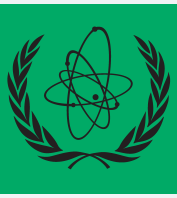
IAEA-TECDOC-1612

Quality Control of Pesticide Products

prepared by the
Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture



IAEA
International Atomic Energy Agency

July 2009

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The originating Section of this publication in the IAEA was:

Food and Environmental Protection Section
International Atomic Energy Agency
Wagramer Strasse 5
P.O. Box 100
A-1400 Vienna, Austria

QUALITY CONTROL OF PESTICIDE PRODUCTS
IAEA, VIENNA, 2009
IAEA-TECDOC-1612
ISBN 978-92-0-104909-4
ISSN 1011-4289

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Printed by the IAEA in Austria
July 2009

FOREWORD

The world population is expected to increase to 8.3 billion people by 2030 (FAO, 2002) and although increasing numbers will be well-fed, the target set in 1996 by the World Food Summit to reduce the number of chronically malnourished people to half by 2015 is unlikely to be met without improved agricultural technology, including the safe and efficient use of pesticides.

The majority of authorized pesticide products are currently undergoing a process of re-registration, particularly in countries where regulatory systems are well established. Only two thirds of these products are expected to meet the stringent new criteria for safety, toxicity and efficacy. As a result high-quality formulations for some pesticides produced in developed countries could be phased out. Inevitably the production and use of these pesticides will be continuing, mostly in developing countries, where pesticide registration systems are not well established. Without strong registration and enforcement standards various negative impacts are likely to re-emerge. For example, uncontrolled technical impurities accompanying any active substance or base material used for the formulation could worsen the toxicicological properties of final products and thereby cause additional adverse health and environmental effects. By the same token, inappropriately defined concentrations can lead to overuse and misuse of pesticides by the user.

Analytical quality control of pesticides analysis is crucial for ensuring their safe and effective use in agriculture. Supervision of a large number of pesticide products required for crop protection in agriculture can only be implemented with support from well-equipped laboratories, operated by trained and experienced staff applying methods suitable for the analysis of numerous active ingredients in a reliable and economical manner. This can only be fully achieved by using “Multi-Pesticide Methods” (MPM), instead of “single-analyte” methods optimized and collaboratively validated for a particular product formulation.

Recognizing the need of regulatory laboratories to improve their position through rationalization of their internal analytical regimes, a coordinated research project (CRP) was initiated within the Joint Programme of FAO and IAEA with the goal to assist national pesticide control agencies to assure the quality of pesticide products and hence supporting national legislation and regulations concerned with food quality and environmental protection. The CRP was designed with the help of consulting specialists and started with 15 Contract Holders and three Agreement Holders (see List of Participants).

The first Research Coordination Meeting (RCM) was held in Vienna, Austria, in 2001, the second RCM took place in the Philippines in 2003 and the final RCM was held in Myanmar in 2006. The hospitality of the respective institutes in hosting the RCMs, above all the assistance in the organization and conduct of Ms. Uy from the National Pesticide Analytical Laboratory, Bureau of Plant Industry, Quezon City, Philippines and of Mr. Shwe from the Plant Protection Division, Yangon, Myanmar, and their respective colleagues are appreciated.

The results of the research work reported in this IAEA-TECDOC provide detailed guidance for developing MPM, including practical examples demonstrating the application to the analysis of particular pesticide products. With the detailed description of the underlying principles, this publication may also be used as a training manual for the staff of respective pesticide formulation laboratories.

The main contributors of analytical results and elaborated papers to the IAEA-TECDOC are E. Dudar, Canping Pan, J. Lantos, I. Virtics, E. Karasali, and Zhiqiang Zhou. Their inputs are especially appreciated.

The CRP programme was formulated and initially led by Á. Ambrus, Head of IAEA Agrochemicals Unit until 2004. It was continued and completed by J. Brodesser, as Scientific Secretary. Finally, the IAEA-TECDOC was compiled by Á. Ambrus and the editing done by J. Brodesser.

EDITORIAL NOTE

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SUMMARY

The use of pesticides in agriculture has played an important role in the enhancement of agricultural production and protection of the health of humans and animals. Pesticide use in some regions, e.g. in South-East Asia and Latin America, has increased because of the need for improved agricultural production and reduced post-harvest losses, whereas in other regions it remains at about the same level. At the same time, the growing concern related to food safety by keeping the pesticide residues at the lowest possible level, the principles of Good Agricultural Practice (GAP), including Integrated Pest Management (IPM), have to be observed. This requires high quality pesticide products to assure the producers in the field that their crops are sufficiently protected when the application instructions on the label are followed and the pesticides are applied with calibrated and well-maintained spraying equipment.

Farmers may follow the application and safety instructions but this only holds true as long as the pesticides provide efficient control of pests and plant diseases. This can only be achieved with products of consistently high quality. Products of inferior quality may include imported and locally produced pesticides, especially the so-called generics, but also degraded, expired and other kinds of sub-standard products. Improper dosage and overuse are the consequences in the field. Such pesticides often contribute to the accumulation of obsolete pesticide stocks in developing countries. Regular quality control of pesticides marketed in any country is essential to facilitate their safe and efficient use and for increasing agricultural productivity while at the same time protecting the farmers, consumers and the environment. Therefore, safety of pesticide products remains an issue of great worldwide concern.

