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**Note**

# Determination of sulphur-containing amino acids by quantitative ion-exchange thin-layer chromatography

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Difficulties in the determination of sulphur-containing amino acids in biological materials are mainly due to oxidative losses during acidic hydrolysis. To overcome this problem, methionine and cysteine + cystine are usually converted into stable oxydized derivatives prior to hydrolysis. Methionine sulphone ($MESO_2$) and cysteic acid ($CYSO_3H$) can be separated by ion-exchange column chromatography (for a review, see ref. 1) or thin-layer chromatography (TLC)[2,3]. Detection is usually carried out by means of the ninhydrin reaction. Non-hydrolytic methods are also available, such as reaction with cyanogen bromide combined with gas chromatography[4–7] for methionine, and hydrazinolysis and colorimetry[3,8] for cysteine + cystine.

A procedure combining ion-exchange TLC and video-densitometric quantitation has been developed as a convenient method for the determination of sulphur-containing amino acids. A comparison with other methods was carried out by analysing different legume seeds in which methionine and cysteine + cystine are the nutritionally critical (limiting) amino acids.

## EXPERIMENTAL

### *Sample preparation*

A 200-mg amount of pulverized plant material was oxidized with 2 ml of ice-cold performic acid according to Hirs[9]. After oxidation, the samples were diluted with 6 ml of distilled water and lyophilized. Hydrolysis was carried out with 1.5 ml of 6 *N* hydrochloric acid at 105° for 48 h. The hydrolysates were filtered and the clear filtrates were used directly for the determination of cysteic acid. With soybean samples methionine sulphone can also be determined in this solution but, because of the lower protein concentration in beans, peas and lentils, the hydrolysates of these samples have to be concentrated. The filtrates were evaporated to dryness under vacuum at 40–50° and the residue was redissolved in 400 $\mu$l of distilled water, the solutions obtained being used for the determination of methionine sulphone.

A 2-$\mu$l volume was applied on to the chromatosheet for cysteic acid and 6 $\mu$l for methionine sulphone.

*Chromatography of methionine sulphone*

$Li^+$ (0.6 *M*)–citrate (0.1 *M*) buffer (pH 2.50 ± 0.05) was prepared by mixing lithium citrate tetrahydrate (28.20 g), lithium chloride (12.70 g) and 37% hydrochloric acid (20.1 ml) and diluting to 1000 ml with deionized water. Strongly acidic cation-exchange chromatosheets (20 × 20 cm, Fixion 50 X8 $Li^+$, Chinoin, Nagytétény, Hungary, or Ionex 25 SA-$Li^+$, Macherey, Nagel & Co., Düren, G.F.R.) were used. The chromatosheets were equilibrated by continuous development for 24 h with the eluting buffer diluted 10-fold. The chromatography was carried out at 4° by continuous development for 16 h.

*Chromatography of cysteic acid*

For the separation of cysteic acid an anion-exchange TLC method, described by Ferenczi and Dévényi[10], was used with modification of the eluting buffer. Chromatosheets coated with a layer of anion-exchange resin (Fixion 2-X8 $Ac^-$, Chinoin, or Ionex, Macherey-Nagel & Co.) were used. The sheets were equilibrated by continuous development for 24 h with 0.01 *N* acetic acid. The eluting buffer was $Na^+$ (0.02 *M*)–acetate (0.2 *M*) (pH 3.7) prepared from 10 ml of 0.2 *M* sodium acetate solution and 90 ml of 0.2 *M* acetic acid.

*Staining*

The chromatograms were stained with ninhydrin spray reagent consisting of 100 ml of solution A, 20 ml of solution B and 3 ml of pyridine, where solution A is 1 g of ninhydrin in 100 ml of acetone (freshly prepared) and solution B is 10 g of cadmium acetate in 150 ml of glacial acetic acid plus 100 ml of distilled water.

The addition of pyridine increased the colour yield about 3-fold on the chromatograms developed with low pH buffers in comparison with the original spray[11]. No further increase was observed on chromatosheets developed with a buffer of pH 5.1, illustrating that this effect is due to the shift of pH. After staining, the sheets were dried and kept overnight in the dark.

