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Capillary supercritical fluid chromatography–Fourier transform infrared spectroscopy study of triglycerides and the qualitative analysis of normal and "unsaturated" cheeses

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Abstract

Capillary supercritical fluid chromatography (cSFC)–Fourier transform infrared spectroscopy (FT-IR)–flame ionization detection (FID) has been used to examine the separation of a synthetic mixture of saturated triglycerides. A careful study of the on-line FT-IR spectra of the separated components revealed that relative absorption intensities of the infrared peaks corresponding to the antisymmetric CH_2 stretching and carbonyl (C=O) stretching modes were related to the carbon number of an individual triglyceride.

A comparison of the FT-IR spectra obtained in cSFC–FT-IR-FID separations of a cheese said to be high in unsaturates ("Flora" cheese) and a cheddar cheese showed that the Flora cheese does indeed contain only unsaturated triglycerides. The cheddar cheese contained many more triglyceride components, most of which correspond to saturated species. Comparison with the synthetic saturated triglyceride mixture showed that the speciation of some of the cheddar cheese components can be carried out.

1. Introduction

One of the areas of analytical chemistry where supercritical fluid chromatography (SFC) has already proved to be of considerable value is in the study of food products, particularly fats and oils [1]. These are often mixed materials of some complexity, whose relative involatility and/or thermal lability makes them poor candidates for GC analysis.

Reports by Taylor and coworkers [2,3] have shown that, following SFC, Fourier transform infrared spectroscopic (FT-IR) detection can provide a great deal of structural information on the nature of triglycerides and fatty acids derived, for example, from soya bean oil. In particular, the presence of unsaturation in the hydrocarbon chains gave characteristic infrared absorptions, and it even proved to be possible to identify the geometry associated with specific centres of unsaturation by this means [3].

In our laboratory we have been active in developing an improved on-line capillary SFC (cSFC) system, which has proved to have approximately 25 times greater sensitivity than previous systems, while preserving a linear response to analyte concentration over several orders of magnitude [4]. A number of analytical results using this system have been published, showing that it is compatible with a wide range of analyte types [5–7].

Proot *et al.* [8] have reported a study of

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triglyceride separation by SFC and it therefore seemed appropriate to expand the study of triglycerides and related systems by cSFC–FT-IR. In particular, the growing concern with healthy eating habits has led to an enormous growth in the range of food products which are advertised as being "high in unsaturates". We therefore report a study of a number of saturated triglycerides in a synthetic mixture, in which the possibility of speciation on the basis of infrared intensity measurements is explored, as well as a comparison between the chromatographic and infrared spectroscopic properties of a "natural" cheese (an English cheddar) and a recently-introduced "unsaturated" cheese (marketed in the UK under the "Flora" label).

2. Experimental

The cSFC–FT-IR–flame ionization detection (FID) system used has been described earlier [6,7]. It uses a Brownlee MicroGradient pump (Anachem, Luton, UK) for delivery and pressure programming of the mobile phase. The outlet of the pump was connected via a length of stainless-steel tubing (1.6 mm O.D. × 0.25 mm I.D.) to the injection valve. This was a Valco C14W two-position switching valve (Anachem) with an internal loop (volume 100 or 200 nl), located on the door of an 8500 GC oven (Perkin-Elmer). The SFC capillary column (Lee Scientific, from Dionex, Camberley, UK) was installed inside the GC oven (maintained at 100 or 120°C in the experiments described in this paper) and connected directly to the valve. Samples (100 nl) were introduced on to the column by direct injection. The column outlet was connected to a 50 cm × 50 μm I.D., uncoated, deactivated fused-silica capillary transfer line (S.G.E., Milton Keynes, UK) using a zero dead-volume butt-connector (S.G.E.) which passed through the oven wall and was connected to the FT-IR flow-cell.

