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Rapid and Direct Gas Chromatographic Determination of Oxalic Acid in Urine

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Summary. The known methods of oxalic acid determination are not suitable for reliable, rapid and economical routine analysis. A rapid gas chromatographic method has been developed which dispenses with separation operations and measures oxalic acid as a diethylester by means of back-flushing, and using malonic acid as an internal standard. One determination can be conducted within 6 to 8 min. and preparation of the specimen takes about the same amount of time. If the gas chromatographic conditions are changed and more time is permitted for analysis, numerous other extractable acidic metabolites can also be determined.

Key words: Oxalic acid - Acid metabolites - Urine - Back-flushing - Gas chromatography

Approximately 75% of all kidney stones are composed partly or wholely of calcium oxalate hydrates (14). Oxalate is therefore the most frequent anion encountered. This dominant role of oxalic acid has not yet been properly recognized due to difficulties in measurement. At the 5th Bonn-Vienna Symposium on Urolithiasis, a team was formed to review the known methods of determination of oxalic acid and to test them in independent laboratory experiments. The present paper is a contribution to this undertaking.

State of Current Investigation

A survey of the methods of determination of oxalic acid in biological materials up to 1970 was compiled by Hodgkinson (20). He distinguishes the following 4 groups: direct precipitation, solvent extraction, isotope dilution, and enzymic methods. A more informative categorization, however, should distinguish between the steps of separation and the identification techniques (Fig. 1), as the results obtained from the latter are meaningless if there is uncertainty as to losses or other errors during the separation procedure.

Urine pre-treatment comprises, in general, acidification, usually with hydrochloric acid, to pH 1. Additional steps, such as filtration or boiling in order to decompose the oxaluric acid, can be dispensed with (20). In most methods, the precipitation of $CaC_2O_4 \cdot H_2O$ was used as a separation step. The problems involved here are:

1. Loss of oxalate due to:
a) the presence of precipitation inhibitors such as magnesium-ions, citric acid, polyphosphates, and
b) the variable solubility of calcium oxalate in urine samples.
2. The simultaneous precipitation of components which disturb the subsequent determination of the oxalate.

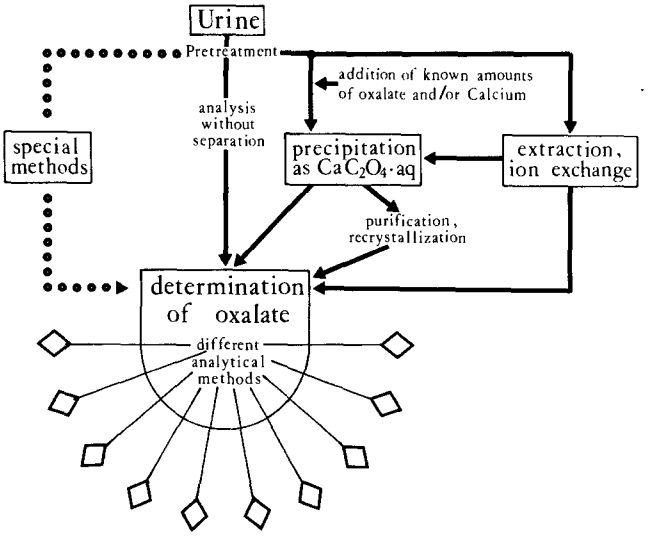

Fig. 1. Analysis procedures for oxalate determination. For methods of final determination of oxalic acid see Table 1

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Table 1. Methods of oxalate determination. (selected publications)

1. Oxidimetric methods:
   a) manganometric determination of the precipitated calcium oxalate: classical method (1); modern variant (22)
   b) cerimetrical (25)

2. Colorimetric methods:
   a) oxalate-indole-reaction (18)
   b) interaction of oxalate on uran/4-(2-pyridylazo)-resorcin-complex (3)
   c) with chromotropic acid after reduction to glycolic acid (11, 21)
   d) with 2. 7-dihydro-naphthalene after reduction to glycol (6)
   e) with resorcin after reduction to glyoxalic acid (fluorimetric) (40), compare (22)

