Determination of Ammonium, Calcium, Magnesium, and Potassium in Silage by Capillary Isotachophoresis

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A method for the simultaneous determination of ammonium, calcium, magnesium, and potassium in silage samples by capillary isotachophoresis was developed. The electrophoretic migration properties of ammonium, calcium, magnesium, potassium, and sodium were investigated in electrolyte systems with leading electrolytes containing the complexing agent crown ether (18-crown-6). It was determined that the electrolyte system consisting of 0.0075 mol/L sulfuric acid, 0.007 mol/L 18-crown-6, 0.1% hydroxypropymethyl cellulose as leading electrolyte, and 0.01 mol/L Bis-tris-propane as the terminating electrolyte is most appropriate for the determination of cations cited above. The results of this capillary isotachophoresis method were compared with atomic absorption spectroscopy, and both methods were found to be in agreement. The precision of the determination including sample pretreatment, expressed by the relative standard deviations was between 4 and 7% and 10% for calcium, magnesium, potassium, and ammonium, respectively. Detection limits were less than 200 ppm in the solid sample.

Keywords: *Capillary isotachophoresis; silage; ammonium; calcium; magnesium; potassium; sodium; minerals*

INTRODUCTION

During the silage fermentation two principal processes are occurring, respiration and proteolysis, which are attributed to the activities of microorganisms and enzymes (Bolsen, 1993; McDonald *et al.*, 1991). Both processes contribute to loss of silage quality. The process of proteolysis is characterized by degradation of plant proteins into peptides, amino acids, amides, and ammonia. The degree of proteolysis is characterized as the *N*-ammonia/*N*-total ratio (Moisio and Heikonen, 1989).

Silage is a source of minerals for animals. Minerals are an essential part of a feed as they take part in many processes in the animal body (McDowell, 1992). For the determination of calcium, magnesium, potassium, and sodium in animal feeds, atomic absorption (AAS) or atomic emission spectrometry is applied most often (Jones *et al.*, 1982).

In our work we used capillary isotachophoresis (ITP) for the simultaneous determination of ammonium, calcium, magnesium, and sodium. ITP is a method of capillary electrophoresis. In ITP a combination of two buffer systems is used to create a state in which the separated zones all move at the same velocity (Foret *et al.*, 1993). Another interesting feature of ITP is the constant concentration in each zone, determined by the composition of leading electrolyte. This technique enables a significant part of the capillary volume (as much as 50%) to fill with the sample, and that is why the ITP has in generally higher concentration sensitivity compared to CZE. The physicochemical properties of zones differs stepwise from zone to zone, which enables the application of an universal detection

(conductivity, potential gradient). The ITP is therefore a very suitable method for the determination of small (non-UV-absorbing) ions in the presence of nonionogenic sample matrix.

Optimization of the isotachophoretic separation conditions of the alkali and alkaline earth metal cations was subjected to a number of previous studies. All approaches focused upon the selectivity enhancement of the two groups of cations which are difficult to separate under noncomplexing conditions in aqueous electrolyte systems. In the first group, potassium, rubidium, cesium, and ammonium have very similar ionic mobilities. The second group, represented by calcium, strontium, magnesium, and sodium, is also difficult to separate when ionic mobility determines their effective mobility (Simunicova *et al.*, 1994). In biological samples the concentrations of rubidium, cesium, and strontium are unimportant compared to those of ammonium, potassium, sodium, calcium, and magnesium. Many authors used ammonium as the leading cation and optimized the separation of alkali and alkaline earth metals. Kaniansky *et al.* (1990) achieved separation of nine alkali and alkaline earth metal cations using polyethylene glycol pseudophase in the leading electrolyte. Valaskova *et al.* (1988) applied (hydroxyethyl)iminodiacetic acid as the complexing counterion for the separation of alkali and alkaline earth metals. Such electrolyte systems are, however, not suitable for the determination of ammonium. Vacik and Muselasova (1985) used the protons as the leading cation. In this paper the separation of ammonium and potassium was not possible because of very similar ionic mobilities of this ion couple. For their separation, a combination of two electrolyte systems can be applied (Matejovic and Polonsky, 1987; Kvasnicka *et al.*, 1993). The electrolyte system where potassium and ammonium can be separated from each other is based on potassium hydroxide as the leading electrolyte, which was adjusted

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to pH greater than 8.3. A higher pH decreases namely the effective mobility of ammonium as a weaker base than potassium, which leads to the separation of both cations. However, the potassium cation cannot be determined in this electrolyte system. The use of an electrolyte system in which the separation of ammonium, potassium, sodium, calcium, and magnesium can be achieved in one run is more advantageous. For this purpose crown ether can be used as the complexing agent for the resolution of potassium and ammonium (Tazaki *et al.,* 1982; Stover, 1984).