*Densitometry and computation of results*

Densitometry was carried out with an automatic video-densitometer[11] (Telechrom OE-976, Eurolab, Munich, G.F.R.). The measurement is based on the video-scanning principle followed by a high-speed integration. The chromatograms were evaluated in the reflectance mode. Two density integrals were determined for each sample: the value for the individual amino acid ($MESO_2$ or $CYSO_3H$) and the total density of ninhydrin-positive material. The latter was used as the total amino acid content. The measured values were transferred to an on-line Hewlett-Packard HP-97 programable calculator and the results were expressed as the percentage by weight of the total amino acid content. The computation of the results included the calculation of a calibration constant for each chromatogram. This constant was determined from a standard sample chromatographed on each chromatosheet. The time required for the quantitative evaluation was about 4 sec per sample, including calculation and printing.

For comparison, methionine was determined by gas–liquid chromatography (GLC) as methylthiocyanate formed in the cyanogen bromide reaction[6], using a Hewlett-Packard 5720A gas chromatograph. Cysteine + cystine was determined as cysteic acid[12] on a Beckman Unichrom amino acid analyser.

## RESULTS AND DISCUSSION

In our previous work[13] we dealt with the cation-exchange TLC separation of aspartic acid, threonine, serine, asparagine, glutamine and glutamic acid. It was found that their separation can be achieved at low pH using an "Li system", *i.e.*, sheets coated with resin in the $Li^+$ form, with lithium citrate buffers for elution. As the chromatographic behaviour of methionine sulphone is similar to that of the above amino acids, we tested the "Li system" for separation.

The pH of the eluting buffer has a marked influence on the separation of $MESO_2$ from these amino acids (Fig. 1). It can be seen that $MESO_2$ cannot be separated satisfactorily from Thr + Ser + Asp at lower pH (2.3–2.4) or from Glu at higher pH (2.6–2.7). Fig. 2 shows the separation of $MESO_2$ (pH 2.5) (as described under Experimental) in hydrolysates of different performic acid-treated samples. It can be seen that even the relatively small amount of $MESO_2$ is well separated from the large excess of Asp + Thr + Ser and Glu present in legume hydrolysates. In the present separation, ninhydrin-positive degradation products of plant materials do not interfere with $MESO_2$. This interference is a source of difficulties in separating $MESO_2$ by silica gel TLC[3]. Linear response curves were obtained in the ranges important for determining sulphur-containing amino acids in plant materials (2–12 $\mu$g of $MESO_2$ and 0.2–1.2 $\mu$g of $CYSO_3H$ per spot).

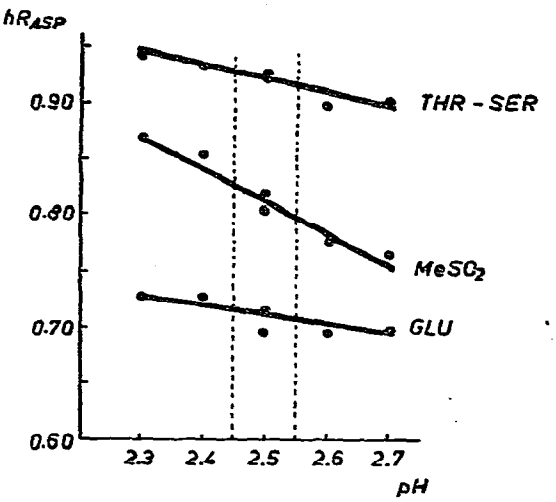


Fig. 1. Effect of the pH of the eluting buffer on the separation of methionine sulphone. Eluting buffer, $Li^+$(0.6 *M*)–citrate (0.1 *M*); continuous development at 4° for 16 h. The results are expressed as migration distances relative to that of Asp ($hR_{Asp}$).

The molar ratio of Glu to Met was determined in two synthetic peptides. The separations are shown in Fig. 2. In ACTH (1–14) a ratio of 1.1:1 was measured and in ACTH (1–32) a ratio of 3.3:1 was found, in agreement with the theoretical values (1:1 and 3:1, respectively).

