

# Oxidation behaviour of vanillin in dairy products

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The stability of vanillin in dairy products was investigated by means of high-performance liquid chromatography. While vanillin was oxidised to vanillic acid in fresh and pasteurised milk, it was stable in all other milk products investigated (UHT milk, pasteurised and UHT cream, yoghurt, curd and butter). The oxidation of vanillin to vanillic acid was shown to be pH-dependent. The relatively thermolabile enzyme xanthine oxidase was shown to be partly responsible for the formation of vanillic acid. However, peroxidase, which is also present in dairy products, did not lead to vanillic acid. Oxidation to the dimeric product, divanillin, occurred in the presence of peroxidase and hydrogen peroxide. The oxidation rate of synthetic vanillin to vanillic acid was very similar to that of vanillin from natural vanilla extracts or vanilla capsules. Both were shown to be stable in dairy products for subvention, such as butter and cream. © 1997 Elsevier Science Ltd

## INTRODUCTION

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is one of the most important food flavours and is also widely used for other purposes, such as in cosmetics and drugs. Many consumers prefer natural compounds instead of synthetic ones. Therefore, both synthetic vanillin and vanillin from natural vanilla are added to food products. Extracts from vanilla (after fermentation and ripening, ready for use) have many technological applications. In addition to vanillin, natural vanilla contains a large number of other organic compounds. About 200 of these constituents have so far been identified, of which only 26 occur in concentrations greater than 1 ppm. The five most important constituents are vanillin itself (2–2.8%), *p*-hydroxybenzaldehyde (about 0.2%), vanillic acid (about 0.2%), *p*-hydroxybenzylmethyl ether (about 0.02%) and acetic acid (about 0.02%) (Anklam, 1993; Klimes & Lamparsky, 1976).

Vanillin (natural and synthetic) is also used as a tracer compound in dairy products under subvention by the European Union. It can be incorporated in butter for sale at reduced prices or for use in pastry products, ice cream or other food in concentrations of 250 ppm for synthetic vanillin or 100 ppm for vanilla capsules or natural vanilla extracts (European Union Commission, 1988).

Recently it was reported that vanillin could be oxidised to vanillic acid in pasteurised cream and that this process would be even faster if natural vanilla instead of vanillin was added (M. Nicolas, unpublished results, 1995).

Whereas the oxidation of vanillin and other phenolic aldehydes with means of oxygen in alkaline solution (Fricko *et al.*, 1980), chlorite (Lindgren & Nilsson, 1973; Jaroszewski, 1981), peracetic acid (Nimz & Schwind, 1979) or potassium bromate (Samaddar & Banerjee, 1982) has been described in the literature, little is known about the enzymatic oxidation of vanillin. The dimeric product, divanillin, was detected after oxidation of vanillin in aqueous solution with peroxidase in the presence of hydrogen peroxide (Baumgartner & Neukom, 1972).

The aim of this study was to investigate the stability of vanillin in various dairy products in order to gain knowledge about its oxidation behaviour and possible oxidation products, depending on matrix effects. The various dairy products (milk, cream, butter, yoghurt, curd) were spiked with synthetic vanillin and natural vanilla extracts or vanilla capsules, in most cases with a concentration of 200 ppm, which is in compliance with the amount used as tracer. High-performance liquid chromatography (HPLC) was the analytical tool.

## MATERIALS AND METHODS

Synthetic vanillin, vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid were purchased from Aldrich. All solvents used, 2-propanol, ethanol and acetonitrile (p.a.), acetic acid and methanol (HPLC grade), were from Merck. Water was deionised using an ion-exchange system (Millipore). Natural vanilla (extracts and capsules) and dairy products (fresh milk, pasteurised milk, UHT milk, pasteurised cream, UHT

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cream, yoghurt, curd, ricotta, mascarpone and butter) were those available from the European market (Belgium, Germany, France, Italy, Switzerland).

### Sample preparation

The various dairy products were spiked with vanillin (synthetic, natural extracts, ground vanilla capsules). The vanilla extracts and vanilla capsules (both Bourbon vanilla) were analysed by HPLC prior to spiking in order to determine the content of vanillin, vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. The spiked samples were stored in the dark at 22°C and 6°C, respectively. Spiking of cream for the long-term study (6 weeks) was performed as follows: synthetic vanillin (200 ppm) was added to pasteurised cream and portions were sterilised in sealed glass bottles in a boiling water bath for 2 h. One sample was analysed immediately after sterilisation in order to evaluate the loss of vanillin during the heating process. The samples were stored in the dark at 6°C. Spiking of butter was carried out with 5 g portions of melted homogenised butter to which synthetic vanillin was added (200 ppm). The spiked butter samples were shaken vigorously and stored in the dark at 6°C. Whole portions were extracted and analysed after storage.