MATERIALS AND METHODS

Apparatus. The volume-coupling isotachophoretic analyzer (Ionosep 900.1, Recman-Laboratorni technika, Ostrava, Czech Republic), equipped with Teflon capillaries (preseparation part 50×1 mm, separation part 150×0.45 mm, detection part 70×0.3 mm). Detection was carried out with a contactless high-frequency conductivity cell.

The separation was carried out by the constant driving current 100 μA, which was reduced to 50 μA before the detection.

Reagents. The following chemicals were used: sulfuric acid (ACS reagent grade, 0.505 N solution in water), hydroxypropylmethyl cellulose (HPMC, 4000) (Aldrich, Steinheim, Germany); 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (Bis-tris-propane, BTP, 99% purity) (Sigma, Deisenhofen, Germany); ammonium chloride (p.a.), lithium citrate (p.a.), acetone (p.a.), chloroform (p.a.) (Lachema, Brno, Czech Republic); potassium and sodium standard solutions for AAS (p.a., 1 g/L in water), calcium and magnesium standard solutions for AAS (p.a., 1 g/L in 2% nitric acid) (Analytika, Prague, Czech Republic).

For the preparation of the buffers, deionized water was used.

The solution of HPMC (1% in water) was purified by shaking with a mix-bed ion exchanger.

Calibration. An external standard calibration method was used with an equimolar mixture of standards of each cation (ammonium, calcium, magnesium, potassium, and sodium). Seven calibration points were measured in the calibration range, 0−0.3 mmol/L.

Procedure. *Homogenization and Storage.* Approximately 2 kg of a sample were taken from different places of a silage pile to obtain an average silage sample. The sample was mixed by hand and then crushed in the laboratory mixer. The samples were then frozen and stored at −20 °C.

Extraction Procedure A. Two hundred grams of a homogenized silage sample was weighed into the 2000 mL widemouth volumetric flask, and 1600 mL of hydrochloric acid (0.1 mol/L) and 4 mL of chloroform were added. After the overnight maceration the flask was filled with distilled water. After the filtration, the solution was diluted with deionized water (20−50-fold) and analyzed.

Extraction Procedure B. Fifty grams of a homogenized silage sample was weighed into the beaker, and 400 mL of hydrochloric acid (0.1 mol/L) was added. The beaker was placed into an ultrasonic bath for 10 min at the room temperature. After this period the solution was filtered under vacuum through a sintered glass filter. Filtration residue was rinsed with 50 mL of distilled water and re-extracted in the same way as the primary silage sample. Joint extracts were diluted to 1 L in a 1000 mL volumetric flask. The solution was diluted (10−25-fold) and analyzed.

Ashing Procedure. In uncovered platinum dishes 20 g of a silage was placed and ashed at 450 °C in a muffle furnace for 10 h. The ash was extracted with 10 mL of 10% HCl in the ultrasonic bath for 10 min. The mixture was then transferred to a 100 mL volumetric flask. After the volumetric flask was diluted to 100 mL volume with deionized water, the extract was filtered. Before the isotachophoresis the diluted extract was neutralized to pH 6−8 with 1 M solution of Bis-tris-propane.