3. Enzymatic methods: (oxalic acid + oxalic decarboxylase $\longrightarrow$ CO_2 + HCOOH)
   a) volumetric determination of CO_2 (28)
   b) automated photometric determination of CO_2 (phenolphthaleine) (24)
   c) photometric determination of formate with NAD/formate dehydrogenase (17)
   d) pH change (15)

4. Atomic absorption spectroscopy:
   Comparison of Ca-values in solution of Ca-oxalate-precipitations at pH 5 and pH 1 (29); compare also (2, 26)

5. Polarographic method:
   Precipitation of $Eu_2(C_2O_4)_3$ with subsequent polarographic Eu-determination (38)

6. Isotope dilution method:
   a) in combination with glycol-determination (19)
   b) in combination with glycolic acid-determination (36)

7. Use of ion-specific electrodes:
   Oxalate electrodes are still being developed, see (7, 20)

8. Distribution chromatography:
   Separation by activated silicic acid and photometry with o-nitro-phenole (4)

9. Ion exchange and gel-chromatography:
   Prefractionation by gel-chromatography and separation with an anion exchanger of high resolving power (5)

10. Gas chromatographic methods:
    Identification as methylester (after lyophilization) (27, 35)
    Identification as ethylester (13, 33, present paper)
    Identification as silylester (9, 34, 39)

The usual precautions are:
to 1a) precipitation by addition of a known amount of oxalate and/or calcium ions;
to 1b) separation of the oxalate in the form of salts with extremely low solubility, e. g., $Eu_2(C_2O_4)_3$ (4);
to 2) the recrystallisation of the precipitates or careful rinsing.

The extraction methods are performed alone or in combination with subsequent precipitation. Diethylether acts incompletely and unspecifically. Ethylacetate (9) and tri-n-butylphosphate (40) are more advantageous.

Special methods such as the above-mentioned precipitation of $Eu_2(C_2O_4)_3$, or the method suggested by Dodds and Gallimore (12), where oxalic

