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ORIGINAL PAPER

Co-crystals of Beeswax and Various Vegetable Waxes with Sterols Studied by X-ray Diffraction and Differential Scanning Calorimetry

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Abstract In this study, mixtures of purified wax and sterols were melted and subsequently cooled. Using X-ray diffraction of the mixed, solid phase, it was shown that for up to 30–40 wt% sterols no measurable re-crystallisation of sterols occurred, i.e. the sterols became dissolved at a molecular level. Probably a form of amorphous co-crystals of sterols and wax is formed if the molecular ratio does not exceed 1:1. Differential scanning calorimetry (DSC) suggests that a minor amount of pure sterols could already be present at lower sterol levels. This may be because of the higher temperature at which the microstructure is probed when using DSC—melting of the wax might lead to crystallisation of the sterols. For application in foods, the structure as probed by X-rays at ambient temperatures is more relevant. When sunflower wax and rice bran wax are used, prevention of sterol crystallisation is even more pronounced, probably because the melting temperatures of these waxes are closer to the melting temperature of sterol crystals. Replacing the beeswax with a saturated fat (heRP70), sunflower oil, or jojoba wax (a liquid wax) substantially increases the amount of crystalline sterols. The difference between the various waxes and fats was qualitatively the same for X-ray diffraction and DSC. Stanols can be incorporated in the same manner and up to similar concentrations. Another insoluble nutritional compound, ursolic acid, has a greater tendency to crystallise in wax. This is probably because the melting temperature of ursolic acid is much higher than that of wax.

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Introduction

Sterols are therapeutically useful materials with efficacy in lowering cholesterol. Incorporation of sterols into various foods can therefore be desirable. However, incorporation of sterols into foods is complicated by the physical properties of the sterols. Because sterols are hardly soluble in fat or oil, large crystals are found in the food products prepared therewith. These crystals are less available for lowering blood cholesterol than so-called molecularly dispersed or "free" sterols. The technique currently used for incorporation of sterols into, e.g., margarines is esterification with fatty acids. As a result, the sterols become oil-soluble and are more bioavailable.

Assuming that esterification is not a desirable solution for all food products, it is important to understand the behaviour of sterol crystallisation in various compositions. Because in plants and fruits sterols are often naturally present in a mixed form with waxes, we investigated the molecular properties of such mixtures. With the application of microcapsules of such mixtures in foods in our minds, we focussed on investigating the extent to which the sterols are molecularly dispersed in the waxes. For the same reason, we attempted where possible to probe the structure at typical food temperatures, i.e. not exceeding ambient temperatures. Waxes may be typically suitable here because of their similar hydrophobicity to sterols and their typical plasticity which leaves a structural "flexibility" for the sterols to fit in the (amorphous) crystal network.

We have also tested the mixing of ursolic acid in beeswax. Ursolic acid is an anti-inflammatory compound similarly poorly soluble in water and oil as sterols. The fact that in plants and fruits ursolic acid, sterols, and waxes are often present in a combined form suggests that the compounds are miscible, i.e. can form a mixture desirable for protection of the plant.

The most commonly used wax in foods is beeswax. Beeswax contains a high proportion of various wax esters: C_{40} to C_{46} molecular species, based on 16:0 and 18:0 fatty acids, some with hydroxyl groups in the omega-2 and omega-3 positions. In addition, some diesters with up to 64 carbons may be present, together with triesters, hydroxypolyesters, and free acids. The major compound is triacontanyl palmitate. Beeswax crystallises in a orthorhombic structure with some disorder of the layers of molecules (amorphous) [1]. Similar structures are found for most natural plant waxes [2, 3].

In this report various other waxes are tested, including a wax which is liquid at room temperature: jojoba wax. Jojoba wax mainly contains C_{38} to C_{44} esters with one double bond in each alkyl moiety. Two waxes with higher melting point (i.e. "hard" waxes), rice bran wax and sunflower (SF) wax, are also tested. The hardness of wax depends on its composition of various esters—hard waxes are high in esters with two chains of equal and substantial length. Beeswax is high in an ester with one long chain and one shorter chain, making it somewhat softer.

