A Review of the Production of Green Notes

By Cathy Fabre and Gérard Goma, Critt-Bioindustries, Cedex, France

Green notes are present in a wide variety of fresh leaves, vegetables and fruits. These compounds are responsible for a so-called green odor, green aroma, fresh note or green note. Related compounds present in many plants include trans-2-hexenol, cis-2-hexenol, trans-3-hexenol, 1-hexanol, 1-hexanal and cis-2-hexenal; organoleptically, each of these compounds possesses a specific type of green character, which is why they are known collectively as the green notes. Such compounds can be used to sharpen and enhance flavored products, such as those products having fruit flavors.[1,2] Green notes have been isolated from plants or chemically synthesized: they are present in plant essential oils and have been obtained by steam distillation of plant material. However, the supply of flavor materials for the food industry is mainly based on the biosynthetic capabilities of plants. Cultivation problems, environmental defects and other reasons have led to a decreasing availability of some plant resources. These developments are accompanied by an increasing industrial requirement and various legal restrictions concerning the use of natural and chemosynthetic compounds. This is especially evident in the US market. Natural flavors are perceived as being better than the synthetic varieties and are therefore able to command a higher price. In 1995, the worldwide market for natural green notes was estimated at US$20-40 million per annum. The increasing demand for these natural compounds now exceeds their supply from traditional sources; this has motivated research efforts toward finding alternative natural ways of obtaining these materials.

Moreover, with the recent surge of interest in biotechnology it is easy to understand how flavor-producing microorganisms have turned from being laboratory curiosities into potential new sources of natural flavor compounds. Green notes also have been biosynthetically produced as reported by several authors.[1,3,4,5]

Biosynthetic Pathways of Green Notes

The biosynthetic pathway to the green notes is present in many plant tissues such as leaves, fruits, and vegetables and involves the action of three enzymes (a lipoxygenase, a hydroperoxide lyase and an oxidoreductase).The lipoxygenase-catalysed oxidation of fatty acids containing a 1,4-pentadiene sequence into positionally specific acyl hydroperoxides is a well-documented enzymatic reaction.[6] The hydroperoxide lyase cleaves the hydroperoxide to produce a C-6 unsaturated aldehyde. Then aldehyde isomerase, when present in the environment and under certain conditions, catalyses the formation of an aldehyde which is transformed into an alcohol by the action of alcohol dehydrogenase.

A process using crude enzyme preparations from different biological sources might increase the conversion of fatty acid precursors into the desired volatile products.[6] Except for the hydroperoxide lyases, these enzymes are commercialized; in order to improve the action of the hydroperoxide lyases, it is possible to add vegetal homogenates containing sufficient quantities of this enzyme.[7] A process using a crude vegetal preparation of non-purified enzymes could be used to efficiently convert the fatty acids into volatile compounds. However, because of the lack of available lyases, there is no an enzymatic system for carrying out green note industrial production.[8]

Precursors of the Green Notes

It is well known that unsaturated fatty acids are the precursors of volatile compounds responsible for the characteristic odors of plants ; in particular, linoleic and linolenic acids are present in different vegetal tissues such as thea, grape, soyabean, tobacco and leek. The works of Gonzalez et al.[9] concerning the process of traditional tea maceration revealed that the concentrations of linoleic and linolenic acids decreased while the quantities of hexanal and trans-2-hexenal increased in the volatile fraction. Moreover, the studies of Grosch and Schwarz[10] demonstrated that the fatty acids contained in cucumbers (43% of linolenic acid and 20% of linoleic acide) led to the formation of hexanal and hexenal. Numerous investigations have confirmed this aroma production and revealed the oxidation reaction of the fatty acids in Ginkgo biloba leaves,[11,12] in apples, cabbages, strawberry,[1] rumex, vine leaves and the tops of radish.[13]

The composition of unsaturated fatty acids can vary; the unsaturated fatty acid is provided in a free acid form (e.g. carboxylic acid or salt), as opposed to an esterified form (triacylglycerol). Examples are oleic acid, linoleic acid, linolenic acid, arachidonic acid, eicosapentaenoic acid and ricinoleic acid which are contained in fat or oil coming from vegetal, animal or microbial sources.[14] Among the

