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MULTI-COMPONENT ANALYSES OF HUMAN BODY FLUIDS AND TISSUES IN HEALTH AND DISEASE USING CAPILLARY GAS CHROMATOGRAPHY–MASS SPECTROMETRY AND HIGH-RESOLUTION TWO-DIMENSIONAL ELECTROPHORESIS

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SUMMARY

Glass capillary gas chromatography–mass spectrometry and high resolution two-dimensional electrophoresis have been used in combination to achieve multi-component analyses of human serum, urine, cerebrospinal fluid and various tumours. The analytical system covers both low- and high-molecular-weight constituents, that is, metabolites and proteins. Over 1000 compounds can be separated in a single sample of a few milligrams. The techniques have been used to study changes in the biochemical composition of body fluids and tissues in different human diseases: metabolic disorder, neurological disorders and certain types of cancer. The new multi-component analytical approach, now in its early stages, may have considerable potential in biomedical research and diagnosis.

INTRODUCTION

During the past decade much attention has been paid to "metabolic profiling" of human body fluids and tissues using gas chromatography (GC) and mass spectrometry (MS)[1–3]. This type of technique is suitable for the diagnosis and study of a number of metabolic disorders[2–4], as many of these often result in easily recognizable changes in the pattern of metabolites. Other diseases, *e.g.*, various types of cancer, seem to lead only to small changes in the metabolic profiles. Useful information may nevertheless be extracted, particularly when advanced multivariate data analyses are used to handle the analytical results. Thus, using capillary GC and the SIMCA pattern recognition method[5], we have shown that it is possible to differentiate between various brain tumours based solely upon differences in their chromatographic profiles[6].

Only compounds of low molecular weight (up to about 1000) may be analysed by GC–MS methods. Recent advances in high-performance liquid chromatography (HPLC) may extend the molecular weight range considerably, and may bridge the gap between GC–MS and a new multi-component analytical technique for proteins: high-resolution two-dimensional electrophoresis. The latter method was described by O'Farrell[7] in 1975 and later modified by Anderson and Anderson[8,9] to handle many samples in parallel.

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In our laboratory we now use GC–MS and high-resolution two-dimensional electrophoresis to study body fluids and tissues in health and disease. The results, some of which are presented in this paper, indicate that this new multi-component analytical approach may have considerable potential in biomedical research and diagnosis.

EXPERIMENTAL

Materials

Solvents were redistilled before use. The silylating reagent, bistrimethylsilyltrifluoroacetamide (BSTFA), was obtained from Supelco (U.S.A.). N-Nitrosomethylurea for diazomethane production was obtained from ICN Pharmaceuticals (New York, U.S.A.). Glass capillary columns for gas chromatography were products of LKB (Stockholm, Sweden), Chrompack (Middelburg, The Netherlands) or Hewlett-Packard (U.S.A.) (fused-silica columns). The columns were 25–50 m long, coated with SE-30 or SP-1000.

Acrylamide, bisacrylamide and sodium dodecyl sulphate (SDS) were products of Bio-Rad Labs. (U.S.A.) or Serva (Heidelberg, G.F.R.). Ampholytes for isoelectric focusing were either Ampholines (LKB) or Servalyt (Serva).

All other chemicals were commercially available products of analytical-reagent grade.

Methods

The capillary GC–MS procedures were as described earlier[10–12]. The instruments used where either a Varian 112 GC–MS with Spectrosystem 100 MS computing system (Varian-MAT, Bremen, G.F.R.) or a Finnigan 4021C GC–MS with an Incos Nova 4 computer (Finnigan-MAT, U.S.A., G.F.R.).

The equipment for high-resolution two-dimensional electrophoresis was obtained from Electro-Nucleonics (Oak Ridge, TN, U.S.A.) and was based on the apparatus constructed and described by Anderson and Anderson[8,9]. The analytical procedures were as developed by Andersons group at Argonne National Laboratory (*e.g.*, refs. 8, 9, 13 and 14). In principle, the two-dimensional electrophoresis is based on isoelectric focusing in one dimension followed by SDS electrophoresis in a gradient polyacrylamide gel in the second dimension. All proteins (or rather polypeptide chains, as the protein was treated with 9 *M* urea and mercaptoethanol) are therefore separated according to electrical charge and size (molecular weight). Staining of the separated proteins was achieved with Coomassie Brilliant Blue R-250 (Bio-Rad Labs.).

