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Analytical Raman spectroscopic study of *cacao* seeds and their chemical extracts

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Abstract

A Raman spectroscopic study of *cacao* seeds and their extracts has been successful in the identification of biomarker bands for theobromine, a member of the caffeine group of alkaloids. Unlike caffeine detected in guarana, the spectral signatures of theobromine suggest that it occurs as the free base in *cacao*. No theophylline or caffeine alkaloids were detected in *cacao*. Raman spectroscopic monitoring of the heptane and ethyl acetate organic extracts of pulverised *cacao* seeds demonstrates the removal of the fatty and waxy components whereas the methanol extract is found to remove and concentrate the highly coloured aromatic polyphenolic compounds. The theobromine alkaloid is still detectable in the solid residues from the extractions. This study suggests that Raman spectroscopic monitoring of *cacao* seed processing should be investigated further for the purposes of industrial process analytical control.

Keywords: *Cacao*; Raman spectroscopy; Theobromine; Seed extractions

1. Introduction

Cacao (*Sterculiaceae theobroma cacao* L.), commonly named kakao, cacau, criollo or cacaoyer, is the starting material for chocolate production. It is known in popular medicine as an antiseptic, diuretic and parasiticide [1]. More recently, studies have shown that *cacao* liquor extract acts therapeutically on rats with hepatocarcinogenesis, targetting mainly the tumour marker enzymes and having the potential in decreasing the severity of this disease [2]. Chemically, *cacao* seeds contain 40–50% of fat (*cacao* butter, *oleum theobromatis*, oil of *cacao*), a free alkaloid base theobromine (content 0.9–2.4%), caffeine (0.05–0.35%), starch (1.3–7.5%), a red phenolic pigment, *cacao* red and ca. 30 μg of β-carotene [3–6].

Cacao butter is by far the most important chemical component of cacao, since its physical and chemical characteristics provide specific functional properties of great demand in the food, pharmaceutical and cosmetic industries, related to the fact that *cacao* butter is considered the main transport and suspension medium for *cacao* powder, chocolate liquor, sugar and several other ingredients used in chocolate production [7]. In this sense, the analytical determination of the chemical components in *cacao* is of great importance industrially, especially for the quality control. There are several analytical methods described in the literature for the analysis of the chemical content of *cacao* seeds, including gas and liquid chromatography and mass spectrometry [7–16]; however, all of these are destructive of sample and can be time-consuming. A recent investigation has shown the capability of Raman microscopy in analysing foods in general and in particular the fatty content of *cacao* [17]. However, this study was not specific in analysing the molecular vibrations that could be used

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as biomarkers i.e. the vibrational bands that are characteristic of the main components in each system which are responsible for the spectral signatures. In recent years, we have been able to describe the use of Raman spectroscopy as a powerful tool in the analytical and spectroscopic description of complex chemical systems in archaeological [18], botanical [19,20], and food [21,22] samples; in all of these it has been possible to describe the key vibrational biomarkers of significance for the characterisation of genuine materials and from which process information can follow.

The main purpose of this current study is the identification of key biomolecules using Fourier-transform Raman spectroscopy which has hitherto been described as a successful non-destructive technique employed in analytical investigations of natural products. *Cacao* seeds were extracted and the Raman spectra of the raw materials and of successive solvent extractions were analysed, specifically for the presence of the alkaloid theobromine. The results obtained here will be applicable in several fields for quality control procedures, including the pharmaceutical and food industries, where *cacao* seeds have been used as a raw material for a range of manufactured products.