The FT-IR flow cells have been described previously [9]. They have volumes of 500 and 980 nl, respectively, and an optical path length of 4.5 mm. The cell being used was placed in the sample compartment of a 1760-X FT-IR spectrometer (Perkin-Elmer) fitted with a mercury cadmium telluride (MCT) detector. All experiments were carried out with a spectral resolution of 8 cm^{-1}, and the flow-cell maintained at 24°C. After the flow cell a second transfer line was used to return the sample to the GC oven. This transfer line was butt-connected to a frit restrictor (Lee Scientific) which was then interfaced to the FID system of the gas chromatograph. IR data were processed using the standard GC–IR software of the spectrometer, which enabled Gram–Schmidt reconstruction of the IR chromatograms to be carried out.

cSFC–FT-IR studies were carried out on a synthetic mixture of the following triglycerides: tricaprylin (C_8 hydrocarbon chain), trilaurin (C_{12}), trimyristin (C_{14}), tripalmitin (C_{16}), tristearin (C_{18}) and triarachidin (C_{20}). The samples were obtained from Sigma (Poole, UK) and all were used without further purification. The synthetic mixture was prepared as a solution in dichloromethane.

Samples of cheddar and "Flora" cheeses were purchased from a local supermarket, dissolved in dichloromethane to give a saturated solution and filtered to give a clear solution.

The mobile phase used in all of the experiments described in this paper was 99.99% CO_2 (Air Products, Rotherham, UK).

3. Results and discussion

3.1. Triglyceride mixture

Despite the presence of three polar carboxylic groups, the long hydrocarbon chains ensure that triglycerides are soluble in supercritical CO_2; and cSFC–FT-IR with CO_2 as the mobile phase has been shown to be a practical proposition [3]. As a preliminary to the investigation of some food samples, we have therefore examined a synthetic mixture of saturated triglycerides, as indicated in the Experimental section above. Fig. 1a shows a Gram–Schmidt reconstructed FT-IR chromatogram of this mixture, showing good separation of the components (the chromato-

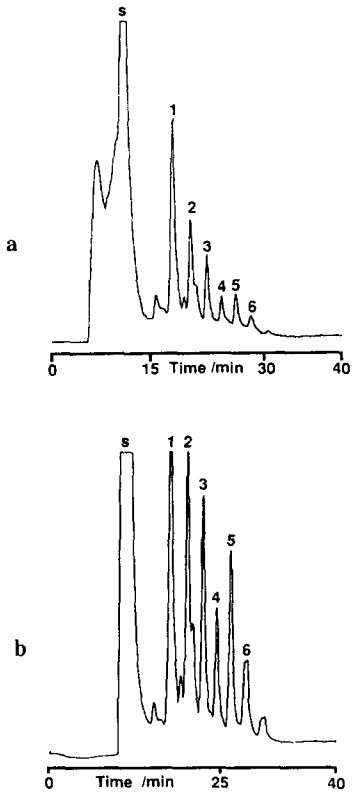

Fig. 1. (a) FT-IR chromatogram of a synthetic mixture of saturated triglycerides, separated by cSFC. Chromatographic conditions: column 10 m × 100 μm; stationary phase SB-Phenyl-5; sample size 100 nl; mobile phase CO_2; temperature 100°C; pressure programme 150 bar (5 min isobaric), then ramp to 350 bar at 10 bar/min, and 10 min at 350 bar. Peaks: 1 = tricaprylin; 2 = trilaurin; 3 = trimyristin; 4 = tripalmitin; 5 = tristearin; 6 = triarachidin. (b) FID chromatogram of a synthetic mixture of saturated triglycerides (details as for 1a).

graphic parameters are summarised in the figure caption). In the same run a subsequent FID chromatogram was obtained, which is shown in Fig. 1b. This confirms our earlier results showing that the effect of the FT-IR flow-cell on chromatographic resolution is negligible [10].

The great advantage of FT-IR detection over FID is that it is possible to retrieve a considerable amount of additional structural information about the separated species from the FT-IR spectra (see, *e.g.*, refs. 3 and 6). In Fig. 2a we show the FT-IR spectra corresponding to the peaks labelled 1, 3 and 5 in Fig. 1, *i.e.* tricaprylin (C_8 hydrocarbon chains), trimyristin (C_{14}) and tristearin (C_{18}). All show an excellent signal-to-noise ratio, and a number of bands characteristic of the fatty acid chains, *i.e.* CH_2 antisymmetric stretch ($\nu_{as}CH_2$) near 2930 cm^{-1}, CH_2 symmetric stretch (ν_sCH_2) near 2865 cm^{-1}, ester C=O stretch (νC=O) at 1748 cm^{-1}, CH_2 symmetric (scissors) deformation (δ_sCH_2) near 1465 cm^{-1} and ester C–O stretch (νC–O) near 1165 cm^{-1}. Note that for tricaprylin, with the shortest hydrocarbon chain and hence the largest proportion of terminal CH_3 groups, the antisymmetric CH_3 stretch ($\nu_{as}CH_3$) is clearly resolved (2966 cm^{-1}). Fig. 2b shows the C–H stretching region for peaks 1 (tricaprylin), 2 (trilaurin), 3 (trimyristin) and 5 (tristearin), in which the CH_3 stretching component (*i.e.* the highest wavenumber band) becomes steadily weaker as the chain length increases. All of these assignments are in good agreement with literature values for solid and conventional solution samples of long-chain esters [11].