RESULTS AND DISCUSSION

Our combined multi-component analytical system covers both low- and high-molecular-weight constituents, *i.e.*, metabolites and proteins. At this early stage of our work with the system, we have attempted to gain some experience with various body fluids and tissues both from normals and from selected patients.

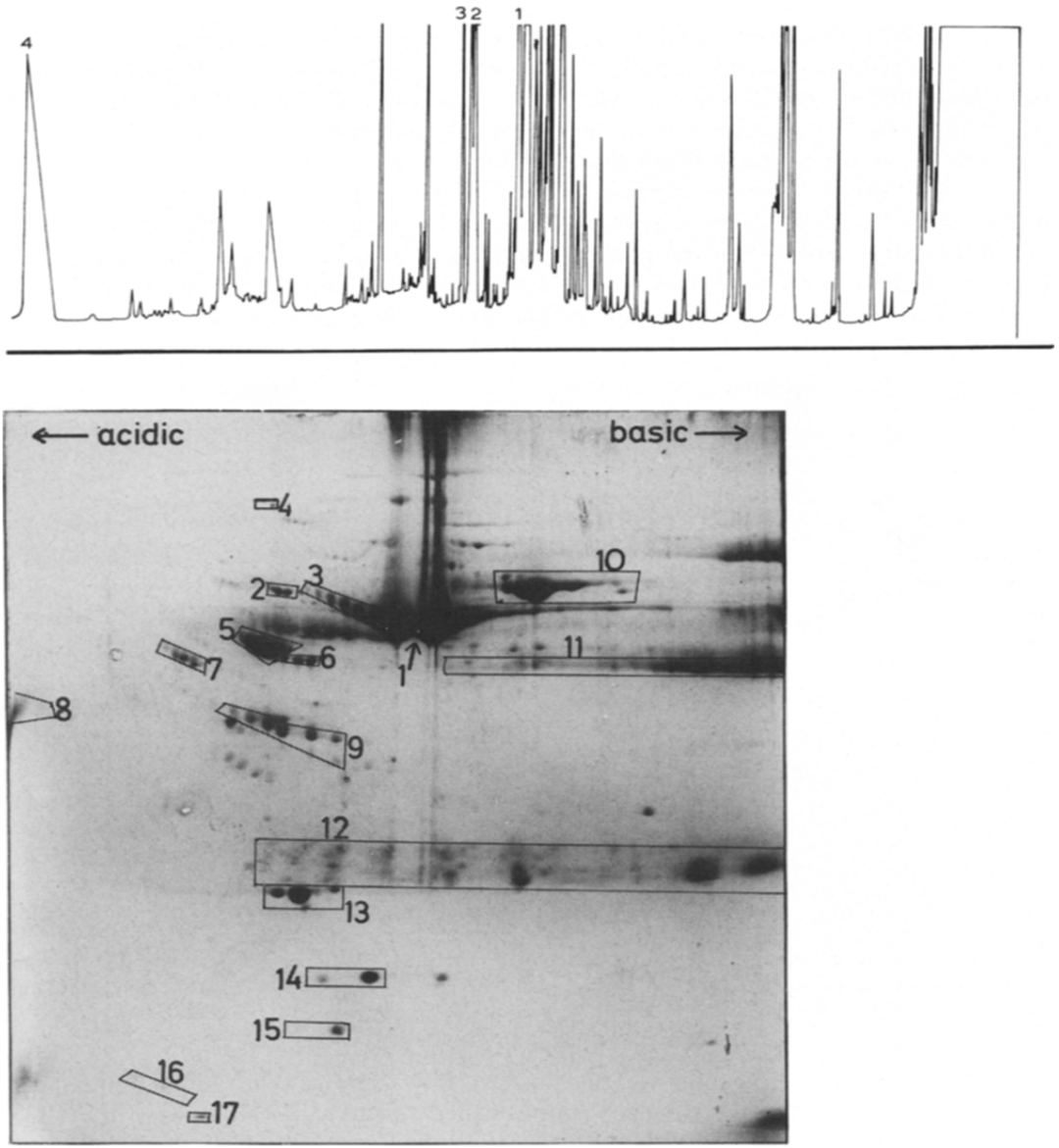

Fig. 1. Metabolic profile of normal serum (top) and protein pattern of the same serum (bottom). The serum (0.3 ml) was evaporated to dryness and refluxed with anhydrous methanol–hydrochloric acid overnight to complete methanolysis. After removal of the solvent and silylation with BSTFA, the compounds were separated in a 25-m SE-30 glass capillary column coupled to a Varian 112 mass spectrometer. The identities of some of the major peaks are: 1 = palmitic acid, 2 = oleic acid, 3 = stearic acid, 4 = cholesterol. High-resolution two-dimensional electrophoresis as described in the text was used to separate the proteins. The identities of some of the proteins are: 1 = albumin; 2 = α_1-B-glycoprotein; 3 = hemopexin; 4 = α_1-antitrypsin dimer; 5 = α_1-antitrypsin; 6 = Gc-globulin; 7 = α_2-SH-glycoprotein; 8 = α_1-acid-glycoprotein; 9 = haptoglobin β-chain; 10 = transferrin; 11 = IgG γ-chain; 12 = IgG light chains; 13 = Apo A-I lipoprotein (HDL); 14 = haptoglobin α^2-chain; 15 = prealbumin; 16 = lipoprotein (LDL); 17 = Apo A-II lipoprotein (HDL) (see ref. 13).

