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Synthesis and characterisation of starch phosphates

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ABSTRACT

Monostarch monophosphates with different phosphorus contents were prepared via reacting starch with a mixture of primary and secondary sodium phosphates under weak acidic conditions in a semi-dry process. For a structural characterisation of starch phosphates ^{31}P NMR and FT Raman spectra were recorded. The degrees of substitution (DS_P) of modified starch were determined using a standard wet chemistry method. By recording the FT Raman spectra of starch phosphates a characteristic band at ~975 cm^{-1} was found whose intensity depends on the degree of phosphorylation of starch. The integrated areas of this C–O–P stretching vibration were plotted versus the degree of substitution DS_P of starch phosphates obtained from standard photometric method. A strong linear correlation was derived which is suitable to determine the level of phosphorylation of starch phosphates with unknown DS_P value by Raman spectroscopy as a rapid and non-destructive method.

1. Introduction

Modified starches such as starch phosphates are important products with manifold properties which are used as additives in food and non-food applications. In the food industry, low substituted starch phosphates are applied as thickening agent, emulsifier and stabilizer in a wide variety of products (BeMiller & Whistler, 2009; Tomašik & Schilling, 2004; Whistler & Paschall, 1967). Phosphorylated starch is also used as paper coating and wet-end paper additive improving the mechanical properties and filler retention of paper (Hullinger & Carrasco, 1964; Kerr & Cleveland, 1964), and as sizing agent in textile industry (Bode & Olcott, 1963). Because of the anionic character starch phosphates can also be employed as flocculating agents and ion exchangers (Meiczinger, Dencs, Marton, & Dencs, 2005). Cross-linked and highly substituted starch phosphates are very swellable products which may be used as water absorbing and water storing materials (hydrogels, superabsorbants) for different applications in cosmetics, pharmacy, or agriculture (Milloch, 1965; Passauer, Liebner, & Fischer, 2009; Volkert, Fischer, Loth, Langer, & Engelhardt, 2006).

The degree of substitution of phosphate groups DS_P significantly affects the properties of starch phosphates e.g. solubility, swelling properties, rheomechanical characteristics, stability of dispersions (e.g. retrogradation, syneresis), and transparency of solutions or dispersions which are desired for different applications (BeMiller & Whistler, 2009; Sitohy & Ramadan, 2001; Tomašik & Schilling, 2004; Whistler & Paschall, 1967). Consequently, DS_P is an important parameter to characterise starch phosphates. It is commonly determined by using wet chemical and photometric methods (Wongsagonsup, Shobsngob, Oonkhanond, & Varvanit, 2005). This requires a comparatively elaborative and time consuming sample preparation especially for removing residues of non-reacted phosphates by washing or dialyzing starch phosphates with water and precipitating the starch derivative from the aqueous solution. Furthermore, the partially crystalline and semi-crystalline character of the starch granules makes them resistant to dissolution. Hence, starch phosphates are difficult to purify from non-reacted residuals of inorganic phosphates and the phosphorus content of phosphorylated starch is supposed to be overestimated. As an alternative, an analytical method without or little sample preparation and a directly determination of the degree of chemical modification without an interference of residual impurities of the derivatisation reagents is very attractive.

A spectroscopic method for the characterisation of starch and its derivatives which can be used with little or no sample preparation is Raman spectroscopy (Phillips, Pan, Liu, & Corke, 1998; Phillips, Xing, Liu, Chong, & Corke, 1999; Pigorsch, 2009). Raman spectra can be collected from very small sample amounts and water does not generally interfere with Raman analysis. Furthermore, Raman spectroscopy is less susceptible to interference from residues of derivatisation reagents or impurities since each compound and binding form displays a special vibrational spectrum. Since Raman band intensities have a linear dependence on the compound present in a sample, FT Raman spectroscopy has been used as a quantitative analytic tool in polysaccharide chemistry (Phillips et al., 1998, 1999). In conjunction with the investigation

