

# Analysis of Carious Dentine using FTIR and ToF-SIMS

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## Abstract

Apart from the Maillard reaction, other processes, such as esterification, take place in carious tissue. The aim of the present study was to analyse sound and carious dentine in terms of ester groups and their reaction with hydrazine derivative using Fourier Transform Infrared Spectroscopy (FTIR) and Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS). Carious and sound dentine from human premolars were excavated in three series (Experimental Parts I-III) and separated into inner and outer layers of carious dentine. The excavated tooth material was analysed with FTIR (Part I). Carious and sound dentine were also exposed to different chemical treatments and analysed with FTIR-Attenuated Total Reflectance (FTIR-ATR; Part II) and ToF-SIMS (Part III). The FTIR absorption spectra showed that the carious tissue contained ester groups, not detected in sound dentine. The results also indicated a higher occurrence of ester groups in the inner dental caries layer than in the outer ones. Potential binding to these ester groups by hydrazine derivative was observed after different chemical treatments with both FTIR-ATR and ToF-SIMS. The results of the present study revealed ester groups unique to the carious dentine which, after reaction with hydrazine derivative, form a covalent bond not found in sound dentine. The staining of carious unique groups would be clinically helpful in detection and prevention unnecessary removal of sound dentine.

*Key Words: Caries removal, Dental caries, Hydrazine derivative, Staining*

## Introduction

The pathogenesis of dental caries is a consequence of the metabolism of dietary fermentable sugars by the cariogenic bacterial species [1-4]. The cariogenic micro-organisms constitute a complex oral microflora with both acidogenic and aciduric properties [2]. When present in the metabolically active biofilm, covering the tooth surface, the underlying tooth surface may gradually become chemically modified and over time this may result in the net loss of mineral.

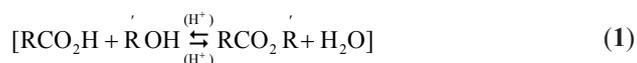
From a clinical point of view, dental caries has been described as a soft, yellowish-brown discoloration of the dentine [5]. Previous investigations of dental caries have revealed reactions between proteins and sugars in producing advanced glycation end products (AGEs; generally called Maillard reactions). It has been suggested that they are responsible for the typical discoloration of dentine, a non-enzymatic crosslink reaction, when reacting with both glucose and glucose amine [6,7]. Other authors have explained the discoloration as an effect of the reaction between carbohydrate fermentation products and carious dentine [8].

Furthermore, differences within carious tissues have previously been described when dental caries was divided into an outer and inner layer adjacent to sound dentine [9]. The outer carious layer is more necrotic, to a higher degree infected with bacteria, physiologically uncalcifiable and Fuchin-stainable, compared with the inner layer) [10]. The outer softer portion needs to be removed before restauration is applied, while the inner harder part has the capacity to remineralize [11-13].

In addition to the Maillard reactions, other organic molecular alterations may occur in the carious tissue. The presence of esters in dental caries has been investigated and it has been found that esterases are more common in carious

tissue than in intact tissue [14]. In addition, esters deriving from bacterial lipid components such as cholesterol esters [15,16] have been recognised in both sound and carious dentine [17].

Experimental evidence of the presence of esters in dental caries is still lacking and possible differences in the esters in the outer and inner layers of dental caries have not been investigated. It can be hypothesised that the esterification of the carboxylic side-chains of aspartate and glutamate residues facing an acidic environment has occurred, catalysed by the acidic environment (lactic acid). The esterification between these free carboxylic ends and small carbohydrates can take place in a process similar to Fischer esterification {1} and is unique to the carious lesion [18].



Fourier Transform Infrared Spectrum (FTIR) analyses have been frequently used to observe chemical alterations after different treatments, such as mechanical, chemo mechanical and etching with or without further adhesives exposed to dentine and carious tissue [19-26]. This technique enables analyses of protein linkage to the crystals without further purification.

Time-of-flight Secondary Ion Mass Spectrometry (ToF-SIMS) enables the analysis of the chemical composition without the previous separation of the components. This technique has also been used for the surface analysis of dental hard tissues in terms of the chemical composition and to study chemical changes in sound dentine [27].

The aim of this study was to investigate the presence of ester groups in the outer and inner layers of carious dentine using FTIR and ToF-SIMS. A further aim was to confirm by

staining the existence of esters, by modifying the ester groups with a hydrazine derivative, to detect the reaction product by means of FTIR-ATR and ToF-SIMS.