Serum

Typical multi-component patterns of low- and high-molecular-weight constituents of a normal serum sample are shown in Fig. 1. About 30–40 proteins have been identified by Anderson and Anderson[13]. Our studies[15] and those of Latner *et al.*[16,17] on sera from patients with various myelomas have added new information. We now know the position of all the main immunoglobulin types.

Fig. 2 shows the protein pattern of serum from a patient suffering from cancer of the colon. The first serum sample was collected during an early stage of his disease and a second sample when the patient's condition had deteriorated. Some proteins (marked with arrows) appear as the disease progresses. The clinical interpretation of these changes must, however, await further studies on larger number of patients.

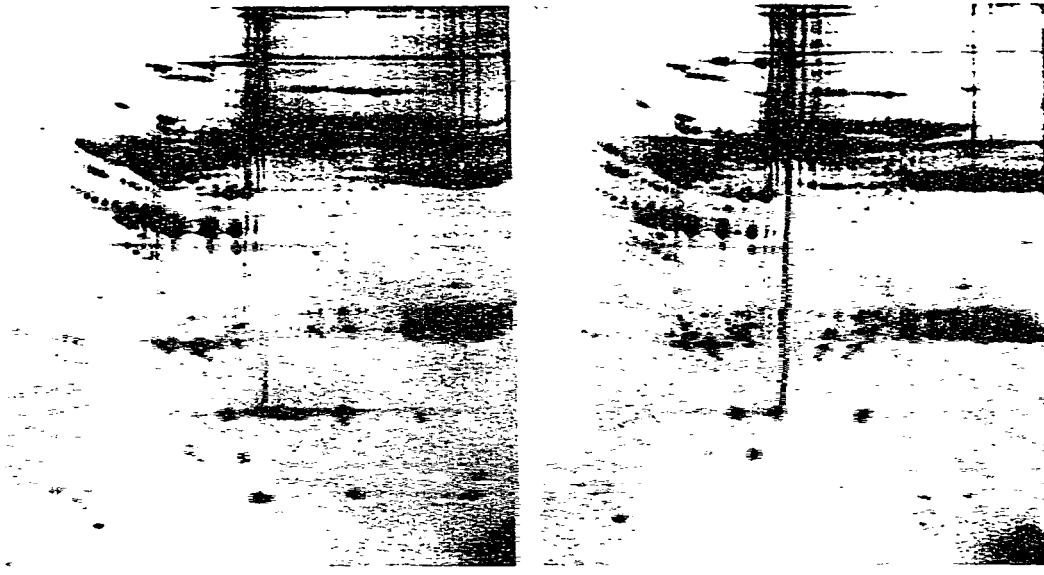

Fig. 2. Two-dimensional pattern of serum proteins from a patient with cancer of the colon. Left: serum collected during an early stage of disease. Right: serum collected 6 months later. Some spots appearing in the light chain region are marked with arrows.