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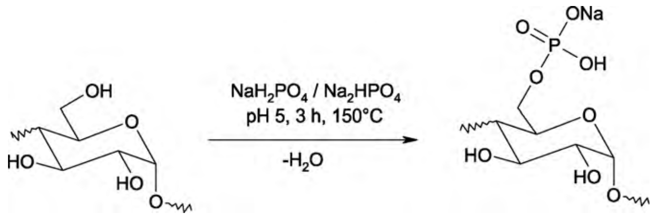

Fig. 1. Synthesis of starch phosphates by esterification of starch with primary and secondary sodium phosphates.

of phosphorus compounds the utilization of FT Raman spectroscopy allows the differentiation of inorganic [e.g. υ(P–O) at 910 cm^{-1}, υ(P=O) at 1170 and 525 cm^{-1}) and organic bonded phosphorus [e.g. υ(C–O–P) at 980–970 cm^{-1}] (Baudler, 1956; Pasterny, Chełmecka, Wrzalika, & Szeja, 2005; Sokrates, 2001). In contrast, the observation of changes in the FT IR spectra of starch phosphates due to the phosphorylation is hampered by broad overlapping bands of starch backbone (Heinze, Klemm, Unger, & Pieschel, 2003; Passauer, Liebner, & Fischer, 2006). Consequently, Raman spectroscopy should be a suitable method for the quantitative characterisation of monostarch monophosphates with different DS_P values.

2. Materials and methods

For the experiments partly degraded starch (Merck, Darmstadt, Germany), potato starch (Emslandstärke, Emlichheim, Germany), and waxy maize starch (Cerestar, Krefeld, Germany) were used. Sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate were obtained from Sigma–Aldrich (Steinheim, Germany) and sulphuric vanadate-molybdate reagent from Merck (Darmstadt, Germany). All other chemicals used were of laboratory grade.

2.1. Synthesis of starch phosphates

Mono-phosphorylation of starch (Fig. 1) was performed by reacting starch with a mixture of sodium dihydrogen phosphate monohydrate $NaH_2PO_4{\cdot}H_2O$ and disodium hydrogen phosphate dihydrate $Na_2HPO_4{\cdot}2H_2O$ in a semi-dry process (Paschall, 1964).

For the synthesis different amounts of both sodium orthophosphates were used (Table 1) in order to obtain starch phosphates with different DS_P values. Prior to phosphorylation, sodium dihydrogen phosphate monohydrate and disodium hydrogen phosphate dihydrate were dissolved in 20 ml water at 35 °C and the pH was adjusted to 5 by adding a few drops of 3 M aqueous sodium hydroxide solution. 10 g of starch were added to the salt solution and the mixture was stirred for 20 min at ambient temperature. The resulting slurry was vacuum filtrated and the filter cake was crumbled and dried for 24 h at 55 °C. After homogenizing in a ball mill the mixture was dried again at 65 °C for 90 min. For starch phosphorylation, the dried mixture was reacted at 150 °C for 3 h. After cooling down to room temperature the reaction product was suspended and stirred in 50% aqueous methanol for 30 min in order to remove non-reacted orthophosphates, diphosphates and degradation products of starch. The filtered product was dehydrated by washing with absolute ethanol. The resulting paste was suspended/diluted in 200 ml de-ionized water and precipitated with acetone. In order to remove water and acetone the product was suspended in ethanol, than vacuum filtrated and finally dried at 45 °C before grinding.

Table 1
Amounts of sodium orthophosphates used for the phosphorylation of starch.

Molar ratio phosphate/starch	$NaH_2PO_4{\cdot}H_2O^a$ [mol]	$Na_2HPO_4{\cdot}2H_2O^a$ [mol]
0.15:1	0.005	0.0038
0.23:1	0.01	0.0042
0.42:1	0.02	0.0063
0.84:1	0.04	0.012
1.26:1	0.06	0.018
1.63:1	0.08	0.021

[a] Amounts of $NaH_2PO_4{\cdot}H_2O$ and $Na_2HPO_4{\cdot}2H_2O$ related to 10 g of starch.