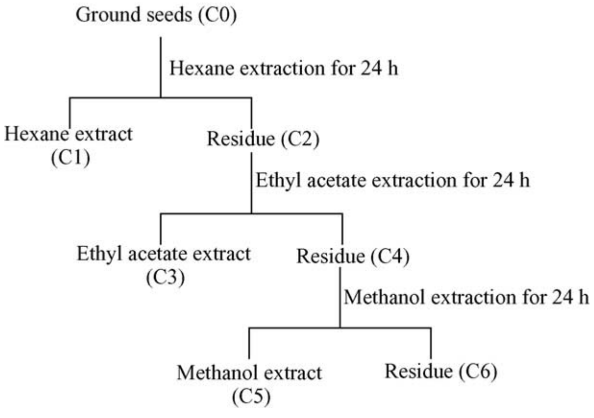
2. Experimental

2.1. Specimens

All the reagents and solvents used were analytical grade purchased from Aldrich. The *cacao* seeds were obtained from Salvador (Bahia, Brazil) and after drying and peeling, were subjected to mechanical grinding.

2.2. Extraction technique

According to Scheme 1, about 50 g of ground *cacao* seeds (C0) were extracted in a Soxhlet apparatus first with hexane, then with ethyl acetate and finally with methanol. All the extracts (C1, C3, and C5, respectively) were then concentrated under reduced pressure. After each extraction, a sample of the seed residue (C2, C4, and C6) was separated and air-dried before analysis.



Scheme 1.

In addition, samples C7 and C8 were supplied as peeled, whole *cacao* seeds and unpeeled, whole seeds, respectively.

2.3. Raman spectroscopy

Fourier-transform Raman spectroscopy was carried out in the macroscopic mode with a specimen footprint of about 100 microns using a Bruker IFS 66 instrument with an FRA 106 Raman module attachment and a Nd^{3+}/YAG laser operating at 1064 nm in the near infrared and InGaAs detector cooled with liquid N_2. To improve the signal-to-noise ratios, 2000 scans were accumulated over a period of about 30 min, using a 4 cm^{-1} spectral resolution. All spectra were obtained several times as replicates from each sample provided to show reproducibility; no changes in band positions and intensities were observed between replicates.

3. Results and discussion

3.1. Raman spectroscopic analysis

The solid samples comprise the whole seed (C8); a peeled whole seed (C7); a ground, dried specimen of the same (C0); the solid residues from heptane (C2); ethyl acetate (C4); and methanol (C6) extractions of the powdered dried seeds. The first heptane extraction would remove the nonpolar fatty compounds (C1), and then the more polar components would be progressively removed from the residues using ethyl acetate and methanol (C3 and C5 samples).

The chemical composition of *cacao* seed shows the following approximate composition (g/100 g): moisture from 4.35 to 7.0%; protein from 14.7 to 20.50%; fat from 46.0 to 56.0%; ash from 3.6 to 5.3%; crude fibre from 4.2 to 8.7%, and carbohydrates from 8.7 to 28.2%. The fat content presents an average composition that is richer in oleic acid (from 35.0 to 41.0%), followed by stearic acid (from 34.0 to 39.9%) and palmitic acid (from 23.0 to 30.0%); some cultivars also present a small content of linoleic acid (about 2.0–3.0%) [6,7]. The Raman spectra of the C0, C7, and C8 samples are shown in Fig. 1. The Raman spectrum of the unpeeled, natural seed consists of several rather broad features which, nevertheless, make an interesting comparison with the peeled kernel and the bulk, ground, dried and seed kernel. The main Raman bands of the C0 sample can be seen in Table 1, together with a tentative vibrational assignment. The FT-Raman spectrum of the latter consists of several C–H stretching vibrations at 3000–2850 cm^{-1}, and some sharp bands which can be considered as key features of the kernel and fat matrix; these include C=O stretching bands at 1743 and 1730 cm^{-1} (shoulder), (C=C) + (C=O) stretching at 1659 cm^{-1} and a broad unresolved aromatic C–CH quadrant stretching mode feature centred at 1609 cm^{-1}. The former Raman band can be assigned to carbonyl ester stretching vibration, and the shoulder