#### Sample preparation prior to HPLC analysis

The sample preparation prior to HPLC analysis was carried out as follows (European Union Commission, 1997; J. Labriijn, unpublished results, 1995). About  $5\text{ g} \pm 0.1\text{ mg}$  of butter or  $10\text{ g} \pm 0.1\text{ mg}$  of the other milk products investigated were weighed into a 100 ml volumetric flask. To each test portion, 75 ml of the extraction solution (ethanol:2-propanol:acetonitrile in the ratio 1:1:2, v/v) were added and shaken vigorously for 15 min. The flask was filled to the mark with the extraction solution; 10 ml of this extract were transferred into a test tube with a plastic stopper. The test tube was placed in the freezer ( $-20^\circ\text{C}$ ) and allowed to stand for about 30 min. This cold extract was centrifuged and then immediately decanted. After having reached room temperature, 5 ml of the decanted solution were placed in a 100 ml volumetric flask which was filled to the mark with HPLC-grade water. The solution was passed through a  $0.45\text{ }\mu\text{m}$  disposable filter prior to injection into the HPLC instrument.

### High-performance liquid chromatography (HPLC)

HPLC analysis was performed after determined time intervals. A high-pressure pump (HP 1050) equipped with a UV detector and data station (HP Vectra cs/165) were used. Separations were performed using an RP18 (Supelcosil) column ( $250\text{ mm} \times 3\text{ mm}$ ;  $5\text{ }\mu\text{m}$  particle size). Operating conditions were as follows: mobile phase, gradient mode (A, methanol; B, 97.8% water/2.2% acetic acid) was 10% A/90% B for 1 min, 70% A/30%

B up to 40 min, 100% A to the end; flow rate  $1\text{ ml min}^{-1}$ ; UV detector set at 283 nm; injection volume  $20\text{ }\mu\text{l}$  loop (automatic or manual).

## RESULTS AND DISCUSSION

### Stability of vanillin in milk

Fresh milk samples were spiked with different concentrations of vanillin. The stability of vanillin was measured after standing for 6 h at room temperature ( $22^\circ\text{C}$ ) in the dark. As shown in Table 1, vanillic acid was formed in all samples analysed. No formation of other components could be observed. The oxidation to vanillic acid is much faster in fresh milk samples spiked with lower concentrations of vanillin. Whereas all vanillin was oxidised in the sample containing 25 ppm vanillin, there was still 90% remaining in the sample consisting of milk with 900 ppm vanillin.

The oxidation process is also time-dependent. After 3 h of storage, the sample containing 25 ppm of vanillin showed an oxidation to vanillic acid of about 50%, whereas all vanillin was transformed into vanillic acid after 6 h.

The same oxidation process could be observed in various pasteurised milk samples spiked with vanillin. As demonstrated in Table 1, similar rates of oxidation were found, which leads to the conclusion that the matrices for oxidation of vanillin seem to be very much alike. The HPLC chromatograms of sample 1 (48 h at  $22^\circ\text{C}$ ) and sample 2 (72 h at  $6^\circ\text{C}$ ) showed, besides vanillin and vanillic acid, many other unidentified components which were probably caused by spoilage of the milk samples.

However, no formation of vanillic acid occurred in UHT milk samples, and the added vanillin could be totally recovered. It is highly probable that the cause of oxidation of vanillin was removed during the heating of the milk, which again leads to the assumption that enzymes could be the responsible factor.

### Stability of vanillin in cream

Various pasteurised cream samples from the European market were spiked with concentrations of about 200 ppm vanillin. The recovery of vanillin was determined after storage for between 6 and 72 h at  $6^\circ\text{C}$  and, for cream 1, also between 6 and 24 h at  $22^\circ\text{C}$ . The results are listed in Table 2.