As our aim is the individual speciation of the separated analytes, it is necessary to examine the spectra for differences. With the exception of the observation of a CH_3 stretch for tricaprylin, the spectra, however, seem at first sight to be regrettably similar. The wavenumber of the carbonyl (C=O) stretch is invariable, and no clear trends appear in the positions of the other well-defined bands, the CH_2 stretches, *i.e.* for tricaprylin 2929 and 2868 cm^{-1} for ν_{as} and ν_s respectively, trimyristin 2935, 2862 cm^{-1} and tristearin 2934, 2862 cm^{-1}. On the other hand, closer investigation of the spectra does reveal significant differences in the relative intensities of some of the bands. This is particularly noticeable for the carbonyl and $\nu_{as}CH_2$ stretches. Each of the triglycerides contains just three carbonyl groups, but differing numbers of CH_2 units in the hydrocarbon chains. In agreement with this, there is a definite increase in the intensity of $\nu_{as}CH_2$ compared to νC=O as the carbon number of the triglyceride increases (νC=O is an effective internal intensity standard). The question that must be addressed in the context of speciation is whether this relationship is sufficiently quantitative to allow identification of individual compounds.

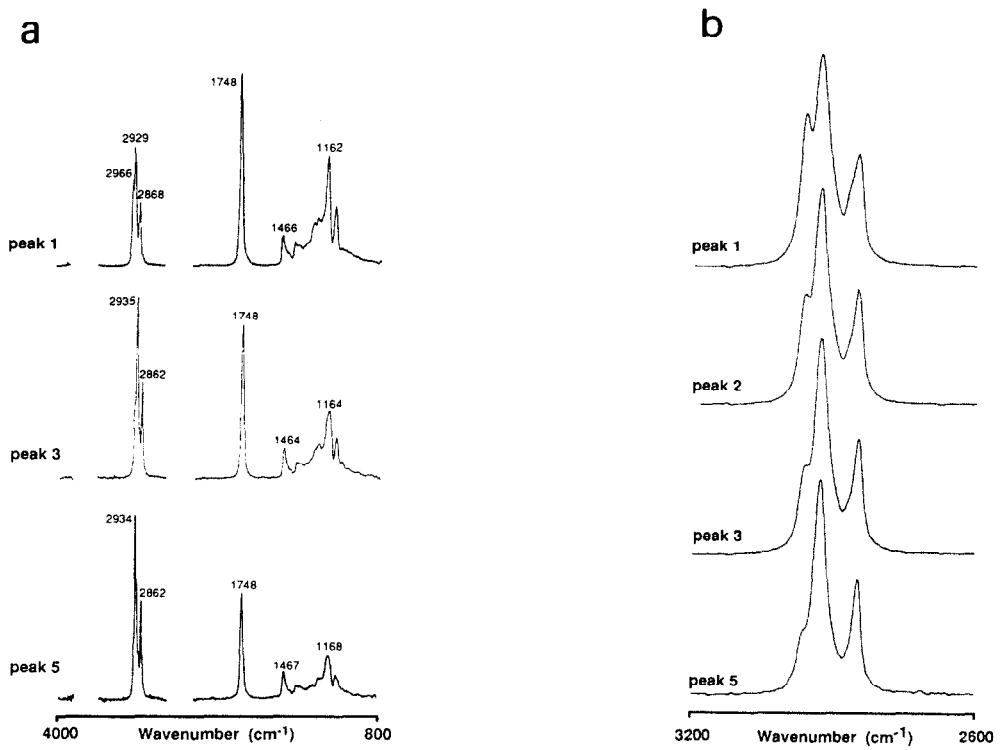

Fig. 2. (a) On-line FT-IR spectra of selected triglycerides from Fig. 1. (i) Tricaprylin (peak 1); (ii) trimyristin (peak 3); (iii) tristearin (peak 5). (b) C–H stretching regions of the FT-IR spectra of (i) tricaprylin; (ii) trilaurin; (iii) trimyristin; (iv) tristearin.