The $MESO_2$ and $CYSO_3H$ contents of plant materials were expressed as percentage by weight of the total amino acid content. As these percentage contents

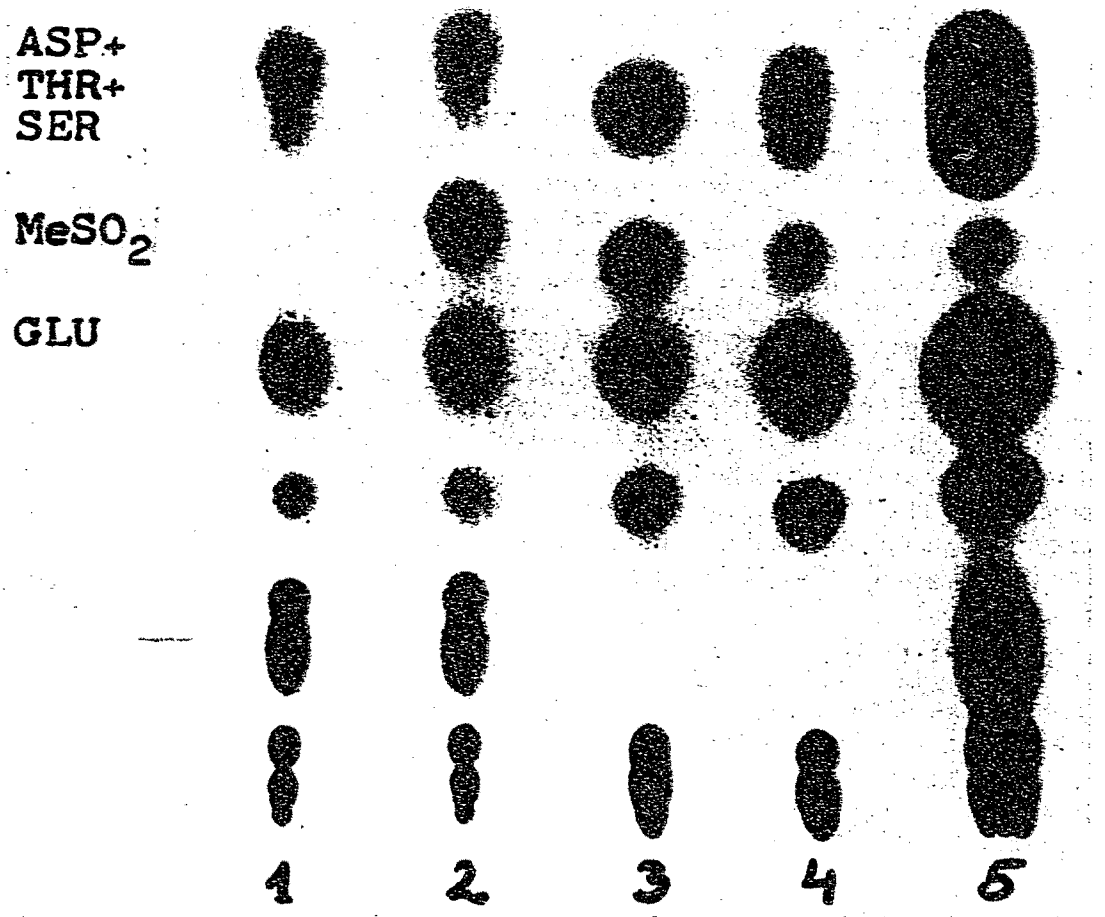


Fig. 2. Separation of methionine sulphone. Samples: 1 = calibration mixture of amino acids without methionine sulphone; 2 = calibration mixture containing methionine sulphone; 3 = hydrolysate of oxidized ACTH (1–14) fragment; 4 = hydrolysate of oxidized ACTH (1–32) fragment; 5 = hydrolysate of oxidized field bean.

are derived from the ratio of two amounts determined on the same sample, it could be expected that they are independent of the sample size in a given range. The "useful range" was found to be 1.5–3.0 mg of plant seed meal for $MESO_2$ and 0.2–0.33 mg for $CYSO_3H$ (per spot). Although these ranges may vary somewhat with the protein content, it can be stated that a sufficiently broad interval exists in which the percentage content can be determined without an exact knowledge of the sample size. Therefore, approximate weighing can be used, which is an important simplification in large-scale routine work.

The reproducibility of the method was determined by analysing amino acid calibration mixtures containing $MESO_2$ (or $CYSO_3H$) and hydrolysates of performic acid-treated plant materials. Standard deviations were determined from 35 parallel measurements (five parallel runs on seven chromatograms, six samples being run on

each chromatosheet, one of them being used for the calculation of the conversion constant). The results are given in Table I. Although the standard deviations are always higher with plant samples, these deviations (less than 6% of the mean) permit one to detect relative differences of 15%.