2.2. ^{31}P NMR spectroscopy

^{31}P NMR spectra of starch phosphates were recorded with a BRUKER DPX 400 spectrometer at 161.67 MHz (D1: 5 s, NS: 700–2000). The corresponding samples were dried by repeatedly washing with absolute ethanol and removal of residual moisture traces by drying the samples in a desiccator in the presence of silica gel under vacuum. For the measurements about 100 μg of dried starch phosphate were added to 750 μl deuterium oxide (>99.8 atom% D; Carl Roth GmbH, Karlsruhe, Germany) in a NMR tube. Phosphoric acid was used as external standard. The spectra were recorded at ambient temperature.

2.3. Colorimetric determination of the P content and calculation of the degree of substitution DS_P

For the determination of the phosphorus content of starch phosphates the vanadomolybdophosphoric acid method was used which was firstly described by Misson (1908). The procedure which was applied in the present work was modified after Wongsagonsup et al. (2005). A mixture of 0.3 g sodium carbonate diluted in 2 ml of boiling water and 0.5 g starch phosphate was dried at 100 °C and consequently ashed in a muffle furnace at 550 °C for 9 h. During the heating of the sample diphosphates and condensed phosphates were formed which were hydrolyzed to orthophosphates by adding 12 ml 4% hydrochloric acid after cooling down the combusted sample in a dessicator. The solution was filled up to a volume of 50 ml with distilled water and filtered. The filtrate was diluted to 250 ml with distilled water and 10 ml thereof were mixed with 2 ml of vanadate-molybdate reagent. The sulphuric ammonium molybdate $(NH_4)_2MoO_4$ is rapidly reacting with orthophosphates to form molybdophosphoric acid $H_3[P(Mo_3O_{10})_4]$. In the presence of vanadium an intensive and stable yellow colour – vanadomolybdophosphoric acid $(H_{3+n}PV_nMo_{12-n}O_{40})$ – is formed, the intensity of which is proportional to the phosphate concentration. This colour complex was quantified photometric. Its absorption maximum is located at 435 nm and was measured with an UV–vis spectrometer SHIMADZU UV-2101PC 45 min after adding the vanadate-molybdate reagent to the samples. The degrees of substitution by phosphate monoester groups were calculated according to

$$DS_P = \frac{162P}{3100 - 103P} \qquad (1)$$

with P being the colorimetric determined percentage of phosphorus content of the monostarch monophosphate, *162* the molar mass of the anhydroglucose unit, 3100 the atomic weight of phosphorus multiplied by 100 and 103 the molar mass of the phosphate substituent ($NaHPO_3$).

2.4. FT Raman spectroscopy and analysis of spectra

The Raman spectra of starch phosphates were recorded with a BRUKER FT Raman spectrometer MultiRAM with air-cooled Nd:YAG laser source operating at $\lambda_{Nd:YAG}$ = 1064 nm and a liquid nitrogen-cooled Ge diode as detector. Dry samples were placed on an aluminium holder and 300 scans were added with 4 cm^{-1} res-

Table 2
Photometric determined degrees of substitution DS_P of starch phosphates.

DS_P			
Molar ratio phosphate/starch (AGU)	Degraded starch	Potato starch	Waxy maize starch
0.15:1	0.04	0.02	0.04
0.23:1	0.04	0.03	0.06
0.42:1	0.06	0.05	0.13
0.84:1	0.12	0.08	0.24
1.26:1	0.19	0.12	0.28
1.63:1	0.22	0.19	0.37

olution for each (data collection time about 10 min). Quantitative analysis was done with the spectroscopy software OPUS/QUANT version 6 from BRUKER by integrating the areas A_{975} of the C–O–P stretching vibration at ∼975 cm^{-1} from vector-normalized spectra (3500–250 cm^{-1}). In order to determine a calibration curve which allows a rapid determination of the phosphorus content of starch phosphates with unknown DS_P, the band areas A_{975} of Raman spectra from analyzed samples were plotted versus the degree of substitution DS_P obtained from colorimetric method.