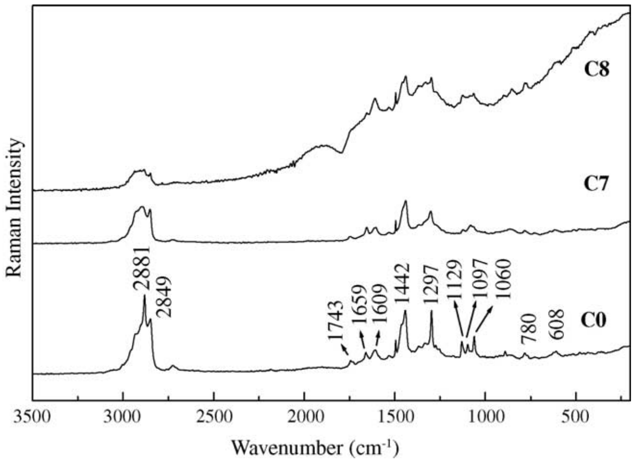


Fig. 1. FT-Raman spectra of C0, C7, and C8 *cacao* samples. See Section 2 and Scheme 1 for details.

at 1730 cm^{-1} can be assigned to carbonyl stretching from the cellulosic structure in the seeds [23]. Strong C–H deformation bands at 1461 and 1442 cm^{-1} and weaker, asymmetric deformations at 1366 and 1332 cm^{-1} are also noted, but the strong, sharp feature at 1297 cm^{-1} and the triplet at 1129, 1097, and 1060 cm^{-1} dominate the central region of this spectrum; the former is probably a CH_2 deformation mode, whereas the latter are assignable to (C–O) + (C=CH) coupled stretching modes. The features at 889 cm^{-1}, sharp, and 780, 760 (broad), 766 (shoulder), and 608 cm^{-1} (broad) are assignable to (C–C) and (CCO) stretching modes. The presence of cellulosic glycosides in the rind of the seeds would also give rise to features in the region of 700–1100 cm^{-1}. Weaker bands below 600 cm^{-1} are complex modes which could contain components from CCO and CCC deformations. The sequence of weaker features at 1531 and 1177 cm^{-1} are reasonably assigned to conjugated C=C and C–C modes from carotenoids in the seed specimen [24].

Table 1
Main Raman wavenumbers (cm^{-1}) from C_0 sample

Wavenumber (cm^{-1})	Tentative assignment
3002 sh	$\nu_{asym}(CH)$
2956 sh	$\nu(CH)$
2929 sh	$\nu(CH)$
2881 vs	$\nu(CH)$
2849 s	$\nu(CH)$
2724 w	
1743 w	$\nu(C{=}O)$ of ester (from fatty acids)
1730 sh	$\nu(C{=}O)$
1659 w	$\nu(C{=}O) + \nu(C{=}C)$
1609 w	$\nu(CCH)$
1531 vw	$\nu(C{=}C)$ in carotenoids and red compounds
1461 sh	$\delta(CH)$
1442 s	$\delta(CH)$
1366 vw	$\delta(CH)$
1332 w	$\delta(CH)$
1297 s	$\delta(CH_2)$
1272 sh	$\delta(CH)$
1170 vw	$\nu(C{-}C)$ in carotenoid
1129 m	$\nu(C{-}O) + \nu(C{-}CH)$
1097 wm	$\nu(C{-}O) + \nu(C{-}CH)$
1060 m	$\nu(C{-}O) + \nu(C{-}CH)$
889 w	$\nu(C{-}C) + \nu(CCO)$
780 w	$\nu(C{-}C) + \nu(CCO)$
766 sh	$\nu(C{-}C) + \nu(CCO)$
620 sh	$\delta(C{=}C{-}C)$
608 w	$\nu(C{-}C) + \nu(CCO)$
478 vw	$\delta(CCO + CCC)$

The peeled, whole kernel by comparison, has a rather broader spectral distribution, but generally, many of the features are the same as those of the whole seed—with bands at 1741, 1655, 1608, 1442, 1367, 1332, 1302, 1274, 1014, 1002, 974, 867, 782, 726, 618, 433, and 365 cm^{-1} (see assignment at Table 1). However, bands at 1400 cm^{-1} and the significant doublets at 1129, 1097 cm^{-1} 1060, and 889 cm^{-1} are now missing. The unpeeled, whole seed of *cacao* gives an even broader, more diffuse spectrum, but the major spectral features noted for the powdered and peeled seeds are still identifiable. However, the (C=O) stretching mode near 1740 cm^{-1} is absent, since there is now a larger contribution from the cellulosic compounds [23], and the (C=C) stretching mode near 1659 cm^{-1} is considerably reduced in intensity. The relative intensity of the strong sharp bands near 1440 and 1297 cm^{-1} is reduced and the spectral signatures of the assigned carotenoid component are somewhat increased here.

