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A Scheme for the Simultaneous Quantitative Determination of Free Amino Acids, Organic Non-amino Acids, Sugars, Nitrogen, and Pectic Substances in Plant Materials

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An analytical scheme for the quantitative determination of the free amino acids, organic non-amino acids, sugars, and nitrogen, and the characterization of the pectic substances is presented. The free amino acids, organic non-amino acids and sugars are extracted with 80 % ethanol and separated by ion exchange. The amino acids are then determined by automatic ion-exchange chromatography and the organic non-amino acids and sugars by linearly temperature-programmed gas chromatography. The pectic substances are extracted from the residue insoluble in 80 % ethanol and fractionated by gel chromatography on Sephadex. Nitrogen is determined by automatic combustion according to Dumas. Statistical data showing the reproducibility of the methods are given.

Investigations on the effects of different variables on the quality of vegetable products often require extensive mapping of their chemical composition. Thus the work is characterized by frequent repetition of sampling procedures and a large number of samples. The investigation described here deals with the development of an analytical scheme for the simultaneous quantitative determination of free amino acids, organic non-amino acids, sugars, and nitrogen and the characterization of the pectic substances (Scheme 1).

METHOD

Free amino acids, organic non-amino acids and sugars

Extraction and fractionation. Free amino acids, organic non-amino acids and sugars were extracted with 80 % ethanol as follows. A chopped vegetable sample was mixed with a sufficient amount of boiling 94 % ethanol to make the final ethanol concentration, as estimated from the water content of the sample, 80 % by volume. The mixture was refluxed for 3 min and allowed to cool to room temperature with occasional shaking.

After 5 h the mixture was filtered. The residue was macerated in 80 % ethanol in a Servall Omni-Mixer homogenizer, the mixture was filtered and the residue extracted with 80 % ethanol at room temperature overnight. The mixture was filtered and the residue washed with absolute ethanol. All the filtrates were combined and diluted to a suitable volume. The alcohol-insoluble solid residue (AIS) was dried under vacuum at 70°C and ground to particle sizes passing through a 60-mesh sieve. The powdered material was analyzed for pectic substances and nitrogen as described below.

The ethanolic filtrate was passed through a column of Dowex 50WX4 (H^+-form, 50—100 mesh). The amino acids were sorbed on the column. The effluent was passed through a column of Dowex 1X4 (CO_3^{2-}-form, 50—100 mesh), which retained the organic non-amino acids. The ion exchangers were washed with sufficient quantities of water to remove sugars and neutral compounds.

Determination of amino acids. Amino acids were eluted from the cation exchanger with 10 % ammonia and the resin was washed with water. The effluent was evaporated to dryness under vacuum in a rotatory evaporator at 40°C. 5 ml of 0.1 N sodium hydroxide was added to the dry residue and the solution was evaporated to dryness as above. The dry residue was dissolved in 0.1 N hydrochloric acid and amino acids in the solution were determined with a Technicon amino acid analyzer using norleucine as internal and external standard.

Determination of organic non-amino acids. Organic non-amino acids were eluted from the anion exchanger with 1.5 N ammonium carbonate and the resin was washed with water. The effluent was evaporated to dryness under vacuum at 40°C. 15 ml of anhydrous methanol-hydrogen chloride (8 % HCl in anhydrous methanol) was added to the dry residue and the solution was allowed to stand overnight protected from moisture. 10 ml of the solution was taken for esterification and 0.1 ml of thionyl chloride was added to it.[1] The mixture was refluxed on a boiling water bath for exactly 10 min. The solution was cooled to room temperature, and the excess reagents were evaporated under vacuum at 20°C. 10 ml of methanol was added to the dry residue and the evaporation was repeated. The methyl esters were quantitatively extracted from the dried residue with five 0.7-ml portions of methanol and determined by a linear temperature programmed gas chromatographic method using methyl myristate as internal standard as described earlier by Salminen and Koivistoinen.[2]

Determination of sugars. The final effluent from the ion exchange resins was used for the determination of sugars by the method of Schoorl-Regenbogen [3] and also by a gas chromatographic method.