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vegetal oils used as an unsaturated fatty acid source, the best known are soya oil, safflower oil, olive oil, sunflower oil, pips of grape oil, corn oil, wheat oil, peanut oil, walnut oil, cottonseed oil, linseed oil and cocoa butter. These unsaturated fatty acids could also have an animal origin and be provided by butterfat, lard, cod-liver oil , sardine, other fish or mammals.[15] It is also possible to transform these oils or fats by chemical or enzymatical hydrolysis in order to obtain free unsaturated fatty acids (Table 1). In the first case, the oil is converted by chemical saponification;[14] in the second case, the preparation of lipases used comes from vegetal, animal or microbial sources[16]. The studies of Luquet et al.[17] revealed that the oxidation of triacylglycerol could be carried out without lipases, only with a crude preparation of soyabean enzymes. The use of such a crude preparation offers new opportunities for industrial applications in aroma production.

Finally, it is important to mention that an excess of substrate could sometimes lead to an inhibition of the enzymes involved; the use of a biphasic system water/hexane or tampon borate/octane decreases such effects and contributes to a better solubility of the fatty acids in the organic phase.[19, 20]

Enzymes Cleaving Fatty Acids

The use of enzymes is of great interest for the aroma industry because the aromatic compounds produced according to an enzymatic process have the benefit of the natural label.

The lipoxygenases: The lipoxygenases catalize, specifically, the dioxygenation of unsaturated fatty acids by molecular oxygen. These enzymes lead to the formation of peroxyl radicals which are converted into hydroperoxides (hydroperoxydienes derivatives). The lipoxygenase activity has been identified in lower and higher vegetal organs.[21] These enzymes are of great interest because of their role in aroma biosynthesis in plants. Moreover, the recent studies of Hsieh[22] revealed that lipoxygenases were present not only in numerous vegetals but also in several animal tissues. The lipoxygenases catalysed the insertion of oxygen in the unsaturated fatty acids in order to form different hydroperoxides according to the insertion position. Depending on the season, the source, the tissue and the environmental parameters, the amount of these enzymes varied considerably.

Table 1. Composition of oils in fatty acids[18]

Substrates	Saturated fatty acids %	Unsaturated fatty acids* %	Mono unsaturated fatty acids %
Canola oil	6	26	58
Safflower oil	9	78	13
Sunflower oil	11	69	20
Corn oil	13	61	25
Olive oil	14	8	77
Soybean oil	15	54	24
Peanut oil	18	34	48
Palm oil	51	10	39
Butterfat	66	2	30
Coconut oil	92	2	6

* Polyunsaturated fatty acids : linoleic acid + linolenic acid

Table 2 : Amount of green notes in the vegetals

Lipoxygenase sources	Products	Concentration	References
Apricot	hexanal	3 mg/g*	Hatanaka et al.[26]
Tomato	hexanal	3,5 mg/g*	Hatanaka et al.[26]
Soya	hexenal	5,8 mg/g*	Hatanaka et al.[26]
Fern	hexenal	3,9 mg/g*	Hatanaka et al.[26]
Apple	hexanol	1,6 mg/l**	Almosnino and Belin[27]

* mg/g : mg of aromatic compound per gram of fresh vegetal substrate
** mg/l : mg of aromatic compound per liter of reactional medium

Lipoxygenases from vegetal sources: Vegetal lipoxygenases catalyze the initiation of lipid peroxidation, the critical first step in the formation of characteristic carbonyl compounds (Table 1). After the action of vegetal lipoxygenases, the linoleic and linolenic acids are converted into 9 or 13-hydroperoxylinoleic acids and 9 or 13-hydroperoxylinolenic acids by molecular oxygen fixation in C9 or C13 position.[21] The cellular concentration in hydroperoxides is often very slight because the products are involved in several transformations. In fact, these hydroperoxides may then decompose to produce short chain carbonyls, resulting in volatile compounds.