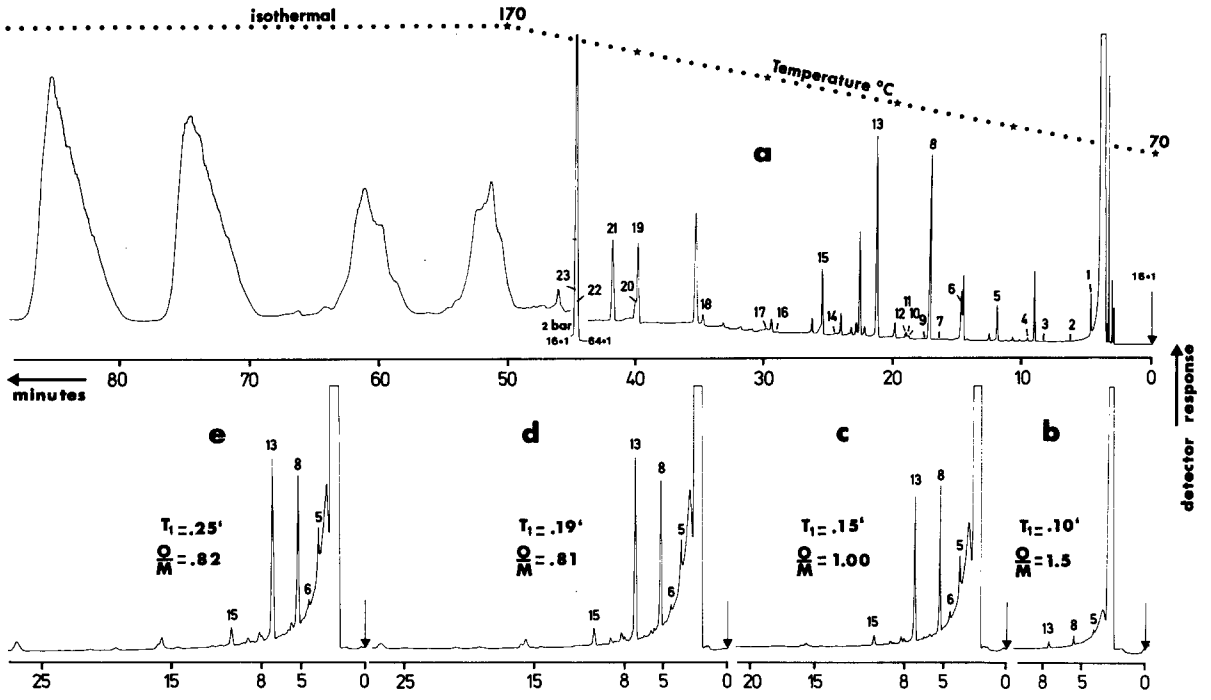

Fig. 2. a 2 µl CH_2Cl_2-extract from 50 ml urine according to Rhode and Zilliken's method (33). 50 m glass capillary column WG-11; splitting ratio 1:100; carrier gas N_2, 1.0-bar, varied up to 2-bar in the annealing period; FID; burning gases: H_2, 0.55-bar; synthetic air, 1.0-bar; injector and detector 210°C. Attenuation 16 x 1; temperature program 70°C + 2°C/min up to 170°C. b to e For each test run: 1.5 µl CH_2Cl_2-extract from 0.5 ml urine (back-flushing). Gas chromatographic methods, if changed: 25 m capillary column WG-11; 0.6-bar N_2. Time T_1 between injection and start of back-flushing in Fig. b to e varied from 0.10 to 0.25 min; oven 130°C, isothermal; injector 250°C; detector 250°C. Attenuation 128 x 1. For peak identification, see text

Table 2. Gas chromatographic determination of oxalic acid. Preparation procedure according to Rhode and Zilliken (32, 33)

1. Adjust 50 ml urine + 1 ml malonic acid[a] with 5n-HCl to pH1.
2. Evaporate to dryness on a rotary evaporator in a 250 ml flat bottom flask at 65°C.
3. Transfer the residue with 3 ml water + 2 ml HGl-conc. through a 250 ml separating funnel.
4. After addition of 4 ml saturated NaCl-solution, 1 g NaCl, 5 ml ethanol, 0.2 ml formic acid and 50 ml ethyl ether, shake the separating funnel for 10 min on the machine.
5. Allow to stand for 10 min, then siphon the watery phase and discard it.
6. Dry the ether-phase with 5 g Na_2SO_4 and transfer in a 100 ml flat bottom flask by rewashing with 10 ml ethanol/ether 1:10.
7. Evaporate in a vacuum desiccator at room temperature. Add 2 ml H_2SO_4-conc. if a volume of ca. 15 ml is reached and evaporate further to a volume of ca. 2 ml.
8. Transfer the concentrate to a 10 ml graduated cylinder, add absolute ethanol up to ca. 3.5 ml and allow to stand overnight.
9. Evaporate the solution on a rotary evaporator up to ca. 0.4 ml at 30°C.
10. Shake after addition of 1 ml ice water and 0.5 ml dichlormethane, allow to stand, isolate the dichlor-methane-phase and analyse.

[a] 140 mg malonic acid/100 ml water. In order to set up a calibration plot, a solution of 160 mg sodium oxalate/100 ml water is used

Table 3. Gas chromatographic determination of oxalic acid. Rapid preparation method

1. Collect 24-hour-urine in a container with 25 ml of 25% HCL or adjust fresh urine with 5 n HCl to a pH of 1.
2. Evaporate 5 ml urine + 1 ml malonate solution[b] + 50 µl antifoam solution[c] in a 100 ml tapered flask on a rotary evaporator to dryness. (Time of distillation ca. 8 min).
3. Dry the flask for an additional hour in a vacuum desiccator over P_2O_5 (Sicapent, E. Merck).
4. Add 10 ml of ethanol/ether-solution[d] and 0.2 ml of H_2SO_4-conc. and let it stand overnight.
5. Filter in a 50 ml tapered flask and rewash with 5 ml ethanol/ether-solution[e]. Concentrate on the rotary evaporator at 30°C to ca. 0.5 ml. (Time of distillation ca. 5 min).
6. Shake after addition of 1 ml ice water and 0.5 ml dichlormethane. Wait 5 min and transfer the dichlor-methane-phase with a pipette to a small stoppered test tube and analyse.