More significantly, however, the bands near 1129, 1097, and 1060 cm^{-1} seen in the bulk, powdered kernel, but not in the peeled whole seed kernel are now present, as are the lower wavenumber bands assignable to glycosidic (COC) and CCO modes. We can interpret this spectral data on the basis of the seed kernel outer layer containing predominantly glycosidic components, with little or no aromatic or (C=C) stretching mode from unsaturated components. It is interesting that these latter components are seen to be identifiable in the bulk seed powder; this could be attributed to a layer of intermediate composition underlying the rind and the kernel, which is relatively poorer in glycosides but has a similar content in aromatic and carotenoid components.

The Raman spectrum of the extracted fat compounds (C1) is shown in Fig. 2 together with the spectrum of the C3 sample. It can be seen that both spectra are very similar and are dominated by the very strong features in the CH_2 and CH_3 symmetric stretching region; other medium intensity bands are present at 1442 cm^{-1} (CH_2 deformation) and 1296 cm^{-1} (in phase methylene twisting motion), whereas medium to weak intensity bands are present at 1744 (C=O stretching) ester, 1659 (C=C stretching), 1130 (C–C stretching),1060 (C–O deformation) and 880 cm^{-1} (C=CH deformation). Previous work carried out on several *Theobromona* species, including *T. cacao*, has shown that the main fat constituents are oleic (C18:1), stearic (C18:0), and palmitic (C16:0) acids [7]. The comparison of our data for *cacao* with recent Raman spectroscopic analytical work on the unsaturates in olive oils [25] shows little resemblance, however, since oleic acid is the major component of olive oil and in *cacao* butter this is present as a relatively minor component. The presence of a

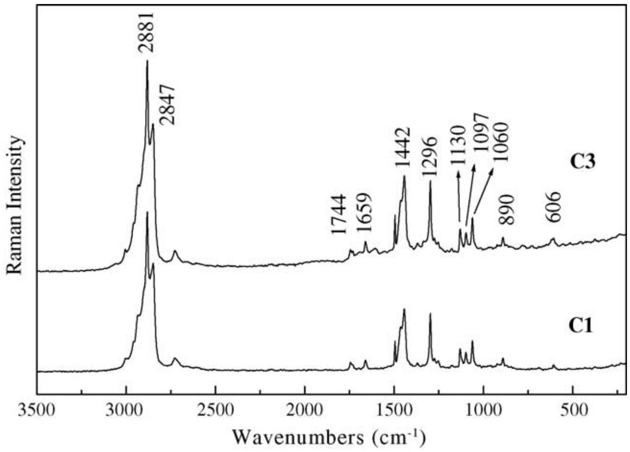


Fig. 2. FT-Raman spectra of C1 and C3 samples. See Section 2 and Scheme 1 for details.

Raman feature at 1655 cm^{-1} could well be a spectral signature of oleic acid but unfortunately several other possible bands cannot be used as key markers due to their accidental coincidence for several organic compounds present. However, the similarity of the Raman spectra of the fat content extracted in sample C1, when compared with the spectrum obtained for sample C3 indicates clearly that the extraction procedure was not completed with only hexane and a more polar solvent was needed to extract the remaining part of the fatty material. This is an interesting conclusion and could be fed back to the industrial process monitoring aspect of this application.