Table 1. Electrolyte Systems Investigated for the Separation of Ammonium, Potassium, Sodium, Calcium, and Magnesium[a]

system	leading cation and counterion	complexing additive
1	0.0075 mol/L H_2SO_4	
2	0.0075 mol/L H_2SO_4	0.001 mol/L Crown ether
3	0.0075 mol/L H_2SO_4	0.003 mol/L Crown ether
4	0.0075 mol/L H_2SO_4	0.005 mol/L Crown ether
5	0.0075 mol/L H_2SO_4	0.007 mol/L Crown ether
6	0.0075 mol/L H_2SO_4	0.009 mol/L Crown ether

[a] In all systems HPMC was added to the leading electrolyte at the concentration of 0.1% (w/w) as the terminating electrolyte 0.01 mol/L BTP was applied. Crown ether = 18-crown-6.
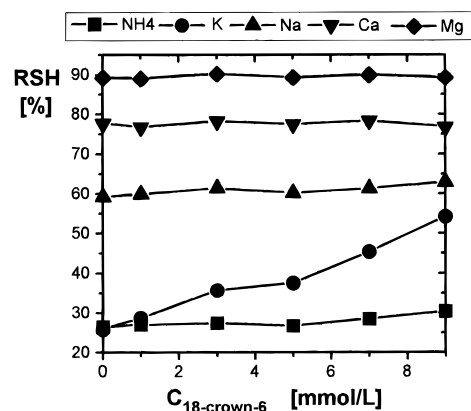

Figure 1. Migration behavior of NH_4^+, K^+, Na^+, Ca^{2+}, and Mg^{2+} in electrolyte systems based on the sulfuric acid with added 18-crown-6. The relative step height, RSH, is related to lithium, which served for this study as an internal standard.

RESULTS AND DISCUSSION

Migration Properties of Ammonium, Calcium, Magnesium, Potassium, and Sodium. The electrolyte system must enable the separation of all cations investigated. In aqueous electrolyte systems, however, the ionic mobilities of ammonium and potassium are very close. During our work we applied the electrolyte system developed by Kvasnicka (1993) using the sulfuric acid as the leading electrolyte. The proton of sulfuric acid serves as the leading cation and sulfate as the complexing anion. The main task of this part of the investigation was to enhance the selectivity of ammonium and potassium which comigrate in a mixed zone. For this purpose the complexing effects of crown ether (18-crown-6) were applied (Tazaki *et al.,* 1982; Stover, 1984). We investigated therefore the separation of all cations in electrolyte systems based on sulfuric acid containing increasing concentrations of crown ether. For the electrolyte systems applied, see Table 1. The results of our investigation are depicted in Figure 1. From this scheme it is apparent that the crown ether present in the leading electrolyte selectively retards potassium while the effective mobilities of other cations remain practically unchanged. This fact enabled us to selectively decrease the effective mobility of potassium. It was possible to move the step of potassium on the isotachopherogram exactly between the step of ammonium and that of sodium (see Figure 2). Only this leading electrolyte (containing 0.007 mol/L 18-crown-6) was chosen for further investigation.

Quantitation. On the basis of the results discussed above, system 5 in Table 1 was chosen for the calibration analysis. The results of calibration analyses are summarized in the Table 2. Correlation coefficients show
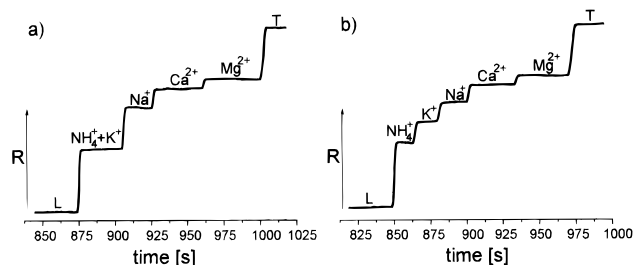
Figure 2. Isotachopherograms of a model mixture of NH^{4+}, K^+, Na^+, Ca^{2+}, and Mg^{2+}. Isotachopherogram a was measured in the electrolyte system presented by Kvasnicka (Table 1, system 1); isotachopherogram b was measured in our developed electrolyte system with 0.007 mol/L 18-crown-6 (Table 1, system 5). L = leading cation (oxonium), T = terminating cation (BTP), R = resistance (detector response).

Table 2. Results of the Calibration Analysis in the System Applied for the Determination of Cations in Real Silage Samples[a]

ion	RSH	calibration equation	R_{xy}
ammonium	35.23 ± 0.31	$y = 83.14x - 0.21$	0.999 75
potasium	46.91 ± 0.40	$y = 95.55x + 0.15$	0.999 77
sodium	57.07 ± 0.79	$y = 101.66x + 2.48$	0.999 90
calcium	66.79 ± 0.55	$y = 177.52x + 0.14$	0.999 58
magnesium	71.95 ± 0.37	$y = 188.25x - 1.72$	0.997 64

[a] RSH = relative step height is related to the step height of terminating ion BTP. R_{xy} = correlation coefficient. y = step length in s. x = concentration of ion in mmol/L.

high linearity of calibration lines and thus can be stated highly stabile isotachophoretic zones.