3. Results and discussion

3.1. *Degree of substitution DS_P*

The colorimetric determined degree of substitution of selected starch phosphates ranged between 0.02 and 0.37 (Table 2), corresponding to different amounts of sodium phosphates which have been used for starch phosphorylation. The DS_P depends on type of starch, whereas the amylopectin rich waxy maize starch possesses the highest phosphorus contents.

Additionally, three further waxy maize starch phosphates which were synthesized in the context of earlier investigations were included in the present work. The samples possess colorimetric determined DS_P values of 0.16 (WMS-Px1), 0.17 (WMS-Px2) and 0.18 (WMS-Px3).

3.2. *^{31}P NMR spectroscopy*

The ^{31}P NMR spectrum of a selected starch phosphate based on partly degraded starch (Fig. 2) shows resonance signals in the range between −5 and 10 ppm indicating the presence of minor portions of starch diphosphates (Heinze et al., 2003) and two broad signals at 3.6 and 4.7 ppm. The latter reveal that under the selected reaction conditions mainly monostarch monophosphates were formed giving resonance signals in the range from 0 to 5 ppm (Lee & Lim, 1998; Sang, Prakash, & Seib, 2007; Santacruz, Andersson, & Åman, 2005). In earlier investigations (Passauer et al., 2006), by means of 2D ^{1}H/^{31}P HMBC NMR spectroscopy and calculations with SPARTAN^{TM} software using α-methyl glucoside and its 2-*O*-, 3-*O*-, and 6-*O*-phosphates as model compounds it was shown that the signal at 3.6 ppm is caused by a primary phosphate group at the C-6 position of the AGU. The signal at 4.7 ppm could obviously be assigned to phosphate groups at C-2 and C-3 positions.

3.3. *FT Raman spectroscopy*

Fig. 3 depicts the normalized FT Raman spectra of waxy maize starch (a), its corresponding phosphate with a DS_P value of 0.37 (b), a mechanically homogenised mixture from waxy maize starch and non-reacted sodium dihydrogen phosphate monohydrate (c), and pure sodium dihydrogen phosphate monohydrate (d). The band positions and assignments are summarized in Table 3.

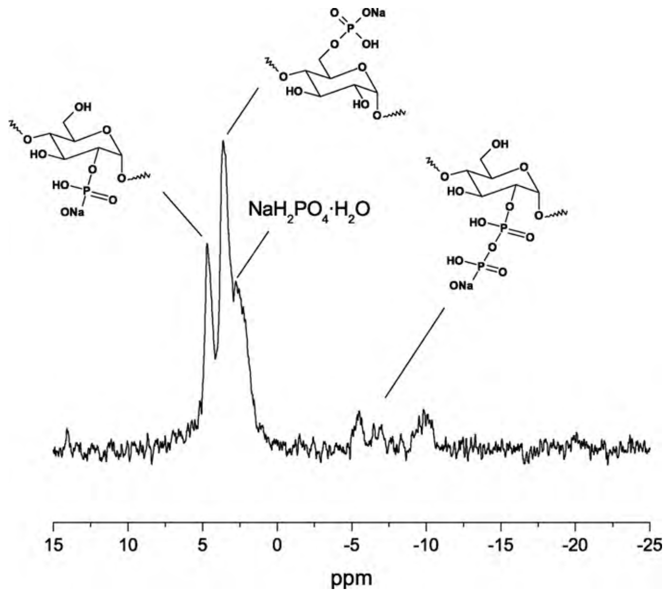

Fig. 2. ^{31}P NMR spectra of monostarch monophosphate from partly degraded starch (DS_P = 0.12).

