VOLATILE PROFILES FOR DISEASE DETECTION IN STORED CARROTS AND POTATOES

by

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ABSTRACT

In Québec, vegetable postharvest losses attributable to diseases are considerable and need immediate attention. The storage systems designed to maintain an optimum environment already exist and are common in modern warehouses. Managing these systems better with monitoring tools capable of detecting stresses in the stored crop before significant losses occur might be the key to loss reduction.

The primary objective of this study was to investigate the possible use of headspace volatile profiles of vegetables to detect storage diseases. The technique was applied to stored carrots (cultivar Charger) and potatoes (cultivar Atlantic).

In order to reproduce ventilated storage conditions, a dynamic headspace analysis method was chosen to collect metabolic volatiles. Small containers filled with sound and disease-inoculated carrot roots or potato tubers were purged continuously with purified air and the volatiles concentrated on polymeric adsorbent (Chromosorb 105) located at the container outlets. Subsequently, the trapped volatiles were thermally desorbed and analyzed by gas chromatography.

Examination of the normal and disease-induced volatile profiles indicated differences in the range of metabolites produced. One compound was unique to each carrot infection caused by either Sclerotinia sclerotiorum or Botrytis cinerea. Pentane and especially dimethyl disulfide dominated the profiles

of potato tubers infected with Erwinia carotovora and Fusarium roseum, but were not detected in the headspace above non-inoculated samples. At least one additional compound was unique to the Fusarium dry rot infection. The metabolic volatiles responsible for the specific responses might serve as disease indicators in commercial storages.

The identity of several normal carrot metabolites were determined by gas chromatography-mass spectrometry. Fewer metabolic volatiles were detected above sound potato tubers.

Vegetable storers, as any other business managers, rely on good information. The exact knowledge of the crop disease status during storage would certainly be an asset to them. The monitoring of headspace volatiles might provide this capability and has potential to become an indispensable management tool.

RESUME

Au Québec, les pertes de légumes en entrepôt causées par la maladie sont considérables et requièrent une attention immédiate. Des systèmes conçus pour maintenir des conditions d'entreposage optimales existent et sont courants dans les entrepôts modernes. Les pertes pourraient être réduites d'une façon certaines par une meilleure gestion de ces systèmes à l'aide d'outils de surveillance capables de détecter les premiers indices de détérioration de la récolte entreposée avant que la situation ne s'aggrave.

L'objectif premier de cette étude était d'examiner la possibilité d'utiliser la fraction volatile des légumes afin de détecter la maladie en entrepôt. Pour cette recherche, la technique fut appliquée à l'entreposage de la carotte (cultivar Charger) et de la pomme de terre (cultivar Atlantic).

Dans le but de reproduire les conditions d'entreposage ventilé, une méthode d'analyse dynamique de l'air ambiant fut choisie pour l'échantillonnage des constituants metaboliques volatils. Des petits contenants remplis de racines de carotte ou de tubercules de pomme de terre sains ou inoculés avec des pathogènes communs furent purgés d'une façon continue avec de l'air purifié. Les composés volatils ainsi entraînées dans l'effluent furent concentrées à l'aide d'un polymère adsorbant et subséquemment libérés sous l'effet de la chaleur pour analyse par chromatographie en phase gaseuse.

L'étude des profils normaux des substances volatiles et de ceux induits par les pathogènes a permis de déceler des différences dans la gamme de métabolites produits. Un composé était unique à chaque infection causée soit par le Sclerotinia sclerotiorum, soit par le Botrytis cinerea. Les composés pentane et particulièrement disulfure de diméthyle dominaient la fraction volatile des tubercules de pomme de terre infectés par Erwinia carotovora et Fusarium roseum, mais n'ont pas été détectés dans l'air ambiant des échantillons non inoculés. Au moins un composé additionnel était unique à la pourriture sèche fusarienne. Les métabolites volatils responsables des réponses spécifiques pourraient servir d'indicateurs de maladie dans les entrepôts commerciaux.

L'identification de plusieurs métabolites normaux de la carotte s'est faite par chromatographie en phase gaseuse combinée à la spectrométrie de masse. Moins de métabolites volatils ont été détectés dans l'air ambiant des tubercules sains de pomme de terre.

Les gérants d'entrepôts de légumes ont besoin d'une information de qualité. Une connaissance exacte du niveau d'infection de la réculte durant l'entreposage serait, pour eux, un atout certain. La surveillance des substances volatiles dans l'air ambiant pourrait leur fournir cette connaissance et a le potential de devenir un outil indispensable de gestion.

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I- INTRODUCTION

1.1 BACKGROUND

Over the years, much research effort in postharvest technology has been devoted in determining optimum storage conditions and how these conditions may be maintained economically. As a result, fruit and especially vegetable crops are commonly stored in large commercial structures that make use of ventilation, refrigeration, CA storage, or other systems to preserve produce quality over longer periods. Yet, despite these technical innovations, losses due to postharvest diseases remain high for certain crops. For example, it is not unusual for Québec's carrot and potato producers to suffer losses caused by decay alone exceeding 10%. And since modern warehouses are larger and often loaded in bulk, the task of detecting disease outbreaks for rapid intervention has become much greater and more crucial.

Existing disease detection methods are obsolete as they did not evolve with storage systems. In fact, most storage managers still rely on changes in crop appearance and odor and on their ability to recognize them to identify disease problems. The option of using multiple sensors or hand-held infrared scanners to pick up the slow temperature rises associated with concentrated microbial activities in the stored crop is available. In all cases, when infections are detected, significant losses would have occurred already. Providing

management with better monitoring tools would be an effective means of reducing postharvest losses and constitute a new research challenge.

1.2 SCOPE

This study considers the monitoring of headspace volatiles above stored vegetables as an alternative disease detection technique. The concept is really a technological improvement of smelling the storage atmosphere. It assumes that the use of precise analytical instruments to follow changes in the volatile profiles produced by the crop could replace the human nose advantageously. The ability to monitor headspace volatiles may: (a) allow early disease detection and identification; (b) give an objective measure of the level of infection; (c) become a source of permanent farm records which enable comparisons of crop performance from year to year; and (d) evolve as an integral component of an automatic control system. Attempts to verify these assumptions have been reported on stored crops such as peanuts, cereal grains, and especially potatoes.

1.3 OBJECTIVES

In this research, volatile monitoring was applied to the storage of carrots and potatoes. The primary goal was to further investigate the possible use of the technique in a storage disease detection system. The specific objectives were to

1) Develop a method of collecting and analyzing headspace volatiles emanating from small ventilated lots of carrots and potatoes.

2) Determine whether this method can detect compounds that are specific to two important diseases for each vegetable. For carrots, the diseases were Sclerotinia sclerotiorum and Botrytis cinerea; whereas Erwinia carotovora and Fusarium roseum were selected for the potato disease trials.

3) Identify some of the volatile metabolites emanating from healthy carrots and potatoes.

II- REVIEW OF LITERATURE

2.1 GENERAL

In Québec, carrots and potatoes are the main vegetable crops in terms of tonnes produced (Anonymous, 1987). According to the 1985 figures for example, the potato crop easily ranks first with 19400 hectares of land that yielded a total of 460000 tonnes. The carrot crop still comes next even though only 4115 hectares were cultivated to produced 102065 tonnes. Both vegetables are stored during the harvest periods and marketed continuously during the rest of the year to meet the year-round demands from consumers and processing industries.

In storage, the quality of the vegetables, and consequently their life, are reduced by moisture loss, physiological breakdown and decay (Raghavan and Gariépy, 1985). Holding temperature, relative humidity and air circulation have a strong influence on these deteriorations. In well designed storage facilities, these factors are controlled to provide the micro-environments that most favor the upkeep of the vegetable quality. Although the environmental conditions required for carrots differ substantially from those prescribed for potatoes, the building structures as well as the handling systems are similar for both vegetables.

2.2 STORAGE REQUIREMENTS

2.2.1 Carrots

A temperature of about 0 °C and a relative humidity (RH) of

93 to 98 % are best to store carrots for about 6 months (Salunkhe and Desai, 1984b). After harvesting, rapid cooling of the roots to the desired storage temperature is required for successful storage (Lougheed and Valk, 1985). This is why cold storages where mechanically refrigerated air is circulated within the storage room are preferred over less efficient storages that operate with outside cold air. The jacket and the Filacell types of cold storages have also been recommended for storing carrots. Their common feature is that very high RH can be maintained. However, higher capital costs made them unpopular among farmers. Carrots are usually stored in stacked pallet boxes (Figure 2.1) and sometimes in bulk bins with roots piled as deep as 3.3 meters and air circulation forced through the pile.

2.2.2 Potatoes

The storage temperature of potatoes depends on their intended end use, duration of storage, and sprout inhibition treatment (Porritt, 1974). Potatoes stored for short periods (up to 10 weeks) are held at temperatures between 7.2 and 10.0 °C (Hall, 1980). For longer periods, seed and table stock should be stored at temperatures between 3.5 and 4.5 °C if sprout inhibitors are not used. However, stock for processing into French fries and potato chips are maintained at 7.5 °C to 10.0 °C during storage (Ryall and Lipton, 1979). At lower temperatures, the tendency of tubers to accumulate reducing

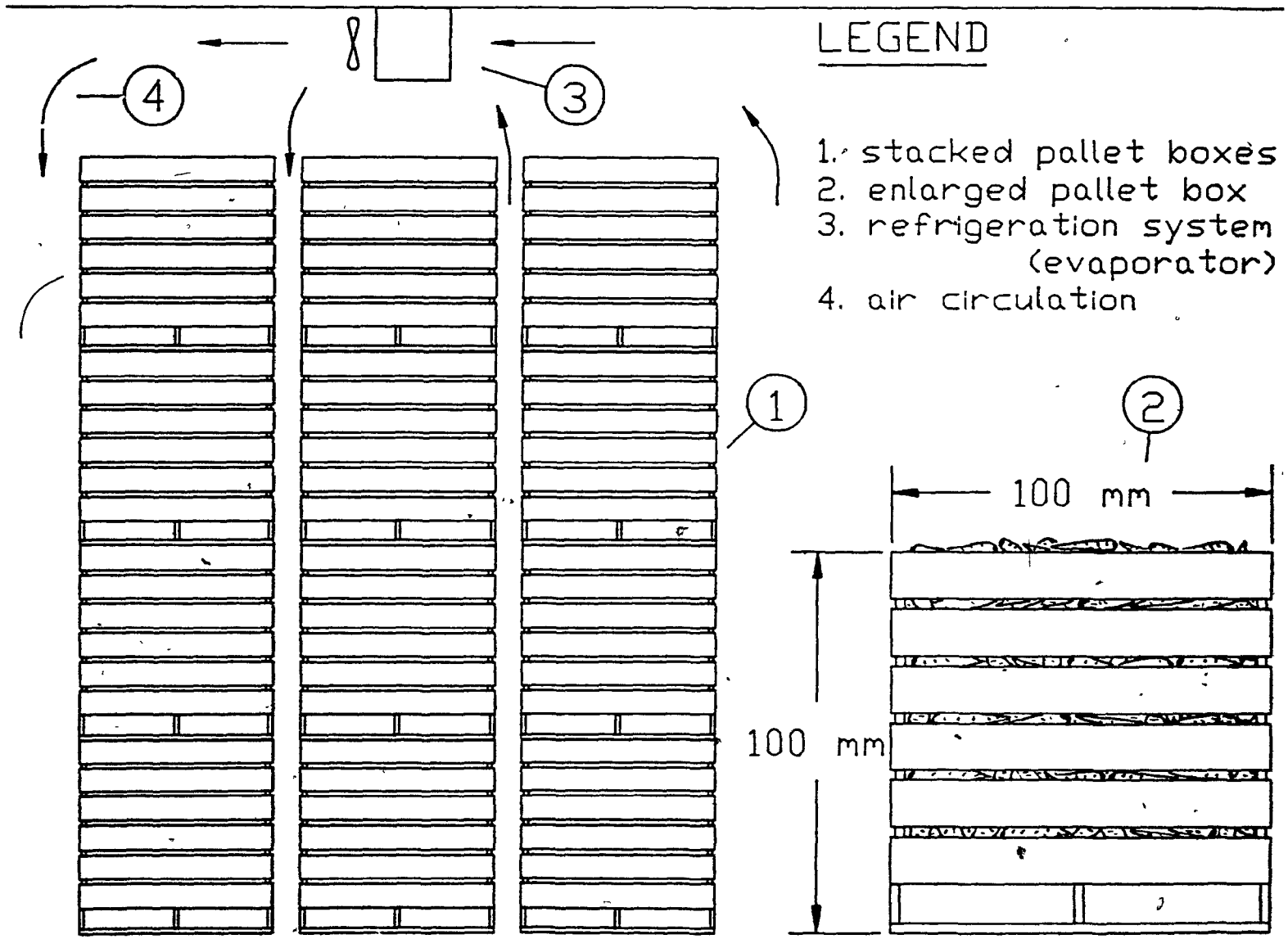


Figure 2.1: Refrigerated pallet box storage.

sugars cause them to fry dark. At all times, high RH (85-90 %) is recommended (Porritt, 1974).

Potatoes, as opposed to carrots, need no rapid cooling after harvesting. On the contrary, it is important to provide a higher temperature (13.3-15.6 °C) and humidity (95 %) environment during a 10-14 day period before getting it to the storage temperature (Porritt, 1974). During this time, referred to as the curing period, suberisation and wound periderm formation take place. These processes reduce the tubers' susceptibility to subsequent storage losses by making their skin firmer and tougher. Usually, fall temperatures in temperate-zone areas are such that these temporary, but necessary, conditions can be obtained without any mechanical refrigeration. Instead, air-cooled storage that operates by controlled ventilation of cold outside air are widely used for storing potatoes (Ryall and Lipton, 1979).

For economy of handling, most potatoes are held in bulk bins although pallet boxes are still used (Porritt, 1974; Figure 2.2). The depth of piles may reach 4.5 m but greater management problems are expected for deeper beds (Bishop and Maunder, 1980). Conventional forced-air ventilation systems supply air from ducts placed on or in the floor. The air travels up through the tuber pile and returns above the pile (Ryall and Lipton, 1979). Ideally, adequate controls and duct layout distribute air of proper temperature and RH to tubers located at any point in

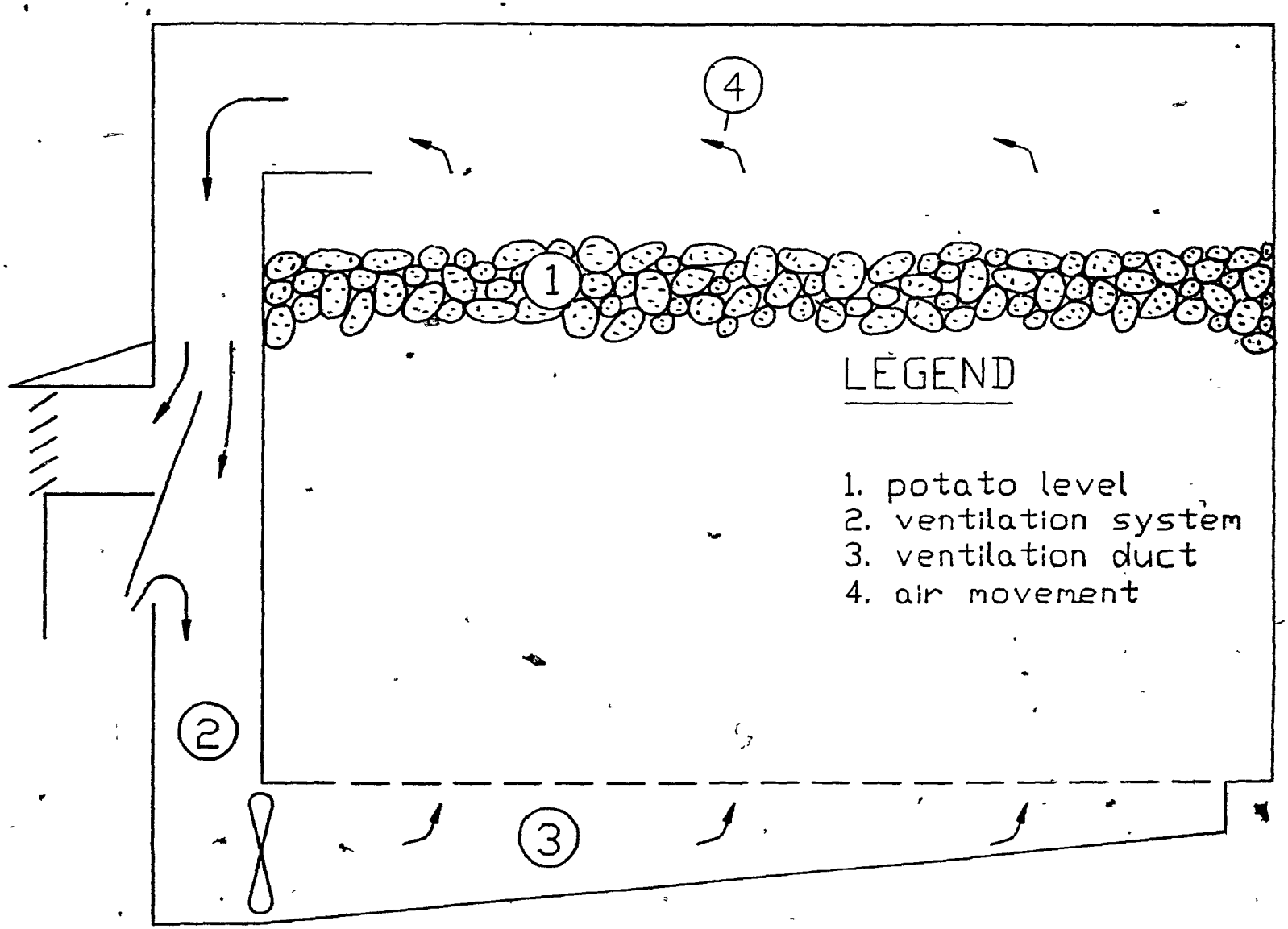


Figure 2.2: Air-cooled bulk storage.

the warehouse (Hall, 1980).

2.3 CAUSES OF POSTHARVEST LOSSES

A major function of any storage system is to keep storage losses as low as possible (Booth and Shaw, 1981). However, even if the optimum storage environment exists, losses will occur. Potato losses have been estimated to vary from 5 to 40 % (Salunkhe and Desai, 1984b). Such a general estimate is not available for carrots. These losses are likely to result from physical, physiological or pathological causes or combinations of all these (Salunkhe and Desai, 1984b).

2.3.1 Physical (or Mechanical) Causes

Losses due to physical or mechanical injury are often overlooked (Booth and Shaw, 1981). Physical injuries and bruising occur in various forms and arises at all stages: from pre-harvest through harvest and handling operations (Salunkhe and Desai, 1984a; Nash, 1978). Soil and crop conditions, temperature, handling care, operation and design of harvesting and handling equipment are factors that influence the amount of damage.

Bruising increases physiological and pathological losses (Salunhke and Desai, 1984a; Lewis and Garrod, 1983; Booth and Shaw, 1981; Nash, 1978; Apeland, 1974). When the skin of the tuber or the root is broken, the barrier against moisture loss and entry of fungi and bacteria is greatly reduced. As a result,

their storage life is shortened.

2.3.2 Physiological Losses

In storage as during growth, vegetable crops are living organisms (Nash, 1978). They respire and give off carbon dioxide, water vapor and heat. Physiological losses, thus, combine natural losses of water from wilting or transpiration and losses due to abnormal disorders arising from exposure to unfavorable storage conditions (Salunkhe and Desai, 1984a). Normal respiration losses significantly reduce produce weight and nutritional value. They account for less than three percent of dry matter in potato tubers during a six month storage period (Anonymous, 1983; Rastovski et al., 1981). On the other hand, sprouting can lead to considerably higher losses of dry matter. The potential of physiological losses are more pronounced with carrots, their rate of respiration being about five times greater than that of potatoes (Nash, 1978). Moisture losses are also likely to cause serious problems considering the thin-skinned nature of these roots. In potatoes, the most obvious undesirable chemical change is the accumulation of sugar (Anonymous, 1983; Booth and Shaw, 1981; Rastovski et al., 1981). Sugar content greatly influences the color of fried products. Finally, the extent of physiological losses (and other types of losses as well) depends on the skill with which the environmental controls in storage are handled (Anonymous, 1983, Rastovski et al., 1981; Nash, 1978; Roberts et al., 1976).

2.2.3 Pathological Losses

2.2.3.1 General

During storage, crops are subjected to a wide range of diseases that develop if conditions are favorable (Salunkhe and Desai, 1984a; Nash, 1978, Eckert, 1975). Infections by fungus and bacteria may give rise to serious losses both in terms of quality and quantity (Salunkhe and Desai, 1984a). In fact, a major portion of the total postharvest losses is attributed to diseases (Eckert and Sommer, 1967). Data available for carrots and potatoes produced and stored in Québec support this statement. The diseases that threaten carrots most in storage are watery soft rot (Sclerotinia sclerotiorum) and gray mold rot (Botrytis cinerea); bacterial soft rot (Erwinia carotovora) and Fusarium dry rot (Fusarium spp.) are the major potato storage diseases.

2.2.3.2 Carrot storage diseases

Losses incurred by Québec's carrot storers have been substantial. From year to year, the percentage of the stored crop wasted is estimated to range from 5 to 30 (personal communication with P. Sauriol, Agronomist, Ministère de l'agriculture des pêcheries et de l'alimentation du Québec or M.A.P.A.Q. in St-Rémi, Québec). In 1984 specifically, a thorough survey was carried out on commercial holdings in the agricultural region no. 7 (M.A.P.A.Q. regions) by the Plant Science Department of Macdonald College. Results revealed that

average losses were of the order of 20 % corresponding to a farm value of about 2 million dollars (personal communication with Dr. R. D. Reeleder, Macdonald College of McGill University in Ste-Anne-de-Bellevue, Québec). In the literature, carrots are reported to be susceptible to bacterial soft rot (Erwinia carotovora), black rot (Stemphylium radicinum), Rhizopus soft rot (Rhizopus tritici, R. stolonifer, or R. oryzae), gray mold rot (Botrytis cinerea), and watery soft rot (Sclerotinia sclerotiorum)(Lewis and Garrod, 1983; Crête, 1980; Ryall and Lipton, 1979). The survey identified the latter two as the main carrot storage diseases in Québec, the predominant one being, watery soft rot.

Across Canada, Watery soft rot is also counted among the diseases responsible for most losses to stored carrots (Crête, 1980). It is caused by the fungus Sclerotinia sclerotiorum which produce a characteristic growth of white cottony mycelium on the host surface. The decaying roots become soft and watery but without sliminess (Crête, 1980; Agrios, 1978). During storage, the disease spreads rapidly from infected roots to adjacent healthy ones and creates pockets of decay. Infections that took place in the field before harvest are largely responsible for postharvest infections (Ryall and Lipton, 1979). Cool and wet harvest conditions favor the development of the fungus. Control measures includes careful sorting, dipping in benomyl, and storage at 0 °C and 95 % RH (Ryall and Lipton, 1979, personal

communication with Dr. R. D. Reeleder).

The carrot is also quite susceptible to gray mold rot (Lewis and Garrod, 1983; Nash, 1978). The pathogen involved is Botrytis cinerea, a fungus that is introduced into storage with soil and enters the root through the crown, the base or injuries. The lesions appear soft and watery at first (Agrios, 1978). As the infection progresses they enlarge, change color to brown and finally dark and become spongy and corklike. Mycelium grows on the surface of the host. Because the pathogen is active at low temperatures, severe losses from molding may occur after prolonged periods of storage (MacNab et al., 1983; Agrios, 1978; Nash, 1978). Rapid cooling after harvest and storage at 0 °C and 95 % RH reduce losses from the disease (Lougheed and Valk, 1985; Ryall and Lipton, 1979). Dipping the roots in a solution of benomyl is also recommended (Nash, 1978; Crisp, 1974; personal communication with Dr. R. D. Reeleder).

2.2.3.3 Potato storage diseases

Loss attributable to diseases is probably the most serious gross postharvest losses in potatoes (Booth and Shaw, 1981). Total wastage has been estimated to vary from 5 to 20 % or more and even reached 50 % when potatoes are stored in extremely poor conditions (Nash, 1978). Data on losses due to diseases applicable for the province of Québec fall within this range. The results of a 1976-77 study conducted in several commercial storages in Québec indicated that wastage amounted to 10-15 %

(Asiedu, 1979). Dramatic losses up to 60 % during the first three months of storage in some warehouses in the province were also reported in stock stored for processing. Bacterial soft rot (Erwinia carotovora) and Fusarium dry rot (Fusarium spp.) were responsible for the losses although in the literature late blight (Phytophthora infestans), leak (Pythium spp.), and ring rot (Corynebacterium sepedonicum) are also recognized as important potato diseases (Logan, 1983; Ryall and Lipton, 1979; Hodgson et al., 1973).

Bacterial soft rot is the most serious of all potato storage diseases (Rich, 1983; Ryall and Lipton, 1979; Nash, 1978). It is capable of spreading from one tuber to another and of causing extensive decay within a few days when conditions are favorable (Rastovski et al., 1981; Pérombelon and Kelman, 1980; Nash, 1978). The pathogen involved is a bacteria, Erwinia carotovora (Hodgson, et al, 1973). It penetrates the tuber usually through the lenticel (Pérombelon and Kelman, 1980) but, unhealed cuts, bruises, heat injury, or lesion caused by other diseases such as late blight or Fusarium dry rot are also sites of infections (Pérombelon and Kelman, 1980; Ryall and Lipton, 1979). Rotting tissues are at first wet, cream colored and turn brown to black after a short time. In the early stages of decay, they are odorless but invasion by secondary organisms transform them into a foul-smelling, slimy mass of bacteria and decomposed flesh (Harrison and Nielsen, 1981; Ryall and Lipton, 1979;

Hodgson et al., 1973). Wet and warm storage environment greatly encourage the development of bacterial soft rotting (Harrison and Nielsen, 1981; Nash, 1978; Roberts et al., 1976). Good ventilation is necessary to prevent massive loss once a wet pocket of rotting tubers is established.

Fusarium dry rot is a disease whose development is slower in storage (Rastovski et al., 1981; Nash, 1978). Nevertheless, it is regarded as one of the most important storage diseases as it may induce great losses (Rich, 1983; Hodgson et al., 1973). Numerous species of the fungus Fusarium are causal agents of the infection, one species being predominant in a given soil or locality (Nielsen, 1981; Ryall and Lipton, 1979). They are wound parasites which infect tubers after harvest through mechanical injuries caused by improper handling (Rich, 1983; Rastovski et al., 1981; Ryall and Lipton, 1979). Fusarium lesions are prime sites of infections for secondary invaders, especially Erwinia spp. which cause rapid rooting and threaten nearby tubers (Nielsen, 1981). The symptoms of Fusarium dry rot varies with the species involved (Rich, 1983; Ryall and Lipton, 1979; Nash, 1978; Busch, 1975; Hodgson et al., 1973). Usually, the surface of the infected tubers is wrinkled, sunken, and may range from light brown to black in color. Affected tubers often develop cavities which may be lined with white, yellow, pink or red Fusarium molds. The primary control measures are prevention of tuber damage, establishment of a curing period to heal

inevitable wounds, and storage in cool and dry conditions (Rich, 1983; Nielsen, 1981; Rastovski et al., 1981; Ryall and Lipton, 1979; Busch, 1975).

2.4 STORAGE MONITORING METHODS

Early detection of storage disease problems is, of course, an essential factor in their control (Varns et al., 1985; Schaper et al., 1984; Waterer and Pritchard, 1984b; Varns and Glynn, 1979; Wilson and Boyd, 1945). Corrective measures exist and could be applied before important losses occur (Schaper et al., 1984; Roberts et al., 1976; Porritt, 1974). At present, however, storage managers have at their disposal little means of effectively monitoring stored crop conditions. The methods that are available operate on different principles and vary in degree of sophistication.

2.4.1 Storage Manager's Inspections

To monitor crop conditions, most storers use their senses as only tools (Schaper et al., 1984; Waterer and Pritchard, 1984b; Varns and Glynn, 1979). Through regular inspections they recognize the subtle changes in crop aspect and odor that are related to quality deteriorations and especially disease infections. Literature on this intuitive method is scarce and the information in this section was mostly gathered through personal communications with managers of warehouses on local farms and in the industry (Maurice Ouellette, potato grower and

storer at St-Léonard d'Aston, Québec; Marcel Michaud, extension engineer, M.A.P.A.Q. in Rimouski, Québec; Pierre Deutsch, Humpty Dumpty Inc. in Montréal, Québec).

During each warehouse visit, the storer checks physiological hints given in by the stored crop that may reveal the presence of diseases. First, as he steps into the warehouse, the sense of smell comes into play. The release of unpleasant odors by decaying produce is recognized as the earliest warning sign. Latter, localized condensation on the crop or the ceiling will often pinpoint the source of these odors. This is because the moisture produced by spoilage and carried away by the ventilation air (Nash, 1978; Roberts et al., 1976) condenses when the moist air encounters a nearby cooler surface. Excessive moisture also causes seepage on the floor or on the side of pallet boxes. Finally, when decay is well advanced, especially in bulk storage, the pile collapses clearly indicating the extent of the infection (Rastovski et al., 1981; Wilson and Boyd, 1945). In today's large commercial storage facilities, although these visual-olfactory signs do lead to the detection of disease problems, significant losses would have occurred already (Schaper et al., 1984; Waterer and Pritchard, 1984b; Varns and Glynn, 1979).

2.4.2 Temperature Sensing

Storage ventilation systems are necessary, in part, to control the crop's temperature (Hall, 1980; Ryall and Lipton,

1979; Nash, 1978). The extent to which they are successful depends on the relevant control equipment and also facilities to measure temperature (Thompson, 1985; Statham, 1983; Rastovski et al., 1981).

In bulk potato storage, multiple-temperature sensors are sometimes used inside the pile to obtain its temperature at several locations (Rastovski et al., 1981; Hall, 1980; Roberts, 1976). Statham (1983) states that the optimum coverage of sensing points equate one per 50-100 tonnes of potatoes. As the breakdown of tubers is invariably accompanied by a marked rise in temperature (Nash, 1978; Wilson and Boyd, 1945), an abnormally high temperature recorded by a sensor could indicate the presence of nearby infection loci (Hall, 1980; Anonymous, 1983). However, the readings are mainly used for control purposes (Rastovski et al., 1981; Bishop and Maunder, 1980, Roberts et al., 1976). The detection capability is an added feature of the temperature recording system but not its primary function. For disease monitoring, the network needs to be much tighter.

Higher density of horizontal temperature sensing points is achieved by thermal infrared scanning (Hyder et al., 1984). As mentioned before, spoilage generates heat and modifies the temperature profile of the pile (Ouellette, 1985; Nash, 1978; Wilson and Boyd, 1945). By scanning the pile with an instrument that measures thermal radiation, localized temperature

variations may be indicative of overheating problems. This technique is commercialized in the United States and provides satisfactory results (personal communication with Larry Hyder, Northwest Ag Consultant, Oregon).

In the Red River Valley (United States), infrared scanning was successfully applied to sugar beet storages (Anonymous, 1978). An imaging thermal equipment was mounted on an aircraft which flew over unventilated sugar beet piles. Sections affected by spoilage were spotted and removed by workers on site.

The practicality of detecting diseases by monitoring temperature is doubtful when the stored crop is well ventilated. Part of the heat generated by the pathogens developing locally in a massive pile of produce is dissipated by the air draught forced through the pile (Rastovski et al., 1981; Nash, 1978; Roberts et al., 1976). The residual heat causes small temperature rise in the immediate vicinity of what is referred to as "hot spot" (Anonymous, 1983; Hall, 1980). Recording these increases in temperature would necessitate a high density of sensing points (more than one per 50-100 tonnes) which is not convenient to cover large warehouses (Hyder et al., 1984; personal communication with Maurice Ouellette). In that perspective, the use of infrared sensors appears as an attractive alternative to temperature probing (Hyder et al., 1984). The technique does outline the entire horizontal temperature distribution although large temperature deviations

are unlikely (Ouellette, 1985). Further more, benefits can be claimed from infrared monitoring only if early detection is achieved. In general, disease detection methods based on slow temperature rise and the appearance of the usual visual-olfactory signs are considered as equally effective (Schaper et al., 1984; Waterer and Pritchard, 1984b; Varns and Glynn, 1979).

2.5 VOLATILE MONITORING

2.5.1 Concept and Practical Aspects

An alternative method of assessing disease status of stored crops is the monitoring of headspace volatiles. The concept assumes that disease infection upsets both quantitatively and qualitatively the equilibrium of the gaseous mixture in the storage atmosphere (Varns and Glynn, 1979). These alterations would result from (a) changes in the normal patterns of metabolite production of the infected organisms (Varns and Glynn, 1979); (b) the elaboration of metabolites by the pathogens involved (Abramson et al., 1980; Lee et al., 1973); or (c) the release of volatile by-products of the host-pathogen interaction (Waterer and Pritchard, 1984b; Richard-Moulard et al., 1976). The ability to register abnormal concentrations of these gases conveyed in the circulating air could possibly provide early detection and identification of diseases in ventilated storages (Schaper et al., 1984; Waterer and Pritchard, 1984b; Varns and Glynn, 1979; Abramson et al., 1980;

Richard-Moulard et al., 1976).

The performance of a storage disease detection system based on headspace volatiles is closely related to the choice of the volatiles monitored and the monitoring strategies employed (Waterer and Pritchard, 1984b; Varns and Glynn, 1979). Volatiles likely to be selected are those that are (a) as disease-specific as possible; (b) produced in sufficient quantities to identify disease stresses at the early stages of development; (c) known not to arise from unrelated sources (ex.: warehouse construction materials, handling equipment, etc); (d) analyzed by methods suitable for on-farm use (Varns and Glynn, 1979); and (e) reliable under different disease development and storage conditions (Waterer and Pritchard, 1984b). To get round the latter criterion, gas concentration ratios rather than absolute values could be measured (Varns et al., 1986; Schaper et al., 1984; Varns and Glynn, 1979). In other words, concentrations of normal volatile metabolites would serve as reference levels to normalize the influence of extraneous factors. This information generated at regular intervals throughout the storage period make up the essence of the storage strategy (Schaper et al., 1984; Waterer and Pritchard, 1984b; Varns and Glynn, 1979). Its implementation requires numerous gas analyses and extensive data processing.

Schaper and co-workers (1984) have developed a computerized gas sampling and analysis system for storages. The

system is controlled by a personal computer and is capable of logging and processing information regarding concentrations of several gases as well as other parameters like storage and climatological conditions, fan status, etc. In preliminary tests, sulfur hexafloride (SF_6) was used to simulate a disease-specific volatile while carbon dioxide (CO_2) served as the primary reference gas. Four thousand hours of operation in commercial potato warehouses confirmed that ratios of gas concentration was a viable approach in the development of procedures mainly because of anemometric effects on storage atmosphere changes (Varns et al., 1986; Schaper et al., 1984). Although this prototype requires further development before it becomes commercially available, it provides exciting insights on future vegetable storage facilities.

Meanwhile, the production of volatile metabolites emanating from perishable commodities must be the subject of continuing research. Key metabolites associated with diseased lots have yet to be identified. The complexity and, therefore, the benefit/cost ratio of monitoring operations are closely linked to the nature of these gases and will largely determine the success of headspace volatiles monitoring as a technique to detect storage diseases.

2.5.2 Volatile Profiles of Stored Agricultural Crops

The volatile profiles of many agricultural crops have been examined to some extent (Heath, 1981; Charalambous, 1978;

Salunkhe and Do, 1976; Self, 1967). However, these studies were conducted within the framework of aroma analysis. Few attempts to define the volatile profiles of healthy agricultural crops during storage has been reported (Abramson et al., 1980; Richard-Moulard et al., 1976; Dravnicks et al., 1973; Meigh et al., 1973; Hougen et al., 1971; Rasekh and Kramer, 1971). Likewise, limited information on the volatile metabolites elaborated by some microorganisms such as bacteria is available (Kaminski et al., 1972; Herrais et al., 1966; Lammana and Mallette, 1959). The first report on host-pathogen interactions was published by Kaminski and co-workers (1973) where odorous volatiles produced by various fungi growing on stored cereals were identified.

Other research work was conducted on cereal grains. Richard-Moulard et al., (1976) observed the production patterns of some volatile compounds associated with fungal growth in stored corn. Their findings suggest that sequential production could serve as early warning of spoilage. Abramson et al., (1980) monitored the amounts of known fungal odor components emanating from small parcels of barley, wheat and oats during several weeks of storage. Correlation between the odor formation and fungal population levels existed.

A headspace analysis technique was proposed as a non-destructive method to detect contamination by Aspergillus flavus and Aspergillus parasiticus in peanut stocks. Lee et al (1973)

observed considerable quantitative differences between the volatile profiles of contaminated and sound stocks.

2.5.3 Volatile Profiles of Stored Carrots and Potatoes

Although headspace analysis techniques have been used to distinguish carrot cultivars, to determine their residual storage life (Rasekh and Kramer, 1971), and in aroma analysis (Simon et al., 1982, 1980a, b; Salunkhe and Do, 1976; Buttery et al., 1968), no experiments on stress detection in stored carrots by means of volatile monitoring have been published to date. Rasekh and Kramer (1971) obtained profiles from 6 carrot cultivars that were composed of 12 unidentified volatile compounds.

Conversely, volatile monitoring for disease detection in stored potatoes was extensively investigated. In both laboratory and commercial environments, Varns and Glynn (1979) observed significant qualitative and quantitative changes in the production patterns of healthy and diseased lots. Laboratory tests revealed that tubers inoculated with soft rot bacteria (Erwinia carotovora var. atrosceptica) produced several compounds at greater rates than healthy tubers. Abnormal concentrations of three compounds (acetone, ethanol and 2-butanone) were recognized as specific response of soft rot infection. Higher levels of these compounds were also measured in commercial storage bins of tubers known to be affected by E. carotovora and Fusarium spp. It appeared feasible to gather

useful information on the pathogen present and its stage of development by following the changes in the volatile fingerprints (Varns, 1983; Varns and Glynn, 1979).

Canadian workers continued work on volatile profiles of diseased potatoes. Waterer and Pritchard (1984b) first repeated the experiment on soft rot infected tubers. They found discrepancies between their results and those reported by Varns and Glynn (1979). With the exception of ethanol, the dominant compounds in the disease-induced volatile profiles were entirely different from those observed by Varns and Glynn (1979). They speculated that dissimilarities between the two studies were related to differences in test conditions and experimental procedures. Waterer and Pritchard (1984a) then compared the volatile production characteristics of ring rot (Corynebacterium sepedonicum) and soft rot infected potatoes. The volatile profiles of the two infections had in common a number of metabolites. However, disease-specific changes in terms of relative concentrations were observed and at least one compound was unique to each infections. Finally, the same technique was used to determine whether infections caused by two varieties of E. carotovora (carotovora versus atroseptica) could be differentiated on the basis of volatile production characteristics. The attempt was not successful (Waterer and Pritchard, 1985).

2.5.4 Headspace Analysis Techniques

The term headspace is defined by Wyllie et al. (1978) as the gaseous mixture surrounding a sample within a closed system in equilibrium. For diverse applications, the headspace contains valuable information about the sample when its composition is revealed, usually by gas chromatography (GC) or gas chromatography-mass spectrometry (GC-MS; Barnes et al., 1981; Charalambous, 1978; Bertsch et al., 1974; Murray, 1977; Zlatkis et al., 1973).

In most headspace studies, sampling is a serious concern for two main reasons. First, the compounds of interest are present in relatively small amounts, often below detection levels of GC instruments. Secondly, these compounds are almost always dominated by water which leads to rapid deterioration of the GC analytical column. The conventional direct injection method using a syringe is therefore inadequate and replaced by methods that incorporate pre-concentration and cleanup procedures (Heath and Reineccius, 1986; Numez et al., 1984; Barns et al., 1981; Tsugita et al., 1979; Wyllie et al., 1978).

The use of adsorbents for the trapping of headspace volatiles is widespread (Sakaki et al., 1984; Cole, 1980; Charalambous, 1978; Murray, 1977). With this technique, sampling and pre-concentration can be achieved in a single step where the volatiles are entrained onto an adsorbent (Numez et al., 1984; Barnes et al., 1981; Bertsch et al., 1974). This adsorbent,

usually a synthetic porous polymer, can be readily and completely desorbed either simply by heating or elution with an appropriate solvent (Barnes et al., 1981). Because porous polymer are hydrophobic, they do not retain large amounts of water (Barnes et al., 1981; Bertsch et al., 1974). The techniques related to the pre-concentration of headspace volatile for GC examination have been reviewed by Numez et al. (1984).

The review includes a compilation of the principle physical characteristics of available porous polymer. A trapping medium is selected on the basis of adsorptive capacity and selectivity, thermal stability, and levels of background when thermal desorption is used (Numez et al., 1984; Barnes et al., 1981; Murray, 1977). Tenax GC is the most widely selected adsorbent because of its high thermal stability (Heath and Reineccius, 1986; Numez et al., 1984). On the other hand, adsorbents of the Chromosorb series have also been favored because they best combine the above selection criteria (Waterer and Pritchard, 1984b; Murray, 1977). The suitable polymeric material is packed into small tubes, often referred to as "traps", which serve as the volatile collection device.

For examination by GC of the adsorbed analytes in trap tubes, Numez et al. (1984) favored thermal over liquid desorption. Several thermal desorption techniques have been proposed (Krost et al., 1982; Young, 1981; Tsugita et al., 1979;

Peterson, 1978; Williams et al., 1978; Murray, 1977; Brown et al., 1971). The one described by Murray (1977) involves the introduction of trap tubes directly into the GC injection port. The desorbed volatiles are momentarily recondensed on a cold pre-column to achieve plug injection onto the GC column. The technique requires little modification to the GC unit and is a simple, inexpensive and effective means of thermal desorption (Numez et al., 1984). Sophisticated thermal desorption units adaptable to GC instruments are commercially available (e.g.: Tekmar Company, Ohio; Supelco Canada Ltd, Ontario).

III- MATERIALS AND METHODS

3.1 GENERAL

A dynamic headspace analysis technique (Numez et al., 1984) was utilized in order to simulate ventilated storage bins. Purified air (the purge gas) was continuously swept through small lots of carrot roots or potato tubers placed inside sealed containers. The emerging gas flow was passed through a suitable polymeric adsorbent and vented to the atmosphere. The headspace volatiles were removed from the gaseous effluent by adsorption onto the trapping medium and subsequently thermally desorbed for examination by gas chromatography (GC).

GC analysis generated chromatograms. These charts, on which volatiles are reproduced as peaks and recognized according to their retention time, were actual graphical displays of volatile profiles from stored carrots and potatoes. The approach followed in this study was to discern the compounds (or the peaks) that were unique to diseases of each vegetable. To achieve this, comparisons of the chromatograms obtained from, for example, healthy and diseased carrots were made on the basis of a match of corresponding peaks. No quantitative analysis was performed. Attempts to identify all volatiles in the profiles were made by gas chromatography-mass spectrometry (GC-MS).

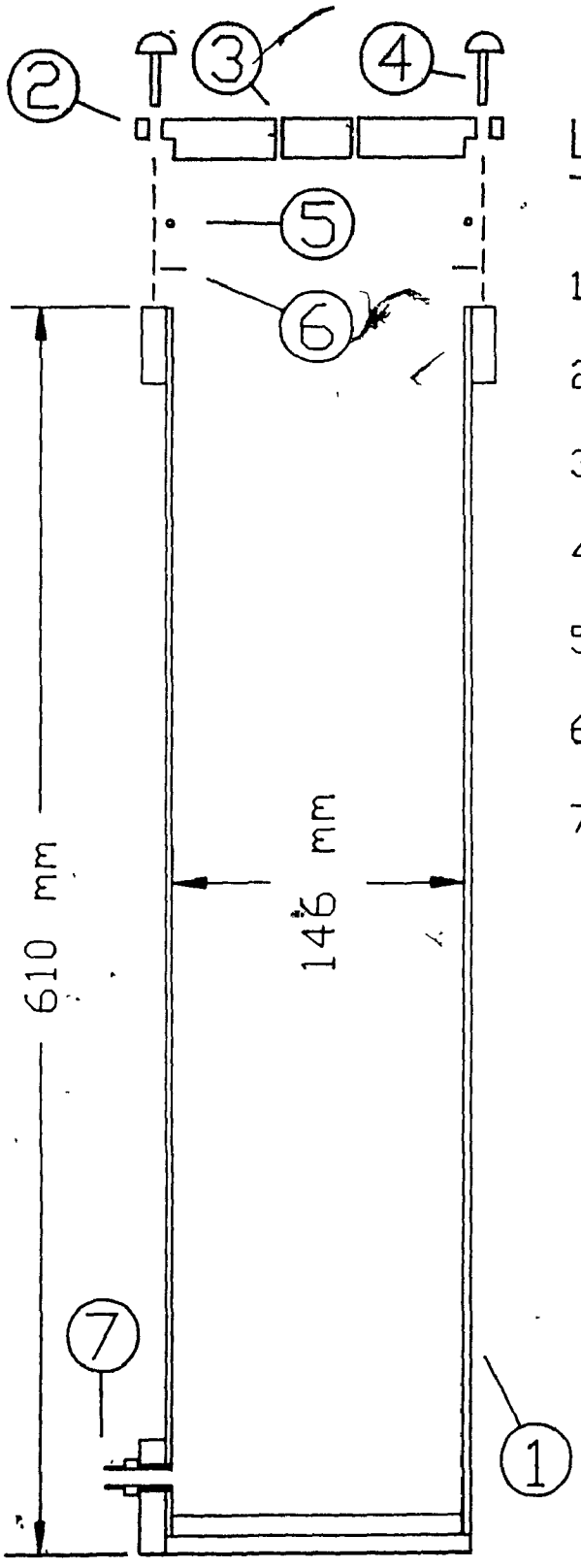
3.2 EXPERIMENTAL SETUP

The experimental setup was designed to achieve comparative

collection of headspace volatiles. Ten acrylic cylindrical containers having a capacity of 7.7 liters each and dimensions as shown in Figure 3.1 were connected to a manifold via individual 3.2 mm O.D. teflon tubings. Purified air originating from a pressurized cylinder was passed through a 6m x 3.2 mm O.D. column of molecular sieve (type 5A; 60/80 mesh) to improve its purity and through a layer of water at the bottom of the manifold for getting the air to saturation levels (Figure 3.2 and Photograph 3.1). The manifold outlets were calibrated to supply each container with an equal rate of air flow. The air was then forced through the produce inside the container and vented to the atmosphere after passage through parallel traps of porous polymer adsorbent. Because the setup was not totally volatile-free, a blank container was used to distinguish the extraneous volatiles from those emanating from the stored produce. In addition to this, a short collection run with all containers left empty was done. The containers and the manifold were housed in a controlled environment cabinet. This design is similar to that of Spence and Tucknott (1983).

3.3 TRAP CONSTRUCTION AND CONDITIONING

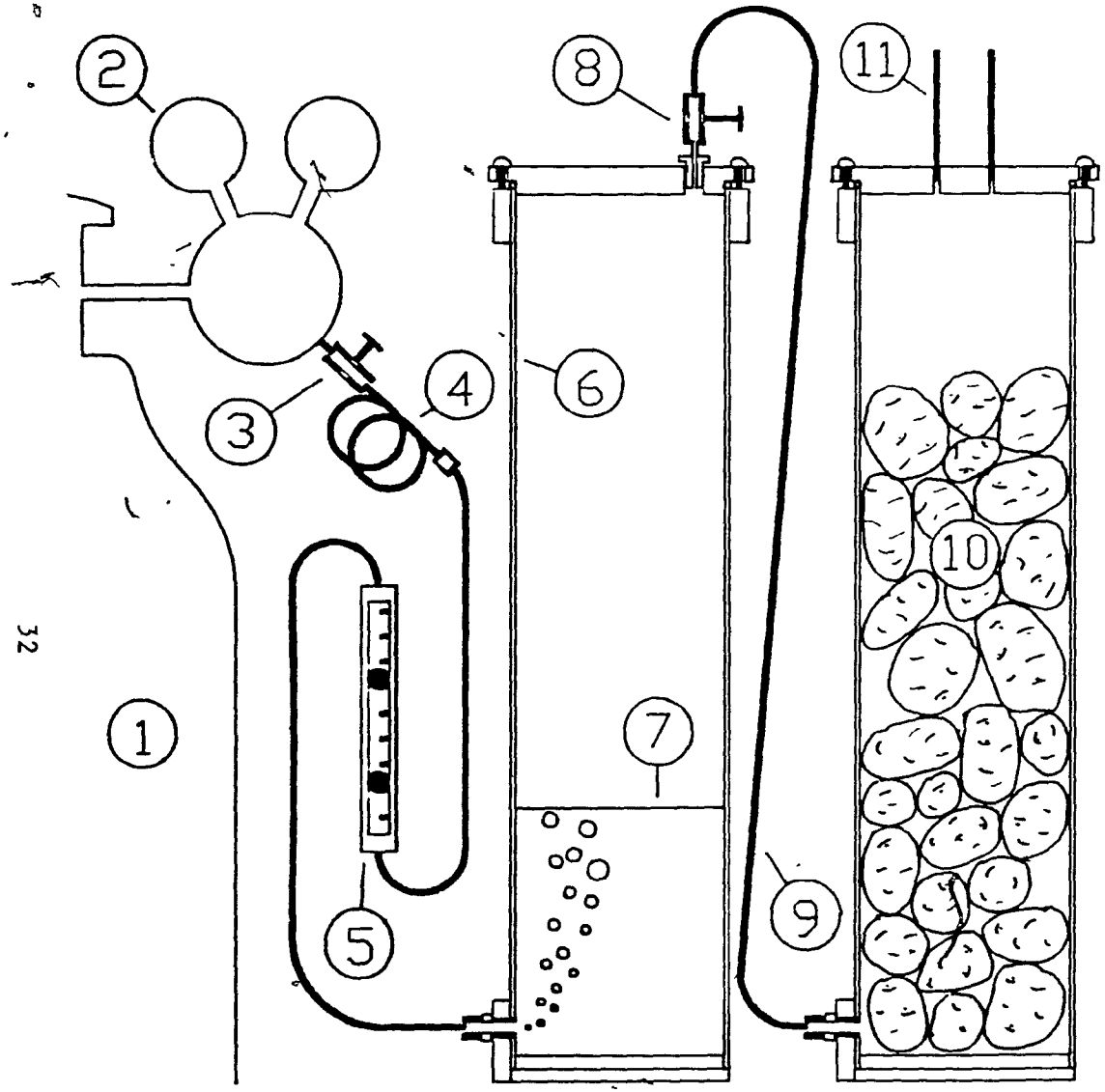
The traps located on the container lids were used to sample and concentrate the volatiles released by the stored produce. They were constructed from stainless steel tubes 88 mm long by 3.2 mm O.D. (Figure 3.3). Outside threads were made at one end



LEGEND

1. acrylic container
2. acrylic cover
3. outlets: locations of traps
4. cover screws
5. rubber seal
6. teflon lining
7. inlet: supply line fitting

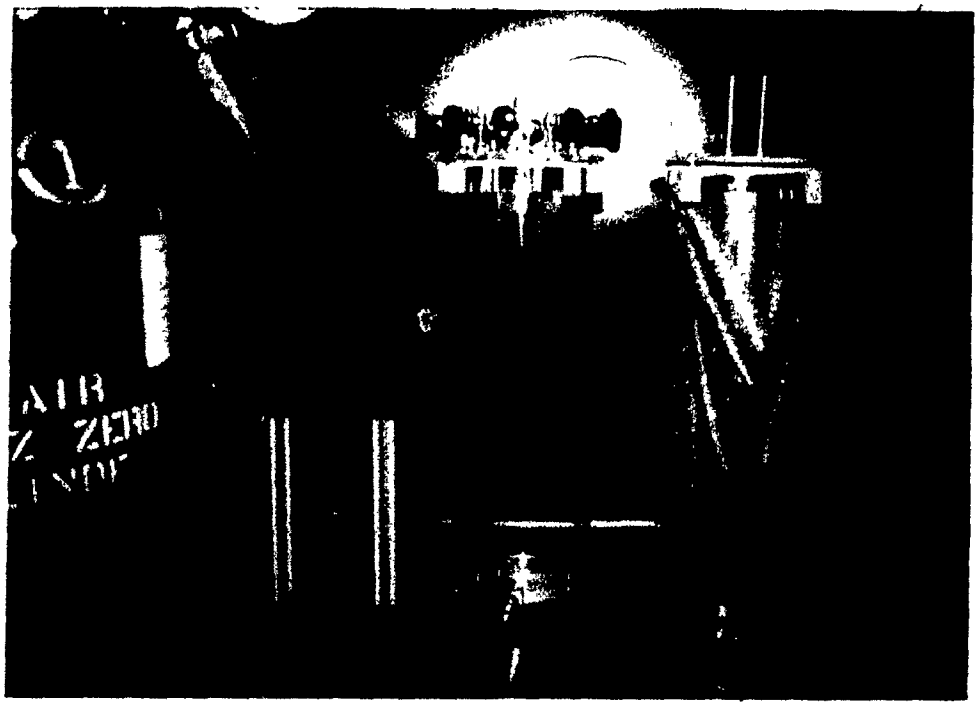
Figure 3.1: Construction details of one 7.7 liter container.



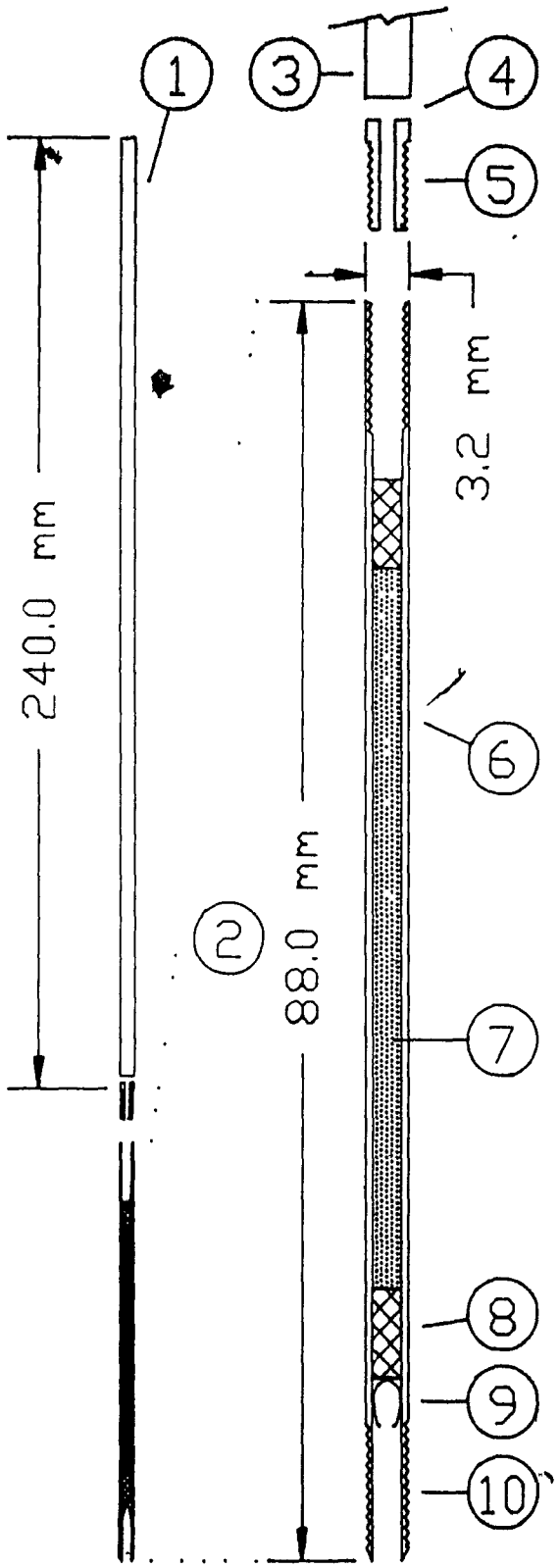
LEGEND

1. pressurized cylinder
2. pressure regulator
3. on/off valve
4. molecular sieve column
5. flow meter
6. manifold
7. water layer
8. regulating valve
9. supply line
10. produce container (one of ten)
11. pair of adsorbent traps

Figure 3.2: Schematic of the experimental setup.



Photograph 3.1: Experimental setup. (Note: column of molecular sieve omitted).



LEGEND

1. Insertion rod and Chromosorb 105 trap
2. enlarged trap
3. insertion rod tip
4. vent holes
5. 3-48 NC threads
6. stainless steel tube
7. Chromosorb 105 packing
8. silanized glass wool
9. retaining wire
10. 6-32 NC threads

Figure 3.3: Chromosorb 105 trap and insertion rod.

so that they could be screwed into the container lids. The other end was tapped to suit the thermal desorption method (see next section). The tubes were packed with 80 mg of 60/80 mesh Chromosorb 105 secured between silanized glass wool pads. A bent short section of stainless steel wire kept the lower pad from moving.

Conditioning was accomplished by heating the porous polymer to an elevated temperature in a non-oxidizing atmosphere. Batches of 20 traps were simultaneously prepared by screwing the tubes onto a manifold block supplied with a stream of oxygen-free Helium (200 ml/min; 10 ml/min per trap) and placed inside an oven. Prior to the beginning of the experiment, the traps were conditioned at 200 °C for 24 hrs. After each use, they were reconditioned for 12-16 hrs at 170 °C.

3.4 THERMAL DESORPTION

3.4.1 The Technique

The headspace volatiles trapped on solid adsorbent in the trap tubes were thermally desorbed for subsequent injection into a GC Unit. For this purpose, the technique described by Murray (1977) was selected and adapted to a Hewlett-Packard 5890A gas chromatograph. Some modifications to the instrument were necessary to allow direct insertion of the trap into the injector port. The volatiles were unloaded from the traps and recondensed onto a cold pre-column for their instant injection

without disturbing the carrier gas flow.

3.4.2 Modifications to the GC Unit

Murray's technique required two main attachments: a trap introducer and a pre-column (Figure 3.4). The introducer consisting of an equilibrium chamber, a seal assembly, a plug valve, and a purge line was simply screwed onto the head of the injector port by the septum retainer nut. The pre-column was a S-curved glass-lined stainless steel tube located inside the oven. Midway along the tube was a 15 mm long packing of 5 % OV-101 on 60/80 mesh Chromosorb-W held between silanized glass wool pads. The pre-column could either be cooled by a hollow probe periodically filled with liquid Nitrogen or heated by a similar probe fitted with a resistive coil controlled by the GC circuitry (Figure 3.5). Both probes were cylindrical, made of brass, and insulated with a 3.2 mm thickness of teflon. A U-shaped notch was machined at one end of each probe to closely fit and surround the pre-column. Access of the probe to the pre-column was given by an opening in the oven lid made by the GC manufacturer in provision of an additional injection port. Details of the attachments and the probes are illustrated in Figures 3.4 and 3.5 (see also Photograph 3.2).

3.4.3 Injection Procedures

The unloading and injection procedures used in this study are described here. The trap stored at 1 °C was brought to ambient temperature, screwed onto an insertion rod (or plunger;

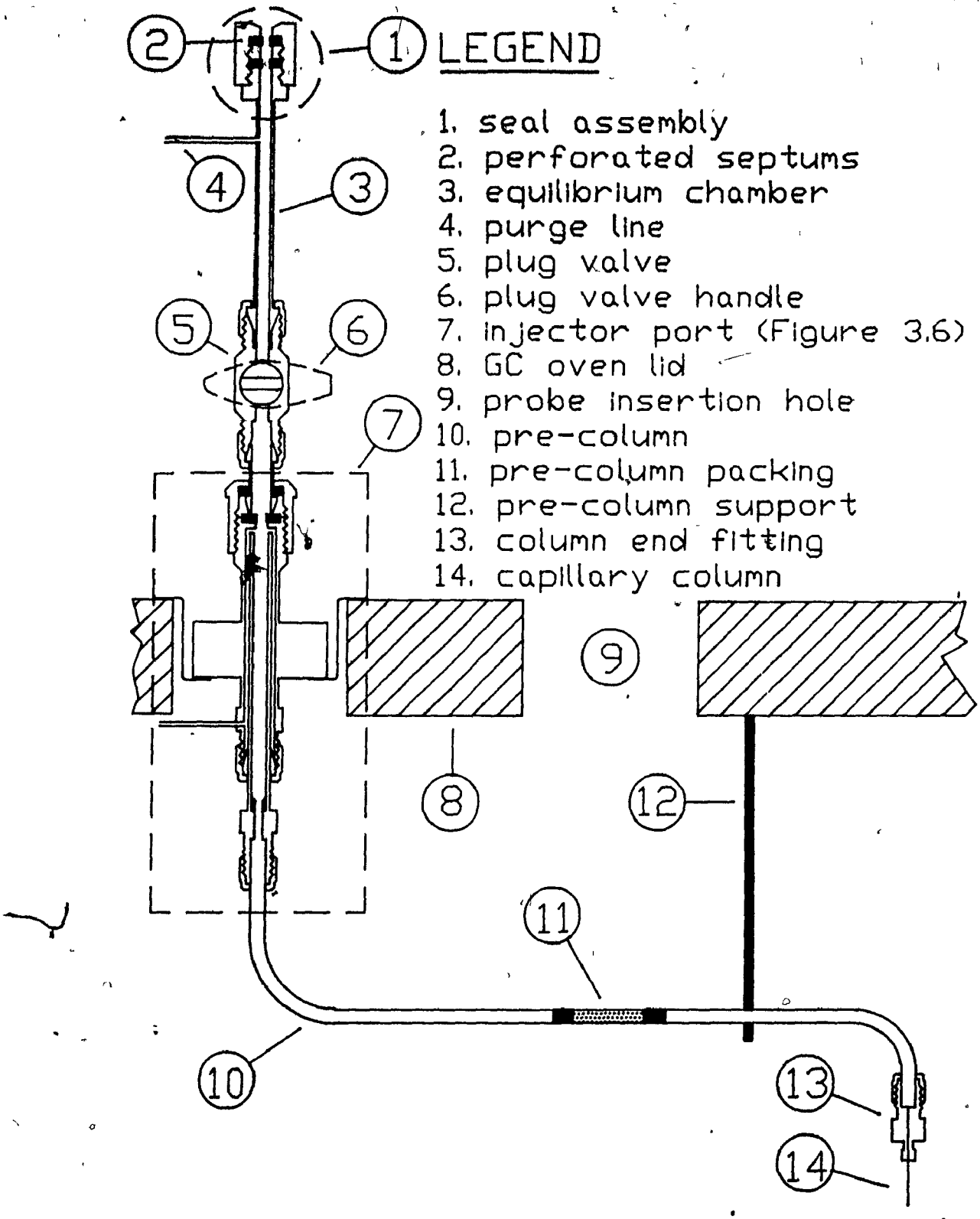
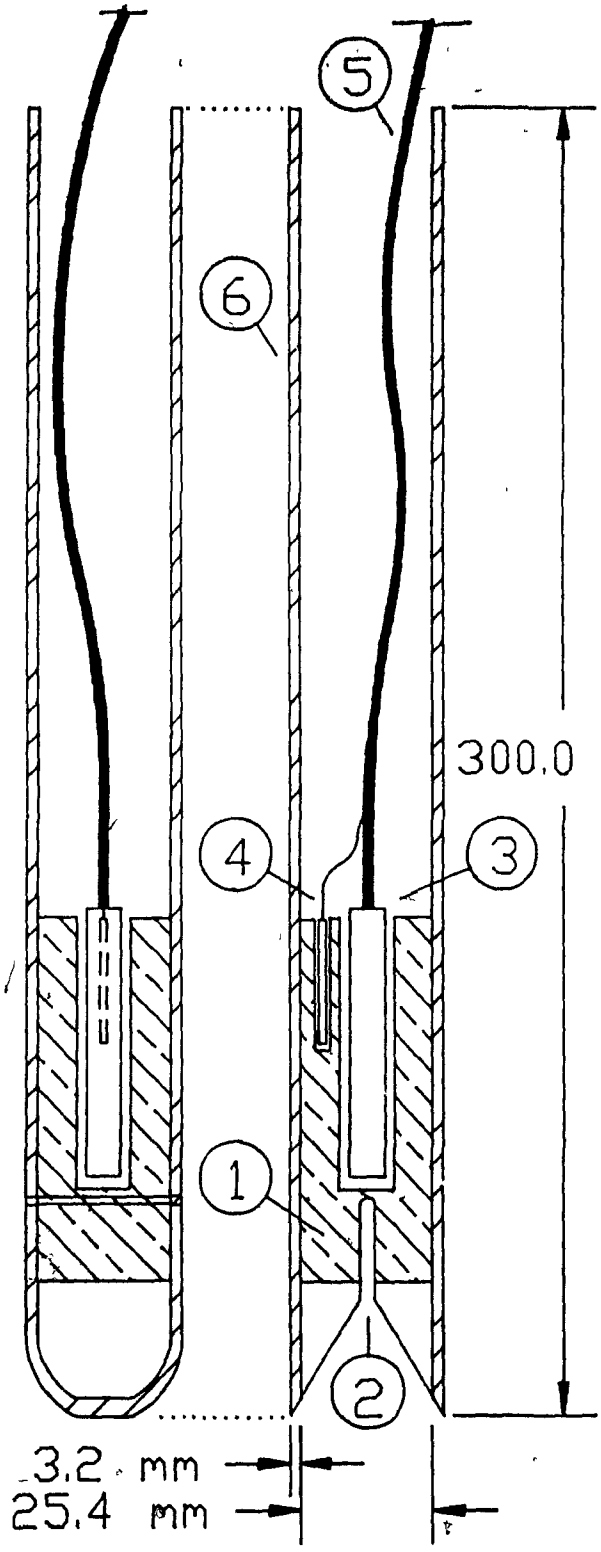


Figure 3.4: Schematic showing the trap introducer, the injector port and the pre-column installed on the GC unit.