## Materials and Methods

### Experimental design

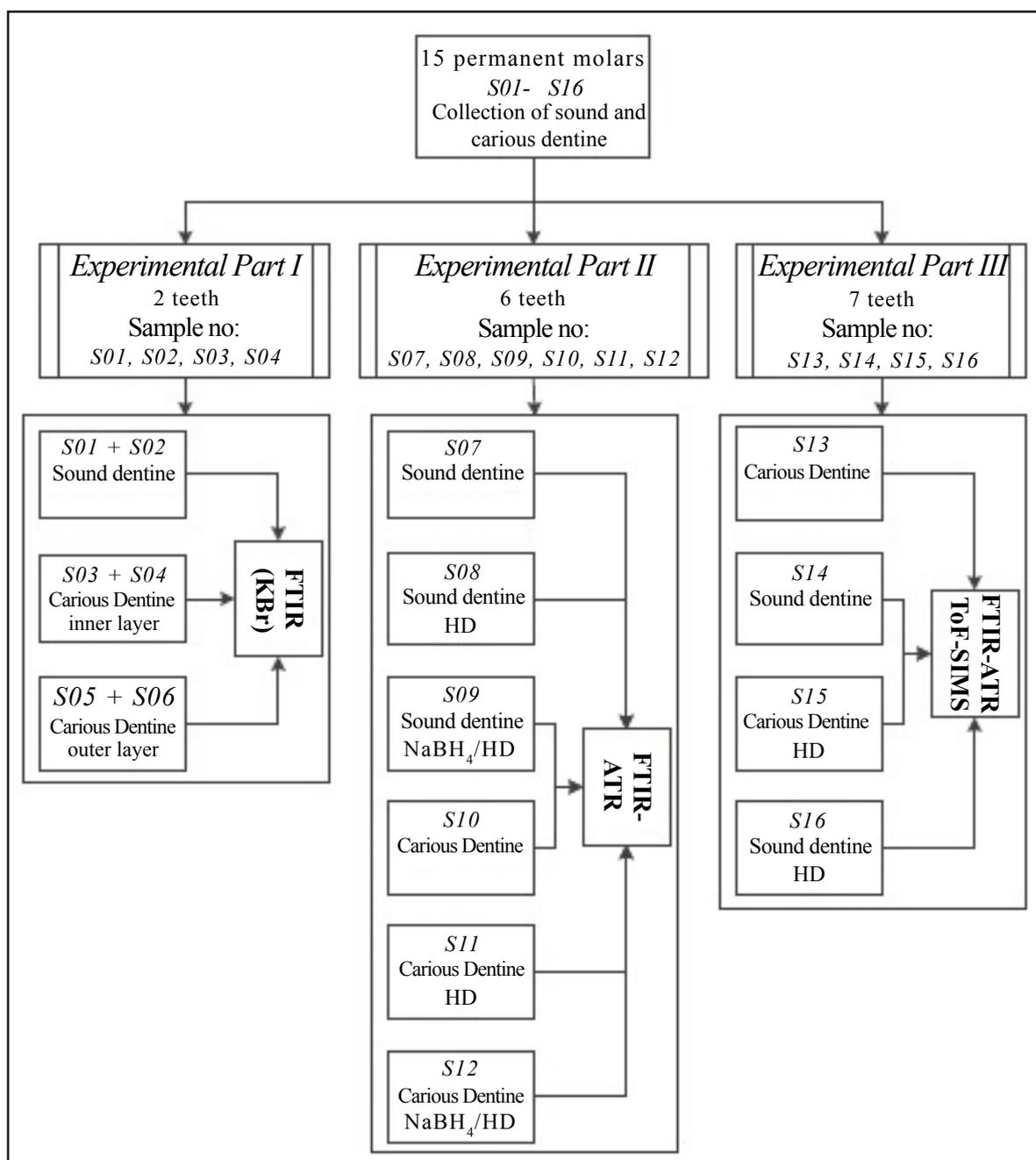
The study was performed in three different experimental parts, *Parts I-III*, which will be described in detail below. An overview of the experimental design is given in *Figure 1* and *Table 1*. A total of 15 permanent molars were used for 16 different samples.

### Collection of sound and carious dentine

Permanent molars extracted for caries-related reasons were collected for the experiments. The teeth were stored separately in purified water (Millipore, Merck Millipore, Billerica, MA, USA) in plastic tubes and stored in a refrigerator (+4°C)

until analysed. All the teeth had open carious lesions and so the carious dentine was accessible without any drilling. The collection of sound and carious dentine was carried out by an experienced clinician (PL) with a hand excavator and by drilling under a normal dental operation light, using magnification glasses. Following the removal of the outermost parts of the carious tissue, two layers of the carious dentine were identified [9]. Tissue from the two layers was collected, rinsed in purified water and stored separately in Eppendorf tubes, which were left to dry in ambient temperature.

The outer carious layer, defined as the dentine which was discoloured and soft, is hereinafter denoted as “carious dentine – outer layer” and the remaining carious dentine is denoted as “carious dentine – inner layer”. After excavation to hard dentine of normal colour, tested using a tactile procedure [28], sound dentine was collected by drilling.



**Figure 1.** Flow chart of the experimental design with the total number of teeth in the three experimental parts (Experimental Parts I-III) and the sample denominations S01-S16. (FTIR=Fourier Transform Infrared Spectroscopy; FTIR-ATR=FTIR Attenuated Total Reflectance analyses; HD=hydrazine derivative; KBr=potassium bromide; ToF-SIMS=Time-of-Flight Secondary Ion Mass Spectrometry).

**Table 1.** Experimental design of the three parts (Parts 1-3) giving information about teeth used, tooth tissue used, treatment and analysis method. (FTIR=Fourier Transform Infrared Spectroscopy; KBr=Potassium Bromide; FTIR-ATR=FTIR Attenuated Total Reflectance analyses; ToF-SIMS=Time-of-Flight Secondary Ion Mass Spectrometry).

Sample	Tooth no.	Part of tissue	Treatment	Analytical method
<b>Experimental part 1</b>				
S01	1	Sound dentine		FTIR (KBr)
S02	2	Sound dentine		FTIR (KBr)
S03	1	Caries – inner	-	FTIR (KBr)
S04	2	Caries – inner	-	FTIR (KBr)
S05	1	Caries – inner	-	FTIR (KBr)
S06	2	Caries – inner	-	FTIR (KBr)
<b>Experimental part 2</b>				
S07	3-4	Sound dentine	Untreated (reference)	FTIR-ATR
S08	3-4	Sound dentine	Hydrazine derivative/NaOH	FTIR-ATR
S09	3-4	Sound dentine	NaBH <sub>4</sub> /hydrazine derivative/ethanol	FTIR-ATR
S10	5-8	Caries – inner	Untreated (reference)	FTIR-ATR
S11	5-8	Caries – inner	Hydrazine derivative/NaOH	FTIR-ATR
S12	5-8	Caries – inner	NaBH <sub>4</sub> /hydrazine derivative/ethanol	FTIR-ATR
<b>Experimental part 3</b>				
S13	9-13	Caries – inner	Untreated (reference)	FTIR-ATR/ToF-SIMS
S14	14-15	Sound dentine	Untreated (reference)	FTIR-ATR/ToF-SIMS
S15	9-13	Caries – inner	Hydrazine derivative/NaOH	FTIR-ATR/ToF-SIMS
S16	14-15	Sound dentine	Hydrazine derivative/NaOH	FTIR-ATR/ToF-SIMS

All the procedures were carried out with the greatest care in order to avoid unwanted hydrolysis of esters in the collected tissue.

#### Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR analyses were performed using a Mattson Cygnus 100 FTIR spectrophotometer with 4 cm<sup>-1</sup> resolution (Thermo Fischer Scientific Inc, USA). The instrument was purged with analytical instrument quality air to remove atmospheric CO<sub>2</sub> and H<sub>2</sub>O, dried and purified with a Balston type 75-60 air purification system. The spectra were baseline corrected using the Omnic FT-IR software (Thermo Nicolet Corp., Madison, WI, USA). For all spectra, the same wave-number positions were chosen. Each spectrum was acquired from 100 scans and the resolution was 4 cm<sup>-1</sup>. To enhance and further survey peaks or specific shoulders, a Fourier Self-Deconvolution technique was used, followed by spectral subtraction with sound dentine set as the reference, using the software for the FTIR instrument.

