations can occur more rapidly, being microbially mediated or acid-catalyzed (18, 19, 27).

Although the finding of hopanes is entirely consistent with those from sedimentary materials, we performed an artificial diagenetic (aging) experiment by saturating alumina and replica pottery fabric with pulque and incubating at 100 °C for 2 wk. The aim was to assess whether the metals present in the ceramic fabric (e.g., aluminum) were able to mediate the proposed transformations, specifically the dehydration of the bacteriohopanpolyols, a commonly used strategy in organic synthesis (28). After artificial aging, the hydrocarbon fraction was extracted and assessed by GC-MS. The distribution obtained showed a remarkable congruence with the hopane distributions observed in the archaeological potsherds (Fig. 5 *A–D*), confirming the ceramic matrix is able to catalyze the diagenetic transformations of bacteriohopanoids during burial. As we have seen with other lipid transformations, heating of pulque vessels during their use could conceivably contribute to the formation of the hopanes observed in the archaeological pottery. The Franciscan friar Sahagún records boiling as part of pulque production during the Aztec period (29), and various early colonial accounts mention a kind of pulque made by fermenting maguey syrup, a concentrate made by boiling ordinary maguey sap (30). Depending on when it was carried out, however, boiling would seem to hinder normal fermentation and is not part of

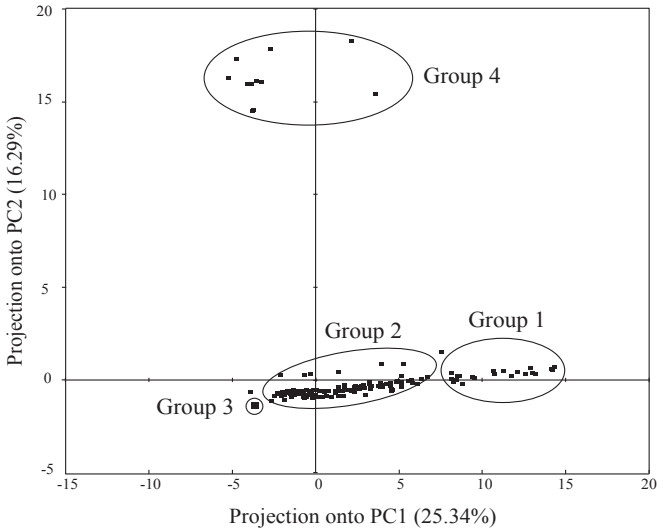

Fig. 4. Biomarker distributions observed in potsherds from Teotihuacan, projected as scores on the first two components from principal components analysis.