Vanillin was shown to be stable in all pasteurised cream samples analysed, even after storage for 3 days, except for the French cream 6 in which oxidation to vanillic acid could be observed. The oxidation rate in this particular sample was much higher even than that of fresh and pasteurised milk samples (Table 1). It has been shown that this French cream sample did not undergo the same heat treatment as the other cream

**Table 1. Stability of vanillin (synthetic) in milk**

Milk sample (from Italy)	Concentration (ppm)	Storage time (h)	Storage temperature (°C)	Recovery of vanillin <sup>a</sup> (%)	Formation of vanillic acid <sup>a</sup> (%)
Fresh 1	904	6	22	91	9
Fresh 1	222	6	22	70	30
Fresh 1	57	6	22	38	62
Fresh 1	25	3	22	50	50
Fresh 1	25	6	22	0	100
Fresh 1	25	18	22	0	100
Pasteurised 1	200	6	22	75	25
Pasteurised 1	200	6	6	74	26
Pasteurised 1	200	24	22	47	53
Pasteurised 1	200	24	6	46	54
Pasteurised 1	200	48	22	HS <sup>b</sup>	HS
Pasteurised 1	200	48	6	10	90
Pasteurised 1	1000	6	22	88	12
Pasteurised 1	1000	72	6	HS	HS
Pasteurised 2	200	6	22	68	32
Pasteurised 2	200	24	22	48	52
Pasteurised 3	200	6	22	75	25
Pasteurised 3	200	24	22	55	45
Pasteurised 4	200	6	22	69	31
Pasteurised 4	200	24	22	52	48
UHT 1	200	6	22	100	0
UHT 1	200	24	22	100	0
UHT 1	200	72	22	100	0
UHT 1	1000	24	22	100	0
UHT 2	200	24	22	100	0
UHT 3	200	24	22	100	0

<sup>a</sup>Relative standard deviation about 5–10%.<sup>b</sup>HS, highly spoiled.**Table 2. Stability of vanillin (synthetic) in various pasteurised cream samples from the European market**

Cream sample <sup>a</sup>	Concentration (ppm)	Storage time (h)	Storage temperature (°C)	Recovery of vanillin <sup>b</sup> (%)	Formation of vanillic acid <sup>b</sup> (%)
Cream 1 (I)	200	6	22	101	0
Cream 1 (I)	200	24	22	98	0
Cream 1 (I)	1000	6	22	90	0
Cream 1 (I)	1000	72	6	105	0
Cream 2 (I)	206	6	6	95	0
Cream 2 (I)	206	72	6	96	0
Cream 3 (I)	210	6	6	102	0
Cream 3 (I)	210	72	6	98	0
Cream 4 (B)	203	6	6	104	0
Cream 4 (B)	203	72	6	102	0
Cream 5 (F)	200	6	6	97	0
Cream 5 (F)	200	72	6	105	0
Cream 6 (F)	203	6	6	22	78
Cream 6 (F)	203	72	6	0	98
Cream 7 (D)	203	6	6	102	0
Cream 7 (D)	203	72	6	103	0
Cream 8 (D)	212	6	6	99	0
Cream 8 (D)	212	72	6	100	0
Cream 9 (CH)	203	6	6	101	0
Cream 9 (CH)	203	72	6	74 <sup>c</sup>	0

<sup>a</sup>I, Italy; B, Belgium; F, France; D, Germany; CH, Switzerland.<sup>b</sup>Relative standard deviation about 5–10%.<sup>c</sup>Highly spoiled.

samples (M. Nicolas, unpublished results, 1996). The degradation of vanillin with the formation of unidentified compounds, but not vanillic acid, in the Swiss cream sample after storage of 72 h was due to spoilage of the sample.

The fact that vanillin was stable in most of the pasteurised cream samples but was rapidly oxidised in the pasteurised milk sample could be due to several reasons: the temperature and time of pasteurisation of milk and cream are different, which leads to the assumption that enzymes responsible for oxidation are inactive in the cream samples; the higher fat content in cream inhibits the enzymes from reaching the potential substrate, vanillin; the pH values of milk and cream are slightly different (the pH-dependence on oxidation of vanillin to vanillic acid is demonstrated below).

### Stability of vanillin in various milk products

The oxidation behaviour of vanillin in various dairy products such as pasteurised yoghurt, curd (Quark, mascarpone, ricotta), butter and cream was investigated after storage for 6 h at 22°C and 24 h at 6°C in the dark. A longer-term study (6 weeks) was also performed with spiked butter and sterilised cream samples. As shown in Table 3, only in one curd sample (Quark 3) could the formation of vanillic acid be observed. In all other samples analysed, vanillin was stable even for a period of 6 weeks in butter and cream (products for subvention).

### Dependence of oxidation behaviour on the pH

Because vanillin was shown to be stable in pasteurised yoghurt samples, products having a similar fat content to the milk samples, it is highly probable that the oxidation process is dependent on pH. For this reason, immediately after the vanillin had been added to the pasteurised milk and cream samples, the pH values (as listed in Table 4) were adjusted by adding hydrochloric acid or sodium hydroxide solution. Analysis by HPLC was performed 6 h later.