The FT-Raman spectra of the extracted samples C2, C4, and C5 are shown in Fig. 3, and the main wavenumbers are displayed at Table 2 together a tentative assignment. All the spectra show a very similar pattern; however, they still retain some critical signatures, despite the onset of spectral broadening. All have lost the C=O and C=C stretching features of lipodial, waxy or fatty compounds (removed by the hexane extraction), but the specimens have still retained some aromatic component character. The strong feature observed at 1440 cm^{-1} in the powdered kernel has now disappeared but that at 1334 cm^{-1} still remains, indicating different chemical sources for these assigned CH_2 and CH_3 vibrational modes. Features such as those at 1608, 1454, 1334, 1204, 1127, 856, 782, 620, and 478 cm^{-1} (see Table 1 for assignments) remain in the solid phase throughout the wet extraction process. It is interesting that at least one of these, viz. 618 cm^{-1}, is a biomarker assigned to theobromine, which confirms that the alkaloid is maintained throughout in the solids subjected to the several sequential extraction processes.

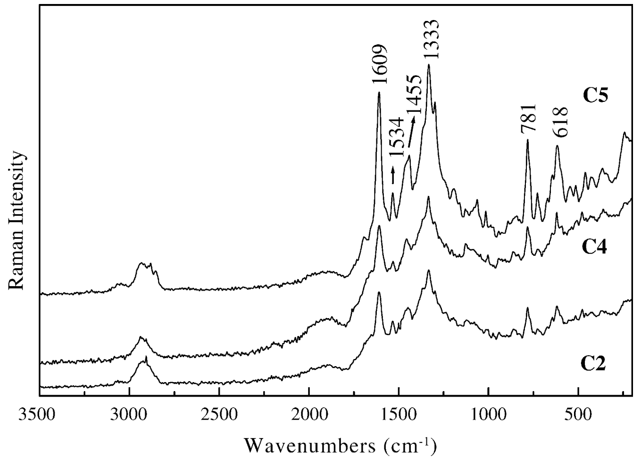


Fig. 3. FT-Raman spectra of C2, C4, and C5 samples. See Section 2 and Scheme 1 for details.

Table 2
Main Raman wavenumbers (cm^{-1}) from C2, C4, and C5 samples displayed at Fig. 3

Wavenumber (cm^{-1})	Tentative assignment
1609 s	ν (C=C)
1534 vw	ν (C=C) in carotenoids
1455 wm	δ (CH)
1332 vs	ν (C–N) symmetric
781 m	δ (O=C–C)
726 w	δ (O=C–C)
618 wm	δ (C=C–C)

w, weak; vw, very weak; wm, weak to medium; s, strong; vs, very strong; sh, shoulder.

3.2. Protocol proposition

Previous results from wet chemical analysis of *cacao* specimens [3–6] indicate that the dried seed powder is relatively rich in theobromine, poorer in caffeine and contains no theophylline. Raman spectral comparison of these three alkaloids [26,27] with the bulk powders shows that theophylline and theobromine can be differentiated using the following spectroscopic protocol:

- Theophylline has a sharp doublet at 1706, 1665 (C=N stretching and C=O asymmetric stretching, respectively) and bands at 1314 (C–N stretching), 1286 (C–N stretching), 1248 (C–N stretching), a weaker doublet at 969 and 928 cm^{-1} (N=C–H deformation and N–CH_3 symmetric stretching, respectively), a singlet at 667 (O=C–N deformation) and a strong sharp band at 555 cm^{-1} (C=C–C deformation).
- Theobromine has bands at 1682 (C=N stretching), 1594 (C=C stretching), 1334 (C–N stretching), 1296 (C–N stretching), 1225 (C–N stretching), 776 (O=C–C deformation), 733 (O=C–N deformation), with a strong band at 620 cm^{-1} (C=C–C deformation); this latter feature is therefore, very characteristic of the free base theobromine alkaloid and has been seen in the solid extracts from the industrial processes. Other possible supporting features are found at 459 and 374 cm^{-1} (N–C–N deformation).

In a recent normal coordinate analysis of caffeine, theobromine, and theophylline, where the authors present Raman and infrared spectra of such a compounds [27] the major difference between the Raman spectra of the solid samples is related to the bands at 550–650 cm^{-1} region, concerning the C=C–C deformation. This vibrational mode can be seen at 625 cm^{-1} for theobromine, and at 550 cm^{-1} for theophylline as strong intensity bands. The significant difference between these two vibrational modes can be understood in terms of the methyl group positions in both structures [27].