#### FTIR Attenuated Total Reflectance (FTIR-ATR)

The FTIR-ATR analyses were performed using a Nicolet 6700 FTIR spectrophotometer (Thermo Nicolet Corp., Madison, WI, USA). A Smart Orbit diamond micro-ATR attachment was used directly to acquire spectra from the samples. The instrument was purged with analytical instrument quality air to remove atmospheric CO<sub>2</sub> and H<sub>2</sub>O, dried and purified with a Balston type 75-60 conditioner.

The dry weight of each sample was approximately 1 mg. Before the analyses, samples representing sound, outer and inner carious dentine respectively were pooled.

#### Time-of-Flight Secondary Ion Mass Spectrometry (ToF-SIMS) analyses

ToF-SIMS was used to analyse the mass differences between sound and carious dentine with or without the addition of hydrazine derivative (Lucifer Yellow CH potassium salt, L0144, www.sigmaldrich.com). ToF-SIMS is based on the separation of charged secondary mass ions emitted from

the sample surface after a pulsed beam of primary ions is projected onto the sample holder (1000 x 1000 μm<sup>2</sup> double adhesive tape of carbon).

The ToF-SIMS analyses were performed in a ToF-SIMS IV (CAMECA/IONTOF GmbH, Münster, Germany). The primary ion was 25 KeV Bi<sup>3+</sup> with a beam current at 0.12 pA. Positive and negative secondary ion mass spectra were recorded from several areas, each one representing one particle of the powdered sample at a size of 200 × 200 μm<sup>2</sup> and separated in a mass analyser and compared with known mass spectra from the Ion Specific application (ION-TOF GmbH, Münster, Germany, ver. 4.1) linked to the ToF-SIMS instrument. The data acquisition time for each secondary ion mass spectrum was 100 seconds. The largest particles of the dry freeze and milled samples with the size of 200 × 200 μm<sup>2</sup>, also one area, with the best signal-to-noise were chosen. Several areas were subjected for each analysis.

#### Experimental Part I

Sound and carious dentine from the outer and inner layers were pooled from two teeth. As a result, six samples [**S01 & S02**=sound dentine; **S03 & S04**=carious dentine inner layer; **S05 & S06**=carious dentine outer layer] were prepared for the FTIR analyses.

The pulverised samples were mixed with potassium bromide (KBr) before subsequent FTIR analyses and pellets with a weight of 100 mg were made.

#### Experimental Part II

Sound dentine was pooled from two teeth (total dry weight (tdw 26 mg) and divided into three samples [**S07-S09**]. Carious dentine was pooled from four teeth (tdw 30 mg) and divided into three samples [**S10-S12**]. Samples **S07** and **S10** were used as references for sound and carious dentine respectively. The samples were repeatedly washed with purified water and dried in a vacuum.

Samples **S08** and **S11**, representing sound and carious dentine from the inner carious layer, were repeatedly washed

with purified water and then mixed with a 13 mM aqueous solution of the hydrazine derivative, and left over night. Thereafter, the samples were washed with a 1 M water solution of NaCl.

The samples were washed twice in a 0.5 M NaOH solution in order to avoid unwanted hydrogen bonding. They were finally washed in purified water and dried in a vacuum. The final steps were used to determine whether or not the bonding was of electrostatic character.

Samples **S09** and **S12**, representing sound and carious dentine from the inner layer, were treated in 0.5 M NaBH<sub>4</sub> in an ethanol solution in order to reduce possible aldehydes and ketones and subsequently washed with 99% ethanol before the addition of a 13 mM aqueous solution of the hydrazine derivative. Finally, the samples were repeatedly washed with purified water before drying in a vacuum.

For the FTIR-ATR analyses, the pulverised samples were pressed between diamond plates before the analyses.

### Experimental Part III

Sound dentine was pooled from two teeth (tdw 35 mg) and divided into two samples [**S14**, **S16**]. Carious dentine from the inner carious layer from five teeth was pooled and divided into two samples [**S13**, **S15**]. Samples **S13** and **S14** were used as references for sound and carious dentine respectively. The reference samples were repeatedly washed with purified water and dried in a vacuum. The reference samples [**S13**, **S14**] were pulverised under liquid nitrogen and dried in a vacuum.

The remaining samples [**S15**, **S16**] were exposed to a 13 mM aqueous solution of the hydrazine derivative for one hour and then washed in a 0.5 M aqueous solution of NaOH and finally washed with purified water before drying in a vacuum. For the FTIR-ATR analyses, the pulverised samples were pressed between diamond plates before the analyses. For the ToF-SIMS analyses, the pulverised samples were applied to double-sided conductive tape and mounted on a sample holder for the instrument.

### Ethical considerations

All the teeth were extracted for clinical reasons and were freely donated by the patients. All the teeth were coded and none of the teeth could therefore be traced to any specific patient. Only tooth number and patient's age were documented.

## Results

### Experimental Part I

The differences in functional groups between the three different tooth tissues, sound dentine [**S01**, **S02**], carious inner dentine [**S03**, **S04**] and carious outer dentine [**S05**, **S06**] analysed by FTIR-KBr, are shown in *Table 2* and *Figures 2 and 3* respectively. None of the samples [**S01-S06**] exhibited a distinct ester carbonyl {C=O stretch} absorption peak around 1740 cm<sup>-1</sup> in the deconvoluted spectra (*Figure 2*). However, shoulders were observed around 1740 cm<sup>-1</sup> in the characteristic peak position of the ester carbonyl group for the inner layer of dental caries [**S03**, **S04**] and in one of the outer layers [**S05**] (*Figure 2*). After spectral subtraction with sound dentine [**S02**], peaks were seen in all the carious samples, **S03-S06**, with the largest amount for the carious inner dentine [**S03**], at 1739 cm<sup>-1</sup> and 1736 cm<sup>-1</sup> respectively (*Figure 3*).