It can be seen from Table 4 that, at lower pH values such as 3.5 and 4, no vanillic acid was formed and the vanillin could be totally recovered. Degradation of vanillin and formation of vanillic acid, however, could be observed above a pH value of 4 in the pasteurised milk sample. As already indicated above, vanillin is much more stable in cream than in milk. Only negligible amounts of vanillic acid in cream at pH values of 6–8 could be detected. At higher pH values (above 8), vanillin was again stable in the milk samples. The dependence of vanillin oxidation on the pH in milk is demonstrated in Fig. 1.

### Stability of natural vanilla in milk and cream

Before adding the vanilla extracts (Bourbon) or appropriate amounts of ground vanilla capsules (Bourbon) to the milk and cream samples, the extracts were analysed by HPLC in order to determine the content of vanillin and the other main constituents and expected oxidation

Table 3. Stability of vanillin in various milk products spiked with 200 ppm vanillin

Sample	Storage time (h)	Storage temperature (°C)	Recovery of vanillin <sup>a</sup> (%)	Formation of vanillic acid <sup>a</sup> (%)
Yoghurt 1–6	6	22	97–98	0
Yoghurt 6	24	6	103	0
Quark 1	6	22	102	0
Quark 2	6	22	100	0
Quark 2	24	6	98	0
Quark 3	6	22	95	5
Quark 3	24	6	87	8
Mascarpone 1	6	22	104	0
Mascarpone 1	24	6	106	0
Ricotta 1	6	22	104	0
Butter 1–3	6	22	99–103	0
Butter 1–3	24	6	98–104	0
Butter 3	6 weeks	6	103	0
UHT cream 1	6	22	103	0
UHT cream 1	24	6	101	0
Sterilised cream 1	6	22	100	0
Sterilised cream 1	6 weeks	6	104	0

<sup>a</sup>Relative standard deviation: 5–10%.

products such as vanillic acid, *p*-hydroxybenzaldehyde and *p*-hydroxybenzoic acid. Taking the sum of these four compounds as 100%, the natural Bourbon vanilla extract and the Bourbon vanilla capsules were found to contain 85–87% vanillin, 6% vanillic acid, 7–9% *p*-hydroxybenzaldehyde and 0% *p*-hydroxybenzoic acid. Suitable amounts of the extracts were then added to the dairy samples to obtain a vanillin concentration of about 200 ppm.

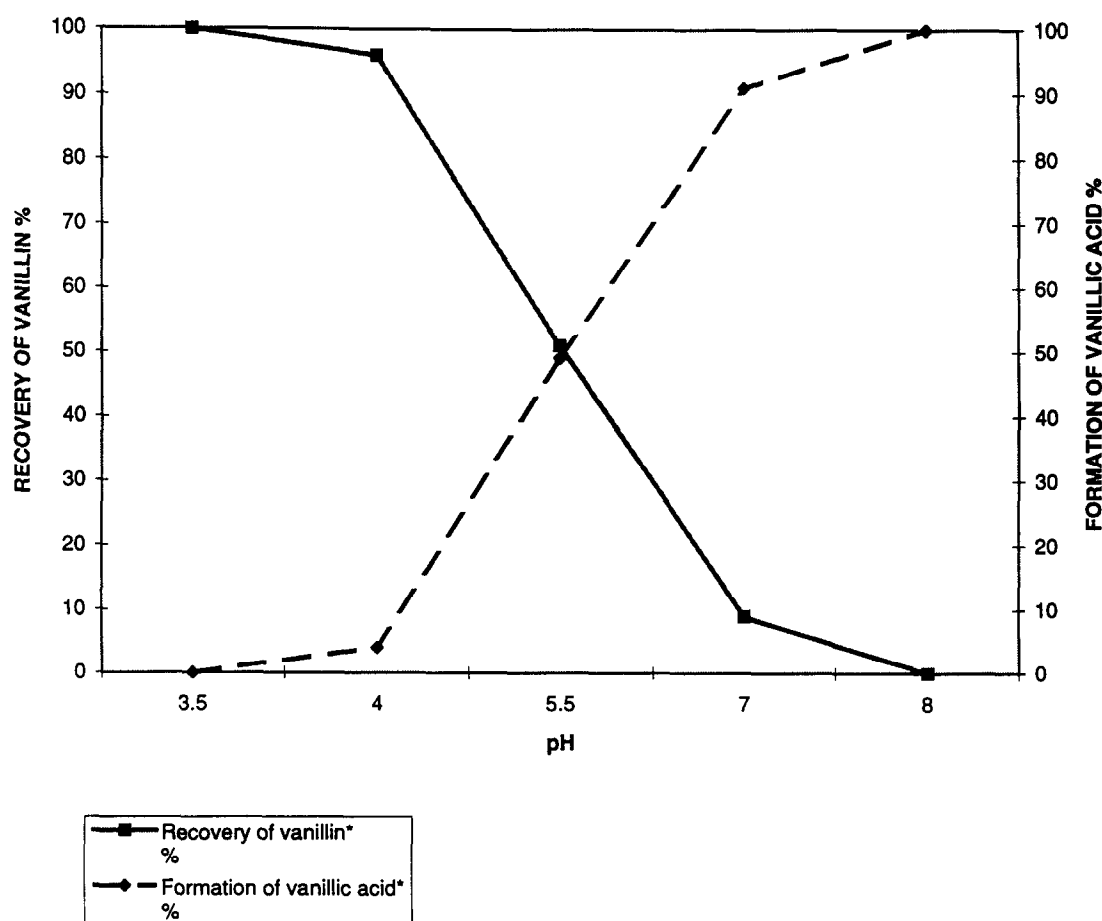
For comparison studies spiking was also performed with synthetic vanillin in the same concentration range.