The spectrum of sodium dihydrogen phosphate monohydrate (Fig. 3d) shows the orthophosphate typical Raman bands υ(P=O) at 1170 and 525 cm^{-1} as well as υ(P–O) at 980 and 910 cm^{-1} (Sokrates, 2001) which can be also found in the spectra of the mixture from non-reacted phosphate and waxy maize starch (Fig. 3c). The absence of this bands especially of the most intensive P–O stretching vibration υ(P–O) at 910 cm^{-1} in the Raman spectra of monostarch monophosphates (Figs. 3b and 4) indicates that most of the non-reacted sodium orthophosphates were removed by purification of the reaction products (see also ^{31}P NMR spectra Fig. 2). Thus, an overestimation of the photometric determined DS_P of starch phosphates due to non-reacted orthophosphates should be out of question.

In the spectra of waxy maize starch (Fig. 3a) the Raman band in the range from 3000 to 2800 cm^{-1} mainly arises from C–H stretch-

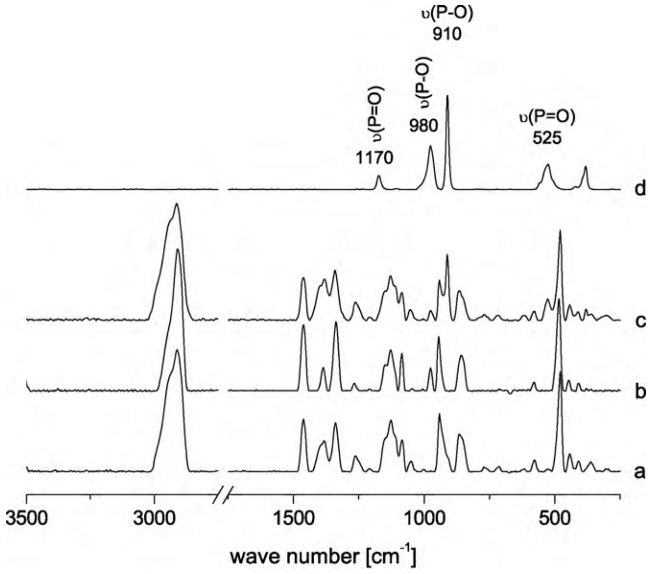

Fig. 3. Raman spectra of (a) waxy maize starch, (b) waxy maize starch phosphate (DS_P = 0.37), (c) homogenized mixture of non-reacted sodium dihydrogen phosphate monohydrate and waxy maize starch (mixture contains 5% P w/w), and (d) pure sodium dihydrogen phosphate monohydrate.

Table 3
Positions and assignments of Raman bands of native starch, monostarch monophosphates, sodium dihydrogen phosphate, and diphosphates.

Band position [cm^{-1}]		Band assignment	Reference
Native starch	Starch phosphate		
2910	2910 vs	υ(C–H)	[a–c]
1460	1460 m	δ(CH$_2$) twisting, CH bending	[a, b, d, e]
1380	1385 m	δ(C–H), CH bending, CH scissoring	[a–c]
1335	1335 s	δ(CH$_2$), C–OH bending	[a, d, e]
1260	1265 m	δ(CH$_2$), CH$_2$OH related mode	[a, b, d]
1205	1205 w	δ(C–H)	[a, b]
1145	1145 m (sh)	υ_a(C–O–C) α-1,4-glycosidic linkage	[a]
1125	1125 s	υ(C–OH) bending, υ(C–O), δ(C–OH)	[a, c]
1085	1085 s	υ(C–O–C) ring mode, C–OH bending	[a–c]
1050	1045 m	δ(C–OH), υ(C–OH)	[a–d]
1000	1005 w	CH$_2$ related mode	[a, d]
	975 s	υ_a(C–O–P)	[f, h]
940	940 s	υ_s(C–O–C) α-1,4-glycosidic linkage	[a–c, e]
865	860 s	υ_s(C–O–C) ring mode, C$_1$–H bending α-configuration	[a, b, e]
715	715···w	υ(C–C)	[b, g]
<600	<600 w	Skeletal + torsional vibrations (ring vibrations)	[e]
475	480 s	Skeletal mode involving (C–O–C) ring mode, δ(C–C–O)	[a, c]
NaH$_2$PO$_4$·H$_2$O and pyrophosphates			
1170	m-w	υ(P=O)	[f]
980	m	υ(P–O)	[f]
1020–920	s	υ_s(P–O–P)	[f]
910	s	υ(P–O)	[f]
730–670	w	υ_s(P–O–P)	[f]
525	m-w	υ(P=O)	[f]