Fig. 3 shows a plot of the triglyceride carbon number (the number of carbon atoms in each hydrocarbon chain) against the ratio of the intensities of the antisymmetric CH_2 stretch and the C=O stretch, the values of which are listed in Table 1. The intensities were simply measured in terms of peak heights as peak area measurements are subject to considerable uncertainty due to the presence of overlapping bands in the CH_2 stretching region.

It will be seen that there is a relationship between the carbon number (n) and the intensity ratio ($\nu_{as}CH_2/\nu C{=}O$) (I) which gives an excellent fit ($R = 1.0$) to the empirical equation

$$I = -0.5664 + 0.1400n$$

Thus a simple measurement of peak heights can

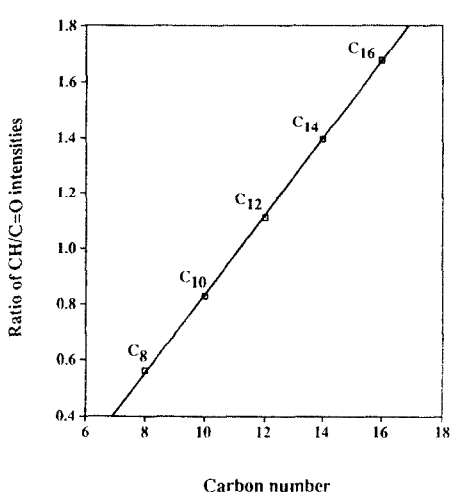

Fig. 3. Plot of intensity ratio, $\nu_{as}CH_2/\nu C{=}O$, *versus* carbon number for some saturated triglycerides.

Table 1
Values of the relative intensities (I) of $\nu_{as}CH_2$ and $\nu C{=}O$ for saturated triglycerides with different hydrocarbon chain lengths (n)

$I[\nu_{as}(CH_2)/\nu(C{=}O)]$	n
0.559	8
0.830	10
1.108	12
1.395	14
1.676	16

be used to identify individual saturated triglycerides from what appear to be very similar infrared spectra.

3.2. *"Natural" and "unsaturated" cheeses*

Current thinking stresses the need for a healthy diet, and an important aspect of this is concerned with the proportions of saturated and unsaturated (mono- and polyunsaturated) fats consumed. Hence it is important to know which triglycerides are present in any foodstuff, and their degrees of unsaturation. In recent times a cheese has come on to the market which claims to be high in unsaturated and low in saturated fats. We have therefore subjected this "Flora" cheese to on-line cSFC–FTIR–FID analysis, and compared it with a conventional, cheddar, cheese.

Fig. 4 shows the cSFC–FTIR chromatograms of the Flora and cheddar cheeses before subtraction of the sloping baseline caused by the increasing CO_2 absorption at higher pressures, and Fig. 5 the FID chromatograms of these cheeses. Both pairs of chromatograms show quite clearly that there is a very marked difference in constitution between the two samples, with a much larger number of components in the "natural" material, and also a wider range of retention times.

In order to probe the degree of unsaturation of the two samples it is of course necessary to record their FT-IR spectra. In Fig. 6 we show the spectra corresponding to peaks 1 and 3 of Fig. 4a. The lower-wavenumber region is very similar in each case, and also very similar to the