For the gas chromatographic determination, a part of the sugar fraction was evaporated to dryness under vacuum at 40°C; 3 ml of absolute ethanol was added and the solution was evaporated to dryness. The dry residue was dissolved in 4 ml of dry pyridine. 2 ml of this solution was pipetted into a 5 ml volumetric flask and 1 ml of a *freshly* prepared solution of arabinose in pyridine (5 mg/ml) was added. 0.1 ml of hexamethyldisilazane and 0.1 ml of trimethylchlorosilane [4] were added to the mixture. The reaction mixture was shaken for 30 sec and allowed to stand 10 min at room temperature, after which the solution was diluted to 5 ml by adding pyridine. The resulting sugar ether solution was chromatographed using a 1-μl sample. The amount of arabinose present as internal standard in each sample was thus always 1 μg.

The apparatus used for gas chromatography was that used earlier in the gas chromatographic analysis of organic non-amino acids.[2] The stationary phase in the two columns was 5 % DC-11 on Chromosorb W HMDS (60—80 mesh). The support and phase were packed into a copper tube 1.5 m long and 1/8 in. in diameter. The carrier gas was nitrogen and its flow rate 40 ml/min in both columns. The flow rate of hydrogen in the detectors was 30 ml/min and that of air about 300 ml/min. The temperature of the injector block was 195°C and that of the detector oven 225°C. The temperature of the column oven was linearly programmed from 65 to 225°C at a rate of 4°C/min.

The standard curves (regression lines) for glucose, fructose, and sucrose were determined as described in the paper relating to the gas chromatographic determination of organic non-amino acids.[2] Because the anomers are separated from one another in this gas chromatographic method for sugars, only the peak area of one anomer was used for calculating the amounts of glucose and fructose.

Pectic substances

Pectic substances were extracted from the solids insoluble in 80 % alcohol (AIS) by a modification of the method described by Kertesz *et al.*[5]

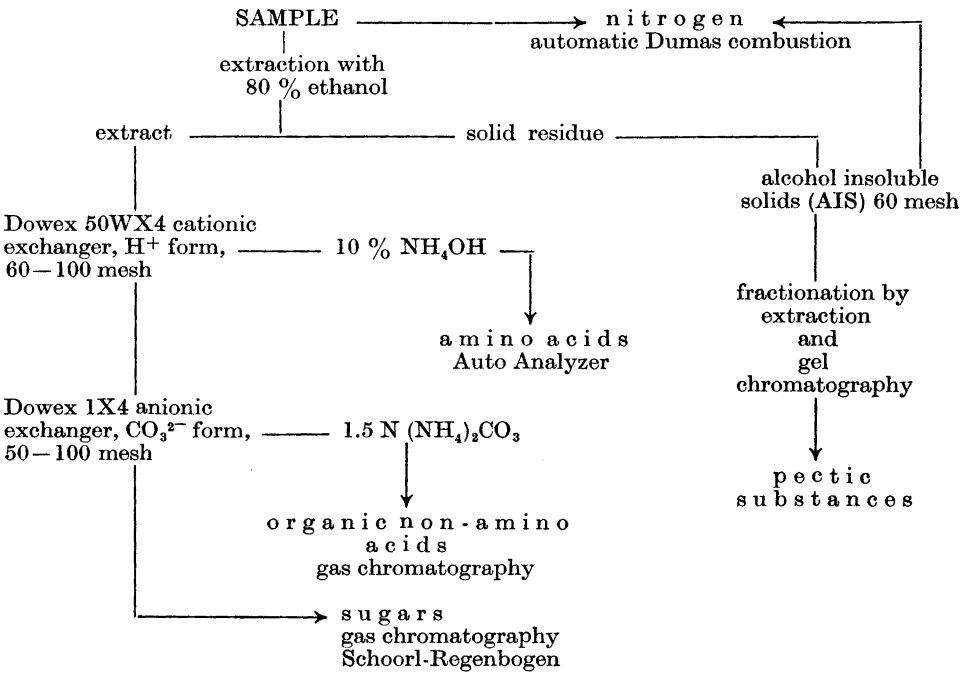
1 g of the dried AIS (particle size ≤60 mesh) was weighed into a centrifuge tube and 50 ml of distilled water was added. The mixture was stirred with a magnetic stirrer for 15 min at room temperature and centrifuged. The clear solution was separated from the solid residue, which was further extracted as above two times, 10 min each time. The water extracts were combined and diluted to 150 ml. The solid residue was extracted with a Calgon solution (0.2 % Calgon and 0.8 % NaCl in distilled water) three times as above. The Calgon extracts were combined and diluted to 150 ml. The residue was extracted three times, 30 min each time, with 0.05 N hydrochlorid acid at room temperature. The acid extracts were combined and diluted to 150 ml.