Preparation of a standard series: instead of urine add 0 to 4 ml oxalate solution[a] + 1 ml 1n-HCl in step 2. Filtration in step 5 can be omitted.

[a] Stock solution I: 148.82 mg dried $Na_2C_2O_4$ pro analysis/100 ml water. Test solution with 0.1 mg $H_2C_2O_4$/ml: stock solution I diluted 1:10.

[b] Stock solution II: 100 ml malonic acid + 2 ml 1n-NaOH/100 ml water. Test solution with 0.1 mg malonic acid/ml: stock solution II diluted 1:10.

[c] Antifoam solution: 2% Silicon-Antifoam (E. Merck Nr. 7743) in dichlor-methane.

[d] Ethanol/ethyl ether 1:1. Dry absolutely by passing through a column with 3 Å molecular sieve (30) or by treating with Na_2SO_4.

[e] Ethanol/ethyl ether 1:10, dried as under d.

acid is extracted and finally precipitated after esterification and distillation, are not in use.

Far more important are the direct methods of analysis (Fig. 1) which dispense with separation steps and therefore avoid oxalate losses. These methods of analysis are usually simple and rapid. The necessary requirement is the use of strictly specific methods of analysis such as the enzymatic decarboxilization of oxalic acid (31, 15) or gas chromatography described below.

Regarding the quantitative determination of oxalic acid, the 10 methods listed in Table 1 are known. However, no truly routine procedure has been found which is reliable, sensitive, economical and easily performed.

Herein a gas chromatographic technique is described which is suitable for routine laboratory tests. Although the cost of a gas chromatograph is high, it is usually part of the basic equipment found in most laboratories. This technique is a modification of Rhode and Zilliken's method (33), which was based on the method developed by Duburque et al. (13). Rhode and Zilliken's preparation technique is illustrated in Table 2, and a gas chromatogram taken according to their instructions is shown in Fig. 2a. Two features can be seen from this illustration:

1. Using this gas chromatographic method, not only oxalic acid, but also many other acidic components of urine can be determined quantitatively.
2. If merely the oxalic acid value is to be determined, however, the time required is too long for a routine test procedure, as the use of the gas chromatograph is costly and much time is wasted on annealing the low-volatile components of urine.

Extension of the Gas Chromatographic Method for the Determination of Different Acidic Metabolites

Rhode and Zilliken's method (33) is applicable when combined with the simplified preparation technique described below or an adaptation of back-flushing, if a broader spectrum of metabolites are to be determined. 41 potential urine components were tested as analytical grade compounds. Using the addition method, 23 of these could be coordinated with the gas chromatographic peaks of urine samples (Fig. 2a).