The limit of detection calculated as the concentration corresponding to the minimal detectable step length (*i.e.* 2 s) was 2.4×10^{-5} mol/L (0.41 mg/L) ammonium, 2.1×10^{-5} mol/L (0.82 mg/L) potassium, 1.9×10^{-5} mol/L (0.44 mg/L) sodium, 1.2×10^{-5} mol/L (0.46 mg/L) calcium, and 1.1×10^{-5} mol/L (0.26 mg/L) magnesium. This corresponds to less than 200 ppm of these cations in silage prepared by the extraction procedure A, after the 20-fold dilution.

Optimization of the Extraction Procedure for Real Samples. Our aim was to develop a quick and a quantitative extraction method. As calcium and magnesium may form water insoluble or partially water soluble salts with many anions, the application of 100 mM hydrochloric acid as the extraction medium was necessary. For the determination of ammonium, potassium, and sodium, water could have been chosen as the extraction medium. The extraction procedure A is a modification of that applied for the determination of volatile fatty acids and other organic acids in silage (Stransky and Dostal, 1986). The overnight maceration extends, however, the time necessary for the sample pretreatment to about 16 h. We have developed therefore another extraction method (method B). The time necessary for this extraction method is less than 30 min. However, this method is more laborious, because the extraction residuum must be reextracted in order to get all minerals into solution. The yield of method B after first extraction is about 92% for magnesium and between 96 and 98% for other cations (related to the concentration found after the second extraction). In this case the amounts determined for all cations in silage samples are practically the same as those obtained after the application of extraction method A. The differences are from 3 to 6%, which is a value comparable with the precision of this method. If reextraction has been made (see Extraction Procedure B under Procedure), the
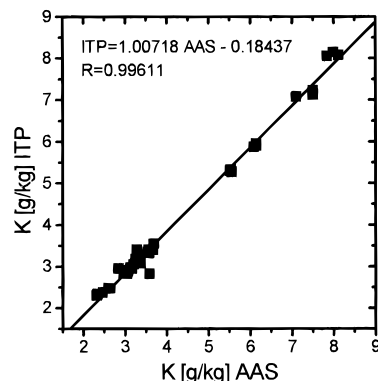

Figure 3. Comparison of ITP and AAS for the determination of potassium. The equation was obtained by regression analysis. R is the correlation coefficient. For both methods, 20-fold diluted extracts prepared by extraction method A were used.
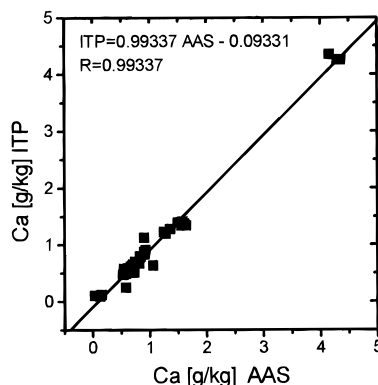

Figure 4. Comparison of ITP and AAS for the determination of calcium. The equation was obtained by regression analysis. R is the correlation coefficient. For both methods, 20-fold diluted extracts prepared by extraction method A were used.

obtained results are systematically from 2 to 6% higher than those from method A. The determined amounts of cations from the ashed samples were used as the basis for the estimation of the extraction yield, which is the most important parameter for the extraction. This procedure unfortunately excludes the determination of ammonium. We have determined that the actual amounts of studied minerals were about 5% (K, Na) or 10% (Ca, Mg) higher in ashed samples compared to nonashed ones prepared by extraction method A. This phenomena can be caused by the fact that part of the cations can remain closed in the sample matrix when a nonashing extraction procedure is applied.