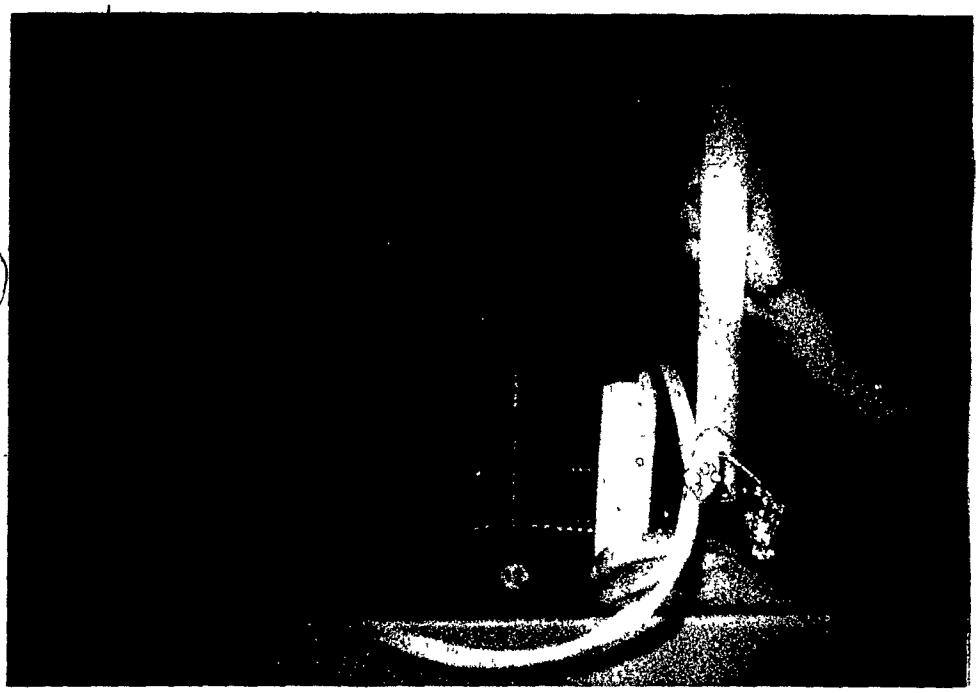
LEGEND

1. brass core
2. 3.2 mm notch fitting pre-column
3. heater, 70 W
4. PRT sensor
5. heater/sensor cables
6. teflon insulation

note:

the design of the cold probe is similar; the heater/sensor assembly is replaced by a reservoir for liquid nitrogen.

Figure 3.5: Front and profile views of the heating probe.

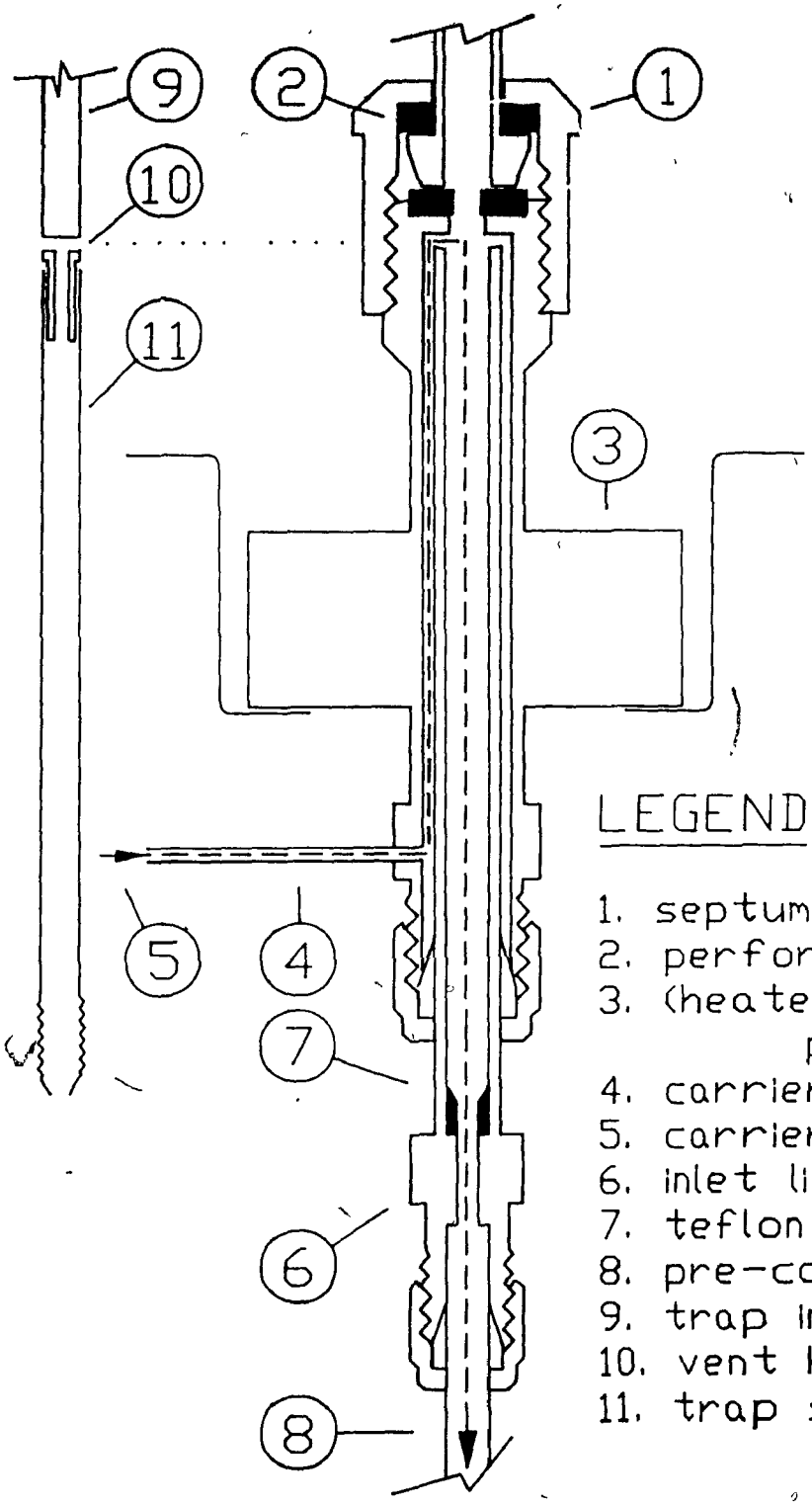


Photograph 3.2: Trap introducer on the GC unit during desorption. The cooling probe replaced by the heating probe.

Figure 3.3) and thrust through the seal assembly into the equilibrium chamber to a point where the vent holes in the plunger were still visible. The purge line was opened for 1 minute to flush air out of the trap with Helium (10 ml/min) at about 25 °C. The trap was pushed further into the equilibrium chamber to enclose the vent holes and the purge line was then closed. The plug valve was opened and the trap lowered against the teflon seat in the heated inlet liner of the injection port (Figure 3.6). To maintain a good seal, a mass (750 g) was placed over the plunger handle. The pre-heated carrier gas flow of Helium (10 ml/min) was thus diverted through the plunger vent holes and through the trap in a backflushing direction. Under the effect of heat, desorption took place and the released volatiles were recondensed onto the pre-column packing through cryogenic cooling. After 6 minutes, the trap was removed from the inlet liner but left inside the oxygen-free equilibrium chamber to cool down and then the plug valve was closed. The cooling probe, inserted through the oven lid, was then quickly replaced by a heating probe to achieve the instant injection of the condensed volatiles. Step-by-step injection procedures are described in Appendix A.

3.5 GC ANALYSIS

Gas chromatographic analyses were performed on a Hewlett-Packard 5980A gas chromatograph with a flame ionisation



LEGEND

1. septum retainer nut
2. perforated septums
3. (heated) injection port weldment
4. carrier gas line
5. carrier gas flow
6. inlet liner
7. teflon seat
8. pre-column
9. trap insertion rod
10. vent holes
11. trap shown outside inlet liner

Figure 3.6: Enlargement of the injector port.

detector. Separation of the volatiles was obtained with a 60 m x 0.75 mm I.D. glass column coated with Supelcowax 10. Conditions of analysis are summarized in Table 3.1. A Hewlett-Packard 3390A reporting integrator recorded the detector output (i.e. chromatograms).

3.6 GC-MS ANALYSIS

Analyses combining gas chromatography and mass spectrometry were carried out to identify the volatiles collected. The GC-MS unit was located in another laboratory (Agriculture Canada Plant Research Center, Ottawa) where a similar volatile trapping system, also based on thermal desorption, was already in operation. However, the thermal desorption unit was designed to accept larger trap tubes than those used in this study. These traps were made of stainless tubes 76.2 mm-long by 6.4 mm O.D., packed with 130 mg of a different polymeric material, 60/80 mesh Tenax GC (Figure 3.7). For GC-MS analysis, the adsorbed samples were transferred from the small traps to the larger ones.

The transfer was accomplished with minor modifications to the original thermal desorption arrangements. The GC column was disconnected and the pre-column replaced by an identical glass-lined stainless steel tube containing no packing (Figure 3.7). Tenax GC traps were screwed onto a reducing union connected to the transfer tube. The same unloading procedures explained

Table 3.1: Gas Chromatographic conditions.

Sample introduction (injector port):	
temperature:	160 C
purge gas:	Helium
purge gas rate:	10 ml/min.
coolant:	liquid Nitrogen
Gases:	
carrier gas:	Helium
carrier flow rate:	10 ml/min.
make-up gas:	Helium
make-up flow rate:	25 ml/min.
air flow rate:	300 ml/min.
Temperature program:	
isothermal at 70 C for 5 min, 70-160 C at 2.5 C/min, and isothermal at 160 C for 4 min.	
Detector:	
type:	flame ionization
temperature:	220 C

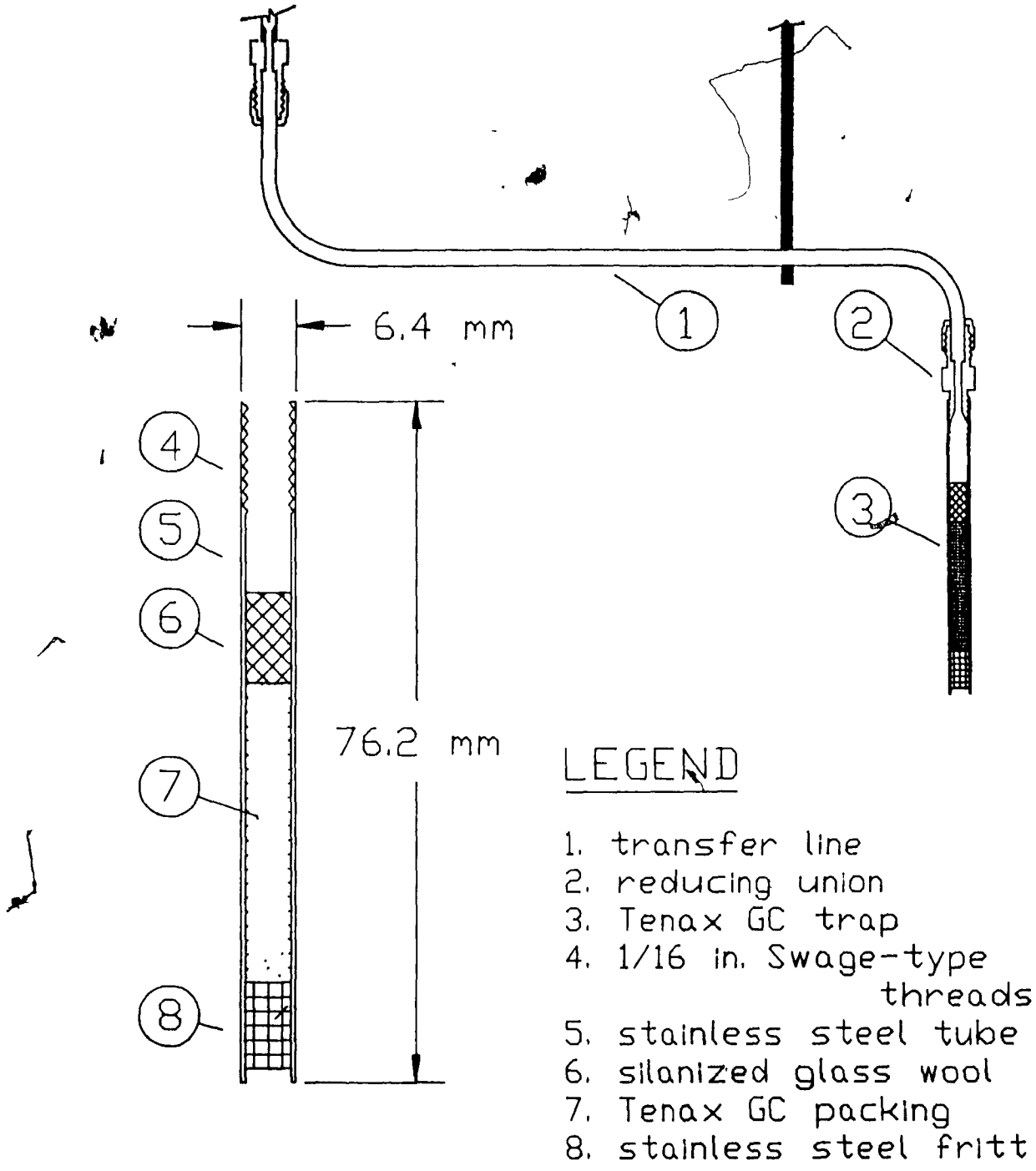


Figure 3.7: Tenax GC trap and transfer line arrangement.

earlier were followed except that neither the cooling or the heating probes were used. The volatiles released from the Chromosorb traps were re-adsorbed in the Tenax GC traps. During the process, the GC oven fan was turned on to maintain the transfer and the Tenax GC trap tubes at room temperature. After adsorption, the Tenax GC traps were wrapped in aluminum foil, placed in individual glass culture tubes that were capped and sent to Ottawa for analysis.

Identification of the compounds were tentative. Their mass spectra were compared with those present in the Finnigan Library (National Bureau of Standards). The degree of certainty in a given match was evaluated considering the index of purity. Identical spectra were given an index of purity approaching 1000, the maximum value (personal communication with P. Lafontaine, GC-MS operator, Agriculture Canada Plant Research Center, Ottawa).

The GC-MS unit was in fact a Varian 3700 gas chromatograph connected to a Finnigan-Mat 312 mass spectrometer with INCOS Data System. Volatile separation was achieved with a shorter column but lined with the same coating, that is a 30 m x 0.75 mm glass column coated with Supelcowax 10. Chromatographic conditions were different than those reported for the GC runs and are summarized in Table 3.2 along with those of the mass spectrometer. Details on the GC-MS unit are described elsewhere (Anonymous, 1986).

Table 3.2: Gas Chromatographic and Mass Spectrometry (GC-MS) conditions.

- Gas Chromatography:

Sample introduction (CDS 320 concentrator):

temperature:	250 C
purge gas:	Helium
purge gas rate:	30 ml/min.

Gases:

carrier gas:	Helium
carrier flow rate:	3 ml/min.

Temperature program:

isothermal at 50 C for 5 min, 50-2000 C at 10 ml/min.

- Mass Spectrometry:

Ion source:

ionizing voltage:	70 EV
accelerating voltage:	3 kV
ionizing multiplier:	2 kV
Mass scan:	40 to 400; 2 sec/scan
Resolution:	1000

Temperature:

ion source:	250 C
capillary interface:	250 C

3.7 VOLATILE COLLECTION

3.7.1 Carrot Experiment

3.7.1.1 Root inoculation

Fungal inocula were used to induce diseases in carrot roots. They were prepared from sectioned roots that were first autoclaved for 30 minutes at 100 kPa and 100 °C. The sterile sections were then inoculated with a 5 mm dia. potato dextrose agar (PDA) disk of actively growing cultures of S. sclerotiorum Pers. ex. Pers. or B. cinerea Pers. ex. Fr. (Dhingra and Sinclair, 1985). These cultures were obtained from the Plant Science Department of Macdonald College of McGill University.

Healthy carrots (Daucus carota) of the cultivar Charger, stored for 5 to 6 months at 1 °C were washed in sterile water. Nine of the ten containers were filled with approximately 3 kg of carrots each. A root section (about 15 mm long) covered with S. sclerotiorum was inserted among the roots in three containers. B. cinerea inoculum was added in a similar fashion to three other containers. The control treatment consisted of three containers of non-inoculated roots. The tenth container was left empty to assess the quality of the purge air during the experiment.

3.7.1.2 Collection procedures

All containers were tightly closed, placed inside the controlled environment cabinet maintained at 3 °C, and connected to the manifold. The flow rate of non-humidified air (i.e. the

manifold contained no water layer) to each container was measured with a ball flowmeter (Figure 3.8) and arbitrarily set to 20 ml/min. The traps screwed into the container lids were replaced every 4 days and the flow rates recalibrated to 20 ml/min. The traps removed were wrapped in Aluminum foil to avoid contamination during handling and stored until analysis at 1 °C. Some traps were sealed with teflon and held at -10 °C inside capped glass culture tubes for longer period of storage. The experiment lasted 32 days and was repeated once.

In the second trial, two pieces of inoculum instead of one were used in the B. cinerea treatment to encourage more disease development. Once the experiment was completed, the degree of infection was assessed on the basis of a count of roots invaded with mycelium.

3.7.2 Potato Experiment

3.7.2.1 Tuber inoculation

Isolates of E. carotovora ver. carotovora and E. roseum var. sambucinum were respectively obtained from the Agriculture Canada Research Station of Summerland, British-Columbia, and the Agricultural Canada Research Station of Charlottetown, Prince-Edward Island. The bacteria, maintained on nutrient agar slants at 5 °C until then, were streaked on a nutrient agar Petri plate and incubated at room temperature for 48 hrs. The plate was then flooded with 10 ml of sterile water and the suspension added to 50 ml of nutrient broth. This diluted suspension was left at

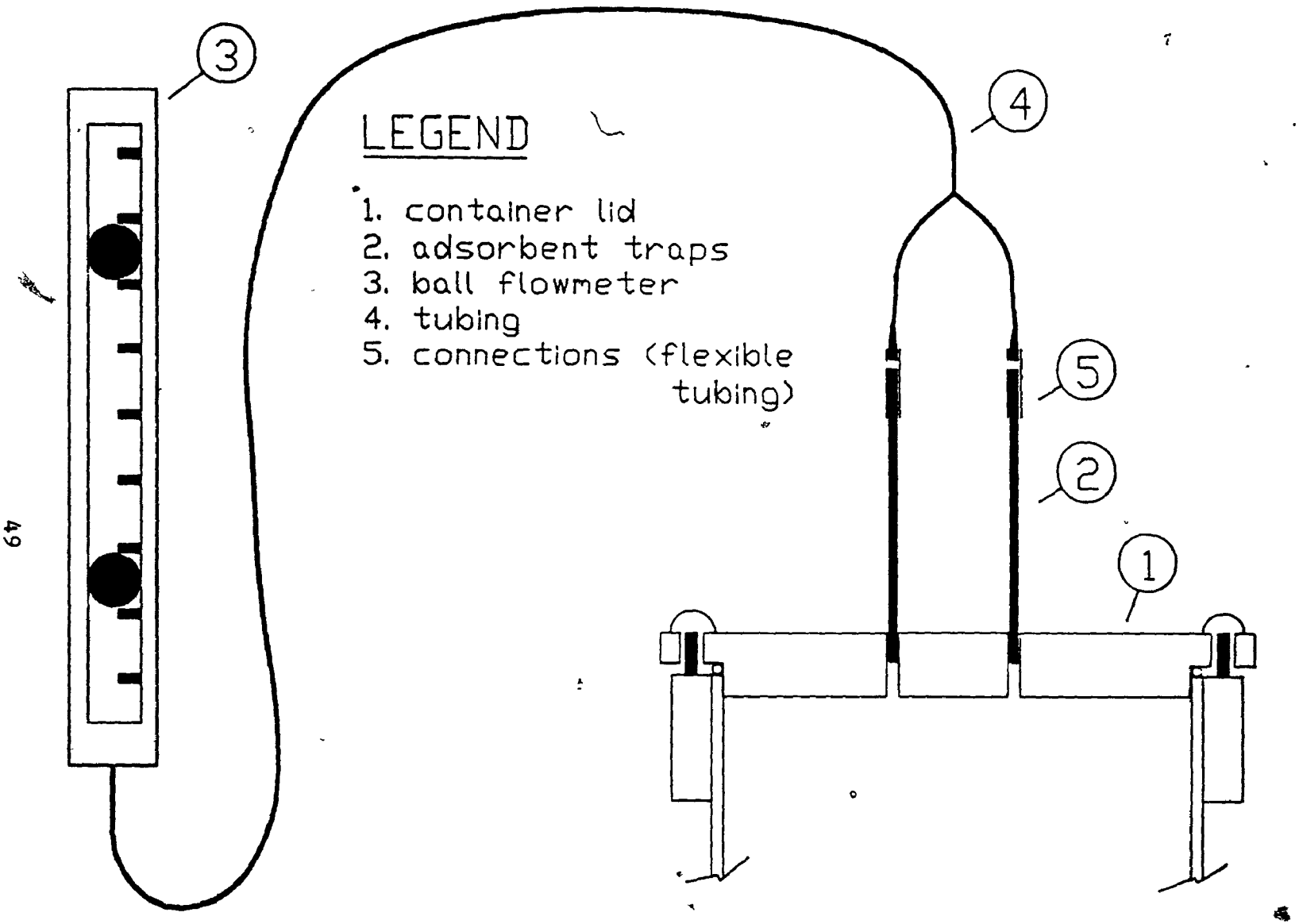


Figure 3.8: Measurement of air flow rate to individual containers.

room temperature on an orbital shaker for 72 hrs. The inoculum was prepared by diluting 40 ml of the bacterial culture in 60 ml of sterile distilled water. At that time, the concentration was approximately 10^9 bacteria/ml.

The inoculum of the fungus F. roseum, which was kept before use at 5 °C on PDA slants, was prepared by transferring a fraction of the original culture onto a fresh PDA Petri plate and held at room temperature for 5 days. One hundred ml of potato dextrose broth was inoculated with a 5 mm dia. PDA disk of the fungal culture. After 96 hrs on an orbital shaker at room temperature, the fungal suspension was filtered through two layers of cheese cloth and centrifuged for 20 minutes. The supernatant was discarded and the spores were re-suspended in sterile water to a concentration of 10^6 spores/ml (Dhingra and Sinclair, 1985).

Healthy potatoes (Solanum tuberosum) of the cultivar Atlantic, stored for 1 to 3 months at 15 °C, were washed and rinsed in sterile water. Six kg of the potatoes were inoculated with the bacterial culture by injecting 1 ml of the inoculum in each tuber with a syringe, fitted with a 38 mm long needle (22 gauge; BD no. 5156), at about 25 puncture points (Waterer and Pritchard, 1984b). The tubers were then placed in three containers (2 kg/container). The same procedure was used to prepare three replicates with the fungal inoculum. As controls, three containers of tubers wounded with syringe injections of

sterile water were used. Again, the tenth container was not filled for air quality control purposes.

3.7.2.2 collection procedures

The containers were not immediately sealed. Rather, they were left open at room temperature in the dark; and misted water was applied on the tubers every 8 hrs to keep them moist. After 48 hrs, the lids were put on the containers which were transferred into the cabinet maintained at 15 °C and connected to the manifold. In this case, the manifold was partly filled with sterile water to humidify the purge air. The volatile collection period and the trap handling were the same as described for the carrot experiment. The 16 day long experiment was conducted twice.

After each trial, the damage caused by the diseases was evaluated on the basis of affected tissues. Each tuber was sliced and the flesh visually inspected. A number from 1 to 4 was assigned depending on whether less than 25%, 25% to 50%, 50% to 75%, or more than 75% of the area of the transversal cut appeared to be affected.

3.7.2.3 CO_2 monitoring

For the potato trials, the air flow rate to individual containers was adjusted to avoid excessive carbon dioxide (CO_2) accumulation in the tuber headspace. Based on a CO_2 balance at steady-state (i.e. CO_2 production rate versus CO_2 removal rate), a flow rate of 35 ml over 2 kg of potato tubers would limit the

concentration of CO_2 below 0.3%. The actual respiration rate of the tubers in terms of CO_2 produced was required in the calculation and was measured with the electronic sensor developed by Forcier et al. (1987).

CO_2 levels in one container/treatment were determined after 4,8, and 16 days. The headspace air, sampled with a 1 ml syringe through a septum into the lids, was analyzed with a Fisher-Hamilton gas partitioner (model 2A) coupled to a Hewlett-Packard reporting integrator (model 3390A).

IV - RESULTS AND DISCUSSION

4.1 INTRODUCTION

In this study, the headspace above stored carrots and potatoes was analyzed. The purpose of the exercise was to explore further the use of volatile constituents of the storage atmosphere to detect vegetable storage diseases, with a specific emphasis on identifying those that would be specific to each infection. Being the first of its kind in the Agricultural Engineering Department of Macdonald College, the headspace volatile sampling system had to be developed and the analysis techniques implemented for this research work. For this reason, the chapter begins with a discussion on the performance of the volatile collection and analysis technique used.

4.2 EVALUATION OF THE VOLATILE COLLECTION AND ANALYSIS TECHNIQUE

4.2.1 Method of Collection

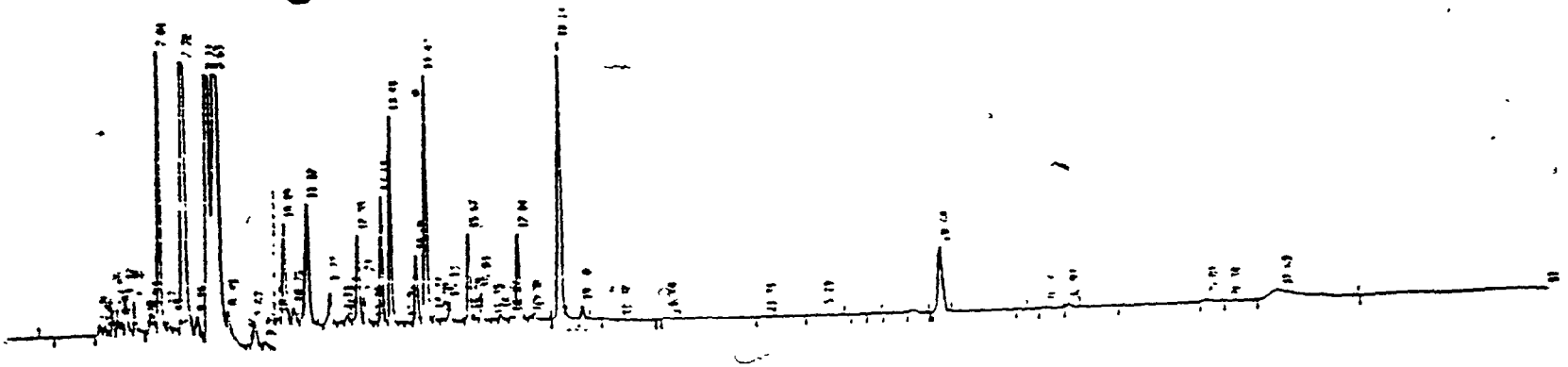
4.2.1.1 Experimental setup

The experimental setup designed for this study permitted the simultaneous collection of headspace volatiles from 9 samples. The samples were small lots of either carrots or potatoes and the experiment consisted of three treatments: two different diseases and one control; 3 replicates per treatment. A continuous flow of air over the produce simulated ventilated storage bins. This arrangement produced comparative volatile profiles from vegetables stored in controlled conditions.

Some variations in the volatile profiles collected were caused by the experimental setup itself resulting from uneven supply of air to individual containers. In the dynamic headspace analysis technique utilized, the headspace vapors were entrained by the purge gas and concentrated onto porous polymer traps. The composition of the trapped vapors are known to vary with the flow rate of the purge gas and the time of collection (Wyllie et al., 1978). In this study, imprecisions in the flow metering system were of the order of 10%. In the potato experiment for example, an air flow to each container adjusted to a rate of 35 ml/min could result in differences in the total volume of air supplied to each container of approximately 40 liters after the 96 hr collection period. Theoretically, a deficit of 40 liters in sampling volume could keep the concentration of certain compounds below the detection limit of the analysis in one replicate while being detectable in others. However, the main effects of uneven distribution of air were variations in the general aspect of the chromatograms in terms of the relative size of peaks (Figure 4.1). This is because in GC analysis, the area under the peaks can be correlated to the compound concentration (Kaiser and Debbrecht, 1977).

In this study, these apparent quantitative variations had no deleterious effect for two reasons. First, the key compounds turned out to be present in concentrations high enough to make their detection independent of flow rate inaccuracies. Secondly,

Chromatogram A:



Chromatogram B:

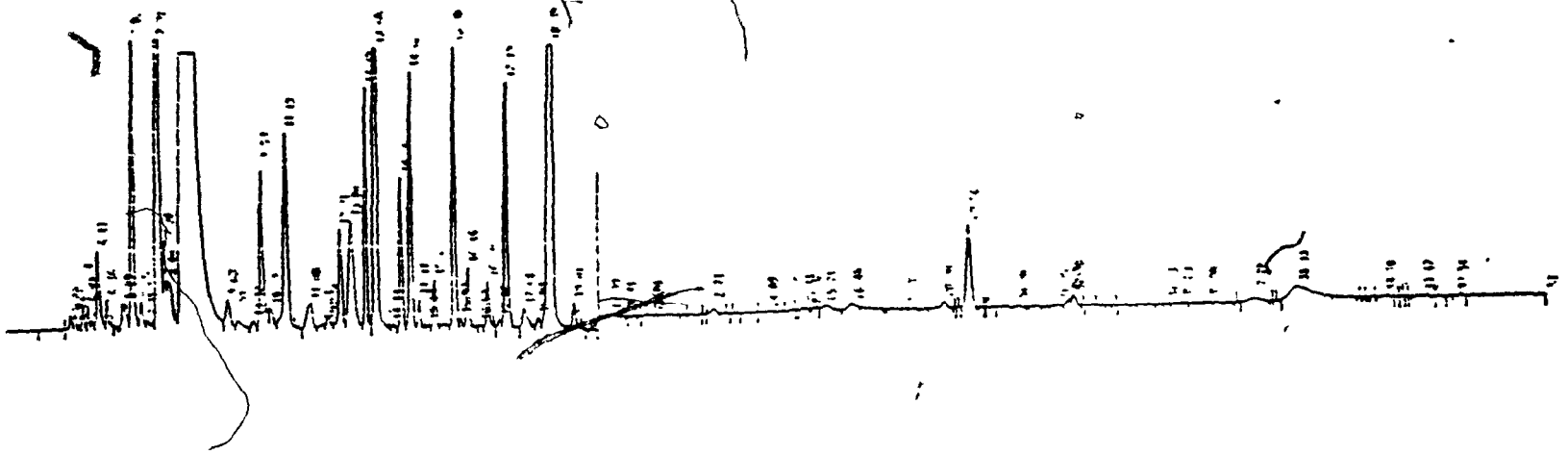


Figure 4.1: Chromatograms obtained from two replicates of the same carrot treatment show difference in appearance partly due to uneven supply of air.

many volatiles almost certainly reached the saturation levels of the trap during the 96 hr collection period. Therefore, after passage of a given volume of air, the adsorbed amount of these volatiles remained unchanged. If a similar headspace analysis technique were to be used in quantitative studies, the flow metering system should be improved to ensure an even air distribution to each sample and the retention capacity of the trap respected.

The material of which the containers were made, acrylic resin, has several advantages. It can be sawed, holes can be drilled and threads tapped in it with ordinary hand tools; separate pieces can be easily glued together with solvent joints. The end product were containers light in weight, more resistant to impact than if they were made of glass while being transparent, a requirement for visual inspection of the stored produce throughout the experiments. The main disadvantage of acrylic resin was the fact that this material was not totally volatile-free although it is considered chemically inert for most applications (see next section).

4.2.1.2 Traps

The fundamental components of the headspace analysis technique were the traps. Their function was to sample and pre-concentrate the volatiles emanating from the respiring material placed inside the containers. The porous polymer conveniently retained the adsorbed volatiles until thermal desorption without

any special handling care. Some loaded traps were put away for long periods (up to 7 months) without any apparent loss of analytes. The trapping medium being enclosed in a rigid stainless steel tube, its accidental disturbance by poor handling was nearly impossible. Repetitive adsorption, desorption and reconditioning in prolonged routine use had no noticeable effect on the trap performance since the major peaks on the chromatograms were reproducible.

After collection, the content of the traps consisted not only of volatile metabolites emanating from the sample under study. Other compounds released by the material which the experimental setup was made of, produced following thermal or oxidation breakdown of the porous polymer during the analysis procedure (Murray, 1977), or present as impurities in the purge gas were also trapped. These compounds from external sources were classified as the background of the system.

In any headspace analysis study, the background must be determined in order to separate the extraneous volatile from those of interest. Figure 4.2 shows typical background pertinent to this study. Chromatogram A was the background of a clean trap before volatile collection (i.e. after reconditioning). Chromatograms B and C were the backgrounds after a 96 hr collection period in the carrot and potato experiments respectively. The latter two chromatograms, obtained from the tenth container (i.e. the one left empty), differed slightly

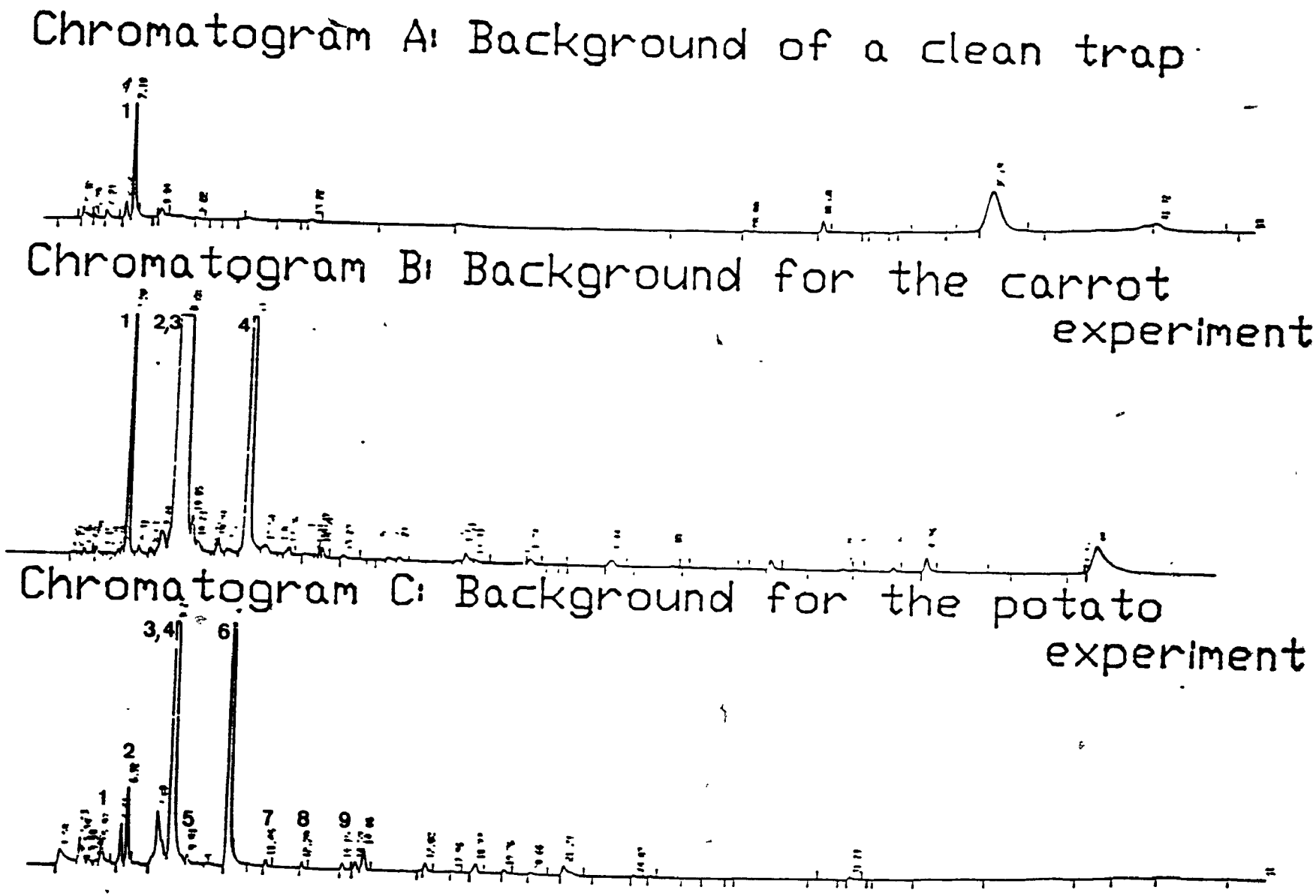


Figure 4.2: Three chromatograms representing the background of the volatiles collection system.

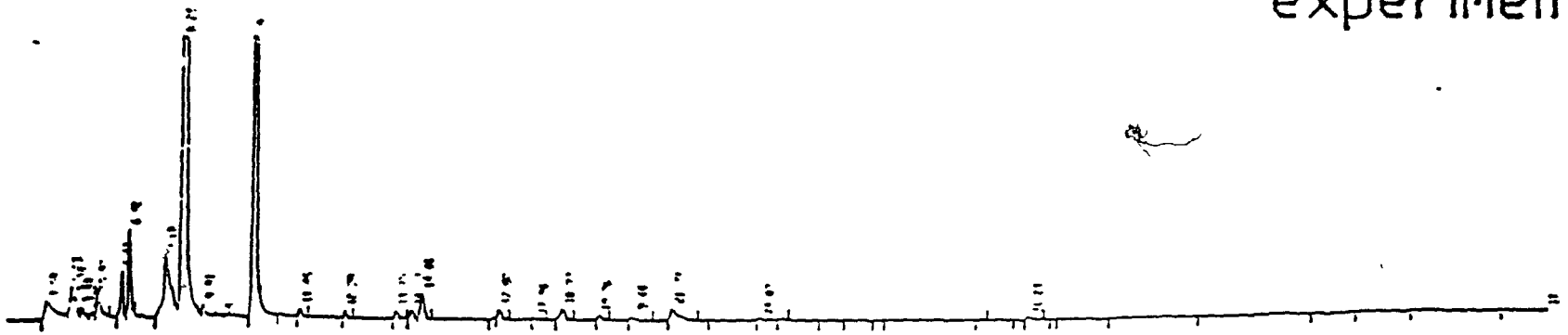
Table 4.1: Tentative identification of the major background components (as referred to in Figure 4.2).

Chromatogram	No.	Compound
A: clean trap		
	1	acetone
B: carrot background		
	1	acetone
	2	dichloro methane
	3	ethanol
	4	2-methyl-2-propenic acid, methyl ester
C: potato background		
	1	acethaldehyde
	2	acetone
	3	dichloro-methane
	4	ethanol
	5	benzene
	6	2-methyl-2-propenoic acid, methyl ester
	7	toluene
	8	hexanal
	9	ethyl benzene

even though they were characterized as part of the same experimental setup. Differences could have been partly due to the slow purging and chemical breakdown of the system since the carrot trials were conducted a few months before the potato experiment. The main background components are listed in Table 4.1.

High backgrounds are to be avoided or minimized in a study of this nature. The perception of the actual profiles of volatiles under study could be confused in a background dominated chromatogram. This situation occurred in the potato experiment where the profiles of healthy tubers (control) were eclipsed by the background (Figure 4.3). The opposite prevailed in the carrot experiment (Figure 4.4). In addition, compounds found in the background might have originated from the produce under observation. For example, acetone, reported to be produced by stored potato tubers (Waterer and Pritchard, 1985; 1984a, b; Varns and Glynn, 1979), was dominant in the background chromatogram of this study (Figure 4.2). These findings stress the importance of maintaining the background signal to the lowest possible level. The remedy, besides using a trapping medium that gives acceptably low and consistent backgrounds (Murray, 1977), would have been to build the experimental setup only with materials which are as chemically inert as possible (i.e. glass, stainless steel, etc) and circulate in it a purge gas of high purity. Varns and Glynn (1979) found it necessary to

Chromatogram A: Background for the potato experiment



Chromatogram B: Volatile profile of healthy potato tubers (control)

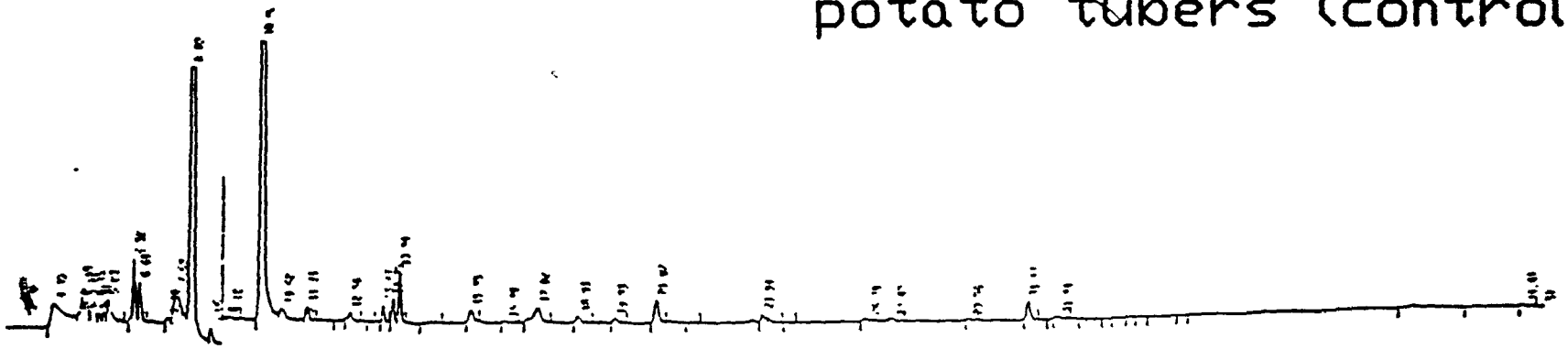
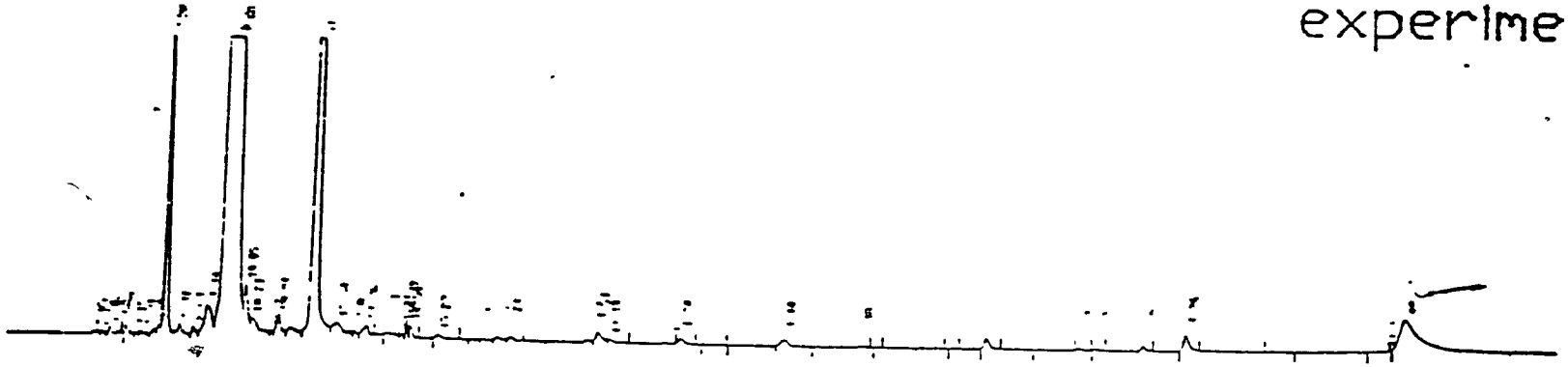


Figure 4.3: Chromatograms show the potato volatile profiles dominated by the background components.

Chromatogram A: Background for the carrot experiment



Chromatogram B: Volatile profile of healthy carrot roots (control)

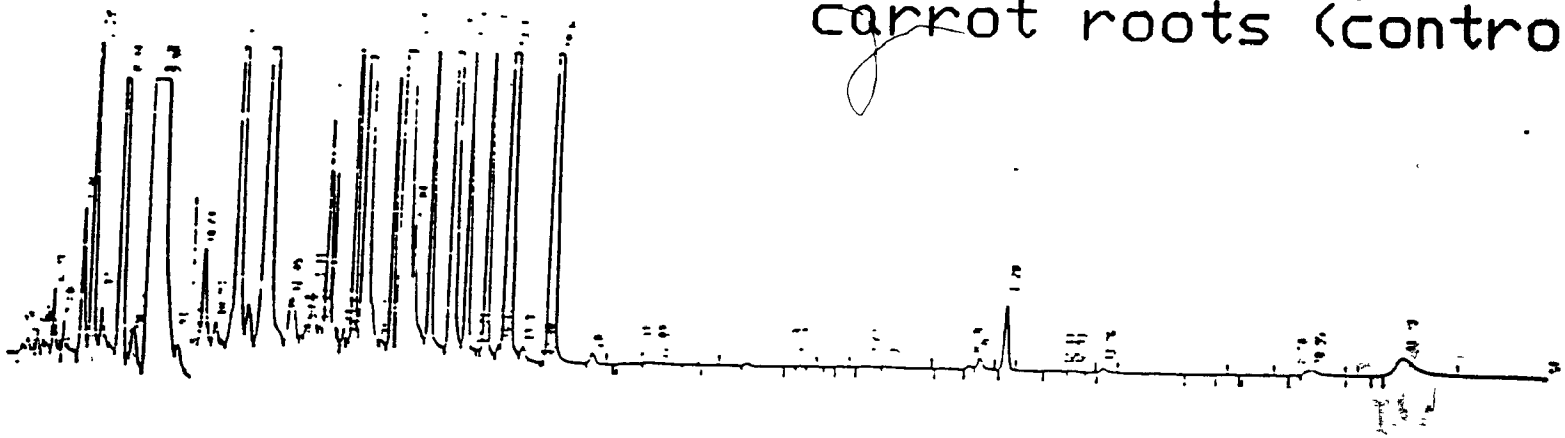


Figure 4.4: Chromatograms show the carrot volatile profiles dominated by the carrot volatiles.

clean the equipment components they used by placing them in a vacuum oven to reduce the background.

Assuming that a volatile monitoring system was installed in a commercial storage site, the background should inevitably be considered. As opposed to laboratory testing, the unwanted compounds could not be eliminated by the selection of appropriate construction materials. Rather, the choice of the volatiles monitored would be a determinant factor. As pointed out by Varns and Glynn (1979), key volatiles should not be common enough to arise from external influences (e.g. outside ventilation, potato handling equipment, etc). As future storage monitoring systems are likely to be controlled by computers (Rowe et al., 1986; Schaper et al., 1984; Hunter and Rowe, 1982), the information could be processed by suitable software programming to filter out the background. Such features in data processing are routinely used in mass spectrometry analysis (personal communication with P. Lafontaine).

4.2.2 Methods of Analysis

4.2.2.1 GC Analysis

The merits attributed to the technique of thermal desorption of the traps by their insertion in the GC unit (Numez et al., 1984; Murray, 1977) were verified in this study. Similar attachments as those described by Murray (1977) were constructed from common materials at relatively low costs. Once the GC unit

was adapted to receive them, the installation of the introducer and the pre-column required no more time than it normally takes to replace a GC column. This feature came handy when ordinary direct injections (i.e. with a syringe) were necessary. The actual injection procedures took about 11 minutes to complete (see Appendix A). In routine operation, some steps were sometimes inadvertently inverted leading to a miscarried injection. As the volatiles from one sample were collected on two traps (i.e. two traps per container), these injections were repeated with the second trap. If not used, some backup traps were stored for ulterior analysis. There was no evidence suggesting GC column deterioration as verified with the manufacturer column test mix even though well over 300 analyses were performed with this technique. Commercially available thermal desorption units are designed to perform sensibly the same injection procedures automatically but their acquisition is obviously more expensive.

Other aspects concerning the injection technique should be discussed. The seal assembly (Figure 4.5) was subjected to excessive wear and the perforated septums had to be replaced several times. The rough surface offered by the outside threads on the traps were largely responsible for the rapid deterioration of the seal. In addition, as the septums wore down, small fragments were introduced with the traps and became contaminants (see below). The teflon seat at the bottom of the

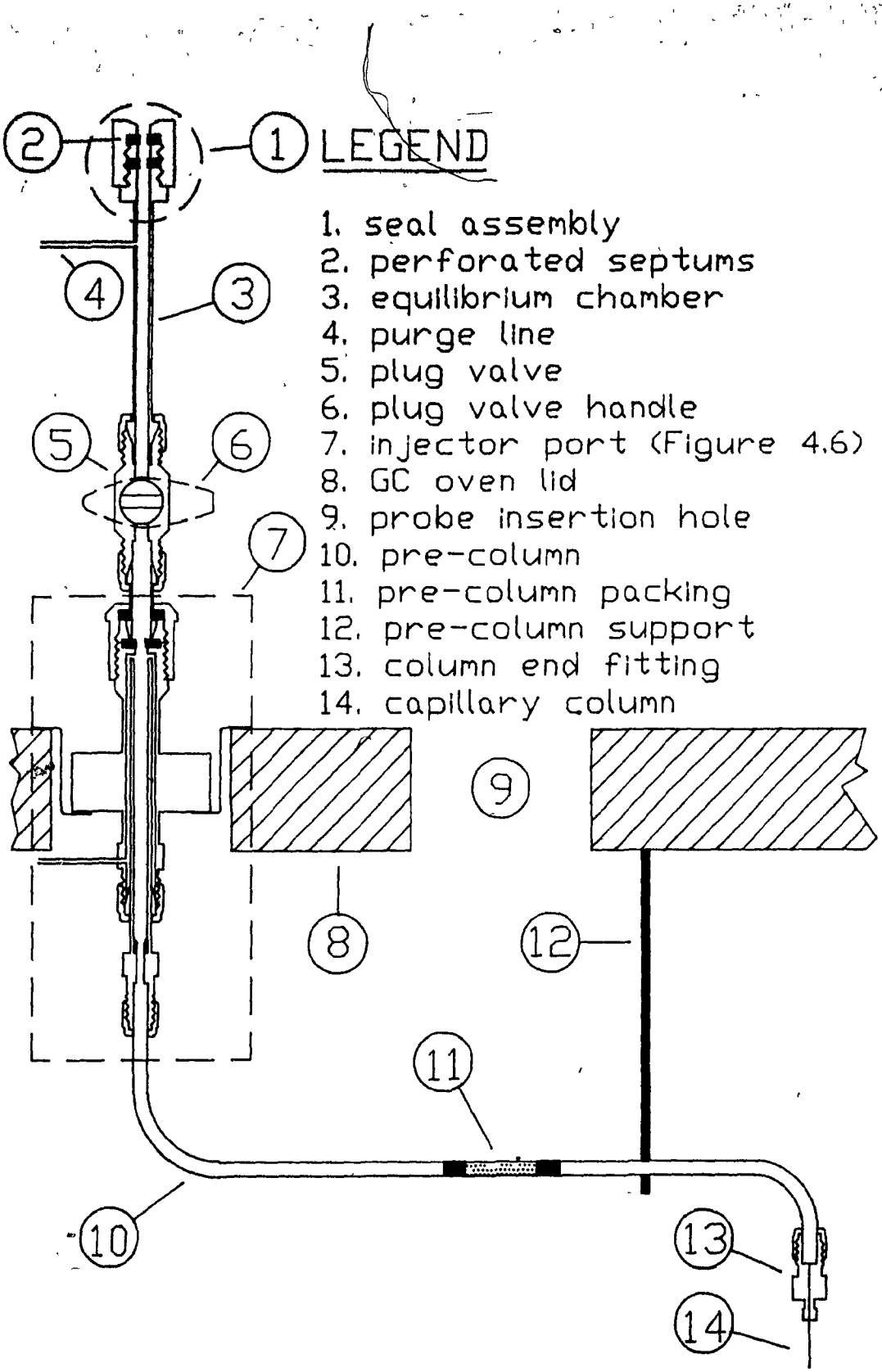


Figure 4.5: Schematic of the trap introducer.

inlet liner (Figure 4.6) also caused some concern. Although the traps were strongly pressed against it during desorption, a perfect seal was not necessarily obtained. Leakage would cause residues on the outside surface of the traps to be drawn along with the leaking portion of the carrier gas flow and to contaminate the analyte. This explained why the traps had to be wrapped in aluminum foil to avoid direct hand contact or contamination from other sources. The insertion of the traps in the carrier gas flow had another noticeable effect. Contrary to what Murray (1977) reported, when the traps were pushed down into or removed from the inlet liner, the carrier gas flow was markedly disturbed. These disturbances being unequal from trap to trap caused the retention time of the volatiles, which is a function of the carrier gas flow rate, to vary from one analysis to another. The solution to this problem was to wait until the carrier gas flow had re-established before injecting the analytes onto the column. These minor defects had little or no influence on the quality of the analysis.

The technique consumed large quantities of liquid Nitrogen. For each injection, about 0.5 liter of N_2 was required to recondense the volatiles unloaded from the traps. When supplies of N_2 must be purchased, the cost per analysis may be high. The teflon insulation on the cryogenically cooled probe split several times. The insulation should have been thicker or replaced by a more resistant material. The hot probe, on the

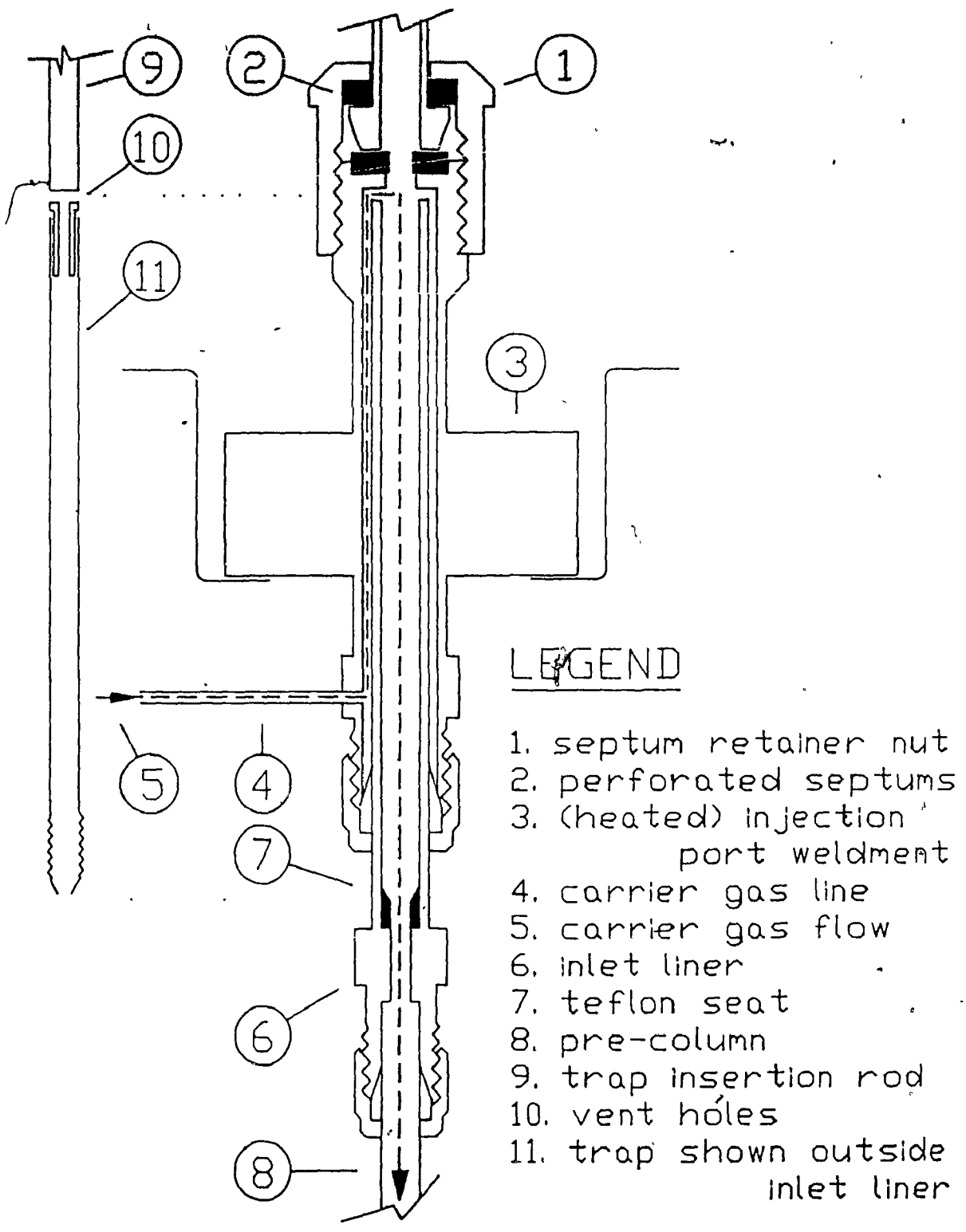


Figure 4.6: Schematic of the injector port. The teflon seat is located near the bottom of the inlet liner.

other hand, performed well throughout the study.

4.2.2.2 GC-MS analysis

For examination by GC-MS, the volatiles collected on traps of Chromosorb 105 were transferred on similar traps but packed instead with Tenax GC. As opposed to Chromosorb 105 which had no specific adsorptive properties (Murray, 1977), Tenax GC was known to exhibit selective adsorption of certain classes of compounds, especially highly volatile compounds (Numez et al., 1984). Consequently, poor recovery or complete loss of several volatiles occurred in the transfer, making their identification impossible by this method. Ideally, the analytes should have been transferred on Chromosorb 105 packing. However, the conjuncture at that time was such that Tenax GC traps had to be used.

4.3 DISEASE SPECIFIC RESPONSES

In this study, special attention has been focused on volatiles which could be considered as disease indicators in stored carrots and potatoes. The evolution of these compounds in the storage atmosphere could then provide warning of disease infections and also be useful in identifying the pathogens involved in order to initiate proper corrective measures. Compounds showing greater potential were those specifically produced by diseased roots or tubers and not by healthy ones. They were recognized by comparison of the volatile profiles

obtained from the inoculated and non-inoculated treatments.

4.3.1 Disease Indicators in Stored Carrots

Comparisons of the carrot volatile profiles revealed that 4 compounds were of significant importance. Compounds **c** and **d**, represented as peaks in the chromatograms shown in Figure 4.7 were only detected in the headspace of carrots infected with S. sclerotiorum and B. cinerea, respectively. Compound **c**, identified as dichloro benzene, appeared 16-20 days after the beginning of the experiment and remained detectable until the end. Compound **d**, whose identity was not determined, was detected right from the first analysis but faded away and completely disappeared after 24 days in the first trial and after 12 days in the second trial. These production sequences confirmed visual observations indicating that B. cinerea did not stay metabolically active in the environment provided in the experiment, while conditions were more favorable to the growth of S. sclerotiorum. The difference in the time appearance (or disappearance) of the compounds of interest between the two trials were due to an accidental rise in the cabinet temperature (up to 30 °C) that occurred during the first few days of trial 1. Compounds **a** and **b** (Figure 4.7) were found in all profiles. The former was part of the background and its identity, 1,3,5-tris(methylene)-cycliheptane, is only relevant to this study. On the other hand, the latter represented a volatile identified as methyl(1-methyethenyl)-benzene and evolved from inoculated and

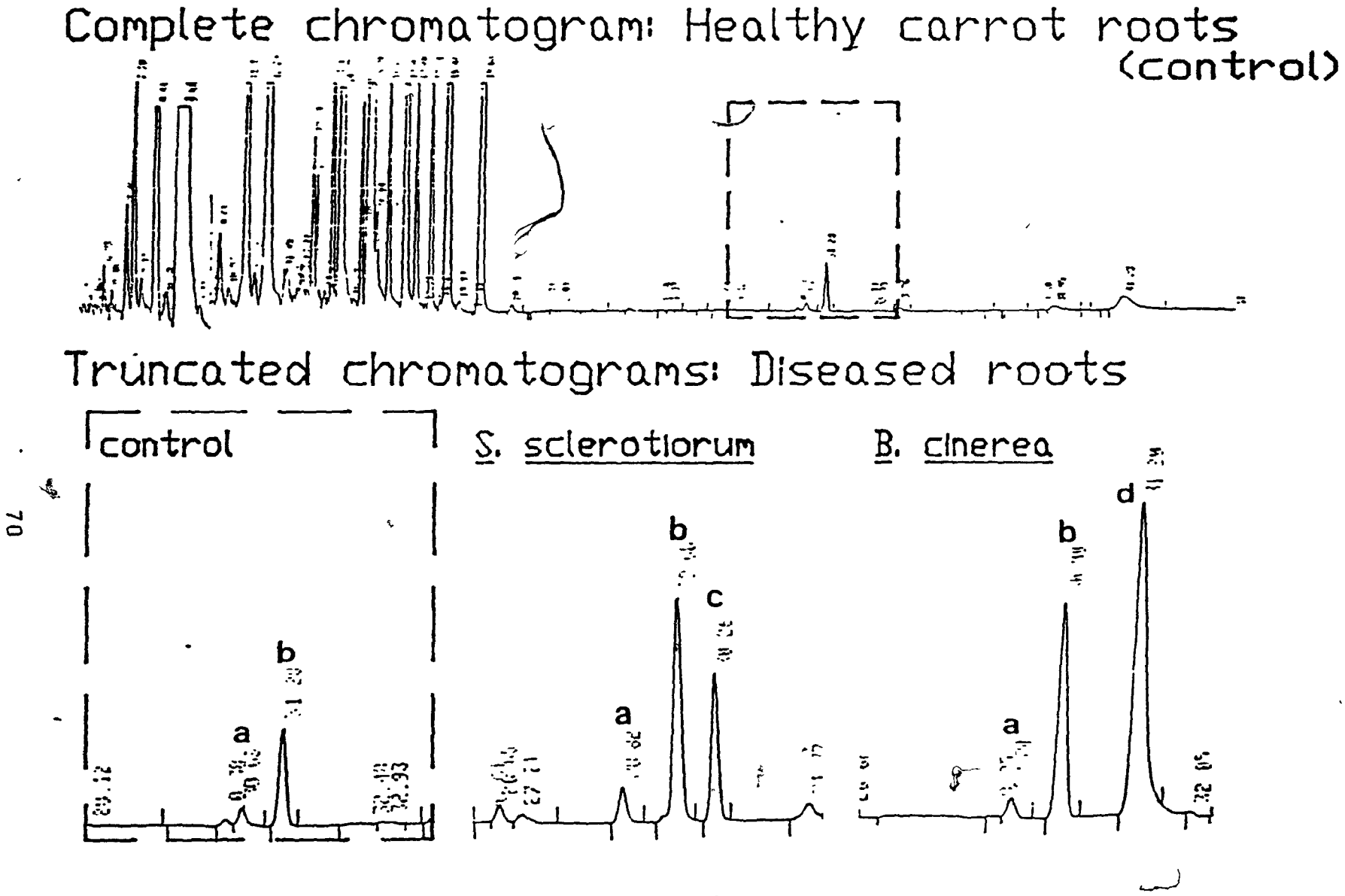


Figure 4.7: Complete and truncated chromatograms illustrating disease-specific responses from inoculated carrot roots.

non-inoculated roots. Both compounds served as reference marks on the chromatograms. The responses were consistent in all replicates throughout the experiment.

Here is an example of how a disease monitoring system might operate. Compounds a and b would keep the same function and act as reference gases. The role of compound a would be the one of an internal standard to check for leaks or other defects in the volatile collection apparatus. Compound b, a normal metabolite of stored carrots, would be used to normalize changing storage conditions related to, for example, management practices and external climatic influences on the storage atmosphere (Schaper et al., 1984). Finally, the concentration of the disease specific gases, compounds c and d, would indicate the degree of disease infection. The data collected would be processed and presented in terms of gas concentration ratios (Schaper et al., 1984; Varns and Glynn, 1979).

The damage caused by the fungi as assessed after the completion of the experiment is reported in Table 4.2. In the control treatment, some roots showed signs of decay caused by Alternaria dauci and Penicillium spp. Minor secondary invasions by Erwinia spp. were also noticed. Carrot dormancy was broken as indicated by the growth of adventitious roots and leaflets. Handling of the carrots at room temperature for several hours prior to the experiment and their storage at 3 °C (instead of the recommended 1 °C), combined with the fact that the carrots

Table 4.2: Number and percentage of healthy and diseased carrot roots per treatment after the completion of each trial.

	Number of roots:							
	-diseased:		-healthy:		-other[1]:		Total:	
Treatment:	1[2]	2	1	2	1	2	1	2
control:								
1[3]	0	0	35	41	1	0	36	41
2	0	0	36	32	3	0	39	32
3	0	0	39	29	2	1	41	30
total:	0	0	110	102	6	1	116	103
s.d.[4]:	0	0	2.0	6.2	1.0	0.6	-	-
%[5]:	0	0	95	99	5	1	100	100
S. sclerotiorum:								
1	35	32	4	3	0	0	39	35
2	34	29	3	4	0	0	37	33
3	33	33	6	3	0	0	39	36
total:	102	94	13	10	0	0	115	104
s.d.:	1.0	2.1	1.5	0.6	0	0	-	-
%:	89	90	11	10	0	0	100	100
B. cinerea:								
1	10	9	26	27	0	0	36	36
2	11	11	28	23	0	0	39	34
3	10	10	28	25	0	1	38	36
total:	31	30	82	75	0	1	113	106
s.d.:	0.6	1.0	1.2	2	0	0.6	-	-
%:	27	28	73	71	0	1	100	100

1: roots invaded by other microorganisms.
2: trial number.
3: replicate number.
4: standard deviation.
5: percentage of the total number of carrots in a given treatment.

utilized were approaching the end of their storage life, may have triggered this reaction. The invasion by unwanted microorganisms was limited and did not cause noticeable shifts in the volatile profiles. Visual inspections revealed that the interruption of the dormancy affected all nine samples equally. Data in Table 4.2 also show that variation in disease progression among the replicates within the same treatment was minimal.