The degree of molecular dispersability in commercial food-grade beeswax, and various other carriers, is estimated using X-ray diffraction and calorimetry (DSC). As a comparison, we will also measure mixtures of sterols in vegetable oil, and stanols or ursolic acid (nutritional compounds with similar physicochemical character) in waxes.

Literature on the subject of mixing "insoluble" compounds, for example sterols, in a waxy format suitable for inclusion in pharmaceuticals or food is scarce. Jenning and Gohla [4] describe solid lipid nanoparticles (SLN) made of beeswax. SLN are usually glyceride beads stabilised by block polymers and phospholipids, but here waxes are tested for their ability to protect retinol (which is hydrophobic, but with some water solubility) against chemical breakdown. It is argued that the uniform orthorhombic structure of waxes does not allow easy inclusion of compounds like retinol and hence does not lead to protection. Glycerides, for example glyceryl monostearate or glycerol behenate, form more suitable matrices for inclusion. Bodmeier et al. [5] describe wax microparticles containing water-insoluble ingredients. The particles were made by an O/W type melt dispersion technique using an emulsifier. Ibuprofen was the main model functional ingredient to encapsulate. However, the authors did not consider that the functional ingredient might be molecularly dispersed.

Materials and Methods

Preparation of Samples

White beeswax was provided by Fagron (Cera Alba, #7323). Refined SF oil was obtained from Albert Heijn ("Zonnebloemolie"), pure jojoba wax ("jojoba olie") from "de tuinen", Rijswijk. SF wax, rice bran wax, heRP70 (high-melting-point rapeseed oil), sterols, stanols, and ursolic acid (an internally produced apple waste measured to contain approximately 1/3 ursolic acid by weight, and a pure extract) were isolated/supplied internally by Unilever R&D Vlaardingen).

Analysis of the heRP70 indicated the fatty acid composition was: C16:0 ~5%, C18:0 ~40%, C20:0 ~9%, C22:0 ~43% with more than 99.5% total saturated fats.

The specific waxes used have following composition [6]. The Jojoba wax is liquid (melting point 7 °C) because of high levels of alcohols esterified with long-chain fatty acids (more than 98%) of C18:1, C20:1, and 22:1. Rice bran wax and SF wax have the highest melting temperature, because of the highest levels or long-chain esters. Rice bran wax contains esters of fatty acids (C26:0–C30:0 carbon atoms) and long-chain alcohols (C26–C30). SF wax is C16:0–C20:0 fatty acids linked to C_{30}–C_{34} alcohols, giving C_{46}–C_{54} molecular species. As already mentioned, the wax esters of beeswax are slightly shorter (hence the lower melting temperature), consisting of C_{40}–C_{46} molecular species. The total level free fatty acids in beeswax is, at maximum, 9–10%.

Sterols are a subgroup of steroids with a hydroxyl group at the 3-position of the A-ring. Stanols are the hydrogenated variant of sterols. Ursolic acid is a pentacyclic triterpene acid with a molecular weight of 456.68. Some minor compounds, for example fatty acids, will always be present in such compounds at a maximum level of 2%.

The basic preparation procedure was as follows. The mixture of nutritional compound and wax or oil was heated while stirring until a clear liquid was obtained. Depending on the ratio, this was obtained between 60 and 160 °C. The mixture was then allowed to solidify at room temperature.

X-ray Diffraction

The X-ray diffraction was performed as follows. Mixtures were poured (hot) into a sample holder. After solidifying for at least 24 h, data were collected with a Philips APD (automatic powder diffractometer) equipped with a Philips

PW1830 generator, operated at 50 kV and 40 mA. CU Kα radiation was used for the measurements. All X-ray diffraction was performed at room temperature. Crystallographic characteristics of the sample were calculated from corresponding X-ray diffraction spectra using Philips APD software.

We pre-melted all samples, including the pure sample. The reason is given in the next section.