In spite of the introduction of more effective active substances, requiring only a few grams instead of kilograms per hectare, there has been an increase in the amount of pesticide active ingredients used annually. This is because of the availability of cheaper off-patent generic pesticides, which are applied in relatively large quantities per hectare. In many countries, the generic products are formulated in factories of varying size and technical capability. This production to a large extent is not regulated and monitored in the frame of rigorous quality control or surveillance programmes. Furthermore, reduced efficacy of sub-standard generic or obsolete pesticide products induces resistance of pests and increases residue levels on commodities resulting in risks to human health and the environment and wastage of national resources.

The estimated proportion of inferior quality products in developing countries amounts to about 30% [1], posing a serious threat to human health and the environment. There are also indications of an increasing number of counterfeit goods, which can significantly affect the quality of food crops and may cause considerable losses in yield. Due to the variability in formulation processes the proposition that a pesticide product “A” contains the same chemical substance active ingredient as product “B”, which had been cleared toxicologically, does not automatically mean that product “A” has similar toxicological (and efficacy) properties and is equally safe.

Effective pesticide product quality monitoring programmes greatly enhance the national capability to ensure more efficient pest control and reduce undesirable collateral effects on human health and the environment. The efficacy and environmental impact of a pesticide product is affected by several important parameters, including the physical properties of the formulation, the active ingredient content, the procedures and the equipment used for the application in the field. Concerning human health, with special emphasis on operators applying and handling the pesticides, the overall toxicity including any impurities in the technical grade active substances and the composition of the materials used for preparing the formulation are of primary importance. Throughout the world, regulatory authorities require the quality of pesticide products to be monitored, particularly their active ingredient content. At the very least the physical and chemical properties of formulations and active ingredients contents of the pesticide products should be determined.

Registration requirements of pesticides may differ from country to country, but there is the general approach to include data related to impurities of toxicological significance of technical material in the

registration documents. For the evaluation of a particular active ingredient the FAO Manual on Pesticide Specifications [2] requires a ”data profile” of the technical material, i.e., the provision of references to impurities, toxicological and ecotoxicological profiles. The minimum data package requires maximum limits for the content of impurities present at or above 0.1%, and also maximum limits for relevant impurities present at less than 0.1% supported by batch analytical data.

Many pesticides in the so-called high-registration countries are currently undergoing re-registration [3] with 470 pesticide products under review. About 350 products are eventually expected to pass the new safety and efficacy criteria. Inevitably the production and trade of the remaining pesticide formulations will continue, mainly in developing countries. Poor quality such as impurities of technical active substances may add to the acute toxicity of the product, causing further adverse health effects and environmental hazards.

The quality criteria outlined in the new procedure for the development of FAO Pesticide Specifications generally require collaboratively tested and validated methods approved by the Collaborative International Pesticides Analytical Council (CIPAC) or the Association of Official Analytical Chemists (AOAC International). CIPAC/AOAC methods [4] of analysis for pesticide formulations have been validated collaboratively for the quantitative determination of individual active ingredients mostly. In order to assure that the results of monitoring are valid, regulatory authorities require the quality of pesticide products to be monitored by means of fully validated methods such as those published through CIPAC/AOAC. However, there are only very few “multi-analyte” methods designed for simultaneous measurement of a wider range of pesticides. Formulation control laboratories therefore cannot utilize these easily as regular methods of analysis.

FAO specifications are a good starting point for pesticide quality control if other information is not available. However, such a specification is valid only for the particular product but not generally applicable because the nature and levels of impurities depend on the manufacturing process. Some impurities may multiply the acute toxicity compared with a formulation containing pure active substances [5]. To check the impurities, detailed information on the composition of the technical active ingredient should also be made available. However, this information is kept proprietary by the manufacturers. Impurity profiles are considered as intellectual property as it may give insight into the synthesis and technical production pathway. In general, it is treated strictly confidential and not made available to regulatory bodies and control laboratories.

As the raw materials used for the formulation of another (generic) pesticide may be different from those used by the original manufacturer, the applicability of CIPAC/AOAC methods, or any other, should be tested, modified if necessary, validated and verified for a particular formulation before use. Methods for the determination of one single compound are basically not applicable to mixtures (formulations with two or more active ingredients) of that compound together with other pesticides. The same applies to the application of a method for the determination of a chemical in another formulation type. Therefore, extreme precaution should be taken when applying a method to formulations or mixtures for other product(s) than it was originally developed for.

As a result of their independent development and specific interest in method precision, the CIPAC/AOAC methods utilize numerous different chemicals, various chromatographic columns and an extended number of different internal standards. This makes a unified application by laboratories extremely difficult in the daily laboratory practice, where up to several hundred different pesticide products have to be analyzed routinely. As a consequence, laboratories in reality often have to alter the critical parameters of the procedures, which sometimes are done without systematic validation but however claiming that a CIPAC/AOAC method has been used. This may adversely affect the validity and reliability of the analysis results. In addition, only about half of compounds currently in use are covered by CIPAC or AOAC methods. The composition of formulations may also be changed in the course of the years but methods are not revised at the same pace to take these changes into account. Consequently, there is a great need for laboratories to have access to simplified analytical methods that could be used under simplified conditions but at the same quality and reliability of results.