Comparison of the theobromine signatures [26,27] with the Raman spectrum of the dried bulk, powdered seed kernel indicates that there are no signatures present for theophylline (in confirmation of the wet chemical analyses) and that the theobromine bands at 1334 (C–N stretching), 1225 (C–N stretching), 946 ($N–CH_3$ stretching), 776 (O=C–C stretching), 733 (O=C–C deformation), 620 (C=C–C deformation), 509 ($C–N–CH_3$ deformation), and 459 (C–N–C asymmetric deformation) are identifiable. It must be also said, according to Ribeiro-Claro and Amado [28] that the main Raman bands derived from theobromine and observed in the *cacao* spectra can be assigned to the hydrated xanthine derivative species, instead of the dehydrated structure, since this chemical form is more stable and bioavailable.

Finally, the three products of the Soxhlet extraction by progressively more polar solvents, viz heptane, ethyl acetate and methanol (C1, C3, and C5) show a differentiation as follows: the spectra of the heptane and ethyl acetate extracts were very closely similar to each other. Clearly, the modes of (C=O) and (C=C) stretching modes have now been "isolated" from the aromatic components left in the solid products of the extraction process—this confirms their assignment to fatty lipids and waxes. The 1440 cm^{-1} signature is very strong and probably, therefore, can be almost certainly assigned to lipid (CH_2) bending chains. C1 is generally termed the "fatty *cacao* butter" extract and this description fits our assignment from the Raman spectra. The glycosidic triplet at 1129, 1097, and 1061 cm^{-1} and other bands at 890 and 1297 cm^{-1} are clearly also in the "fatty" fraction. No theobromine has been extracted in these two Soxhlet processes; this result should be compared with the polar liquid extract (methanol, C5) the spectrum of which is completely different from those obtained from heptane and ethyl acetate extractions. Here, we see evidence in sample C5 of a very strong aromatic component, including some carotenoid, with a quadrant ring stretching band at 1609 cm^{-1}. The presence of some theobromine is now indicated here as well, with characteristic bands at 618, 781, and 1534 cm^{-1}. The dominant feature in the C5 spectrum, however, is the aromatic ring mode at 1609 cm^{-1} and this correlates well with the evidence of wet chemical analysis that this stage of the process concentrates the polyphenolic colouring matter of an as yet indeterminate structure—in fact, the concentrated extract C5 is red in colour (which is also seen in C8, the outer shell of the *cacao* seeds). Some common vibrational features are also present in several other red pigments, as for example, brazilin (present in brasilwood) [20] and dracorhodin (present in dragonι̌s blood, the pigment from dragon tree) [19], and has been used as the key biomarker to indicate the presence of such a coloured polyphenolic species.

4. Conclusions

Raman spectroscopy has been used successfully to monitor the extraction stages of the processing of *cacao* seeds and the following important information applies:

(i) Theobromine is present in the powdered seed kernels—probably as the free base alkaloid, from vibrational band wavenumbers, in contrast with caffeine in *guarana*, several modes of which are found to shift with molecular coordination probably on account of complexation with tannins and phenolic compounds in the seeds.
(ii) No theophylline or caffeine is detectable in the *cacao* specimens.
(iii) The heptane and ethyl acetate extracts remove fatty and waxy compounds, including the lipids. The methanol extract removes the aromatic polyphenolic compounds.
(iv) Theobromine is detectable in the solid products of the multiple Soxhlet extractions of the *cacao* seeds.
(v) Some features, e.g. the bands at 1744 and 1659 cm^{-1}, (C=O) and (C=C) stretching modes, respectively, are representative of fatty material, mainly the C18:1 (oleic acid), and could be used as a key marker of this chemical species.

This study suggests that Raman spectroscopy could be adopted as a non-destructive and relatively rapid method of *cacao* seed process control and quality monitoring of the extracts.

Acknowledgments

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