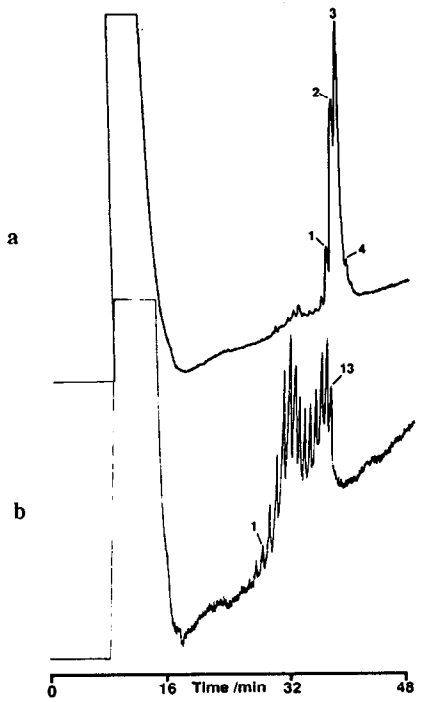

Fig. 4. (a) On-line cSFC–FT-IR chromatogram of "Flora" cheese. Chromatographic conditions: column 10 × 50 μm; stationary phase SB-Phenyl-5; sample size 200 nl; temperature 120°C; pressure programme 90 bar (10 min isobaric) followed by ramp to 400 bar at 10 bar/min. (b) On-line cSFC–FT-IR chromatogram of cheddar cheese (details as for a). Peak nos. referred to in later figures.

FT-IR spectra obtained for the saturated triglycerides (see above). The presence of $\nu C{=}O$ at 1749 cm^{-1} confirms that these are indeed tri-

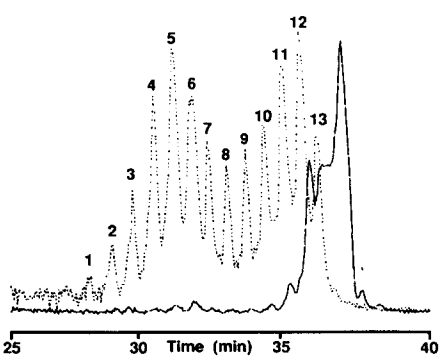

Fig. 5. Comparison of cSFC–FID chromatograms of Flora (solid line) and cheddar cheeses (dotted line) (details as in Fig. 4). Peak nos. referred to in later figures.

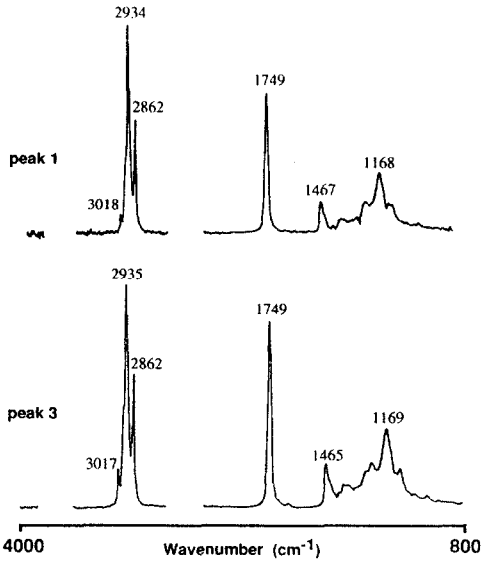

Fig. 6. On-line FT-IR spectra corresponding to selected peaks from the cSFC–FT-IR chromatogram of Flora cheese. (a) Peak 1 (Fig. 4); (b) peak 3 (Fig. 4).

glycerides, rather than, for example, fatty acids, for which νC=O is above 1760 cm^{-1} [3].

Inspection of the C–H stretching region, however, does reveal a significant difference compared to the saturated triglycerides. In addition to $\nu_s CH_2$ (2862 cm^{-1}) and $\nu_{as} CH_2$ (2935 cm^{-1}) a feature is seen above 3000 cm^{-1}. Calvey *et al.* [3] have reported on the FT-IR spectrum of the unsaturated triglyceride, trilinolein, with two double bonds per chain, in the *cis,cis* configuration, and a characteristic ν(=C–H) band at 3016–3018 cm^{-1} (compared to a value of 3010–3012 cm^{-1} for singly, *cis*, unsaturated chains. Thus the triglycerides in Flora cheese appear to contain at least two unsaturated units per hydrocarbon chain, in agreement with advertising claims made for this product.