4.3.2 Disease Indicators in Stored Potatoes

4.3.2.1 Volatile metabolites

In the experiment with potatoes, at least three compounds revealed themselves as potential disease indicators. Compound labelled with letter c (Figure 4.8) was detected in the headspace above tubers infected with Fusarium dry rot and was absent from any other treatment. It is interesting to note that the same compound was also present in the headspace of diseased carrots (Figure 4.9) but its presence in the carrot profiles was erratic and sometimes confused with the background. Other minor compounds produced in lesser quantities were also specific to the Fusarium dry rot treatment. However, compound c clearly predominated and has, therefore, more potential. It was not possible to determine the identity of any of these volatiles. Other research works on the metabolites elaborated by the fungus itself (Greenhalgh et al., 1986; Okazaki, 1976) might provide some indications regarding their nature.

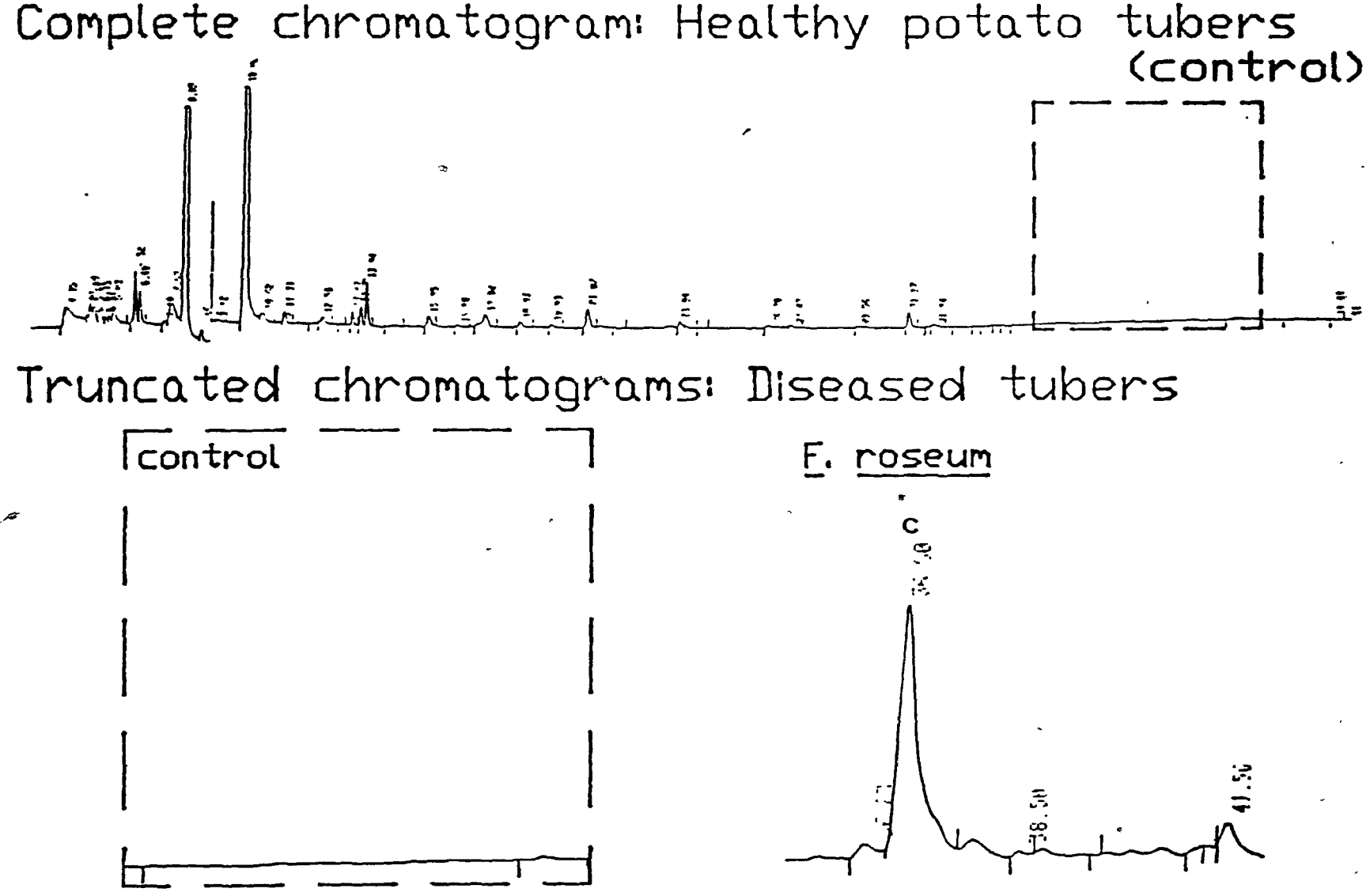


Figure 4.8: Complete and truncated chromatograms illustrating disease-specific from potato tubers inoculated with F. roseum.

Chromatogram: Carrot roots inoculated with *S. sclerotiorum*

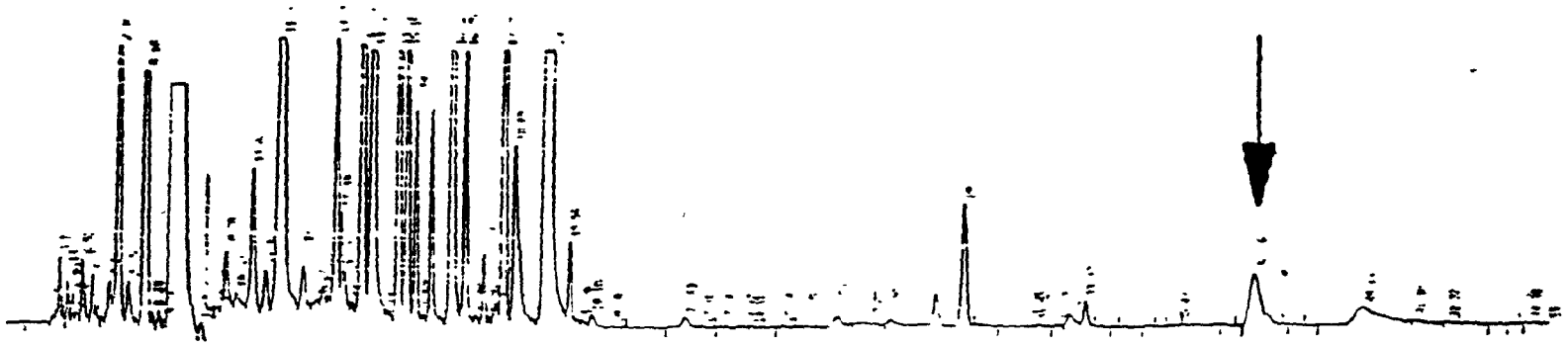


Figure 4.9: Chromatogram from carrot roots inoculated with *S. Sclerotiorum*. Arrow points to a compound also found in the headspace above potato tubers infected with *F. roseum*.

The volatile profiles of tubers inoculated with the bacteria E. carotovora showed limited specificity. Dimethyl disulfide, labelled with letter b in Figure 4.10, was a major volatile in the bacterial soft rot treatment. It was not detected in the headspace above non-inoculated tubers but was present in trace amounts in the Fusarium dry rot treatment during the first trial while becoming a major volatile component in the second trial (Figure 4.11). On the other Hand, pentane, generating peak a, was consistently dominant in the profiles of both diseases. Similar to dimethyl disulfide, pentane was not present in the headspace above the control samples. The compound responsible for the large peak exhibiting excessive tailing (pointed by the arrow in Figure 4.10) characterized the bacteria induced profiles. Its identity was not determined and its chromatographic behavior showed inconsistent retention time. As opposed to the carrot experiment, all volatiles showing specificity in the potato profiles were detected at all analyses.

E. carotovora and F. roseum may not directly induce the production of dimethyl disulfide. E. carotovora infections are usually odorless at the early stages of decay (Harrison and Nielson, 1981). A foul odor develops as rotting tissues are invaded by secondary organisms. The presence of dimethyl disulfide, a highly odoriferous compound, in the headspace above potato tubers would be indicative of advanced decomposition.

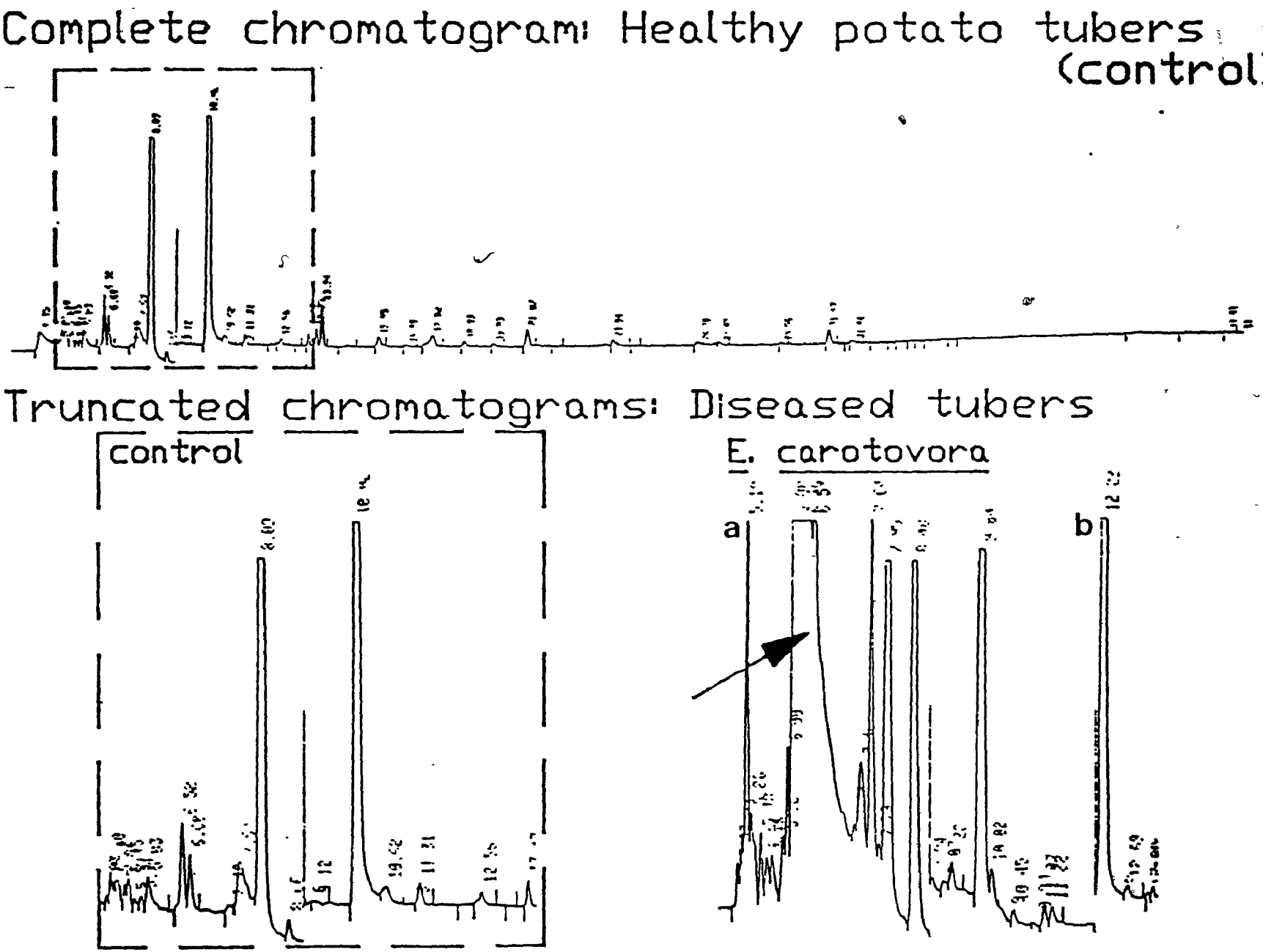
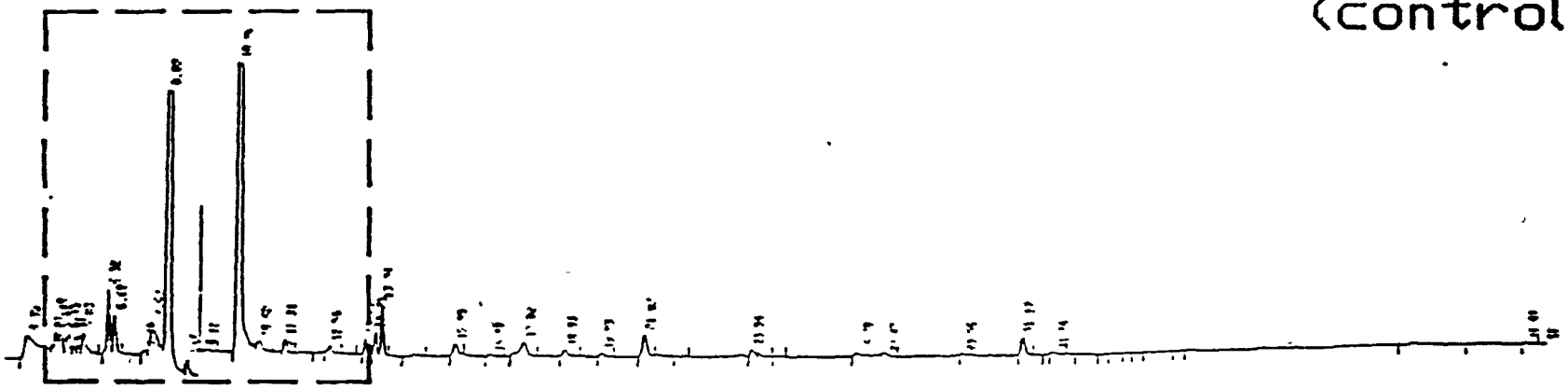


Figure 4.10: Complete and truncated chromatograms illustrating disease-specific responses from potato tubers inoculated with E. carotovora.

Complete chromatogram: Healthy potato tubers (control)



Truncated chromatograms: Diseased tubers

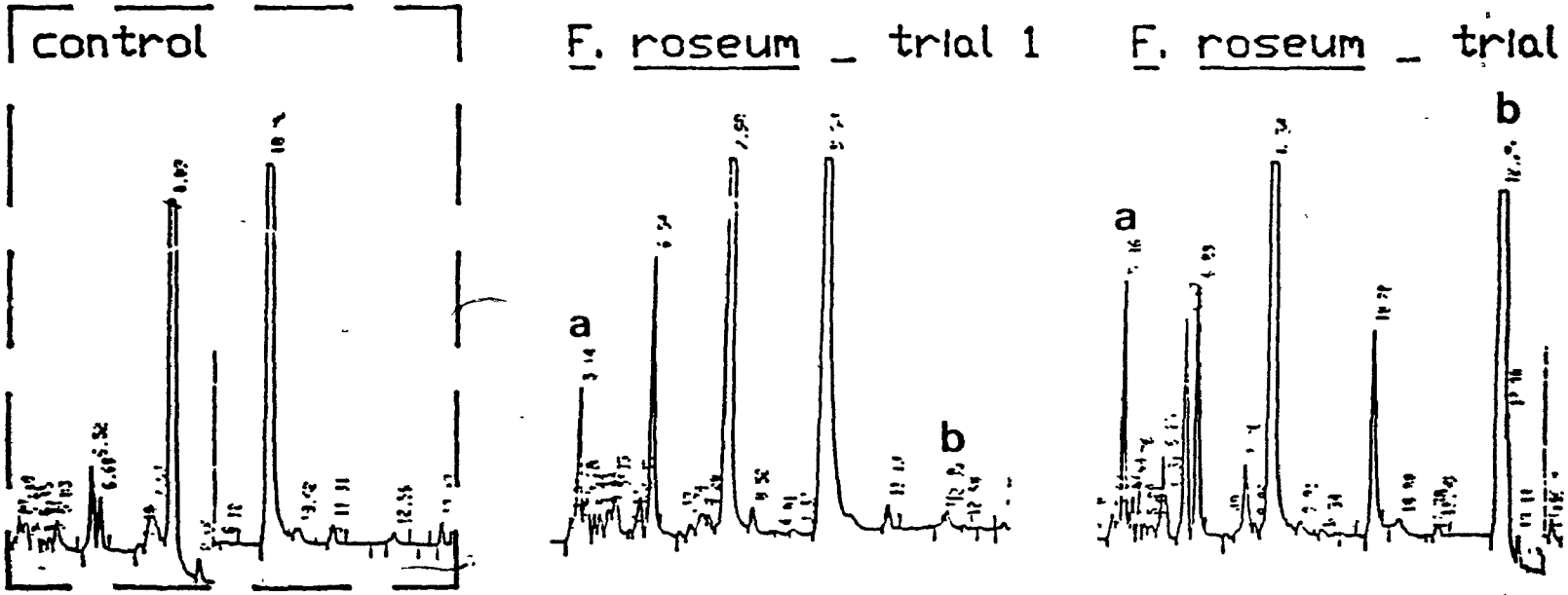


Figure 4.11: Complete and truncated chromatograms illustrating disease-specific responses from potato tubers inoculated with F. roseum in trials 1 and 2.

This hypothesis seems to be supported by the fact that the greater damage caused by F. roseum infection in the second trial was accompanied by an obvious increase in dimethyl disulfide production (see Table 4.3 for the disease damage assessment). As pointed out by Waterer and Pritchard (1984b), since secondary invasion of E. carotovora and F. roseum infected tubers normally occurs under commercial storage conditions (Harrison and Nielsen, 1981; Nielsen, 1981), detection of such volatiles may be a viable means of detecting soft rot or dry rot infections.

Table 4.3 shows compiled data on damages caused by each disease. Disease progression was faster in the second trial and variations among replicates within the same treatment were low. There were some differences in relative disease damage between trials 1 and 2 because the containers in the second trial were left at room temperature for a longer period after inoculation.

Pentane and dimethyl disulfide were not recognized by other workers as an important component in the volatile profiles of E. carotovora infected tubers. Rather, Varns and Glynn (1979) identified acetone, ethanol, and 2-butanone as possible indicators of soft rot infections. The former two were part of the background in this study and, therefore, the significance of their contributions could not be determined. 2-butanone was detected in the headspace above non-inoculated and inoculated tubers. For this reason, it could not be considered as a specific response of soft rot infection. Varns and Glynn (1979)

Table 4.3: Number and percentage of potato tubers per treatment in the given damage categories after the completion of each trial.

	Categories of Infected Area									
	0-25%		25-50%		50-75%		75-100%		Total:	
Treatment:	1[1]	2	1	2	1	2	1	2	1	2
control:										
1[2]	16	13	0	0	0	0	0	0	16	13
2	15	15	0	0	0	0	0	0	15	15
3	13	14	0	0	0	0	0	0	13	14
total:	44	42	0	0	0	0	0	0	44	42
s.d.[3]:	1.5	1.0	0	0	0	0	0	0	-	-
%[4]:	100	100	0	0	0	0	0	0	100	100
E. carotovora:										
1	0	0	5	0	7	2	3	14	15	16
2	0	0	2	1	7	1	8	15	17	18
3	0	0	2	0	5	2	9	15	16	17
total:	0	0	9	1	19	5	20	44	48	50
s.d.:	0	0	1.7	0.6	1.2	0	3.2	0.6	-	-
%:	0	0	19	2	39	10	42	88	100	100
E. roseum										
1	4	2	8	6	3	5	0	2	15	15
2	4	0	4	4	3	5	1	4	12	13
3	4	0	8	4	2	5	0	4	14	13
total:	12	2	20	14	8	15	1	10	41	41
s.d.:	0	1.2	2.3	2.0	1.0	0	0.6	1.2	-	-
%:	29	5	49	34	20	37	2	24	100	100

1: trial.
2: replicate.
3: standard deviation.
4: percentage of the total number of tubers in a given treatment.

did, however, detect a series of sulfureous compounds, including dimethyl disulfide. Waterer and Pritchard's results, on the other hand, showed no presence of dimethyl disulfide. Instead, Waterer and Pritchard (1984a, b) found 2-propanol and two minor unidentified compounds to be unique to the samples from the tubers inoculated with E. carotovora. 2-propanol was not detected in the current study. These discrepancies may have originated from differences between experimental conditions (Waterer and Pritchard, 1984a, b). Each research team adopted its own headspace analysis technique and even used different varieties of potatoes (Chieftan, Russet Burbank, and Atlantic). The choice of headspace analysis technique has been found to have a determinant effect on the results (Ismail et al., 1980; Wyllie et al., 1978) while varietal influences on the volatile production patterns have been observed in carrots (Simon et al., 1982; Rasekh and Kramer, 1971). The disease-specific responses from potatoes and carrots are summarized in Table 4.4.

4.3.2.2 CARBON DIOXIDE MONITORING

The air composition in three containers (one/treatment) were monitored to verify the degree of CO_2 accumulation. The air flow rate to each container was calculated on the basis of a balance of CO_2 at steady state considering that non-inoculated tubers produced 6.1 mg of CO_2/kg/hr. The aim was to keep the CO_2 level below 0.3%. The actual concentrations measured after 4, 8, and 16 days are listed in Table 4.5.

Table 4.4: Disease-specific metabolic volatiles for carrots and potatoes.

Diseases:		Metabolic volatile[1]:
- Carrots:		
S. sclerotiorum:		
	c[2]	dichloro benzene
B. cinerea:		
	d	unidentified
- Potatoes:		
E. carotovora:		
	a[3]	pentane
	b	dimethyl disulfide
F. roseum:		
	a	pentane
	b	dimethyl disulfide
	c	unidentified

1: Identified by GC-MS.
2: As referred to in Figure 4.7.
3: As referred to in Figure 4.8, 4.10, and 4.11.

Table 4.5: Levels of carbon dioxyde in one container per treatment after 4, 8, and 16 days after the beginning of trial.

Treatment:	Percent of CO_2			
	4[1]:	8:	16:	Average:
-Trial 1:				
control:	0.25	0.27	0.22	0.25
E. carotovora:	1.83	1.77	1.43	1.68
F. roseum:	1.30	1.55	1.29	1.38
-Trial 2:				
control:	0.29	0.29	0.38	0.30
E. carotovora:	2.22	2.64	2.55	2.47
F. roseum:	1.77	1.85	2.41	2.01

1; days after the beginning of the experiment.

Some comments regarding differences in CO_2 levels among treatments should be made. As expected, the atmosphere surrounding diseased tubers was richer in CO_2. This is because storage stresses increase the respiration rate of the tubers (Rastovski et al., 1981; Schaper and Varns, 1978; Smith, 1977) and aerobic microorganisms consume oxygen. CO_2 levels seem to correlate well with the relative degree of decay caused by soft rot and Fusarium dry rot infections since both damage and CO_2 concentrations were higher in the second trial (Table 4.6). These data suggest that CO_2 would be a reliable disease indicator. However, CO_2 production has been linked to other potato variables such as maturity and bruising (Rastovski et al., 1981; Ryall and Lipton, 1979; Schaper and Varns, 1978; Smith, 1977). Volatile monitoring for disease detection would be greatly facilitated if it relied on the detection of compounds known to be present in the storage atmosphere only when the corresponding diseases are spreading. As a matter of fact, it is interesting to note that Schaper et al. (1984) have made use of CO_2 as a primary reference gas to normalize storage conditions and not as a disease indicator in their attempt to develop storage sampling procedures for disease detection by volatile monitoring.

Table 4.6: Average levels of carbon dioxyde and percentage of potato tubers in the four damage categories determined in one container per treatment.

	Categories of Infected Area				
	0-25%	25-50%	50-75%	75-100%	CO_2:
Treatment:	%[1]	%	%	%	%[2]
-Trial 1:					
control:					
	100	0	0	0	0.25
E. carotovora:					
	0	33	47	20	1.68
F. roseum:					
	27	53	20	0	1.38
-Trial 2:					
control:					
	100	0	0	0	0.30
E. carotovora:					
	0	0	13	87	2.47
F. roseum:					
	13	40	33	14	2.01

1: percentage of the total number of tubers in replicate 1.
2: average volue for the 16-day long trial.

4.4 PROPOSED MONITORING SCHEME

4.4.1 The Use of Disease Indicators

The ability to determine the exact crop disease status during storage by volatile monitoring would be a definite asset to the storage manager. Decisions concerning short term versus long term storage would be based on an objective prognosis done after harvest. Advance notice would allow the bins presenting the greatest risks to be unloaded first balancing the expected losses with the market opportunities. Data accumulated over the years would aid in correctly assessing the storage potential of the crop in the current year. These examples illustrate that better information about crop conditions would inevitably lead to improved storage management.

Careful selection of the volatiles monitored is primordial if volatile monitoring is to be successful. Analysis of the headspace above stored carrots and potatoes revealed that some metabolic volatiles were unique to disease infections of each vegetables. These gases, because they were disease-specific, satisfied one of the criteria of an ideal disease indicator (see section 2.5.1). The other criteria relate to the actual monitoring operations in commercial storage facilities. Consequently, it has yet to be determined whether the disease-specific gases identified in this study would be suitable for on-farm surveys. Their detection in controlled conditions does suggest that detecting diseases by volatile monitoring is

feasible and that these volatiles should be regarded as potential disease indicators.

Once reliable indicators of disease development have been selected, their production patterns as the infections progress should be studied. Waterer and Pritchard (1985; 1984a, b) observed that the increase of the total concentration of volatiles from diseased potatoes followed the exponential growth of the pathogen population. However, quantitative analyses in this case were based on the headspace above 1 kg of tubers that might not be representative of the atmosphere prevailing in commercial storages. A mass of several tonnes of potatoes provide the opportunity for many infection loci caused by different diseases to develop asynchronously. The model describing the overall volatile production is likely to be more complex. Despite increased difficulties, further research work should be carried out in commercial-size research bins to generate data usable by the industry (personal communication with Dr. M. K. Pritchard, University of Manitoba in Winnipeg, Manitoba).

4.4.2 Sampling of Storage Volatiles

Sampling procedures for monitoring headspace volatiles must not be overlooked. Warehouses, as most confinement structures, are not completely tight. Outside air penetrates in and storage air leaks out. Air infiltrations have been shown to be strongly influenced by the wind (Varns et al., 1986; Schaper et al.,

1984). Measurements of the quantity of key volatiles should be corrected to take into account the dilution effect of the storage atmosphere. The use of concentration ratios (i.e. disease-specific volatiles/normal metabolic volatiles) appears as the most practical approach to alleviate this problem (Schaper et al., 1984; Varns and Glynn, 1979).

The number and the location of sampling points in the warehouse are determinant design parameters. The implementation of sophisticated gas sampling and analysis systems as the one proposed by Schaper and co-workers (1984) is unlikely in the near future. Their conception requires expensive and independent hardware for each warehouse under supervision and their acquisition would be justifiable for large operation only. Rather, a system making use of adsorbent traps, for example, analyzed in local government laboratories is probably more adequate for the present needs in Québec. A similar service, offered at no charge, is routinely used by farmers interested to measure to nutrient value of their soils. Because of limited resources, a given maximum number of samples per warehouse would be assigned (one sample per so many tonnes of produce stored). The turn-around time of the analysis results would be, of course, critical since timely decisions would depend on them. In this perspective, the sampling points need to be carefully considered for adequate coverage of the warehouse. In bulk storage, ventilation systems recirculate the air that was forced

through the pile. A thorough study of the air flow streams in the headspace above the pile should provide some good indication about the best strategic locations. The aim should be concentrated on obtaining the most useful information at minimal costs.

All these considerations emphasize the need for on-going research work in this area. Besides sampling equipment and procedures that have yet to be adapted for on-farm use, storage stresses other than diseases are now attracting researchers' attention. For example, the multi-million potato processing industry is always confronted with the problem of the accumulation of reducing sugars. Volatile monitoring is contemplated as a technique to predict the optimum time for a given stock to be processed (personal communication with L. A. Schaper, Agricultural Engineer, USDA). In addition, the storage of other perishable commodities might benefit from such a technique.

4.5 VOLATILE IDENTIFICATION

GC-MS analyses were carried out to identify the volatiles collected. It is important to stress that the identification was based on a match of the observed mass spectra with those available in the Finnigan Library (National Bureau of Standards). In cases where the spectra of more than one compound provided a reasonably good fit, a situation occurring especially

with compounds of complex molecular structures, the one generating the highest index of purity was selected. In addition, since the GC and GC-MS analysis were performed under different chromatographic conditions, the position of the identified volatiles on the chromatograms in Figure 4.12 and 4.13 are not absolute. However, although tentative, the identities and the relative positions reported here give useful information on the classes of volatiles to be expected in the headspace of stored carrots and potatoes.

4.5.1 Carrot Volatiles

The normal metabolic volatiles detected in the headspace of stored carrots are listed in Table 4.7. Of the 20 volatiles identified, none have been reported in aroma studies (Simon et al., 1982, 1980a, b; Salunkhe and Do, 1976; Buttery et al., 1968). However, in these studies, the volatile constituents were obtained by destructive methods (e.g. steam distillation-extraction; Buttery et al., 1968) of freshly harvested carrots and not from intact roots stored in normal atmosphere for several months.

Here, the term "normal metabolic volatiles" is perhaps misused. As reported before, the dormancy of the carrot roots was broken during the course of the experiment. Sprouting modify the metabolism of stored produce (Salunkhe and Desai, 1984b; Burton, 1982) and consequently probably the metabolic volatile

Chromatogram: Volatile profile of healthy carrot roots (control)

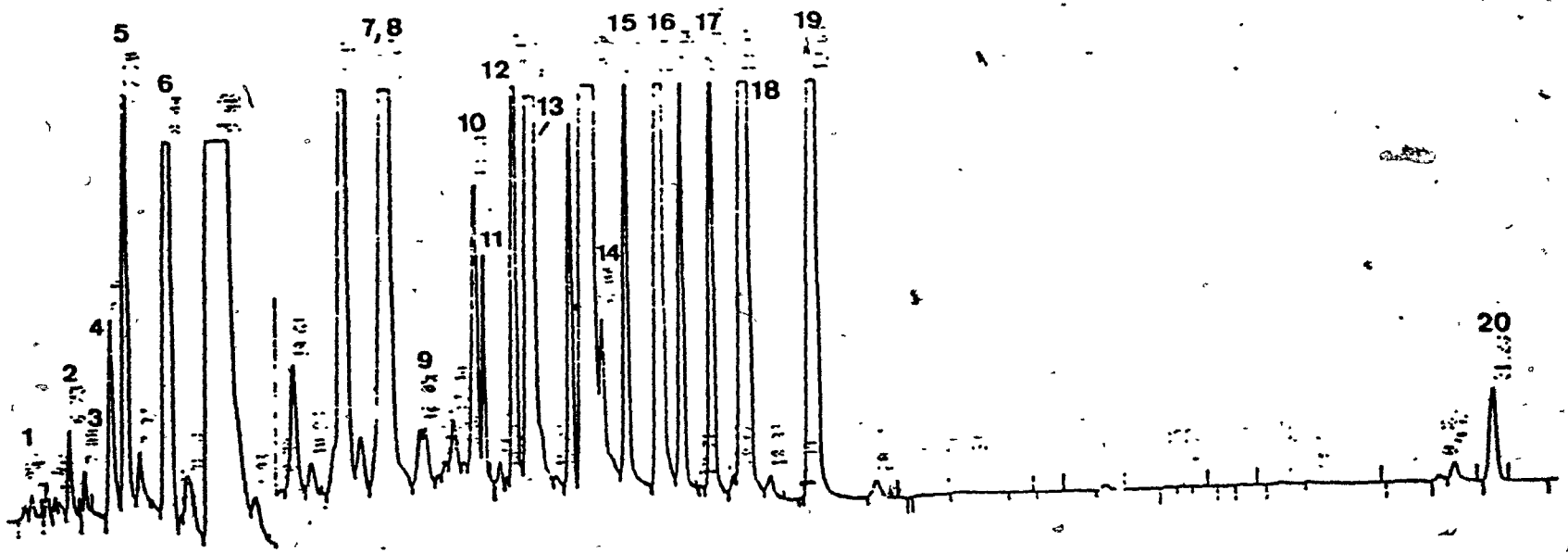


Figure 4.12: Tentative position on the chromatogram of several normal metabolic volatiles of carrot roots (see Table 4.7).

Table 4.7: Tentative identification of several normal metabolic volatiles of carrot roots (as referred to in Figure 4.12).

No.	Compound:
1	pentane
2	heptane
3	1-heptane
4	furan
5	acetone
6	tetrahydro-furan
7	2,6,6-trimethyl-bicyclo[3.1.1] hept-2-ene
8	trichloro-methane
9	1-propanol
10	6,6-dimethyl-2-methylene-bicyclo[3.1.1]heptane
11	4-methylene-1-(methylethyl)-cyclohexene
12	1-butanol
13	3,7,7-trimethyl-bicyclo[4.1.0]heht-3-ene
14	pyridine
15	4-ethyl-1, 4-dimethyl-cyclohexene
16	4-methyl-1-(1-methylethyl)-bicyclo[3.1.0]hex-2-ene
17	3,7,7-trimethyl-bicyclo[4.1.0] hept-3-ene
18	1-methyl-3-(1-methylethyl)-benzene
19	1-methyl-4-(1-methylelidene)-cyclohexene
20	methyl(1-methylethenyl)-benzene

profiles. Thus, in this case, the metabolic volatiles qualified as "normal" refer to those metabolites that were not related to disease development.

4.5.2 Potato Volatiles

The volatile production pattern of stored potatoes differed substantially from the one of stored carrots. The abundance of volatiles emanating from potato tubers in terms of number and apparent concentration was modest when compared to the volatile profiles from carrot roots. This low production of volatile metabolites may have stemmed partly from differences in the rate of respiration, a measure of the produce metabolism (Salunkhe and Desai, 1984b; Burton, 1982). Carrot roots are characterized by a respiration rate several times greater than the one of potato tubers (Forcier et al., 1987; Ryall and Lipton, 1979; Nash, 1978). Nevertheless, the fact that the background overshadowed the volatile profiles from potato tubers evidenced the inadequacy of the headspace analysis technique to collect normal metabolites of stored potatoes. Meigh and co-workers (1973) reported similar difficulties in collecting sufficient yield of metabolic material evolving from intact potato tubers and destructive methods had to be used. The technique described in the current study did register clear specific responses from diseased samples and was therefore suitable for that particular application.

A typical profile of volatiles from non-inoculated potatoes

is shown in Figure 4.13. 2-butanone was the only volatile not found in the background. However, acethaldehyde, ehtanol, and acetone, three background compounds, were detected by Varns and Glynn (1979) and Waterer and Pritchard (1985; 1984a, b) in the headspace of stored potatoes. Benzene, and toluene were also identified as important potato volatile metabolites (Meigh et al., 1973). Because these compounds appeared in the background, it was uncertain whether they also emanated from potato tubers. In any case, the metabolites detected here almost certainly represented only a portion of the entire metabolic profile from carrots and potatoes (Waterer and Pritchard, 1985).

Chromatogram: Volatile profile of healthy potato tubers (control)

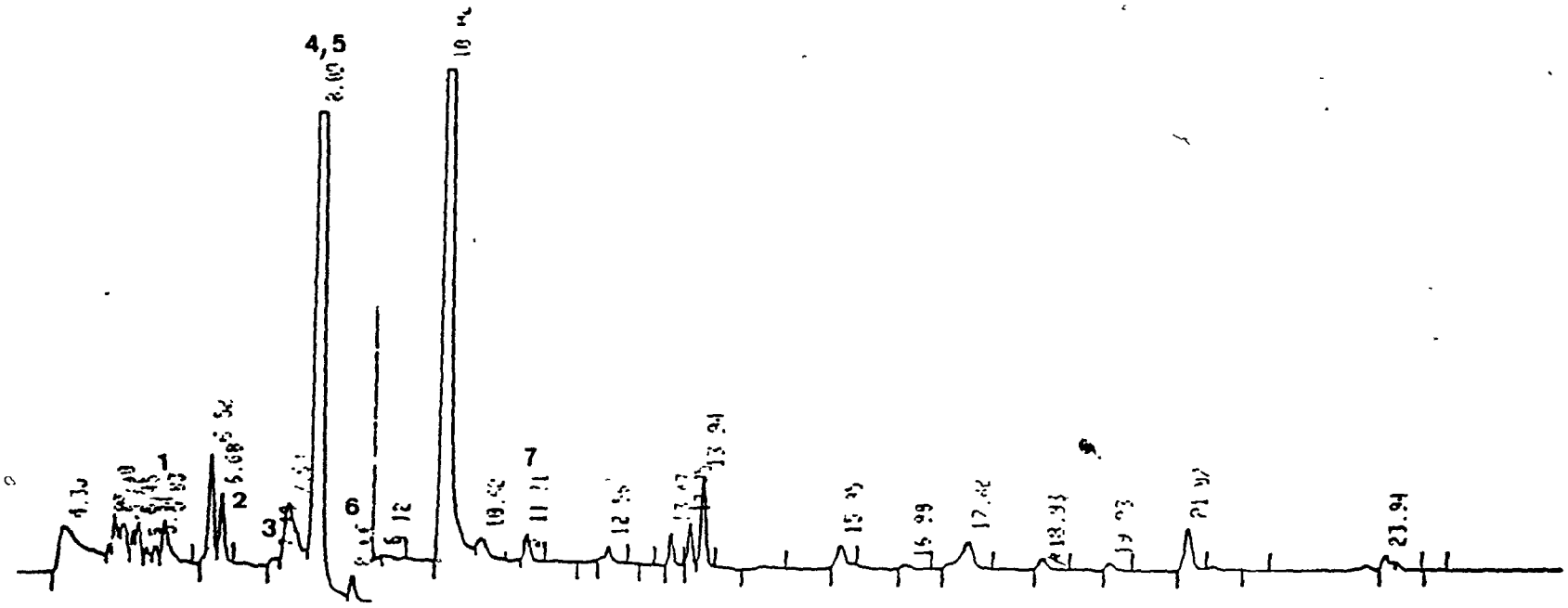


Figure 4.13: Tentative position on the chromatogram of several compounds detected in the headspace above non-inoculated potato tubers (see Table 4.8).

Table 4.8: Tentative identification of several compounds detected in the headspace above non-inoculated potato tubers (as referred to in Figure 4.13).

No.	Compound:
- <u>Some background components</u>:	
1	acethaldehyde
2	acetone
4	dichloro methane
5	ethanol
6	benezene
7	toluene
- <u>Potato volatile</u>:	
3	2-butanone[1]

1: present in trace amounts; detectable by GC-MS.

V- SUMMARY AND CONCLUSIONS

5.1 SUMMARY

The range of metabolic volatiles produced by healthy lots of carrot roots (cultivar Charger) and potato tubers (cultivar Atlantic) were compared to those from samples infected with common storage diseases. The primary objective was to investigate the possible use of headspace volatile profiles in a storage disease detection system.

The experimental setup was designed to simulate ventilated storage bins and to generate comparative volatile profiles. Inoculated and non-inoculated samples (2 infections, 1 control; 3 replicates/treatment) were placed inside individual containers continuously purged with purified air. Traps of polymeric adsorbent were utilized to collect and pre-concentrate the volatiles that were subsequently thermally desorbed for gas chromatographic examination.

The chromatograms obtained were graphical reproductions of the headspace volatile profiles collected. Their study revealed that each host-pathogen combinations gave off at least one metabolite that was not detected in the headspace above the control lots. These metabolic volatiles could eventually act as disease indicators in commercial vegetable warehouses.

Attempts to identify the disease-induced and normal metabolites were made by gas chromatography-mass spectrometry. The analyses, which necessitated the transfer of the analytes

from one trapping medium to another, allowed the provisional identification of 20 normal metabolic volatiles of stored carrots. Conversely, apparently fewer metabolites evolved from stored potatoes since extraneous compounds dominated the observed profiles. Nevertheless, the range of metabolites emanating from potatoes and carrots undoubtedly includes many more compounds. Other techniques of headspace analysis might extend the list of detectable volatiles considerably.

The monitoring of headspace volatiles as a method to detect storage diseases seems feasible. Much of the research in this area is left to be done before it can be used by Québec's vegetable storers. However, even at this early stage of development, one can already appreciate the potential of such a tool as good knowledge of crop conditions during storage is becoming the key factor for improved storage management and postharvest loss reduction.

5.2 CONCLUSIONS

Based on the results observed during the course of this study, the following inferences are applicable to the headspace analysis of stored carrots and potatoes:

1- The technique of headspace analysis developed allowed the comparative collection of headspace volatiles emanating from 9 ventilated samples simultaneously.

2- One metabolic volatile was unique to each carrot fungal

infection. A compound, identified as dichloro benzene, was only detected above roots inoculated with S. sclerotiorum. The identity of the compound responsible for the specific response of roots inoculated with B. cinerea was not determined.

3- The volatile profiles induced by the two potato pathogens, E. carotovora and F. roseum, featured two commonly shared metabolites: pentane and dimethyl disulfide. These compounds were absent from the profiles of non-inoculated tubers. At least one additional compound (unidentified) was unique to the Fusarium dry rot infection.

4- Although about 30 normal carrot volatiles were collected and 20 tentatively identified, the chosen technique of volatile collection was inadequate for the analysis of the headspace above non-inoculated potatoes.

VI- RECOMMENDATIONS FOR FURTHER RESEARCH

The following research will assist towards developing the great potential of headspace volatile monitoring as a technique to detect storage stresses and make it applicable by the industry:

1- The occurrence of extraneous compounds in the storage atmosphere monitored is likely to be inevitable in commercial warehouses. Sampling methods and data processing capabilities should be developed to alleviate their effect on the monitoring system performance.

2- Quantitative analysis should be performed in real-scale environments to determine whether volatile monitoring can provide early disease detection. Such factual information would readily become usable by the industry and sparks its interest.

3- Disease is a classical postharvest stress to which most fruit and vegetable crops are subjected. Other undesirable physiological changes might cause appreciable shifts in the produce normal metabolism. Detecting these shifts by volatile monitoring would widen its applicability and contribute for justification of the cost of the expected extensive installations required.

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APPENDICES

Appendix A: Step-by-step GC injection procedures[1].

Step	Time elapsed (min.)	Description
1	-	Trap brought to ambient temperature.
2	0	**a)** Trap secrewed onto the plunger and pushed through the seal assembly to a point where the vent holes in the plunger are still visible.
		b) Cooling probe inserted onto the pre-column.
note: From this point, liquid N_2 is periodically poured in to the cooling probe to keep it cold.		
3	1	Purge line opened.
4	2	**a)** Trap pushed into the equilibrium chamber to enclose the vent holes.
		b) Purge line closed.
		c) Plug valve opened.
		d) Trap lowered against the teflon seat inside the inlet liner.
		e) Mass (750 g) placed over the plunger handle.
5	8	**a)** Mass removed.
		b) Trap withdrawn from the inlet liner.
		c) Plug valve closed.
6	9	**a)** Cooling probe quickly replaced by heating probe.
		b) GC programmer sequence actuated.
7	10.5	Heating probe removed.

1: Please, refer to Figure A-1.

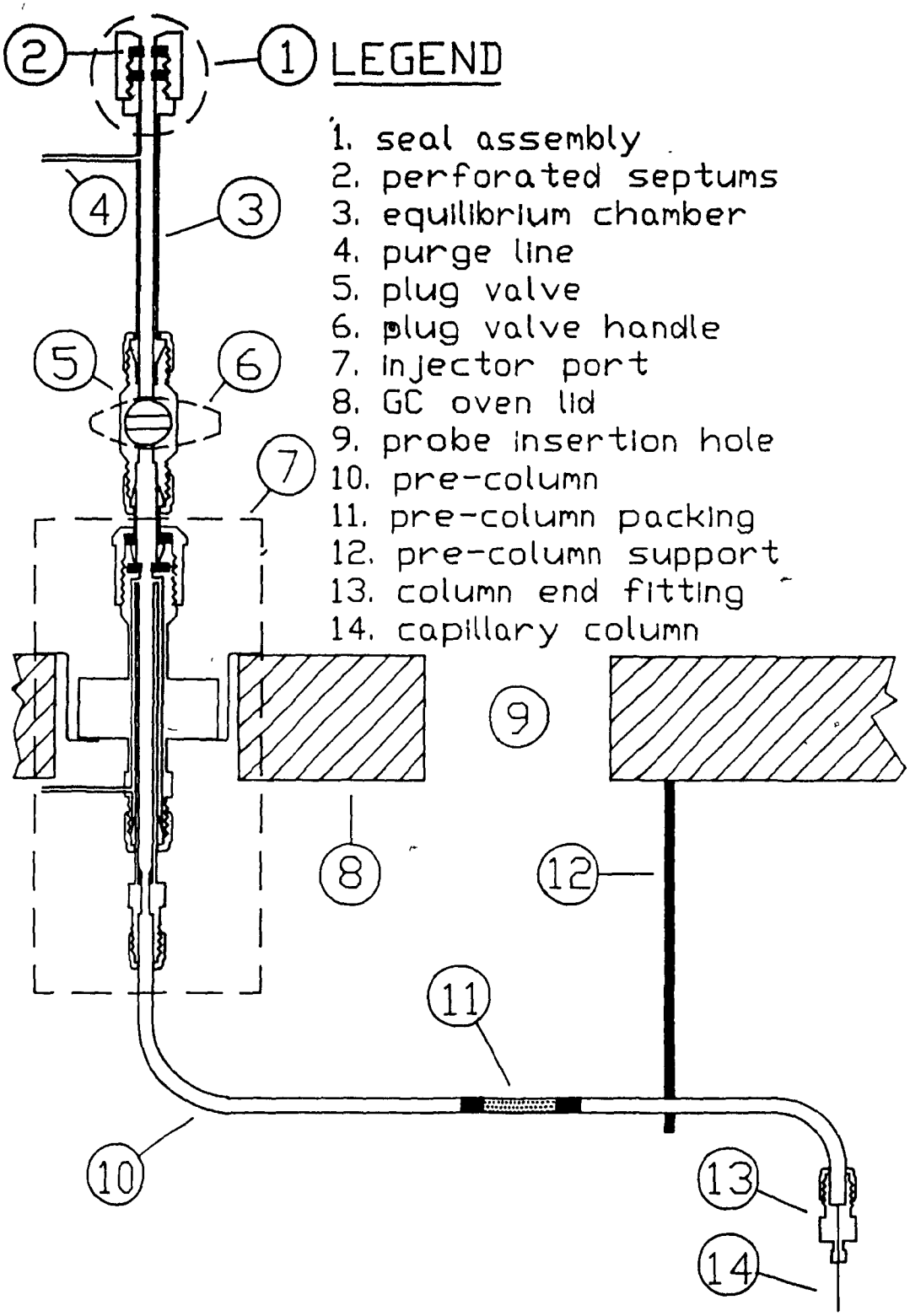

Figure A-1: Schematic of the trap introducer, the injector port and the pre-column.

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Appendix B: Chromatograms for the carrot experiment.

All chromatograms in Appendix B are sorted according to the following numbering system:

v.n1.n2.t.n3.

where v - vegetable:

C for carrot

n1 - trial:

1 or 2.

n2 - analysis:

1,2.3,...or 8.

t - treatment:

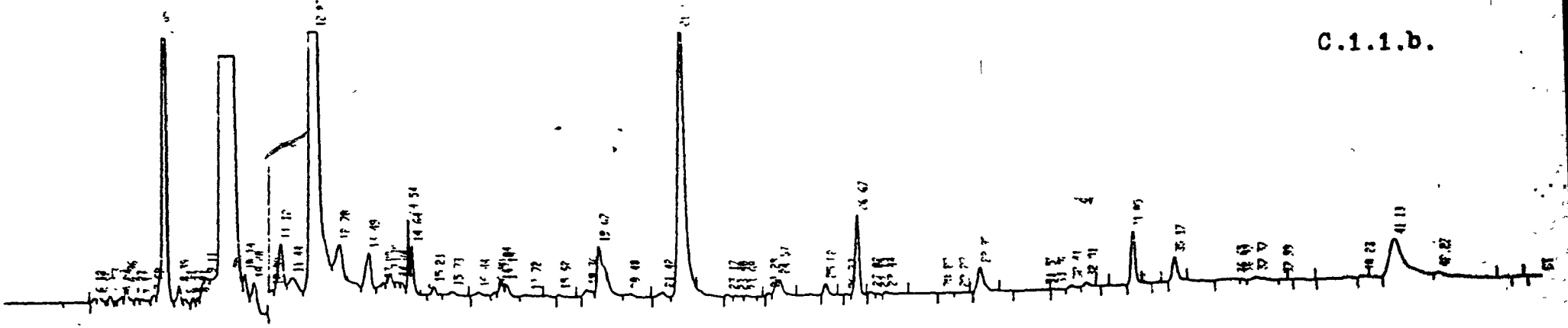
C for control, or

S for Sclerotinia sclerotiorum, or

B for Botrytis cinerea.

n3 - replicate:

1,2, or 3.



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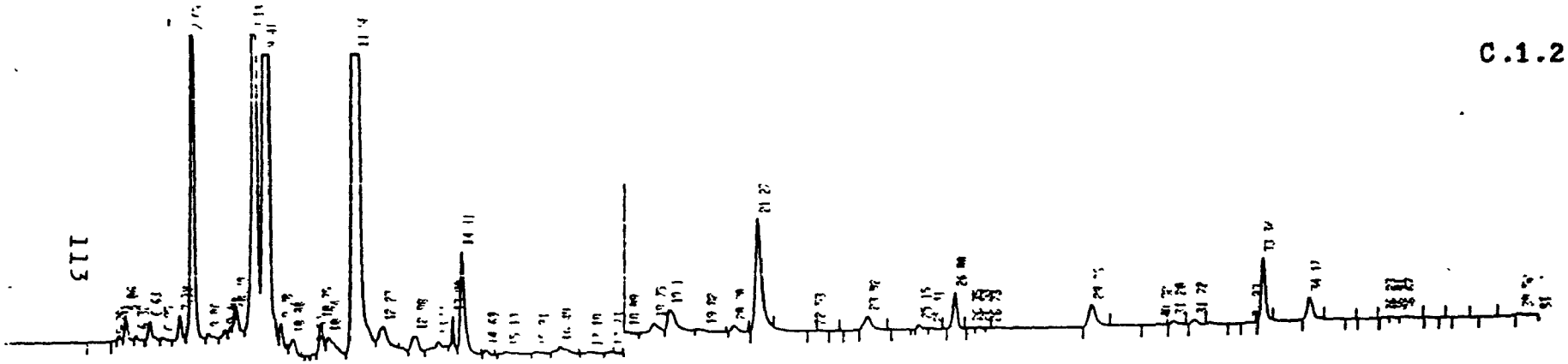

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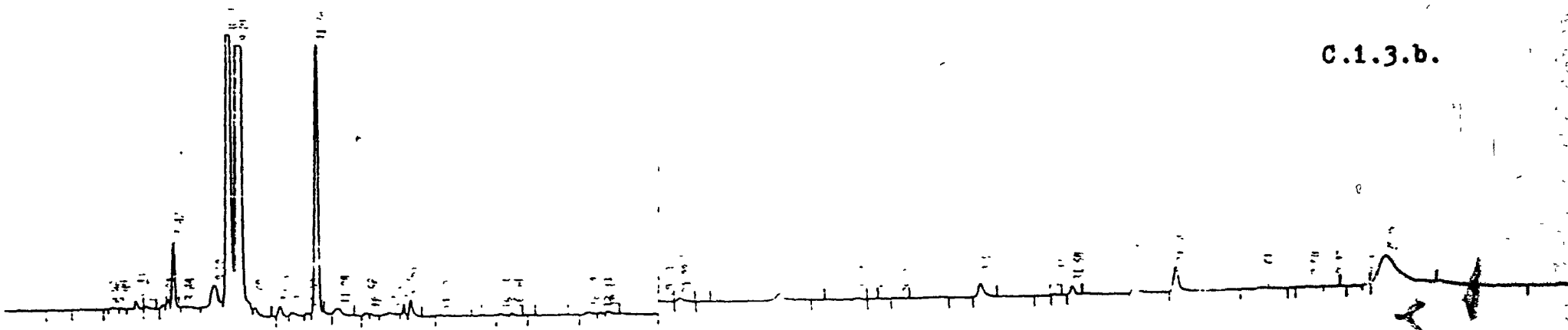

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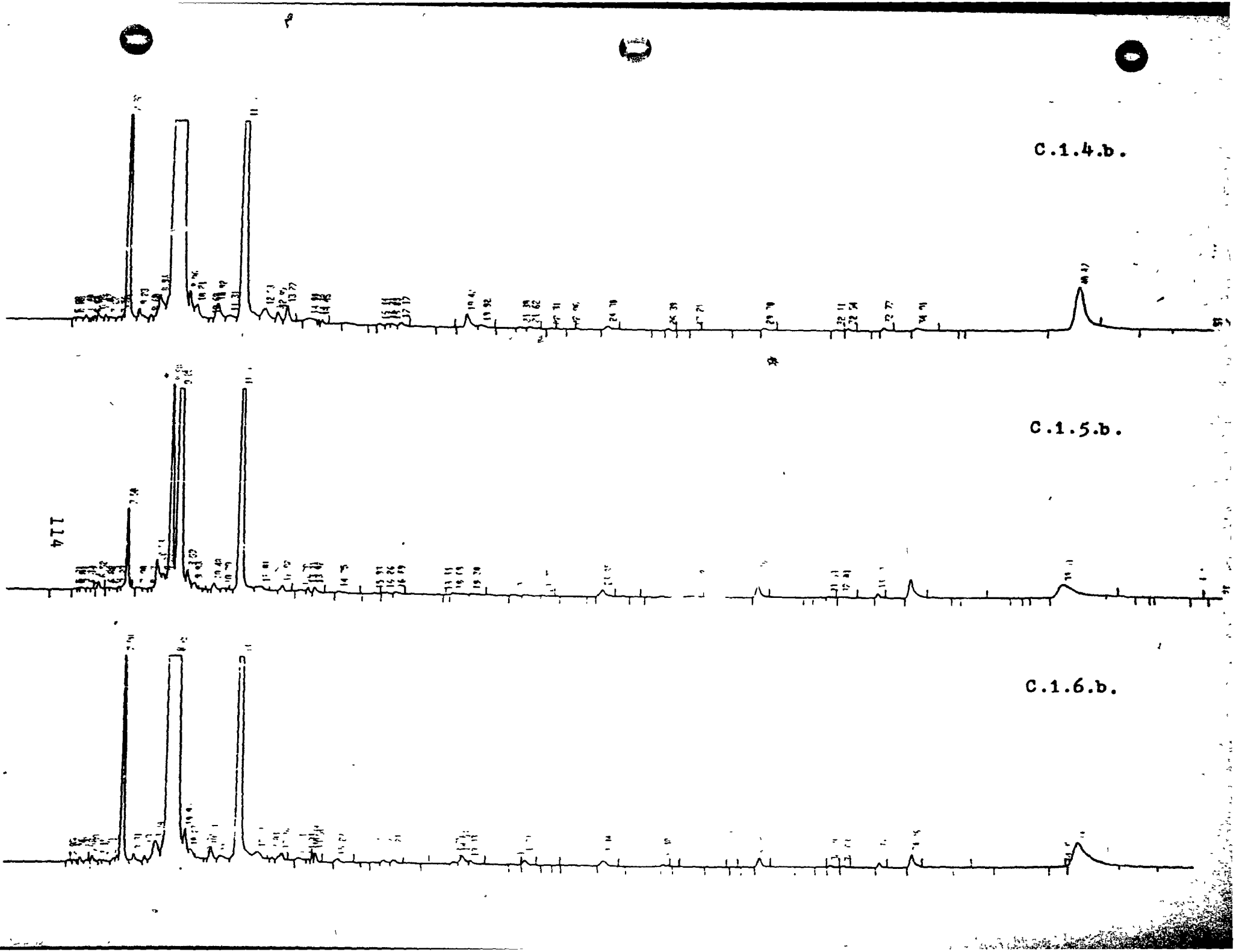
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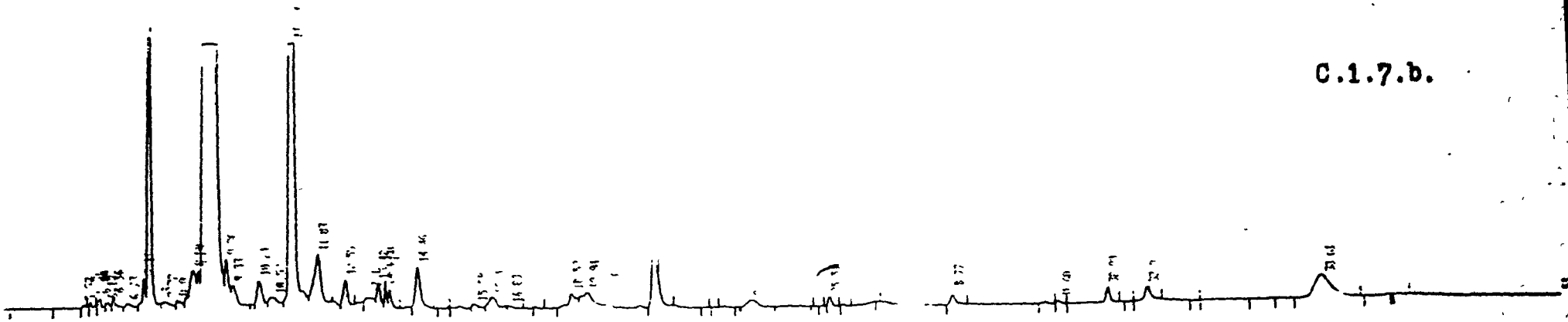
C.1.6.b.
114



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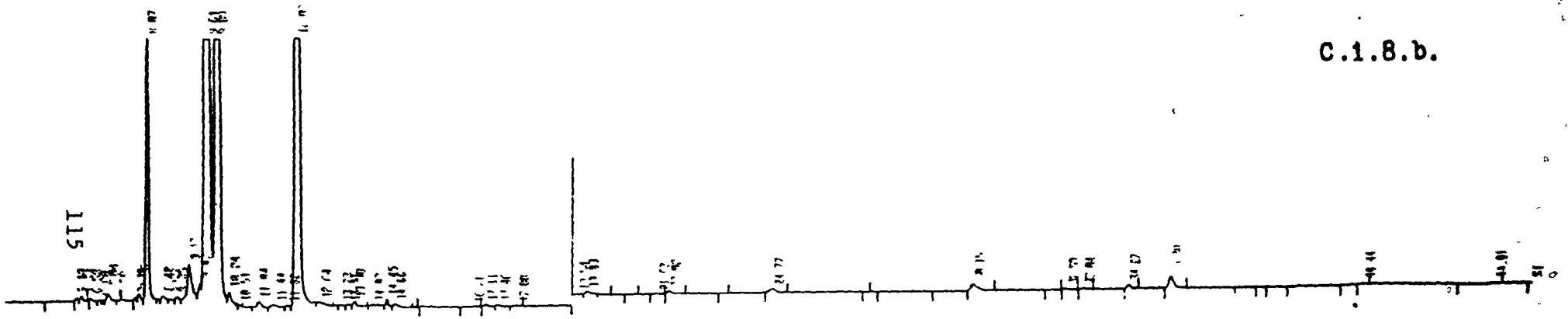

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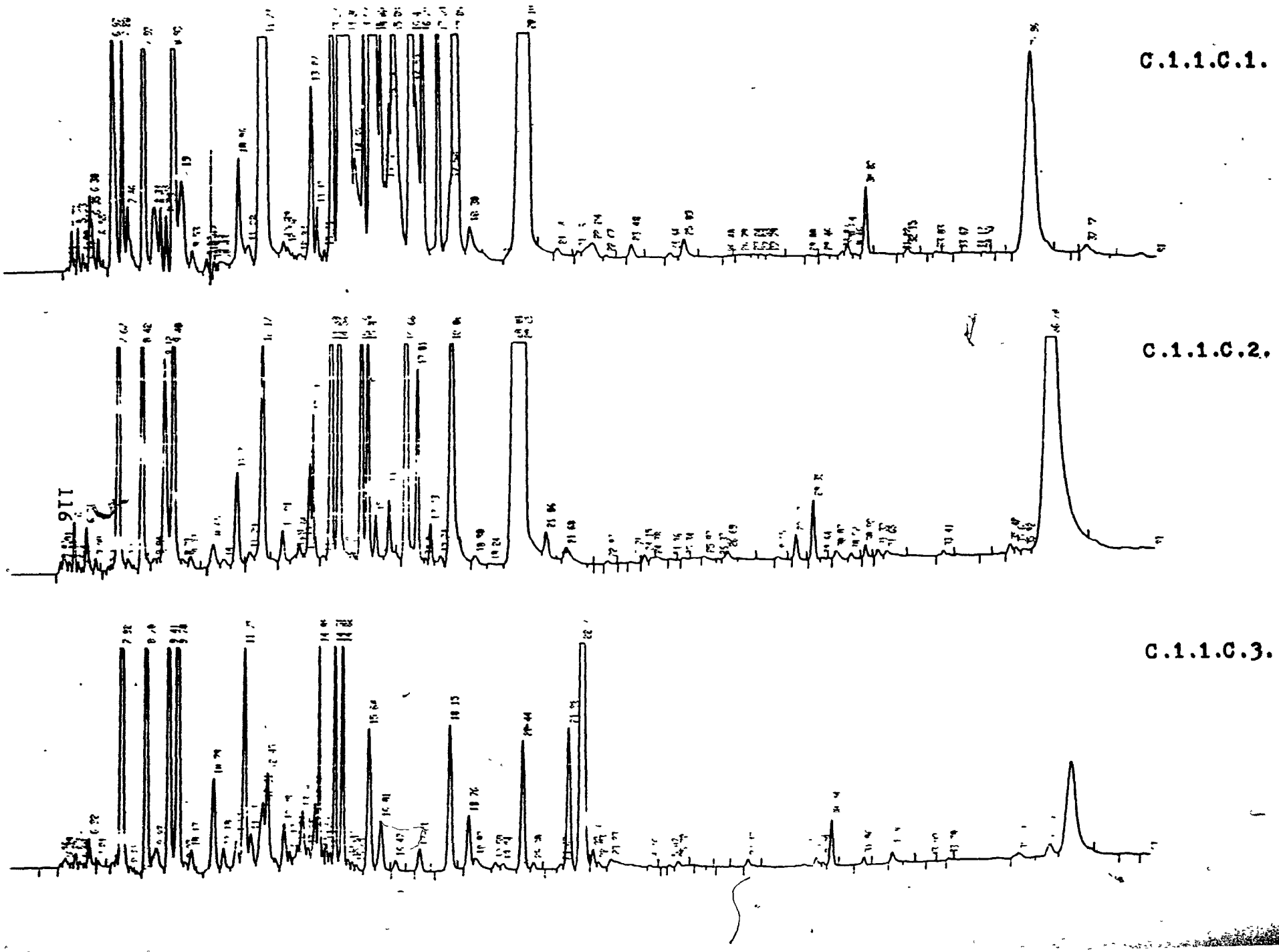
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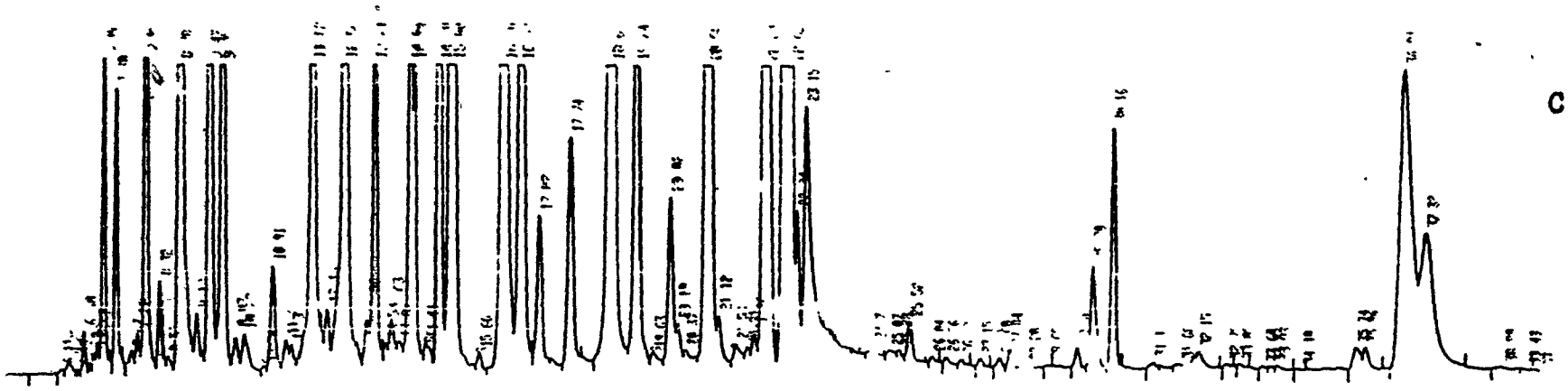

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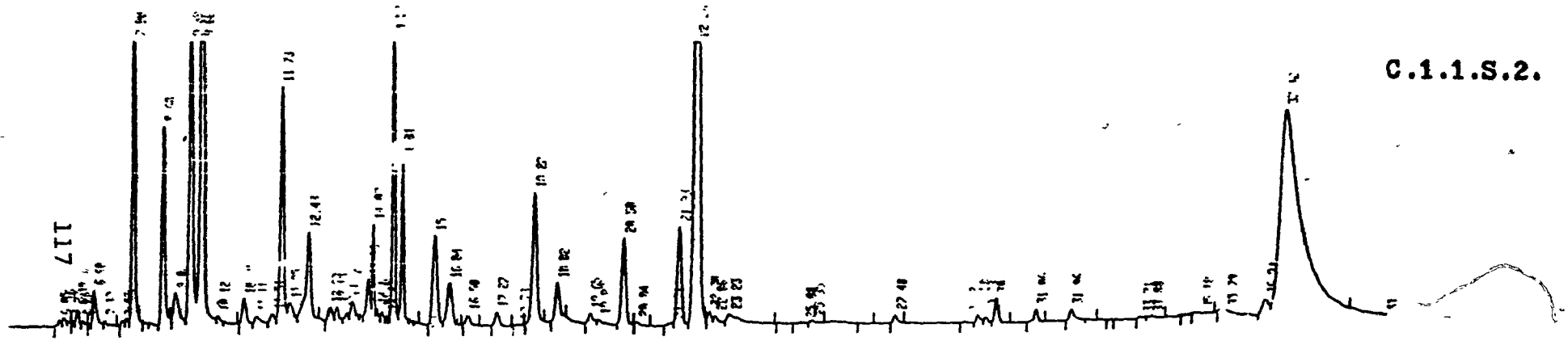