Also the low-molecular-weight pattern may change with development of cancer, although it is our experience (unpublished results) that changes in the GC–MS profiles are small. An example of this is shown in Fig. 3, which shows the organic acid profile of serum collected from the same person several months before and immediately after diagnosis (but before treatment) of cancer mammae. Many more patients and controls, however, are required before one can attribute any significance to the observed, small changes in the metabolic profile.

The question remains of whether it is possible to detect changes in the metabolic profile and protein pattern of serum prior to clinical recognition of cancer. For this purpose the JANUS collection[18] of sera might prove to be of great value. In the JANUS project, sponsored by the Norwegian Cancer Society, serum samples from 64,000 people are collected at 1–3 year intervals, and stored at −25°C. Since this

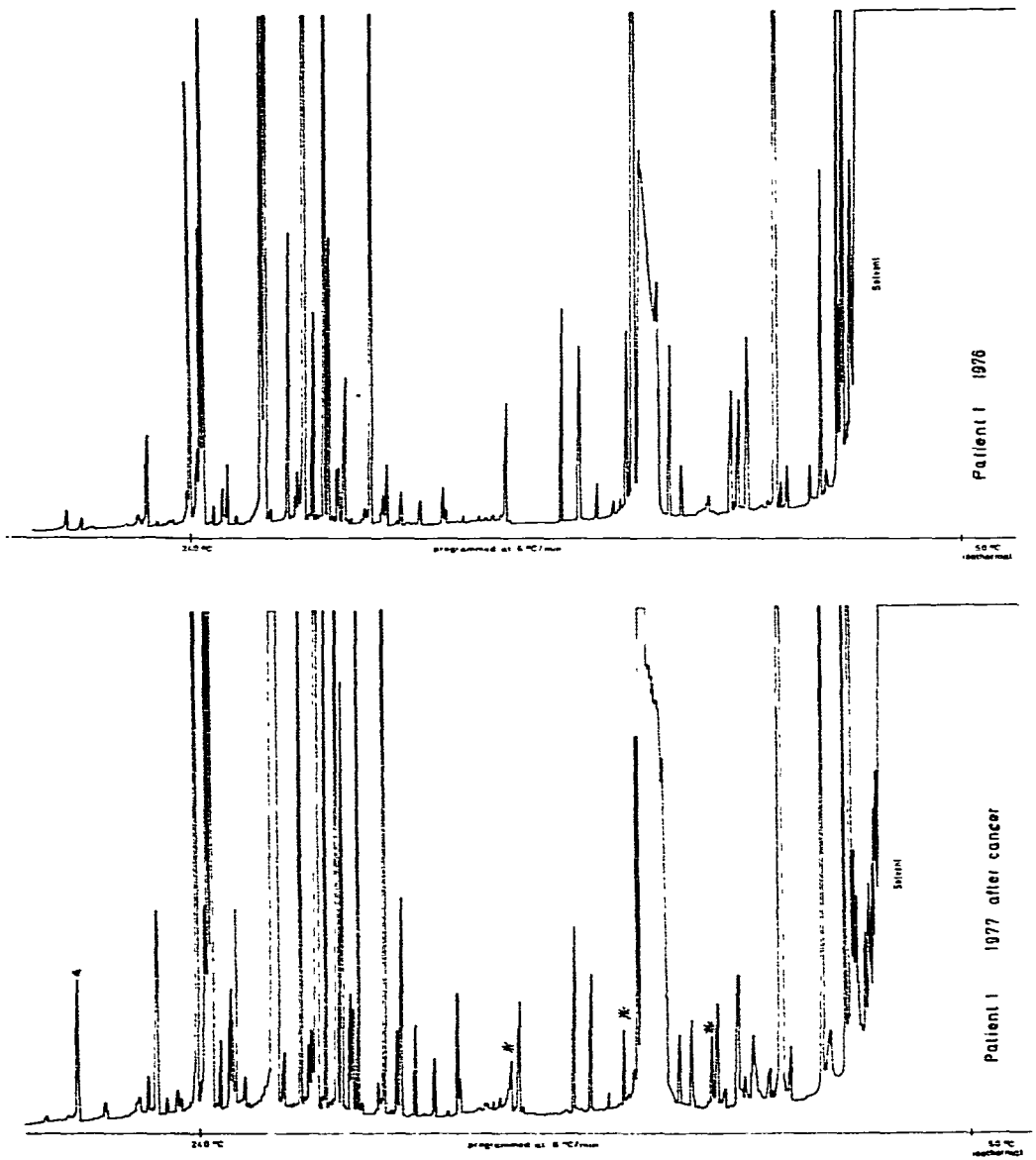

Fig. 3. Organic acid profile of serum from a patient with breast cancer. Top: sample collected 8 months prior to the recognition of the cancer. Bottom: sample collected after diagnosis, but before treatment. The sera were treated with ethanol (final concentration 80%) to precipitate the proteins. After removal of the ethanol *in vacuo*, the residue was acidified and extracted with diethyl ether and methylated with diazomethane. A 25-m SE-30 glass capillary column, programmed from 80 to 250°C, was used to separate the organic acids. Differences are marked with asterisks.

work began in 1973 over 1000 originally healthy persons have developed cancer, and sera obtained 1–8 years prior to recognition of their disease are available. The protein pattern in some of these sera have been examined in a preliminary investigation. Some changes were observed, but a serious problem became evident, namely the

dominance of albumin (*e.g.*, see Fig. 1). Selective removal of this protein and perhaps of some of the other dominant serum proteins prior to the two-dimensional electrophoresis seems to be a necessity. Larger amounts of albumin-free serum may then be applied to the gels, facilitating the detection of less abundant proteins. The chances of detecting tumour-produced proteins in serum may then increase.

Urine

The GC–MS profiles of, *e.g.*, organic acids in urine have been studied in a variety of clinical cases and in many different laboratories[1–4]. An example of such a profile is shown in Fig. 4. The urine appears to contain at least 500 organic acids[19].

After a 1000-fold concentration of urine and analysis of urinary proteins by high-resolution two-dimensional electrophoresis as described by Anderson and coworkers[20,21], several hundred spots are seen. Thus, by combining the two multicomponent analytical techniques, information on a large number of urinary constituents can be obtained. With the experience already gained with the metabolic profiles during the last 15 years, it seems reasonable to expect additional diagnostic information to be obtained by incorporating the protein pattern.