Among the various vegetal sources containing lipoxygenases, it is worth mentioning soya, pea, trunip, tomato, potato, grape, apple, pear, banana,[22] mango,[23] cucumber,[24] raspberry, apricot, peach, melon and acacia.[4] According to the lipoxygenase origin, the green note productions are very different (Table 2). Moreover, cereals contain generally less lipoxygenases than the cabbages. Wheat presents 4 isoenzymes of the lipoxygenase, rice and rye three, corn and oats only two.[25]

It is important to note that when the lipoxygenase activity is very slight in certain vegetal sources, it is not possible to conclude that the lipoxygenase is really missing in these plants; in fact, some vegetal extracts such as onion, parsley, spinach, lettuce, and carrot show an anti-oxydant property which inhibits, for example, the soyabean lipoxygenase.[21]

Depending on the vegetal source, the lipoxygenases show different properties concerning the optimal pH, the substrate specificity and the isomeric structure of reaction products; among the characteristics of the lipoxygenases, the more frequently used are presented in Table 3.

The oxidation of the fatty acids lead to the formation of 9- and 13 hydroperoxydes in variable proportions according to the enzyme source and the environmental parameters.[28] For example, in soya, 13-hydroperoxide is mainly produced when the seeds were incubated in the presence of 100% oxygen as shown in the Table 4. The investigations of Leu[28] also revealed that hydroperoxide formation could be directed toward the 9 or the 13-hydroperoxide by modification of the pH value (respectively at pH = 6,5 or pH = 9). Finally, in the presence of surfactants such

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Table 3. Characteristics of certain vegetal lipoxygenases

Lipoxygenases	optimal pH	optimal T°C	Requirement	References
Ginkgo biloba	6,5-7	-	iron or cobalt	Major and Thomas[11]
Thea *(thea sinensis)*	5,5	< 65°C	presence O_2	Hatanaka and Harada[30]
Bean	6,8	25°C	-	Beaux and Drapron[31]
Birch leaves	7	< 50°C	presence O_2	Hatanaka et al.[26]
Green bean	5,8	-	presence O_2	Lumen et al.[3]
Corn	6-7	50°C	presence O_2	Theerakulkait and Barrett[32]
Wheat, rice	6-7	45°C	presence O_2	Theerakulkait and Barrett[32]
Pea	6-7,5	# 30°C	presence O_2	Theerakulkait and Barrett[32]
Kiwi	6,2	# 30°C	presence O_2	Bisakowski et al.[33]
Oats LP1 et LP2*	6,5	47°C	presence O_2	Doderer et al. [34]
Oats	-	-	+ detergent	Hugues et al.[25]
Soya LP1*	8-9,5	25°C	presence O_2	Gargouri et al.[20]
Soya LP2*	6-7,5	25°C	presence O_2	Grosch and Laskawy[10]
Sunflower	-	35°C	presence O_2	Theerakulkait and Barrett[32]

* LP1 and LP2 : two isoenzymes of the lipoxygenase

Table 4. Proportion of 9 or 13-hydroperoxides according to the amount of oxygen[28]

Amount of O_2	9-hydroperoxide %	13- hydroperoxide %
100%	5	95
1%	50	50

as acetate, the 9-hydroperoxide production from the linoleic acid was improved. However, if the surfactants increased the solubility of fatty acids, they affected not only the activity of lipoxygenases but also their structure. The enzyme conformation in solution was dependant on the primary structure and the links with the environment; a hydrophobe environment caused by the surfactants could modify the protein conformation. An addition of 20 in the medium revealed that such a surfactant was competitive inhibitor of the soyabean lipoxygenase. However, when used in very slight concentration (<1,6 10-3 M), it increased the enzyme activity by stabilization of the protein structure.[29]

The soybean lipoxygenase: Since the discovery of the lipoxygenase in soya, in 1932, several investigations have contributed to the determination not only of the physicochemical characteristics, but also of this enzymes function.[31]

Soyabean represented the best source of lipoxygenase; 3 isoenzymes of lipoxygenase, called lipoxygenases L1, L2 et L3, have been isolated and purified.[34,35] These three enzymes were classified according to their optimal pH of activity and their specificity in the hydroperoxide formation as shown by Table 5.[10,19,36]

Each isoenzyme could be involved in the green note production, but several studies have shown that only the lipoxygenases L2 and L3 were responsible for the volatile compound formation.[35] The works of Moreira et al.[37] confirmed that the lipoxygenase L2 seemed to be the most efficient enzyme in the hexanal synthesis.