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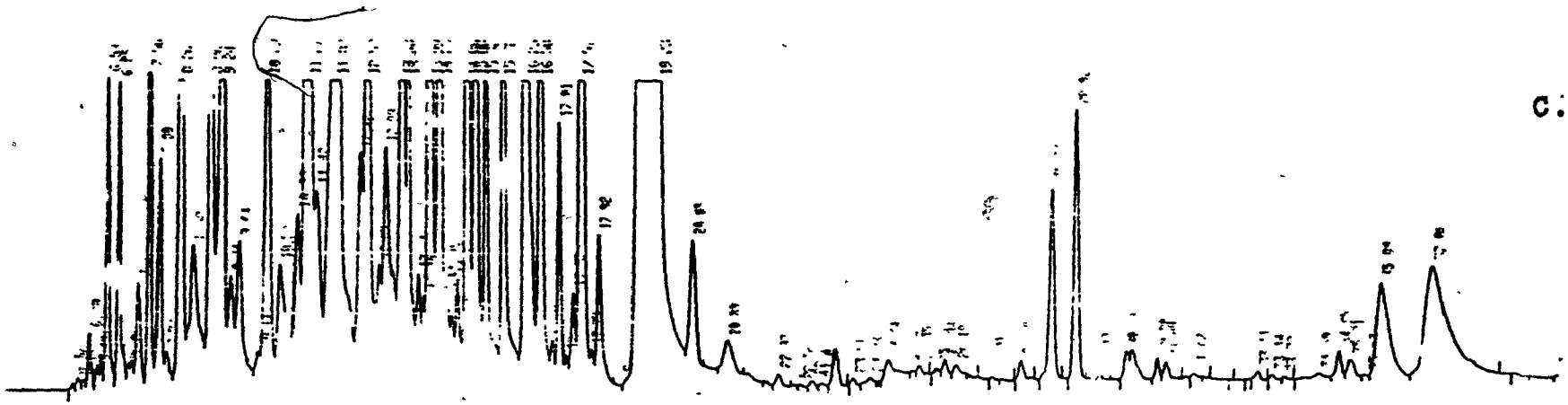

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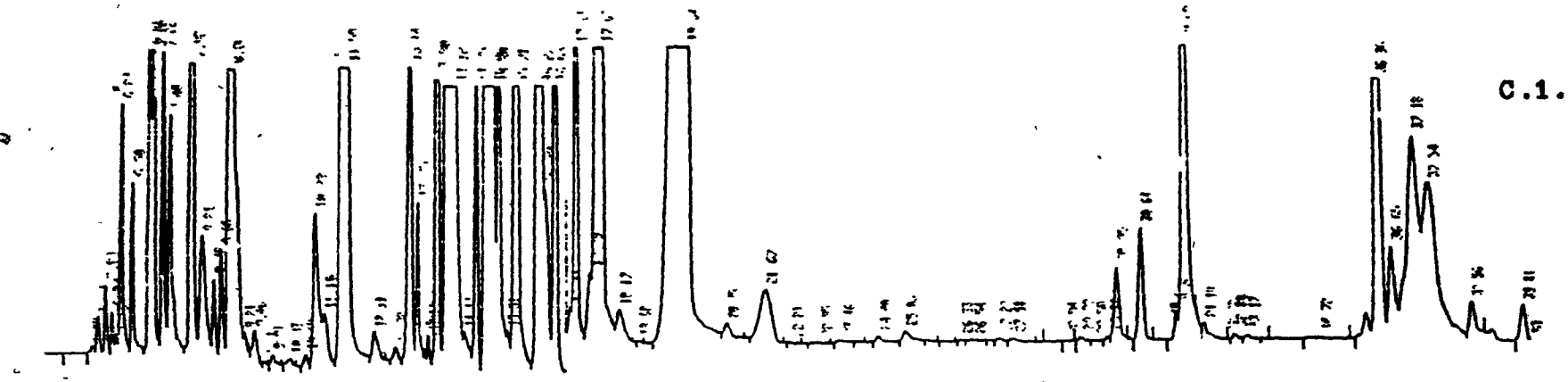

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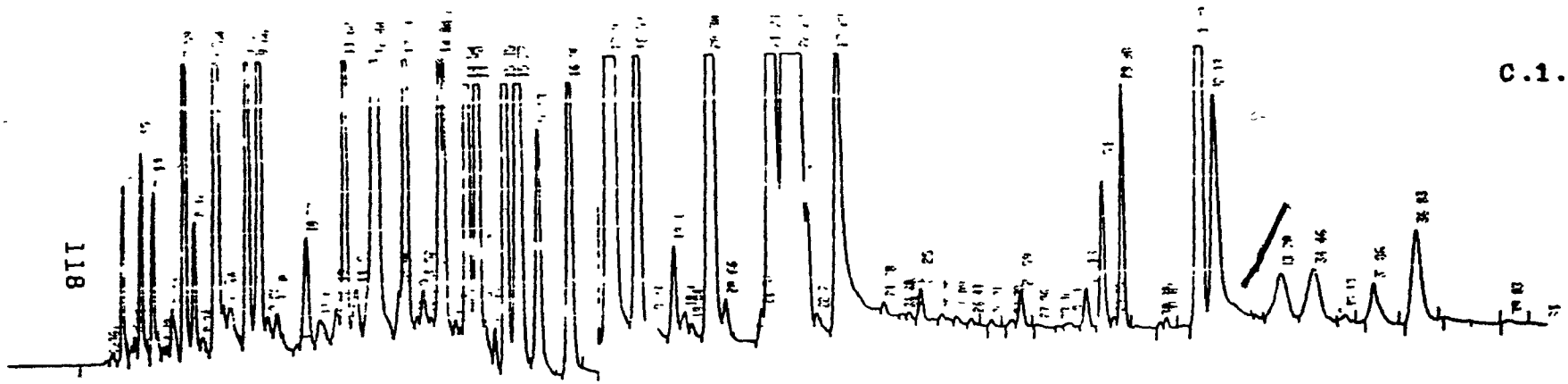

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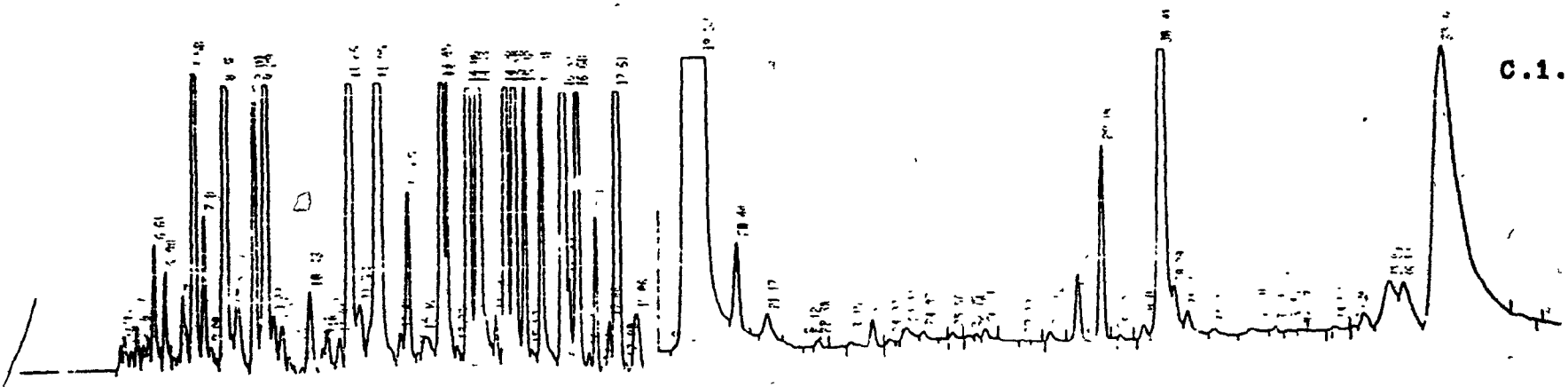

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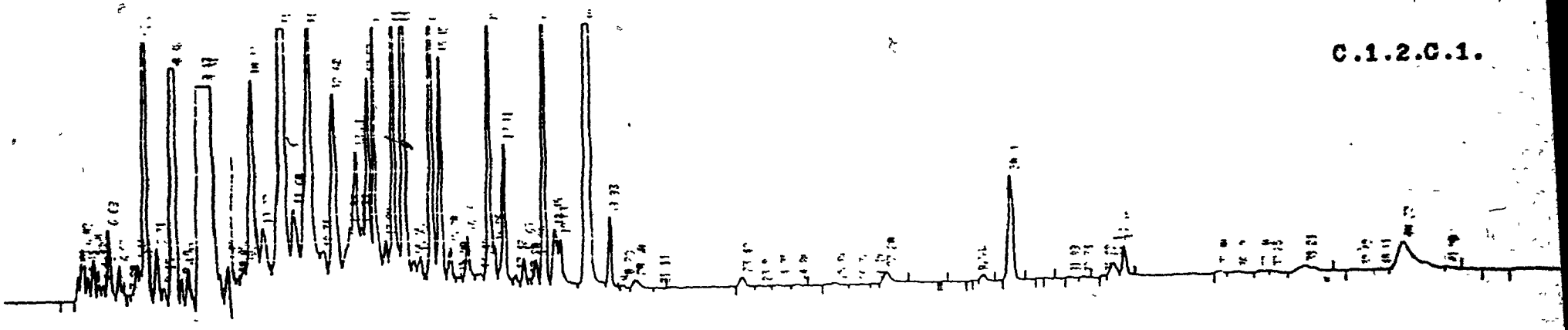

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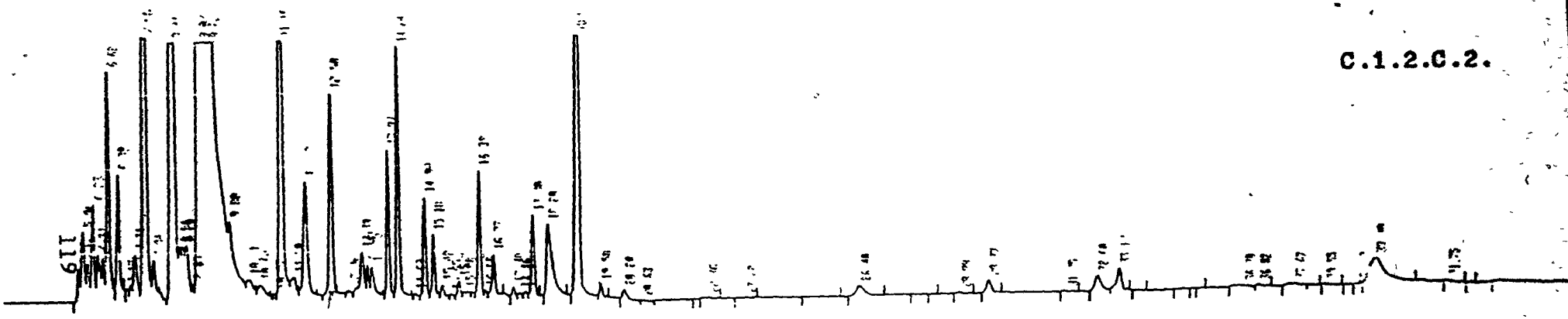

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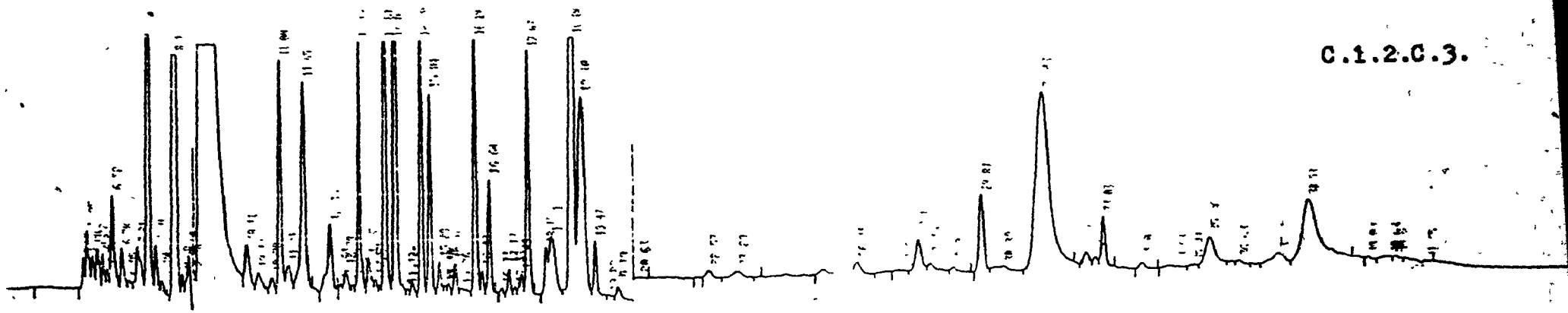

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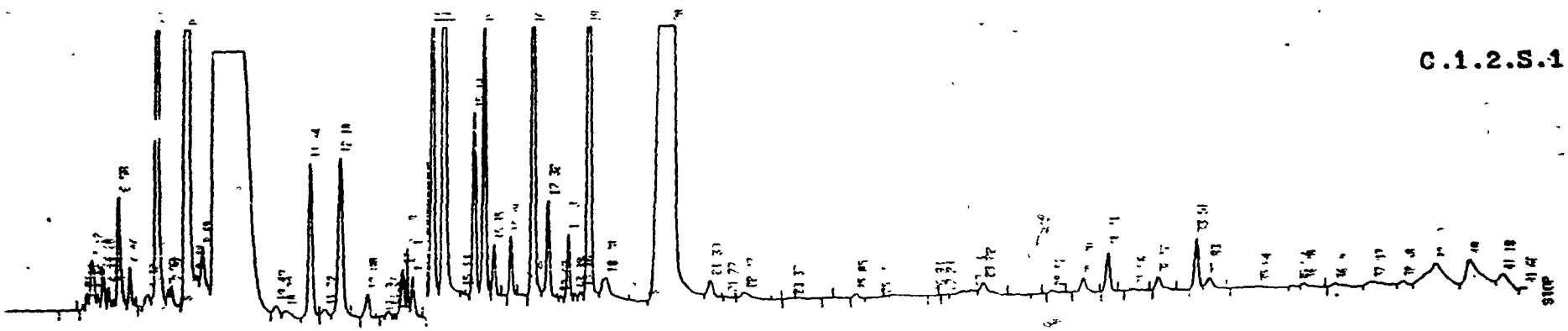
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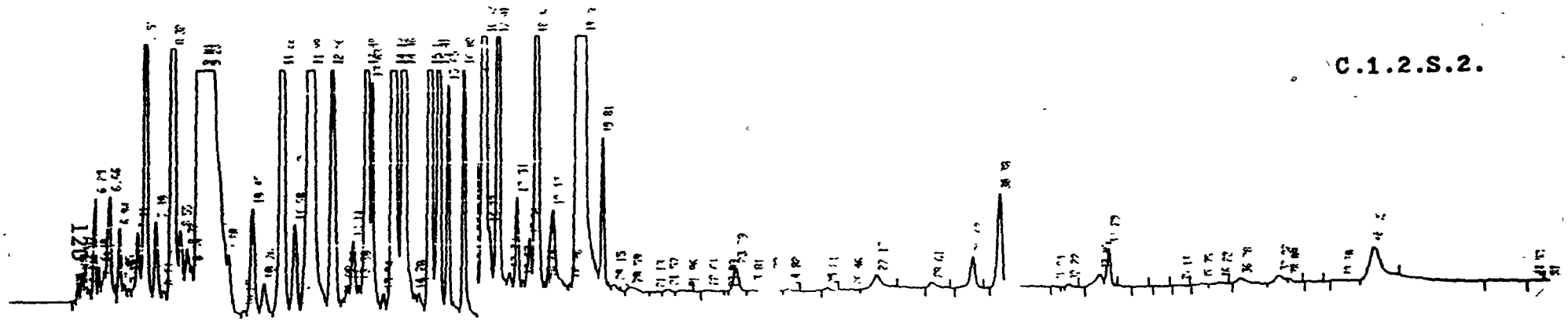
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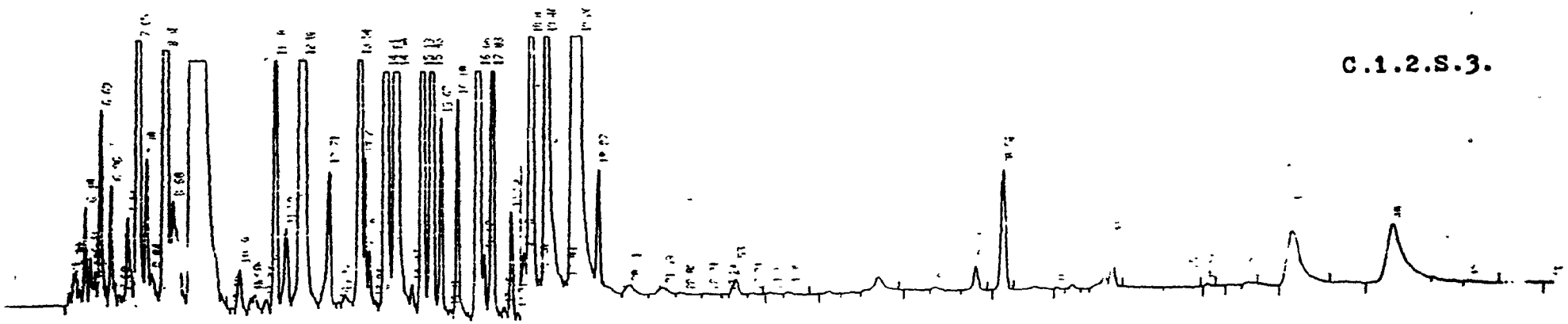
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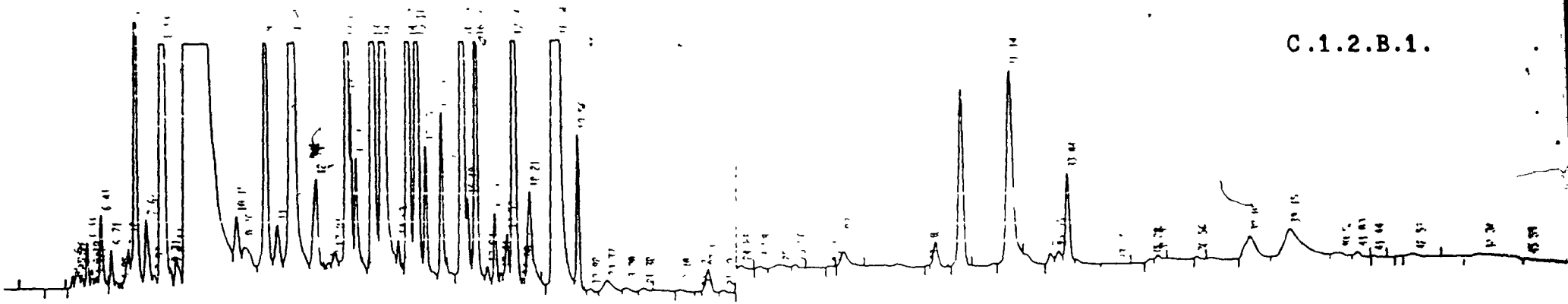

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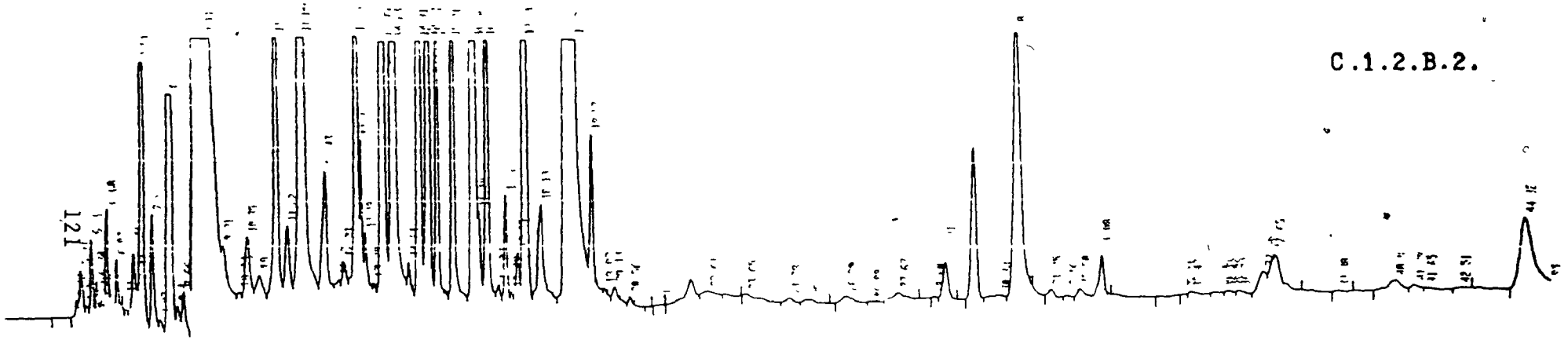

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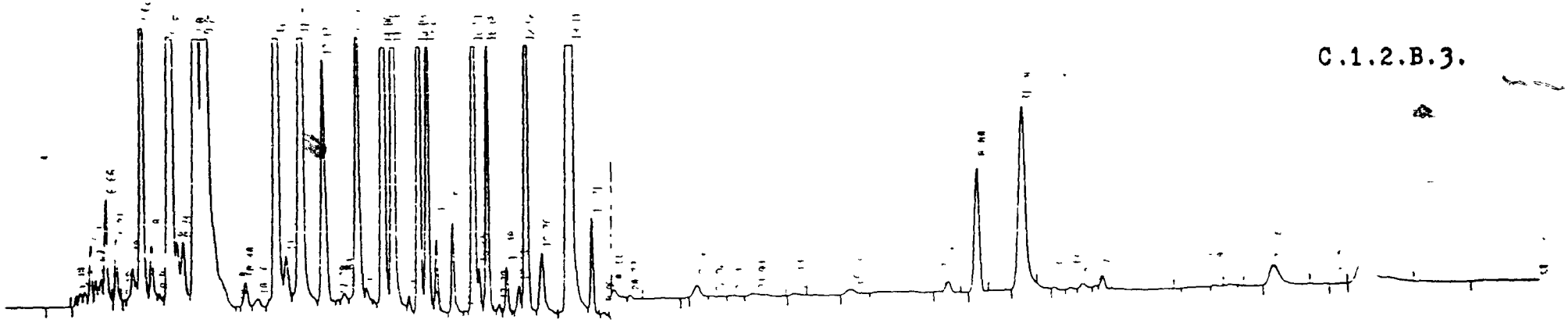

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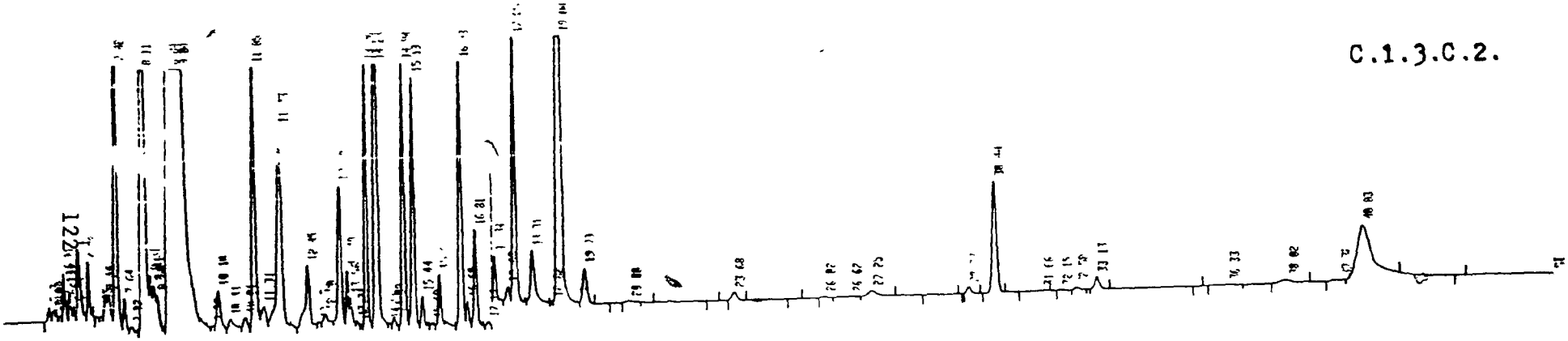

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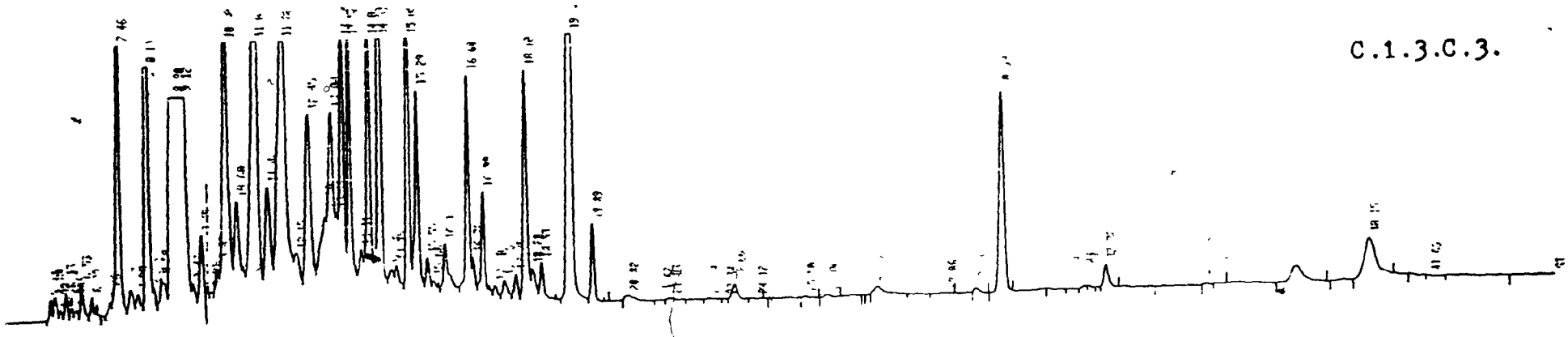

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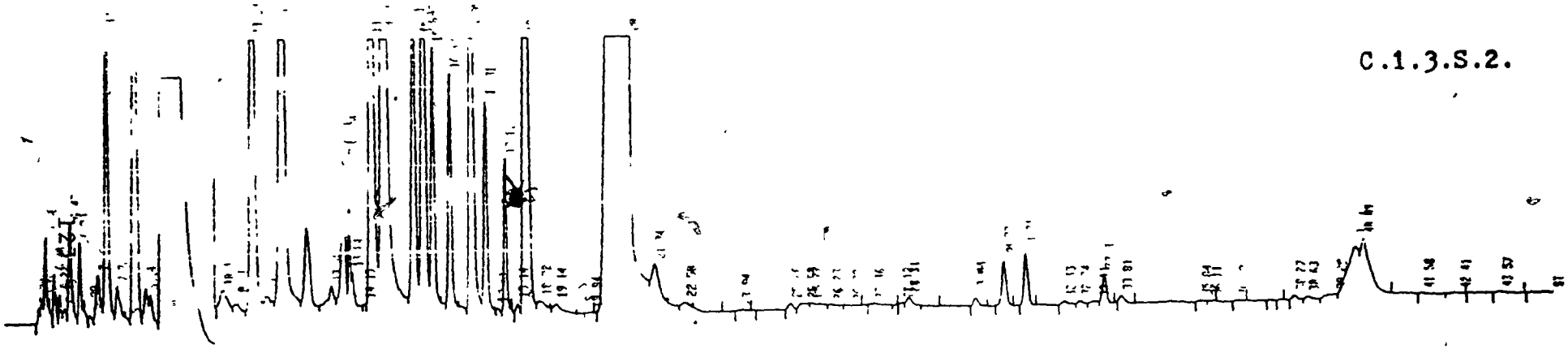

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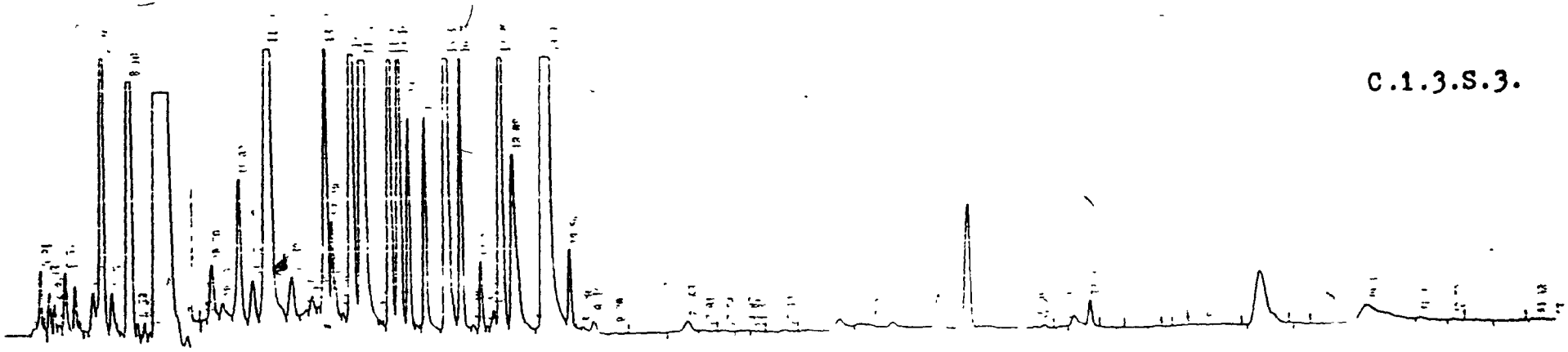

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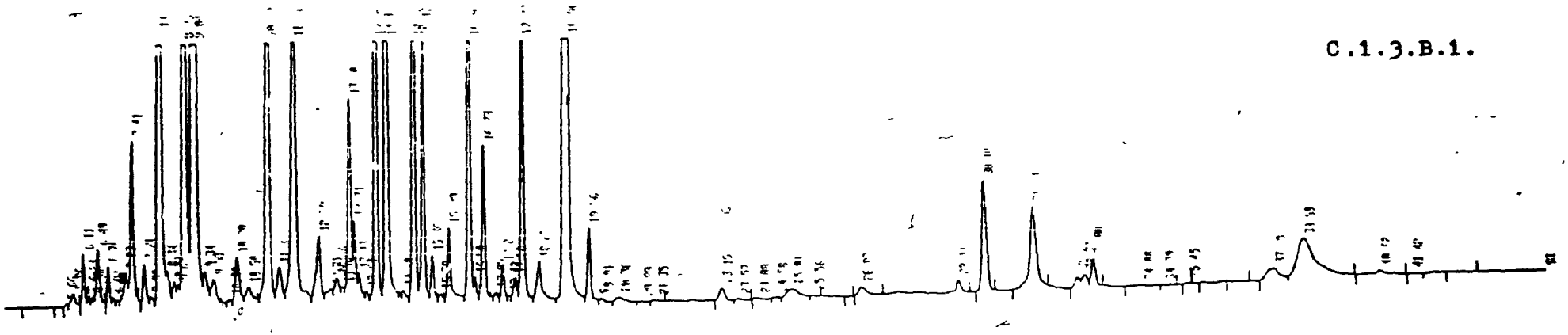

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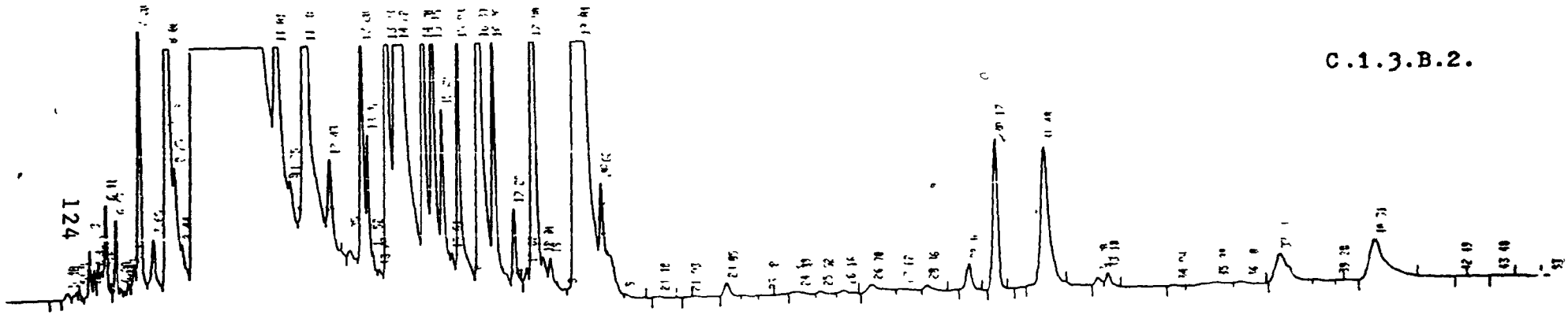

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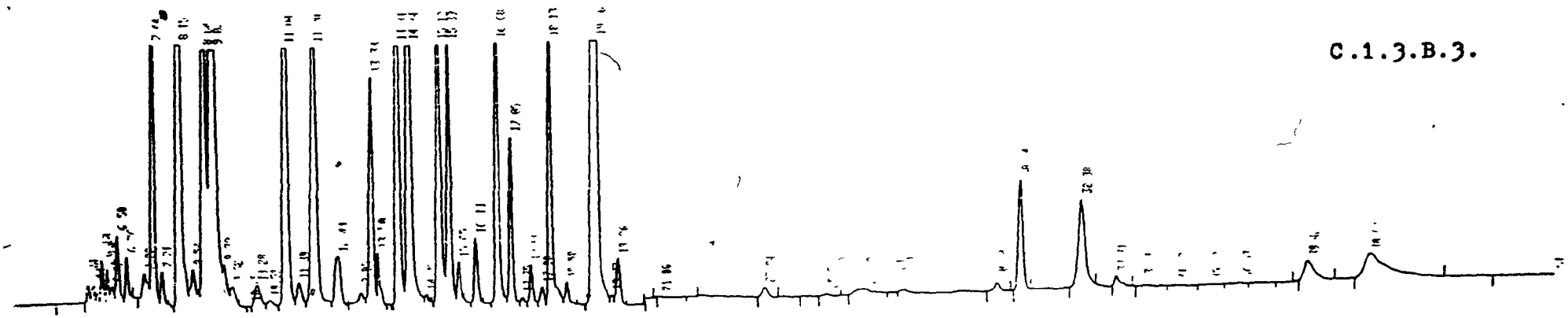

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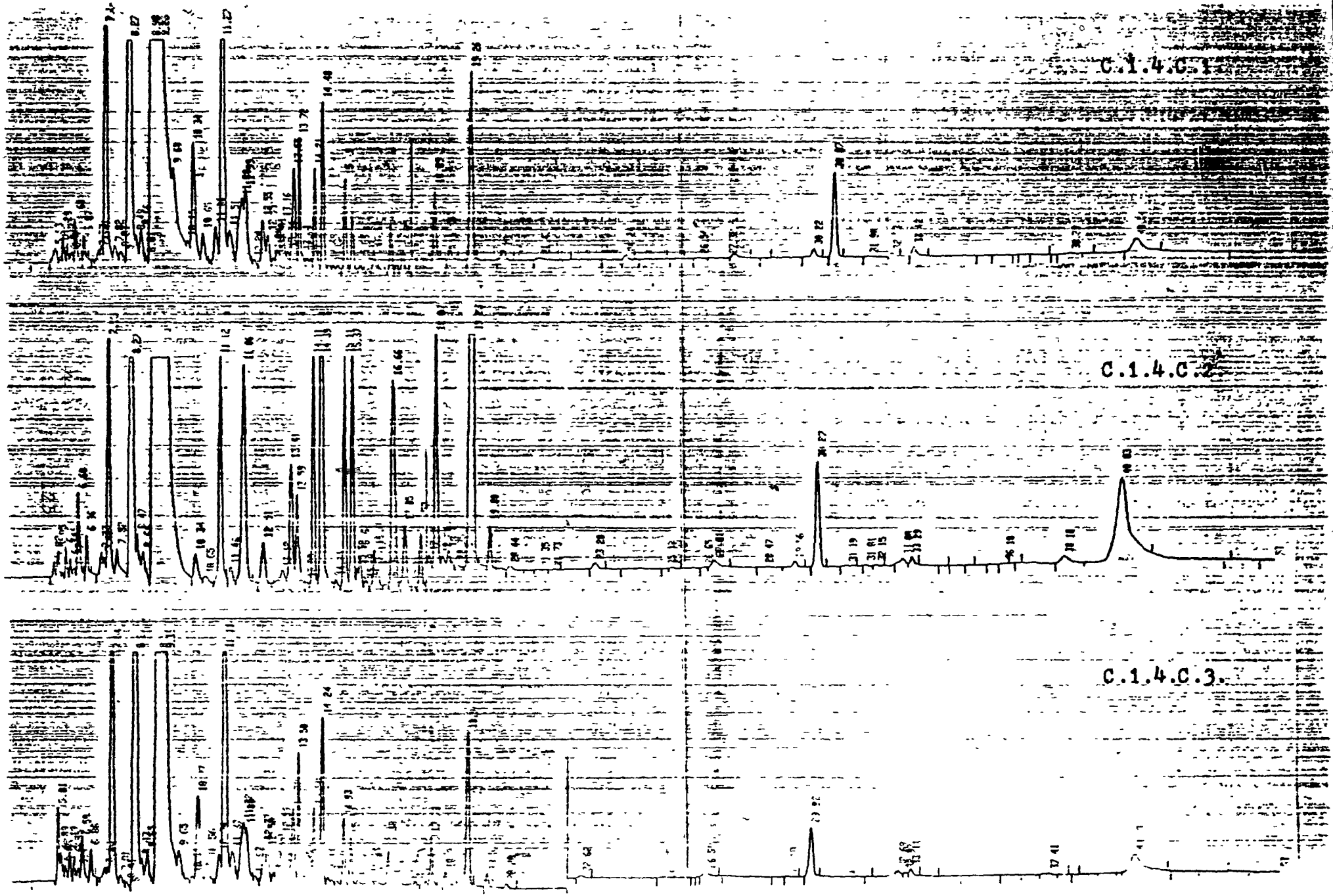
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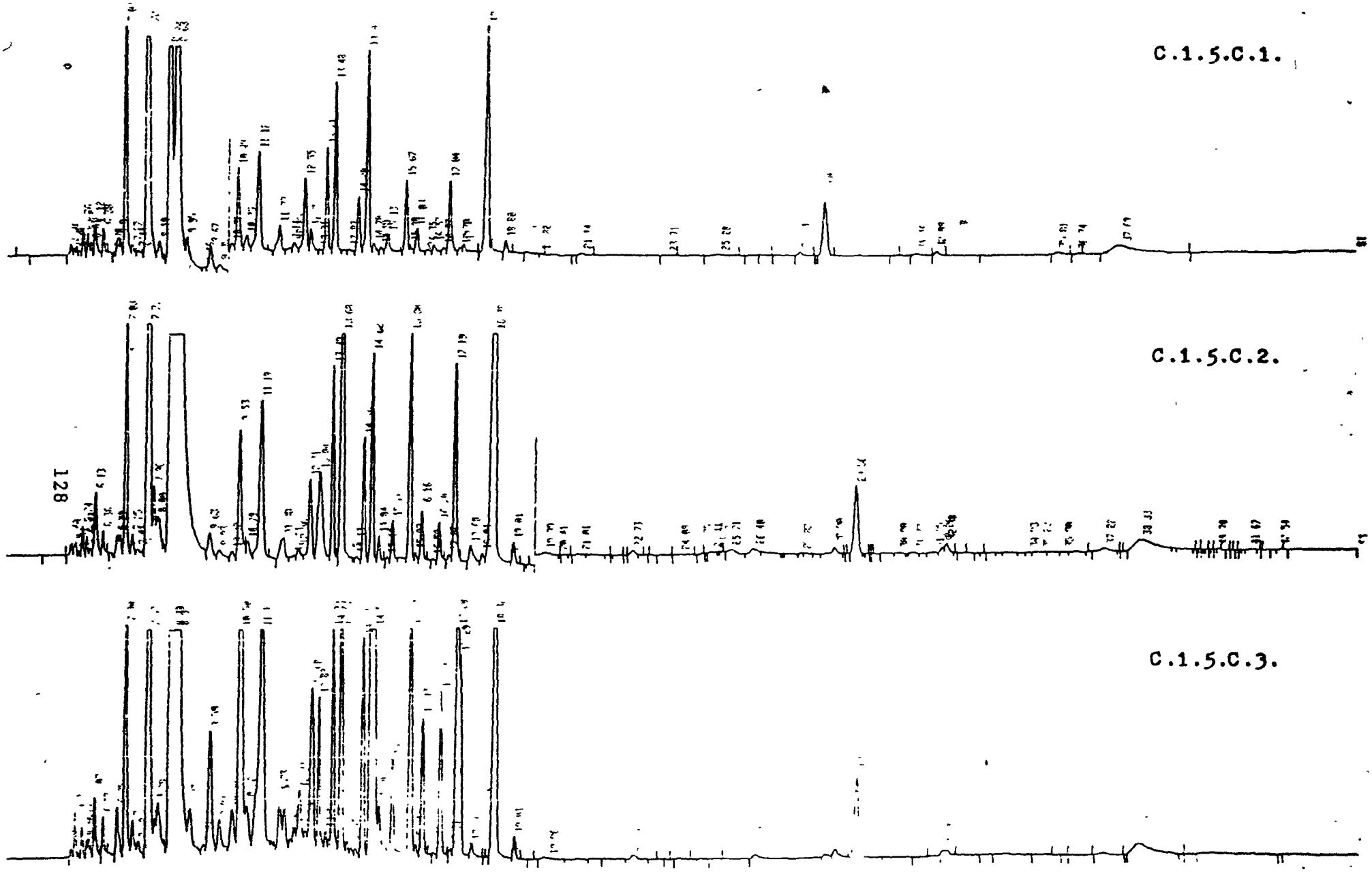
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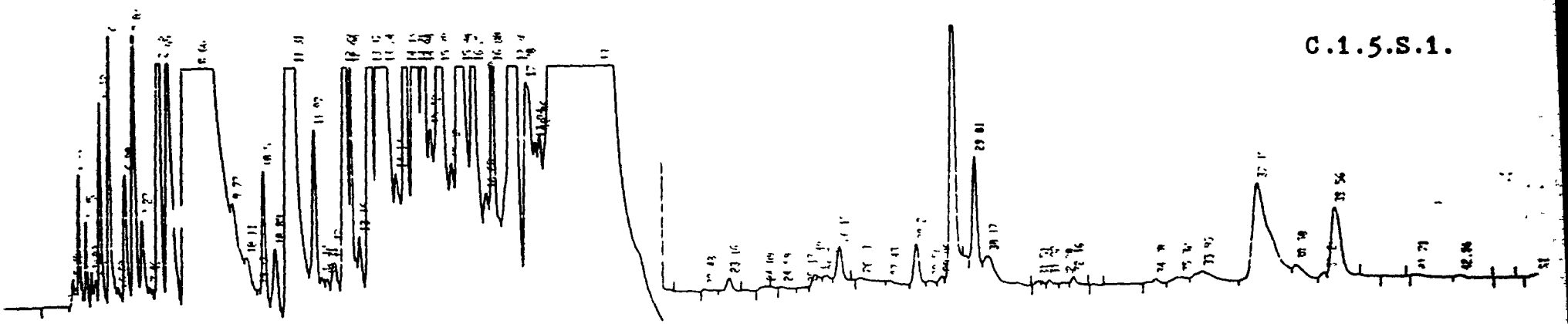

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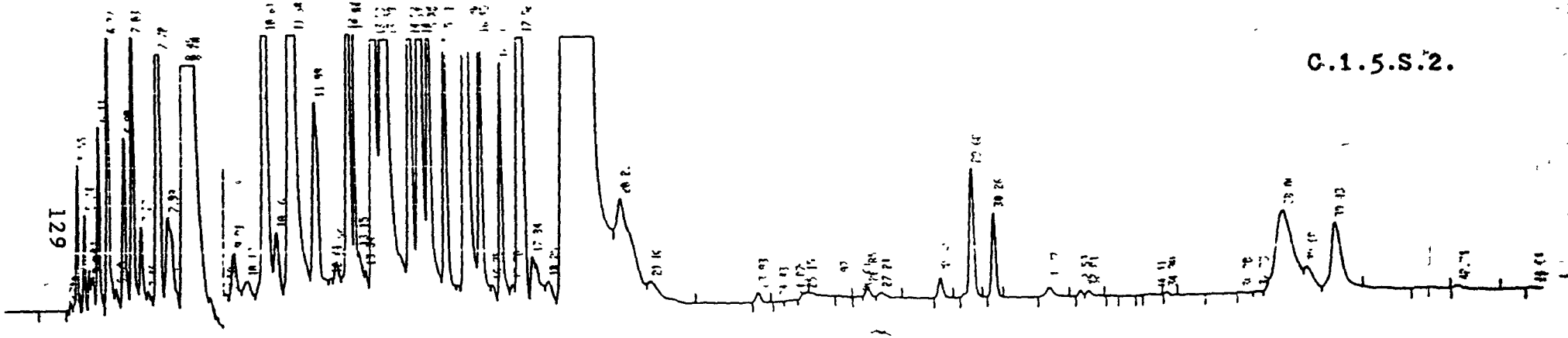

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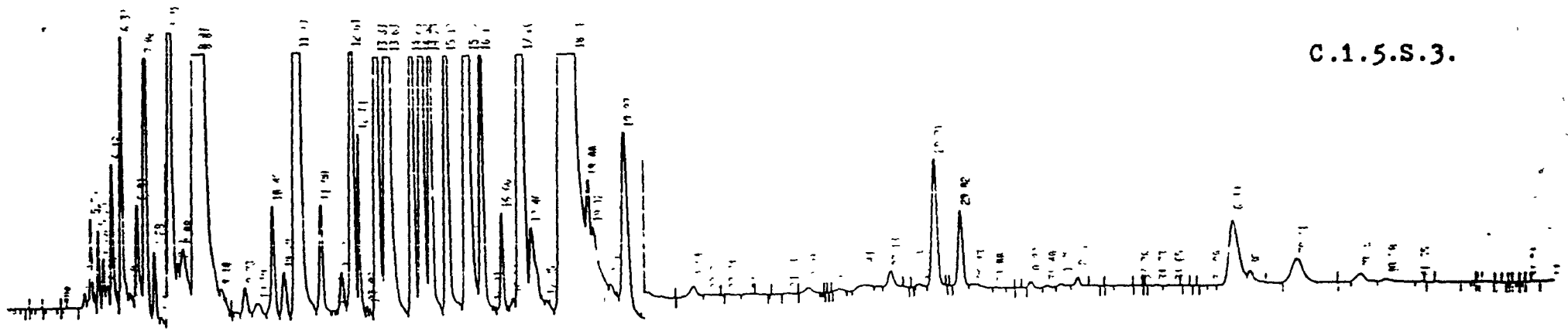

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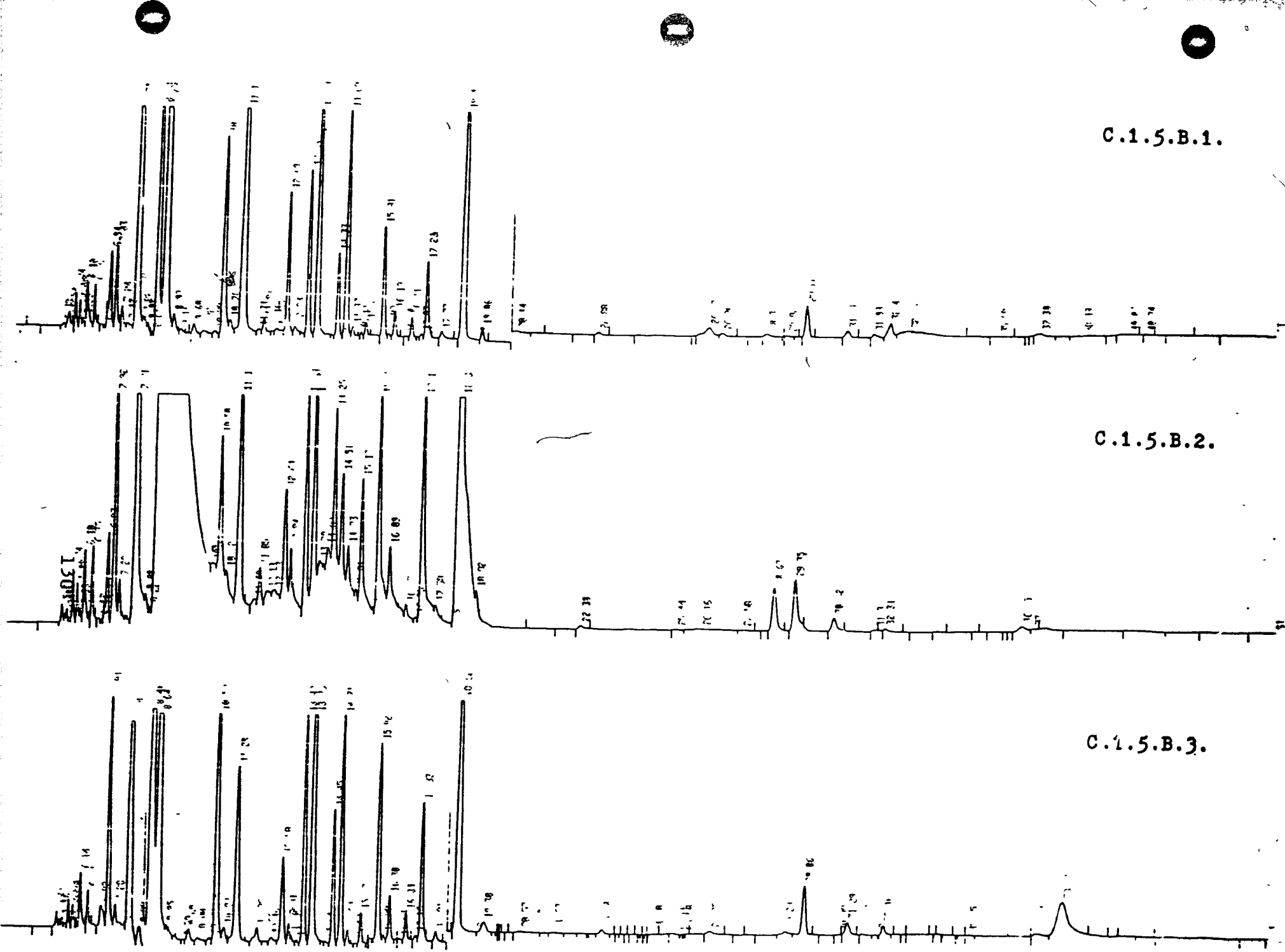
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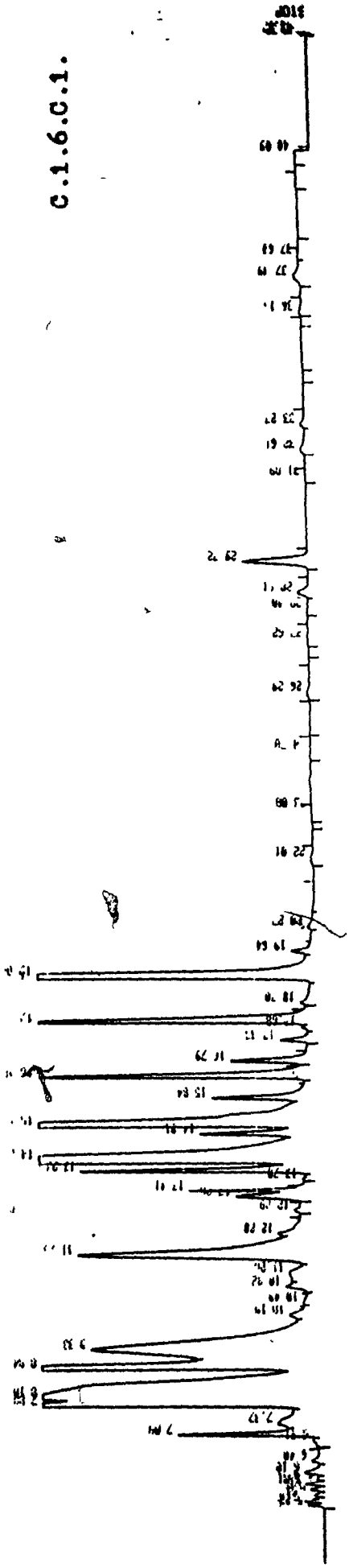
C.1.5.B.2.
C.1.5.B.3.

C.1.6.C.1.



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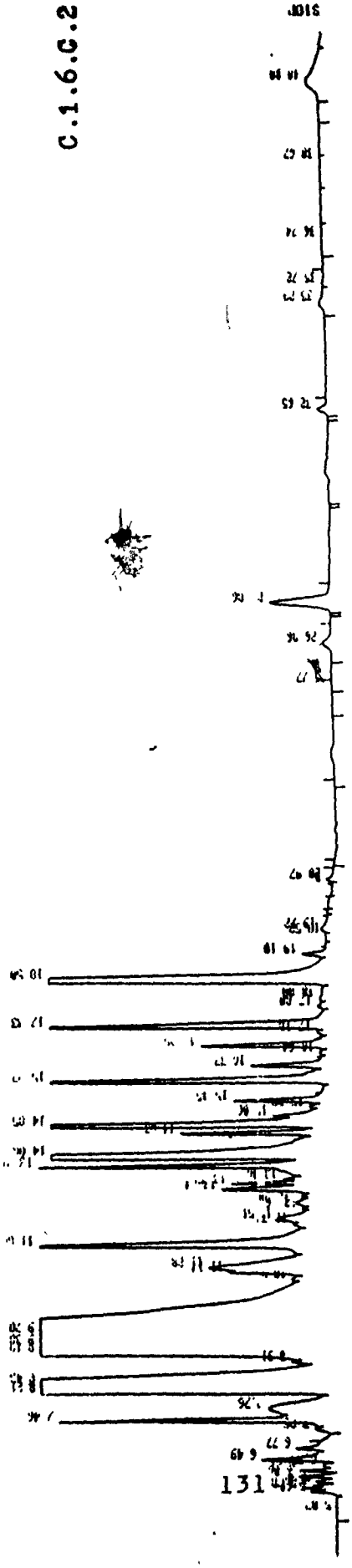

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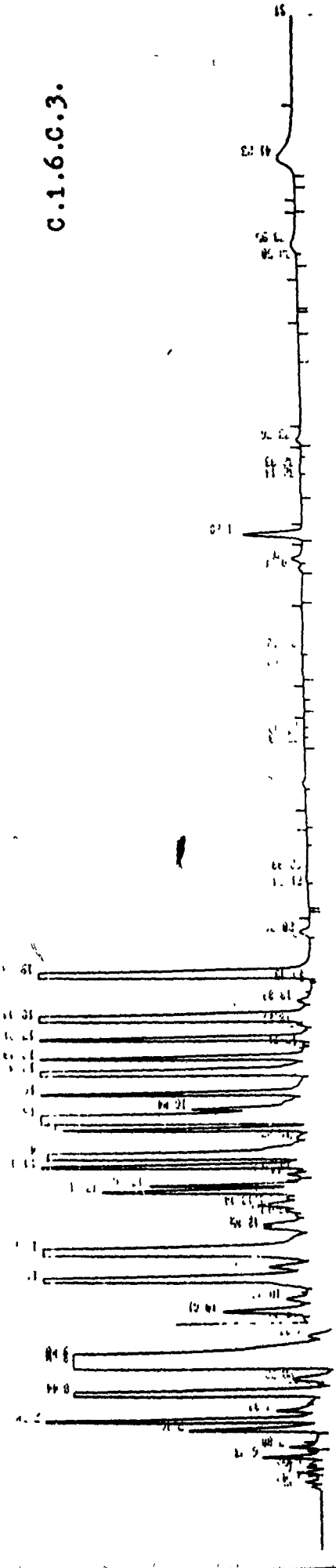

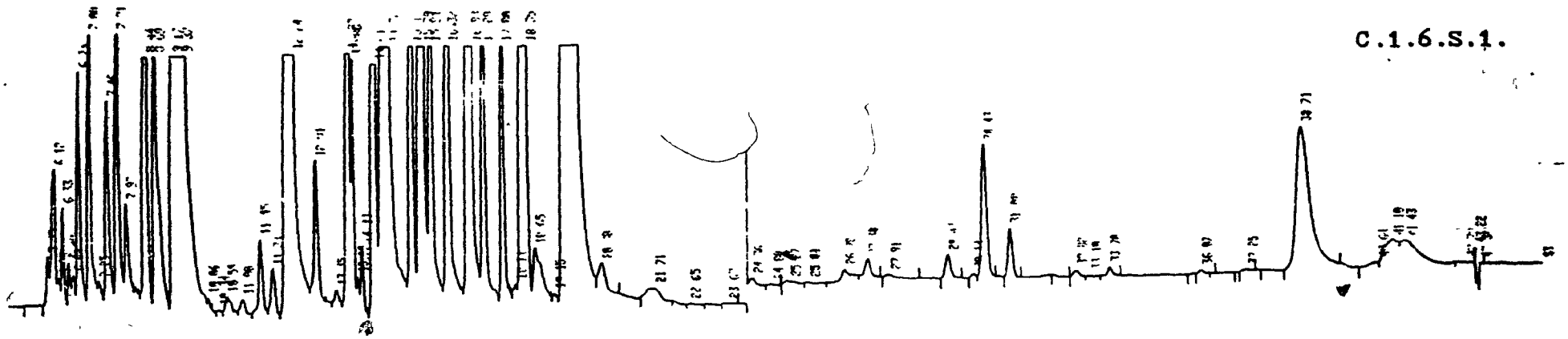
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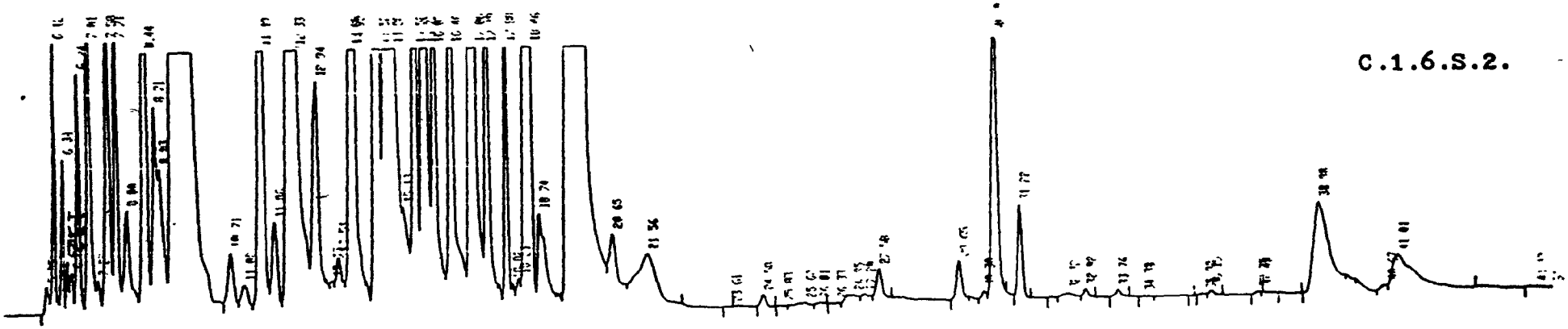
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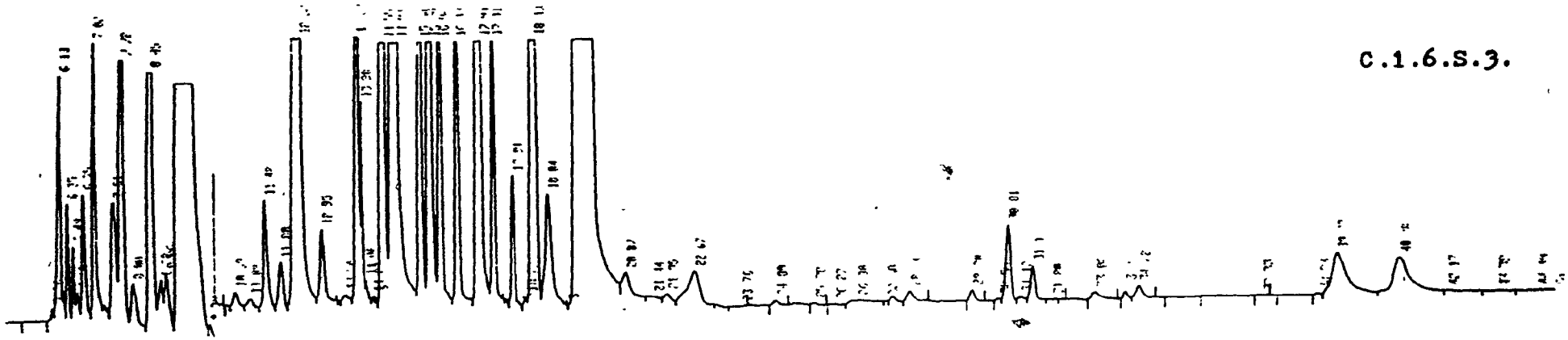
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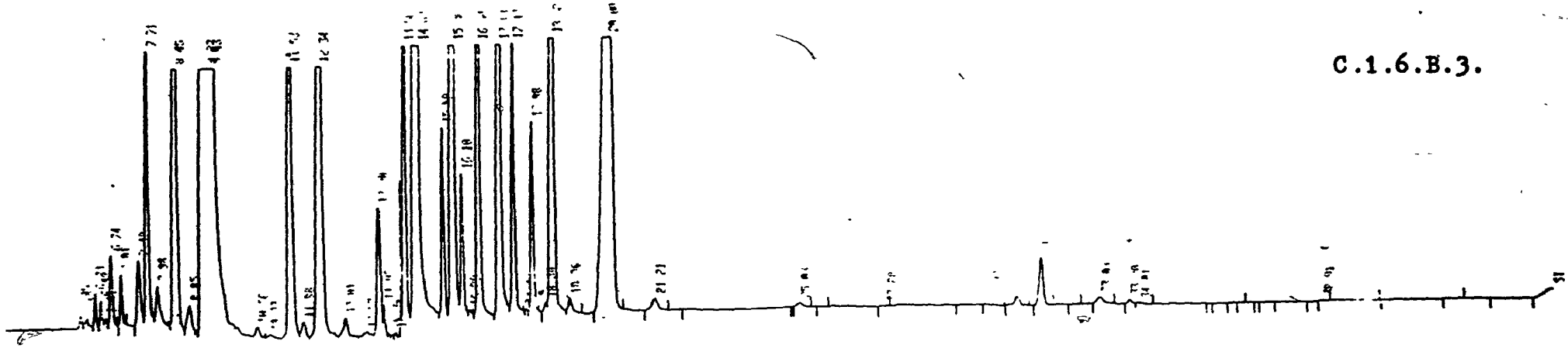
C.1.6.B.1.

C.1.6.B.2.