The analysis by HPLC was performed after 6–72 h of storage of the spiked samples in the dark at 6°C. The results obtained with natural vanilla were very similar to those obtained after addition of synthetic vanillin. Again, the formation of vanillic acid from vanillin could be observed. In addition, *p*-hydroxybenzaldehyde underwent oxidation to *p*-hydroxybenzoic acid. This is illustrated in Fig. 2, which shows the chromatograms of extracts from natural Bourbon vanilla capsules and from the latter added to pasteurised milk and extracted 24 h later.

**Table 4. Stability of vanillin (200 ppm) in pasteurised milk and cream at different pH values (storage 6 h at 22°C)**

Sample	pH	Recovery of vanillin <sup>a</sup> (%)	Formation of vanillic acid <sup>a</sup> (%)
Milk pasteurised	3.5	100	0
Milk pasteurised	4	96	4
Milk pasteurised	5.5	51	49
Milk pasteurised	7	9	91
Milk pasteurised	8	0	100
Milk pasteurised	9.5	51	49
Cream pasteurised	3.5	100	0
Cream pasteurised	4	100	0
Cream pasteurised	5.5	96	4
Cream pasteurised	7	100	0
Cream pasteurised	8	97	3
Cream pasteurised	9.5	100	0

<sup>a</sup>Relative standard deviation: 5–10%



**Fig. 1.** Degradation of vanillin and formation of vanillic acid in pasteurised milk at different pH values.

The yields of vanillin and *p*-hydroxybenzaldehyde and the formation rates of their oxidation products are listed in Table 5. As can be seen, there is no difference between the oxidation behaviour of vanillin and *p*-hydroxybenzaldehyde from vanilla extracts and from vanilla capsules. The vanilla extract was made on the basis of ethanol/water. Because no significant difference in the oxidation rate of vanillin could be observed, the conclusion could be drawn that the alcoholic solution had no effect on the enzyme(s) that are possibly responsible for the oxidation.

Comparing the rates of oxidation, it can be seen that the degradation of vanillin derived from natural vanilla was slightly slower than that of synthetic vanillin. This can be explained by the presence of other phenolic compounds, such as *p*-hydroxybenzaldehyde, which can be oxidised as well. As demonstrated in Table 5, *p*-hydroxybenzaldehyde was completely transformed into *p*-hydroxybenzoic acid.

#### Influence of peroxidase and xanthine oxidase on oxidation behaviour

In order to investigate the effect of enzymes on the oxidation behaviour of vanillin in fresh and pasteurised milk, small amounts of peroxidase or xanthine oxidase were added to samples of UHT milk and pasteurised

cream (in which vanillin was shown to be stable) spiked with synthetic vanillin.

No oxidation product could be detected after the addition of peroxidase. Vanillin could be totally recovered even after storage for 24 h at 22°C or for 48 h at 6°C. As already mentioned in the introduction, the oxidation of vanillin in aqueous solutions leads to the dimeric product divanillin in the presence of peroxidase and hydrogen peroxide (Baumgartner & Neukom, 1972). The same product could be determined in our study after addition of peroxidase and hydrogen peroxide to UHT milk. As well as vanillin and a small amount of divanillin, other unidentified compounds were formed. However, no formation of vanillic acid could be observed.