Comparison of ITP and AAS. Atomic absorption spectrometry is a well-established method for the determination of alkali and alkaline earth metals. In our work this technique was used as a reference method for the accuracy determination. Comparison was carried out with 45 different samples. The obtained data were handled by regression analysis to evaluate the relationship between ITP and AAS. Results of these comparisons are depicted on Figures 3−5. Excellent agreement of both methods for the determination of potassium (ITP = 1.007AAS − 0.184, R = 0.996 11) and calcium (ITP = 0.993AAS − 0.093, R = 0.993 37) can be stated. Magnesium content determined by isotachophoresis (see Figure 5) gives higher results than AAS (ITP = 1.155AAS − 0.010, R = 0.992 37). The comparison of the determinations of sodium content was not done, because the found amounts of sodium determined by isotachophoresis were near the detection limit in practically all samples and so the quantitation was not possible with
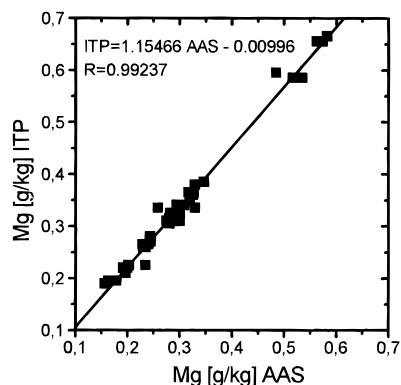
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Figure 5. Comparison of ITP and AAS for the determination of magnesium. The equation was obtained by regression analysis. R is the correlation coefficient. For both methods 20 fold diluted extracts prepared by the extraction method A were used.

Table 3. Results of Determinations of Cations in Real Silage Samples

sample	ammonium (g/kg)	potassium (g/kg)	calcium (g/kg)	magnesium (g/kg)
corn silage 1	0.46 ± 0.07	3.13 ± 0.14	0.93 ± 0.03	0.46 ± 0.01
corn silage 2	0.35 ± 0.04	3.32 ± 0.10	0.54 ± 0.02	0.23 ± 0.01
grass silage	0.50 ± 0.05	7.32 ± 0.26	1.29 ± 0.06	0.30 ± 0.01
clover silage	0.67 ± 0.09	3.55 ± 0.15	0.83 ± 0.05	0.20 ± 0.01
lucerne silage	1.81 ± 0.21	8.85 ± 0.22	4.84 ± 0.13	0.82 ± 0.03

[a] Values are average from three separate determinations $\pm$ standard deviation.
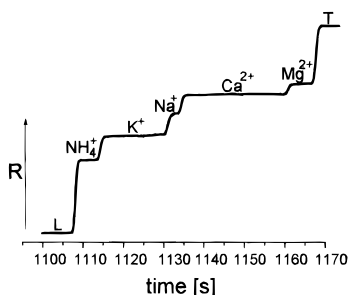

Figure 6. Isotachopherogram of a real silage sample. Sample of a corn silage was prepared by extraction procedure B. L = leading cation (oxonium), T = terminating cation (BTP), $R =$ resistance (detector response).

acceptable reliability. In addition sodium contents in silage found by isotachophoresis were mostly multiples higher than those obtained by AAS. This fact indicates the possibility that sodium comigrates with a compound from the sample matrix (probably with an amine) if a nonashing extraction procedure has been applied. This is not the case if an ashed sample was analyzed, because the ashing procedure mineralizes all organic compounds. On the other hand it is well-known fact that the plant materials are poor sodium sources (Belitz and Grosch, 1982).

Content of Cations in Real Samples. Quantitative results of analysis of silage samples are summarized in Table 3. Extracts for the isotachophoretic analysis were prepared by extraction method B. A typical record of a real silage sample is shown in Figure 6.

The precision of the ITP determination of ammonium, potassium, calcium, and magnesium, given by the relative standard deviation from 10 independent determinations (including sample pretreatment), were between 4 and 7% and 10% for calcium, magnesium, potassium, and ammonium, respectively. Recoveries of these cations when added to the samples varied between

90 and 95% for ammonium, 65 and 70% for sodium, and 95 and 104% for other cations.

