Cacao usage by the earliest Maya civilization

Foaming chocolate prepared in spouted vessels made a delectable Preclassic drink.

The Maya archaeological site at Colha in northern Belize, Central America, has yielded several spouted ceramic vessels that contain residues from the preparation of food and beverages. Here we analyse dry residue samples by using high-performance liquid chromatography coupled to atmospheric-pressure chemical-ionization mass spectrometry, and show that chocolate (*Theobroma cacao*) was consumed by the Preclassic Maya as early as 600 BC, pushing back the earliest chemical evidence of cacao use by some 1,000 years. Our application of this new and highly sensitive analytical technique could be extended to the identification of other ancient foods and beverages.

The site at Colha is known for its specialized production of lithic tools[1] and for its collection of intact, spouted vessels[2], which were manufactured only during the Preclassic period (900 BC to AD 250)[3]. These have generally been recovered from burial sites associated with elite individuals and are relatively rare, typically being found with other serving vessels such as bowls, dishes and plates, and were probably used to dispense liquid from the spout, in much the same way as a teapot.

Based on epigraphic analysis of vessels dating from the Classic (AD 250–900) period and documents written at the time of the Spanish Conquest, liquid chocolate was frothed to produce a foam — considered by the Maya and the Aztecs to be the most desirable part of the drink[4,5] — by pouring the liquid from one vessel into another[4]. In the earlier Preclassic spouted jars (Fig. 1), frothing would also have been accomplished by introducing air through the spout, enabling the vessel to be used for preparing, as well as pouring, liquid chocolate.

At the time of the Spanish Conquest, chocolate was consumed with most meals and was usually mixed with another ingredient (for example, water, maize, chilli and/or honey) and in different proportions to produce a variety of drinks[5]. Our aim, however, was to confirm the existence of cacao residues in spouted vessels, rather than to investigate the presence of other components.

We used high-performance liquid chromatography (HPLC) coupled to atmospheric-pressure chemical-ionization mass spectrometry (APCI MS) to analyse each of the samples collected from the 14 vessels recovered from a series of burials at Colha[6,7]. All of these vessels date to between 600 BC and AD 250 (ref. 2). The procedure involved withdrawing about 500 mg from each sample vial, and adding 3 ml distilled water at 80 °C to solubilize the materials. Before analysis, we passed each sample through membrane filters to eliminate particulate matter.

Cacao has a unique chemical composition of over 500 different compounds, including members of the methylxanthine class (primarily theobromine, with a lower concentration of caffeine). As *T. cacao* is the only Mesoamerican plant that contains theobromine as the primary methylxanthine[8], this compound can be used as a marker for the presence of cacao. For example, HPLC coupled to thermospray mass spectroscopy has revealed cacao residues in ceramic vessels found in an Early Classic (AD 460–480) tomb at the Maya site of Rio Azul in northeastern Guatemala[8–10].