On the other hand, the addition of xanthine oxidase to pasteurised cream samples or UHT milk spiked with vanillin led to the formation of vanillic acid. However, only small amounts of vanillic acid (about 10%) were formed in 6 h and about 90% of vanillin could be retained. Comparing these values with those of sample 6 (French cream sample in which vanillic acid formation was observed, as indicated in Table 2), it can be seen that the latter were about 80% for vanillic acid and 20% for vanillin. It has been shown that this particular French cream sample was heat-treated at much lower temperatures than is usual for pasteurised creams (M.

Table 5. Stability of vanillin (natural and synthetic) in pasteurised milk and cream

Sample	Concentration (ppm)	Storage time (h)	Temperature (°C)	V (%)	VA (%)	pHB (%)	pHBA (%)
<i>Vanilla from vanilla capsules ( Bourbon )</i>							
Milk	340	6	22	69	21	0	10
		24	22	47	43	0	10
		24	6	54	38	0	8
		48	6	23	67	0	0
<i>Vanilla extract (commercial, Bourbon)</i>							
Milk	230	6	22	66	20	0	13
		24	22	34	58	0	9
		24	6	47	47	0	0
		48	6	33	77	0	0
Cream (I)	200	6	6	85	6	9	0
		72	6	84	6	10	0
Cream (F)	200	6	6	36	55	0	9
		24	6	12	82	0	6
		72	6	0	98	0	2
<i>Synthetic vanillin</i>							
Milk	200	6	22	68	32	—	—
		24	22	40	60	—	—
		24	6	66	34	—	—
		48	6	9	91	—	—
Cream (I)	200	6	6	100	0	—	—
		72	6	100	0	—	—
Cream (F)	200	6	6	22	78	—	—
		24	6	0	100	—	—
		72	6	0	100	—	—

V, vanillin; VA, vanillic acid; pHB, *p*-hydroxybenzaldehyde; pHBA, *p*-hydroxybenzoic acid. V + VA + pHB + pHBA = 100%. F, France; I, Italy.