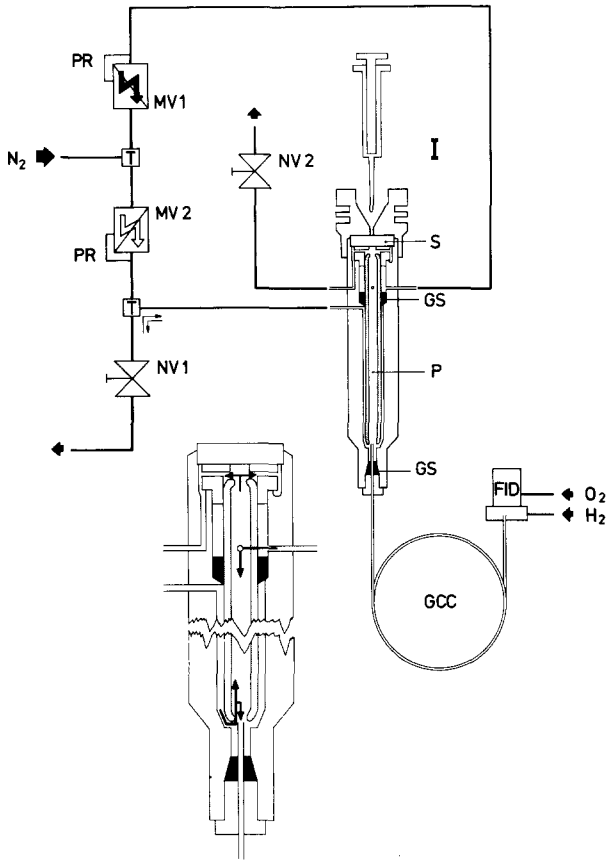

Fig. 3. Carrier gas stream in precolumn forward and back-flushing.
I, injector; s, septum; sc, screw cap with gas outlets for back-flushing; p, glass precolumn with 25% OV-101 on Volaspher (E. Merck); gs, graphite seals; gcc, glass capillary column, 25 m WG-11; fid, flame ionization detector; t, T-piece; mv1, time switch-controlled, closed if out of circuit; mv2, magnetic valves, opened if out of circuit; pr, by-pass capillary with constant gas flow of 1-3 ml/min for suppression of back diffusion; nv1, needle valve with gas flow of ca 20 ml/min (splitter); nv2, needle valve: outlet of the unwanted sample fraction by back-flushing

18 of the substances tested could not be eluted under the given analytical conditions. All of the compounds identified were ethyl esters or diethyl esters of (the numbers refer to Fig. 2a):
1 = butyric acid (formic to propionic acid are within the solvent peak); 2 = n-valeric acid; 3 = n-caproic acid; 4 = pyruvic acid; 5 = lactic acid; 6 = glycolic acid; 7 = acetoacetic acid; 8 = oxalic acid; 9 = glyoxylic acid; 10 = β-hydroxybutyric acid; 11 = i-valeric acid; 12 = methylmalonic acid; 13 = malonic acid (used as an internal standard, not present in urine(33); 14 = fumaric acid; 15 = succinic acid; 16 = maleic acid; 17 = glutaric acid; 18 = adipic acid; 19 = carbolic acid; 20 = pimelic acid; 21 = malic acid; 22 = α-ketoglutaric acid; 23 = p-cresol.

When a larger portion of the right-hand side of Fig. 2a is to be analysed, some additional or other internal standards should be used with retention times closer to the metabolites to be determined. Dicarboxylic acids are again advantageous for this purpose.

The compounds investigated which could not be identified under the conditions shown in Figure 2a are:
citric acid, uric acid, benzoic acid, salicylic acid, p-hydroxybenzoic acid, 4-hydroxy-3-methoxymandelic acid, trans-aconitic acid, indoxylsulfuric acid, glucuronic acid, amino acids such as glycine, alanine, leucine, glutamic acid, hippuric acid, creatine, creatinine and ethanolamines.

The following authors have used gas chromatography in the investigation of urine metabolites other than oxalic acid (8, 9, 16, 35, 37).

Rapid Method of Oxalic Acid Determination

For a satisfactory, rapid, routine preparation method, the procedure must be simplified (cf. Table 2), and the time required for a gas chromatographic analysis must be drastically reduced.

1. Preparation of the Specimen. It has been seen that the time-consuming extraction procedure (Table 2, steps 4-6) can be omitted when the quantity of urine to be analysed is reduced to about 10%. Thus the time required for evaporation and other processes is shortened and the method can be applied to cases where only small amounts of urine are available (Table 3).

2. Gas Chromatographical Back-flushing. With conventional gas chromatography, the specimen is injected into the injecting block and evaporated, and then forced through the capillary column by a carrier gas. With back-flushing (Fig. 3) (10), most of the carrier gas is used to flush the precolumn before the unwanted, difficult to volatilize compounds enter the separating column. This means that the gas chromatographical system is cleaned (septum rinsing) and prepared for the next analysis while the previous analysis is being performed. Moreover, the pressure of the carrier gas can be varied (Fig. 3), although this is not necessary for oxalate determination. The timer required for switching the magnetic valve, as well as the suggested adaptations to the gas chromatograph, can be constructed quite easily and inexpensively. Switch plans and copies of plates are available from the author.