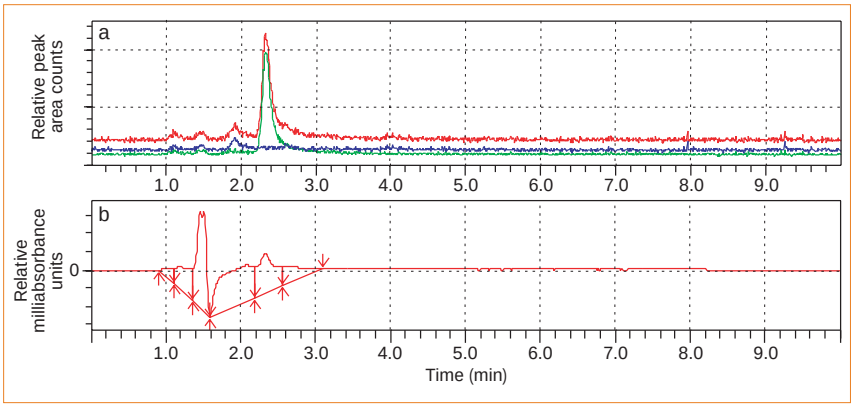

Figure 2 Chromatographic analysis of residue from Maya cooking vessels. **a,** Mass-spectroscopy chromatogram of dry extract from vessel 13. Red, total-ion chromatogram; green, selected-ion monitoring (SIM) at $m/z = 181$ for theobromine; blue, SIM at $m/z = 195$ for caffeine. For the mass spectrum of the peak at 2.6 min, as well as details of methods and standards, see supplementary information. **b,** Ultraviolet chromatogram of the same extract, showing a peak at 2.6 min, which is consistent with results in **a**. Dashed lines are integration markers; arrows indicate the starts and ends of peaks. The peak at around 1.5 min is due to the chromatography solvent. The peak for caffeine is not evident as its concentration in cocoa is about 10 times lower than that of theobromine.

Figure 1 Early Maya use of cacao (*Theobroma cacao*): spouted vessel no. 13, which was found to contain cocoa residue. This vessel is one of 14 excavated from Colha in northern Belize, dating to between 600 BC and AD 250.

For APCI MS, the probe was operated in positive-ion mode to monitor for peaks at $m/z = 181$ (theobromine) and $m/z = 195$ (caffeine), with the ultraviolet detector set at 270 nm. The results of the HPLC MS confirmed the existence of theobromine in 3 of the 14 samples analysed. Peaks from the extract of the residue from vessel 13 (Fig. 1) were evident in the total-ion and ultraviolet chromatograms and the selected-ion-monitoring trace at $m/z = 181$ (Fig. 2; for peak calibration for standards, see supplementary information) and was confirmed by the fact that the mass spectrum (see supplementary information) and ultraviolet chromatogram of this peak show the same retention time as theobromine (Fig. 2b).

To our knowledge, this is the first time that this new technique has been used to analyse dry residues from the interior surfaces of prehistoric pottery. The presence of cacao in Maya spouted vessels at Colha indicates that its usage pre-dates evidence from Rio Azul by almost a millennium. We now know that the Maya had a long, continuous history of preparing and consuming liquid chocolate from the Preclassic period through to the Spanish Conquest. Cacao wood charcoal dating to the same period has been found at several sites in the region[11], further supporting the idea that cacao drinking has its roots in the Preclassic, and indicating that this part of northern Belize may have been one of the main production areas for cacao during this period.

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Cell biology

Targeted transfection by femtosecond laser

The challenge for successful delivery of foreign DNA into cells *in vitro*, a key technique in cell and molecular biology with important biomedical implications, is to improve transfection efficiency while leaving the cell's architecture intact. Here we show that a variety of mammalian cells can be directly transfected with DNA without perturbing their structure by first creating a tiny, localized perforation in the membrane using ultrashort (femtosecond), high-intensity, near-infrared laser pulses. Not only does this superior optical technique give high transfection efficiency and cell survival, but it also allows simultaneous evaluation of the integration and expression of the introduced gene.

Previous techniques that have been developed for transfection of cells with DNA[1] include carrier-mediated transfer[2] and transfer by plasma-membrane permeabilization[3], as well as direct transfer[4], but the efficiency of targeted DNA delivery by these methods may not be optimal. Moreover, none allows contact-free, non-disruptive, stable transfection of individual cells and concomitant evaluation *in situ* of transgene expression.

We directed a high-intensity (10^{12} W cm^{-2}), near-infrared, femtosecond-pulsed laser beam (wavelength, 800 nm) from an 80-MHz titanium–sapphire laser, with a mean power of 50–100 mW and tightly focused using a high-numerical-aperture objective, at a sub-femtolitre focal volume at the cell membrane. This resulted in the formation of a single, site-specific, transient perforation in the cell membrane through which DNA could enter. This mode of targeted transfection differs from the less precise nanosecond-pulsed, ultraviolet (355 nm) lasers used previously[5] and which were found to disrupt cellular integrity[6].