Table II gives the results for the methionine contents of 16 legume samples.

TABLE I

REPRODUCIBILITY OF TLC–VIDEODENSITOMETRIC METHOD

Each value is the average ± standard deviation calculated from five determinations.

| *Experiment No.* | *Methionine (%)* | | *Cysteine/cystine (%)* | |
|---|---|---|---|---|
| | *Standard amino acid mixture (N.V.* 5.00)*** | *Field beans (N.V. 1.18)**** | *Standard amino acid mixture (N.V. 2.00)*** | *Peas (N.V. 0.86)§* |
| 1 | 5.01 ± 0.09 | 1.16 ± 0.05 | 1.96 ± 0.06 | 0.88 ± 0.04 |
| 2 | 5.03 ± 0.07 | 1.19 ± 0.04 | 1.99 ± 0.03 | 0.85 ± 0.06 |
| 3 | 5.13 ± 0.16 | 1.23 ± 0.05 | 2.03 ± 0.06 | 0.82 ± 0.03 |
| 4 | 5.09 ± 0.13 | 1.25 ± 0.06 | 2.00 ± 0.02 | 0.83 ± 0.03 |
| 5 | 5.09 ± 0.12 | 1.16 ± 0.03 | 2.02 ± 0.04 | 0.86 ± 0.03 |
| 6 | 5.12 ± 0.08 | 1.18 ± 0.04 | 1.97 ± 0.04 | 0.85 ± 0.04 |
| 7 | 4.94 ± 0.08 | 1.24 ± 0.05 | 2.01 ± 0.05 | 0.87 ± 0.05 |
| Coefficient of variation (%) | 1.4–3.1 | 2.5–5.0 | 1.6–3.6 | 2.6–5.6 |

* N.V. = nominal value.

** $MESO_2$ and $CYSO_3H$ were added to standard amino acid mixture in the concentrations given. Values expressed as % (w/w) of the total amino acid content.

*** Determined by the cyanogen bromide GLC method and expressed as % (w/w) of the protein content (calculated as Kjeldahl N × 6.25).

§ Determined by amino acid analyser as cysteic acid and expressed as % (w/w) of the protein content.

TABLE II

METHIONINE CONTENT OF PLANT SEED SAMPLES AS DETERMINED BY DIFFERENT METHODS

Results expressed as % (w/w) of methionine in the protein.

| *Sample* | *BrCN GLC method* | *Video-densitometry* |
|---|---|---|
| Field beans | 1.18 | 1.1 |
| Bengal gram | 1.33 | 1.2 |
| Urd beans | 1.63 | 1.7 |
| Mong beans | 1.04 | 1.1 |
| Kidney beans | 1.19 | 1.3 |
| Beas | 0.72 | 0.8 |
| Cowpeas | 0.63 | 0.7 |
| Pigeon peas | 1.15 | 1.1 |
| Lentils (J-11) | 0.87 | 1.0 |
| Lentils (J-17) | 0.83 | 0.8 |
| Lentils (J-19) | 0.85 | 0.9 |
| Soybeans (Wells) | 1.53 | 1.6 |
| Soybeans (Altona) | 1.08 | 1.2 |
| Soybeans (Wilkin) | 1.17 | 1.3 |
| Soybeans (Traverse) | 1.21 | 1.2 |

For comparative purposes, the samples were also analysed by the cyanogen bromide GLC method, and the results were expressed in percentage by weight of the protein content (Kjeldahl nitrogen value × 6.25).

The results of the cystine determination are compared with amino acid analyser results in Table III.

TABLE III

CYSTEINE + CYSTINE CONTENT OF PEA SEED SAMPLES DETERMINED AS CYSTEIC ACID BY DIFFERENT METHODS

Results expressed as % (w/w) of the total amino acid content.

| *Sample* | *Amino acid analyzer (%)* | *Video-densitometry (%)* |
|---|---|---|
| J-63 | 1.35 | 1.3 |
| J-64 | 0.86 | 0.8 |
| J-66 | 0.85 | 0.9 |
| J-74 | 1.11 | 1.0 |
| J-80 | 1.16 | 1.1 |
| J-83 | 0.99 | 0.9 |

The samples were selected to represent different methionine and cystine contents, respectively. It can be seen that the results of the procedure described here were in generally good agreement with those of the other methods examined for the analysis of crude samples such as plant seeds.

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