In Figs. 2b to e, gas chromatograms obtained

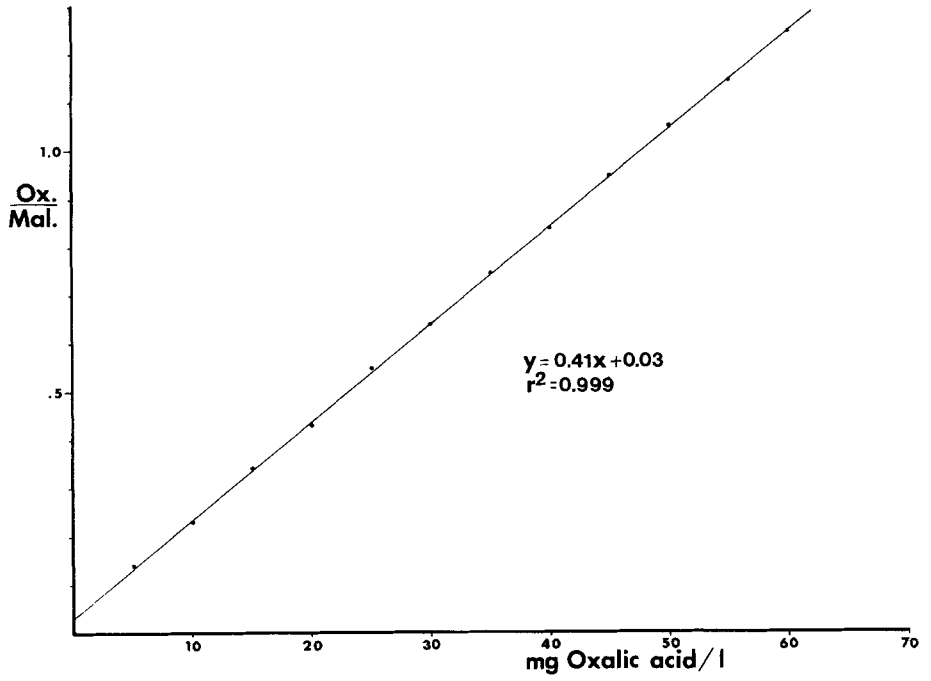

Fig. 4. Calibration plot for oxalic acid with malonic acid as internal standard. Ordinate: oxalic acid/malonic acid ratio

using the rapid urine preparation technique and back-flushing are shown. One analysis consisting of oxalic acid and malonic acid determination can be carried out within 6 to 8 min. Another advantage is that a temperature program can be omitted; the retention times in the isothermal runs remain constant even in simply constructed systems, and no time is lost by recooling down to the initial temperature. The minimal amount of high-volatility compounds also transmitted does not disturb subsequent analyses. The pressure programming illustrated in Fig. 2a to 2 can be dispensed without disadvantages.

In these graphs the time between injection and continuation of back-flushing (T_1) has been varied. T_1 = 0.1 min: parts of the solvent and the compound to be analysed are lost. T_1 = 0.15 min: one part of the internal standard malonic acid is still ineffective, as can be seen from the comparison with the following graphs. These graphs reflect the optimal time of back-flushing: T_1 = 0.19 min, where the relationship between oxalic acid and malonic acid reaches a constant value.

Fig. 4 shows a calibrating plot. In capillary column chromatography it is usually sufficient to measure only the peak heights in order to obtain the quotients O/M (oxalic acid/malonic acid). The amount of malonic acid should be increased, however, if a higher percentage of oxalic acid is to be expected, as in specific metabolic disturbances. Unfortunately, aqueous solutions of malonic acid are not stable, and must therefore be newly prepared each week. An equivalent substitute has not yet been found, although pelagonic acid is suitable according to the retention time. However, because of its volatibility, it can only be added after urine evaporation, and therefore does not compensate for evaporation losses as does malonic acid.