Using Chinese hamster ovarian (CHO) and rat–kangaroo kidney epithelial (PtK2) cells, we studied the process of transfection mediated by intense near-infrared femtosecond laser pulses. Cells were suspended inside a sterile miniaturized cell chamber in 0.5 ml culture medium containing 0.2 μg plasmid DNA vector pEGFP-N1 (4.7 kilobases) encoding enhanced green fluorescent protein (EGFP)[7]. Transmission images of cells were obtained at low power (<5 μW), and the near-infrared laser beam was then focused (under the same microscope) on the edge of the membrane of a target cell, which was exposed to an enhanced mean laser power of 50–100 mW for 16 ms so that transfection could occur. More than 200 cells of each type were targeted in each of 18 replicate experiments; it took 10–15 s to prepare for the transfection of each cell.

We assessed the integration and expression efficiency of the EGFP gene *in situ* by time-lapse two-photon fluorescence imaging[8] at a mean laser power of <1 mW over a period of 72 h, as well as by two-photon fluorescence-lifetime imaging (TPFLIM)[9]. Figure 1 shows that diffraction-limited focusing of intense femtosecond near-infrared laser pulses selectively facilitates transfection of the target cells, but not of the adjacent cells. Expression of EGFP in the transfected cell is also demonstrated by TPFLIM, and the measured fluorescence lifetime of about 2.4 ns is consistent with that reported for mammalian cells expressing EGFP[10].

Irrespective of cell type, the transfection achieved by this technique was invariably 100%. This high level of selective and total transfection, without any detrimental effects on growth and division, and with virtually no cell death or sign of apoptosis, together with the ability to determine expression by fluorescence-intensity imaging and TPFLIM with the same microscope, demonstrate the potential of this non-disruptive technique in transfection and expression studies. The ability to transfer foreign DNA safely and efficiently into specific cell types (including stem cells) — circumventing the need for mechanical, electrical or chemical means — will be an encouraging advance for a range of ventures, including targeted gene therapy and DNA vaccination.

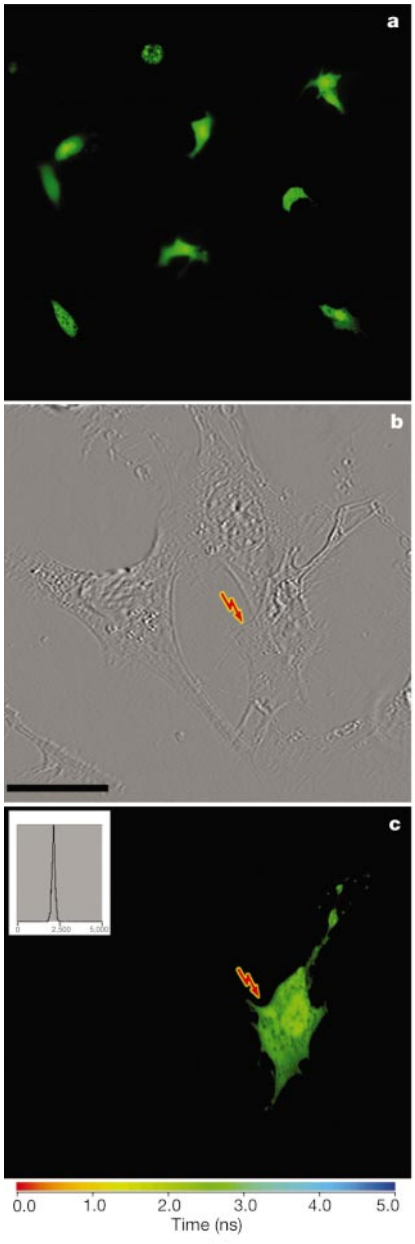

Figure 1 Analysis of the targeted transfection of Chinese hamster ovarian (CHO) cells with a plasmid encoding enhanced green fluorescent protein (EGFP) by *in situ* visualization, and measurement of its expression by near-infrared, two-photon-excitation-evoked, real EGFP fluorescence detection and fluorescence-lifetime imaging. **a,** Real EGFP fluorescence image of several CHO cells transfected with the pEGFP-N1 plasmid. **b,** Transmission image of a single transfected CHO cell (arrow). **c,** Two-photon fluorescence-lifetime image of the same cell expressing EGFP; colour scale indicates the fluorescence lifetime (τ) between 0 and 5 nanoseconds. Inset, distribution of fluorescence lifetime (in picoseconds) of EGFP throughout the entire transfected cell. Scale bar, 25 μm.

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