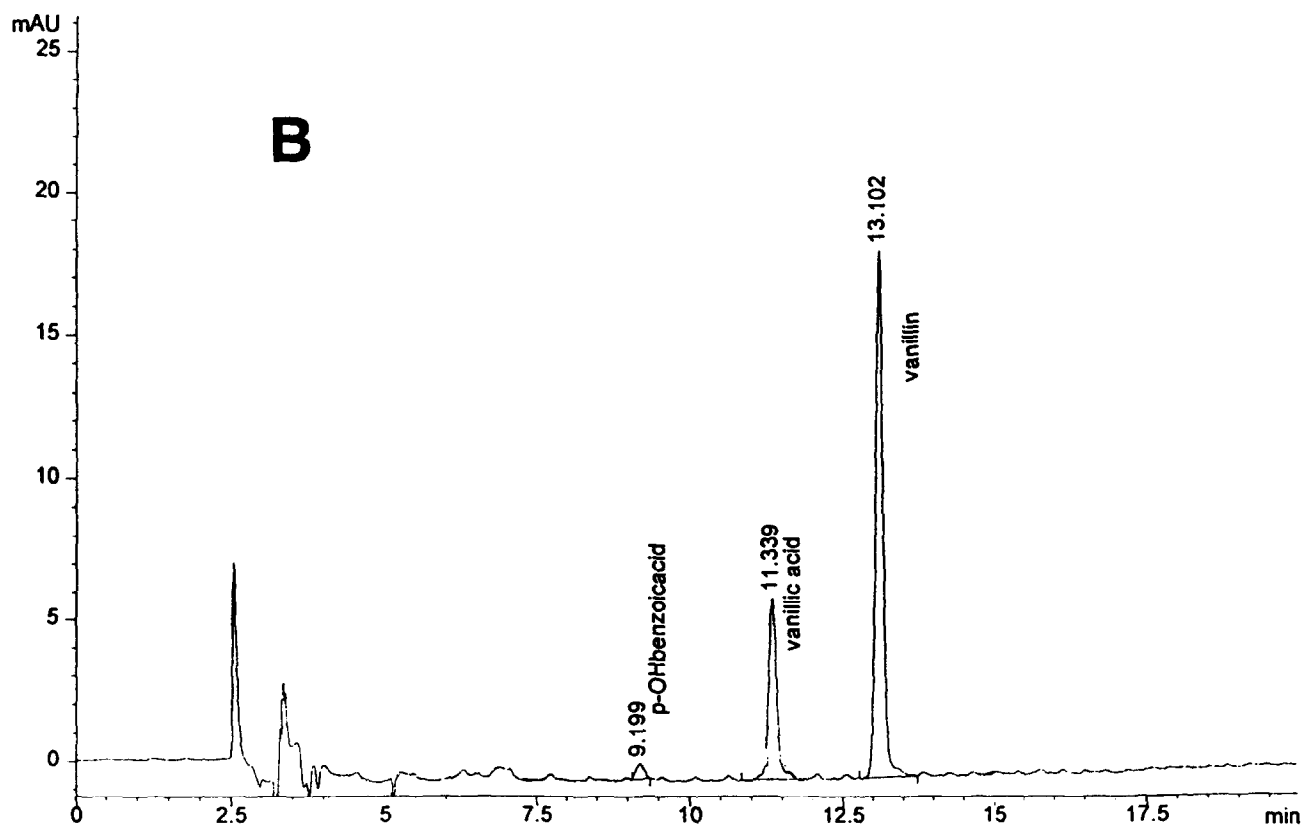
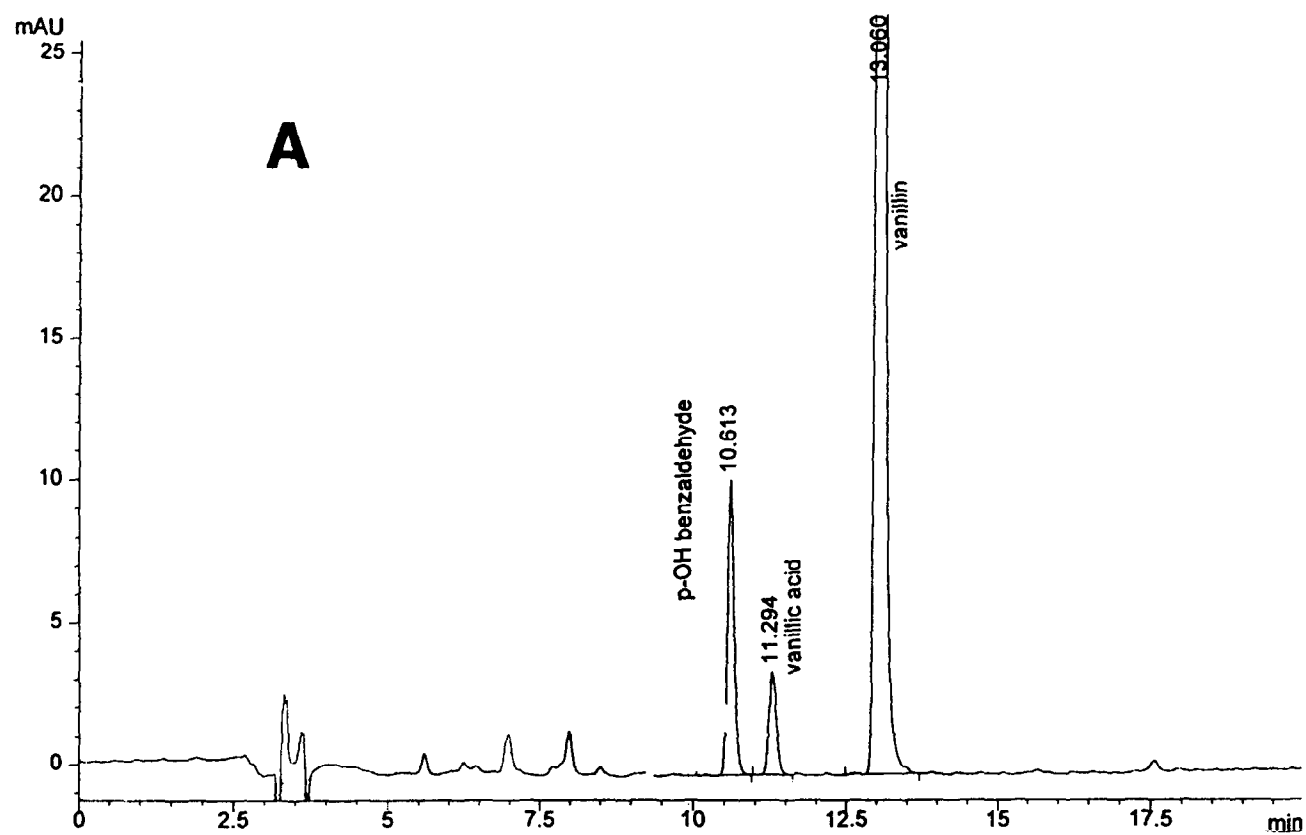


Fig. 2. Chromatograms of natural vanilla: (A) natural vanilla extract; (B) extract from pasteurised milk 24 h after addition of the natural vanilla extract.