Reliability of the Method. Five 5ml portions of one urine specimen were prepared according to Table 3 and analysed: 31 ± 0.5 mg oxalic acid/1; coefficient of variability 1.6%. Five 5 ml portions of the same urine were mixed with 0.1 mg oxalic acid and treated in the same manner: 50.9 ± 0.8 mg oxalic acid/1; coefficient of variability 1.6%. Recovery rate 99.6 ± 1.7%. Double determinations under routine conditions performed by a laboratory technician showed an average deviation of ± 1.97% (n = 40).

The method presented here fulfills the above-mentioned criteria for a reliable routine procedure. Two technicians, one for the preparation and one for the gas chromatograph, can perform up to 60 determinations per day. The quantity of urine to be analysed can be reduced to about 0.5 ml. In principle, this method can also be applied to the analysis of sera, but this has not yet been tested adequately. It should be emphasized that gas chromatographic back-flushing is of great importance for a variety of biological problems in addition to the procedure discussed.

REFERENCES

1. Archer, H.E., Dormer, A.E., Scowen, E.F., Watts, R.W.E.: Studies on the urinary excretion of oxalate by normal subjects. Clinical Science 17, 405 (1957)
2. Baadenhuijsen, H.: Precision of urinary oxalate determination. Clinical Chemistry 21, 1692 (1975)
3. Baadenhuijsen, H., Jansen, A.P.: Colorimetric determination of urinary oxalate recovered as calcium oxalate. Clinica Chimica Acta 62, 315 (1975)
4. Barness, L.A., Morrow III, G., Nocho, R.E., Maresca, R.: Silicic acid chromatography of organic acids in blood cells and biological fluids. Clinical Chemistry 16, 20 (1970)
5. Burtis, C.A., Goldstein, G., Scott, Ch.D.: Fractionation of human urine by gel chromatography. Clinical Chemistry 16, 201 (1970)
6. Calkins, V.P.: Microdetermination of glycolic and oxalic acids. Industrial and Engineering Chemistry, Analytical Edition 15, 762 (1943)
7. Cammann, K.: Das Arbeiten mit ionenselektiven Elektroden. Berlin, Heidelberg, New York: Springer 1977
8. Chalmers, R.A., Watts, R.W.E.: Quantitative studies on the urinary excretion of unconjugated aromatic acids in phenylketonuria. Clinical Chemistry 55, 281 (1974)
9. Dalgliesh, C.E., Horning, E.C., Horning, M.C., Knox, K.L., Yarger, K.: A gas-liquid-chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extracts. Biochemical Journal 792 (1966)
10. Deans, D.R.: A new technique for heart cutting in gas chromatography. Chromatographia 1, 18 (1968)
11. Dempsey, E.F., Forbes, A.P., Melick, R.A., Henneman, P.H.: Urinary oxalate excretion. Metabolism 9, 52 (1960)
12. Dodds, E.C., Gallimore, E.J.: The determination of small quantities of oxalic acid. Biochemical Journal 26, 1242 (1932)
13. Duburque, M.Th., Mélon, J.-M., Thomas, J., Thomas, E., Pierre, R., Charransol, G., Desgrez, P.: Dosage et identification de l'acide oxalique dans les milieux biologiques. Annales de Biologie Clinique 28, 95 (1970)
14. Eisen, M., Dosch, W., Schäfer, L., Hohenfellner, R.M.: Statistics of Urolithiasis. Proceedings of an International Symposium on Urolithiasis Research held in Davos, Switzerland, March 29th - April 1st 1976. Eds. H. Fleisch, W.G. Robertson, L.H. Smith and W. Vahlensieck. p. 429. New York: Plenum Press 1976
15. Hallson, P.C., Rose, G.A.: A simplified and rapid enzymatic method for determination of urinary oxalate. Clinica Chimica Acta 55, 29 (1974)
16. Hammond, K.B., Goodman, S.E.: A gas chromatographic procedure for detection of pathological organic aciduria. Clinical Chemistry 16, 212 (1970)
17. Hatch, M., Bourke, E., Costello, L.: New enzymatic method for serum oxalate determination. Clinical Chemistry 23, 76 (1977)
18. Hausman, E.R., McAnally, J.S., Lewis, G.T.: Determination of oxalate in urine. Clinical Chemistry 2, 439 (1956)
19. Hockaday, T.D.R., Frederick, E.W., Clayton, J.E., Smith, L.H.: Studies on primary hyperoxaluria. II. Urinary oxalate glycolate and glyoxalate measurements by isotope dilution methods. Journal of Laboratory and Clinical Medicine 65, 677 (1965)
20. Hodgkinson, A.: Determination of oxalic acid in biological material. Clinical Chemistry 16, 547 (1970)
21. Hodgkinson, A., Zarembski, P.M.: The determination of oxalic acid in urine. Analyst 86, 16 (1961)
22. Husdan, H., Leung, M., Oreopoulos, D., Rapoport, A.: Modified method for urinary oxalate. Clinical Chemistry 22, 1538 (1976)
23. Knappwost, A., Fraber, R.: Theoretische Grundlagen und Praxis einer verläßlichen Oxalatbestimmung im Harn. Fortschritte der Urologie und Nephrologie 9, 139 (1977)
24. Knowles, C.F., Hodgkinson, A.: Automated enzymic determination of oxalic acid in human serum. Analyst 97, 474 (1972)
25. Koch, G.H., Strong, F.M.: Determination of oxalate in urine. Annals of Biochemistry 27, 162 (1969)
26. Koehl, C., Abecassis, J.: Determination of oxalic acid in urine by atomic absorption spectrophotometry. Clinical Chemistry 22, 71 (1976)
27. Lee, J.M.L., Stanley, R.W.: A rapid gas-liquid chromatographic method for determining oxalic acid in biological materials. Journal of Chromatography 76, 242 (1973)
28. Mayer, G.G., Markow, D., Karp, F.: Enzymatic oxalate determination in urine. Clinical Chemistry 9 334 (1963)
29. Menache, R.: Routine micromethod for determination of oxalic acid in urine by atomic abdorption spectrophotometry. Clinical Chemistry 20, 1444 (1974)
30. Merck, E.: Trocknen im Labor. Darmstadt 1978
31. Ribeiro, M.E., Elliot, J.S.: Direct enzymatic determination of urinary oxalate. Investigative Urology 2, 78 (1964)
32. Rohde, M.: Personal communication
33. Rohde, M., Zilliken, F.: Methodik der Oxal-