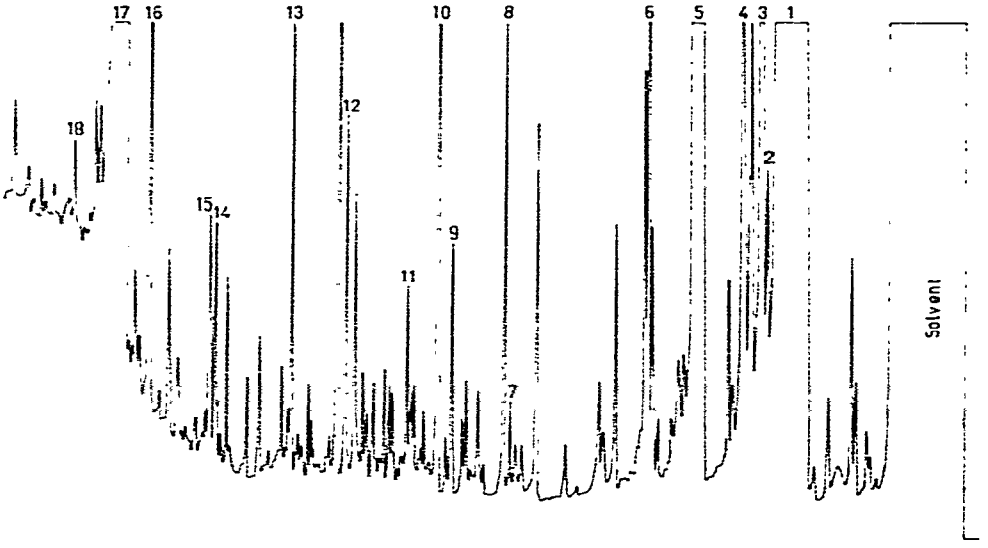

Fig. 4. Profile of urinary organic acids in a patient with ketoacidosis. The urine was acidified, extracted with diethyl ether and methylated with diazomethane. Separation and identification of the methyl esters were effected in an SP-1000 glass capillary column (25 m) coupled to a Varian 112 GC–MS instrument. Peaks: 1 = lactic acid; 2 = 3-hydroxyisovaleric acid; 3 = 2-hydroxybutyric acid; 4 = acetoacetic acid; 5 = 3-hydroxybutyric acid; 6 = 3-hydroxyisobutyric acid; 7 = adipic acid; 8 = methyladipic acid; 9 = dimethyladipic acid; 10 = eicosan (internal standard); 11 = *p*-cresol; 12 = 2,5-furandicarboxylic acid; 13 = citric acid; 14 = metabolite from Fenemal; 15 = homovanillic acid + furoylglycine; 16 = *p*-hydroxyphenylacetic acid; 17 = hippuric acid; 18 = coffeine.

Cerebrospinal fluid (CSF)

Two-dimensional electrophoresis of CSF has been described by Merril and coworkers[22,23]. Using a highly sensitive silver staining technique, only a four-fold concentration of the liquid was required. In our studies we use Sartorius Colloid Bags

(Sartorius, Göttingen, G.F.R.) to concentrate the CSF 100-fold. To illustrate differences that may occur in various neurological conditions, the protein patterns in a case of meningoencephalitis and a case of multiple sclerosis are shown in Fig. 5. Although one does not know at the present stage, it may turn out that the two-dimensional protein patterns of CSF carry valuable diagnostic information.

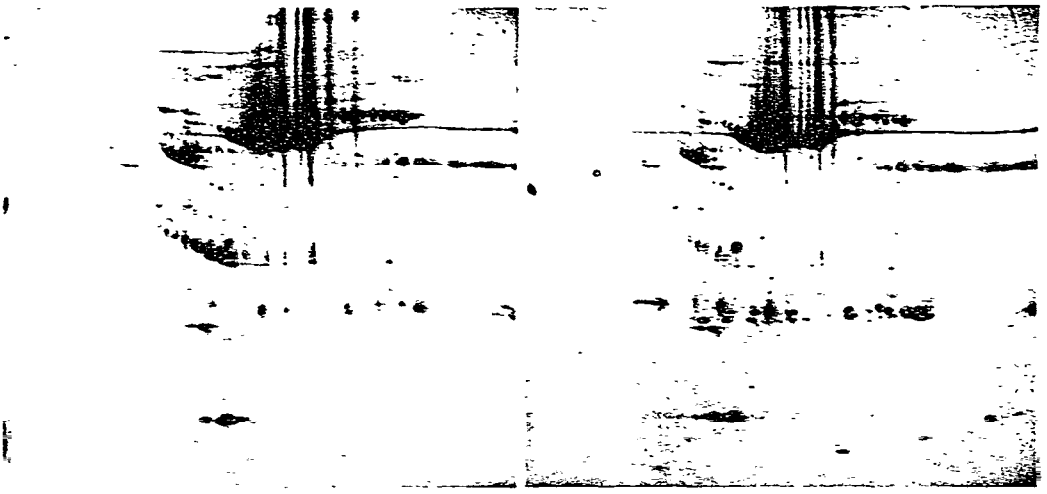

Fig. 5. Two-dimensional protein pattern of cerebrospinal fluid from a patient with meningoencephalitis (left) and from a patient with multiple sclerosis (right). Note marked changes especially in the light chain region (arrow).

Tissue analyses

The great advantage from the analytical point of view with tissues is that there is no single component, such as albumin in serum, that totally dominates the picture. Because of this, many more protein spots are seen in the electrophoresis method, and also the GC–MS profiles of low-molecular-weight constituents give true multi-component analytical results.