### Experimental Part II

Representative recorded spectra of sound and carious dentine are presented in *Figures 4 a-e*. Spectra were recorded from 4000 to 500 cm<sup>-1</sup> (normalized to 1018 cm<sup>-1</sup> phosphate group), which spans the fingerprint regions of both mineral and proteins.

The FTIR spectrum of sound dentine [**S07-S09**] showed typical absorbance bands at 3300 cm<sup>-1</sup>, representing hydroxyl and amine groups {O-H stretch, N-H stretch}, and some minor absorbancies at 3085 cm<sup>-1</sup>, 2970 cm<sup>-1</sup>, 2940 cm<sup>-1</sup> and 2885 cm<sup>-1</sup> {-CH<sub>2</sub>- stretch, CH stretch} (*Figure 4a*).

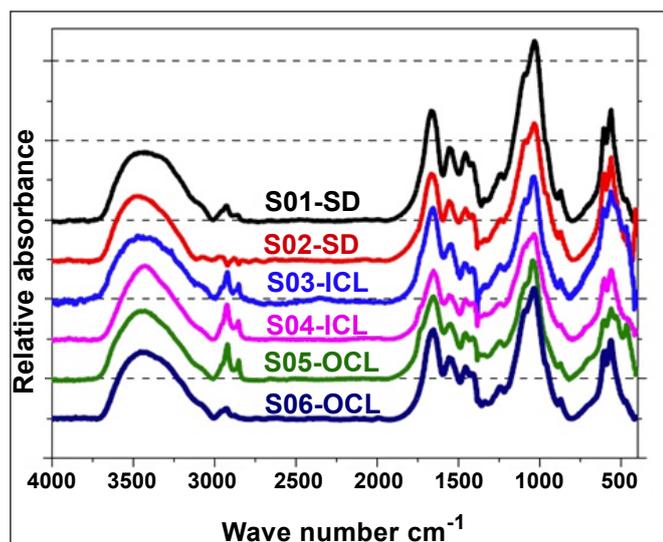
The lower region of the IR active bands associated with the dentine proteins, are represented by amide I (1650 cm<sup>-1</sup>), amide II (1550 cm<sup>-1</sup>) and amide III (1250 cm<sup>-1</sup>) (*Figure 4b*). Other bands corresponding to dentine proteins are the -CH<sub>2</sub>-bending vibrations at 1450 cm<sup>-1</sup> and 1417 cm<sup>-1</sup>. However, the most intense bands, corresponding to the mineral phase {PO<sub>4</sub><sup>3-</sup>, CO<sub>3</sub><sup>2-</sup>} of sound dentine samples [**S07-S09**], were found at 1100-900 cm<sup>-1</sup>.

No spectral changes among the dentine samples after the different treatments, including the addition of hydrazine derivative, were observed (*Figure 4b*).

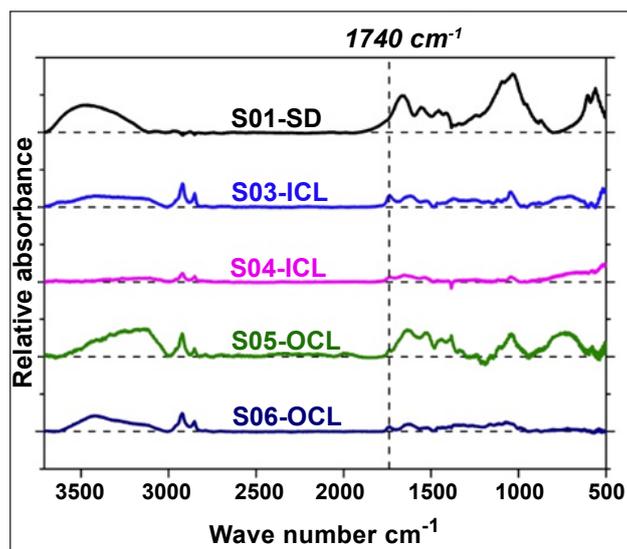
For carious dentine [**S10-S12**], absorbance bands at the higher frequencies were almost identical to those found for sound dentine. However, for all three carious dentine samples [**S10-S12**], unique absorbancies at 2858 cm<sup>-1</sup> {C-H stretch},

**Table 2.** Summary of suggested assignments of the major FTIR peaks detected for sound and carious dentine (references [16,25,29,31,34]). Collagen: amide I (1650 cm<sup>-1</sup>), amide II (1540 cm<sup>-1</sup>) and amide III (1240 cm<sup>-1</sup>); C-H: (1450 cm<sup>-1</sup>); mineral: 1100-900 cm<sup>-1</sup> (POH 1145 cm<sup>-1</sup>) (PO 1100 – 961-957 cm<sup>-1</sup>) C=O 890-860 cm<sup>-1</sup>, CO<sub>3</sub><sup>2-</sup> substitution of OH- (1500-1400 and 3600-3400) cm<sup>-1</sup>. (S=stretch; SE=stretch of esters; Sound=sound dentine; Caries I-layer= inner carious dentine; Caries O-layer=outer carious dentine).