The difference between Flora and cheddar cheeses is highlighted by Fig. 7, where the C–H stretching regions in the FT-IR spectra, corresponding to peak 3 in Fig. 4a of Flora (Fig. 7b) and to peak in 4 in Fig. 5 of cheddar (Fig. 7a), are compared. Two observations can be made on these spectra. First, it can be suggested that the hydrocarbon chain lengths are relatively modest, as the CH_3 antisymmetric stretches are clearly

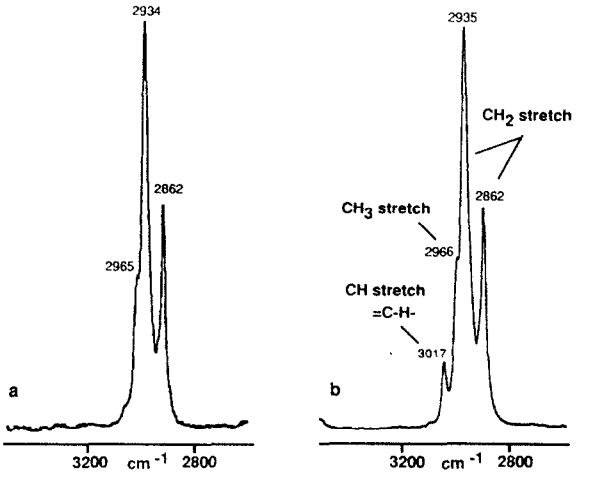

Fig. 7. C–H stretching region of the FT-IR spectra corresponding to (a) peak 4 of cheddar (Fig. 5) and (b) peak 3 of Flora (Fig. 4).

visible as high-wavenumber shoulders (2965 cm^{-1}) on the $\nu_{as}(CH_2)$ peak near 2935 cm^{-1}. Second, it is clear that this triglyceride from the cheddar cheese is completely saturated, while peak 3 for Flora (Fig. 7b), as was the case for peaks 1 and 3 (Fig. 6), contains at least two units of unsaturation per hydrocarbon chain.

Fig. 8 shows FT-IR spectra corresponding to two of the components of the cheddar cheese.

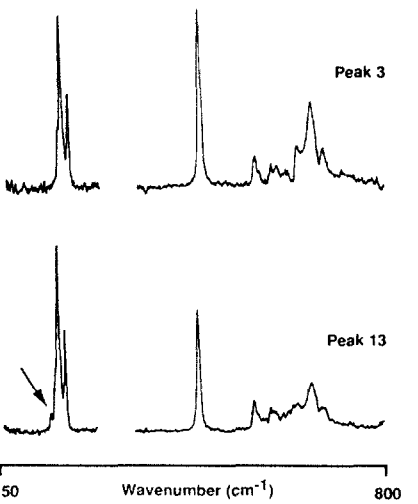

Fig. 8. On-line FT-IR spectra corresponding to selected peaks from the cSFC–FT-IR chromatogram of cheddar cheese. (a) Peak 3 (Fig. 5); (b) peak 13 (Fig. 5).

The early-eluting peak 3 shows no absorption above 3000 cm^{-1}, and is therefore a saturated triglyceride (characteristic νC=O band at 1748 cm^{-1}), and comparison of the relative peak heights for $\nu_{as}CH_2$ and νC=O shows that it is most likely to be trilaurin (see Fig. 3). The late-eluting component (peak 13) is an unsaturated triglyceride, whose FT-IR spectrum is very similar to that of the species corresponding to peak 1 of the Flora cheese.

Thus, cSFC–FT-IR can, even in the absence of any complementary spectroscopic detection, provide a considerable amount of information as to the chemical constitution of cheeses, with the possibility of individual speciation in some cases.

4. Conclusions

cSFC–FT-IR has been confirmed as an excellent technique for the analysis of fats and oils, and especially triglycerides. Despite the apparent similarity of the FT-IR spectra of saturated triglycerides, careful comparison of diagnostic peak intensities can give information as to their individual identities.

The identification of unsaturation in triglycerides is a straightforward process, and further work is in progress to extend the possibilities of specific triglyceride identification to unsaturated species. In the example studied in this paper, it became clear that at least one advertising claim could be substantiated scientifically!

Despite the successes of FT-IR alone as a detection system, it will clearly be advantageous to obtain more detailed information about the molecular masses and structures of analytes, as can be done by the addition of a mass-spectrometric detector after the FT-IR flow-cell. Preliminary results [12] show that the resultant "multiply-hyphenated" system can be constructed, and that it will be an extremely powerful analytical tool.

5. Acknowledgements

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