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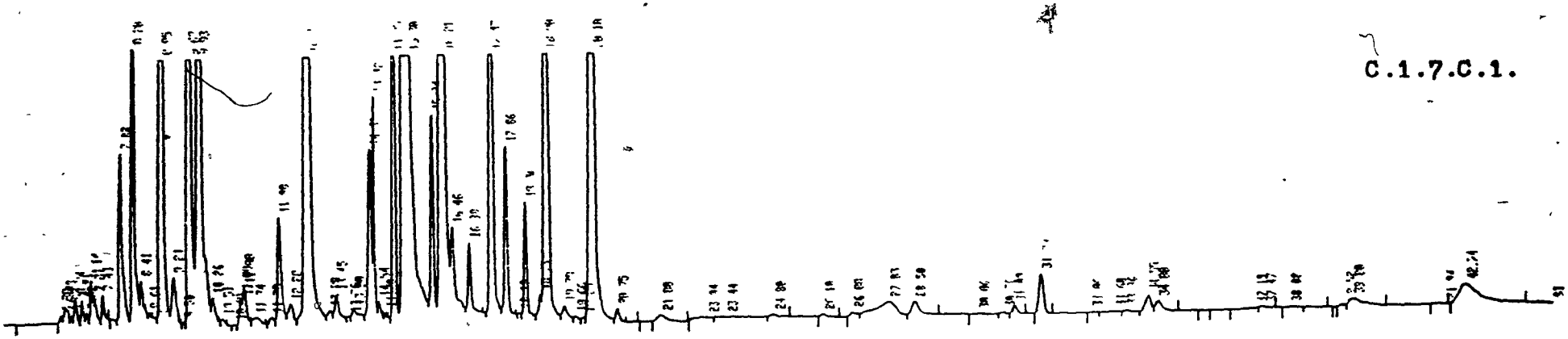

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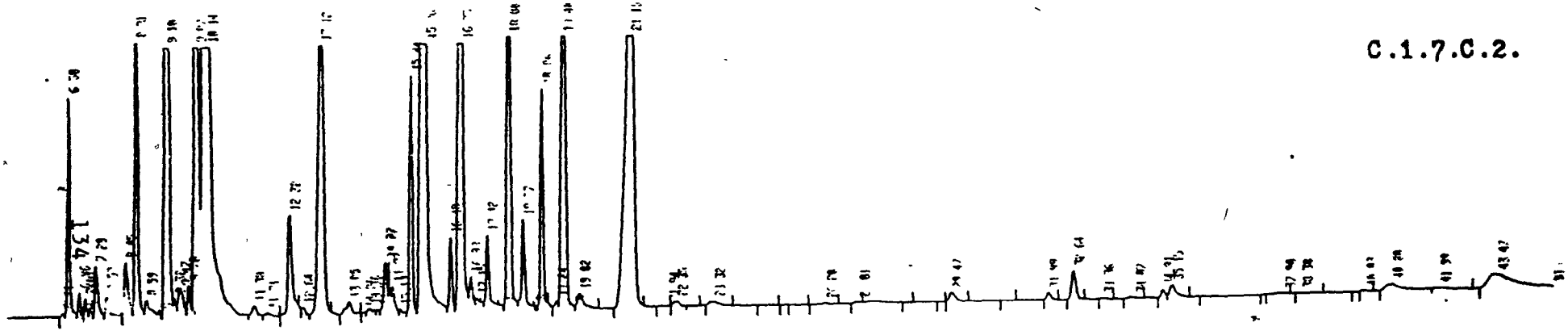

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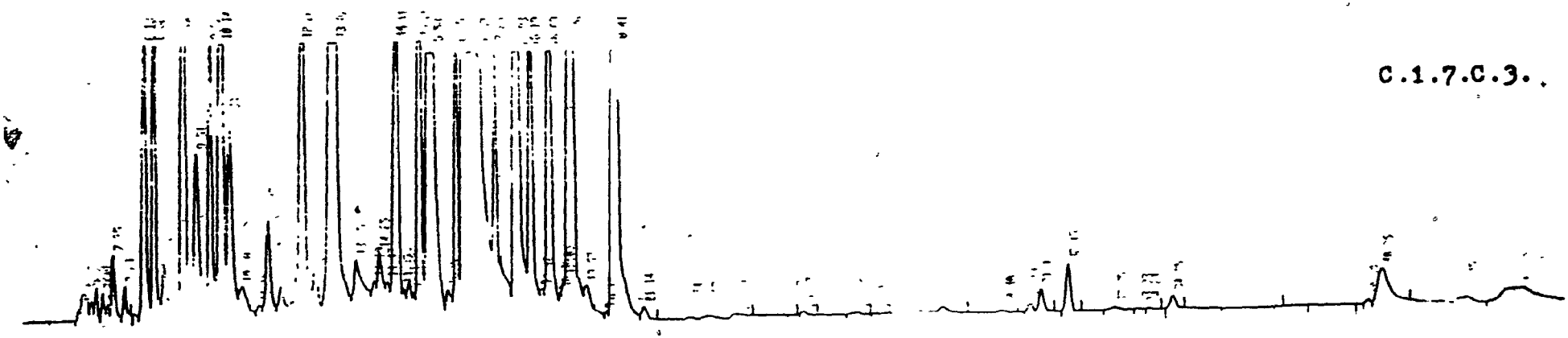

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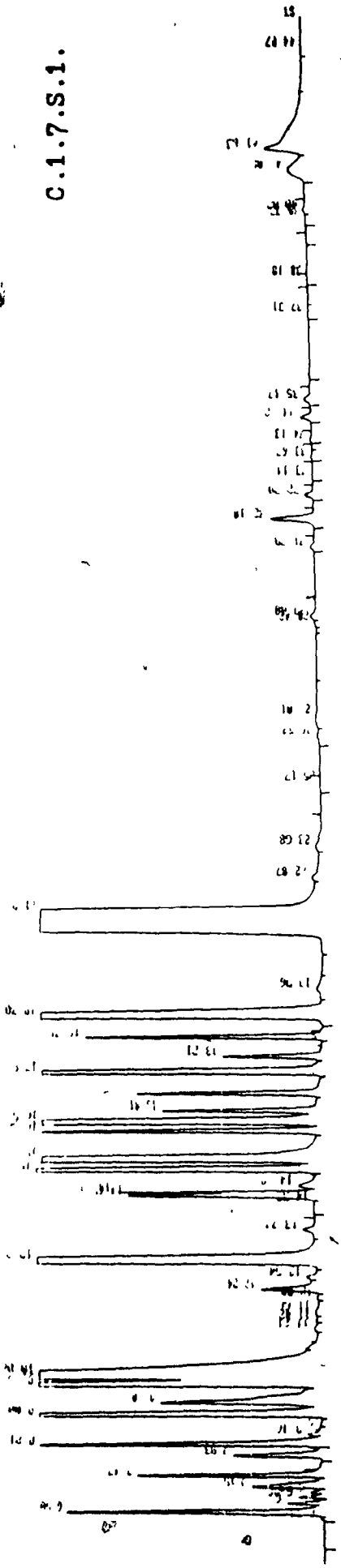
C.1.7.S.1.



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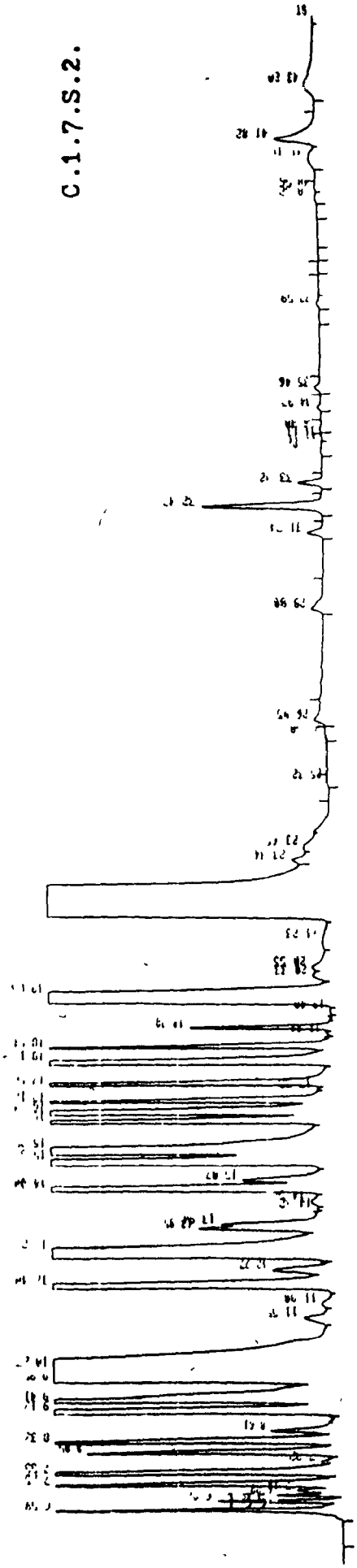

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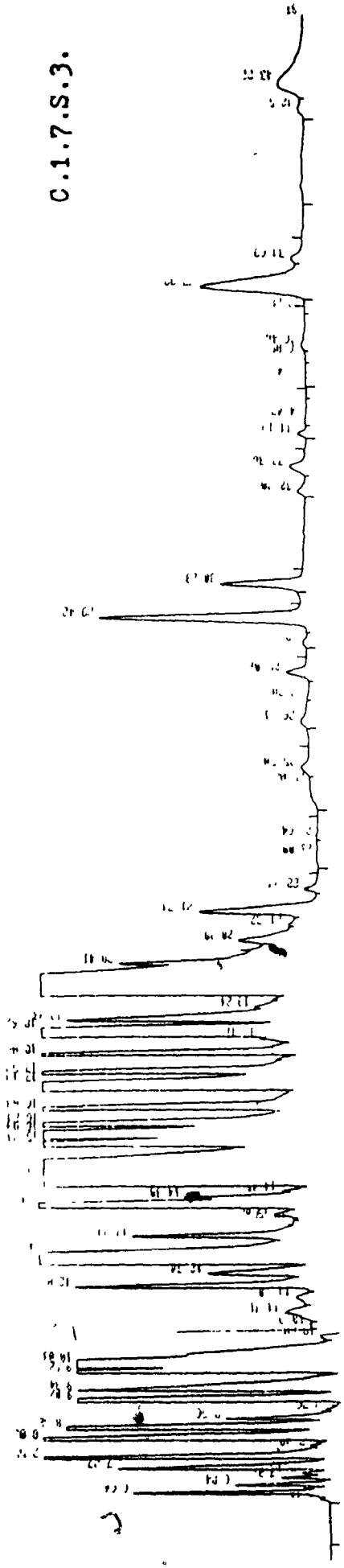

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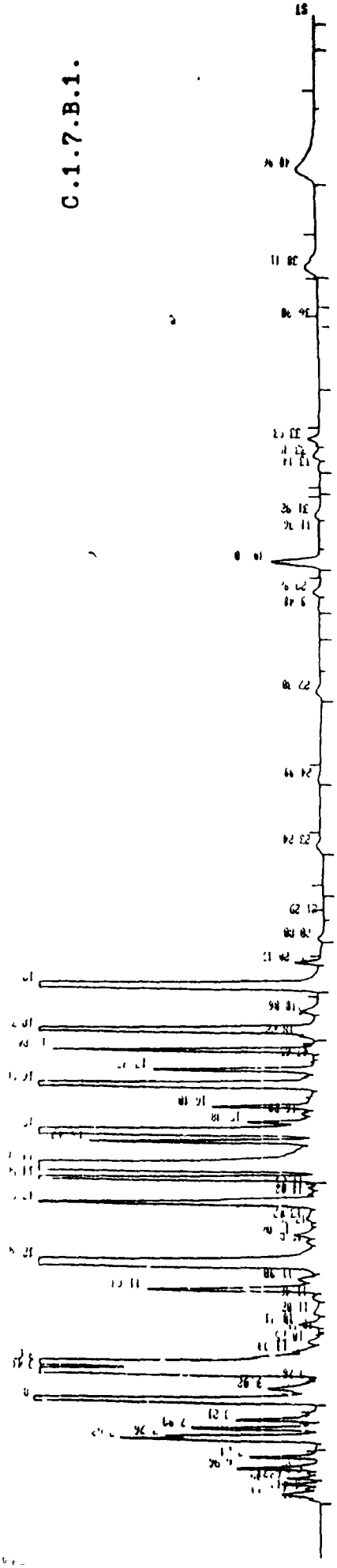

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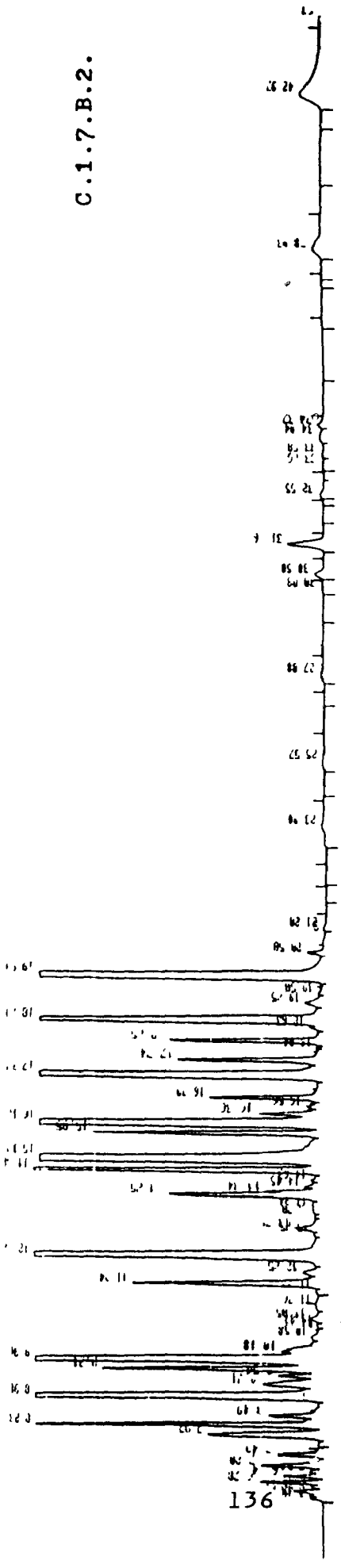

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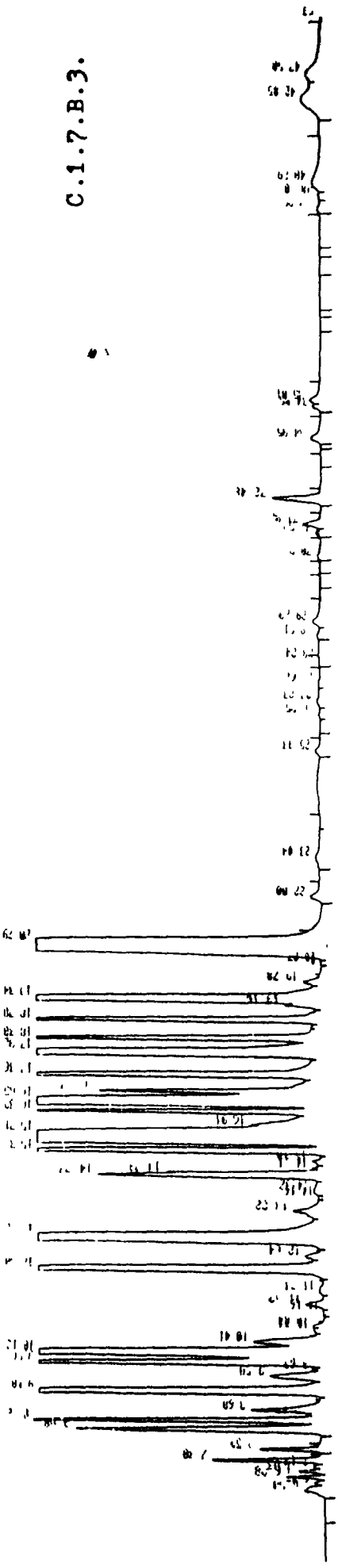

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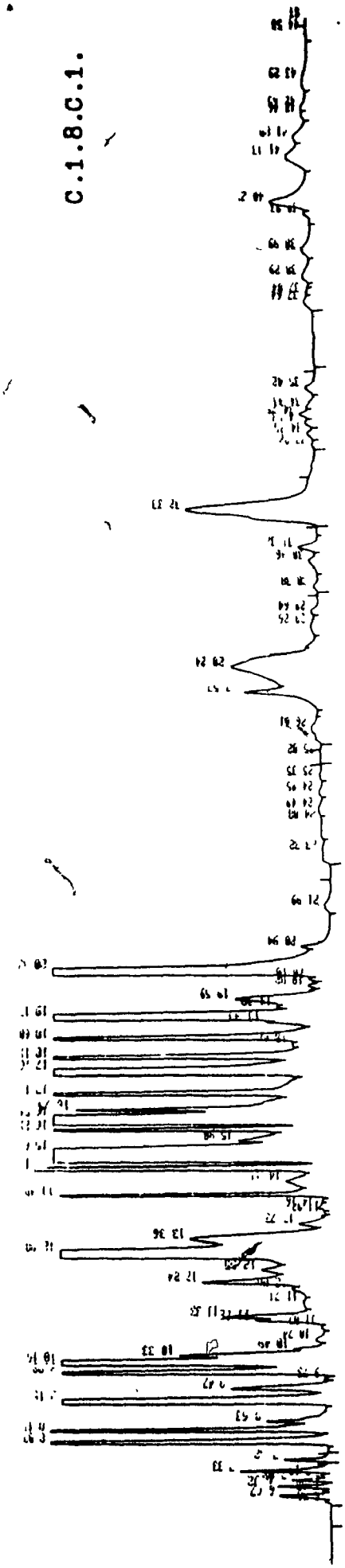

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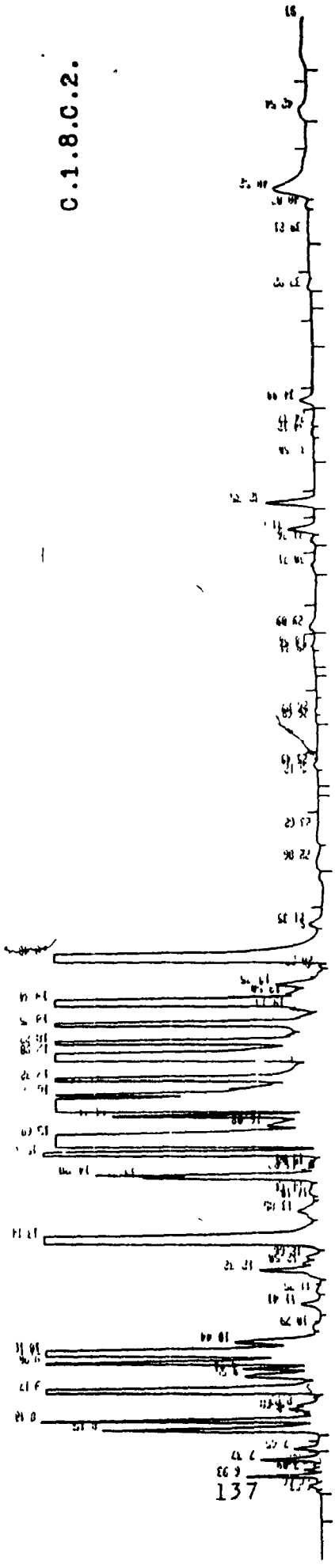

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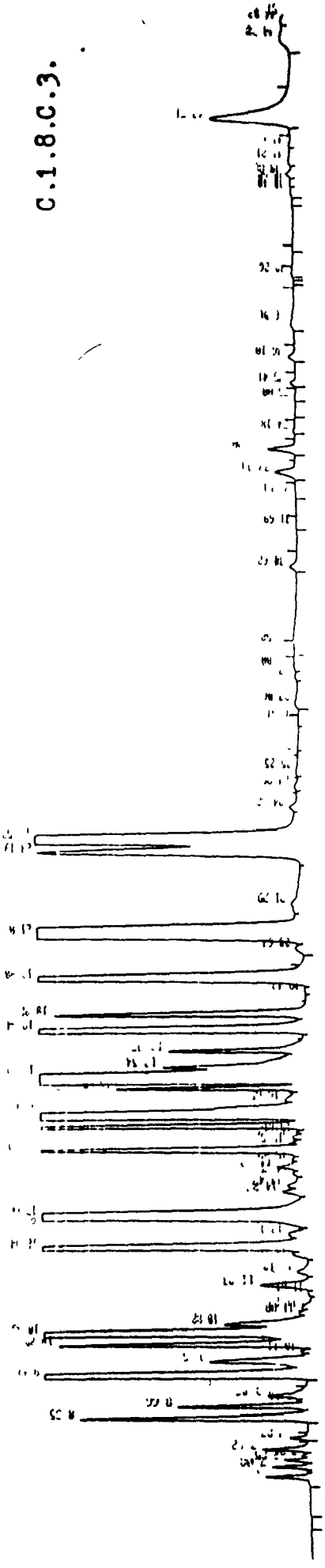

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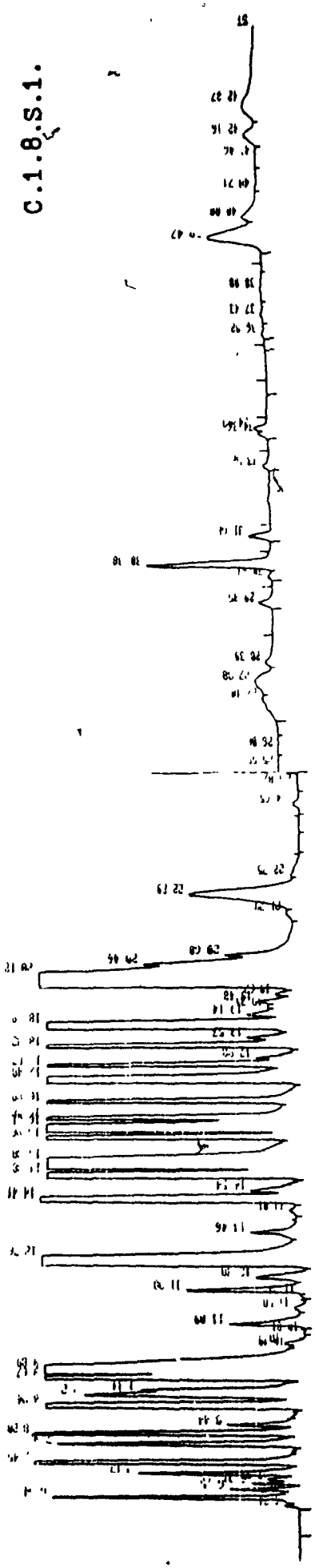

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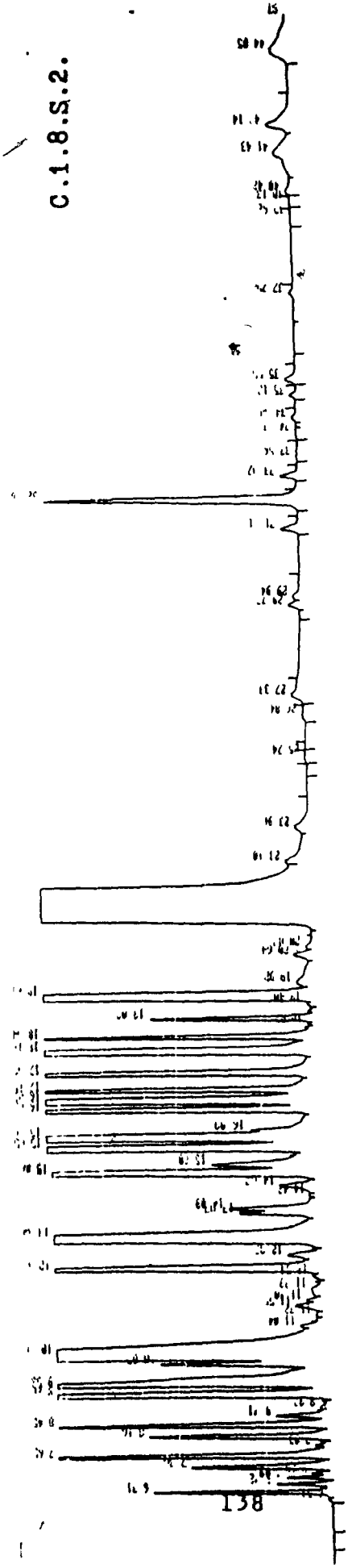

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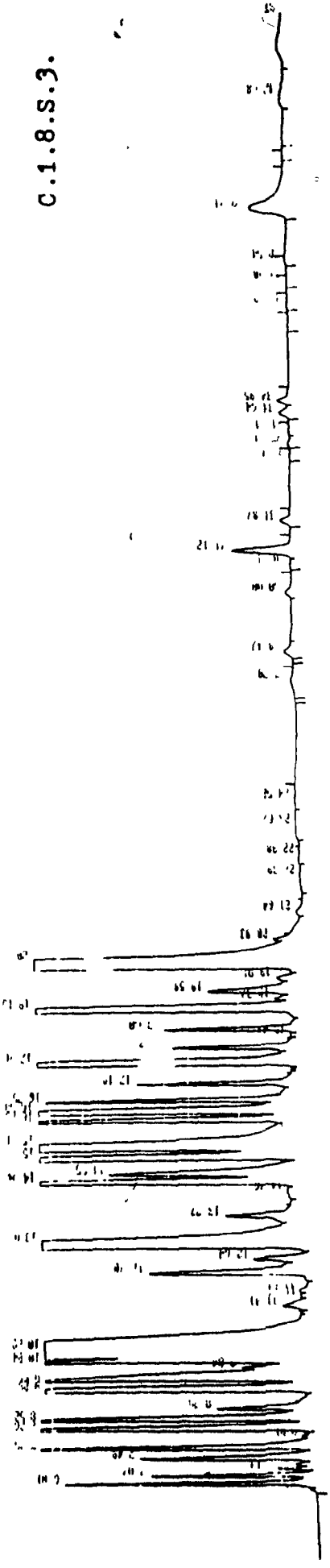

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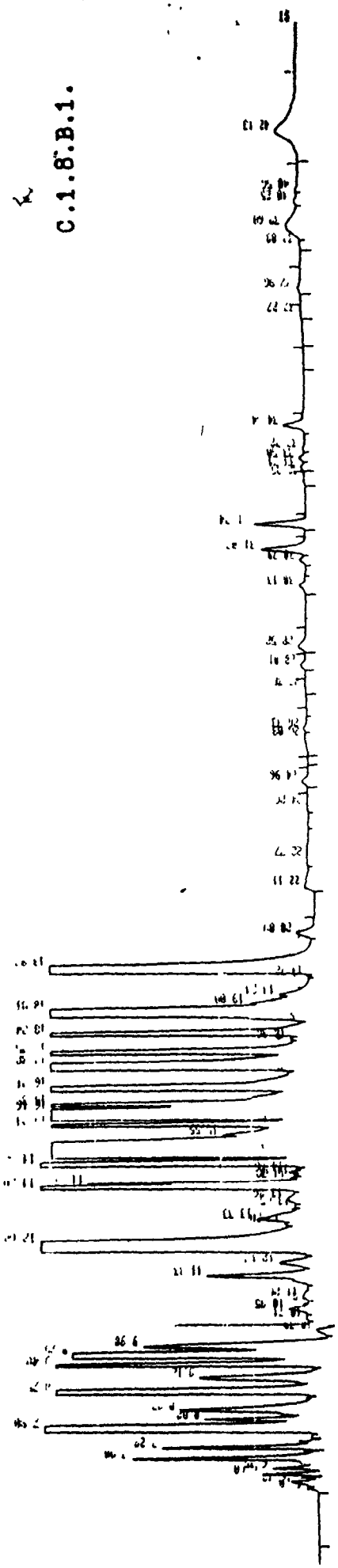

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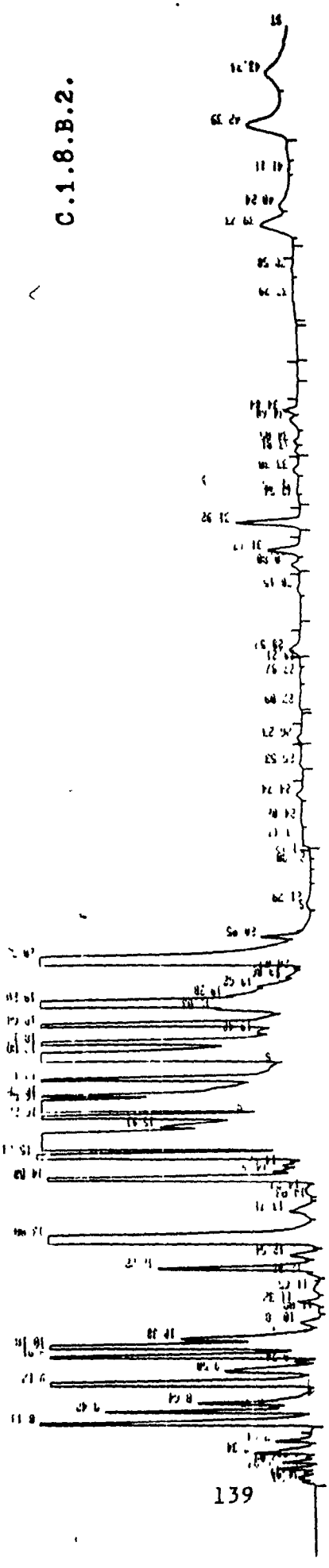

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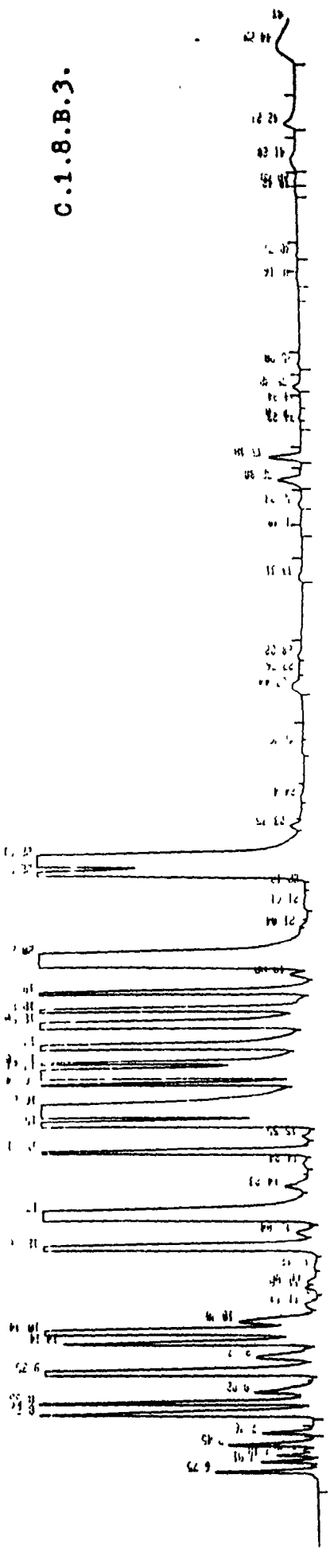

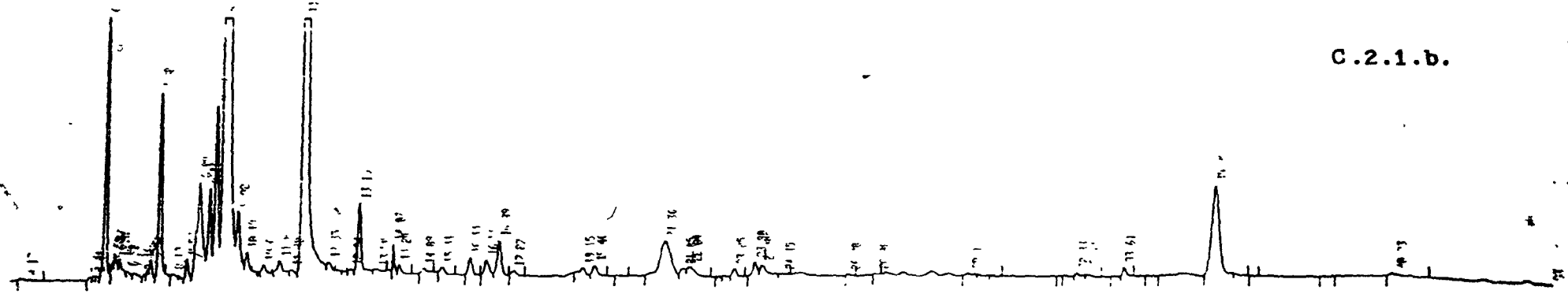

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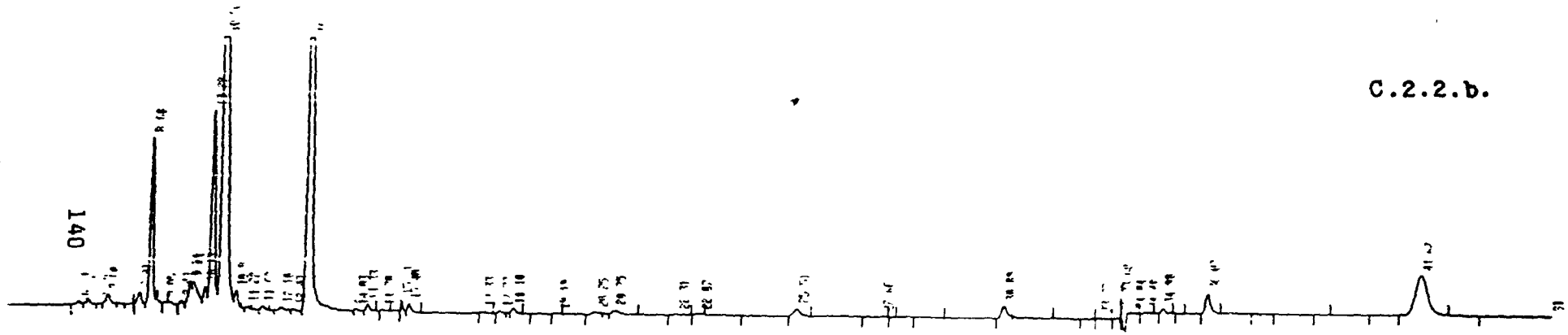

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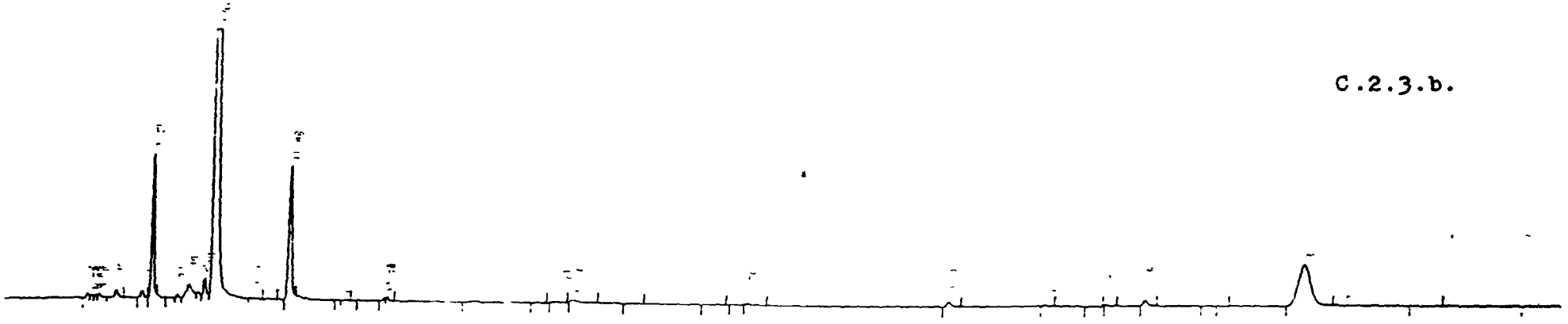

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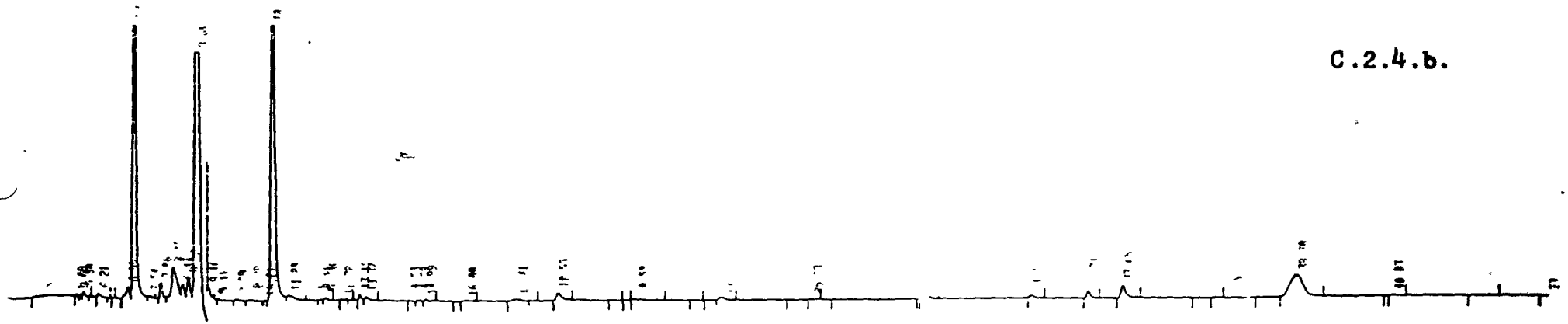

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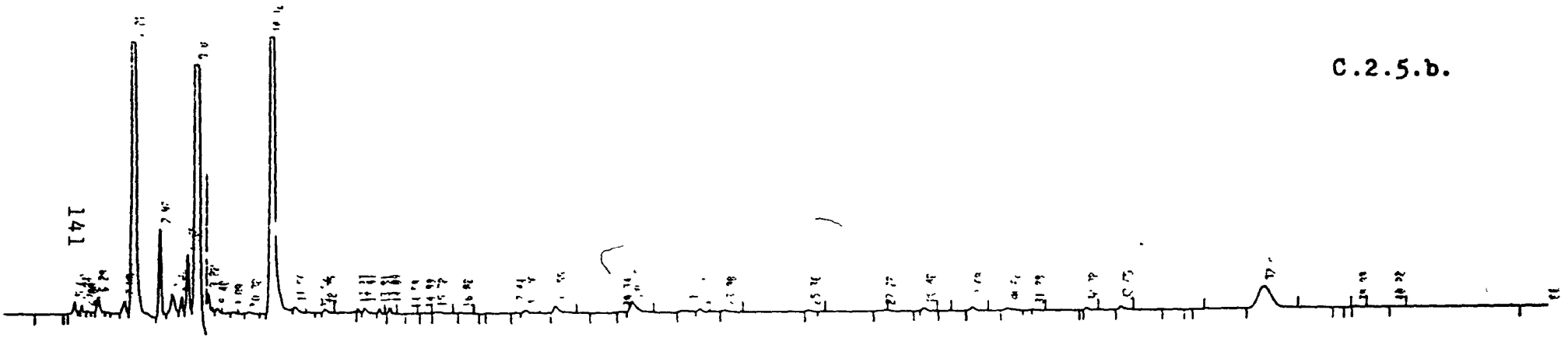

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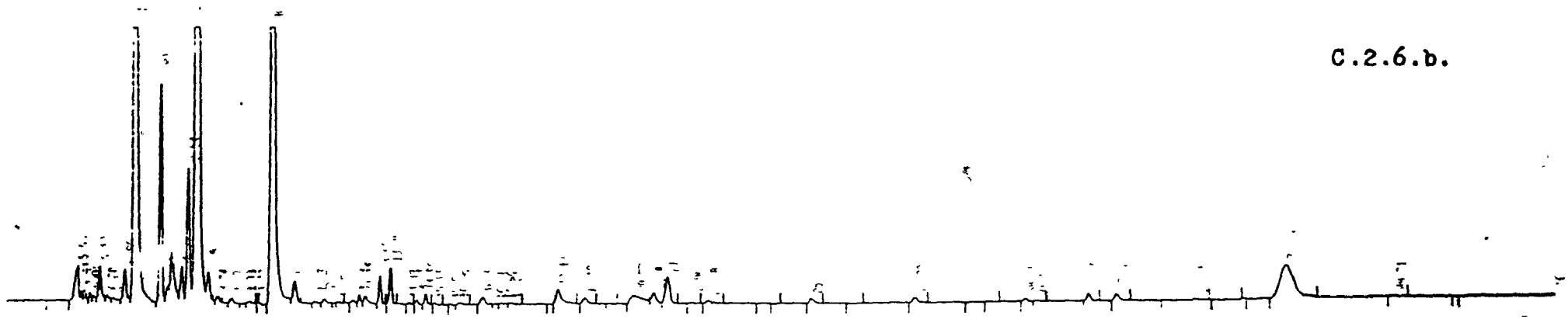

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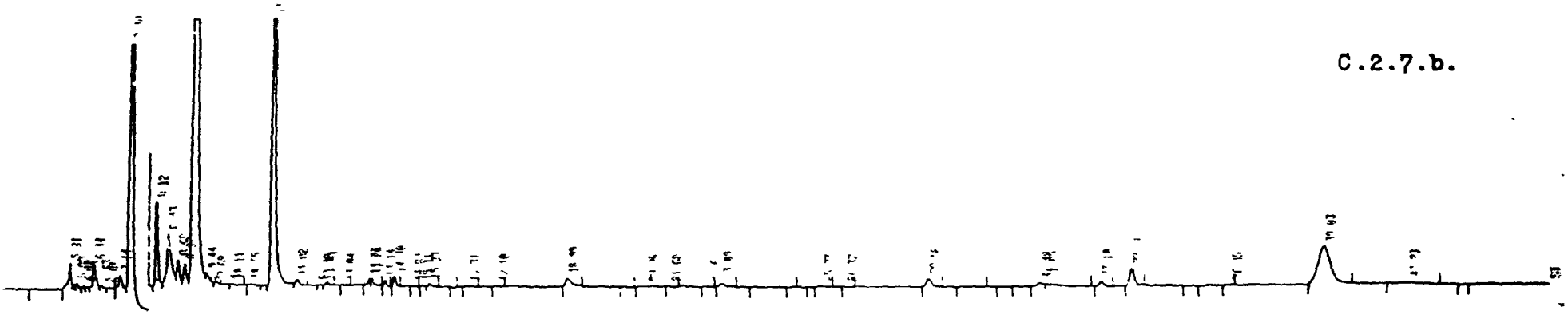

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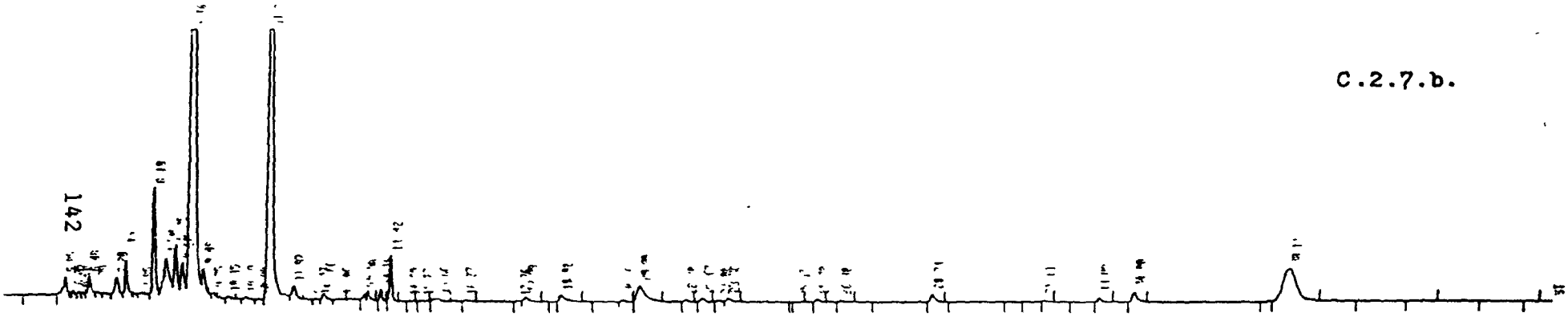

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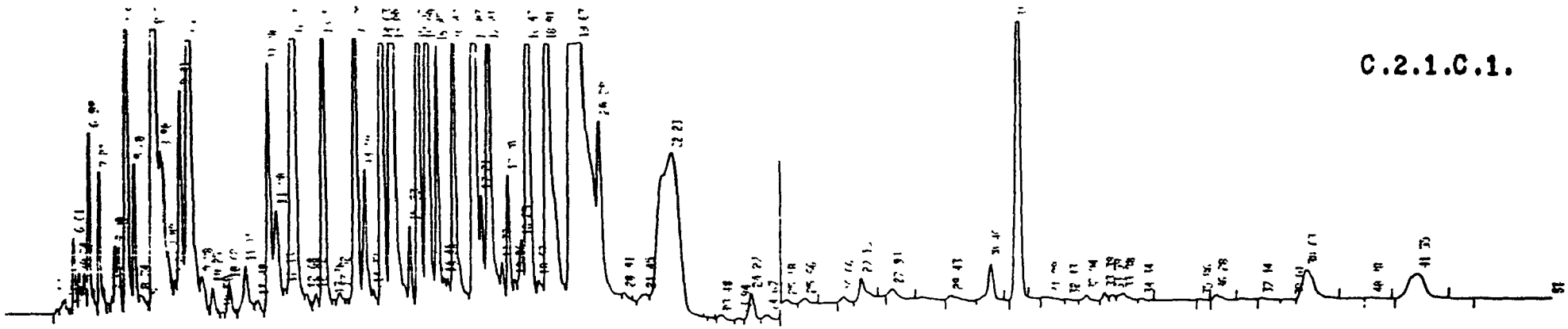

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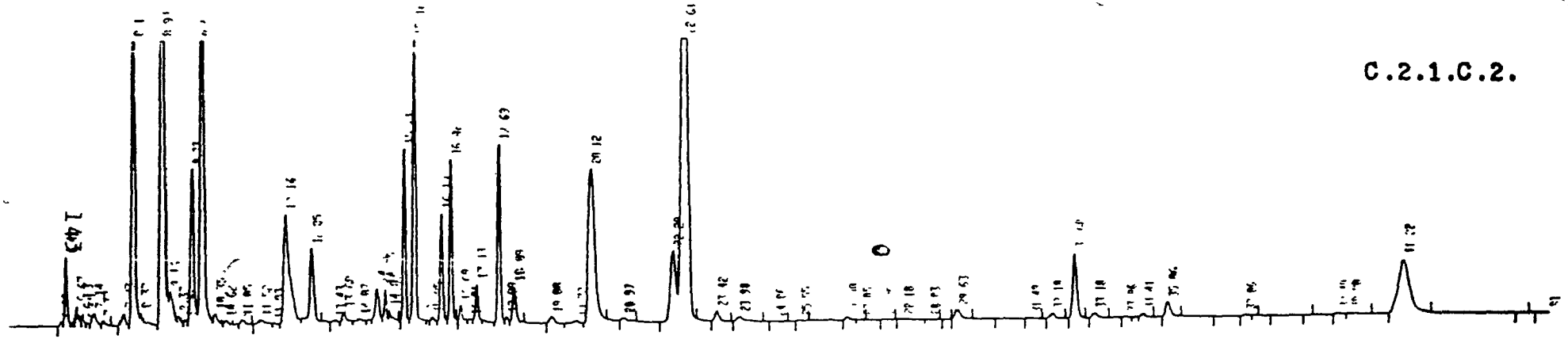

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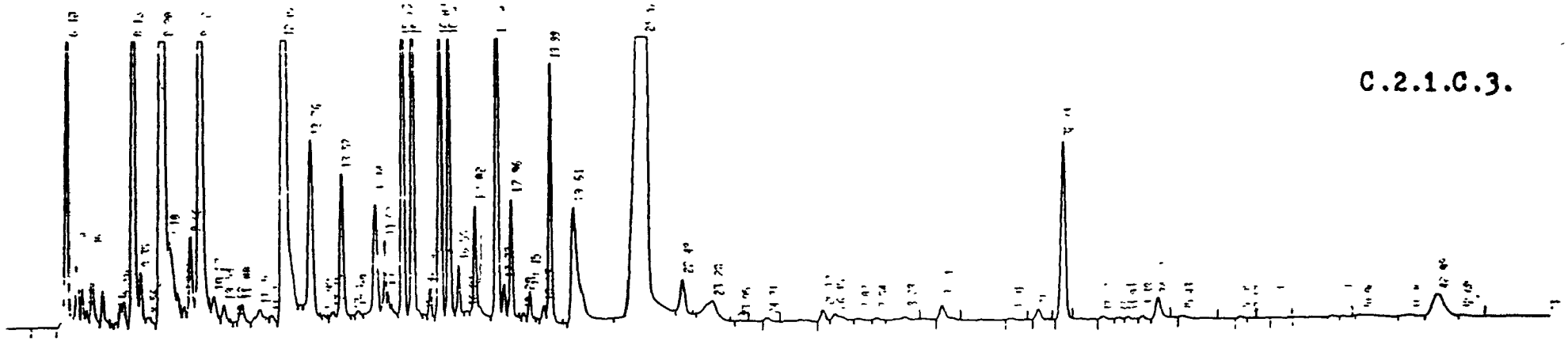

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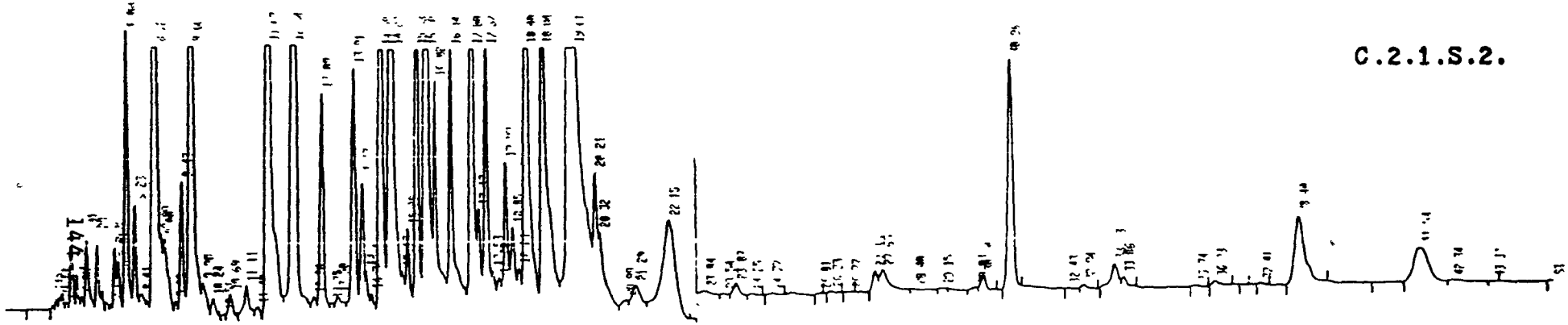
C.2.1.S.1.



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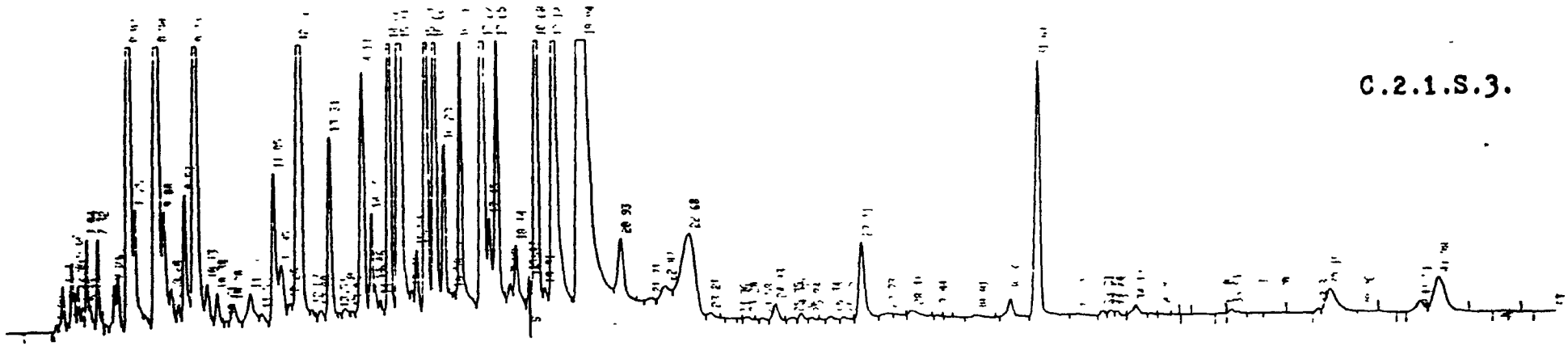

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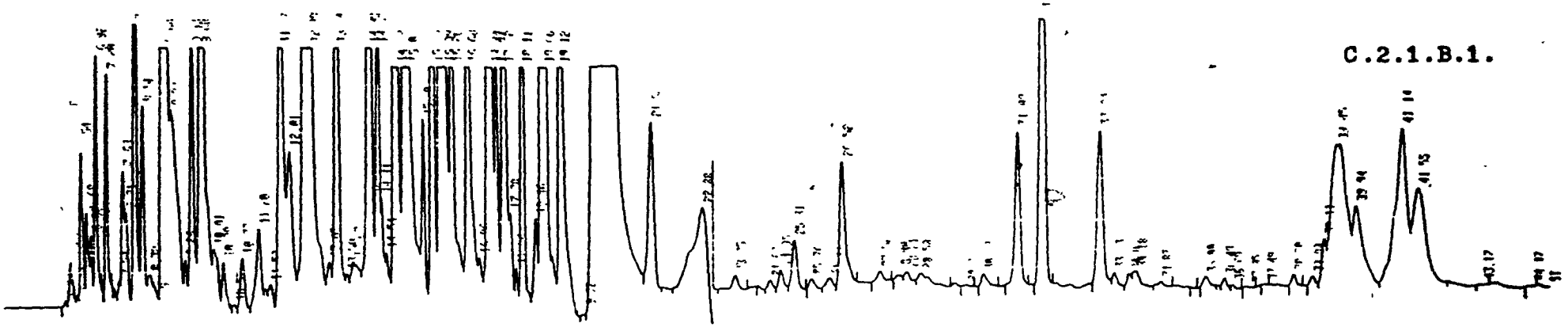

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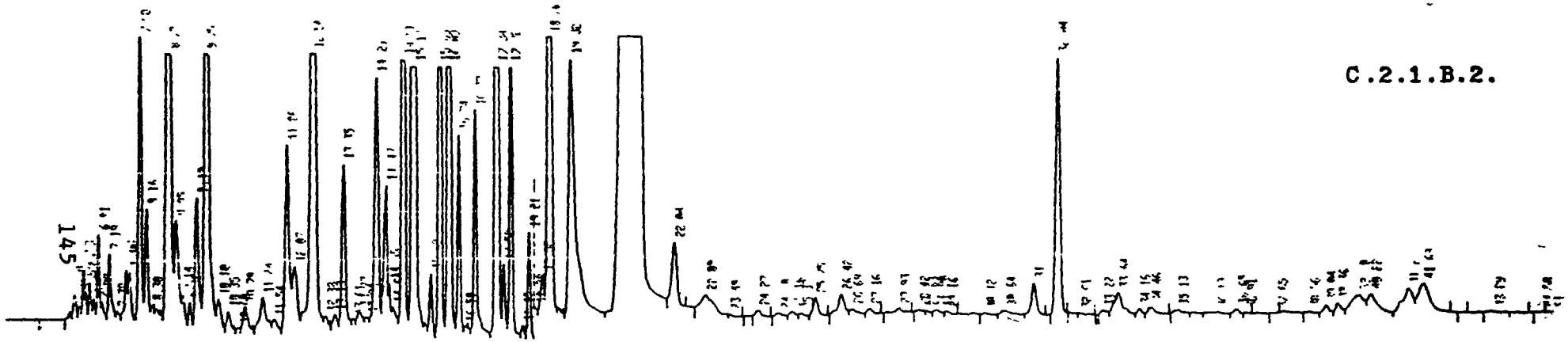

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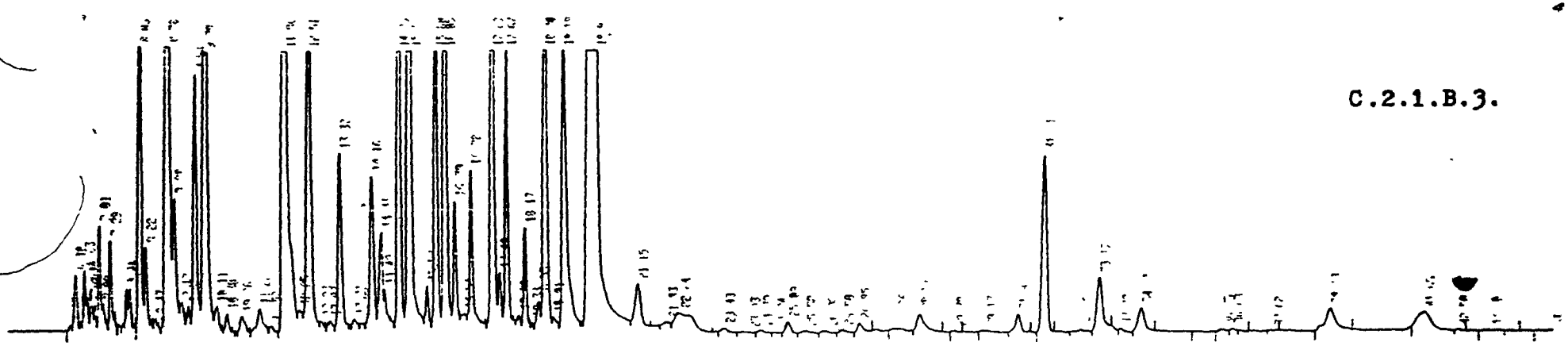

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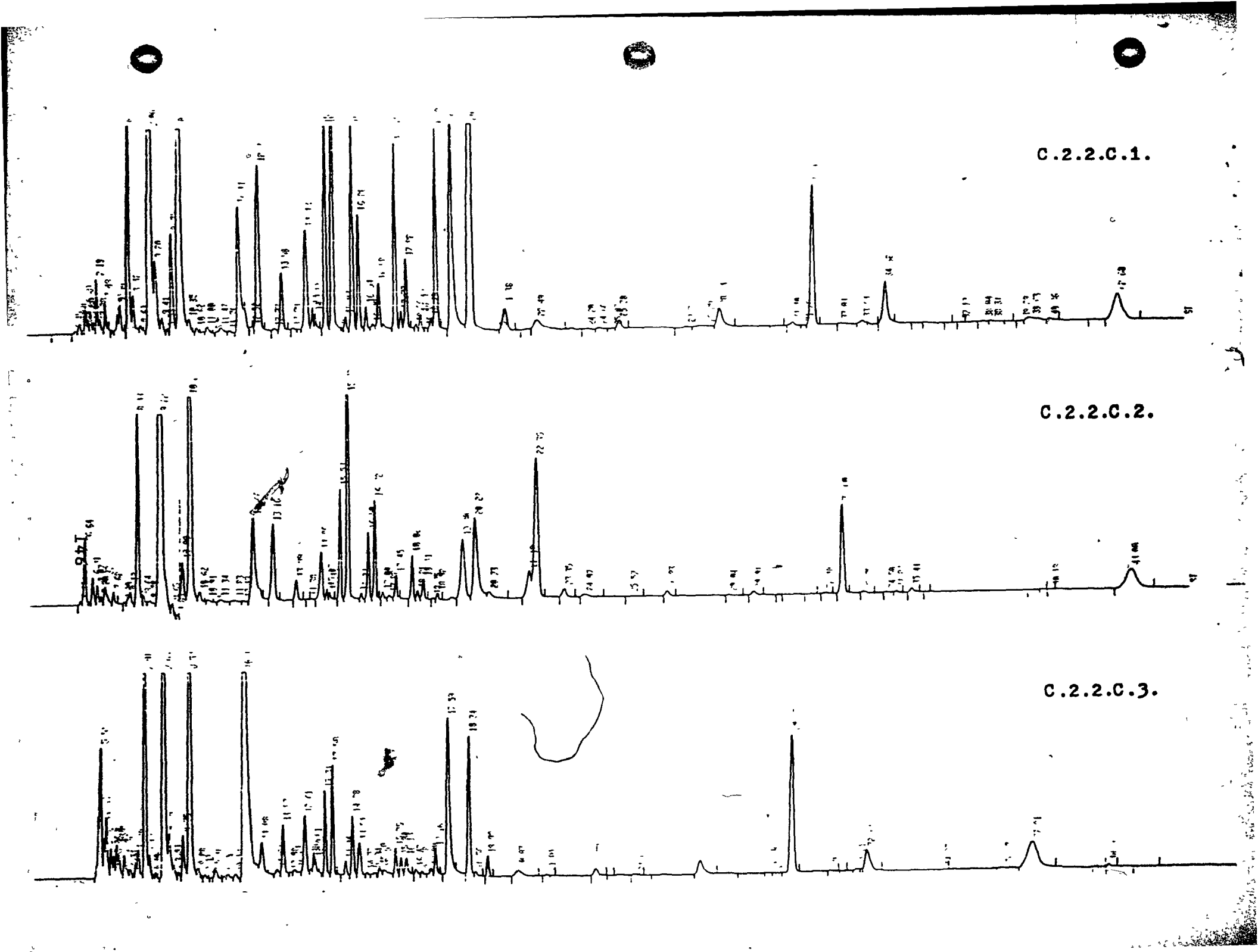
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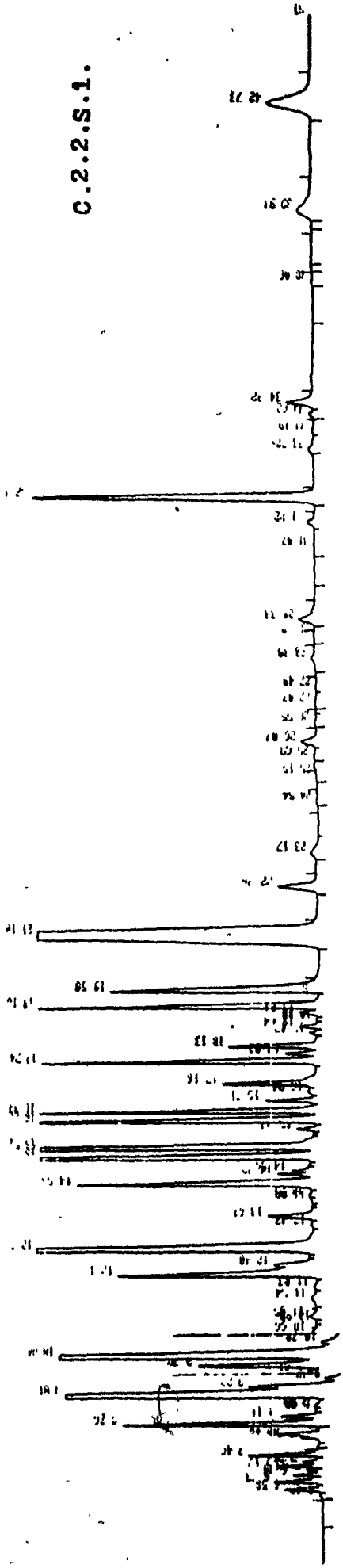
C.2.2.C.3.

C.2.2.S.1.



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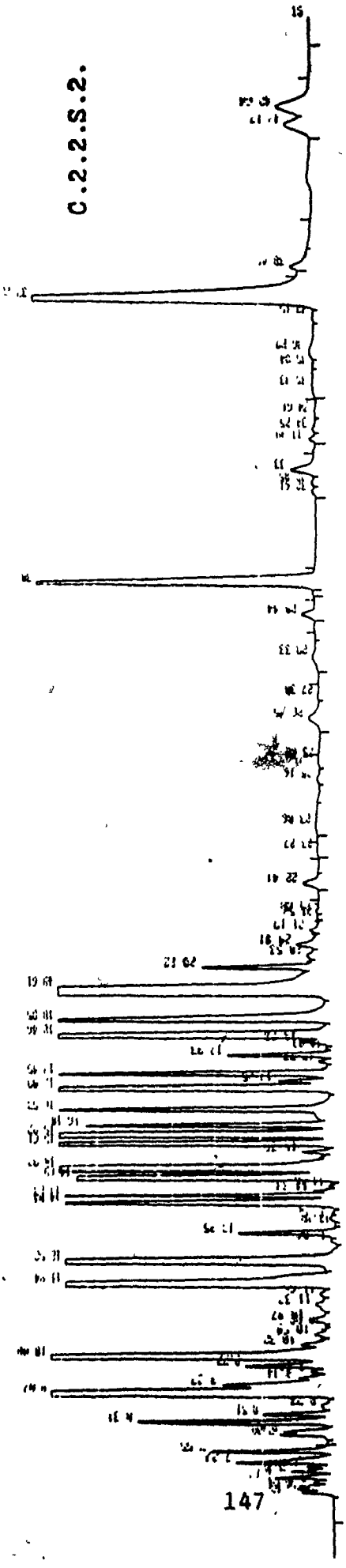

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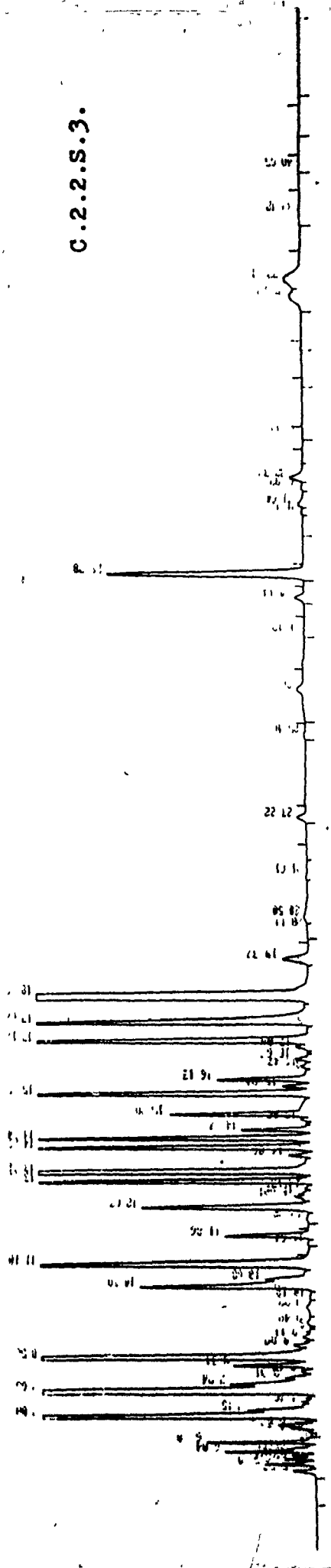

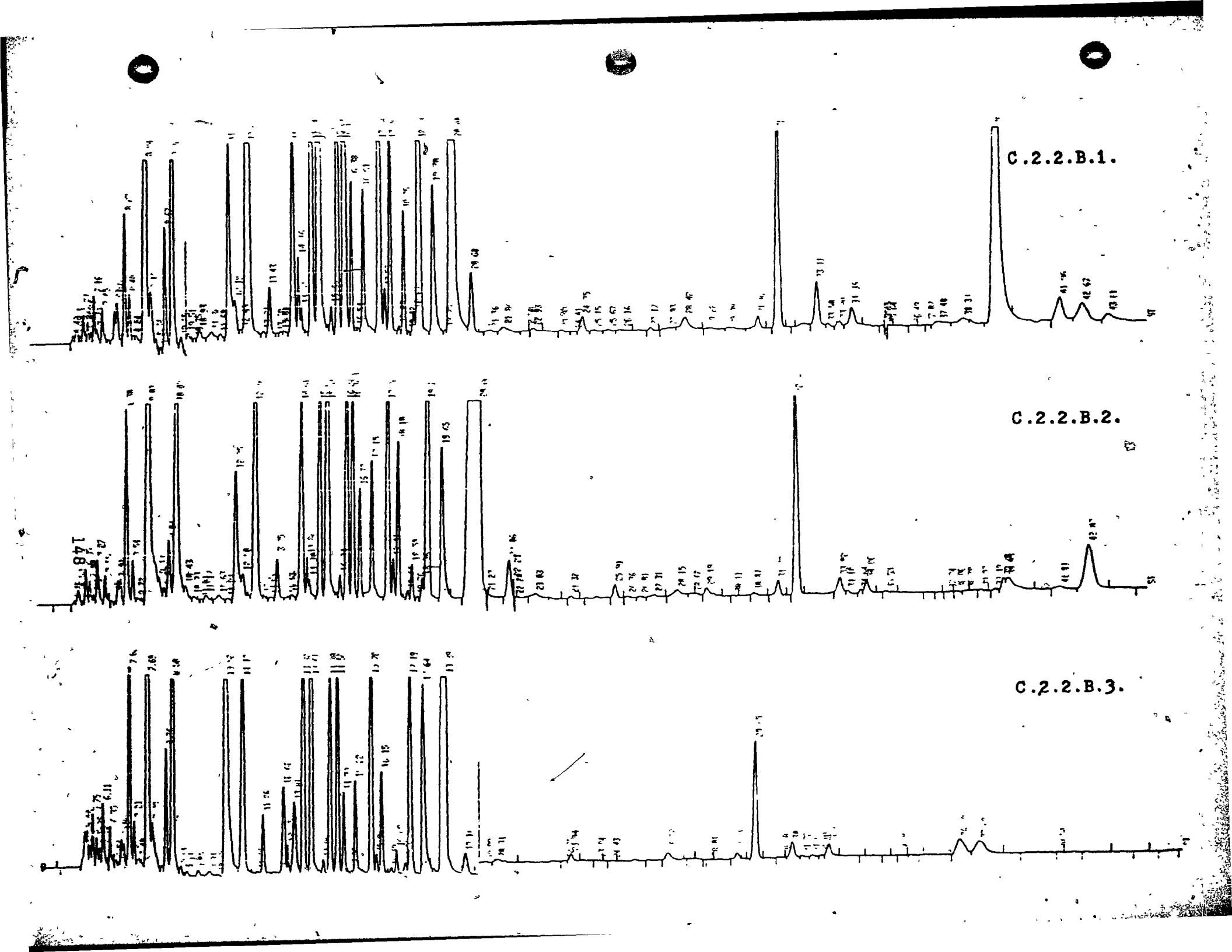
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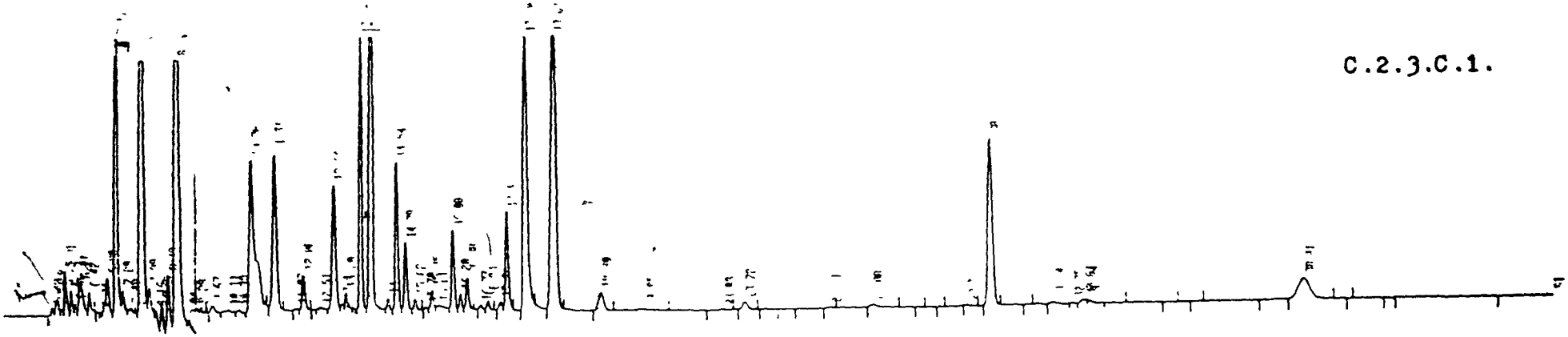
C.2.2.B.2.
C.2.2.B.3.



