An EPR study of free radical generation during maceration of uncooked vegetables

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Abstract: Electron paramagnetic resonance (EPR) spectroscopy has been used to study free radicals formed in a range of plant tissues as a result of physical damage, with the objective of gaining some insight into the free radical chemistry that is initiated when uncooked vegetable products (eg salads) are eaten. Chemical spin traps were used to aid the detection of unstable free radicals; more stable radicals were detected directly. Commonly observed 'stable' species were the monodehydroascorbate radical, which has a characteristic doublet spectrum, and a single-peak resonance, which is presumed to come from free radical centres stabilised in macromolecules. In mushrooms (Agaricus spp), spintrapping experiments using either \( \alpha-(4\text{-pyridyl-1-oxide})\text{-N-t-butyl-nitrotrone} \) (4-POBN) or phenyl-t-butyl-nitrotrone (PBN) showed the formation of large quantities of adducts of the radical from 4-hydroxymethylbenzene diazonium salts. Pleurotus species, in contrast, gave signals consistent with the formation of unidentified C-centred radicals. With other foodstuffs, reaction with 4-POBN was complex and signals from 4-POBN* and \( \text{CO}_2^- \) adducts were observed along with the t-butylhydroxy-nitrooxide radical (an adduct breakdown product). Investigation of carrot hypocotyl rootstock in the presence of 5-(diethoxypyridyl), 5-methyl-1-pyrroline-N-oxide (DEPMPO) revealed adducts of \( \cdot\text{OH} \) and unidentified C-centred radicals. Free radical interactions between food components were suppressed by the suppression of the signal from the 4-POBN adducts of lettuce by onion, garlic, satsuma or vinaigrette, but not by olive oil. In addition, an appreciable decrease in spectral intensity of the 4-POBN adduct from lettuce was observed in the presence of saliva, which suggests that saliva contains free radical scavengers which are able to compete successfully with the spin trap.

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INTRODUCTION

The consumption of diets containing substantial quantities of fruit and vegetables is associated with reduced risks for the development of several types of cancer,1,2 and heart disease.3,4 Oxygen-derived free radicals have been implicated in many disease processes,5 and Bolton-Smith et al.6 have shown that there is an inverse relationship between the intake of antioxidant vitamins and the risk of coronary heart disease, particularly in men. Consequently, considerable scientific activity is being aimed at evaluating the protective modes of action of vitamins and other antioxidant molecules on mammalian cells,7 and dietary supplementation is now common in developed countries.

Plants represent a major source of vitamins and other antioxidants such as polyphenols in unsupplemented diets, and there is now considerable interest in the genetic manipulation of crops in order to design foods and diets for optimal health effects.8 In addition to the production of enhanced levels of selected vitamins, maximisation of the total antioxidant capacity of foods is becoming recognised as an important quality criterion.9

In addition to the production of antioxidant molecules as aids to survival in an aerobic environment under solar radiation, plants make use of free radicals in normal metabolic processes such as photosynthesis10 and in their defences against (potentially) pathogenic organisms and predators.11 In the latter case, free radical production is initiated in response to cellular damage and, consequently, similar reactions should occur when the product is eaten. Little attention has, however, been given to the generation of free radicals during food consumption, yet these free radical reactions might be expected to occur to a substantial extent during the digestive process and may also be relevant to diseases of the digestive tract.

The present paper reports observations using EPR spectroscopy to investigate free radical generation on maceration of a range of vegetable products that are commonly consumed in an uncooked state. The mini-
beadbeater used for maceration is considered to provide a reasonable simulation of mouth chewing in that the food particles are crushed by physical impact and there is no external heating. These measurements thus represent a crude simulation of eating individual ingredients of a salad. In addition, some preliminary measurements on interactions between various food components are reported along with observations on the influence of saliva on the course of these reactions.