One of our main projects, in collaboration with scientists and surgeons of the Norwegian Radium Hospital and the Norwegian Cancer Society, is to search for tumour-produced metabolites and proteins. For this purpose both GC–MS and high-resolution two-dimensional electrophoresis are used. A biopsy of the tumour and of control tissue (cells from which the tumour originated) and serum from each patient are analysed. By comparing the multi-component analytical data of the tumour with the control tissue, it is possible to detect tumour-characteristic proteins and metabolites and also to examine for the presence of these compounds in serum. Sera collected in earlier years (JANUS collection[18]) may then be analysed to detect at what time prior to clinical diagnosis the cancer metabolites and/or proteins appear. To exemplify the complexity of the patterns, Fig. 6 shows the protein patterns of a biopsy from breast cancer, of a brain tumour and the capillary GC profile of the latter. With all these analytical data available, there is at least a possibility that new information on cancer may be obtained.

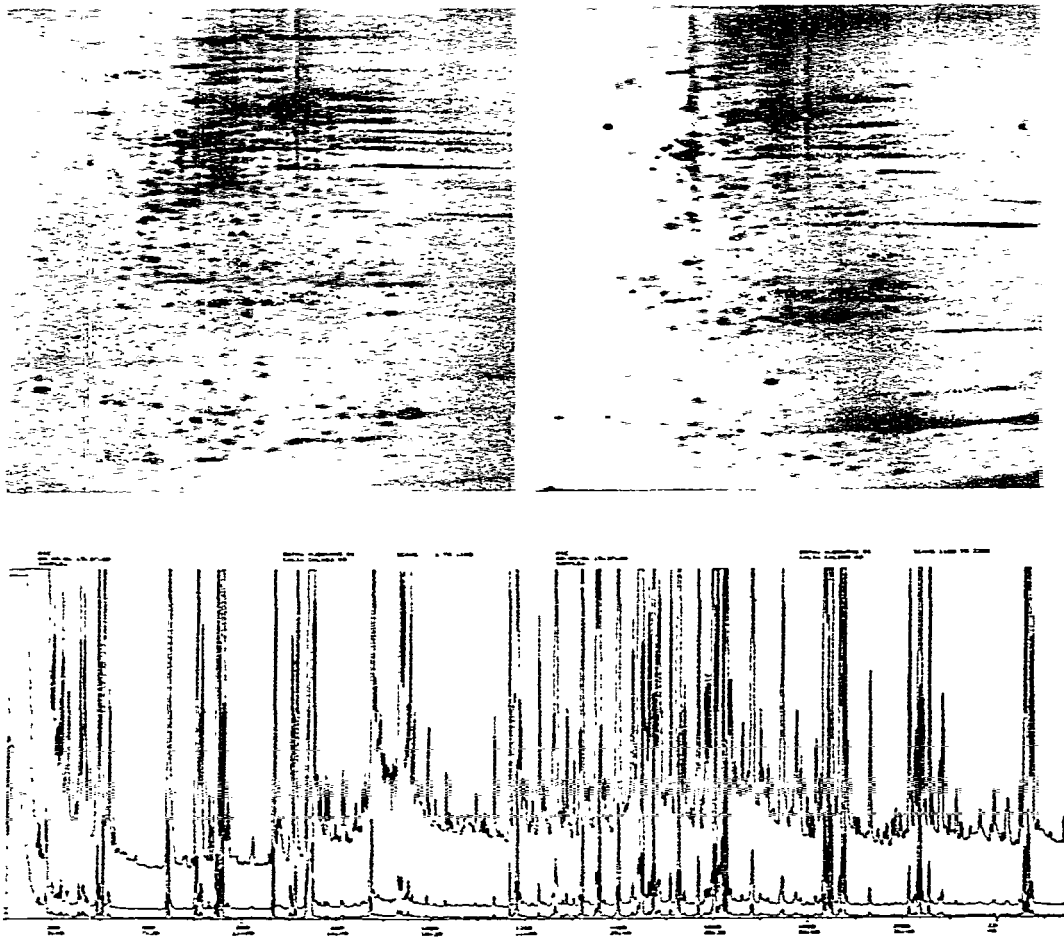

Fig. 6. Two-dimensional protein pattern and metabolic profile of biopsies from breast cancer and brain tumour. Top left, breast cancer; top right, pituitary tumour; bottom, GC–MS profile of the pituitary tumour. Experimental condition as in Fig. 1, except that a Finnigan 4021C GC–MS with an Incos Nova 4 data system was used to record the profile of the metabolites. No attempts were made to identify the constituents.

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