Differential Scanning Calorimetry (DSC)

DSC was performed as follows. Approximately 15 mg of the flakes of the sterol/wax mixture were put in a stainless steel sample pan. The apparatus for measuring the heat exchange upon heating and cooling was a Perkin–Elmer power-compensated Pyris-1, equipped with a controlled cooling accessory using liquid nitrogen as cooling agent. Standard scan rate was 10 °/s. It should be said that in the range of 5–30 °/s we sometimes noted a difference in the first heating run between 105 and 180 °C. We also found this difference for pure sterols (without wax), hence we assume that for relatively old samples the sterols behave differently at different scan rates. In the cooling run and the second heating run this difference was no longer observed. For this paper we only used these curves, because we were then sure the samples had been pretreated similarly. Also, for the X-ray diffraction we only measured pre-melted samples, again to have the same sample history.

Generally, a faster scan speed increased the signal, but reduced the resolution. A scan rate of 10 °/s turned out to be a workable compromise between the two.

The measurement temperature in principle affects the X-ray diffraction patterns, and hence, possibly, the relationship between X-ray diffraction and DSC (where we effectively probe the microstructure at higher temperatures).

Results and Discussion

Sterol Incorporation Measured by X-ray Diffraction

In order to investigate the pure sterol/beeswax mixed crystal, a series of X-ray diffraction experiments was performed. The results are presented in Figs. 1–3. From Fig. 1, we clearly see some crystalline material at large reflection angles (2θ). The two largest peaks on the right probably arise from the beeswax (amorphous) crystalline material, which is the most abundant material in the sample. At small angles we observe at least four minor peaks. Considering these are not observed for the 0% sterol sample, we assume they are because of the presence of the

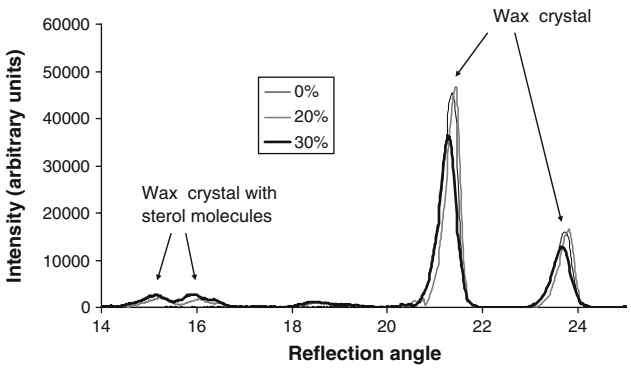

Fig. 1 X-ray analysis of various percentages (0–30%) of sterols in beeswax

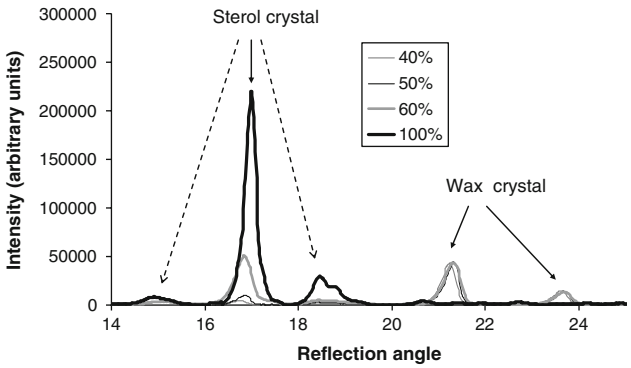

Fig. 2 X-ray analysis of various percentages (40–100%) of free sterols in beeswax. Note the enlargement of the vertical axis scale compared with Fig. 1

sterols. As we see in Fig. 2, the spectra for higher (>30%) contents of sterols and for the 100% sterol sample are very different. The 100% sterol sample gives one clear peak at an angle of $2\theta \approx 17$, and at least two minor peaks at 15 and 18.5. We therefore assume that any sterol crystals in the beeswax with the same microstructure (i.e. pure sterol crystals), would give peaks at the same place but with lower intensity depending on the concentration in the beeswax.

To be able to see judge better what happens between 30 and 40% sterols, we enlarged the area between $2\theta \approx 14$ and 20 from Figs. 1 and 2, in Fig. 3. It turns out that the peak at $2\theta \approx 16$ for 20 and 30% sterols has completely disappeared at 40%. The peak is replaced by a sharp peak at approximately $2\theta \approx 16.8$. This is the major peak in the 100% sterol sample, which is absent in the 30% sample. From this we can conclude that the minor peaks in Fig. 1 result from a crystalline material that does not consist entirely of sterols. They can only arise from wax crystals contaminated with sterols, or vice versa. More importantly, we can conclude there is a step-change in amount and type of crystals going from 30 to 40%.