Key to intensities: vs, very strong; s, strong; m, moderate; w, weak; sh, shoulder.
Key to references: (a) Pigorsch, 2009; (b) Cael et al., 1973; (c) Schuster et al., 2000; (d) Sekkal et al., 1995; (e) Tu et al., 1979; (f) Sokrates, 2001, (g) Phillips et al., 1999; (h) Baudler, 1956.

ing vibration (Cael, Koenig, & Blackwell, 1973; Pigorsch, 2009; Schuster, Ehmoser, Gapes, & Lendl, 2000). The finger print region from 1600 to 250 cm^{-1} is dominated from C–H and CH$_2$ deformation vibrations (1480–1230 cm^{-1}) (Cael et al., 1973; Pigorsch, 2009; Schuster et al., 2000; Sekkal, Dincq, Legrand, & Huvenne, 1995; Tu, Lee, & Milanovich, 1979), C–O–C stretching vibration (α-1,4-glycosidic linkage) at 1150 cm^{-1} (antisymmetric) (Pigorsch, 2009) and 940 cm^{-1} (symmetric) (Cael et al., 1973; Pigorsch, 2009; Schuster et al., 2000; Tu et al., 1979), C–OH stretching and deformation vibrations at 1125 and 1050 cm^{-1} (Cael et al., 1973; Pigorsch, 2009; Schuster et al., 2000; Sekkal et al., 1995), as well as C–O–C stretching vibration of the α-glucose ring at 1085 cm^{-1} and 855 cm^{-1} (Cael et al., 1973; Pigorsch, 2009; Schuster et al., 2000; Sekkal et al., 1995). The most characteristic band in the Raman spectra of starch is located at 480 cm^{-1} which is assigned to the symmetric C–O–C vibration of the α-glucose ring (Cael et al., 1973; Pigorsch, 2009; Schuster et al., 2000). In comparison to other polysaccharides such as cellulose (Schenzel & Fischer, 2001) it is the most intensive peak within the finger print region of Raman spectra. The Raman band at 715 cm^{-1} is primarily attributed to C–C stretching vibrations (Cael et al., 1973; Phillips et al., 1999).