CONCLUSIONS

The results show that the ITP technique is suitable for the determination of ammonium (*N*-ammonium), calcium, magnesium, and potassium in silage. Determination of sodium in silage extracts is not possible, because of the comigration of sodium with an unknown compound from the sample matrix. Using extraction method B (see Materials and Methods), it is possible to obtain results in less than 1 h including the sample pretreatment. The advantage of the ITP methods is the possibility to determine all cations mentioned above in one analysis. The use of capillary isotachophoresis as the separation method offers a solution to very different separation problems. For example, after the replacement of the buffers and polarity reversal, it is possible to carry out the determination of volatile fatty acids, other organic acids, and preservatives in silage extracts (Stransky and Dostal, 1986).

ABBREVIATIONS USED

AAS, atomic absorption spectrometry; BTP, Bis-tris-propane; HPMC, hydroxy-propylmethyl cellulose; ITP, isotachophoresis; RSH, relative step height (ratio of step height of analyzed compound and the one of a selected standard or terminating ion).

LITERATURE CITED

Belitz, H. D.; Grosch, W. Mineralstoffe. In *Lehrbuch der Lebensmittelchemie*; Springer: Berlin, Germany, 1982; pp 323−329.

Bolsen, K. K. The basic principles of silage - with emphasis on fermentation and additives. Proceedings. VI. International symposium Roughage Preservation, VUVZ Pohorelice, Czech Republic, 1993; pp 51−67.

Foret, F.; Krivankova, L.; Bocek, P. Principles of Capillary Electrophoretic Techniques. In *Capillary zone electrophoresis*; Radola, B. J., Ed.; VCH: Weinheim, 1993; Chapter 5.

Jones, J. W.; Capar, S. G.; O'Haver, T. C. Critical evaluation of a multielement scheme using plasma emmision and hydride evolution atomic absorption spectrometry for the analysis of plant and animal tissues. *Analyst* **1982**, *107* (1273), 353−377.

Kaniansky, D.; Zelensky, I.; Valaskova, I.; Marak, J.; Zelenska, V. Isotachophoretic separation of alkali and alkaline earth metal cations in water-polyethylene glycol mixtures. *J. Chromatogr.* **1990**, *502*, 143−153.

Kvasnicka, F.; Parkin, G.; Harvey, C. Capillary isotachophoresis as a new tool in sugar factory analysis. *Int. Sugar J.* **1993**, *95*, 451−458.

Matejovic, I.; Polonsky, J. Isotachophoretic analysis of cations important in plant nutrition. *J. Chromatogr.* **1987**, *390*, 155−160.

McDonald, P.; Henderson, A. R.; Heron, S. J. E. Plant Enzymes, Microorganisms. In *The biochemistry of silages*, 2nd ed.; Willey J. and Sons Ltd. Bucks, UK, 1991; Chapters 3 and 4.

McDowell, L. R. Calcium and phosphorous, Sodium and chlorine, Potassium, Magnesium. In *Minerals in animal and human nutrition;* Tony, J., Eds.; Academic Press, Inc: San Diego, CA, 1992; Chapters 2−5.

Moisio, T.; Heikonen, M. A titration method for silage assessment. *Anim. Feed Sci. Technol.* **1989**, *22*, 341−353.

Stover, F. S. Isotachophoresis of metal-neutral ligand complexes: 18-crown-6 ether complexes with alkali metals. *J. Chromatogr.* **1984**, *298*, 203−210.

Stransky, Z.; Dostal, V. Applications. *Analytical isotachophoresis in agricultural laboratories*; Textbook of Palacky University: Olomouc, Czech Republic, 1986; pp 64−67.

Simunicova, E.; Kaniansky, D.; Loksikova, K. Separation of alkali and alkaline earth metal and ammonium cations by capillary zone electrophoresis with indirect UV absorbance detection. *J. Chromatogr. A* **1994**, *665*, 203−209.

Tazaki, M.; Takagi, M.; Ueno, K. Use of uncharged neutral ligands in the isotachophoretic analysis of alkali and alkaline-earth metals. *Chem. Lett.* **1982**, *5*, 639−640.

Vacik, J.; Muselasova, I. Isotachophoretic determination of anionic and cationic species in well and surface waters. *J. Chromatogr.* **1985**, *320*, 199−203.

Valaskova, I.; Zelensky, I.; Zelenska, V.; Komarnicki, A.; Kaniansky, D. Hydroxyethyliminodiacetic acid in the isotachophoretic separation of alkali cations. *Collect. Czech. Chem. Commun.* **1988**, *53*, 515−525.