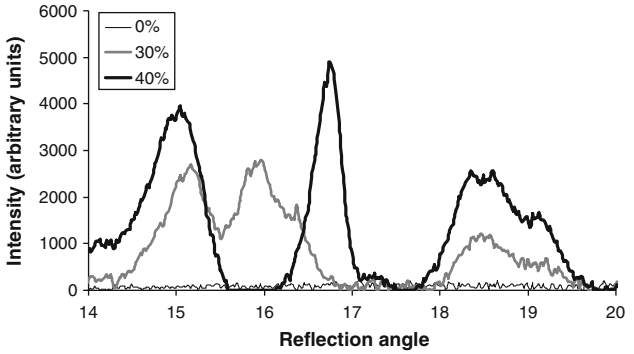

Fig. 3 X-ray analysis of 0, 30, and 40% of sterols in beeswax. Note the substantial amplification of both axis scales compared with Figs. 1 and 2

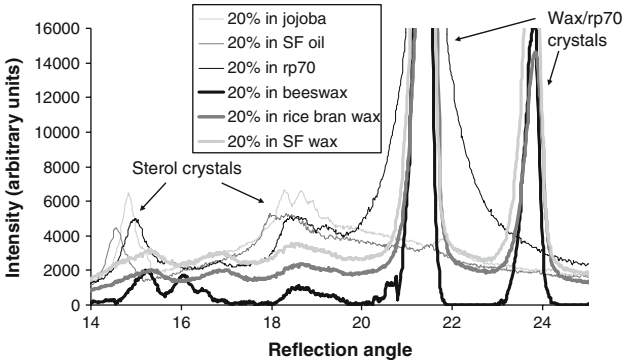

Fig. 4 X-ray analysis of 20% of sterols in various carriers. Note the different vertical axis scale compared with Fig. 2

If we consider the ratio of sterol to beeswax in terms of numbers of molecules instead of weight, we can show that roughly between 30 and 40% the molecular ratio is approximately 1:1 (levels of impurities are not exactly known so a more exact figure cannot be given). For this we used 415 as the molecular weight of the sterols and 676 as that of the beeswax. We can imagine that below 30–40% the sterol and beeswax molecules take alternate positions in some kind of amorphous crystal. If this is true, any sterol molecule that is added to the mixture at a concentration higher than 30–40%, will automatically be in contact with another sterol molecule, and hence will exist in a sterol crystal form. Apparently sterols mix very well with beeswax.

In principle, the resulting sterol/wax formulation could be emulsified, micronised, or ground to give smaller particles. As such, the mixture could be transformed into carriers of the nutritional compounds for slow release in the gastro-intestinal tract.

Note that the X-ray spectra of sterol/beeswax mixtures stored in the fridge for at least weeks were identical with those of freshly made mixtures. This rules out any slow (re)crystallisation processes within such a time span.

To test the flexibility of the types of structures capable of molecularly incorporating such high (20–30%) amounts of sterol crystals, we repeated the above X-ray analysis with other carriers. The carriers tested were SF oil, SF wax, jojoba wax, and heRP70. The results of these experiments are shown in Fig. 4.

According to Fig. 4, for at least three of the alternative carriers (heRP70, SF oil, and jojoba wax) substantially more crystalline material is observed in the range of angles corresponding to the sterol crystals compared with the beeswax "reference". The rice bran wax and SF wax profiles have the lowest peaks which are closest to those from beeswax. This suggests that, in addition to beeswax, rice bran and SF wax might be other waxes capable of incorporating sterols and acting as carriers.

Sterol Incorporation Measured by DSC

The presence of sterol crystals in wax was further analysed by use of DSC. After first preheating to 150 °C, the samples were cooled and reheated. The results of this cooling step and the second heating step with the various percentages of sterols in beeswax are shown in Figs. 5 and 6. The same samples were tested as in the X-ray analysis.