EXPERIMENTAL
Materials
Cabbage (Brassica oleracea), carrot (Daucus carota), celery (Apium graveolens), coriander (Coriandrum sativum), cress (probably Brassica spp), cucumber (Cucumis sativus), garlic (Allium sativum), lettuce (Lactuca sativa), mushrooms (Pleurotus ostreatus (Polyporaceae) and various varieties of cultivated mushrooms (Agaricus bisporus), onion (Allium cepa), parsley (Petroselinum) and satsuma (Citrus reticulata), along with vinegarette and olive oil, were purchased from local food retailers and used within 48h. Saliva was obtained fresh from one of us (SMG) and used immediately. The spin traps 2-(4-pyridyl-1-oxide)-N-t-butyl-nitroxide (4-POBN) and phenyl-t-butyl-nitroxide (PBN) from Sigma Chemical Co (Poole, UK), 13C-PBN from OMRF Spin Trap Source (Oklahoma City, OK, USA) and 5-(diethoxyphosphoryl)-methyl-1-pyrrolone-N-oxide (DEPMPO) from Calbiochem-Novabiochem (UK) Ltd (Beeston, UK) were all used without further purification, as no EPR signals were observed from solutions of the spin traps alone.

Sample preparation
Solutions (ca 100 mM) of spin traps were made up in deionised water and used immediately or from small aliquots previously stored in the dark at −15°C. Samples containing approximately 50mg of tissue were macerated in a mini-beadbeater (Biospec Products, Bartlesville, OK, USA) in 250μl of deionised water or spin trap solution for 20s, after which the liquid was decanted immediately into a quartz flat cell for EPR analysis. For the measurements involving dressings or saliva, ca 250μl aliquots were added to the intact tissue along with the spin trap solution before maceration.

EPR spectroscopy
All measurements were performed at ambient temperature (20 ± 2°C) on a Bruker ESP300E (Bruker UK Ltd, Coventry, UK) computer-controlled spectrometer operating at X-band frequencies and using an ER4103TM/9202 cylindrical cavity. Microwave generation was by means of a klystron (ER041MR), and the frequency was measured with a built-in frequency counter. Spectra were recorded routinely at 1024 points as first derivatives of the absorption using 10mW microwave power, 100kHz modulation frequency and 0.1mT modulation amplitude. Sweep widths of 10mT were used for experiments involving 4-POBN or PBN as spin trap and 20mT for those with DEPMPO, in each case centred on 348mT. Other experimental parameters, such as receiver gain, conversion time, time constant and number of scans over which the spectra were accumulated, were optimised for individual spectra. Where spectral intensity permitted, however, additional spectra were recorded as second derivatives with lower modulation amplitudes in order to improve the resolution of small hyperfine splittings. Spectral analyses were confirmed by simulation using the Bruker SimFonia software package (Bruker Instruments, Inc, Billerica, MA, USA).

RESULTS AND DISCUSSION
Three types of EPR response were observed from macerated tissues: (i) a spectrum was observed in a specimen macerated in the absence of a spin trap; (ii) a spectrum was observed from a sample macerated in the presence of a spin trap; or (iii) no spectrum was observed either with or without the presence of a spin trap. There were, however, considerable differences between apparently similar samples. These were investigated more thoroughly with carrot hypocotyl rootstock and lettuce leaves, where the EPR responses varied greatly between samples. For this reason the results are presented in terms of the different types of EPR response that were observed.

Signals produced in the absence of spin traps
On maceration in water, samples of cabbage leaf, carrot hypocotyl rootstock, celery stalk, cress shoots, cucumber fruit and parsley leaf produced a doublet spectrum with a splitting of 0.18mT (Fig 1(a)), which is characteristic of the monodehydroascorbate (‘ascorbate’) radical. Similar observations have been reported for other types of biological tissue; for example, Büttnner and Jurkiewicz considered this radical to be a marker of oxidative stress. The spectra of the ascorbated radical persisted in the presence of spin traps, demonstrating that the spin traps used in

Figure 1. Representative EPR spectra obtained in the absence of any spin trap: (a) monodehydroascorbate radical in macerated cabbage; (b) single-peak radical signal in macerated carrot.
the present experiments were ineffective as scavengers of this radical. In several samples a relatively broad single-peak resonance (peak-to-peak width ca 0.4 mT) was also seen (Fig 1(b)), often obscuring the spectrum of the ascorbate radical. Such signals are common in biological tissues (see eg Ref 14), especially when stressed by either biotic\textsuperscript{15} or abiotic\textsuperscript{16} challenges. It is probable that a considerable proportion of these signals corresponds to stabilised free radical centres in macromolecular species, but there is also the possibility of rapid turnover of unstable species.\textsuperscript{17}