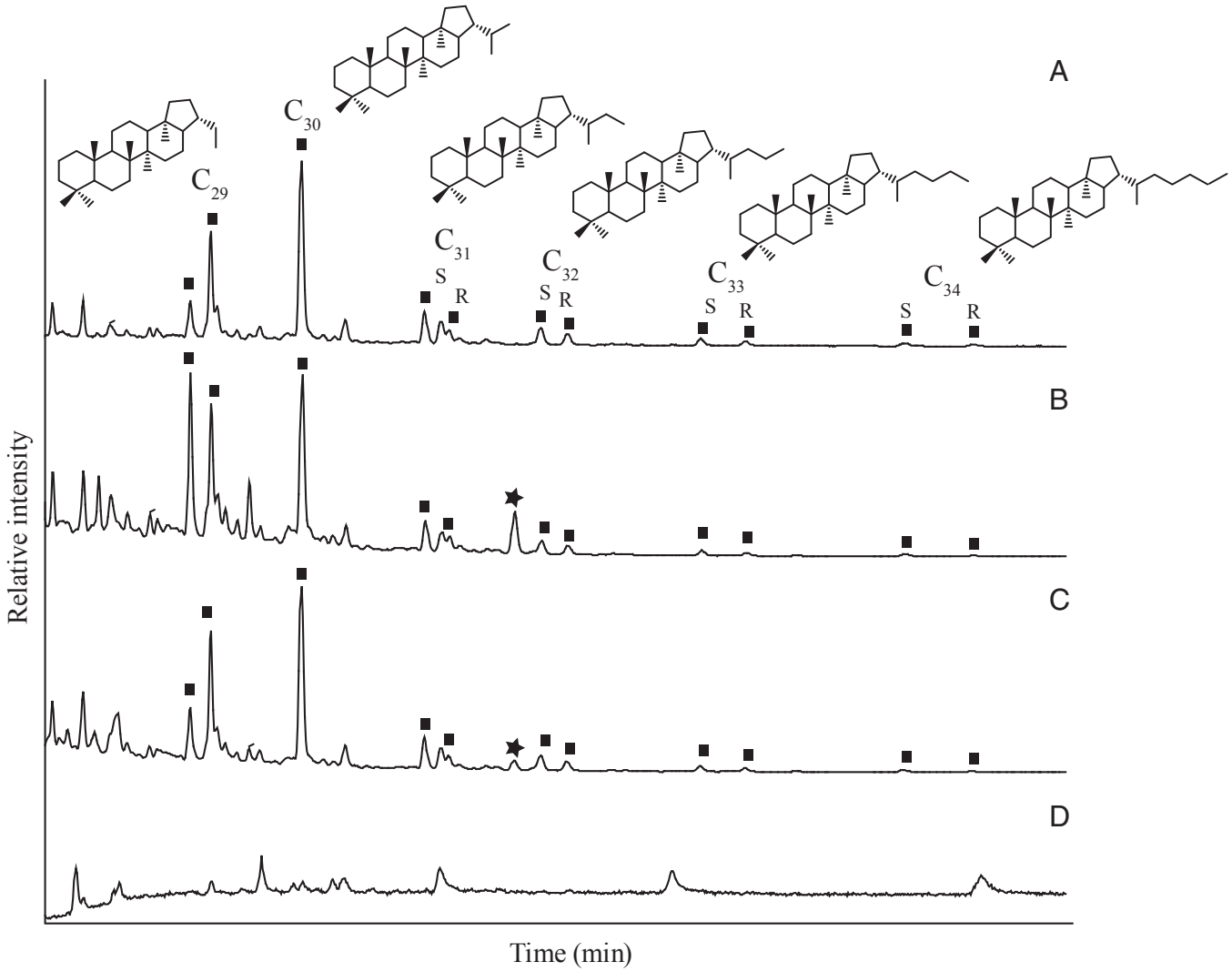

Fig. 5. GC-MS selected ion monitoring (*m/z* 191) showing hopane distributions (C_{29}–C_{34}) in (*A*) amphora from La Ventilla (LV-100), (*B*) replica pot and (*C*) alumina saturated with pulque after incubation, and (*D*) alumina (blank). Chromatographic peak denoted by a filled star corresponds to diploptene, another bacteriohopanoid present in pulque; however, it is highly susceptible to degradation and is thus absent in the archaeological amphorae.

the process recorded in major ethnographies describing pulque making in modern Central Mexico (31, 32).

These findings provide compelling evidence for the use of ceramic vessels to contain pulque in the locality of La Ventilla around A.D. 200–550, at the height of Teotihuacan's growth and power (1). The vessel forms from which as "hopane-bearing" potsherds derived, that is, amphorae and ollas, would have been ideal for processing/storing liquids, with the presence of pine resin being entirely consistent with postfiring waterproofing. Given the presence of hopanes, bacterial markers of pulque, exclusively in this group of vessels, there is little doubt that pulque was produced at Teotihuacan. Only a more comprehensive survey of absorbed organic residues of pottery samples, coupled with systematic analysis of the spatial and temporal distribution of relevant artifact classes (particularly specialized ceramic vessels such as amphorae and stone tools used in sap extraction) will ultimately establish the level and intensity of pulque production within the ancient city. Notwithstanding this, our results are a critical first step, providing the first direct chemical evidence to the authors' knowledge for the production of an alcoholic beverage in Prehispanic Mesoamerica and a foundation for fresh insights into how the nutritional needs of a major prehistoric city were met in an environmental zone in which critical shortfalls in other food crops were probably all too common.

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