The following properties of the pectin in AIS as well as in the water, Calgon solution, and hydrochloric acid extracts were determined; uronic acid content by the carbazole method of Bitter and Muir [6] and degree of esterification by the titrimetric method of Gee *et al.*[7] The molecular weights of the extracted pectic substances were estimated by gel chromatography [8] on Sephadex G-25 and G-100 columns, using dextrans (Pharmacia, Uppsala) and polyethyleneglycols of known molecular weight as reference substances. A 0.1 % sodium chloride solution was used for elution.

Nitrogen

Nitrogen determinations were done on the fresh plant material as well as on the dried AIS with an automatic Coleman Nitrogen Analyzer II, Model 29 A, according to the combustion principle of Dumas.

Scheme 1.



RESULTS AND DISCUSSION

Extraction with 80 % ethanol has been extensively used for the separation of free amino acids, organic non-amino acids, and sugars from plant material, even though the extraction conditions, such as time and temperature, have varied. In our method the enzymes of chopped plant material are inactivated by rapid heating in boiling ethanol (*cf.* Ref. 9); the final extraction is performed at room temperature to minimize possible denaturation reactions, such as esterification and glycoside formation. Amino acids, organic non-amino acids, and sugars in the ethanol extract are isolated in separate fractions by ion exhangers. The method is in common use (*cf.* Ref. 10) and has been described by many investigators; in general, our procedure follows the modification outlined by Kliewer.[11]

The amino acids were quantitatively bound on Dowex 50 resin and, with the exception of glutamic acid, were also quantitatively eluted from it with 10 % ammonia (Table 1). After 6 h contact with the ion exchanger, the

Table 1. The recovery of amino acids in the ion exchange procedure.

Amino acid	Recovery %[a]	Relative standard deviation[b]
Aspartic acid	101.9	3.3
Threonine	105.2	1.8
Serine	105.3	5.7
Glutamic acid	75.4	5.1
Proline	98.5	1.5
Glycine	103.3	2.0
Alanine	104.4	3.1
Valine	105.2	3.1
Cystine	100.2	3.3
Methionine	95.6	6.4
Isoleucine	105.1	2.9
Leucine	105.0	5.1
Tyrosine	99.7	3.3
Phenylalanine	102.2	3.3
Lysine	101.2	3.5
Histidine	103.6	2.8
Arginine	95.6	5.1

[a] Mean value from four determinations.
[b] Reproducibility of the determinations with the amino acid analyzer at the 0.1 μmole level.