The basic features of Figs. 5 and 6 are the same. At low temperatures we see the two peaks of the beeswax (around 50–65 °C), while at high temperatures we see various sterol peaks, depending on the ratio of sterol to wax. For pure sterol, there is one major melting peak at 137 °C (second heating). For 40 and 60%, there are minor peaks around 100–110 °C that result from the presence of sterol crystals. For 20 and 30%, we cannot distinguish any peaks on this scale. Later (Fig. 8) it will be shown that even for 20% sterols in beeswax, there is a melting peak of sterol crystals. We conclude from this that the "threshold" level of sterols in wax that are supposedly still molecularly dispersed, is lower for DSC than for X-ray diffraction. The reason for this could be that DSC probes the structure at

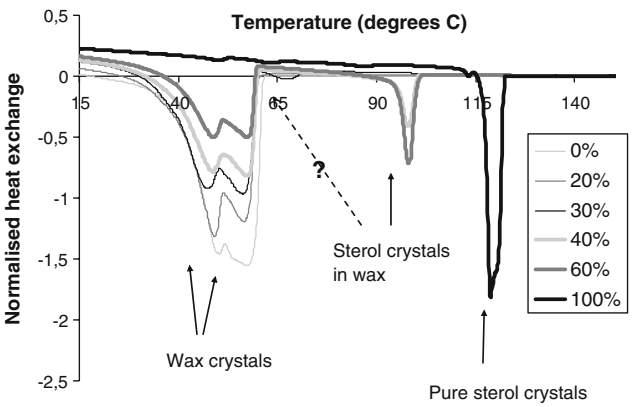

Fig. 5 DSC (calorimetry) analysis of various percentages of sterols in beeswax. Cooling of samples from Figs. 1 and 2. Heat exchange is in J g^{-1}

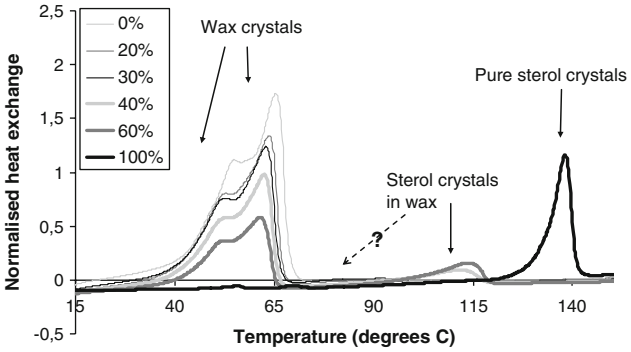

Fig. 6 DSC (calorimetry) analysis of various percentages of sterols in beeswax. Second heating of samples from Fig. 5. Heat exchange is in J g^{-1}

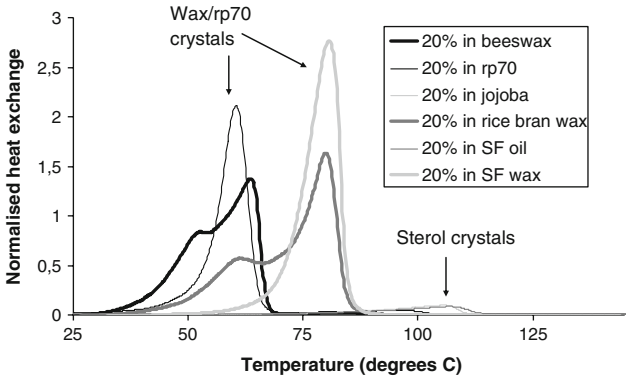

Fig. 7 DSC (calorimetry) analysis of 20% sterols in various carriers. Second heating of samples from Fig. 4. Heat exchange is in J g^{-1}

higher temperatures, and sterol crystals are formed because the wax has melted out.