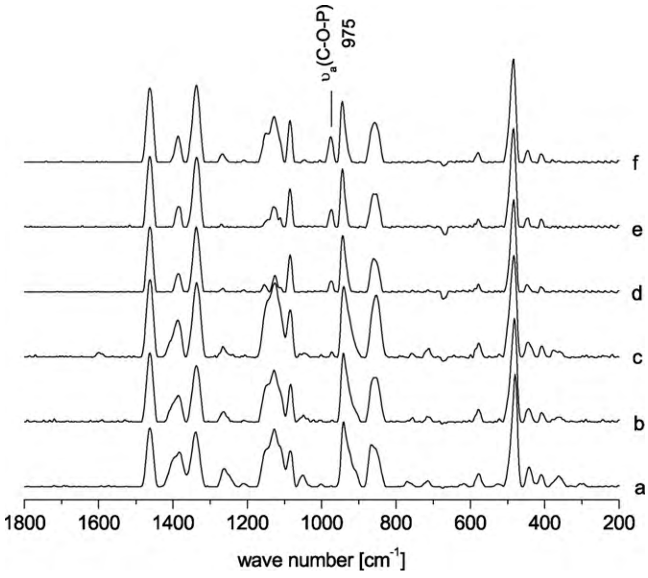

Fig. 4. Raman spectra of waxy maize starch phosphates (a) WMS-P-1 (DS$_P$ = 0.04), (b) WMS-P-2 (DS$_P$ = 0.06), (c) WMS-P-3 (DS$_P$ = 0.13), (d) WMS-P-4 (DS$_P$ = 0.24), (e) WMS-P-5 (DS$_P$ = 0.28), and (f) WMS-P-6 (DS$_P$ = 0.37).

After phosphorylation of starch, a new band appeared in the range from 980 to 970 cm^{-1} (Fig. 3b), which is assigned to the antisymmetric C–O–P stretching vibration (Baudler, 1956; Pasterny et al., 2005; Sokrates, 2001). This band indicates organically bonded phosphate groups which were introduced into the starch molecule by esterification of the hydroxyl groups of the anyhdroglucose units (AGU) of starch with sodium orthophosphates. It could be observed that with increasing total DS$_P$ the C–O–P band is shifted to slightly higher wave numbers (Fig. 4). The P=O stretching vibration of alkyl phosphates with the structure (RO)(OH)$_2$P=O which is located at 1250 cm^{-1} (Baudler, 1956; Sokrates, 2001) is interfered by CH$_2$ deformation vibration of starch backbone. Furthermore, the P–O stretching vibration of organically bonded phosphates which occurs in a region from 1335 to 1080 cm^{-1} (Sokrates, 2001) is dominated from antisymmetric C–O–C stretching vibration (α-1,4-glycosidic linkage; 1150 cm^{-1}) and C–OH stretching vibrations (1125 and 1050 cm^{-1}) of starch backbone and could not be observed.

In consequence of the phosphorylation, minor changes in starch typical Raman bands were found (Fig. 3b), e.g. the decreasing intensity of the C–OH band at 1050 cm^{-1} with increasing DS$_P$ of starch phosphate (see also Fig. 4) what is due to the substitution of the hydrogen atoms from the hydroxyl groups of the AGU by phosphate groups.

Spectral vibrations such as υ_s(P–O–P) representing pyrophosphates and starch diphosphates which lie in regions from 1020

Table 4
Photometric determined degrees of substitution DS_P and peak areas A_{975} of υ_a(C–O–P) of selected starch phosphate samples which were used for correlation analysis.

Sample	Starting material	DS_P	Peak area A_{975} υ(C–O–P)
PS-P-1	Potato starch	0.02	0.51
PS-P-2a	Potato starch	0.03	0.55
PS-P-2b	Potato starch	0.03	0.69
WMS-P-1	Waxy maize starch	0.03	0.50
DS-P-2	Degraded starch	0.04	0.78
WMS-P-2	Waxy maize starch	0.06	1.05
PS-P-3	Potato starch	0.08	1.51
PS-P-5a	Potato starch	0.12	1.90
PS-P-5b	Potato starch	0.12	2.20
WMS-P-3	Waxy maize starch	0.13	2.25
WMS-P-x1a	Waxy maize starch	0.16	2.63
WMS-P-x1b	Waxy maize starch	0.16	2.80
WMS-P-x2a	Waxy maize starch	0.17	3.03
WMS-P-x2b	Waxy maize starch	0.17	3.10
WMS-P-x3	Waxy maize starch	0.18	3.15
PS-P-6a	Potato starch	0.19	3.22
PS-P-6b	Potato starch	0.19	3.45
WMS-P5	Waxy maize starch	0.28	4.45
WMS-P6a	Waxy maize starch	0.37	5.61
WMS-P6b	Waxy maize starch	0.37	5.80

to 920 cm^{-1} and 730 to 670 cm^{-1} (Sokrates, 2001) could not be identified in the Raman spectra of starch phosphates (Fig. 4). Corresponding with the results from ^{31}P NMR measurements, it could be assumed that during starch phosphorylation under the selected reaction conditions mainly monostarch monophosphates were formed.