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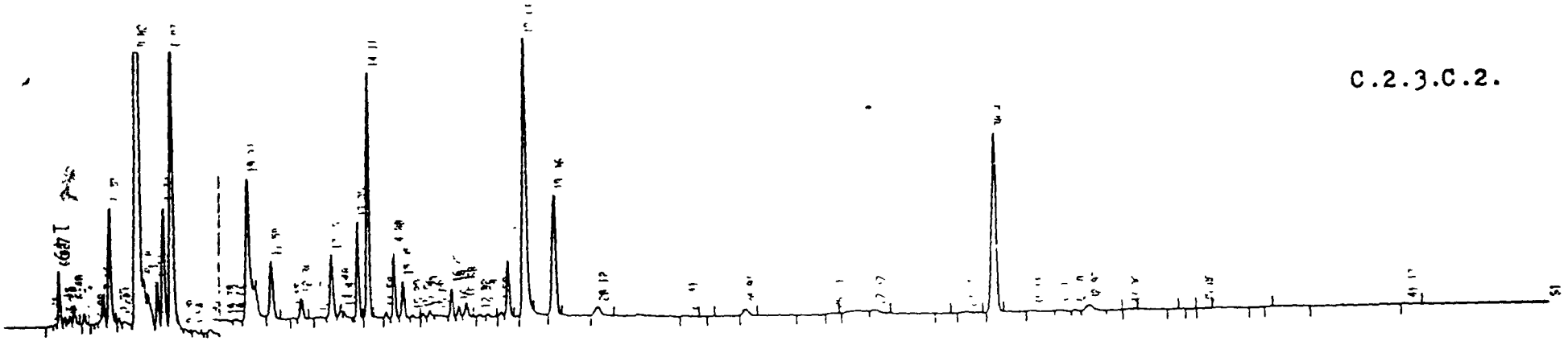

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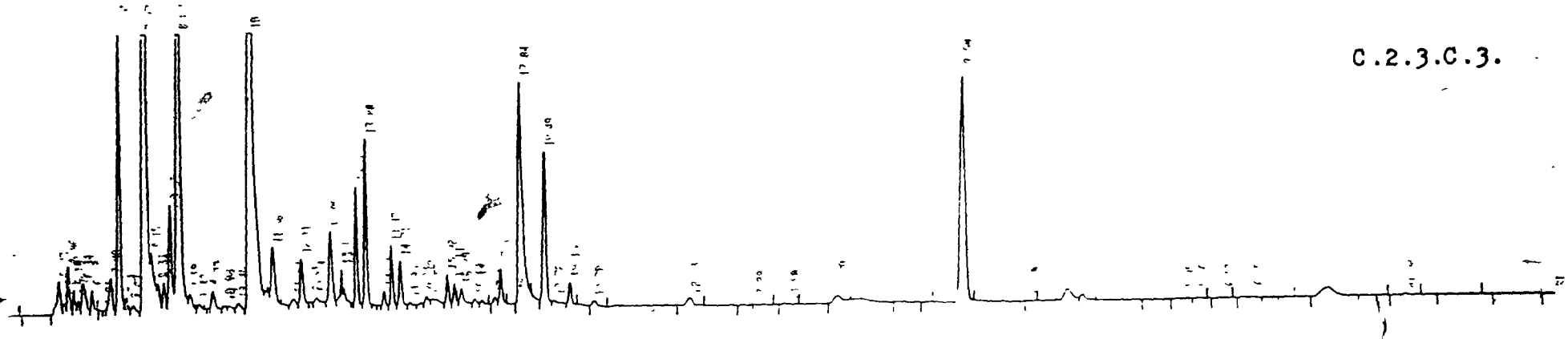

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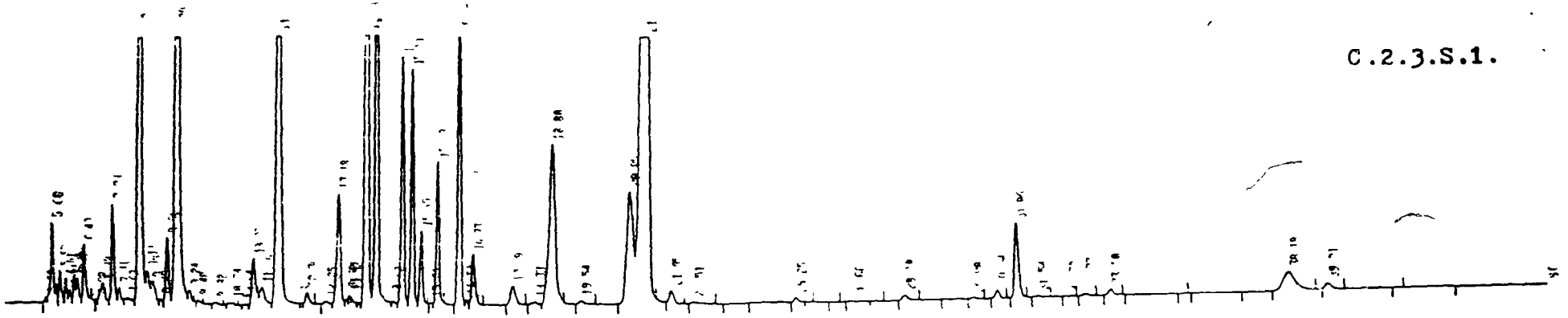

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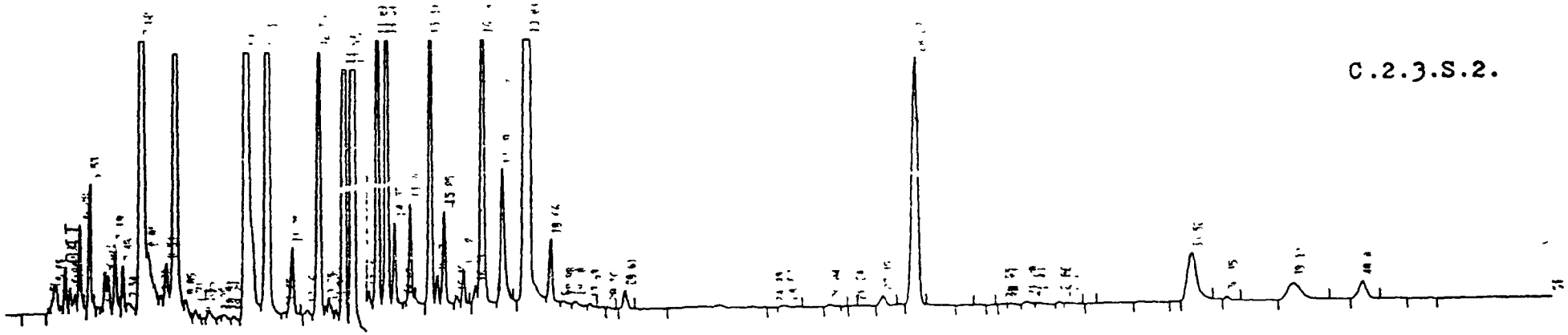

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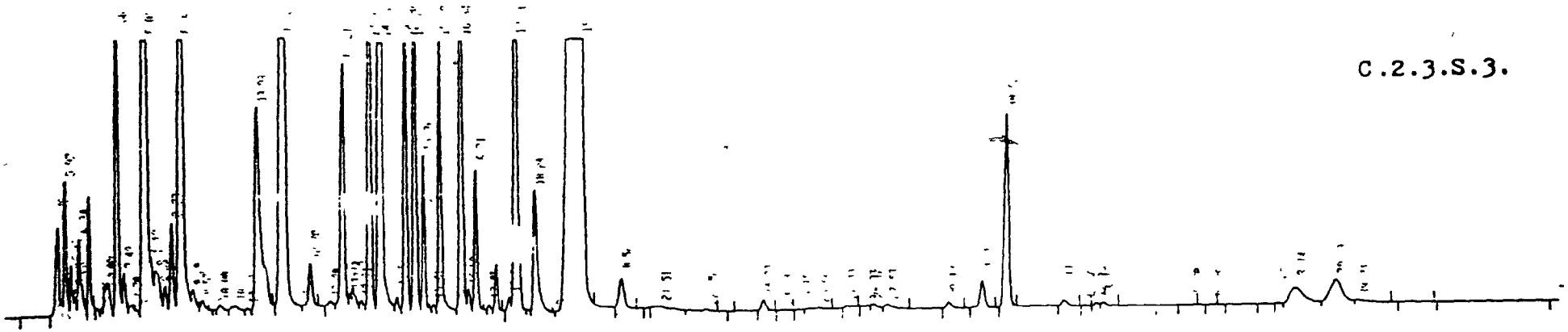
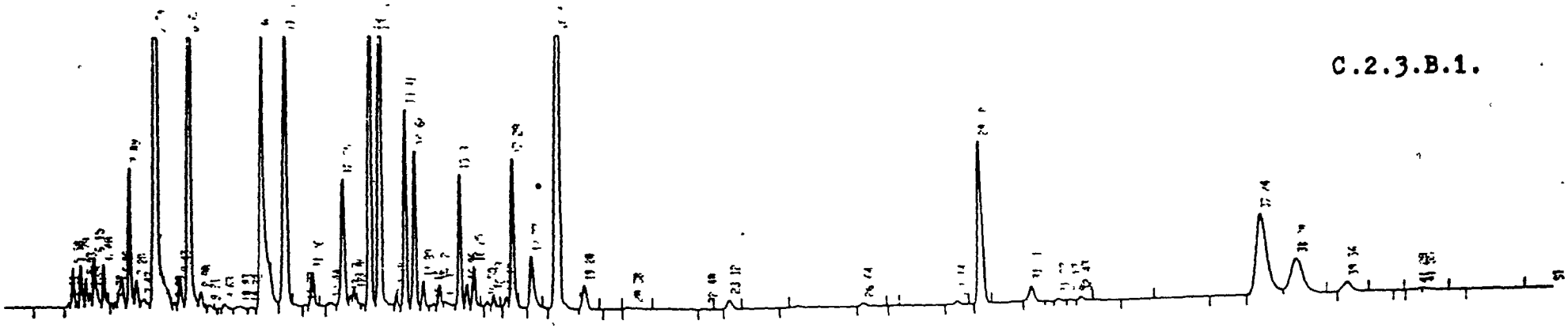


C.2.3.S.3.



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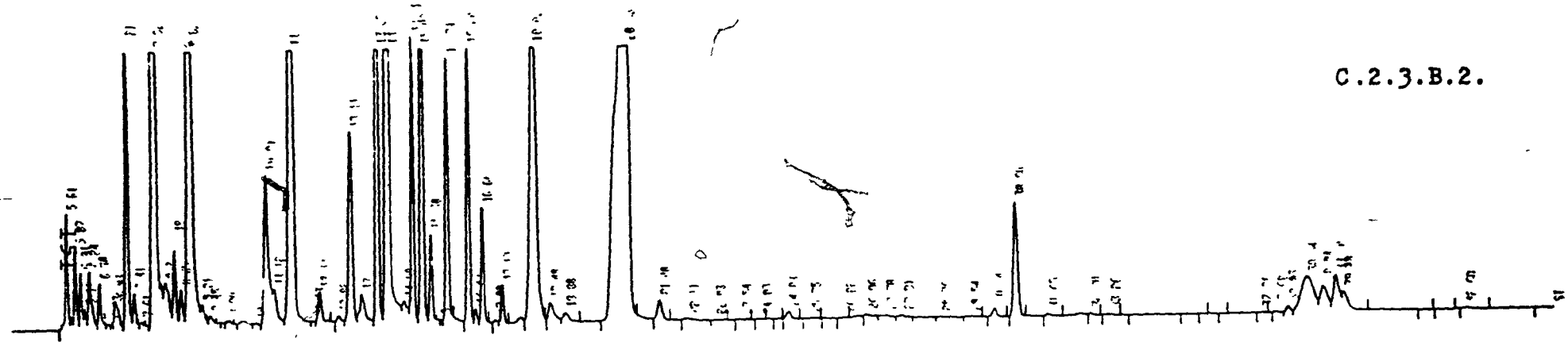

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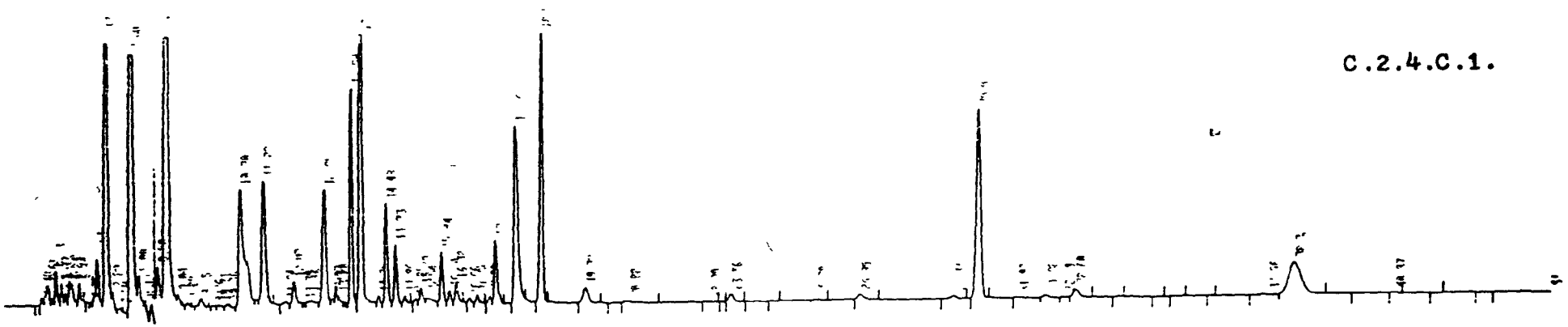
C.2.3.B.3.



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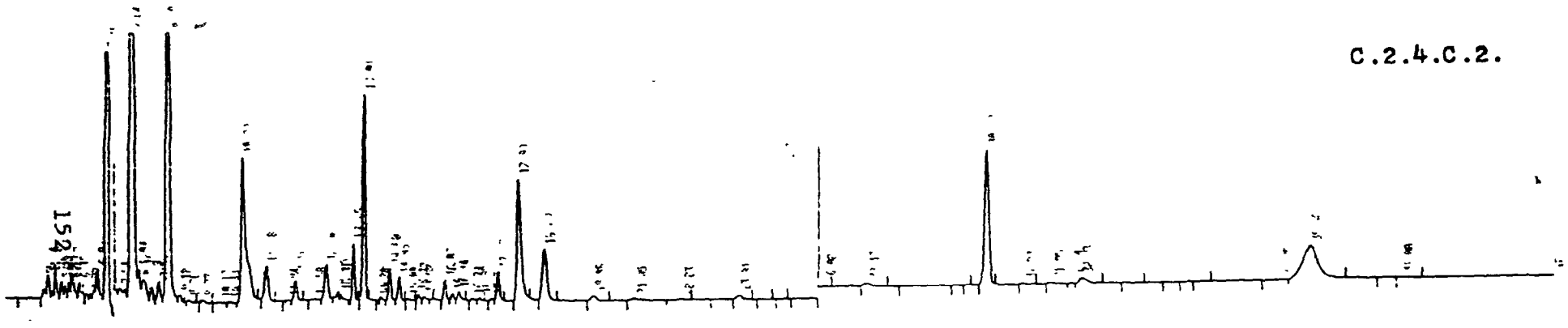

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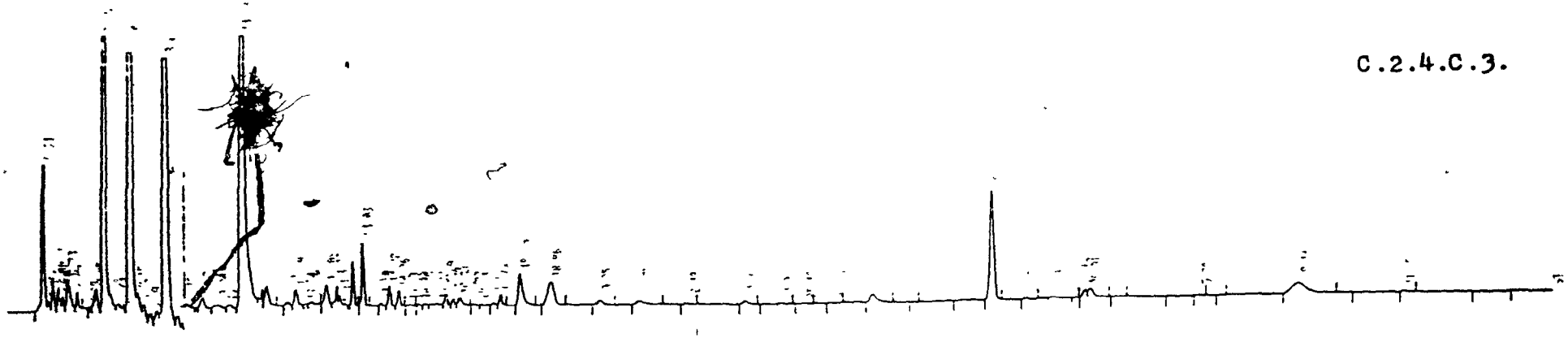

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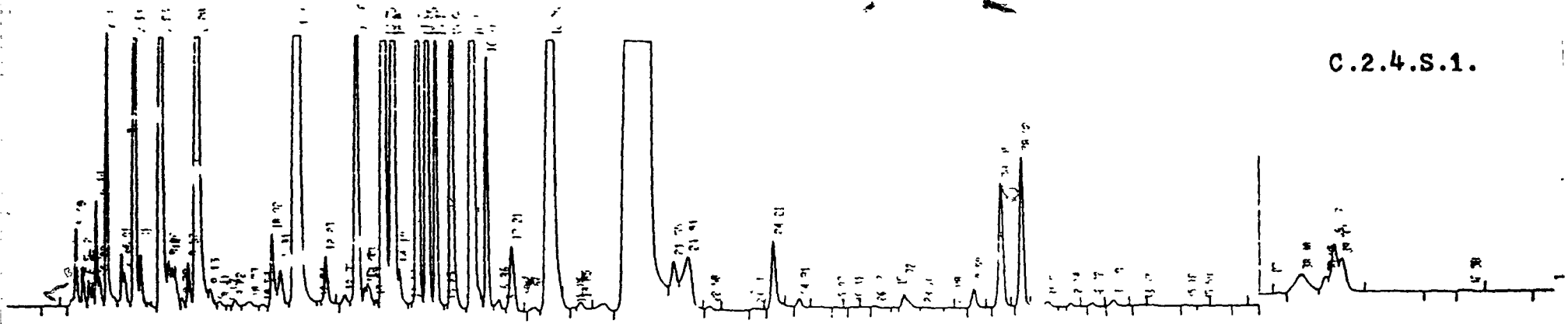

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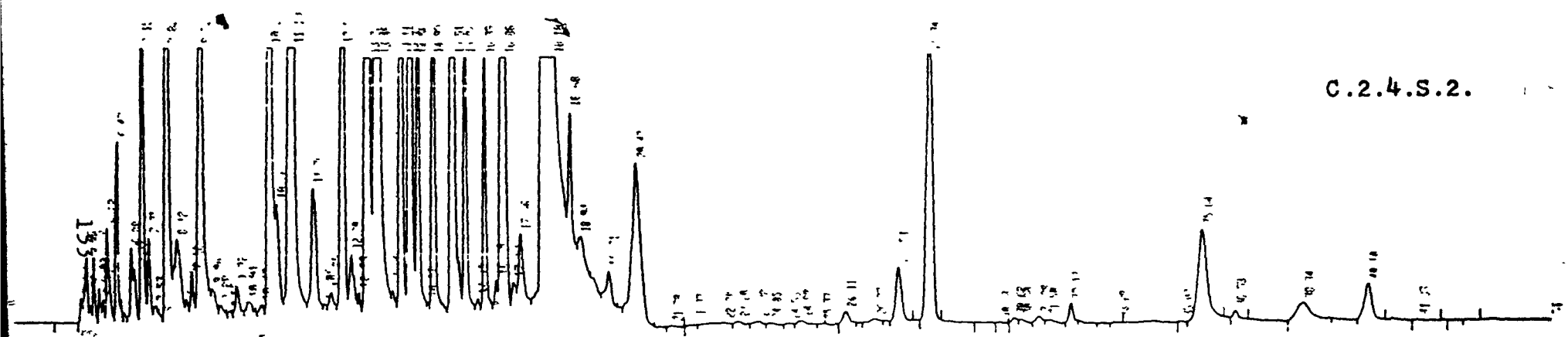

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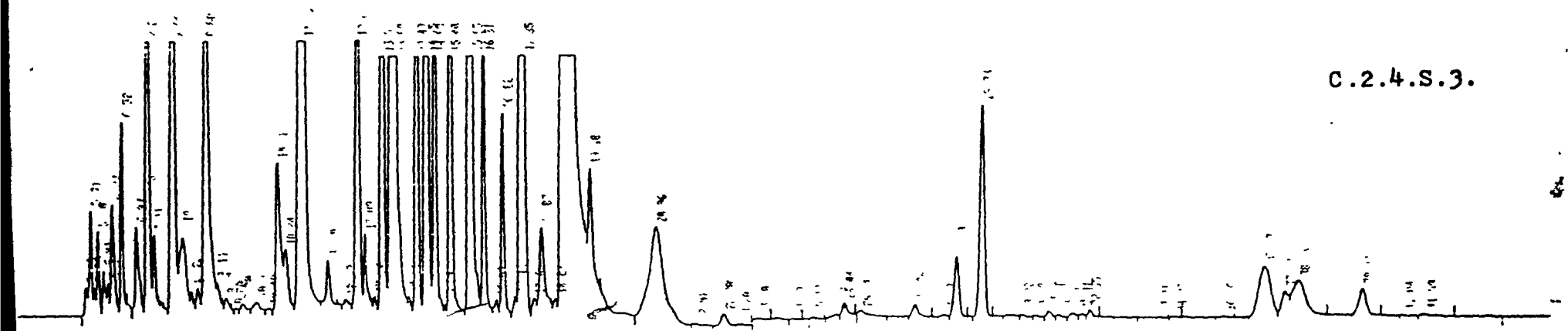

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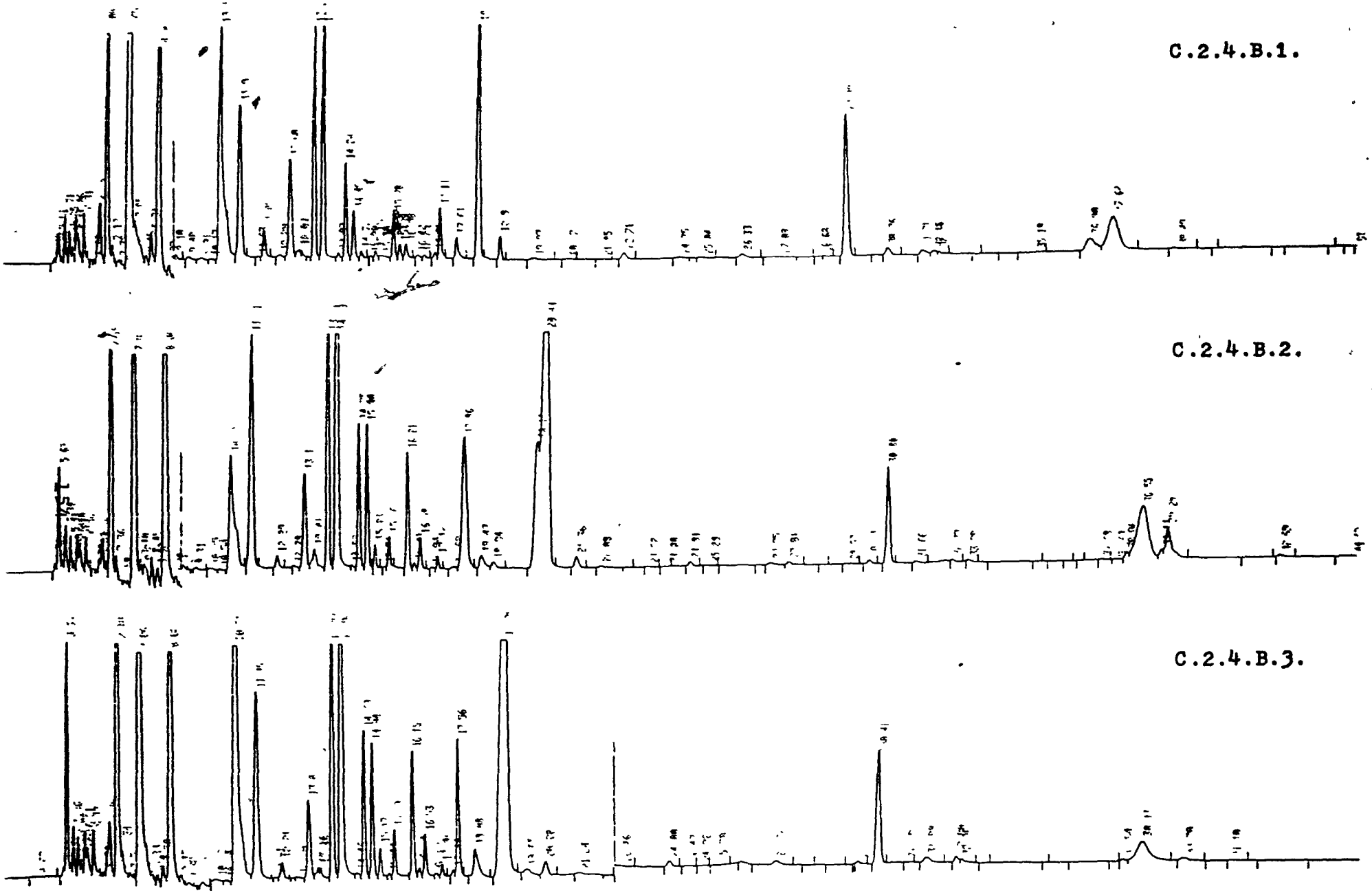
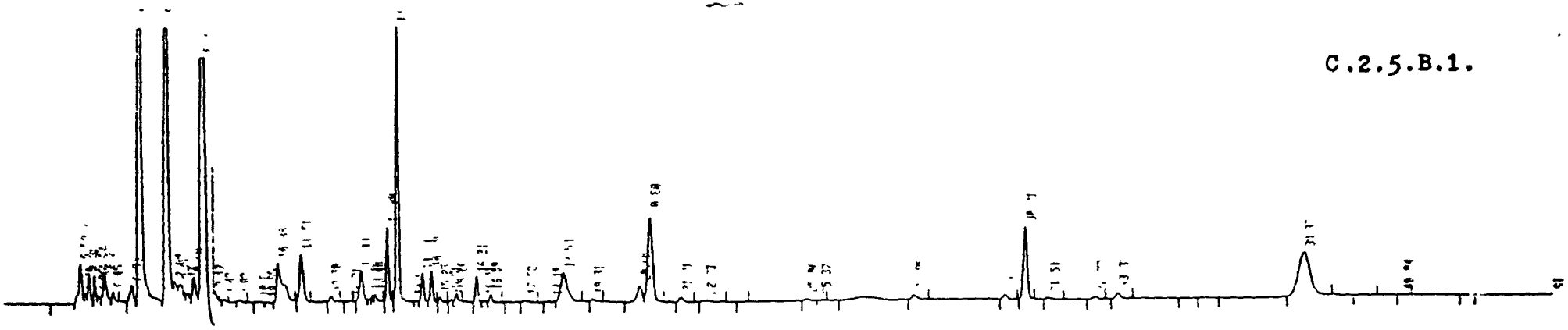
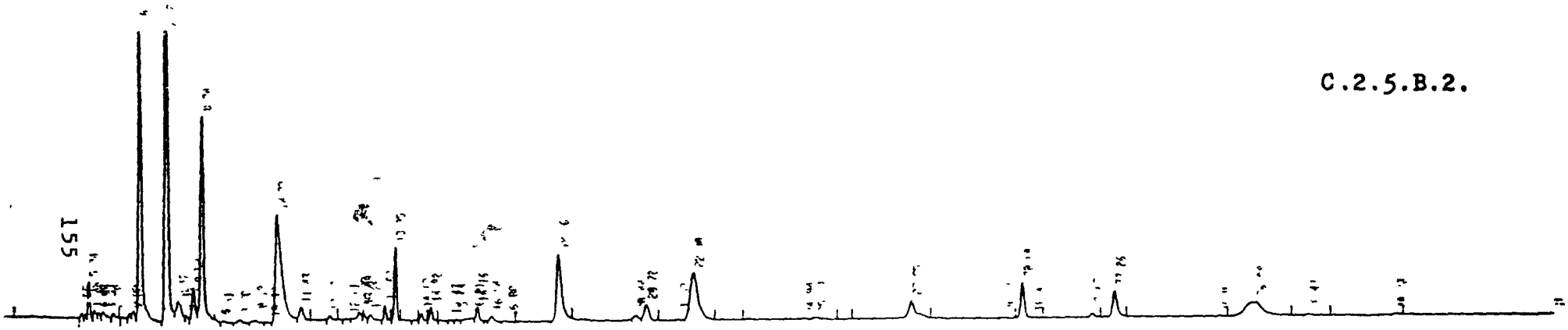

C.2.4.B.1.
C.2.4.B.2.
C.2.4.B.3.



C.2.5.B.1.



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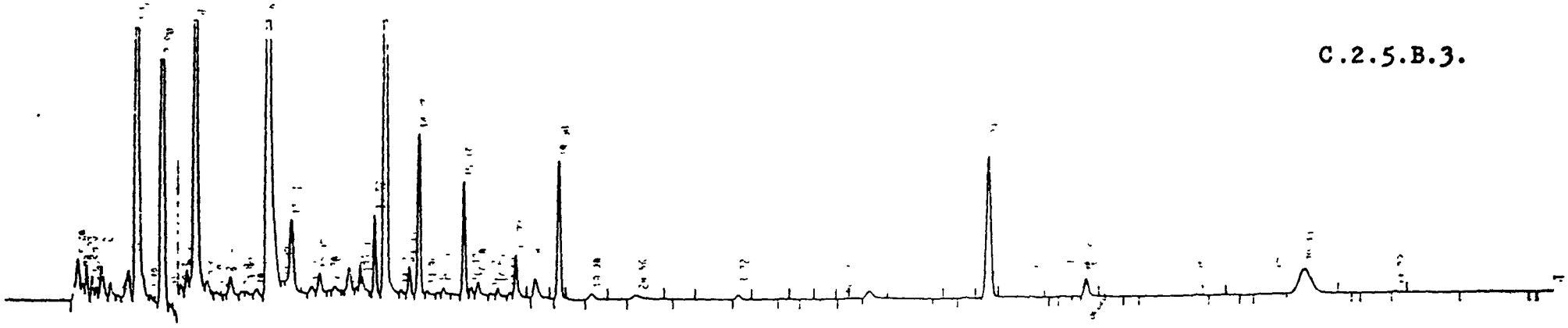

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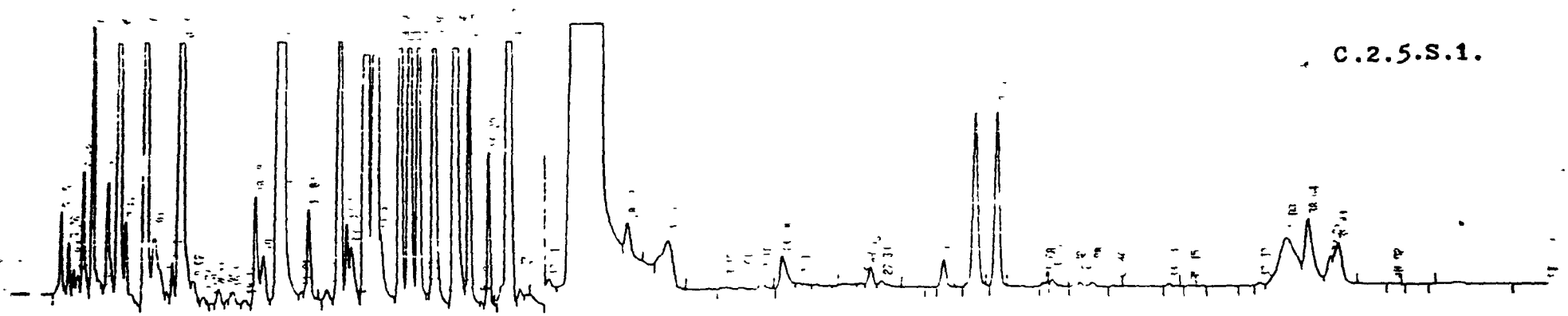

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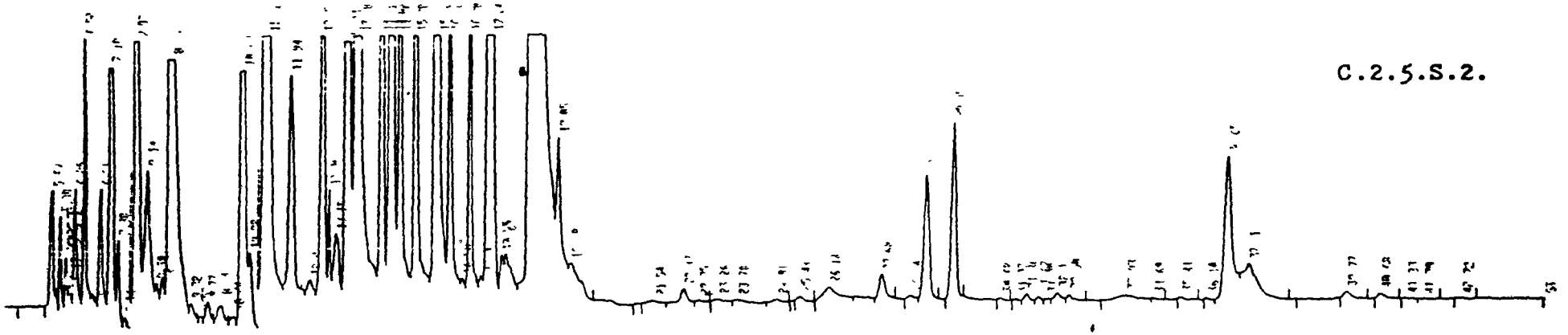

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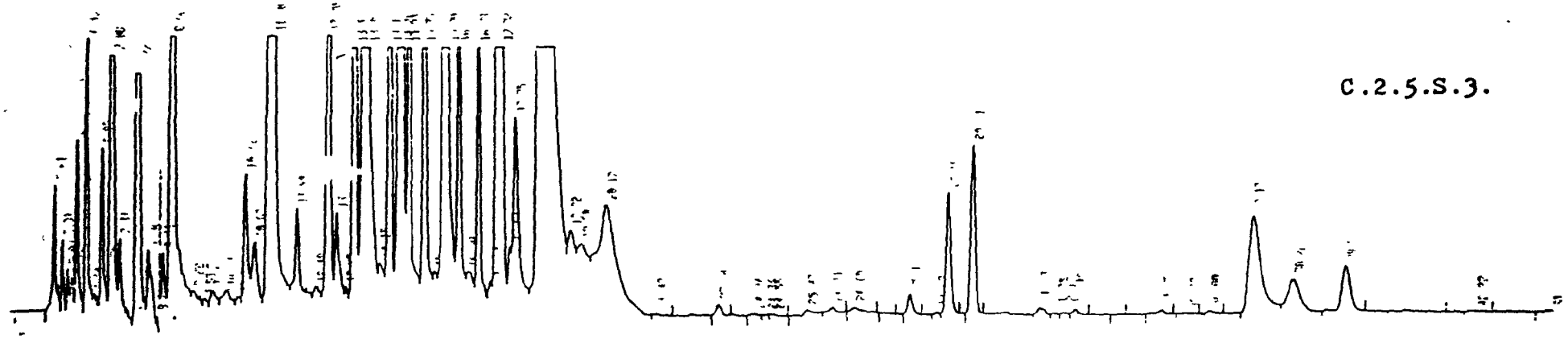

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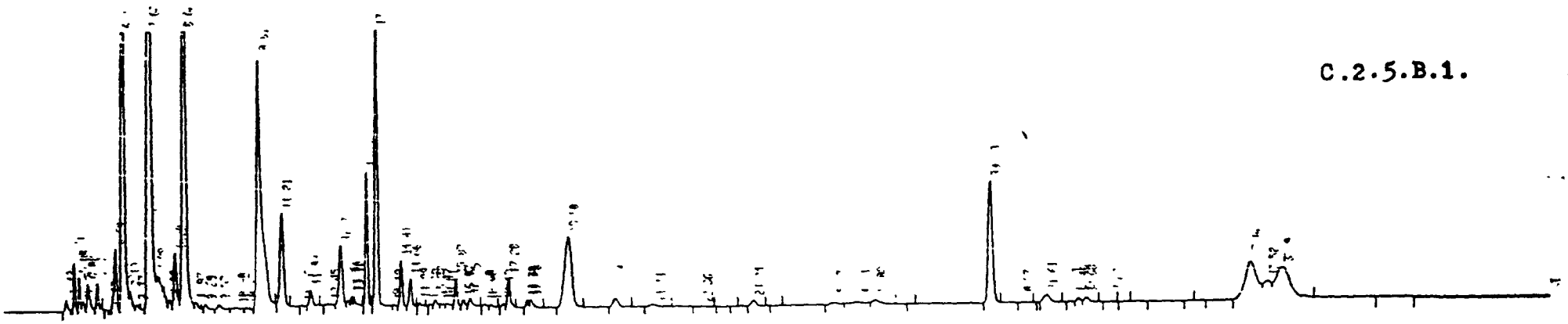

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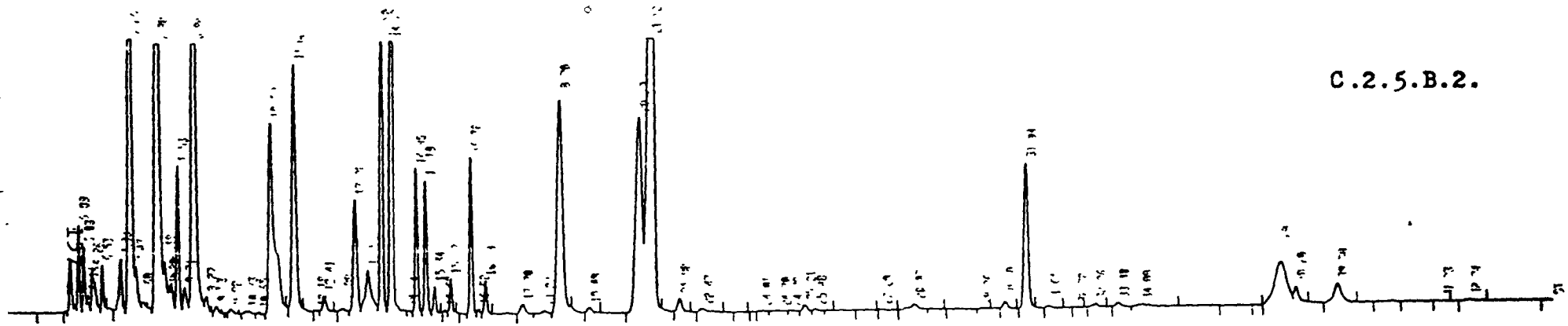

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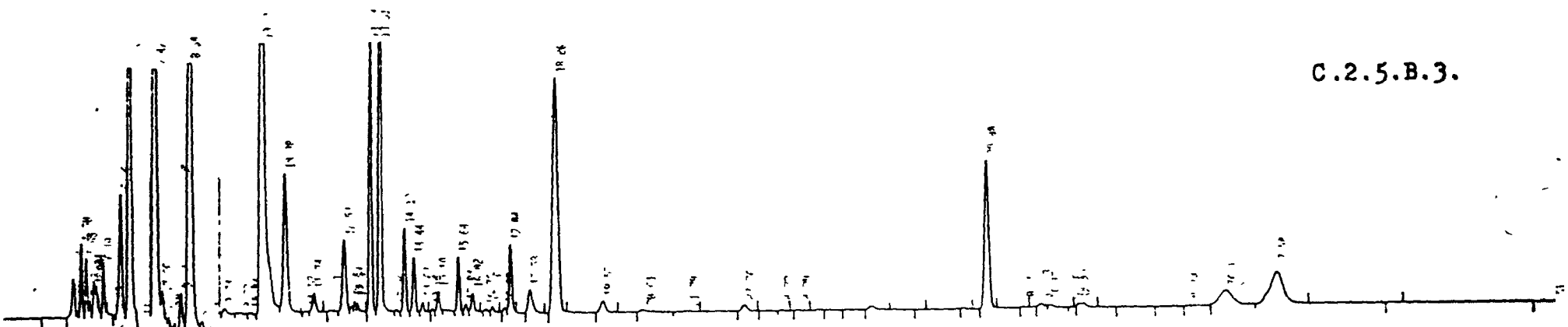

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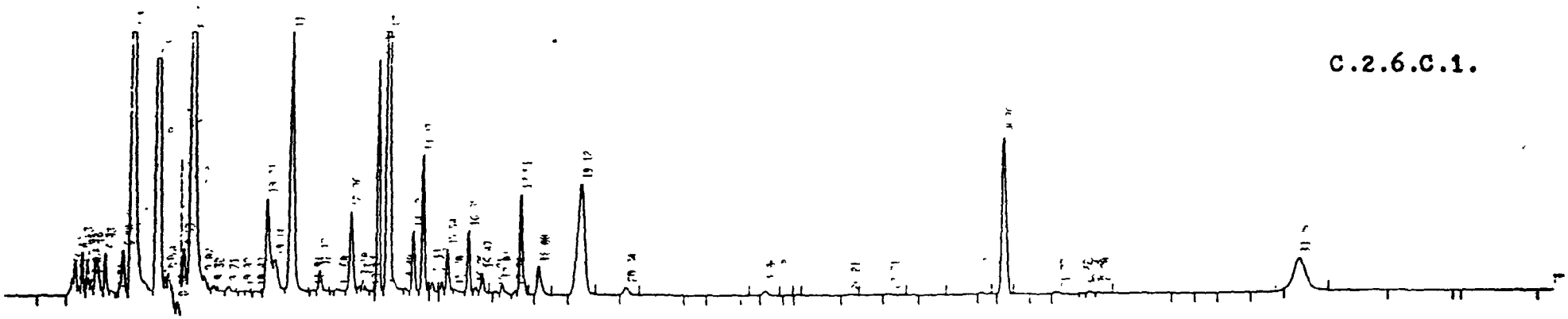

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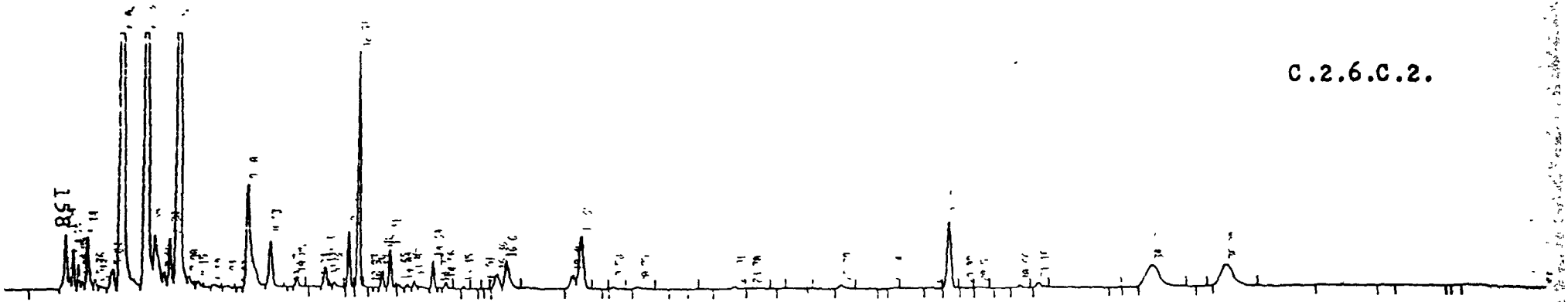

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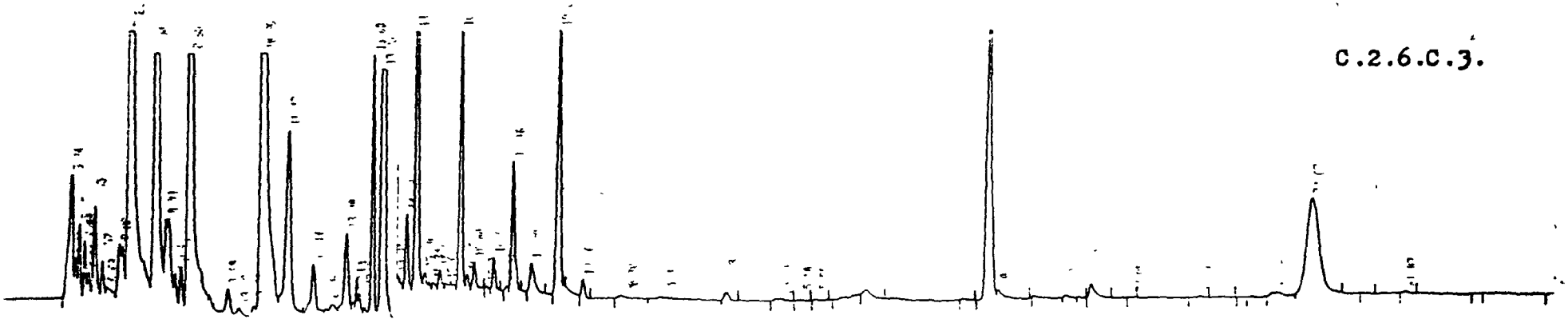

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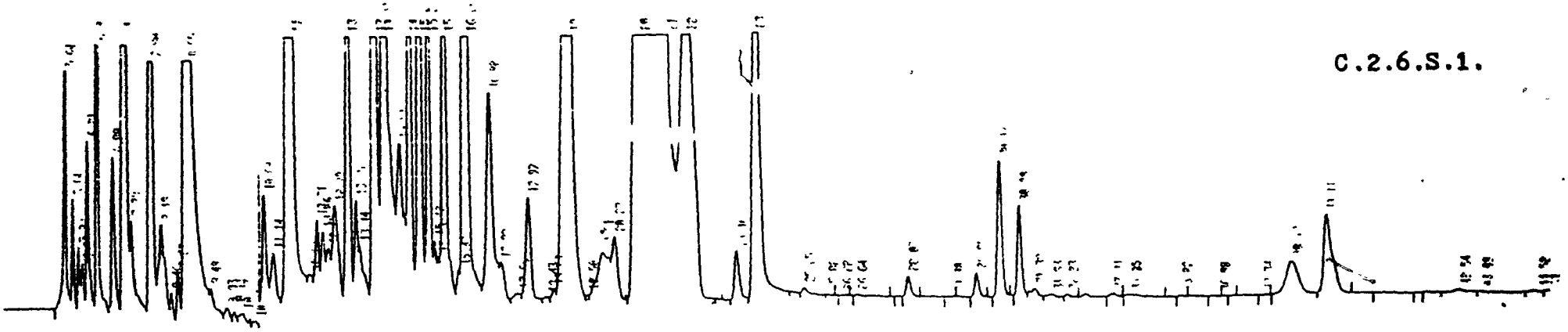
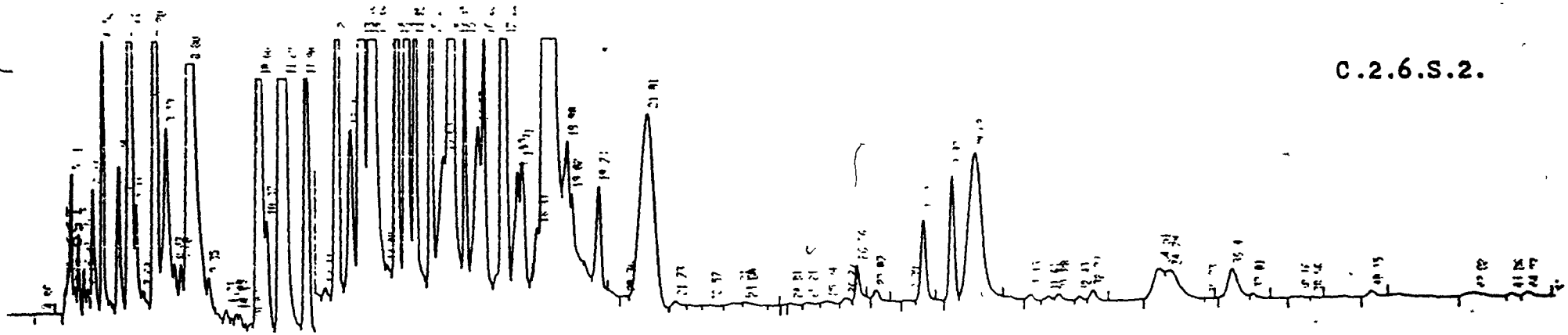


C.2.6.S.1.



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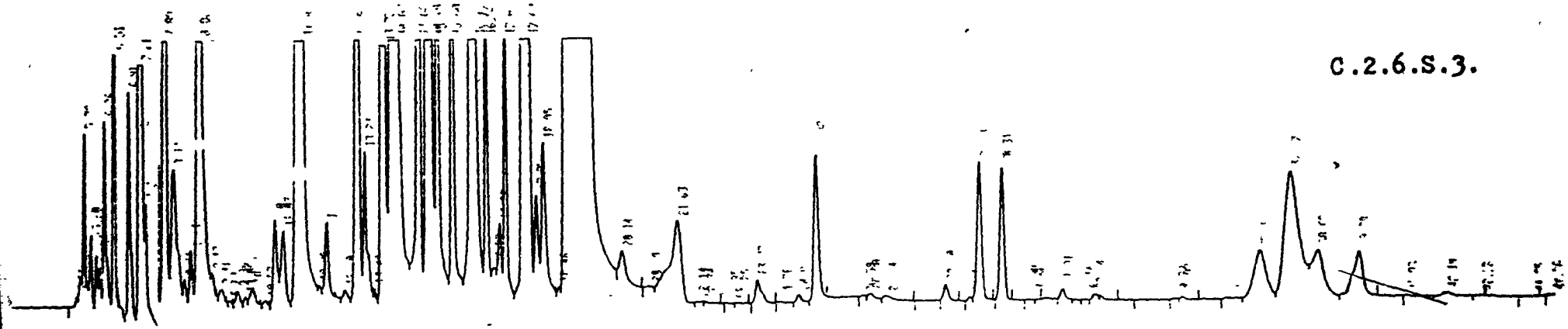

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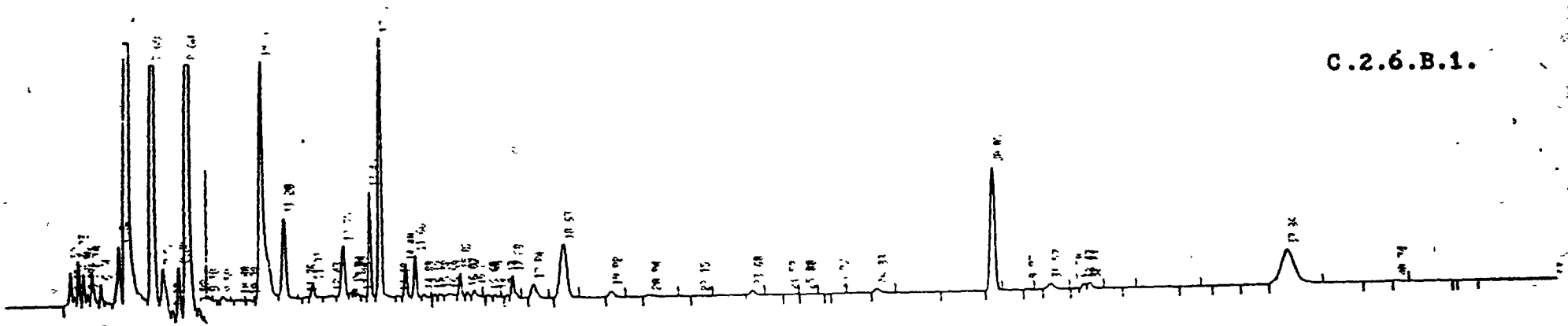

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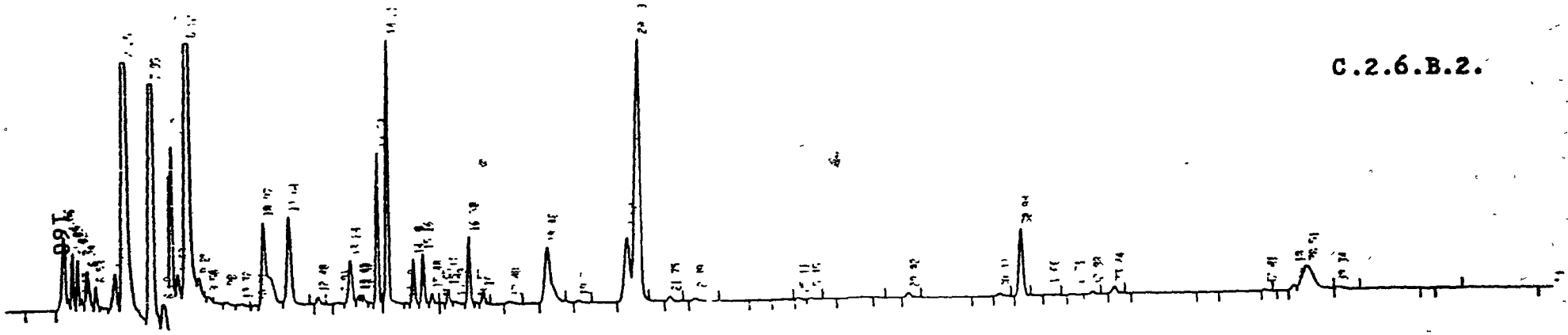

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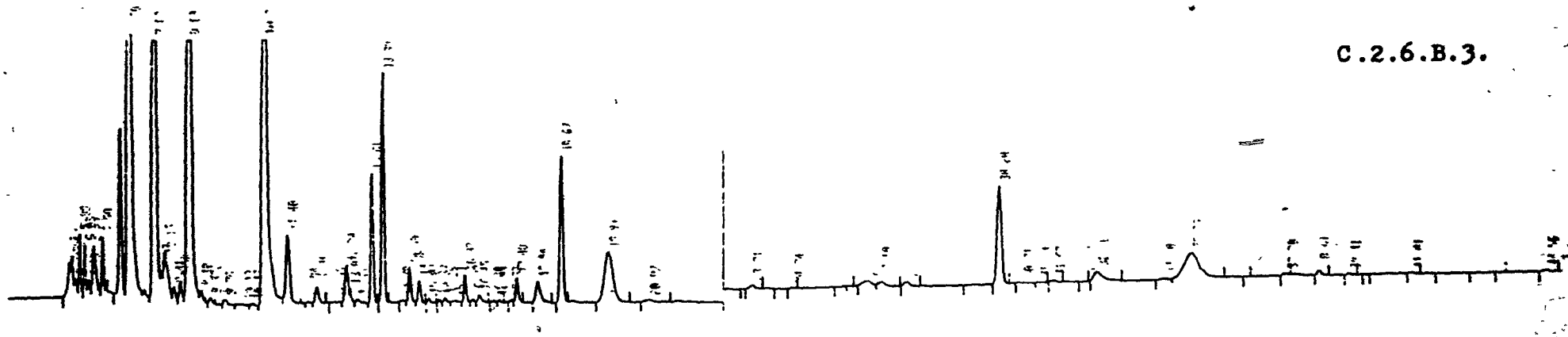

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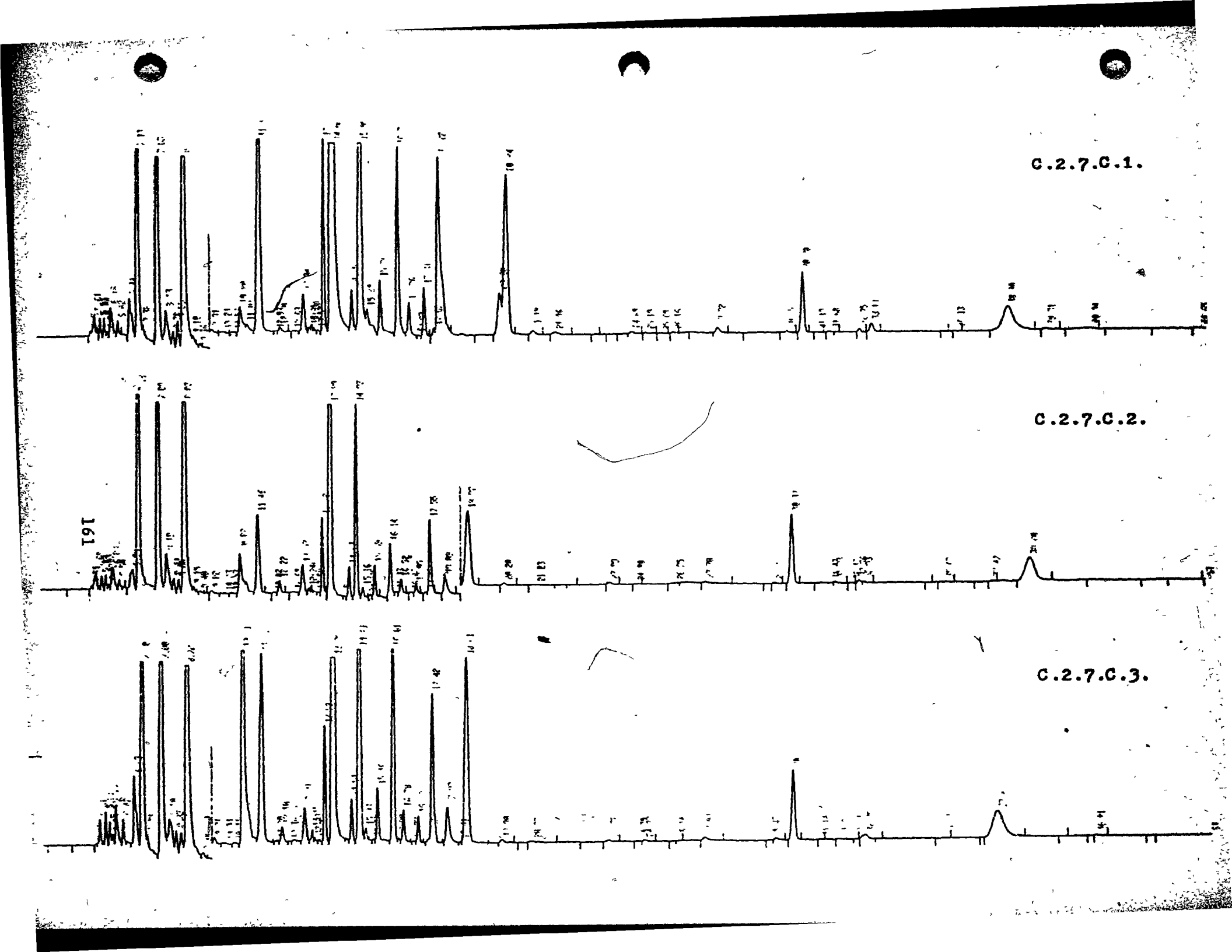
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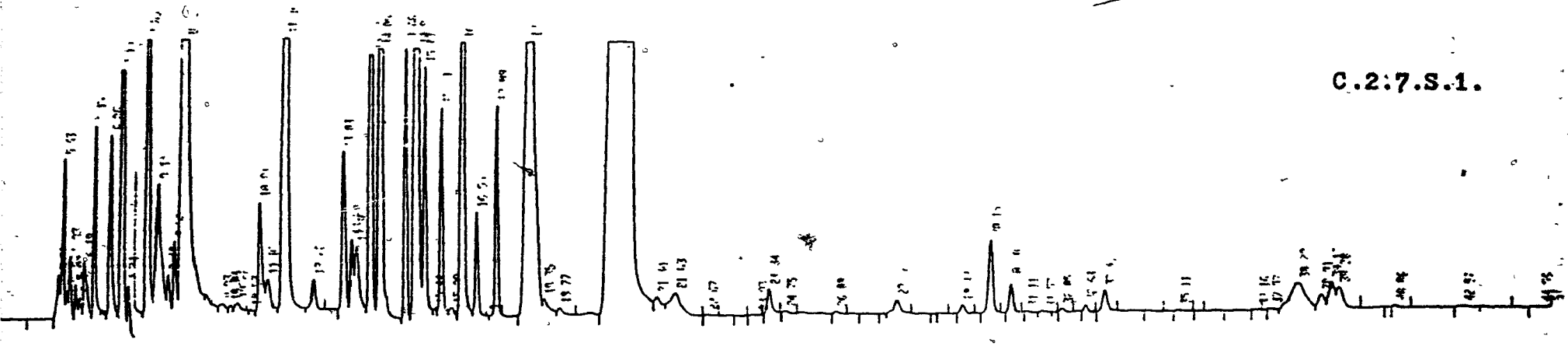
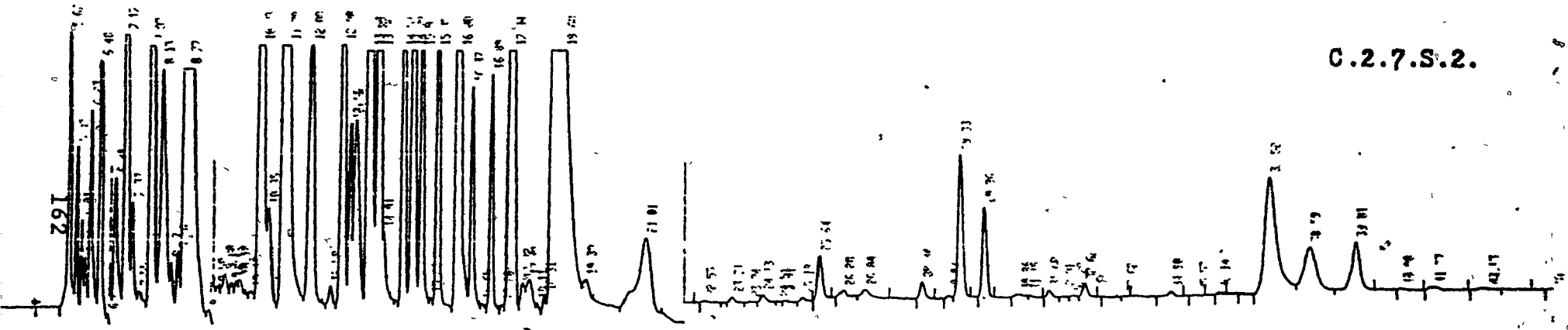
C.2.7.C.2.
C.2.7.C.3.



C.2:7.S.1.