The investigations carried out with deficient mutants for one of these lipoxygenases revealed that the isoenzyme L2 was responsible for the n-hexanal formation in soyabean homogenates.[38] The results presented in Table 6 showed that without lipoxygenase L3, the n-hexanal production was improved. It was also demonstrated that an enrichment in L3 of a soyabean homogenate decreased the green note production.[34] This enzyme led to an inhibition of the hexanal synthesis, but the mecanisms involved were still obscure. In order to elucidate this observation, Hildebrand et al.[39] have proposed a hypothesis which explained that the lipoxygenase L3 could achieve the hexanal production by conversion of hydroperoxylinoleic acid into non-available compounds for the hydroperoxide lyase.

The works of Grosch and Laskawy[40] revealed that the isoenzymes L2 and L3 in the soya formed significatively more volatile compounds than the L1 lipoxygenase. The different products obtained in each case were reported in Table 7.

The use of soyabean as a lipoxygenase source led to the production of the hydroperoxides corresponding to the linoleic and linolenic acids coming from sunflower oil and linseed oil respectively.[6] Concerning the thermostability of these isoenzymes, the L2 seemed to be less stable than the

Table 5. Characteristics of the isoenzymes contained in soya

Isoenzyme	optimal pH for activity	oxidation of linoleic acid	oxidation of linolenic acid
L1	9	C*13	-
L2	6,5	C*9 et C*13 (50/50)	C*9 et C*16
L3	6,5	C*9 et C*13 (65/35)	C*9 et C*16

*C9, C13 or C16 : number of the carbon concerned in the oxidation

Table 6. Study of the 3 isoenzymes of the lipoxygenase[38]

Soyabean	n-hexanal production %
Wilde	100
Deficient in L1L3	300
Deficient in L1L2	18
Deficient in L2L3	180

L1 (by a factor of 36). Moreover, a comparison between the activity of L1 and those of a crude preparation of soyabean revealed that the lipoxygenases contained in soya flour were more stable than the purified enzyme L1. In fact, they reacted on a lipidic substrate in a water poor medium.[17] Finally, the presence of alcohol could led to an inhibition of these isoenzymes.[41]

The lipoxygenase of the thea (Thea sinensis): There have been numerous studies concerning the green notes produced in the macerated black thea.[9,12,30] The enzymatic system responsible for the cis-3-hexenal and the trans-2-hexenal production in the thea leaves was localized in the chloroplats.[42] A recent study, revealing the presence of three isoenzymes of the thea lipoxygenase, showed a simultaneous increase of both lipid concentration and lipoxygenase activity during the thea maturation.[43]

Lipoxygenases contained in the birch leaves presented properties similar to those of the thea. They were situated in the chloroplast lamella and also required the presence of oxygen.

Lipoxygenases from animal sources: In contrast to the vegetal lipoxygenases which mainly degraded the linoleic and linolenic acids, animal lipoxygenases acted on substrates such as arachidonic acid (C20:4), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6), which is very abundant in fish tissue in particular.[15,22,41] These lipoxygenases were inactivated by alcohols according to a process similar to that involved for thesoyabean lipoxygenases.[30,41] Hatanaka and Harada[30] have demonstrated that pheromones in certain insects consisted of hexanal or hexenal. These results suggested the existence of lipoxygenase in such tissues.

Finally, the studies of Yoshino et al.[45] revealed the presence of a lipoxygenase responsible for the n-hexanal production in the liver and the lungs of mice.

Lipoxygenases from microbial sources: Plants are currently the only natural source of flavoring compounds. The majority of these compounds are contained in essential or volatile oils. Unfortunately, these volatiles constitute only a small fraction of the weight of the plant. In addition, the mechanisms of flavor formation in plants have yet to be elucidated, thus ruling out the possibility of increasing their