**Signals obtained in the presence of spin traps**

In ‘spin-trapping’ experiments a diamagnetic molecule (spin trap) reacts with an unstable free radical to produce a more stable radical (radical adduct) which can be characterised by physical techniques such as EPR spectroscopy. In the case of nitrones, nitrooxide radicals are formed by the addition of a free radical to the \(x\)-carbon. Spectra from adducts of 4-POBN and PBN typically consist of triplets from interaction of the unpaired electron with the \(^{14}\text{N} (I=1)\) nucleus. Usually, each of these peaks is further split into a doublet as a result of interaction with the \(^{1}\text{H} (I=\frac{1}{2})\) nucleus on the \(x\)-carbon atom to which the trapped radical is bound. Occasionally, additional hyperfine splittings from more remote \(^{1}\text{H}\) atoms can also be resolved, when spectra are recorded with low modulation amplitudes. Spectra from radical adducts of DEPMPO contain a doublet splitting from \(^{31}\text{P} (I=\frac{1}{2})\) in addition to the \(^{14}\text{N}\) and \(^{1}\text{H}\) hyperfine structure seen with 4-POBN and PBN.

**Signals obtained from mushrooms in the presence of 4-POBN or PBN**

Spin trap adducts were observed with all mushroom samples when they were macerated in the presence of 4-POBN, but the parameters were different for *Pleurotus* and *Agaricus* spp. Spectra of the adducts are shown in Figs 2(a) and 2(b), where the hyperfine parameters for *P ostreatus* are \(a(^{14}\text{N})=1.58\text{ mT}\) and \(a(^{1}\text{H})=0.24\text{ mT}\) and those for *A bisporus* are \(a(^{14}\text{N})=1.56\text{ mT}\) and \(a(^{1}\text{H})=0.42\text{ mT}\). Similar results were obtained with *A bisporus* when PBN was used as the spin trap (not shown); in this case the hyperfine parameters were \(a(^{14}\text{N})=1.60\text{ mT}\) and \(a(^{1}\text{H})=0.42\text{ mT}\). These latter values closely match those reported by Hiramoto et al\textsuperscript{18} for PBN adducts of the radical formed from 4-hydroxymethylbenzene diazoniump salts, a carcinogen found in *A bisporus*. The parameters for the *P ostreatus* adducts are similar to those reported for several C-centred radical adducts of POBN.\textsuperscript{19}

The high intensity of the spectrum from *A bisporus* allowed the resolution at high gain of additional satellite peaks with 1–3% of the intensity of the main peaks. Hyperfine splittings were 0.80 and 0.50 mT for the 4-POBN adduct (Fig 2(c)) and 0.80 and 0.52 mT for the PBN adduct (not shown). These almost certainly originate from \(^{13}\text{C}\) nuclei \((I=\frac{1}{2},\) natural abundance 1.1%) close to the N—O group, but it is not immediately clear how they should be assigned. By using PBN specifically labelled with \(^{13}\text{C}\) on the \(x\)-carbon atom, an adduct spectrum (Fig 2(d)) was generated with \(a(^{13}\text{C})=0.61\text{ mT}\) from this carbon and weak side peaks with \(a(^{13}\text{C})=0.80\) and 0.53 mT which correspond, therefore, to the natural abundance isotopes in the \(x\)-butyl group (the larger splitting with the smaller intensity is assigned to the tertiary carbon, and the smaller splitting with the larger intensity to the three equivalent methyl groups).

**Signals from other foodstuffs in the presence of 4-POBN**

When macerated in the presence of 4-POBN, carrot hypocotyl rootstock, celery petiole, coriander leaf, cucumber fruit and lettuce leaf gave EPR spectra of radical adducts that were similar to those described for *P oleatus*. Spectral responses, however, differed for apparently similar species, presumably as a result of factors such as age, genotype, husbandry, etc, which were outside the control of the experimenters in this work. We have performed more detailed investigations on lettuce leaves and carrot hypocotyl rootstock as representatives of photosynthetic and non-photosynthetic...
thetic tissues respectively. The results are summarised in the following paragraphs.