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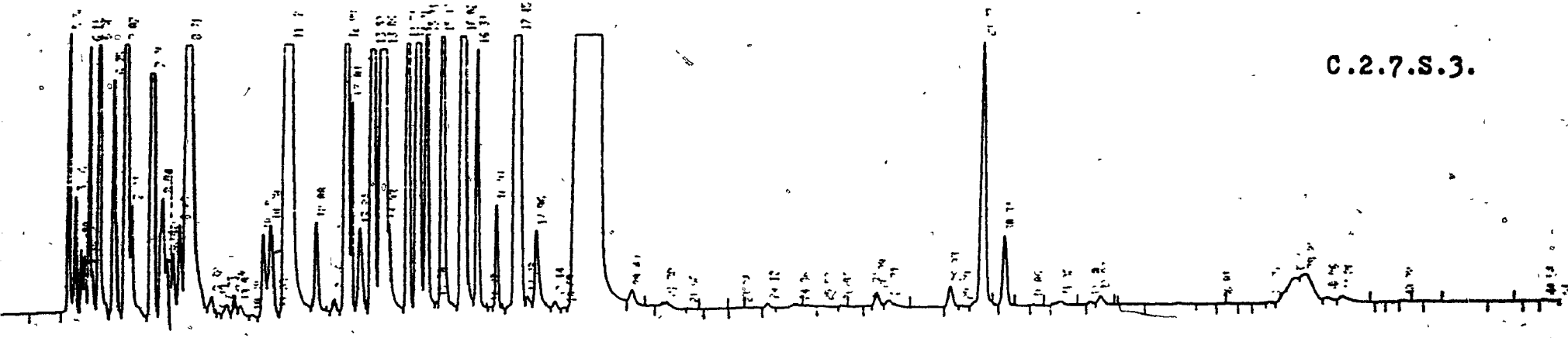

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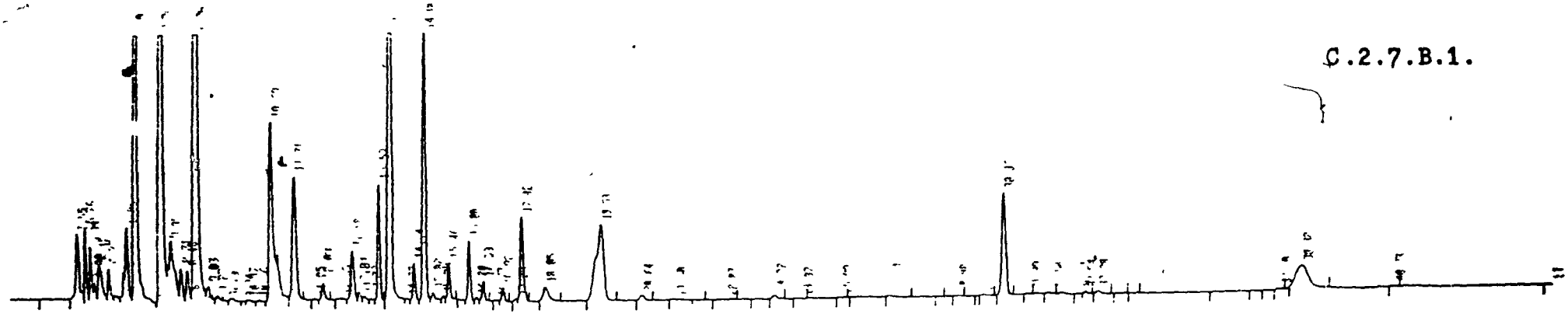

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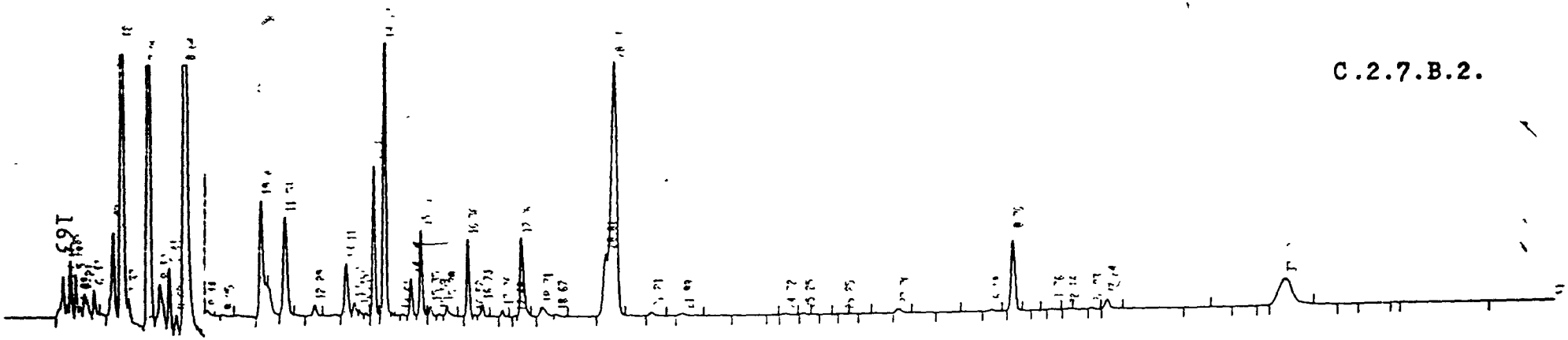

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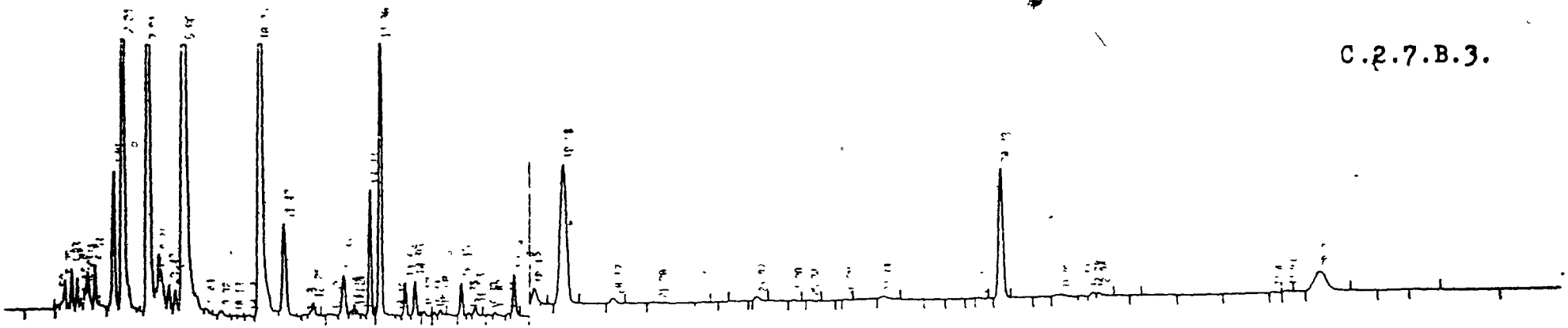
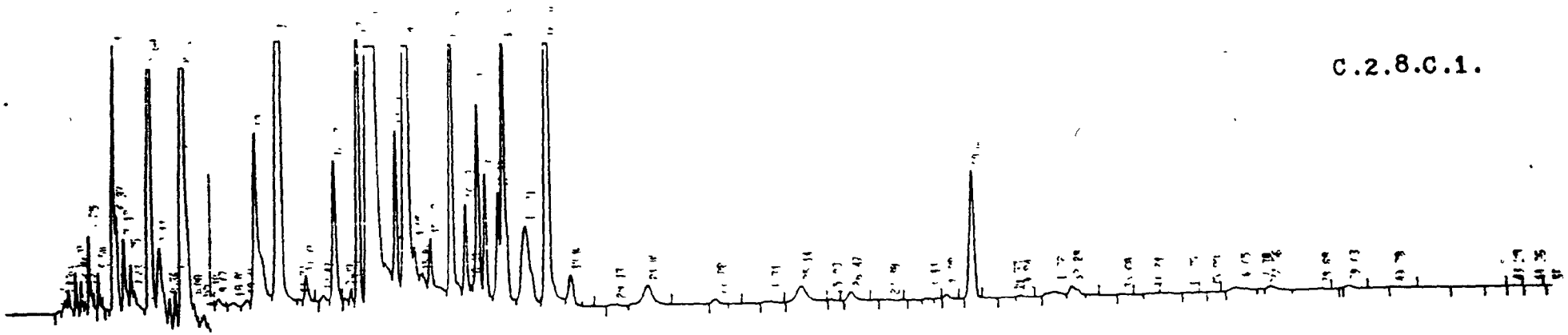
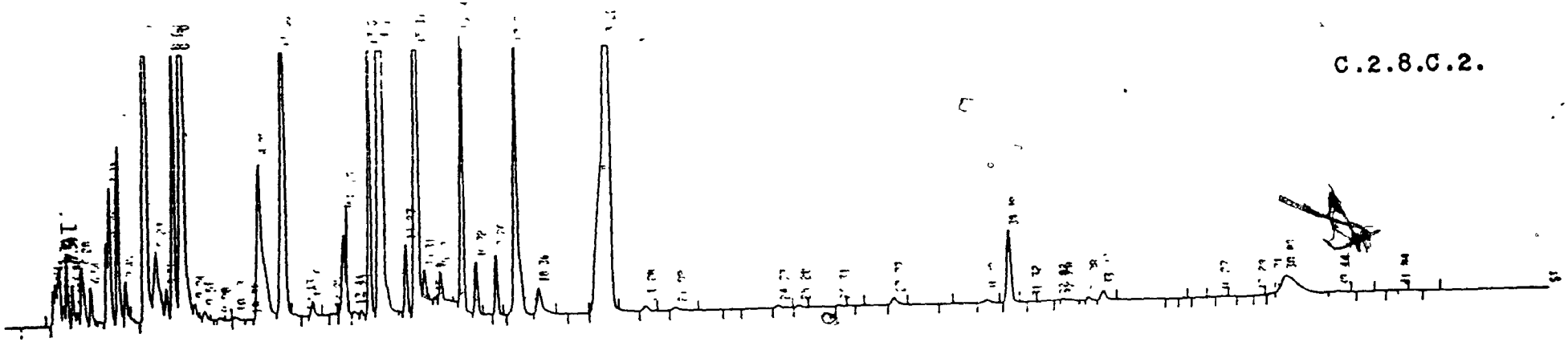


C.2.7.B.3.



C.2.8.C.1.



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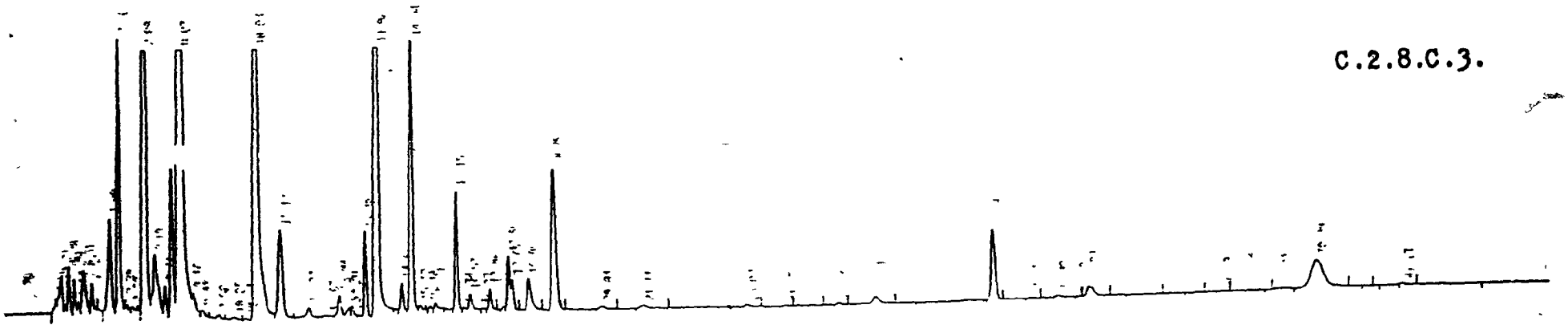
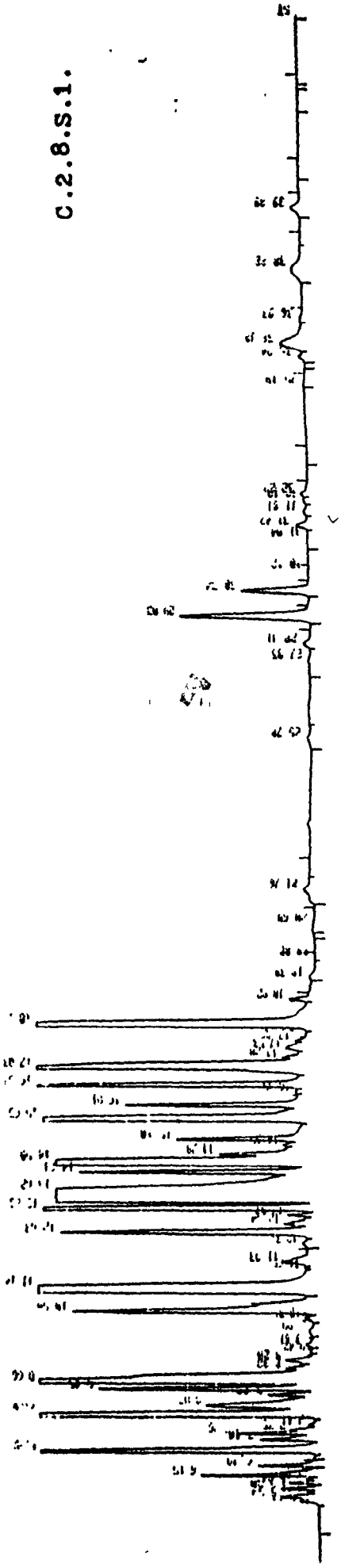


C.2.8.C.3.

C.2.8.S.1.



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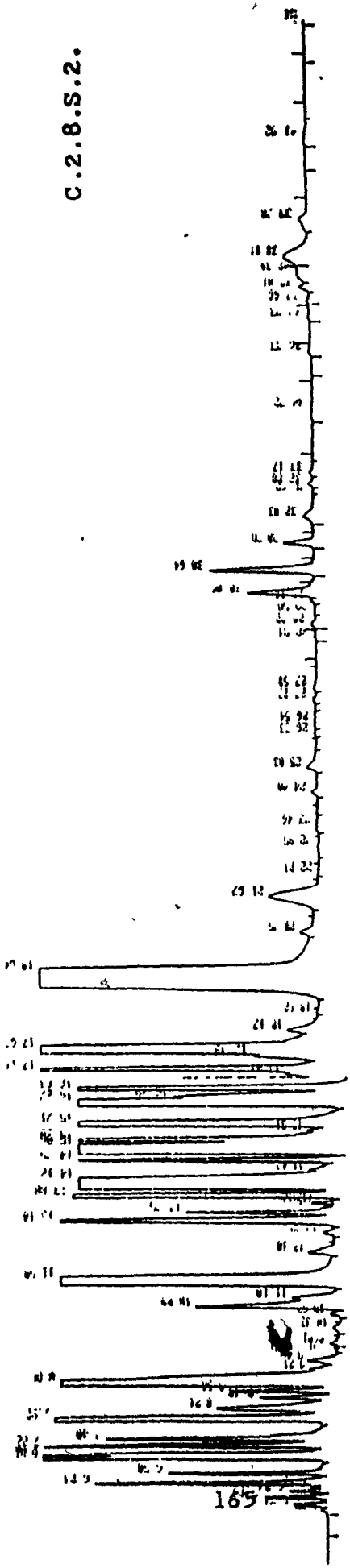

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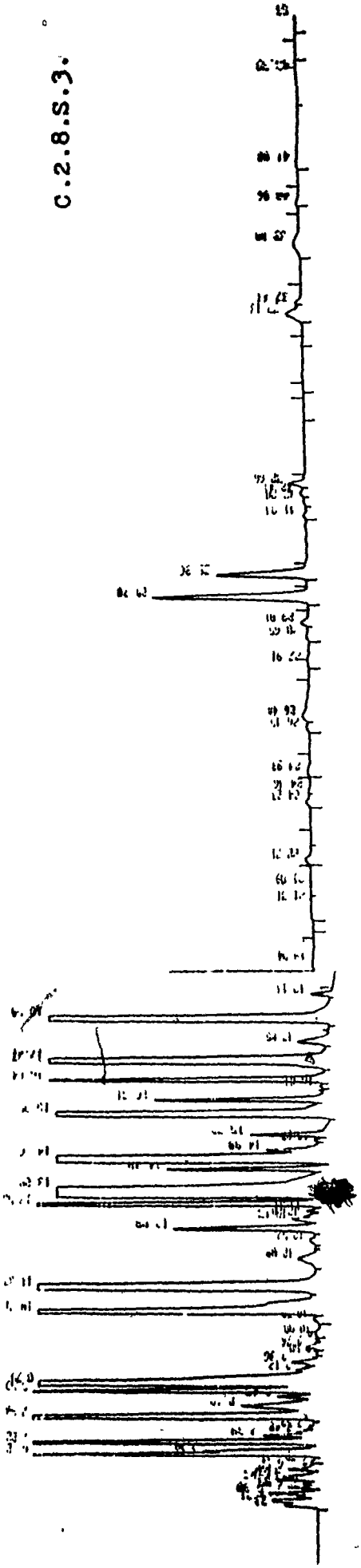
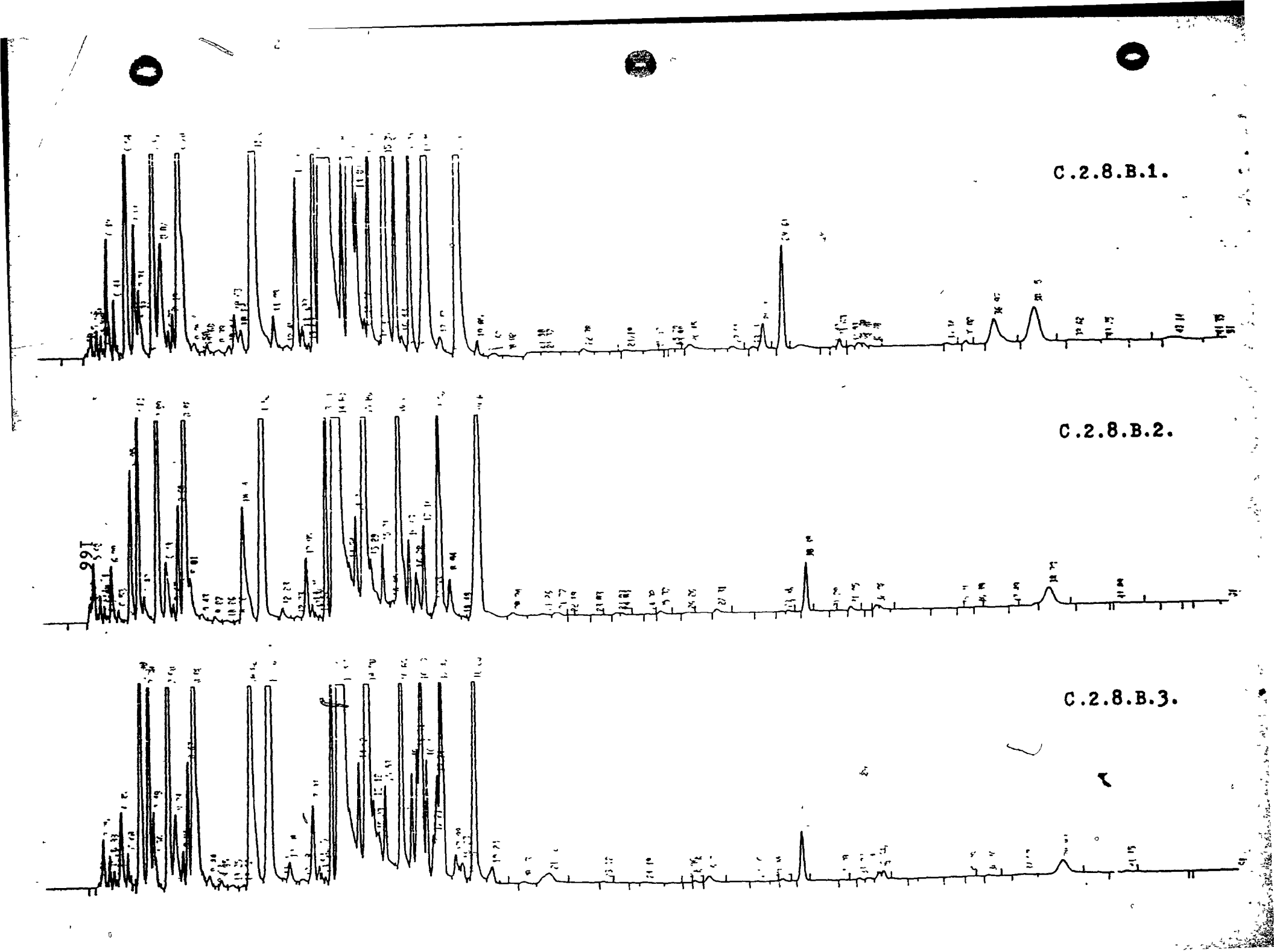



C.2.8.B.1.
C.2.8.B.2.
C.2.8.B.3.

Appendix C: Chromatograms for the potato experiment.

All chromatograms in Appendix C are sorted according to the following numbering system:

v.n1.n2.t.n3.

where v - vegetable

P for potato

n1 - trial:

1 or 2.

n2 - analysis:

1,2.3, or 4.

t - treatment:

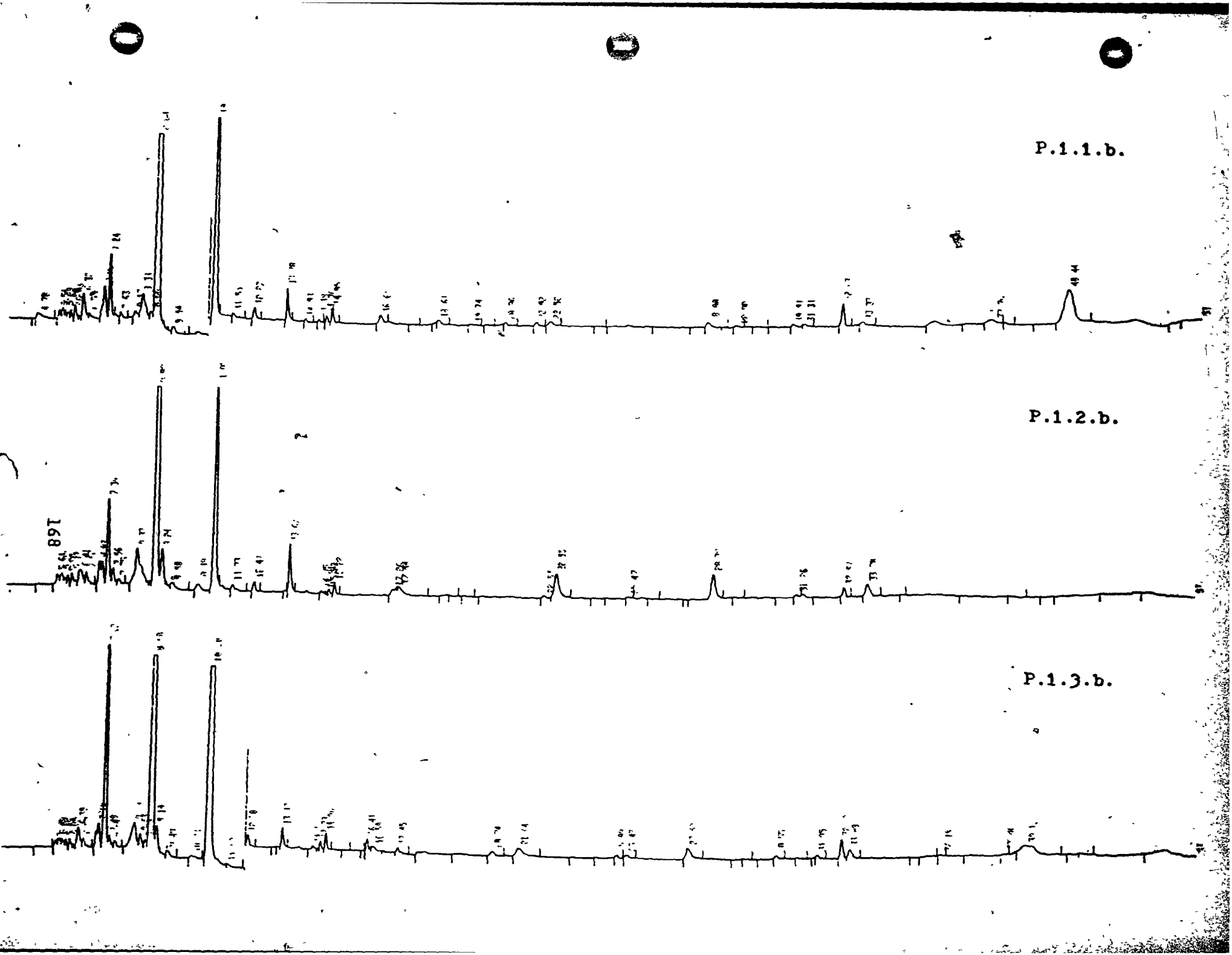
C for control, or

E for Erwinia carotovora, or

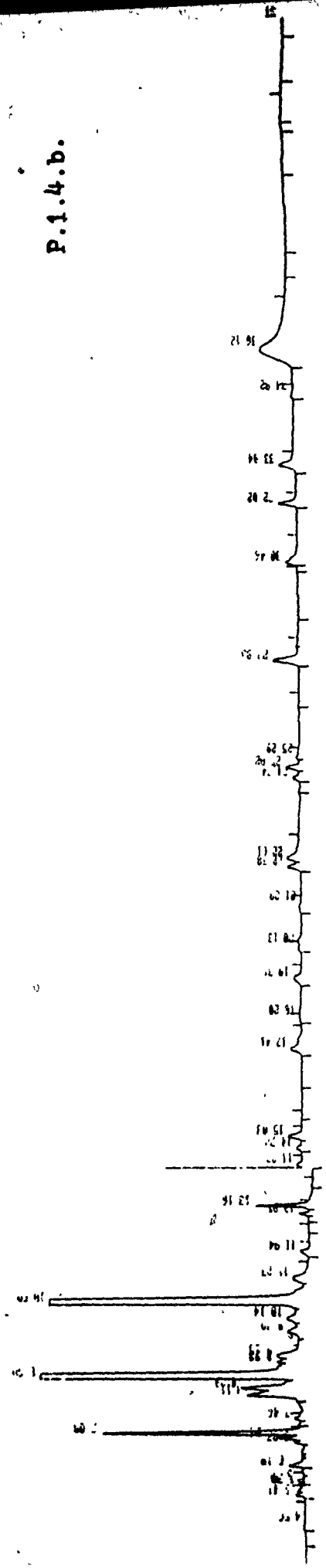
F for Fusarium roseum.

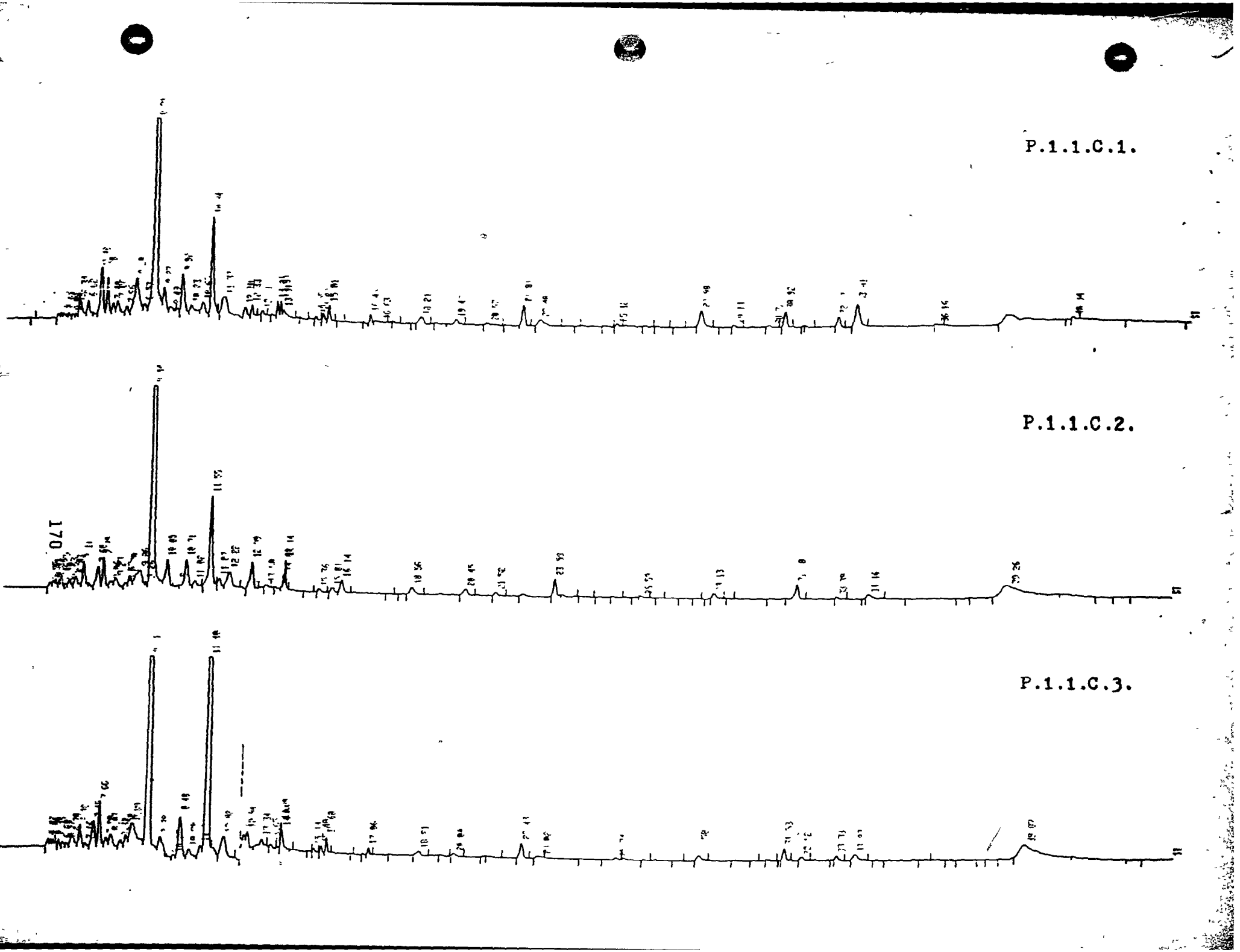
n3 - replicate:

1,2, or 3.


P.1.1.b.
P.1.2.b.
P.1.3.b.

P.1.4.b.

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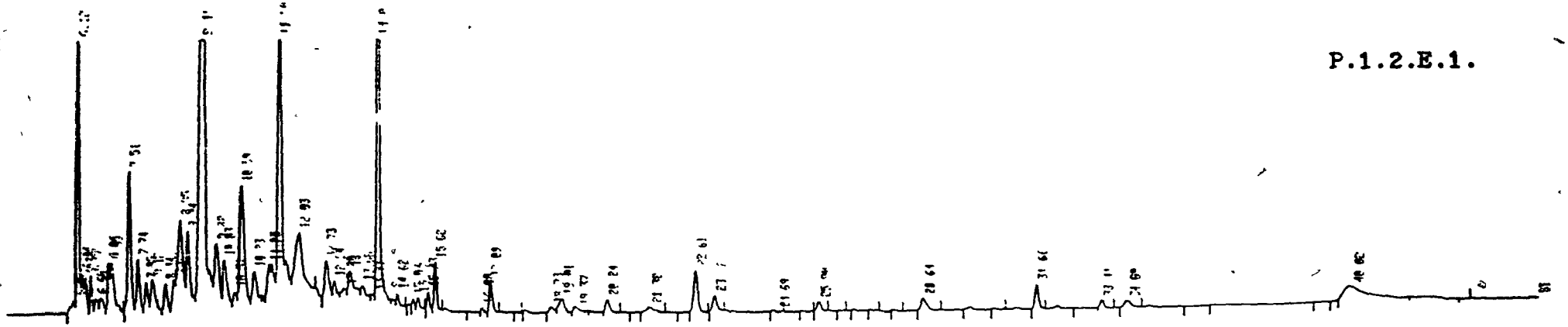
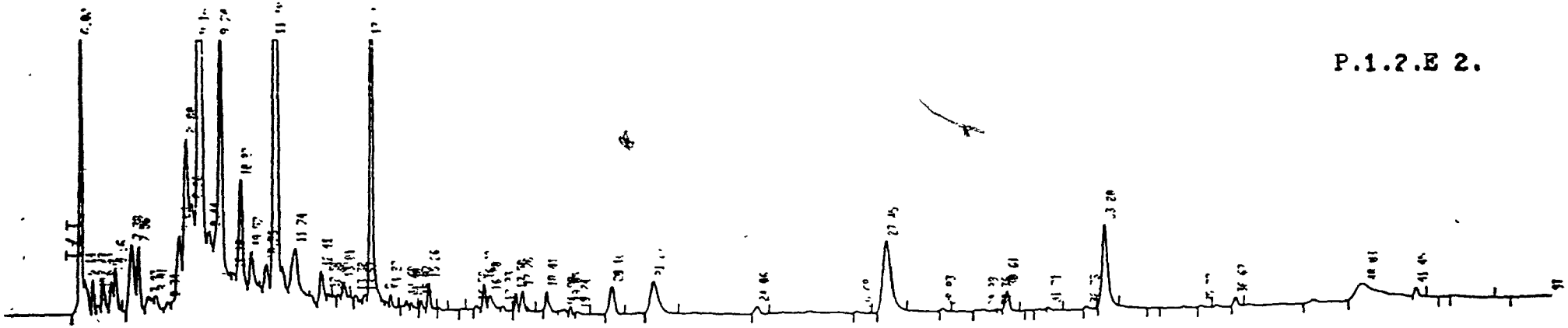
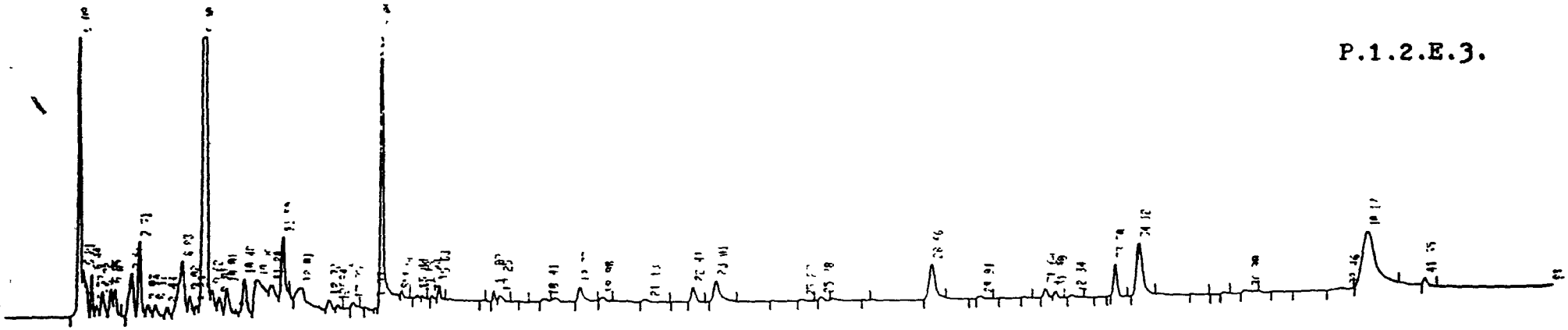
P.1.1.C.2.
P.1.1.C.3.



P.1.2.E.1.



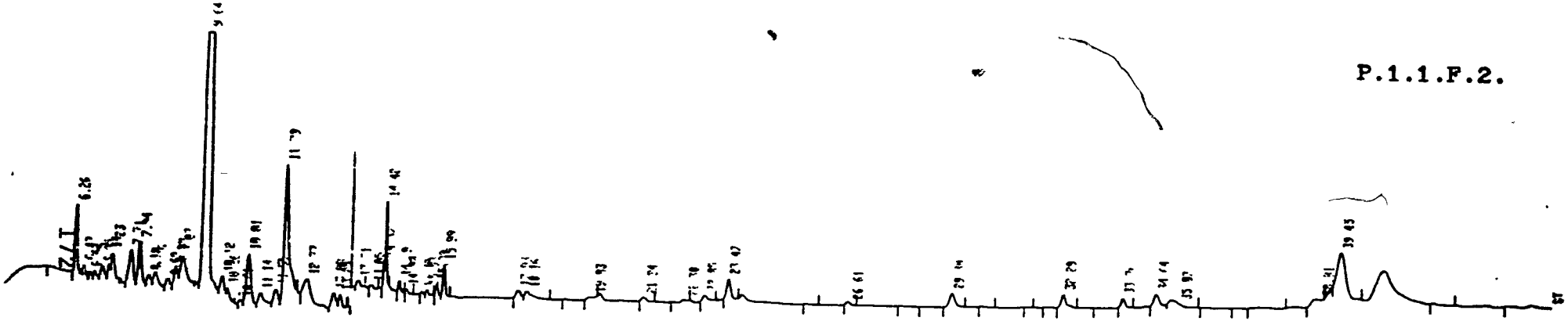
P.1.2.E 2.



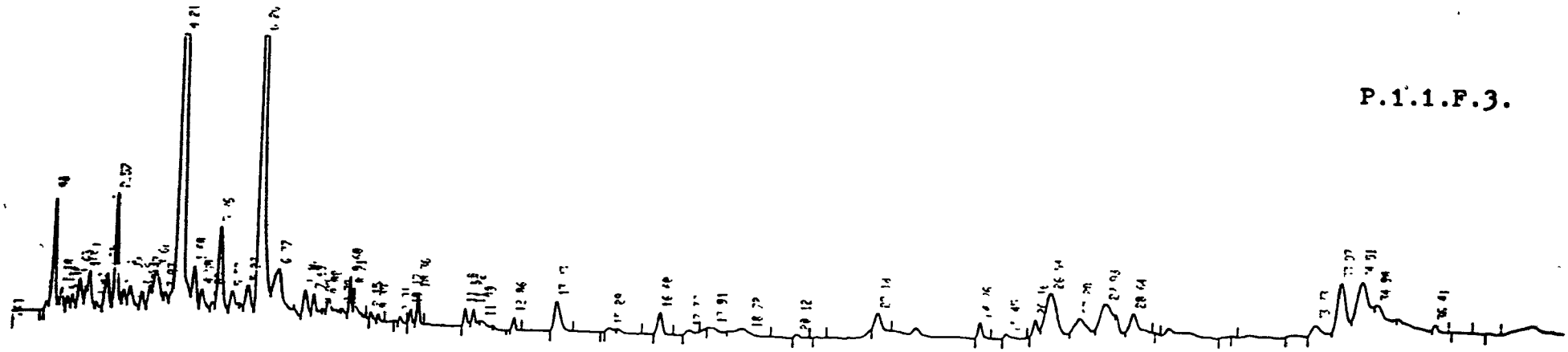
P.1.2.E.3.

P.1.1.F.1.

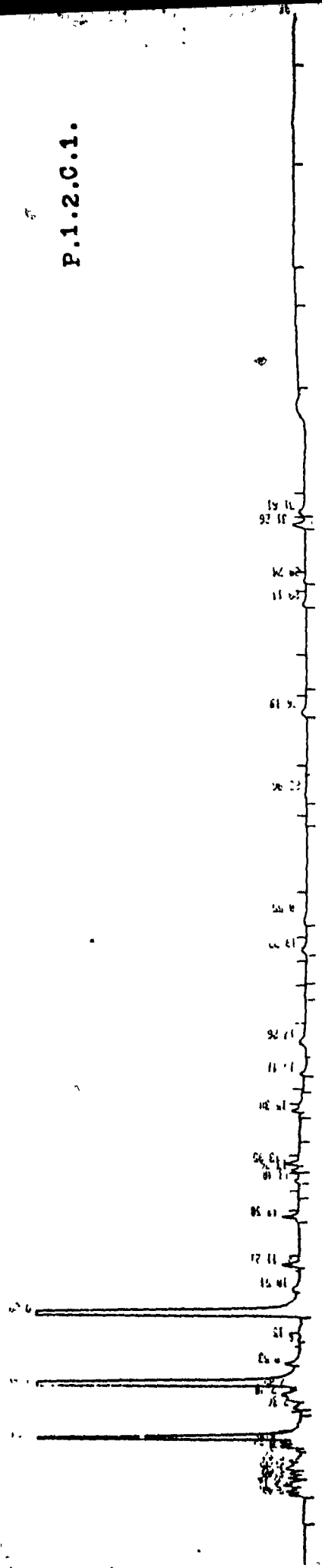
P.1.1.F.2.



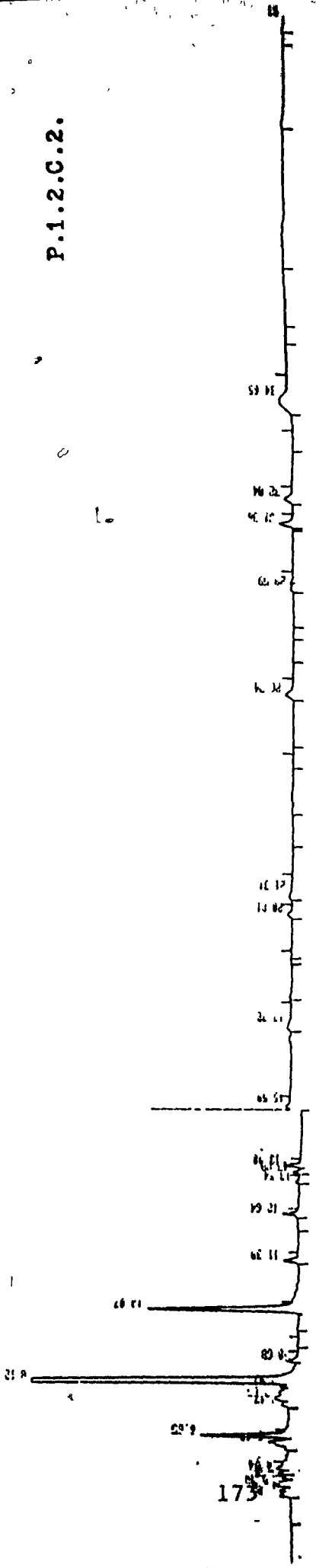
P.1.1.F.3.



P.1.2.C.1.



P.1.2.C.2.



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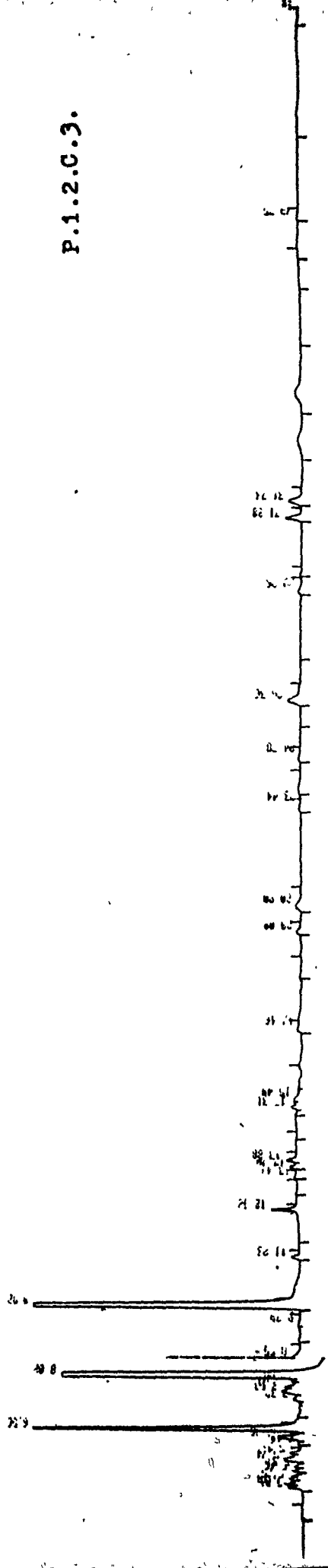
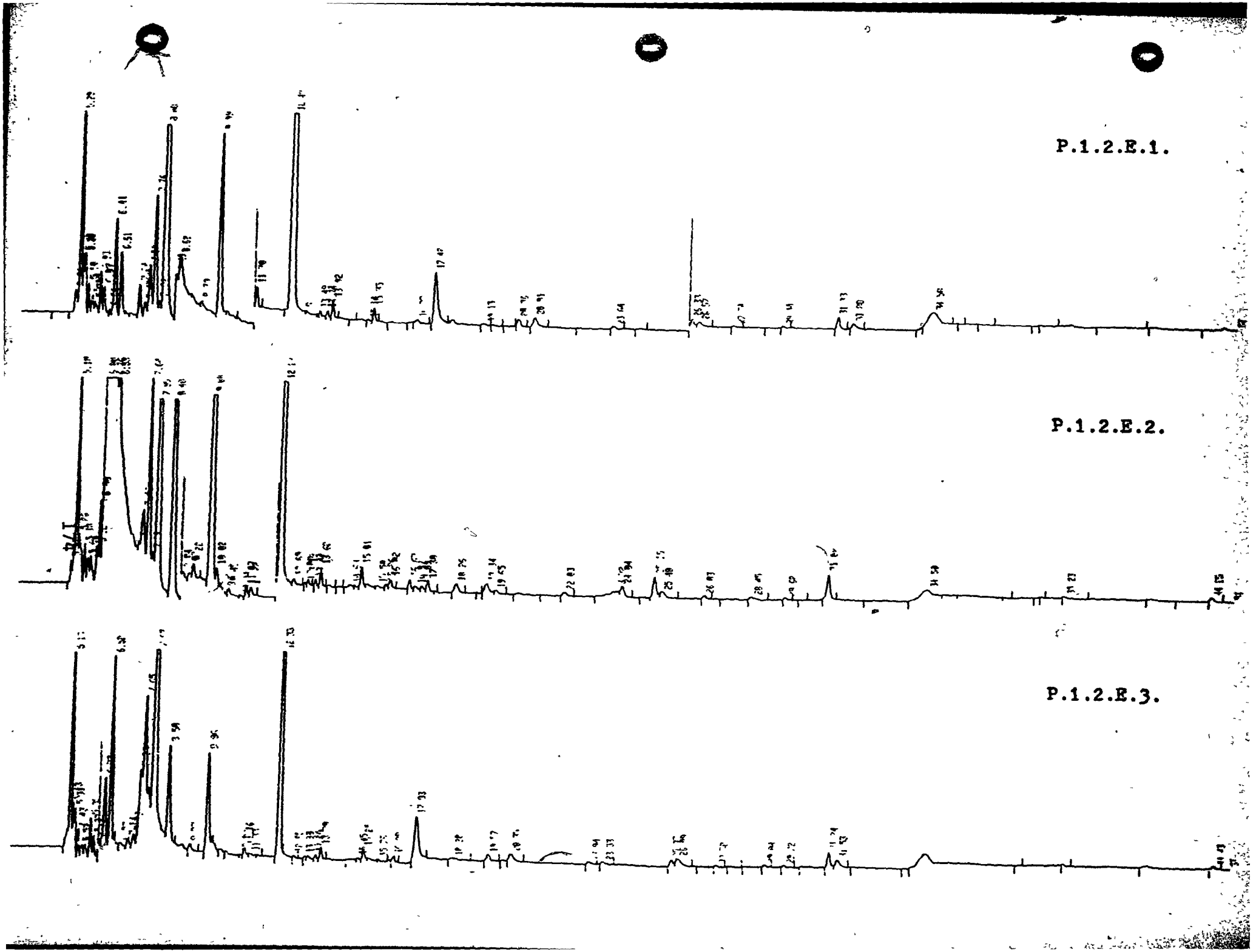
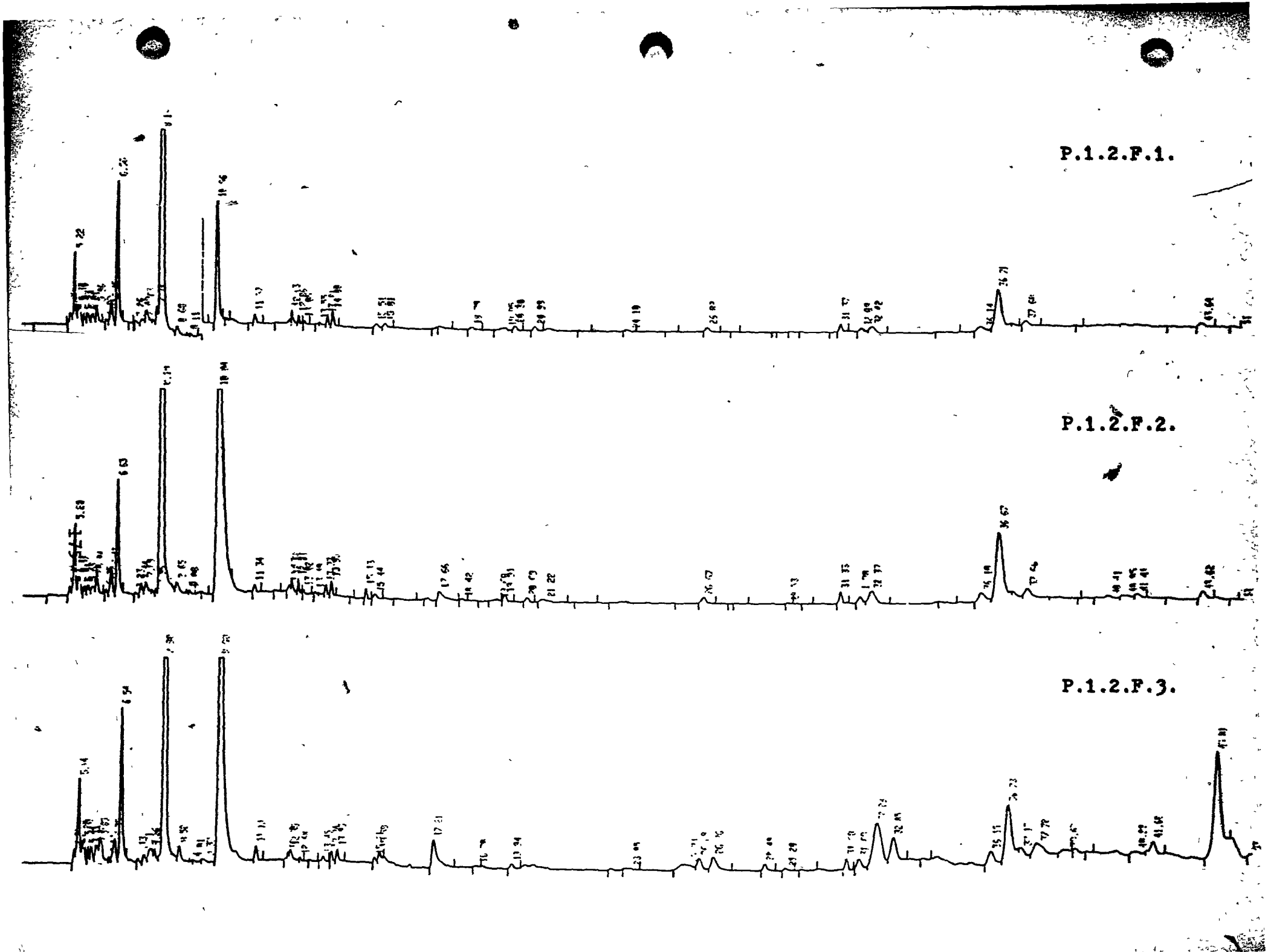



P.1.2.E.1.
P.1.2.E.2.
P.1.2.E.3.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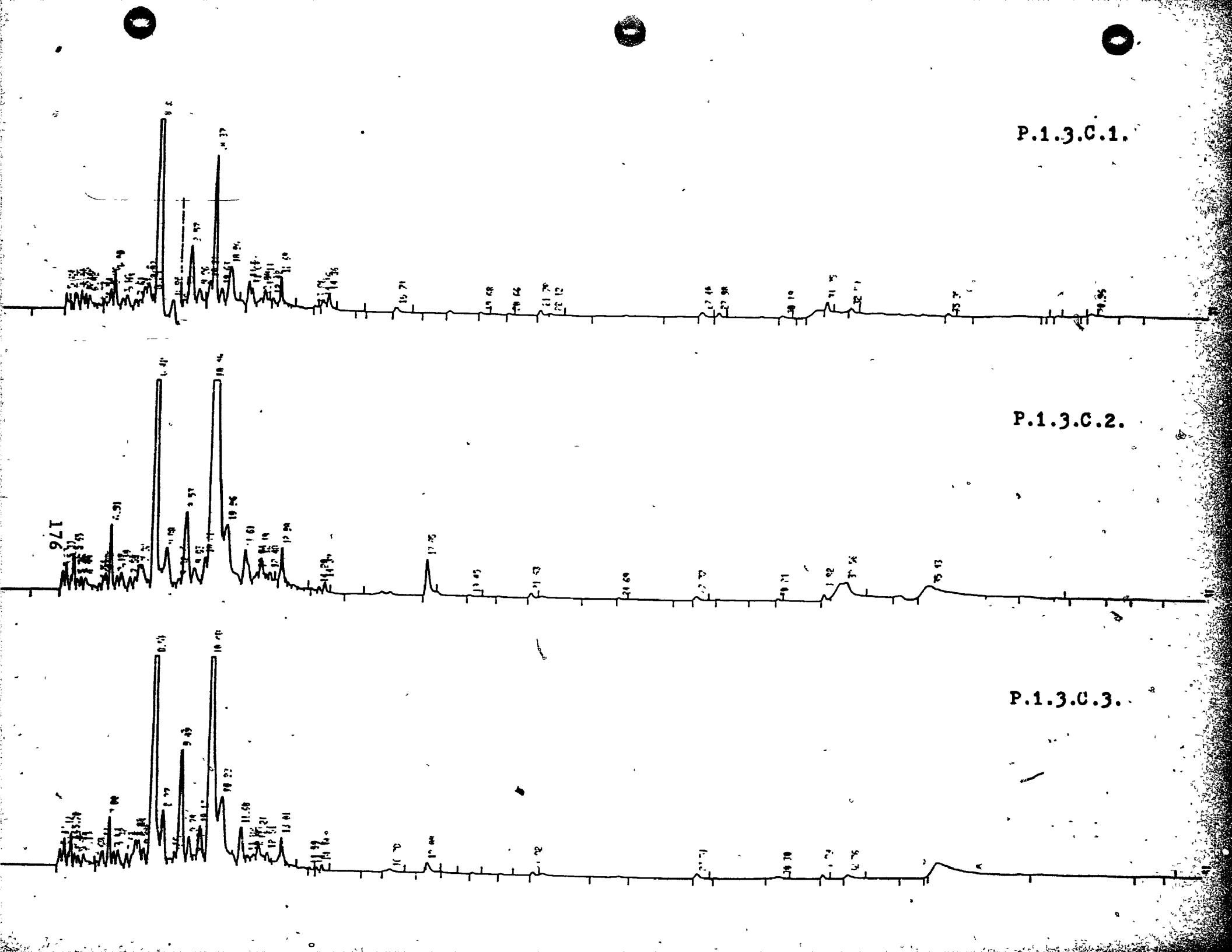
P.1.2.F.3.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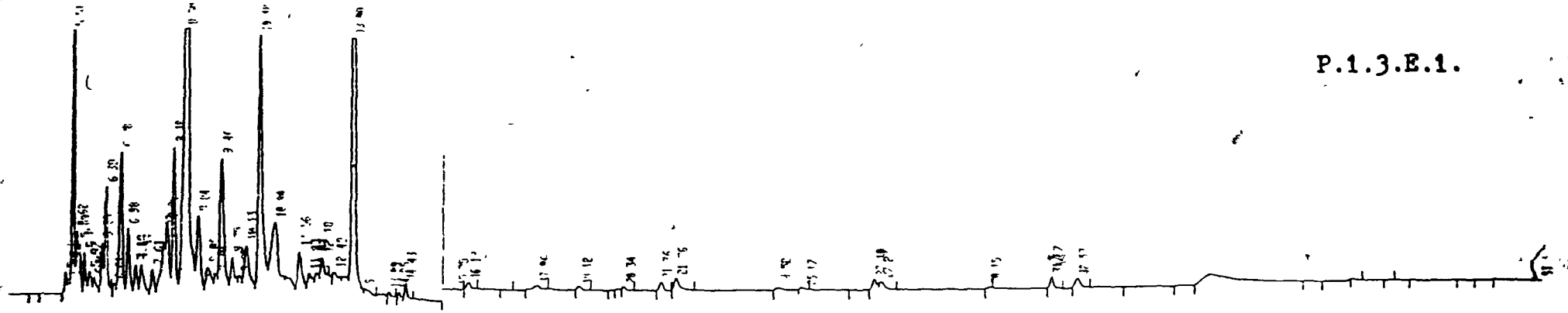

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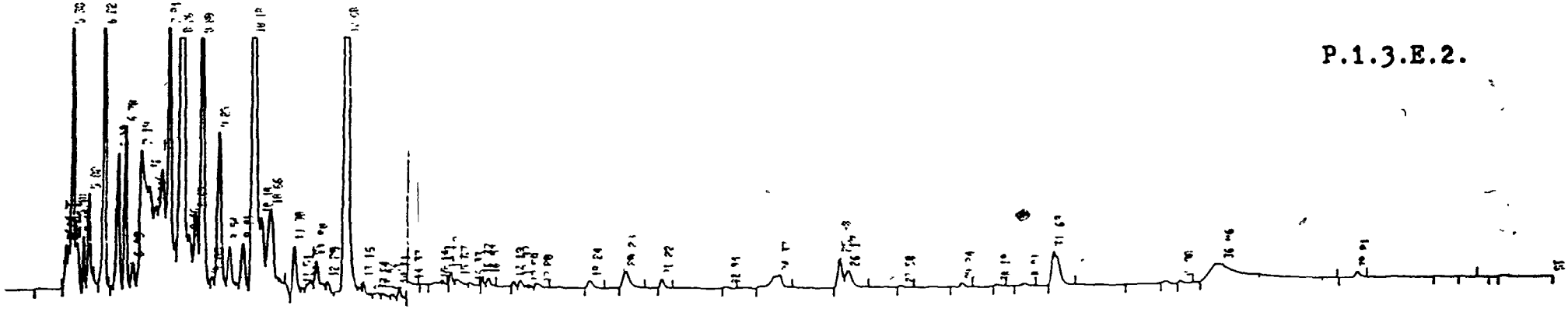

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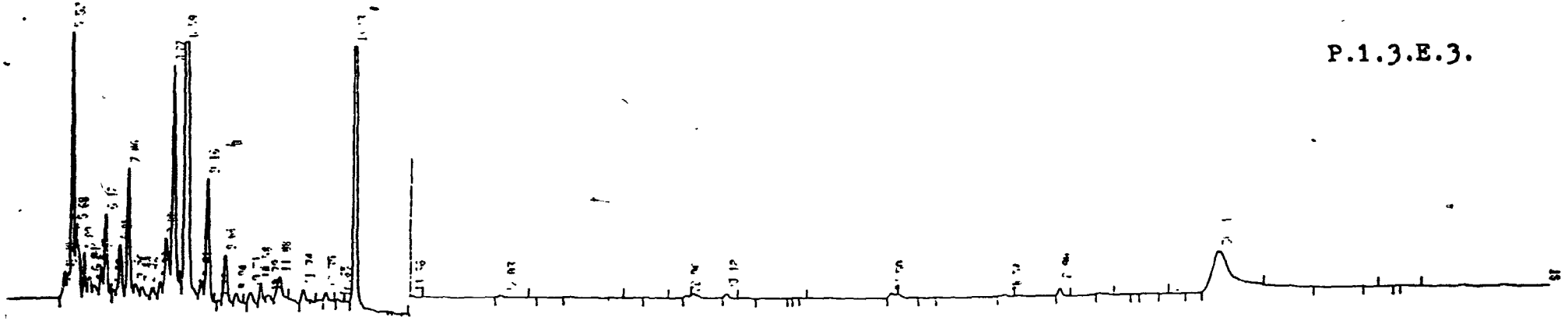

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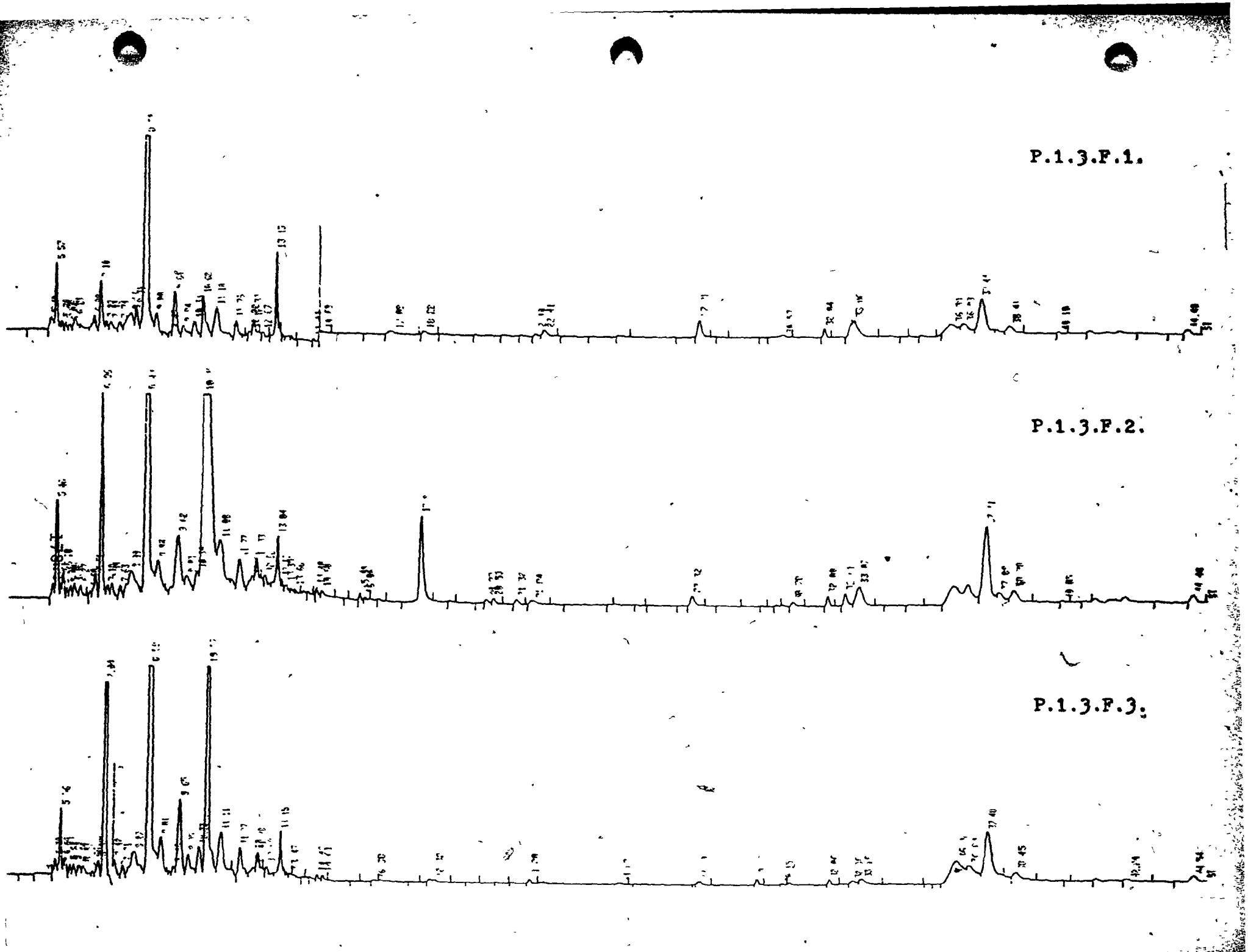

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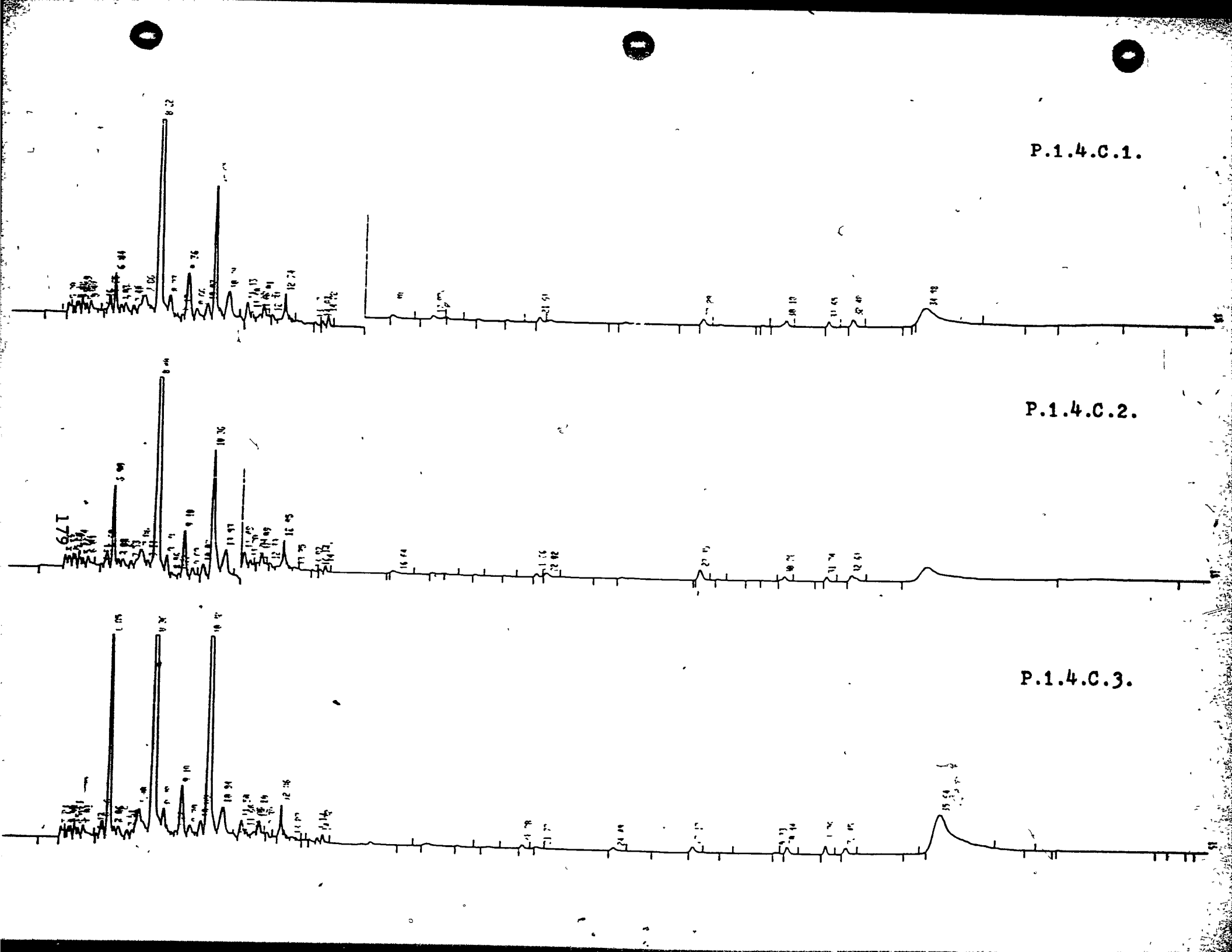
P.1.3.F.2.