Table 7. Compounds obtained by the 3 isoenzymes of the soyabean lipoxygenase

Compounds	L1 %	L2 %	L3 %
Acetaldehyde	-	1,5	-
Propanal	18	46	41
trans-2-pentanal	5	8	11
trans-2-hexenal	77	9	9
trans-2-cis 6-nonadienal	-	-	2,5
trans-2-cis-4-heptadienal	-	20,5	20
3,5 octadien-2-one	-	8	8

yields. These limitations, combined with the fact that plant growth is subject to climatic, seasonal and geographical variations, have led to an almost total dependence on chemical synthesis for the production of flavor compounds. Chemical processes are slowly giving way to microbial processes, which provide several advantages over their chemical and botanical counterparts. Many microorganisms are capable of de novo synthesis of flavoring compounds, thus demonstrating their ability to perform conversions which would require multiple chemical steps. In addition to opening up new possibilities for rapid and controlled production of flavoring materials, microorganisms provide simple systems for studying the biosynthetic pathways involved in the formation of many important flavoring compounds. Microbial systems are used to catalyze specific steps. The stereospecificity of microbial enzyme systems has led to their increased use in the resolution of optical isomers created by chemical processes. Microorganisms are also an economical source of enzymes which can be utilized to enhance the flavor of many food systems.

Concerning the green notes, little is known of the microbial lipoxygenase. This enzyme, isolated from *Aspergillus*, seemed to be responsible for n-hexanal and n-hexanol production.[46] Enzymatic systems involved in the production of both these compounds have been identified in the yeasts *Pichia*, *Torulopsis*, *Candida* et *Hansenula*.[31,47] Moreover, it was important to mention that from fatty acids, such as linoleic and linolenic acids, the peroxides led to the formation of the cis-3-hexenal or the hexanal, respectively. In the presence of linolenic acid as substrate, the cis-3-hexenal, initially produced, is unstable and is transformed into trans-2-hexenal by the action of an isomerase. The simultaneous addition of yeasts and substrate in the medium led to the formation of 85% of cis-3-hexenol. To obtain pure hexanol, only linoleic acid must be introduced with the yeasts.

Finally, lipoxygenase activity was reported in the fungus *Gaeumannomyces graminis*, *Saprolegna parasitica*, *Lagenidium giganteum* and *Pityrosporum orbiculare*. The enzyme was also present in mushrooms including *Psalliota campestris* and *Agaricus campestris*.[33] This study revealed in particular that two isoenzymes of this lipoxygenase existed in *Fusarium oxysporium*. Their maximal activities were obtained at a pH equal to 8 and 10, respectively.

Hydroperoxide Lyases

Origin and specificity of the hydroperoxide lyases: Numerous studies have described n-hexanal formation by

the action of lipoxygenase on linoleic acid, but few study the mechanism by which hydroperoxides are transformed into hexanal have been elucidated.[48] Generally, it is agreed that unsaturated acids were converted into peroxides by a lipoxygenase then opened by a hydroperide lyase which cleaved these compounds to obtain volatile aldehydes in C6.[13,27] Hydroperoxide lyase activity was reported in cucumber, watermelon, tomato, pear and thea.[14] This enzyme is specific to the 13-hydroperoxide in tomato, soya and thea, specific to the 9-hydroperoxide in pear and of both compounds in cucumber.[5,6,34]

Among the enzymes involved in green note synthesis, only the hydroperoxide lyases were not available on the market. Green note processes also required the use of vegetal homogenates containing this enzyme.[7] Table 8 reported the concentrations of hexanal and hexenal depending on the hydroperoxide lyase origin.

Relationships between lipoxygenase and hydroperoxide lyase: The studies by Hatanaka et al.[49] reported the inhibition of lipoxygenase by linolenic acid and the corresponding 13-hydroperoxide affected both the activity of the lipoxygenase as well as that of the hydroperoxide lyase. The transformation of linoleic acid into hexanal is also reduced by 40% in simultaneous presence of these two inhibitors.

Alcohol dehydrogenases: The alcohol dehydrogenases, responsible for the reduction aldehydes in corresponding alcohols, played a major role in the green note synthesis. Their ability to produce alcohols oriented the processes toward hexanol or hexenol production for example.[6] However, it was particularly difficult to obtain the cis-3-hexenol by biological or chemical pathways. The works of Brunerie[13] described a process for this alcohol formation involving both an enzymatic system to oxidase the fatty acids and a yeast to transform the accumulated cis-3-hexenal into cis-3-hexenol. First, the cis-3-hexenal increased to a maximal concentration before being converted progressively into trans-2-hexenal by isomerization. It was well known that yeasts such as *Saccharomyces cerevisiae* were able to convert cis-3-hexenal into cis-3-hexenol, a more stable compound. In such conditions, no isomerization in trans-2-hexenol was observed.[13] The use of this baker's yeast led to hexanol formation (see Table 8) in the proportion of 100% and 60% from linoleic and linolenic acids, respectively.[5] In order to limit the isomerization of cis-3-hexenal in trans-2-hexenal, it was important to carry out the addition of yeasts in the reactional medium just an hour after the hydroperoxides cleaving.