3.4. Raman spectroscopic quantification of the degree of substitution DS_P

Examination of Fig. 4 which depicts the Raman spectra of phosphates from waxy maize starch shows that the C–O–P Raman band (980–970 cm^{-1}) increases in intensity and width significantly as the amount of phosphorylation of starch increases.

Hence, this band seems to provide the most suitable marker band to determine DS_P values of starch phosphates with Raman spectroscopy all the more this peak is not interfered by vibrations of starch backbone. In order to validate this assumption the C–O–P band of selected starch phosphate samples was integrated after vector-normalization of the Raman spectra whereas the symmetric C–O–C stretching vibration of the α-glucose ring at 480 cm^{-1} was used as internal standard. After integration the peak areas A_{975} of the C–O–P Raman band were obtained which were plotted versus the colorimetric determined DS_P values of starch phosphates. The samples which were selected for correlation analysis are specified in Table 4.

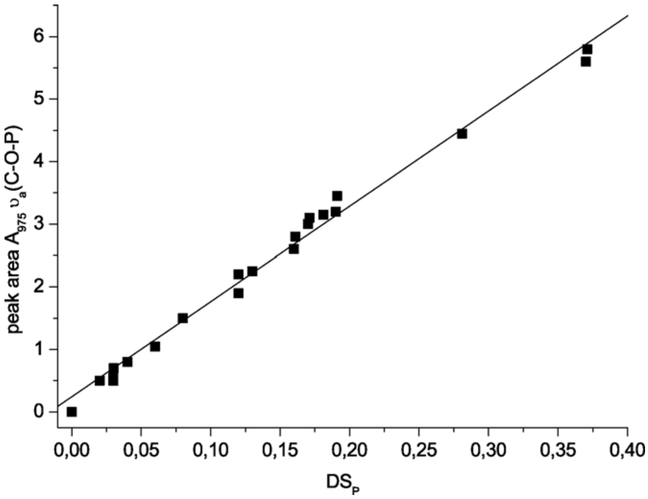

Fig. 5. Correlation between the degree of substitution DS_P (determination via UV–vis) and the area of the υ_a(C–O–P) Raman band; linear regression: $y = 0.2427 + 15.225x$ with $R^2 = 0.9908$.

A strong linear correlation between the C–O–P peak integral and DS_P values with a coefficient of determination of 0.9908 was obtained (Fig. 5). That finding indicates the strong linear relationship between the observed intensity changes in the Raman band at 980–970 cm^{-1} and the DS_P and confirms the suitability of FT Raman spectroscopy for quantifying the DS_P values of low substituted starch phosphates.

Generally, this alternative for the quantification of DS values by Raman method exhibits some advantages over wet chemistry methods. The Raman technique requires almost no sample preparation and could be used as quick analysis tool after a calibration curve has been developed. Furthermore, the Raman method is non-destructive and only small sample amounts are needed.

4. Conclusions

In the present paper a novel method for the determination of the degree of substitution of monostarch monophosphates by using FT Raman spectroscopy is demonstrated. As the observation of changes in the FT IR spectra due to phosphorylation of starch is hampered by broad overlapping bands of starch backbone, Raman spectroscopy was proven to be most suitable to reveal and quantify phosphate groups in modified starch. The observed intensity changes of the C–O–P Raman band at ~975 cm^{-1} yielded a linear relation with the colorimetric determined degree of substitution DS_P of monostarch monophosphates with a correlation coefficient of 0.9908. Thus, the DS_P can be exactly derived from the integrated area of the C–O–P band in the Raman spectra.

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