Let us now discuss the results of the second heating of the samples prepared with 20% sterols in various other carriers. Figure 7 and, especially, Fig. 8 show the same basic features as the X-ray data—an increase of the amount of sterol crystals is observed upon replacement of beeswax

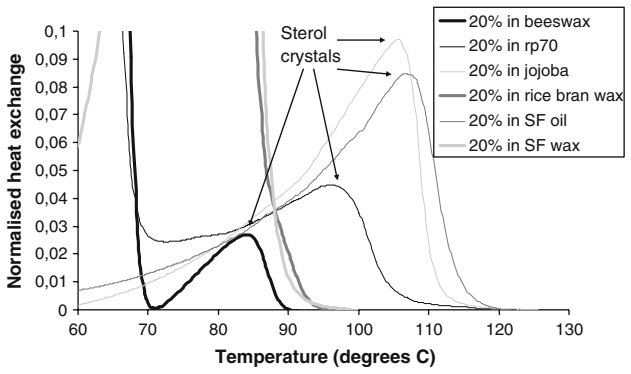

Fig. 8 DSC (calorimetry) analysis of 20% sterols in various carriers. Same data as in Fig. 7, but with substantial amplification of the horizontal and vertical axes. Heat exchange is in J g^{-1}

with heRP70, SF oil, or jojoba wax, with the exception of SF and rice bran wax. We cannot even detect the melting of the sterol crystals in the SF and rice bran wax. This suggests that molecular incorporation is improved with SF or rice bran wax. It is known that a mixed crystal is more easily formed between crystals with similar saturation and chain length, which correlates with similar melting behaviour [7]. Because melting of rice bran and SF wax occurs at higher temperatures than that of beeswax, this could be the explanation of improved molecular incorporation. On the other hand, it could be argued that increased hardness of waxes usually correlates with improved compatibility of wax esters, which would hence not allow inclusion of other compounds. Apparently the effect of the similarity of melting temperature dominates over this effect.

Stanol Incorporation Measured by X-ray Diffraction and DSC

Stanols are very similar to sterols with regard to molecular structure, cholesterol-lowering effect, and (lack of) solubility in oil and water. We have also tested the incorporation of stanols in the beeswax using the same procedure. Results of the X-ray and DSC analyses are shown in Figs. 9 and 10, respectively.

The X-ray analysis in Fig. 9 shows already at 20% stanols in beeswax, various peaks can originate from crystal stanols. Hence, the molecular dispersability of stanols in beeswax seems to be slightly worse than that of sterols.

Figure 10 shows similar behaviour of stanols in beeswax compared with sterols. However, the melting peak of the stanols is somewhat less pronounced than that of the sterols in Fig. 8, probably because stanols have a lower melting temperature than sterols.

We conclude from Figs. 9 and 10 that stanols can probably be incorporated in beeswax in a similar way to

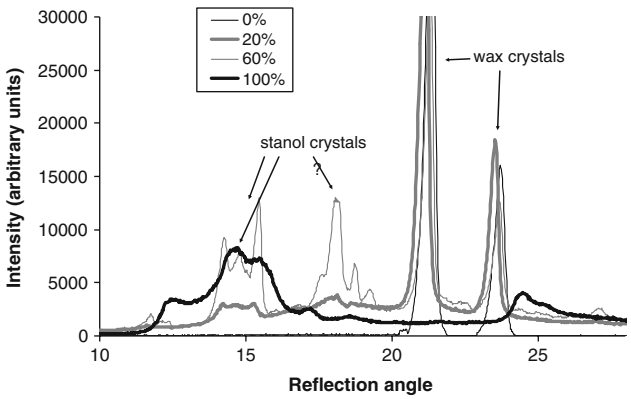

Fig. 9 X-ray analysis of various concentrations of stanols in beeswax

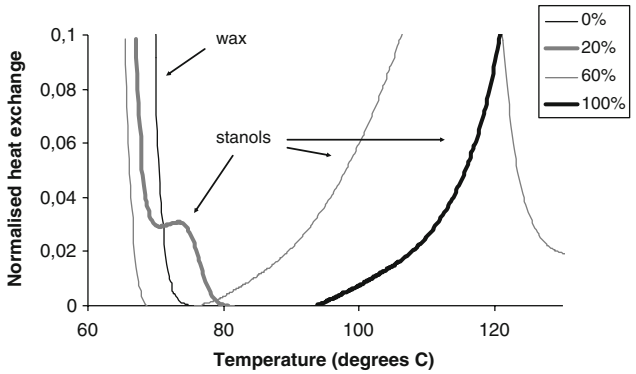

Fig. 10 DSC analysis of various concentrations of stanols in beeswax. Same scale as Fig. 8. Heat exchange is in J g^{-1}

sterols (the X-ray diffraction results show an increase in crystallisation whereas the DSC results show a decrease). On the basis of the similar molecular structures we would expect this behaviour.