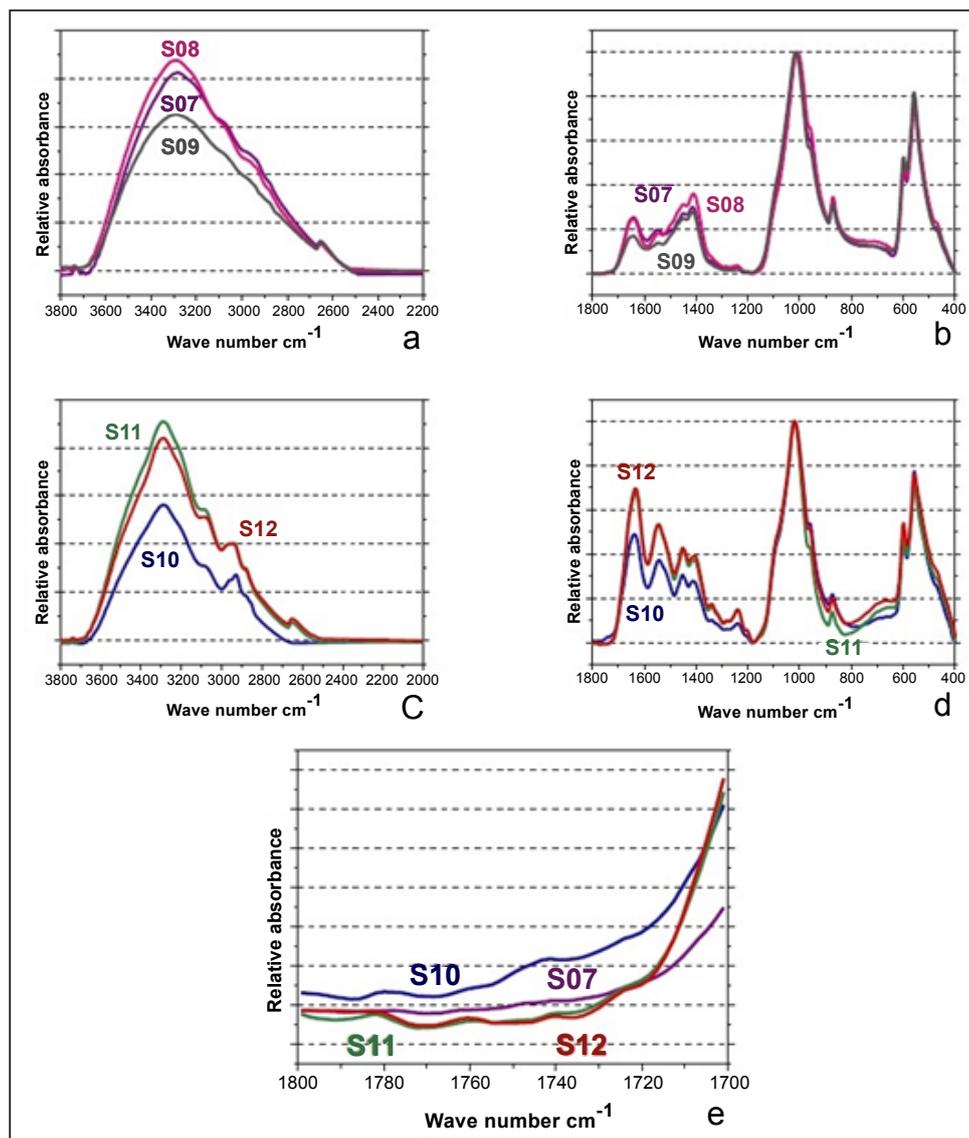
Sample number	Dentine sample	N-H	C-H S	C=O SE	AMIDE I	AMIDE II	CH <sub>2</sub>	AMIDE III	V <sub>1</sub> V <sub>3</sub> PO <sub>4</sub>	V <sub>2</sub> CO <sub>3</sub>
S01	Sound	3500-3380	2297	-	1668	1552	1456	1238	1032	872
S02	Sound	3500-3380	2941	-	1653	1558	1456	1244	1034	872
S03	Carious I-layer	3500-3380	2920	1736	1657	1558	1456	1238	1038	870
S04	Carious I-layer	3500-3380	2924	1739	1653	1545	1462	1242	1034	872
S05	Carious O-layer	3500-3380	2920	1736	1655	1550	1456	1240	1045	872
S06	Carious O-layer	3500-3380	2924	1726	1653	1552	1456	1238	1034	872



**Figure 2.** Deconvoluted FTIR spectra from 4000-500  $\text{cm}^{-1}$  of sound dentine [S01-SD & S02-SD], carious dentine from the inner layer [S03-ICL & S04-ICL] and carious dentine from the outer layer [S05-OCL & S06-OCL].



**Figure 3.** FTIR spectra of the amide I band, from the samples in Fig. 2, representing carious tissue from the inner layer [S03-ICL & S04-ICL] and outer layer [S05-OCL & S06-OCL] after the subtraction of sound dentine [S02-SD]. The shoulders at 1740  $\text{cm}^{-1}$  are marked with a dotted vertical line.



**Figure 4a-e.** (a) FTIR-ATR spectra at 3800-2200  $\text{cm}^{-1}$  of sound dentine [S07], after treatment with NaOH/hydrazine derivative [S08] and after treatment with NaBH<sub>4</sub>/hydrazine derivative [S09]. (b) FTIR-ATR spectra from 1800-400  $\text{cm}^{-1}$  of the same samples as in Fig. 4a. (c) FTIR-ATR spectra of inner layer of carious dentine [S10], after treatment with NaOH/hydrazine derivative [S11] and after treatment with NaBH<sub>4</sub>/hydrazine derivative [S12] at 3800-2000  $\text{cm}^{-1}$ . (d) FTIR-ATR spectra of the same samples as in Fig. 4c from 1800-400  $\text{cm}^{-1}$ . (e) Enlargement of the FTIR-ATR spectra in the region of 1800-1700  $\text{cm}^{-1}$  for samples S07, S10, S11 and S12.

as well as more intense bands at 2967  $\text{cm}^{-1}$  and 2939  $\text{cm}^{-1}$  {-CH<sub>2</sub>- stretch, C-H stretch}, were observed (Fig. 4c). In samples **S11-S12**, these two latter peaks diminished after reaction with the hydrazine derivative. Another characteristic feature of carious dentine was a lower shift of the band at 3095  $\text{cm}^{-1}$  to 3085  $\text{cm}^{-1}$  [**S07-S09**].

When comparing the amide I band at 1650  $\text{cm}^{-1}$  with the phosphate band at 1100  $\text{cm}^{-1}$  of sound dentine (Figure 4b) and of carious dentine, an interesting difference in the mineral/protein ratio was observed, with lower values for the carious dentine (Figure 4d). The lower region of the spectra, the amide I band, was shifted from 1660  $\text{cm}^{-1}$  for sound dentine to 1650  $\text{cm}^{-1}$  for carious dentine (Table 2; Figures 4b and 4d). A shift in the intensity from the sound dentine [**S07-S09**] and the carious dentine [**S10-S12**] at 1450  $\text{cm}^{-1}$  and 1417  $\text{cm}^{-1}$  was also found (Figures 4b & 4d).

A total of four unique absorbancies for carious dentine compared with sound dentine were found at 1740  $\text{cm}^{-1}$  {carbonyl esters C=O}, 1340  $\text{cm}^{-1}$  {C-H deformation; C-N stretch of primary, secondary and tertiary aromatic amines}, 1286  $\text{cm}^{-1}$  {C-H deformation} and 1210  $\text{cm}^{-1}$  {C-C(=O)-O stretch} (Figure 4d). The bands at 1286 and 1210  $\text{cm}^{-1}$  [**S11, S12**] appeared to be enhanced after reaction with the hydrazine derivative in comparison to the caries reference sample [**S10**].