Many of the carrot and lettuce samples produced a dodecet signal (Fig 3(a)), which is similar to that reported by McCormick et al.\(^{20}\) for the 4-POBN adduct of the 4-POBN• radical, which has parameters \(a(14\text{N})=1.49\text{ mT}\), \(a(14\text{N})=0.185\text{ mT}\) and \(a(1\text{H})=0.18\text{ mT}\). Gildeuwel et al.\(^{21}\) reported a spectrum from lettuce macerated in the presence of 4-POBN with two components, one having \(a(14\text{N})=1.492\text{ mT}\), \(a(1\text{H})=0.515\text{ mT}\) and \(a(1\text{H})=0.050\text{ mT}\) and the other having \(a(14\text{N})=1.493\text{ mT}\), \(a(1\text{H})=0.185\text{ mT}\) and \(a(1\text{H})\) (two equivalent) = 0.041 mT. This analysis is not unique, however, and their spectrum can be simulated by a single component with \(a(14\text{N})=1.493\text{ mT}\), \(a(14\text{N})=0.182\text{ mT}\), \(a(1\text{H})=0.145\text{ mT}\) and \(a(1\text{H})=0.050\text{ mT}\). The similarity of the \(a(14\text{N})\) parameters to those reported by McCormick et al.\(^{20}\) suggests that this might also correspond to the 4-POBN• radical adduct; the resolution of the additional \(a(1\text{H})\) splitting is almost certainly a function of the modulation amplitudes used (0.025 mT in Ref 21, 0.09 mT in Ref 20).

The dodecet signal was unstable and decayed to reveal a sextet spectrum (Fig 4) with \(a(14\text{N})=1.59\text{ mT}\) and \(a(1\text{H})=0.32\text{ mT}\). This signal was relatively stable and its parameters are similar to those reported for the \(\text{CO}_2^+\) adduct of 4-POBN.\(^{19}\)

In carrot and lettuce samples where the dodecet signal was absent, a quartet signal was often seen immediately after macerating the samples. This quartet, which is shown in Fig 3(b), along with the sextet signal described above, disappeared within 1 h of extraction. The spectral parameters are \(a(14\text{N})\approx a(1\text{H})\approx 1.45\text{ mT}\) and probably correspond to the \(t\)-butylhydronitroxide radical, which could be formed as a result of the breakdown of 4-POBN adducts; a similar spectrum has been reported for breakdown of PBN adducts.\(^{22}\) At the present time we have no information on the reasons for the adducts’ apparent instability in some specimens but not in others.

**Signals obtained from carrot in the presence of DEPMPO**

There are relatively small differences in the hyperfine splittings of different types of 4-POBN adducts, so DEPMPO was also used as a spin trap for macerated carrot tissue in an attempt to identify the radical(s) produced. Representative spectra obtained over a 6 day period are shown in Fig 5. The initial spectrum (Fig 5(a)) consisted of two components, one having \(a(13\text{P})=4.86\text{ mT}\), \(a(14\text{N})=1.47\text{ mT}\) and \(a(1\text{H})=2.06\text{ mT}\), characteristic of adducts of C-centred radicals, the other having \(a(13\text{P})=4.75\text{ mT}\), \(a(14\text{N})=1.39\text{ mT}\) and \(a(1\text{H})=1.38\text{ mT}\), which correspond to the adduct of the 'OH radical.\(^{23,24}\) The intensity of the 'OH adduct increased progressively over the first 3 days, after which it stabilised, then decreased slowly; nevertheless, it still produced an intense signal after 6 days. The intensity of the C-centred adduct, in contrast, decreased steadily and had virtually disappeared after 4 days. During this period a third component with approximate values \(a(13\text{P})=4.61\text{ mT}\), \(a(14\text{N})=1.41\text{ mT}\) and \(a(1\text{H})=2.13\text{ mT}\) was resolved (Fig 5(b)), and this probably corresponds to a second C-centred adduct. It is not possible to say with certainty whether this was generated during the incubation period or was simply obscured by the original C-centred adduct during the early measurements; it may correspond to the radical that was assigned to \(\text{CO}_2^+\) when 4-POBN was used, but we have no additional evidence to substantiate this. During the later stages of the experiment the signal from this second C-centred radical adduct decreased.
in intensity more rapidly than that of the 'OH adduct, but it was still clearly visible after 6 days (Fig 5(c)). Additional hyperfine structure, presumably originating from protons on the methyl group and the C3 and C4 positions of the pyrrole ring, was revealed in the spectrum of the 'OH adduct when it was recorded with low modulation amplitude (Figs 6(a) and 6(b)). Although this hyperfine structure is superficially similar to that obtained from the reaction of DEPMPO with 'OH (generated from the reaction of H₂O₂ with Fe(II)), the splittings (average 0.037 mT) are appreciably smaller than those obtained with the simple Fenton system (average 0.045 mT) (Goodman BA, unpublished). It appears, however, that there may be considerable variation in the number of peaks and magnitudes of these small splittings, because Fréjaville et al.²⁴ have reported three equivalent 'H atoms with a splitting of 0.027 mT in DEPMPO-'OH.