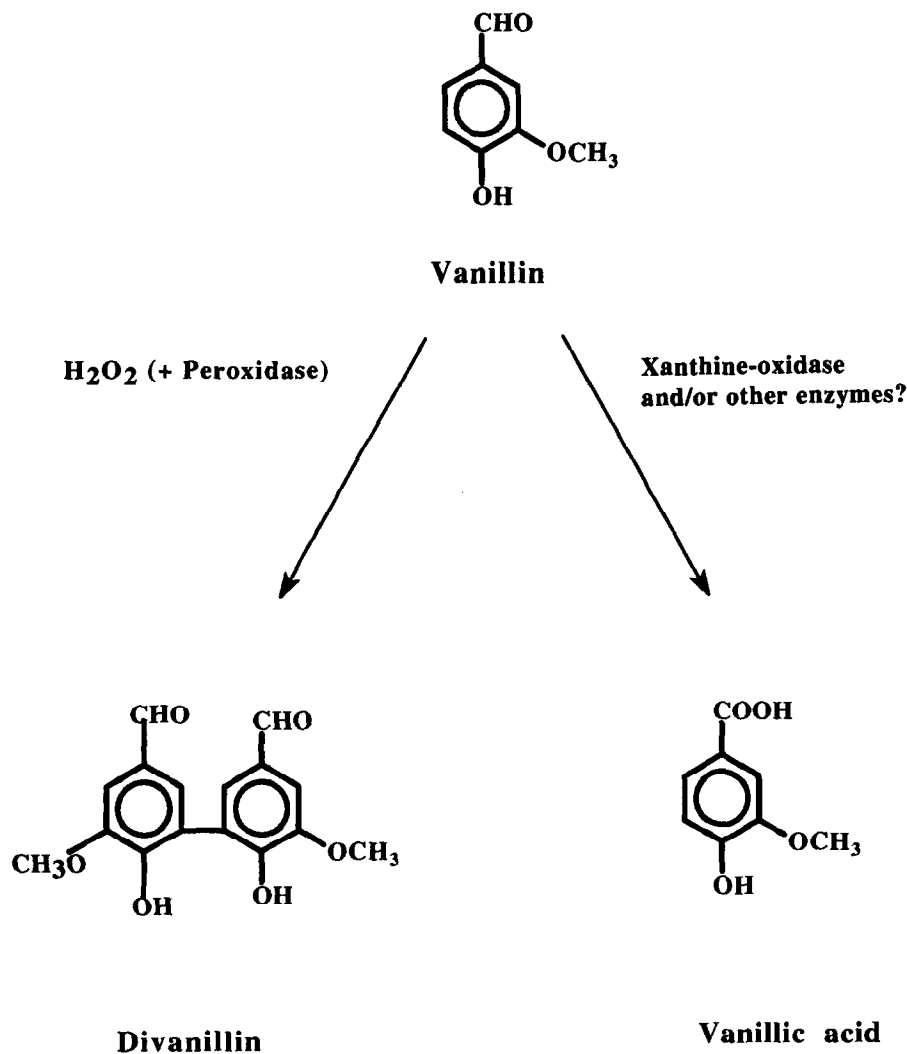


Fig. 3. Oxidation scheme of vanillin, depending on the enzymatic system.

Nicolas, unpublished results, 1996), and xanthine oxidase and other milk enzymes could be still active.

The different oxidation pathways are demonstrated in Fig. 3. Depending on the enzymatic system, the two oxidation products of vanillin—divanillin and vanillic acid—can be observed.

However, xanthine oxidase does not seem to be the only driving force for the oxidation of vanillin to vanillic acid in milk products. Even increasing the amounts of added xanthine oxidase or combining xanthine oxidase with peroxidase did not lead to faster oxidation.

Most probably there are other enzymes that are responsible for the oxidation to vanillic acid in fresh and pasteurised milk. Further investigations with other enzymes are under way.

## CONCLUSION

Milk enzymes such as peroxidase and xanthine oxidase can lead to two oxidation products of vanillin—divanillin and vanillic acid. While divanillin can be obtained

in the presence of peroxidase and hydrogen peroxide, it is not formed only by peroxidase. However, addition of vanillin to fresh and pasteurised milk leads to the formation of vanillic acid. This oxidation could be partly caused by xanthine oxidase. The oxidation of vanillin in fresh milk samples was shown to be pH-dependent.

In all other dairy products, vanillin was shown to be stable (with the exception of one French pasteurised cream sample, for which the heat treatment was not appropriate, and of one curd sample).

The oxidation rate of synthetic vanillin to vanillic acid is very similar to that of vanillin from natural vanilla extracts or vanilla capsules. They are both stable in dairy products for substitution such as butter and cream.

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