The shoulders found at 1740  $\text{cm}^{-1}$  in the reference sample [**S10**] were diminished after reaction with the hydrazine derivative [**S11, S12**], when the proportions of the intensities of the 1780  $\text{cm}^{-1}$  peak with the 1740  $\text{cm}^{-1}$  peak for each sample were compared (Figure 4e). The spectra of the reference dentine sample [**S07**] were added to the plot for comparison with the samples with no ester carbonyl shoulders.

The untreated dentine sample maintained these peaks, whereas in **S11** (hydrazine derivative/NaOH) and **S12** (reduction by NaBH<sub>4</sub> of aldehydes/ketones, thereby forming alcohol functional groups followed by the addition of the hydrazine derivative) the 2900  $\text{cm}^{-1}$  absorbancies were reduced or lost. The dentine samples did not indicate any differences between untreated [**S07**] or treated samples [**S08, S09**] respectively (Figure 4a).

### Experimental Part III

The results from the original FTIR-ATR spectra of the inner layer of carious dentine [**S13**], compared with Part II [**S10**], is shown in Figures 5a and 5b. The two samples produced similar results, showing the carbonyl esters in the region of

1740  $\text{cm}^{-1}$ . No shoulder was detected for the sound dentine sample [**S14**].

From the ToF-SIMS analyses, only the positive secondary ion mass spectra are shown, as the negative spectra displayed basically the same pattern. When the result from the carious sample treated with the hydrazine derivative was analysed with ToF-SIMS (Figure 6a), higher masses were detected after reaction [**S15**] compared with before [**S13**]. No differences were observed before [**S14**] and after [**S16**] the treatment of sound dentine with the hydrazine derivative (Figure 6b). These spectra showed that the carious dentine tissue reference had the largest peak at 652.56 mass/charge (m/z), whereas the carious dentine treated with the hydrazine derivative held masses up to 1505.56 m/z. The hydrazine derivative had no masses higher than 600 m/z. Fragmentation was observed after the reaction between the hydrazine derivative and caries [**S15**], where 106 mass units were repeatedly lost. This was not found for sound dentine after the hydrazine treatment [**S16**].

## Discussion

The main finding in this study was that ester groups were observed in the carious dentine but not in the sound dentine and that these could be used for binding to a hydrazine derivative (Lucifer Yellow). Although previous work have shown that the amine function is altered and outer and inner layers of carious dentine are distinct from each other [9,10], this is the first time in which a similar alteration for the carboxyl function has been shown and staining of these unique functional groups in inner carious infected dentine, i.e., ester groups, has been proven to be possible.

The exact proportions of organic and inorganic content in the sound and carious dentine of the permanent molars were unknown. As the chemical alterations studied in carious dentine have taken place in the organic component, the exact proportions are of little relevance to the esterification process.

The majority of known analytical techniques, for the analysis of functional groups (e. g. esters), require proteins to be extracted from the calcified tissue. The two methods used in the present study, FTIR and ToF-SIMS, have been shown to be useful for the analysis of chemical alterations in sound and carious dentine, without extensive sample preparation and protein purification [25,29]. ToF-SIMS is a newer

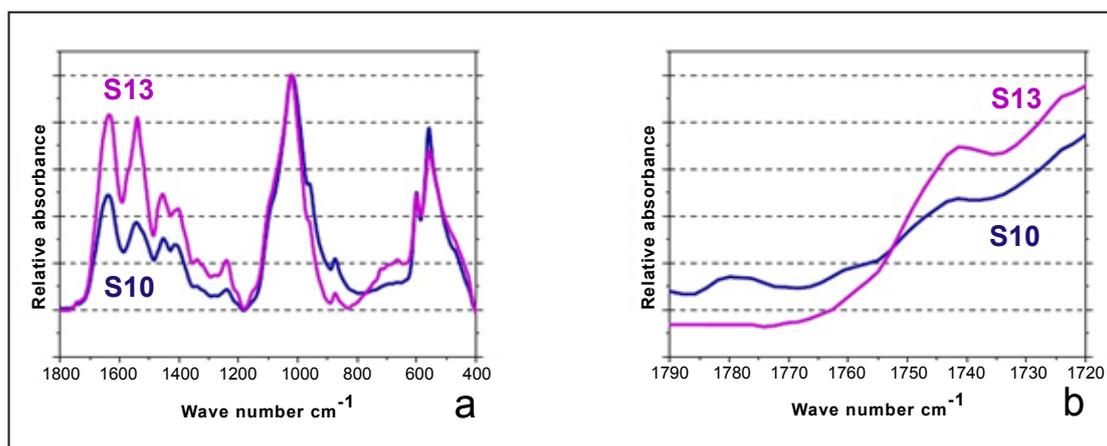
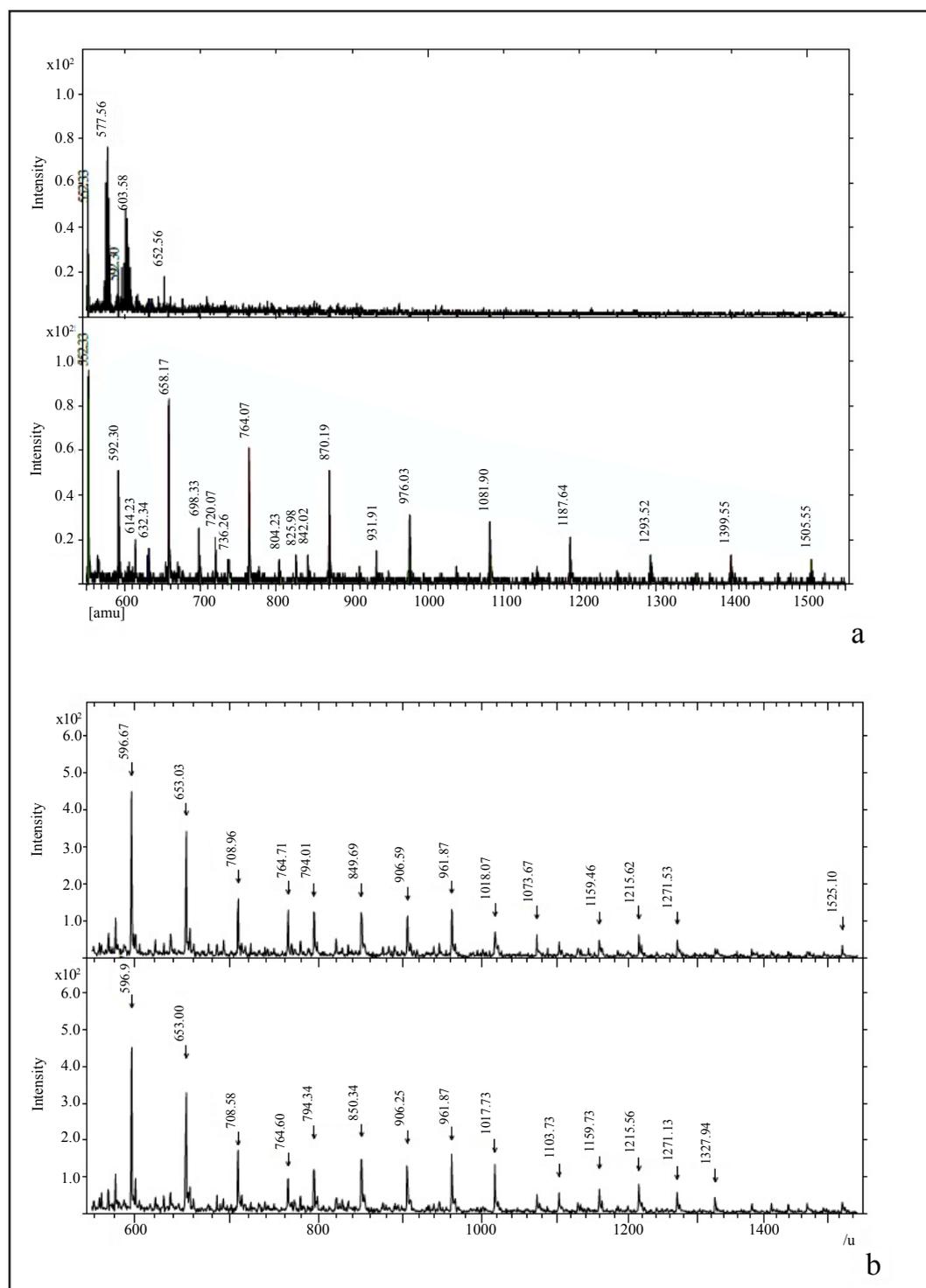