recovery of glutamic acid was 76 %. Plaisted[12] has shown that the recovery of glutamic acid depends on the length of time the resin and glutamic acid have been in contact; after 24 h 52 % of the glutamic acid was recovered (*cf.* Ref. 13). According to Plaisted, glutamic acid in the presence of 80 % ethanol and a cation exchange resin was converted in limited quantities to its γ-monoethyl ester, which is ninhydrin-active. In our chromatograms there was no extra peak due to any ester of glutamic acid. On the other

hand, Hamilton [14] has shown that glutamic acid and its γ-monoethyl ester are not separated from one another in a chromatographic system similar to the Technicon system. Thus it appears that in cases where glutamic acid is of particular interest, amino acid analysis should be carried out on the plain plant extract without any preceding ion exchange purification. In the amino acid analysis outlined above there is also another point worthy of consideration. Asparagine and glutamine run together throughout the 21 h residence time in the Technicon amino acid analyzer and their separation from thereonine and serine is not satisfactory. This is a serious handicap as far as free amino acids of plant material are concerned, since asparagine and glutamine very often are the main components. Boulter [15] has shown that in some cases these amides and threonine and serine can be simultaneously assayed by hydrolyzing the sample (*cf.* Ref. 16) or by varying the chromatographic conditions.

The recoveries of organic non-amino acids eluted from the Dowex 1 column are shown in Table 2. These data are in agreement with the paper chromato-

Table 2. The recovery of organic non-amino acids in the ion exchange procedure.

Acid	Recovery %[a]
Oxalic	81.4±11.2
Fumaric	98.7± 2.5
Succinic	99.5± 0.7
Malic	114.3± 0.7
trans-Aconitic	76.9± 9.3
Citric	125.1± 1.9

[a] Mean value of duplicate determinations and the deviation from the mean. For the reproducibility of the gas chromatographic determination of the acids, see Ref. 2.

graphic findings of Bryant and Overell.[17] Kuksis and Prioreschi [18] have recently presented a method for the isolation of Krebs cycle acids from tissues for subsequent gas chromatographic analysis.

The ion exchange procedure has been approved by AOAC [19] as an official clarification method for sugars extracted from plant materials. It is, however, necessary to check the quality of the ion exchange resins to avoid degradation or isomerization of the sugars.[20,21] In the gas chromatographic method used for the sugar determinations, both glucose and fructose gave two peaks (Fig. 1). The relative retention times of the glucose peaks with respect to arabinose, which was used as internal standard, were 1.39 and 1.54 after equilibration of the glucose in water solution, and the peak areas were 39.0±0.7 % and 61.0±0.7 %, respectively, of their combined area. Obviously, the slower main component, which was used for the quantitative determination of glucose, is β-glucopyranose.[4] The equation of the calibration line (*i.e.* the linear regression equation, *cf.* Ref. 2) was $y=-0.019+0.517x$, where $y=A_{\beta\text{-g}}/A_{\text{std}}$ is the ratio of the peak area for β-glucose to the peak area

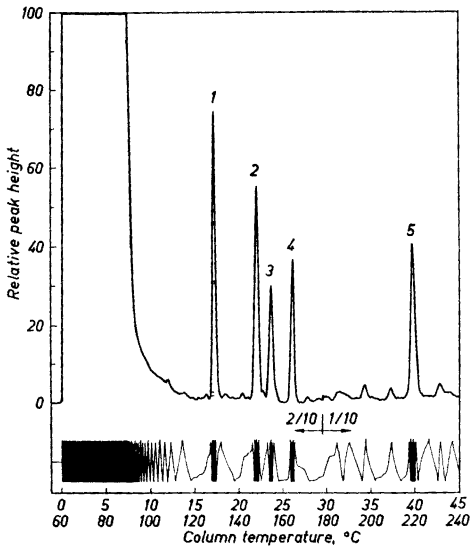


Fig. 1. Linear temperature-programmed gas chromatogram of trimethylsilyl ether derivatives of sugars. Column size: 1.5 m × 1/8 in. Column packing: 5 % DC-11 on Chromosorb W HMDS (60–80 mesh). Column temperature: 65°–225°C programmed 4°C/min. Nitrogen flow rate: 40 ml/min. Sugars: 1, arabinose (internal standard); 2, fructose; 3, fructose + glucose; 4, glucose; 5, sucrose.