Ursolic Acid Incorporation Measured by X-ray Diffraction

The flexibility of the beeswax to molecularly mix with oil-insoluble and water-insoluble compounds was further tested with ursolic acid. We tested the incorporation of an apple extract containing approximately 1/3 ursolic acid, and pure ursolic acid, in beeswax. The results of the X-ray analysis is shown in Fig. 11.

The melting point of ursolic acid is approximately 290 °C. This complicates incorporation in the procedure we used, because beeswax melts at much lower temperatures. Our intention was to heat the mixture to sufficiently high temperatures to melt all crystals. However, in the case of the apple extract this resulted in a burning reaction, leading to the formation of green and brown sediment and various off-flavours.

In Fig. 11, we see that both the 10 and 37.5% samples show peaks at an angle of approximately 15°. From the profiles of the pure samples we conclude that these peaks

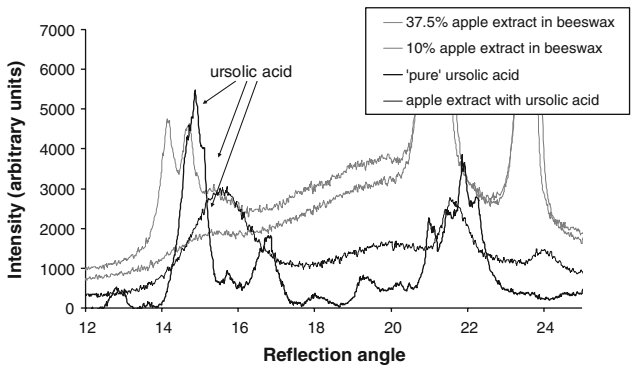

Fig. 11 X-ray analysis of various samples of ursolic acid in beeswax

can be attributed to ursolic acid crystals. Note that the profile of the peaks of the 10 and 37.5% samples are different: at 10% there is only one smooth peak whereas at 37.5% there are at least two sharp peaks. We conclude that although, by use of the current procedure, we cannot fully prevent formation of ursolic acid crystals, at low concentrations (10%) they may be of a different type, possibly some kind of mixed crystal.

Interestingly, we can observe in the X-ray profile of the 100% apple extract sample two small peaks at angles corresponding to wax crystal peaks. This means that in the apple extract some wax is present. This is not surprising, because the apple extract is an extract from the apple skin which contains (besides ursolic acid) apple waxes. In addition to this, apple extracts could contain primary alcohols or terpenes, which can plastify plant wax. This could be reflected in a weakened crystal behaviour of the waxes in the higher angle range.

Conclusions

By X-ray diffraction it was shown that sterols are mostly molecularly dispersed up to concentrations of about 30–40%. Probably some form of molecularly mixed crystals of wax and sterols are formed if the molecular ratio does not exceed 1:1, corresponding to a concentration between 30 and 40 wt%.

Differential scanning calorimetry suggests that some minor amount of pure sterols could be formed at lower sterol levels. This discrepancy may be because of the higher temperature at which the microstructure is probed when using DSC.

Sunflower wax and rice bran wax can replace beeswax in the above formulation. Molecular incorporation is even improved because the melting temperatures of these waxes are closer to the melting temperature of sterol crystals. Replacing the beeswax with heRP70, SF oil, or jojoba wax (a liquid wax) significantly increases the amount of crystalline material in the formulation.

Stanols can be incorporated in the same manner and up to similar concentrations. Another insoluble health compound, ursolic acid, can be incorporated at lower concentrations. This may have something to do with sample impurities in the apple extract used.

In principle, the mixtures could be transformed into carriers of nutritional compounds for slow release in the gastro-intestinal tract.

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