Green-Note Processes

Most of the green-note processes known today corresponded to a simple mixture between a natural source of fatty acids and an homogenate of lipoxygenase combined with hydroperoxide lyase coming from vegetal origin. Such processes were novel because of the low amount of water: the biosynthesis of these aldehydes was realized in a medium characterized by a high viscosity and the presence of concentrated compounds.[2]

Consequently, these processes required good homogenization in order to obtain sufficient extraction of the enzymes contained in the vegetal substrate. The plants were also mixed in a stirring reactor to accelerate the lipoxygenase and the hydroperoxide lyase liberation by shearing action.[14]

Few performances of green-note in industrial production have ever been reported as being effective in the literature. Only some estimations are known today (Table 9).

The control of certain parameters plays a leading part in green note synthesis. The amount of unsaturated fatty acids contained in the vegetal substrate used, has a significant influence on aroma biosynthesis yield. The constitutive fatty acids susceptible to conversion into aldeydes C6 are generally present in low concentration. Thus it was necessary to add fatty acids as precursors to enhance the production.[13] However, an excess of linolenic acid inhibited the involved lipoxygenases.[50] The control of the fatty acid amount seemed to be determinant. Moreover, a decrease in the temperature considerably affected the kinetic of the green-note formation, but improved the cis-3-hexenal synthesis relative to that of the trans-2-hexenal.

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Address correspondence to Cathy Fabre, CRITT Bio-Industries, INSA, CBGD, Hall Gilberd Durand, Département de Génie Biochimique et Alimentaire, UMR CNRS 5504, Institut des Sciences Appliquées, Complexe Scientifique de Rangueil, 31077 Toulouse Cedex, France.

Table 8. Concentrations of hexanal and hexenal depending on the origin of the hydroperoxide lyase[6]

Lyase origin	[Hexanal]* mg/Kg	[Hexenal]** mg/Kg
Alfalfa	1360	210
Celery	160	< 10
Fennel	120	-
Pea	250	< 10
Radish	230	10
Soya	250	< 10
Tomato	510	< 10
Apple	380	220
Banana	310	130
Grape	330	-
Kiwi	270	240
Orange	610	230
Papaya	730	110
Apricot	-	230
Pear	330	-
Strawberry	370	< 10
Raspberry	400	< 10
Cucumber	-	280
Pepper (green)	-	370

* Hexanal: obtained from linoleic acid
** Hexenal: cis-3-hexenal + trans-2-hexenal obtained from linolenic acid

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Table 9. Green note production

Substrate	LPO + HPO* sources	ADH** sources	Aroma production	Reference
Linoleic acid	Strawberry homogenates	-	cis-3-hexenal 28 % trans-2-hexenal 68 % cis-3-hexenol 4 %	Patent[1] US N°4 806379
Fatty acids	Leaves of radish	Yeasts	cis-3-hexenol 0,55 g/Kg	Patent[50]EP0481147
Safflower oil	Soya flour	-	hexanal 0,92 g/Kg	Patent[2] N°FR2 696192
Linseed oil Sunflower oil	Soya flour	-	hexanal 5 g/Kg hexanol 5 g/Kg cis-3-hexenol 4,2 g/Kg trans-2-hexenal 1,5 g/Kg	Patent[5] N∞EP 597069
Fatty acids	Soya	-	cis-3-hexenal 5,8 g/Kg	Hatanaka et al.[26]
Linolenic acid Linolenic acid	Watermelon Alfalfa	Yeasts *S. cerevisiae*	cis-3-hexenol 1 g/Kg hexenal 100 %	Patent[14]S N°9526413 Patent[14]US N∞9526413

* LPO lipoxygenase and HPO hydroperoxide lyase ** ADH : alcohol dehydrogenase
Note: aromatic compound concentrations are given in gram per Kilo of fresh vegetal substrate (g/Kg)

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