for the internal standard, and $x = W_g/W_{std}$ is the ratio of the total weight of glucose divided by the weight of standard substance. The correlation coefficient for the two variables was 0.985, which differs significantly from zero ($P < 0.001$). The relative retention times of the fructose peaks were, after equilibration in water solution, 1.30 and 1.39. The peak areas as percentages of their combined area were 89.3±3.9 % and 10.7±3.9 %. Fructose was quantitatively determined on the basis of the more rapidly eluted main component. For the standard line, the equation $y = 0.008 + 0.809x$ was obtained, where $y = A_{f1}/A_{std}$ is the ratio of the peak area of the fructose component first eluted to the peak area of the standard, and $x = W_f/W_{std}$ is the ratio of the total weight of fructose to the weight of the standard. The correlation coefficient for the variables is 0.979, which differs significantly from zero ($P < 0.001$). Sweeley *et al.*[4] obtained only one peak for fructose on chromatography on a SE-52 column. The relative retention time of sucrose was 2.33, and the equation of the standard line, obtained as above, $y = -0.002 + 0.779x$. The correlation coefficient is 0.781, which differs significantly from zero ($P < 0.01$). In the above regression equations, the constant term is close to zero. Thus, use can be made of the so called K-value method, in which K is the regression coefficient (*cf.* Ref. 2), for the determination of sugars.

In Table 3 the efficiencies of the methods used for the extraction of pectic substances are compared. It is seen that in our modification about 90 % of the pectic fraction soluble in the solvent in question is extracted by water and Calgon solution and about 80 % by hydrochloric acid. The reproducibilities of the methods for characterizing pectin were as follows. The relative standard deviation of the uronic acid determination [4] was 1.6 %, and that of the determination of degree of esterification [7] 0.6 %. These relative standard

Table 3. The recovery of pectic substances in repeated extractions.

Extraction solvent	Number of extraction	AUA[a] μg/ml	Recovery %	Recovery in first 3 extractions, %
Water	I	1137.5	83.3	
	II	116.0	8.5	
	III	66.0	4.8	96.6
	IV	23.5	1.7	
	V	13.0	1.0	
	VI	9.0	0.7	
	Σ	1365.0	100.0	
Calgon solution	I	89.5	9.9	
	II	575.0	63.6	
	III	187.5	20.7	94.2
	IV	25.5	2.8	
	V	17.8	2.0	
	VI	9.5	1.0	
	Σ	904.8	100.0	
Hydrochloric acid	I	86.5	37.5	
	II	66.5	28.8	
	III	32.0	13.9	80.2
	IV	16.3	7.0	
	V	13.5	5.8	
	VI	16.3	7.0	
	Σ	231.0	100.0	

[a] AUA = anhydrouronic acid content.

deviation values were calculated from 10 determinations on a commercial water-soluble pectin preparation (Fluka, purum), which had a uronic acid content of 67.5 % and a degree of esterification of 72.1 %. When the corresponding relative standard deviations were estimated from analyses made on the only partly water-soluble AIS fraction ($\leq$60 mesh) obtained from vegetable material (carrot), which had uronic acid content of 27.4 % and a degree of esterification of 98.6 %, the values 15.1 % and 0.2 %, respectively, were obtained.

The reproducibility of automatic nitrogen determinations expressed as the relative standard deviation was 4.6—9.1. % depending on the plant material and its nitrogen content (*cf.* Refs. 22, 23). The nitrogen determinations were made both on fresh plant material and on the AIS fraction (scheme 1); the difference between the nitrogen contents gives the soluble nitrogen. Part of this consists of free amino acid nitrogen, the amount of which can be calculated from the amino acid chromatogram.

The analytical scheme presented provides also possibilities for the investigation of other groups of compounds than those mentioned here; *e.g.*, the 80 % ethanol extract is a suitable starting point for the analysis of polyphenolic compounds and the AIS fraction for the investigation of starch and the amino acid compositions of proteins.

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