P.1.3.F.3.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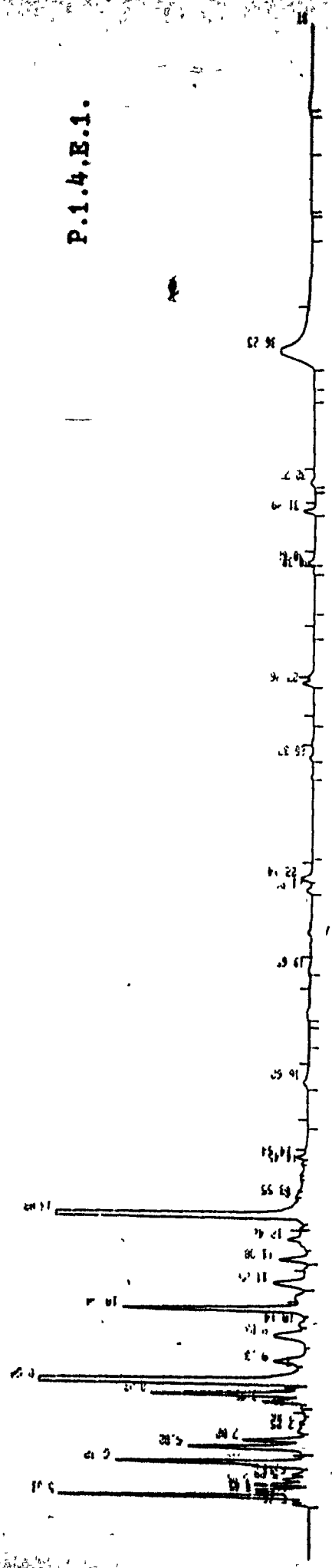
P.1.4.E.1.



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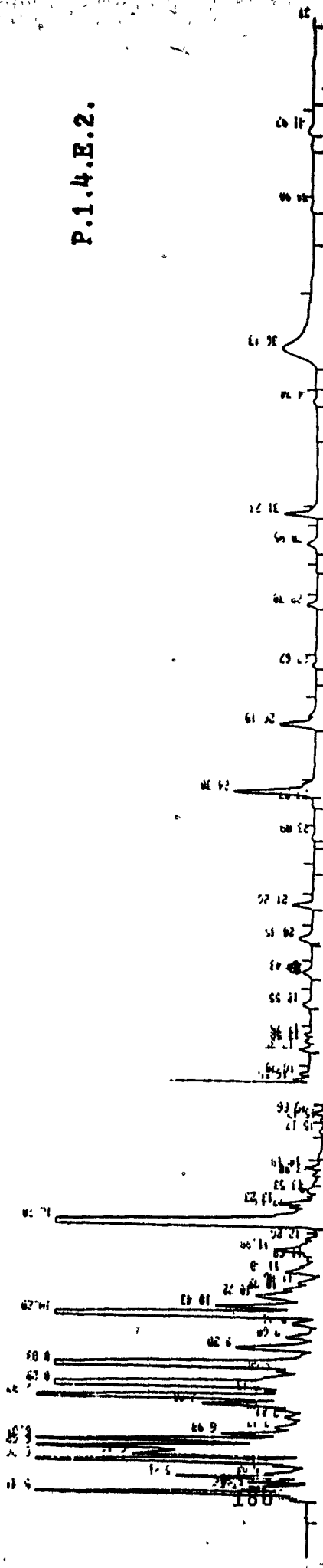

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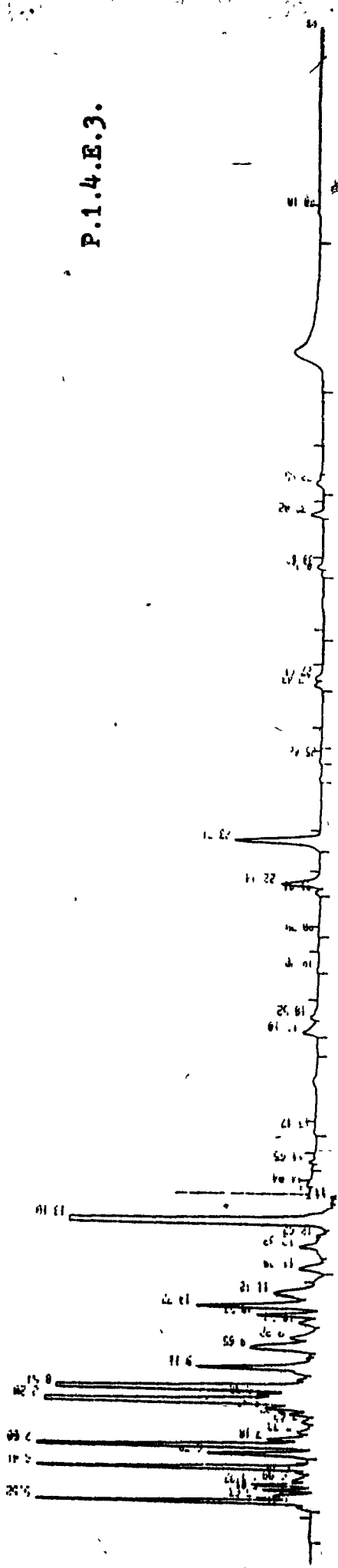

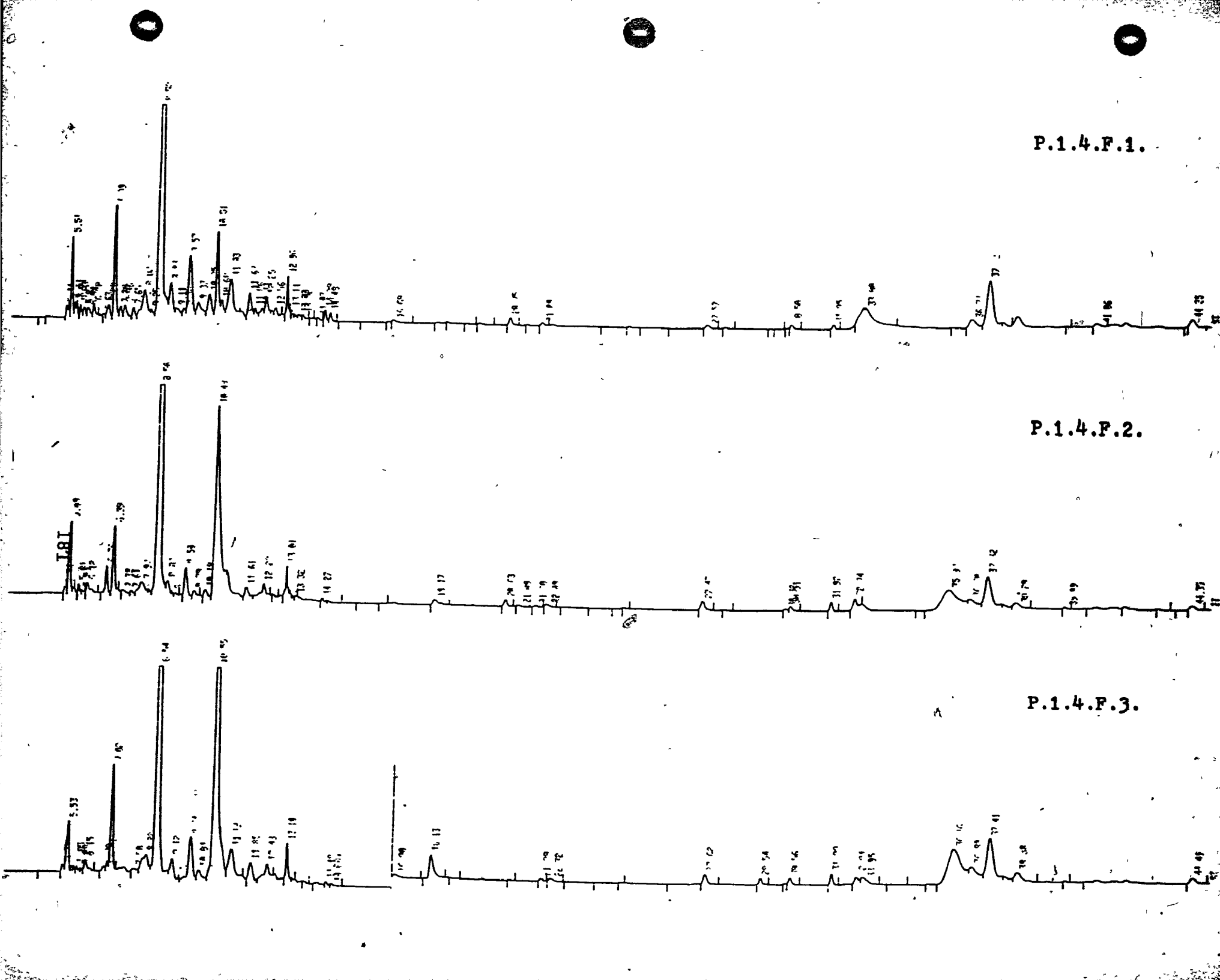
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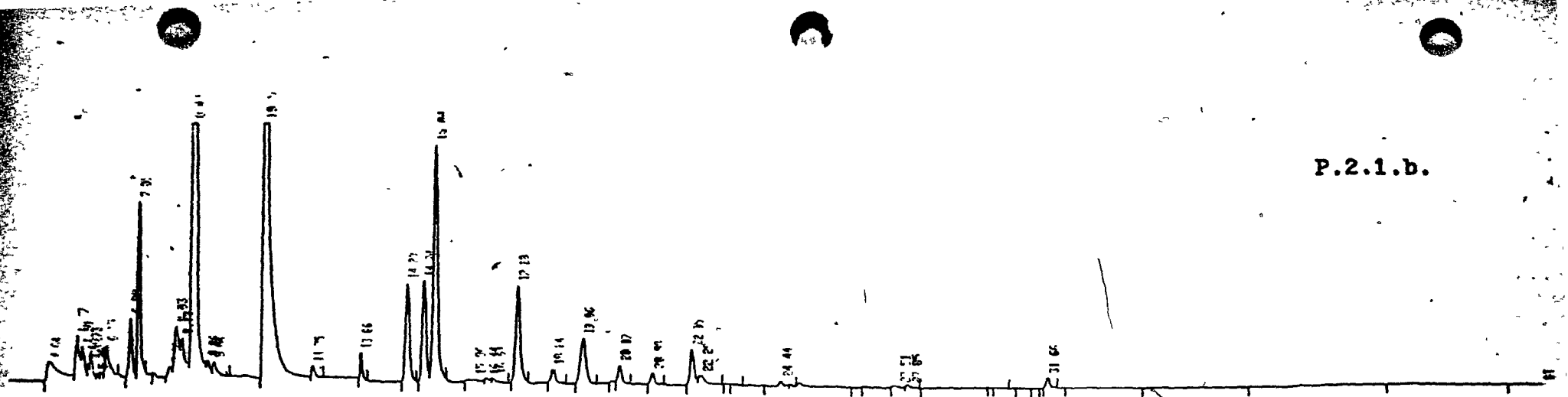

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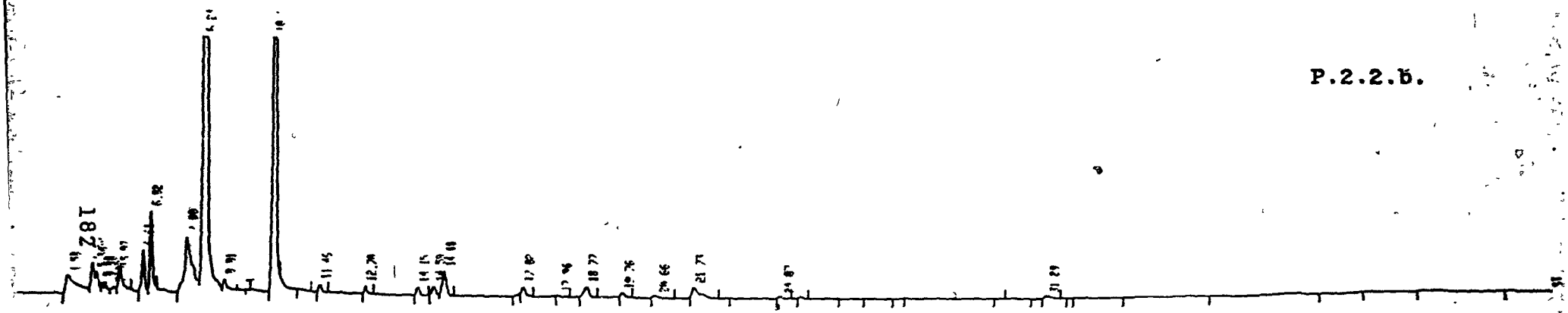

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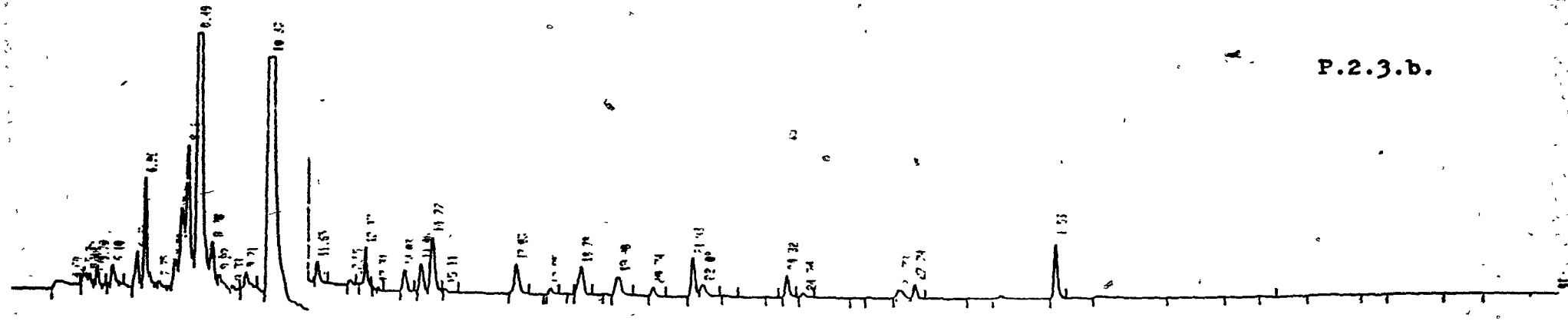
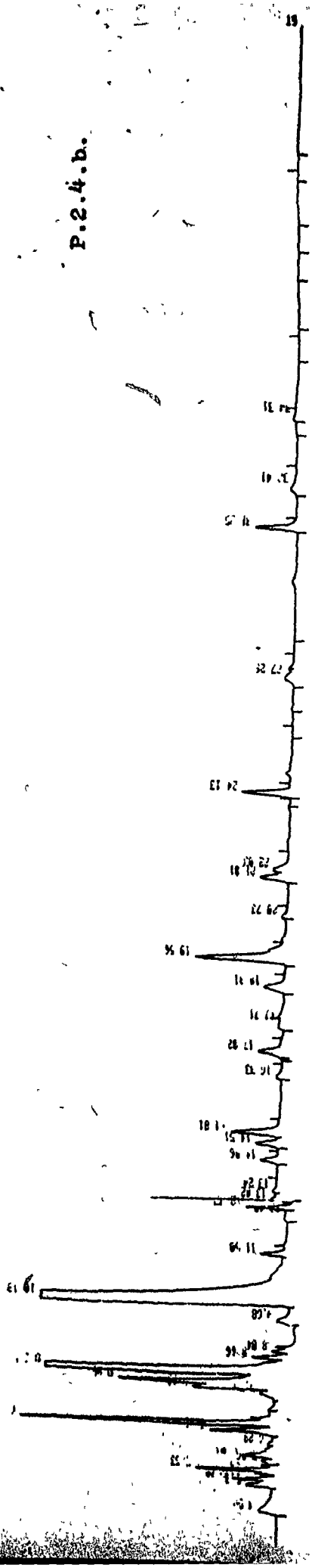
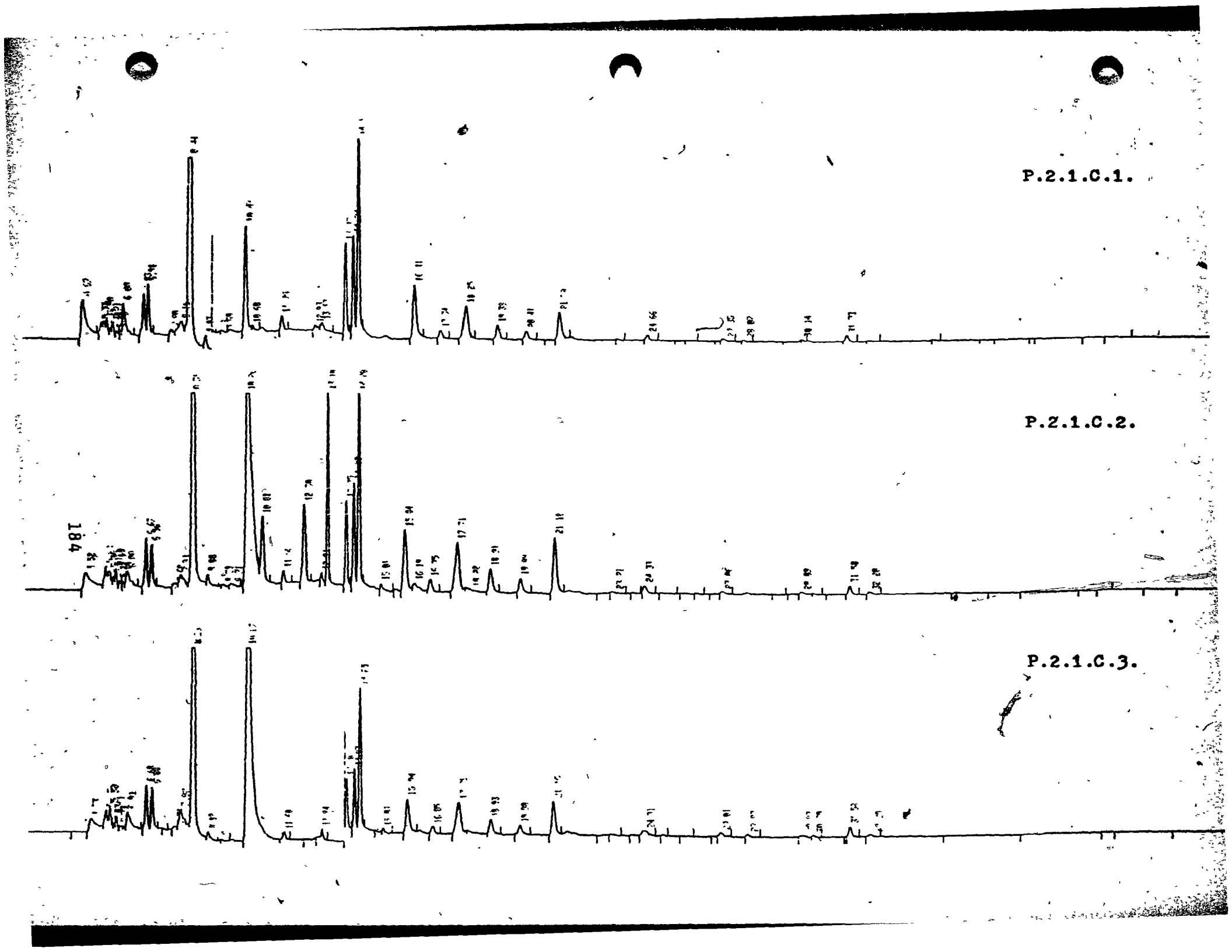


P.2.3.b.

P.2.4.b.

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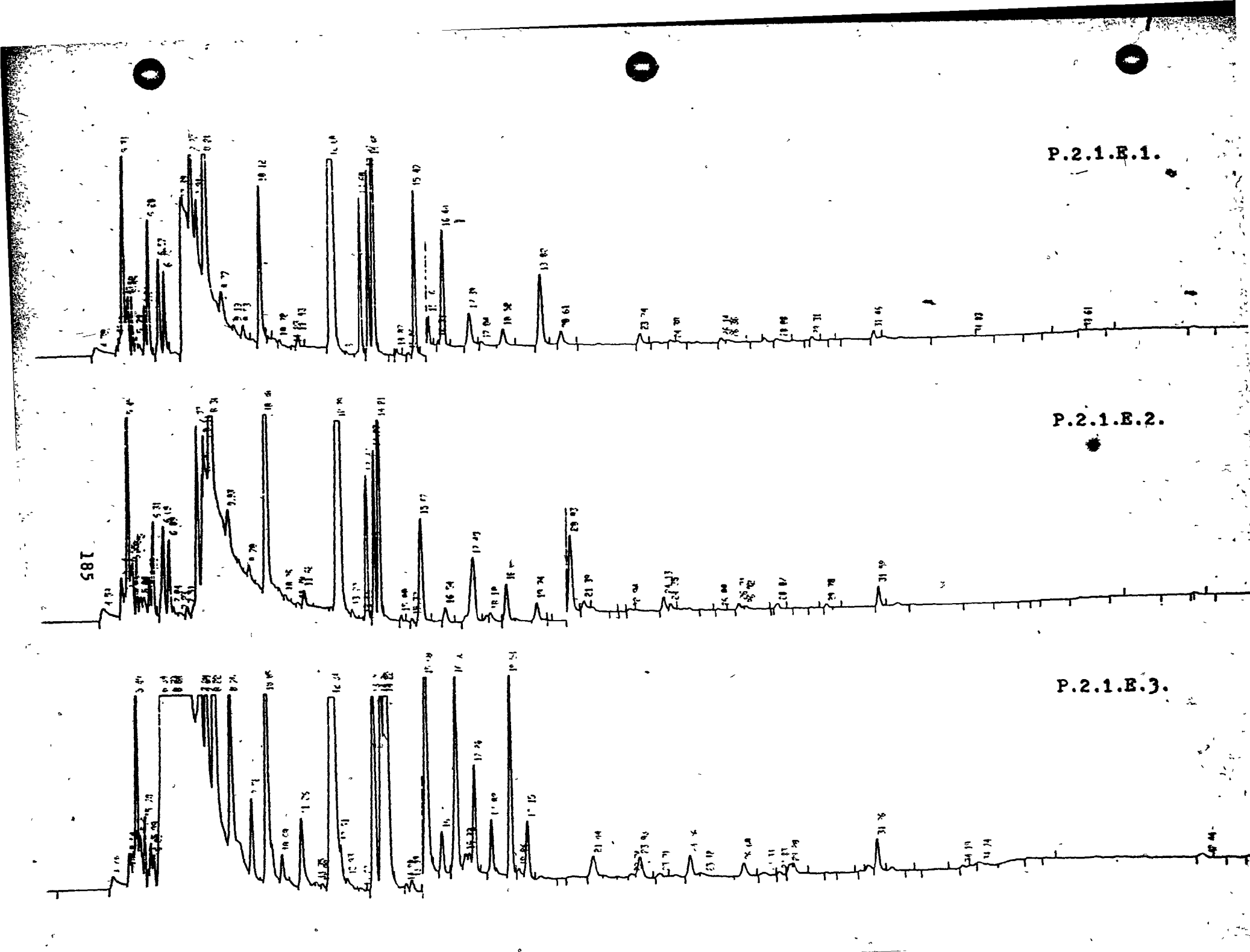
P.2.1.C.2.
184
P.2.1.C.3.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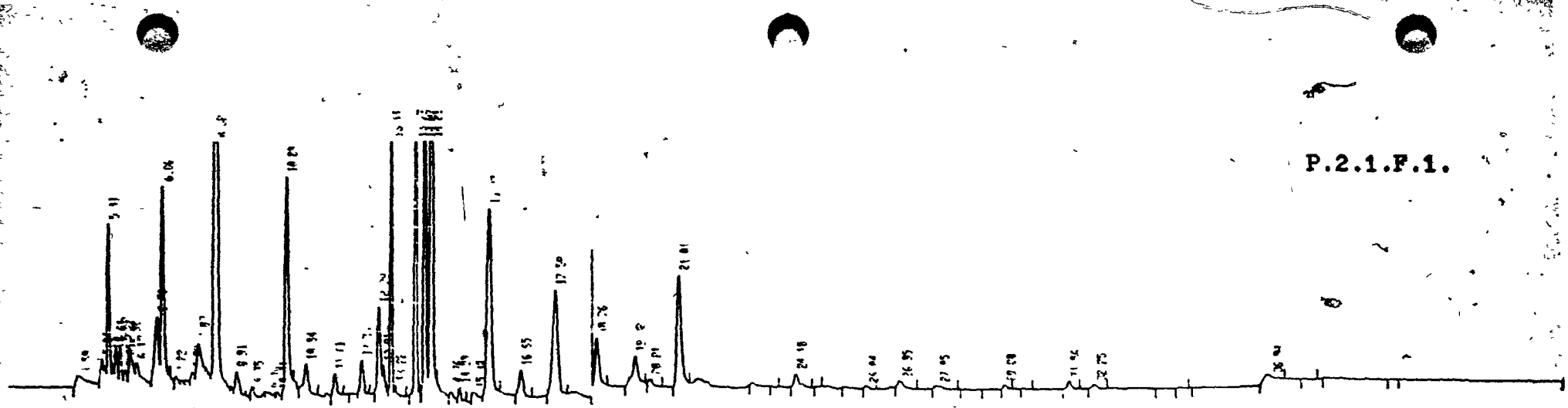
P.2.1.E.3.



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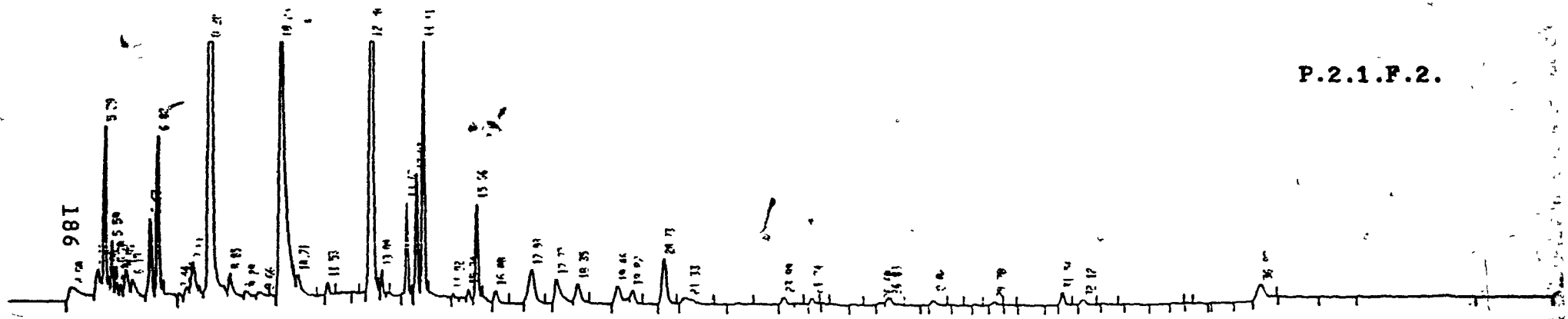

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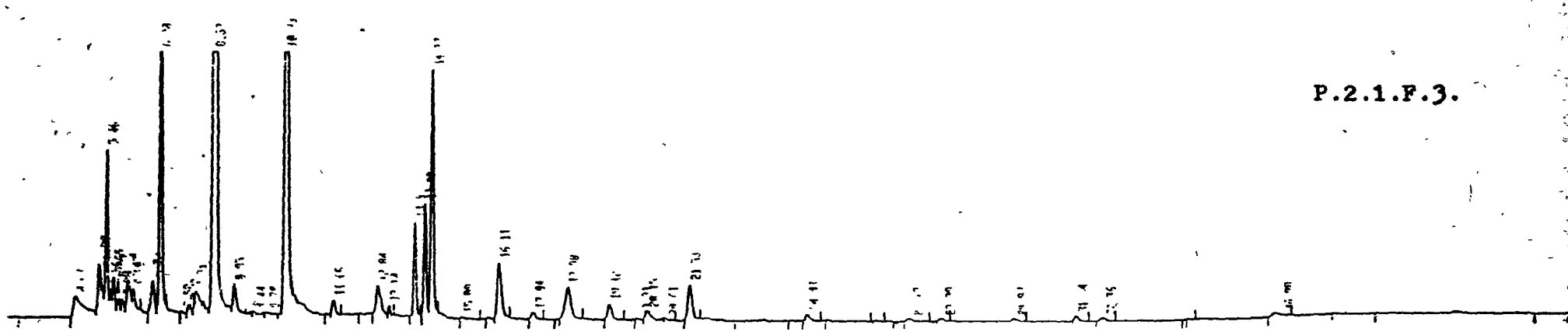

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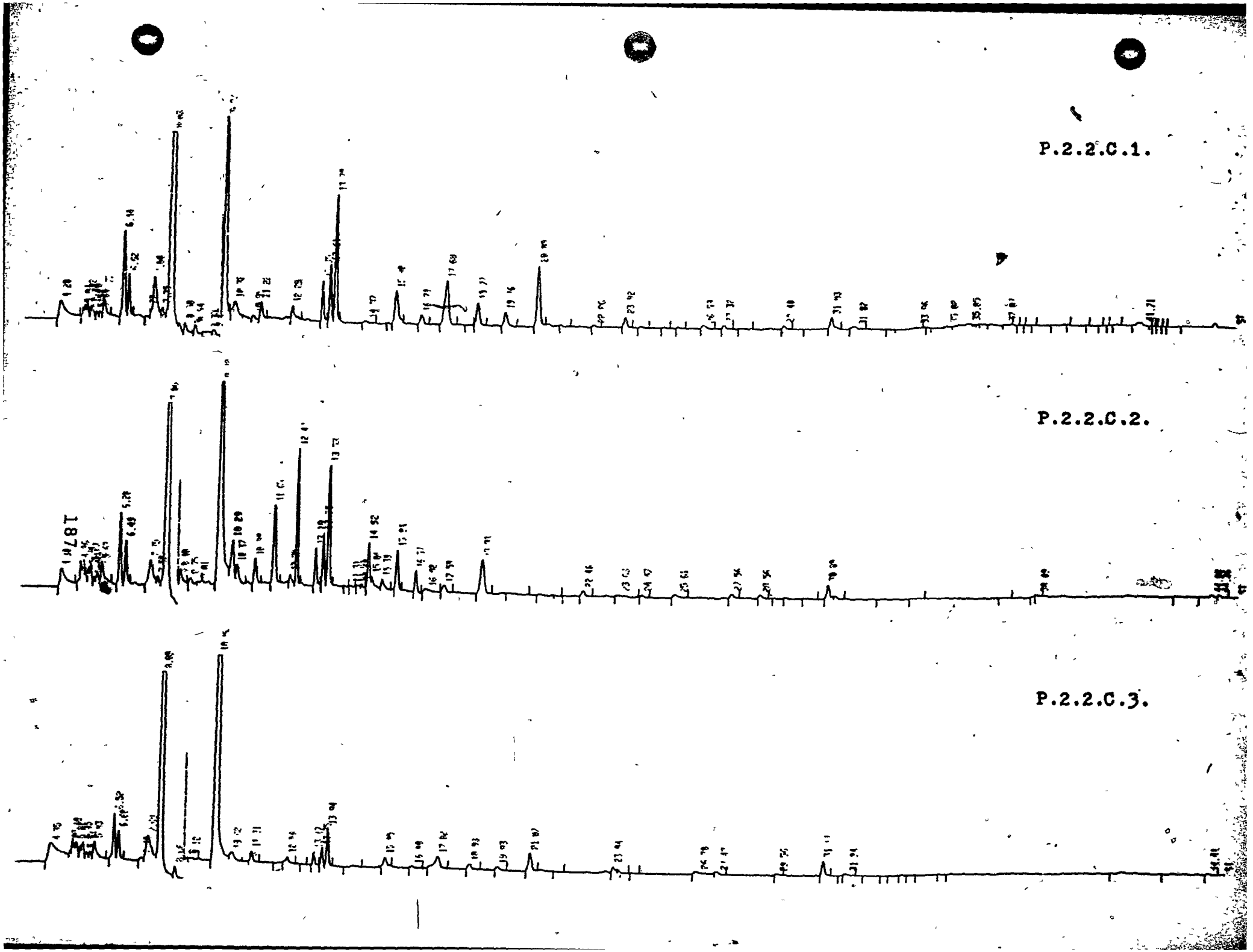
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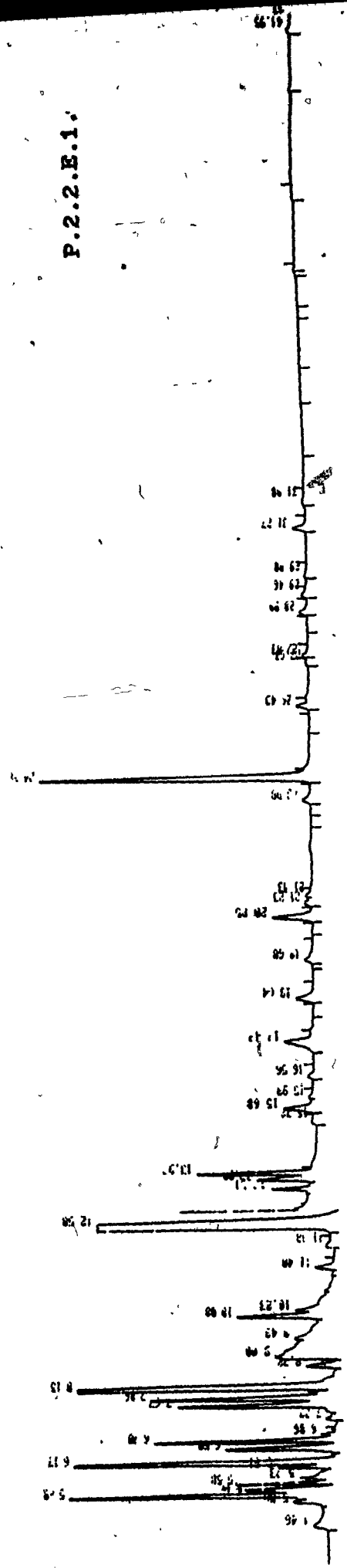
P.2.2.E.1.



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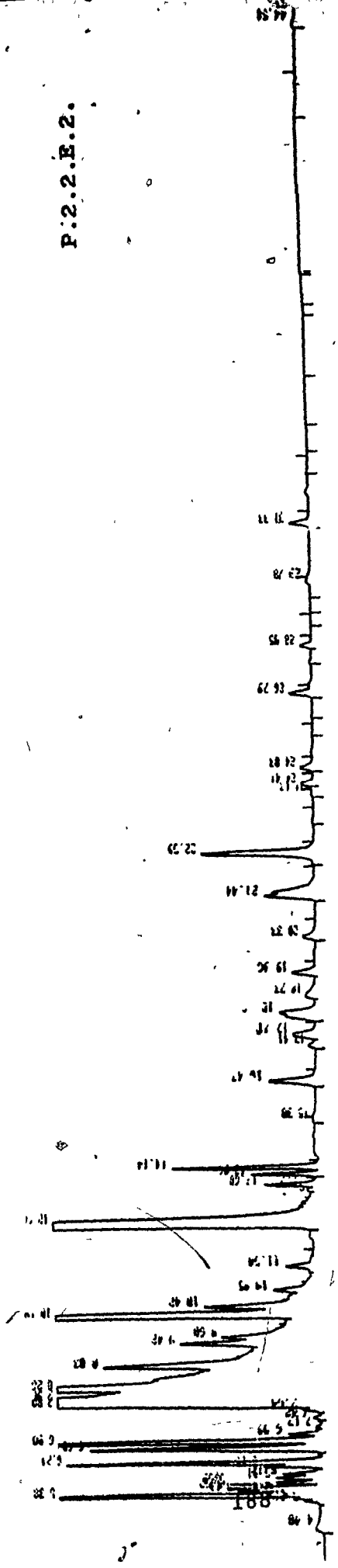

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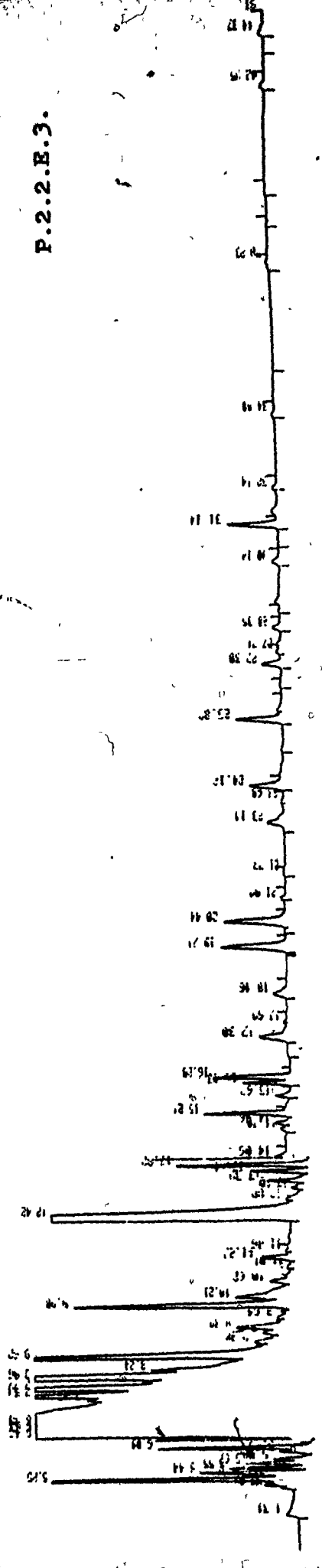

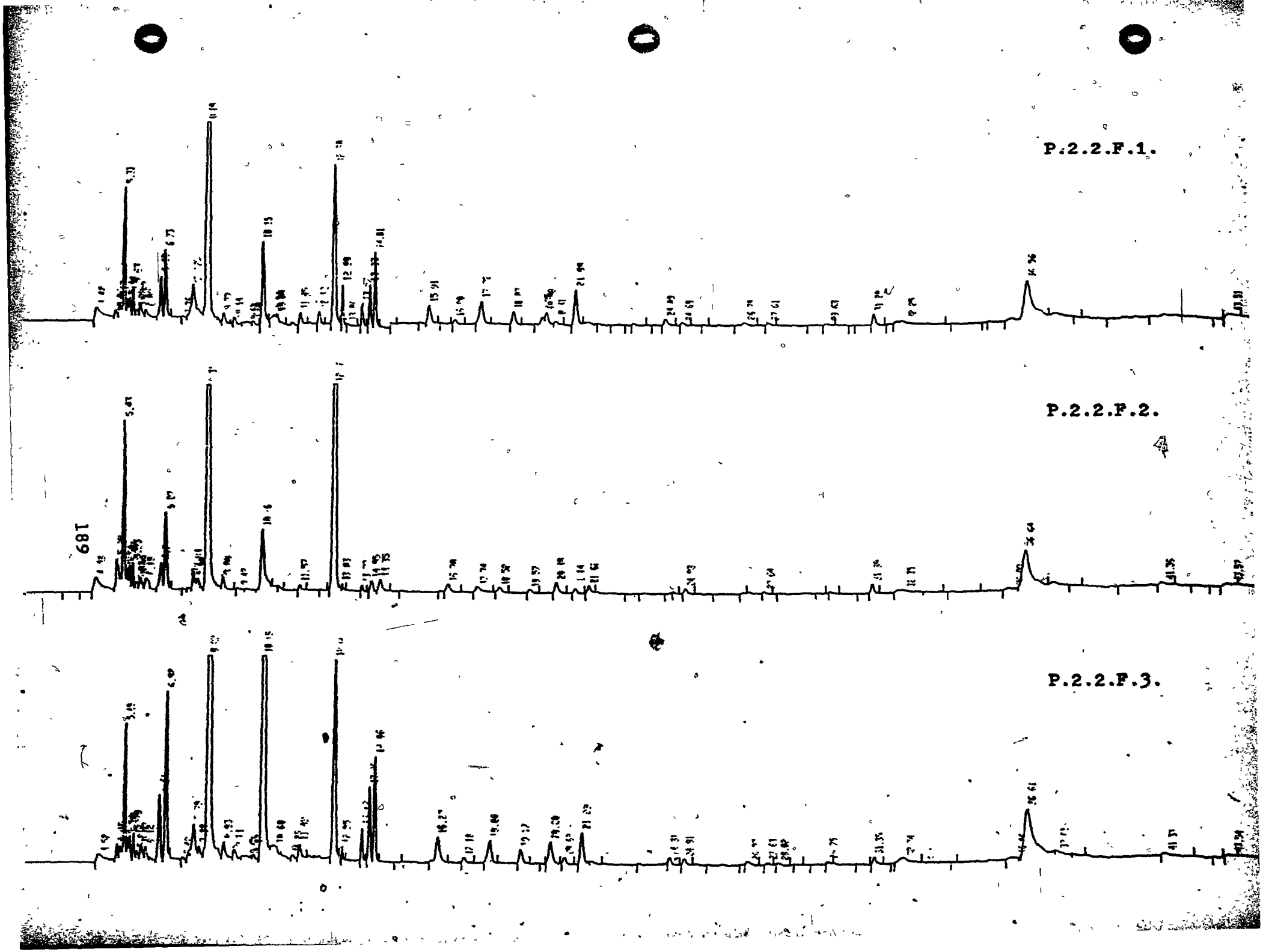
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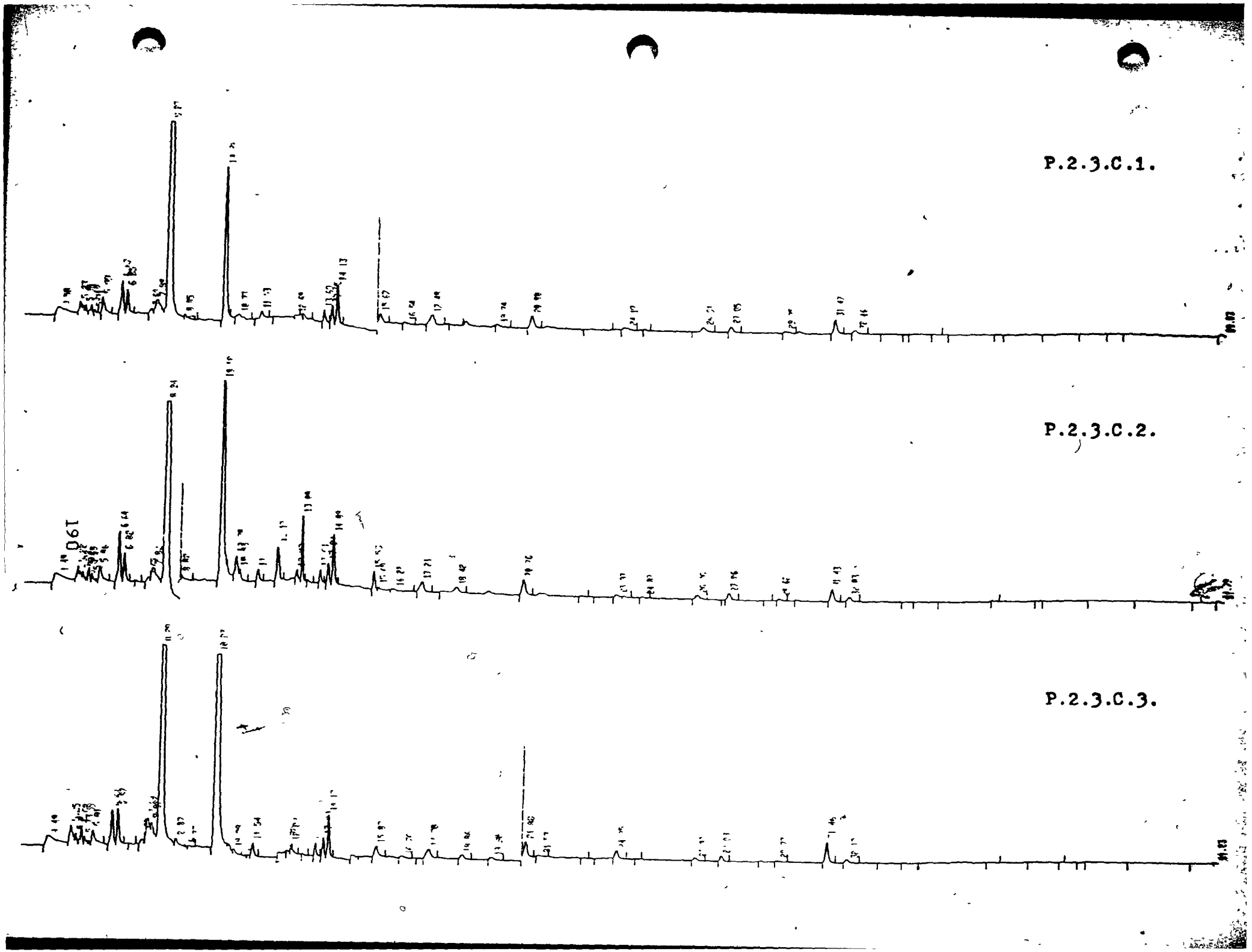
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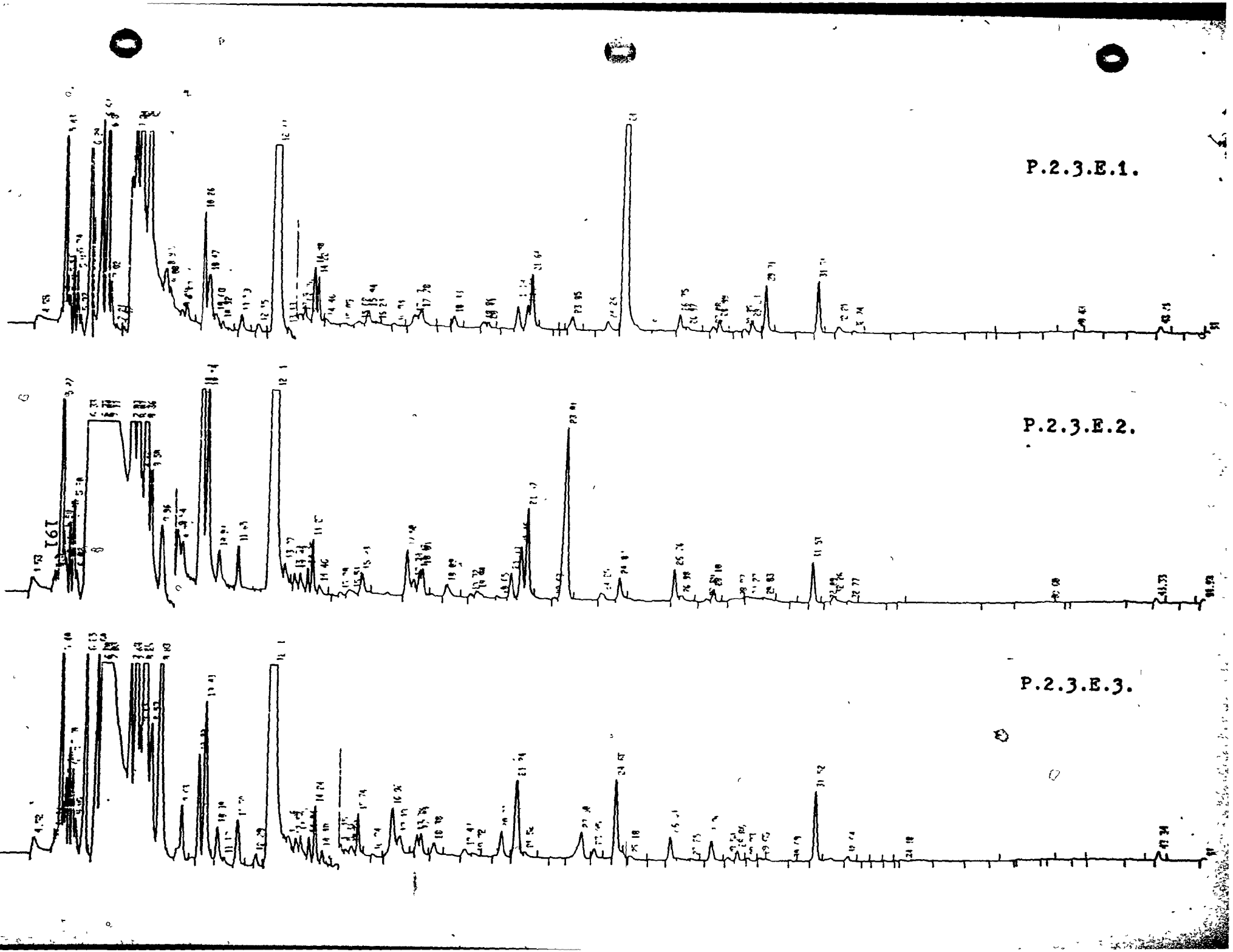
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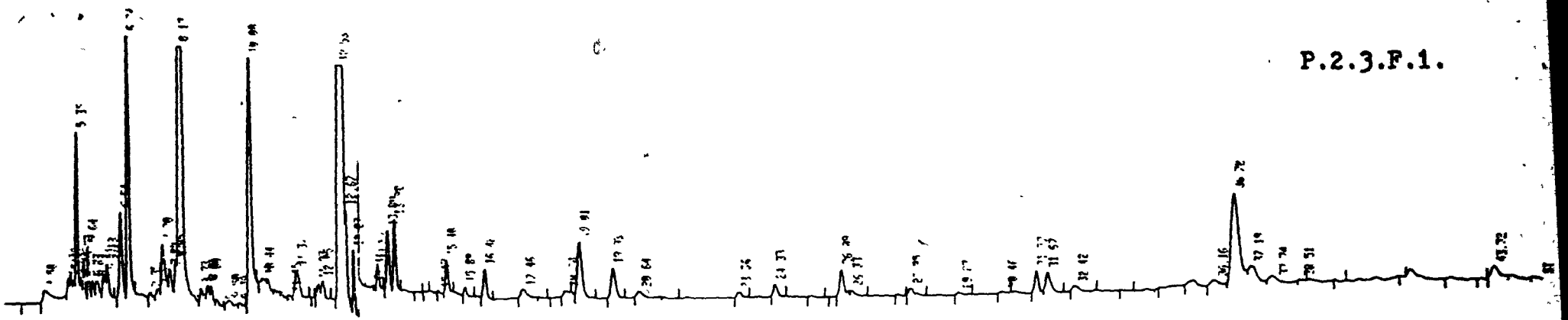

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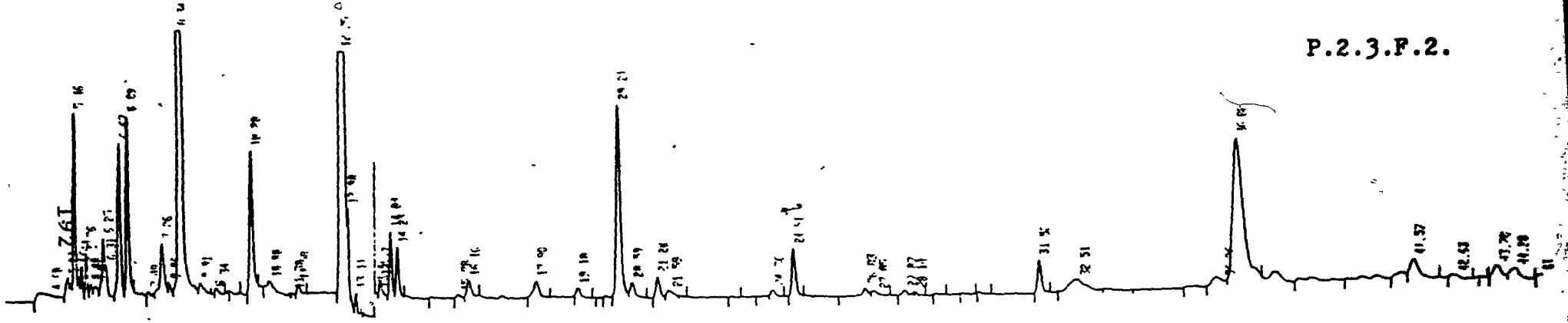

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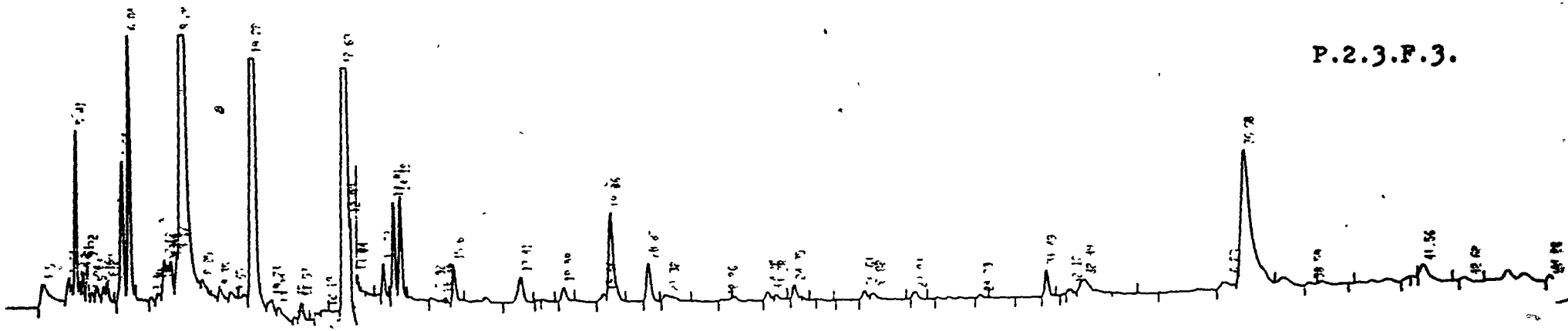

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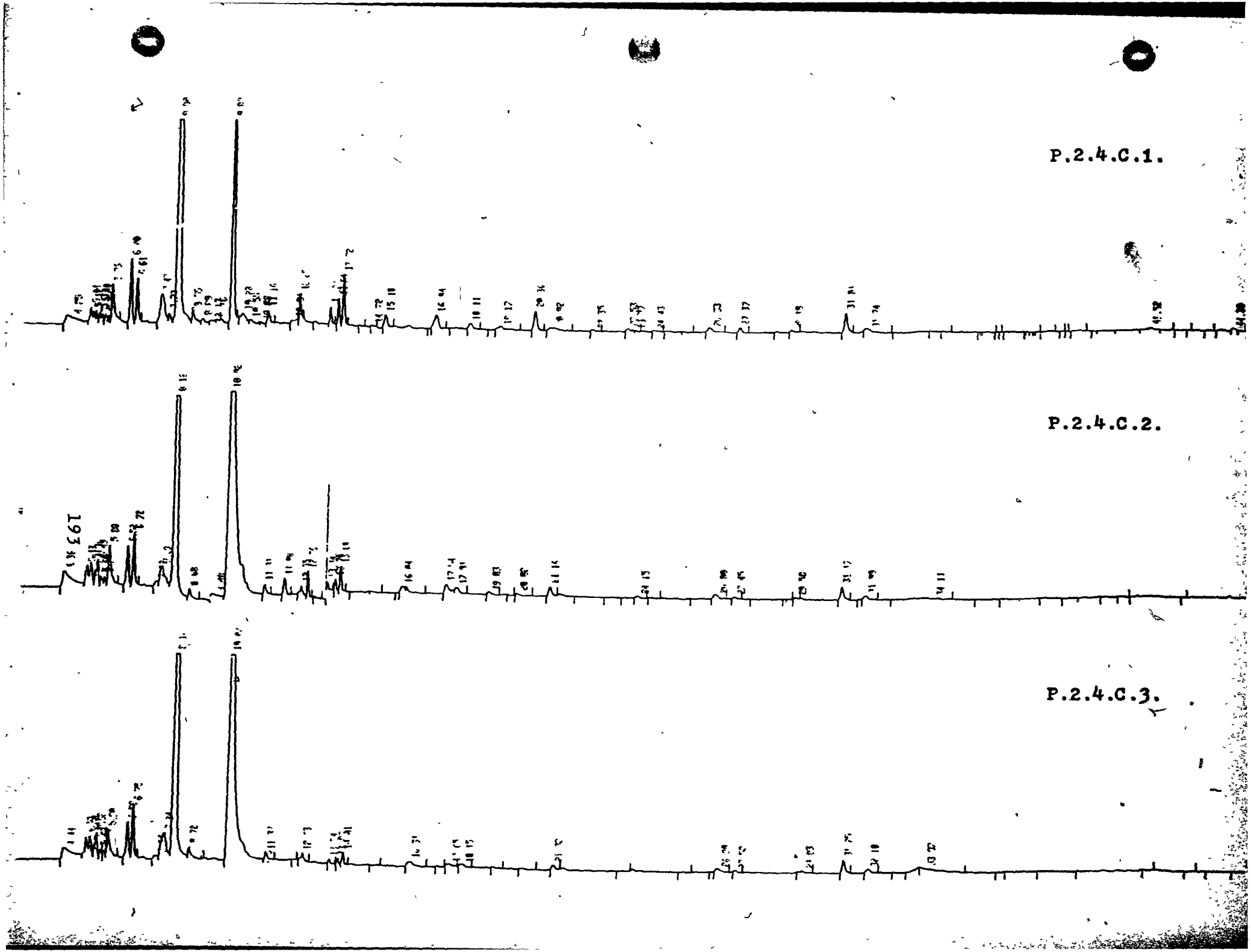
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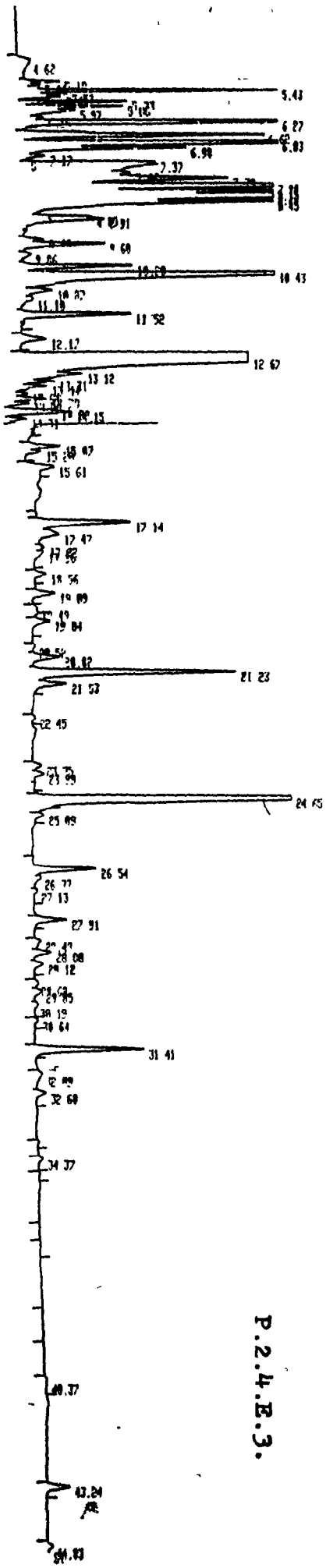

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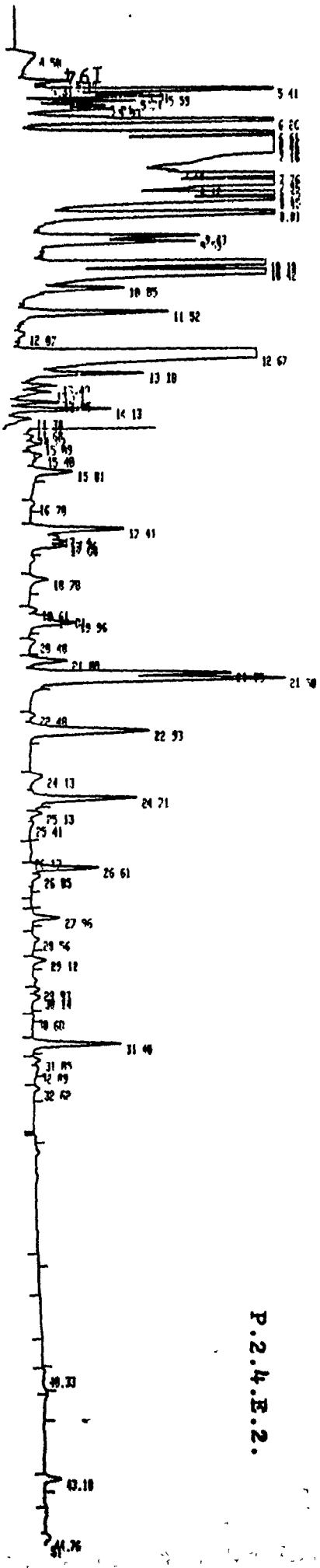

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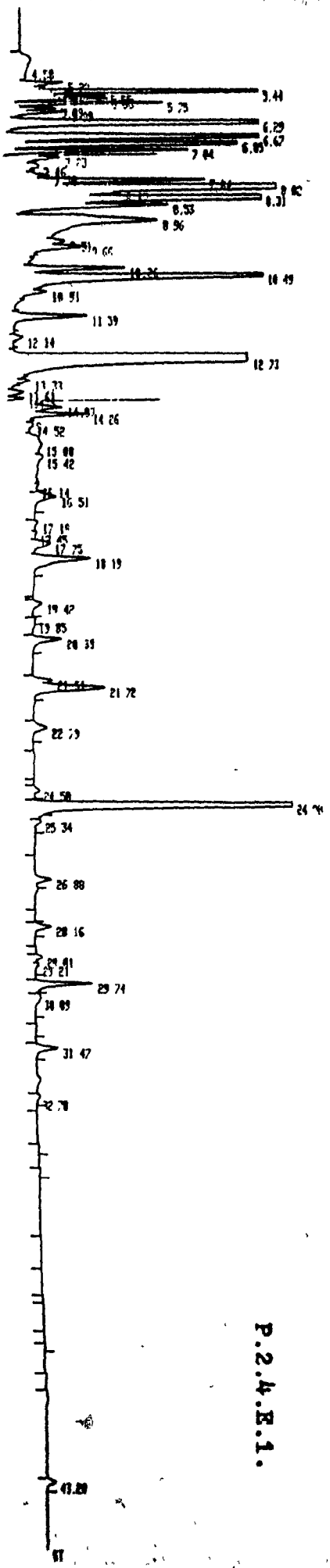

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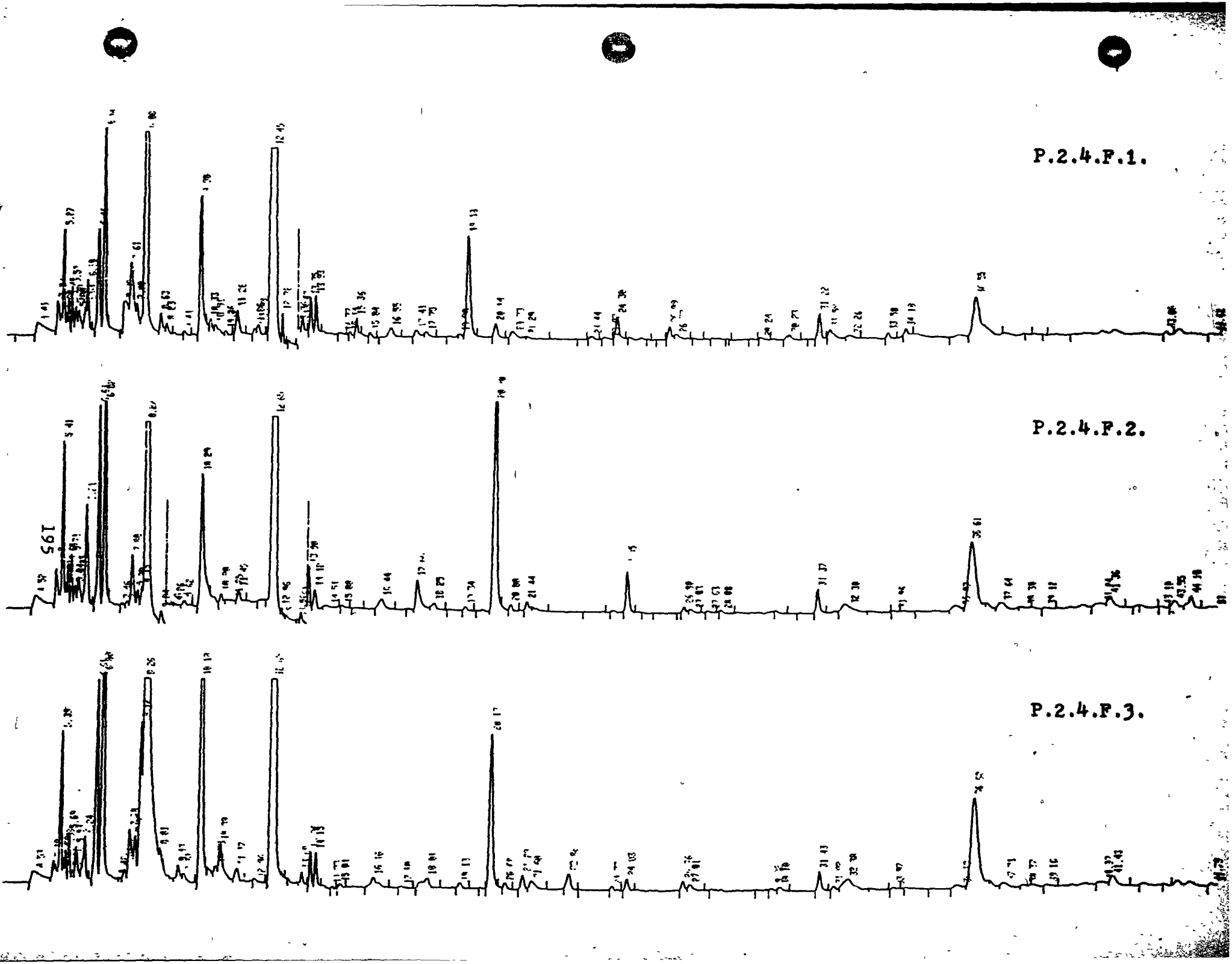
P.2.4.F.1.
P.2.4.F.2.
P.2.4.F.3.