Figure 5a-b. (a) FTIR-ATR spectra of untreated inner layer of carious dentine from Part II [**S10**] and Part III [**S13**]. (b) Enlargement of the region of 1790-1720  $\text{cm}^{-1}$  from the FTIR-ATR spectra of the untreated inner layer of carious dentine from Part II [**S10**] and Part III [**S13**].



**Figure 6a-b.** (a) TOF-SIMS (positive ion spectra;  $m/z$ ) of the inner layer of carious dentine before [S13] and after [S15] treatment with NaOH/hydrazine derivative. (b) TOF-SIMS (positive ion spectra;  $m/z$ ) of sound dentine before [S14] and after [S16] treatment with NaOH/hydrazine derivative.

method and adds important information about the binding after treatment of the tissue with the hydrazine derivative. The chemical modification of the ester groups can be carried out through a reaction with a hydrazine derivative [18]. It is plausible to assume that chemical changes in carious dentine, for example, would be detected using this technique [27].

The excavation technique used for the different samples can be questioned, as it must be regarded as fairly crude based on visual and tactile judgement. However, all the samples were collected by one of the authors under standardised conditions according to previously described techniques [12-13, 30].

The presence of esters has previously been reported and it was suggested that the hydroxyl groups are acylated in

carious dentine, which modifies the collagen to a collagenase-resistant form [8]. In addition, esters derived from bacterial lipid components have been identified in both sound and carious dentine [17] and larger amounts of esterases have been found in carious tooth tissue compared with sound tooth tissue [14].

The data from Part I showed a greater presence of ester groups, around  $1740\text{ cm}^{-1}$  at the amide I band, in the inner layer of carious dentine samples compared with the samples from the outer layer of carious dentine, but no esters were seen in the sound tissue. This can be explained by the fact that esters are influenced by their environment, such as access to water and changes in acidity. In a less humid environment,

more esters are formed, which is a favourable condition for esterification in the carious lesion, as the water content is reduced going from the outer to the inner tooth surface. The pH decrease from the outer to the inner layer may also influence the ester formation in the different carious layers [5]. These aspects correspond well with our finding of larger amounts in the inner samples and no esters in the sound dentine from the tooth with a carious lesion. The studies of sound dentine correspond well with earlier findings, where no shoulders around  $1740\text{ cm}^{-1}$  were identified [20-25,31]. The second characteristic absorption region of esters at  $1300\text{--}1050\text{ cm}^{-1}$  is more difficult to interpret, as other functional groups also absorb in that region. The peak at  $1739\text{--}1736\text{ cm}^{-1}$  for both the inner and outer layers of carious dentine is considered to be a true peak, as its position corresponds to the known characteristic peak position of the ester carbonyl group [19].

The Fourier Self-Deconvolution technique was used to enhance the presence of carbonyl bands in the spectra of Part I [32]. Adding constants to each spectrum separated the data in order to visualise all the spectra within the same figure. For this reason, the absorbance that is shown is therefore a relative absorbance. As false peaks can appear in deconvoluted spectra, the spectra subtraction technique was used on each FTIR spectrum showing true peaks, e.g. the presence of carbonyl groups.

The demineralisation process occurring in the carious tissue is shown as the difference in the mineral/protein ratio (absorbance at  $1650\text{ cm}^{-1}$  and at  $1020\text{ cm}^{-1}$ ) found between sound and carious dentine. Furthermore, the observed absorbancies between  $1450\text{ cm}^{-1}$  and  $1417\text{ cm}^{-1}$  for sound and carious dentine indicate differences between these two tissues. It is suggested that the higher absorbance at  $1450\text{ cm}^{-1}$  for the carious dentine is a consequence of an increase in the number of aliphatic side-groups of various amino acid residues [29]. These bands have been reported to belong to a  $\text{CH}_2$ -bending vibration at  $1450\text{ cm}^{-1}$  and  $1406\text{ cm}^{-1}$  [25]. An increase in the proportion of aliphatic amino acids with the increase in  $\text{CH}_2$ -CH- vibrations could be an effect of the demineralisation process.