Samples that produced no EPR signal
Onion and garlic bulbs produced no EPR signal under any of the experimental conditions used. Of the various tissues investigated, these were the only ones which consistently failed to generate a spectrum.

Effects of interactions with other salad components on free radical generation in lettuce
We have made some preliminary investigations on the role of chemical interactions between different salad components in free radical generation. Initially, we considered interactions of lettuce with vinaigrette and olive oil salad dressings and with satsuma, which was considered to be representative of citrus components in salads. The EPR spectra obtained on macerating these mixtures in the presence of 4-POBN are shown in Figs 7(b)–7(d) along with that of undressed lettuce in Fig 7(a). Vinaigrette and satsuma completely suppressed formation of the EPR signal, whereas olive oil had no effect. It is also interesting that the ascorbate radical signal was not seen with the sample containing satsuma. In addition, lettuce macerated with small amounts of either onion or garlic in the presence of 4-POBN produced no signal (see eg Fig 7(e)). Thus components in vinaigrette, satsuma, onion and garlic either compete successfully with 4-POBN for the lettuce-derived radicals or react with the 4-POBN adducts of these radicals. In either event, they all must possess potent free radical scavengers.

Effect of saliva on free radical generation
In order to estimate the possible significance of these free radical reactions in a more realistic dietary situation, the lettuce/spin trap maceration was also performed in the presence of saliva. The EPR spectrum (Fig 7(f)) shows that saliva caused a major reduction in the intensity of the 4-POBN adduct signal, demonstrating that it also contains powerful free radical scavengers. A major proportion of this may
be uric acid, since the total antioxidant activity of saliva has been correlated with uric acid content.25

**GENERAL DISCUSSION**

These experiments show that a range of biologically viable foodstuffs generated free radicals when macerated; similar free radical generation would therefore be expected to occur when these food products are eaten. The foodstuffs investigated here fall into two groups.

In one group the relatively stable ascorbate radical was seen, and this is formed as a result of the one-electron oxidation of ascorbic acid. The antioxidant (free radical-scavenging) properties of ascorbic acid are well known,26 and the ascorbate radical probably represents the product of such reactions in damaged tissue. It is interesting to note that the simultaneous presence of the ascorbate radical and free radical adducts of spin traps in the same sample was rare. This suggests that either ascorbic acid is a better free radical scavenger than the spin traps or that the adducts of the spin traps are unstable in the presence of ascorbic acid. These possibilities are further supported by the observation that free radical adducts of 4-POBN were not seen when lettuce was macerated in the presence of satsuma, which is known to have an appreciable ascorbic acid content.27

In the second group of samples the results were dominated by unstable free radicals. 'OH and C-centred radical species were identified as major components in macerated carrot when DEPMPO was used as spin trap, and results obtained with 4-POBN and tissues from several different plant species are consistent with the presence of C-centred radicals.

In detailed studies of lettuce leaves and carrot hypocotyl rootstock, we found variable behaviour from sample to sample. The adduct of the 4-POBN radical was often seen at the beginning of the measurements. This radical can be generated by the action of peroxidases on 4-POBN in the presence of H2O2,20 either as a result of atom extraction from 4-POBN by the 'OH radical or through direct oxidation of 4-POBN by peroxidases. The formation of the t-butylhydroxynitroxide radical, which was also sometimes seen in early measurements, may also result from the reaction of 'OH with 4-POBN. In this case the observed radical results from breakdown of the initial radical adduct.22 Thus the reaction of 'OH with 4-POBN may not be straightforward and could result in the generation of at least two EPR-detectable species that are not simple hydroxyl radical adducts of the spin trap.