säurebestimmung im Urin und Tagesprofile der Oxalatausscheidung. Fortschritte der Urologie und Nephrologie 9, 142 (1977)
34. Rowland, M., Riegelman, S.: Use of carbon disulfide as a solvent for the silylation of submicrogram amounts of carboxylic acids. Annals of Biochemistry 20, 463 (1967)
35. Rumsey, T.S., Noller, C.H.: A study of the quantitative measurement of certain metabolic acids by gas-liquid chromatography. Journal of Chromatography 24, 325 (1966)
36. Johansson, St., Tabova, R.: Determination of oxalic and glycolic acid with isotope dilution methods and studies on the determination of glyoxylic acid. Biochemical Medicine 11, 1 (1974)
37. Thompson, J.A., Markey, S.P.: Quantitative metabolic profiling of urinary organic acids by gas chromatography-mass spectrometry: comparison of isolation methods. Analytical Chemistry 47, 1313 (1975)
38. Vittu, Ch., Lemahieu, J.C.: Détermination de loxalurie par méthode polarographique. Annales de Biologie Clinique 23, 913 (1965)
39. Nicolai, H. von, Zilliken, F.: Gaschromatographische Bestimmung von Oxalsäure, Malonsäure und Bernsteinsäure aus biologischem Material. Journal of Chromatography 92, 431 (1974)
40. Zarembski, P.M., Hodgkinson, A.: The fluorimetric determination of oxalic acid in blood and other biological material. Biochemical Journal 96, 717 (1965)

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