Carious dentine [S10-S12] had more accentuated peaks at  $3000\text{--}2800\text{ cm}^{-1}$  in comparison to dentine samples [S07-S09], which might be a consequence of lipids and/or bacteria cells, but it could also be an effect of an increase in the aliphatic content [16,26]. After reaction with the hydrazine derivative, the double peaks at  $2900\text{ cm}^{-1}$  appeared to be reduced.

Unique peaks of carious dentine [S10-S12] were seen at  $1740$ ,  $1340$ ,  $1286$  and  $1210\text{ cm}^{-1}$ . The peaks at  $1740$ ,  $1286$  and  $1210\text{ cm}^{-1}$  appeared to be affected by the reaction. The bands at  $1286$  and at  $1210\text{ cm}^{-1}$  also appeared to be diminished. The  $1286$  and  $1210\text{ cm}^{-1}$  bands exist in all carious dentine, independent of the treatments, and an increase is therefore difficult to predict. One explanation could be that more amide groups are formed as a result of the hydrazine derivative reacting with esters and forming new peptides (amides).

Collagen is reported to exhibit a series of unique IR absorptions between  $1300$  and  $1000\text{ cm}^{-1}$  [16]. This could possibly explain the absorbancies of C-C(=O)-O stretch at  $1210\text{--}1190\text{ cm}^{-1}$  and/or  $1299\text{--}1250\text{ cm}^{-1}$ , unique to carious

dentine found in this study. The  $1338\text{ cm}^{-1}$  peak has been reported to be linked to the degradation of collagen [33] and/or to represent peaks of free amino acids at  $1417$ ,  $1286$  and  $1210\text{ cm}^{-1}$  [19]. Carbonyl absorbancies at  $1340\text{ cm}^{-1}$  could be related to the C-O stretch of COOH (free amino acid) or of an ester at  $1340\text{ cm}^{-1}$  [19]. Furthermore, the most intense absorbance of the hydrazine derivative is at  $1230\text{--}1120\text{ cm}^{-1}$  and it is therefore hidden under the strong absorbance of the mineral peaks.

Normally saturated esters absorb between  $1750$  and  $1735\text{ cm}^{-1}$ , indicating that the signals viewed in the subtraction spectra at  $1739\text{--}1736\text{ cm}^{-1}$  are assigned to normal saturated esters [19]. Moreover, amino acid esters arising from lactones were reported to absorb at  $1735\text{--}1720\text{ cm}^{-1}$  by IR (KBr) and could therefore also be responsible for the signals observed at  $\sim 1740\text{ cm}^{-1}$  [19].

The absorbancies of the new amides formed in the reaction between the esters and the hydrazine derivative would be hidden under the amide bands at  $1650$ ,  $1550$  and  $1250\text{ cm}^{-1}$ . The observed decrease in the absorbance of the carbonyl ester at  $1740\text{ cm}^{-1}$  after the treatment of the carious dentine with the hydrazine derivative suggests that a reaction has taken place.

The enlargement of the wavelength area at about  $1800\text{--}1700\text{ cm}^{-1}$  showed that a reaction had taken place between carious dentine and the hydrazine derivative, as the spectral features associated with the carbonyl esters {C=O} at  $1740\text{ cm}^{-1}$  were changed, i.e. reduced or lost.

In order to confirm the presence of carbonyl esters in the samples [S13-S16] before the reaction to hydrazine derivative in alkaline solution, the FTIR-ATR technique was used prior to the ToF-SIMS analysis. The ToF SIMS analysis revealed new masses that were larger after the treatment with hydrazine derivative than before, compared with the non-treated carious tissue. The largest masses of all the samples were found for the inner layer of carious dentine with hydrazine derivative in positive mode at  $975$ ,  $1081$ ,  $1187$ ,  $1293$ ,  $1399$  and  $1505\text{ m/z}$ .

Due to the experimental conditions, the electrostatic binding was excluded by rinsing with sodium hydroxide, thereby minimising unwanted hydrogen bonding with the hydrazine derivative. As a result, observed masses larger than  $652.56\text{ m/z}$ , the untreated inner layer of the carious dentine sample, strongly indicated that the hydrazine derivative is covalently bound to the tissue.

Both sound and carious dentine were powdered and both therefore contained mineral and proteins. This explains why the observed mass difference for the carious dentine treated with hydrazine derivative not could be due to the alkaline treatment. The mineral therefore appeared to be unaffected by the different treatments in carious tissue.

The observed pattern in the mass spectra, caused by defragmentation corresponding to  $\text{C}_5\text{NO}_2$ , could be ascribed to the hydrazine derivative covalently bound to the carious tissue. This pattern was not detected for the sound dentine or the untreated carious samples respectively.

In the case of sound dentine, the positive mass spectra after different treatments were similar to the positive mass spectra of untreated sound dentine samples. Both untreated healthy dentine and treated healthy dentine (with NaOH/hydrazine derivative) held repeated fragmentation with masses of  $56$

m/z, larger than the intact mass of Lucifer Yellow (521 g/mol), which correlates with CaO of the mineral. This is suggested to be a consequence of the hydrazine derivative not reacting with sound dentine. Accordingly, the hydrazine derivative selectively reacts with carious dentine tissue. Furthermore, an alkaline solution affects sound dentine more than carious dentine, which could be attributed to a larger number of crystallites in sound dentine.

### Conclusion

The results indicate that ester groups are unique to carious tissue and are not found in sound dentine. The ester functions reacted with the hydrazine derivative (Lucifer Yellow), forming a new stable covalent bond in the carious dentine.

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### Clinical Relevance

This work focuses on the chemical evidence behind the visual discoloration observed by the clinician. The finding of specific binding of a hydrazine derivative to the innermost carious dentine would help clinicians to detect the unstained and slightly harder carious tissue, before filling therapy. The staining of carious dentine would be clinically useful, as normal dentine is not stained by the hydrazine derivative, thus preventing the un-necessary removal of sound dentine.

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