In the case of carrot, measurements using DEPMPO as spin trap clearly demonstrated that hydroxyl free radical generation took place over an extended period of time after maceration. This indicates that the production of free radicals was not simply the consequence of the fission of chemical bonds during the maceration process and supports the evidence from the reaction of 4-POBN with lettuce that enzymatic processes are involved in free radical generation.

Also, although the basic assumption of spin trapping is that an unstable free radical reacts with a spin trap to form a (more) stable radical (radical adduct) which can then be characterised, different radical adducts display different stabilities, and spin traps may have some preference for reaction with certain radicals over others. Both of these problems have been encountered in the present work. The most obvious case of adduct instability is the observation of the formation of the t-butylhydroxynitroxide radical in some of the spin-trapping experiments using 4-POBN. However, other less dramatic cases are also seen. For example, when lettuce was macerated in the presence of 4-POBN, the intensity of the initial spectral component from the 4-POBN adduct decreased with time to reveal the spectrum of a C-centred radical. In contrast, with carrot the 'OH adduct of DEPMPO was stable over several days, during which period reactions involving C-centred radical adducts were observed. None of the three spin traps used in the present work appeared able to scavenge the ascorbate radical or the radical responsible for the broader single-peak resonance in the absence of spin traps. Similar observations have been made in model coffee systems.28

The absence of any EPR signal from onion and garlic in the absence or presence of spin traps is probably the consequence of the existence of strong free radical scavengers in members of the Allium genus. These scavengers are probably sulphur-containing compounds,20 although polyphenolic antioxidants are also present in substantial amounts; oxidation of sulphur compounds may result in the production of dimeric species, in an analogous manner to glutathione, and hence not produce radicals for detection by EPR. Garlic has been associated with

![Figure 7. Effects of second components on the EPR spectra of lettuce macerated in the presence of 25 mM 4-POBN. (a) no additive; (b) vinaigrette; (c) olive oil; (d) satsuma; (e) garlic; (f) saliva. Spectral parameters are the same as those for Fig 4.](image-url)
tumour inhibition properties\textsuperscript{30,31} and beneficial effects on the cardiovascular system, which have been attributed to its strong antioxidant activity.\textsuperscript{32} The presence of antioxidant free radical scavengers is probably the explanation for the observation that small quantities of onion or garlic tissue completely suppressed the generation of EPR signals from lettuce macerated in the presence of 4-POBN. The observation that vinaigrette, but not olive oil, also suppressed the formation of EPR signals with macerated lettuce suggests that the former, but not the latter, dressing possesses stronger free radical-scavenging properties than 4-POBN.

The presence of saliva brought about a major reduction in intensity of the EPR signal from macerated lettuce, which indicates that saliva also possesses strong free radical-scavenging properties. Thus free radical scavenging may represent an important role for saliva in the digestion process, beyond that of initial tissue breakdown. The reducing (antioxidant) capacity of saliva has been shown to decrease with age and with the presence of certain diseases and clinical disorders.\textsuperscript{33} Thus, if free radical generation during the digestion of foods has health implications, it would be expected to become more significant with increasing age of the consumer, especially at times of impaired health.

CONCLUSIONS

When many plant food products in biologically viable states are physically damaged, as would occur on chewing, they generate free radicals, which can be detected using EPR spectroscopic techniques. Our results suggest that the generation of these reactive oxygen species is not simply the consequence of chemical bond breaking during tissue maceration and that enzymatic processes make an appreciable contribution. Thus radical reactions might be expected to occur through the digestive system and not just in the mouth.

Free radical generation in food products is influenced by interactions with other food components, so it is important to consider whole meals and not just individual foodstuffs if a true picture of free radical generation in the digestive tract of the consumer is to be obtained. It should also be recognised that such reactions may have a role in both the nutrition and control of micro-organisms in the digestive tract. Our measurements also indicate that saliva may have an important function as a free radical scavenger if free radical control is not achieved through an appropriately balanced diet. Thorough mastication of food may therefore have positive benefits in controlling free radical generation in the stomach and maybe other parts of the digestive tract.

In view of the recognised importance of free radical reactions in health and nutrition, more extensive investigations of their generation during food preparation and digestion need to be performed in order to develop improved knowledge of the processes by which plant food products make a positive contribution to human health.

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