The results of measurements have to provide reliable information and the laboratory should be able to prove the correctness of measurements with documented evidence. Analysts carry great responsibility to produce correct and timely analytical results, and are fully accountable for the quality of their work. The accuracy and precision of the analytical results may be assured by proficient analysts applying properly validated methods fit for the purpose. It has become a global requirement that this should be done by laboratories accredited according to the relevant international standards. The International Standard ISO/IEC 17025:2005 [6] contains the general requirements for the technical competence to carry out tests that laboratories have to meet in order to demonstrate that they operate under a well-defined and consistent quality system and they are able to transparently generate valid results.

In this regard analytical procedures must be thoroughly validated before use according to a suitable protocol. Methods must be fully documented and staff adequately trained in their use. The analytical method should be fit for its intended purpose and fulfill the quality requirements in terms of accuracy, precision, sensitivity and specificity. The laboratory shall have quality control procedures for monitoring the batch-to-batch quality, accuracy and precision of the analyses undertaken, e.g., control charts should be used to ensure that the procedures are under proper statistical control.

There are some principal differences between the "multi-residue methods" (MRM) widely used in trace analysis and the "multi-pesticide methods" (MPM) applicable for pesticide formulations. In the latter case, only one to three active substances are to be expected in a test sample at usually high concentration (g/kg) and known origin and identity. The chromatographic separation and detection conditions are optimized only for a few substances then. The driving force in the development has been the pesticide industry's particular interest in specific methods for their respective formulations.

In order to assist Member States in strengthening their pesticide quality control capabilities especially in terms of routine pesticide formulation surveillance, a coordinated research project was proposed by the Joint FAO/IAEA Programme. The aim was developing and validating principles of MPM, suitable for the analysis of various types of pesticides with the same instrument setup, and to verify the practical applicability of the procedures under different laboratory conditions.

Recognizing the need of regulatory laboratories to improve their position, the CRP in particular was aimed at assisting national pesticide control agencies to assure the quality of pesticide products and to support legislation concerned with food quality and environmental protection, by:

- Elaborating the principles of method development and validation of pesticide formulation control multi-methods.
- Testing the repeatability and reproducibility of the individual processes in the participating laboratories.
- Elaborating/adapting chromatographic methods, which are suitable for the determination of the active ingredient(s) of several classes of pesticide formulations.
- Validating analytical methods for quality control of pesticides of local and regional importance.
- Demonstrating the practical applicability of the multi-pesticide methods in formulation control analysis.

The goal was to implement practical GC or HPLC methods with a limited number of chromatographic columns and mobile phases, normally using internal standards under individually optimized conditions. Thereby the time and chemicals required for the analyses and the need for frequent changes of columns should be reduced considerably. Before use, the applicability of chromatographic conditions including selectivity of the separation, specificity of detection, repeatability and reproducibility of measurements were to be tested and validated under the particular conditions of the laboratory in order to ensure the accuracy and precision of the results.

The CRP was implemented in 2000, the research work done from 2001 until 2006. This TecDoc summarizes the outcomes and results in four main sections and annexes:

- General documents providing guidance to analysts performing single laboratory validation of methods for pesticide formulation or technical active substance control.
- Summary of results obtained by the participants regarding the optimization of chromatographic conditions for the determination of active ingredients of pesticides.
- Multi-pesticide methods developed for illustrating the practical applicability of the elaborated procedures.
- Reports on the synthesis, identification and characterisation of impurities eventually occurring in technical products.
- IR, UV, NMR, MS spectra, GC and HPLC chromatograms of representative compounds (Annexes).

Initially, 16 laboratories from 14 countries took part in the practical implementation of the programme. The main achievements of the joint work are presented and summarized in this TecDoc.

The main achievements of the laboratories participating in the CRP were as follows:

- Stepwise procedures were developed for the elaboration and validation of MPM based on GC and HPLC separation techniques. Their practical applications are demonstrated here by means of worked-out examples.
- Internal quality control procedures were introduced and applied for demonstrating the suitability of the operation conditions and reliability of the results. Test mixtures for verifying performance of the whole chromatographic system were applied with specified performance criteria for inertness and stability of the system and goodness of calibration.
- The participants introduced and applied proper methods for the estimation of the uncertainty of measurement data and the statistical evaluation of results.
- The configurations of gas chromatographs were updated in seven laboratories, where necessary, e.g., to replace packed columns with wide bore capillary columns. Wide bore capillary columns are more inert and have much higher theoretical plate numbers resulting in better separation of eluting peaks than packed columns.
- The chromatographic conditions of the MPM were tested for over 70 active substances, and repeatability and reproducibility values were found acceptable for most of the compounds. Problems observed were mainly due to deficiencies in instrument configuration or operating conditions.

The main outcomes in terms of universal applicability for laboratories dealing with pesticide formulation control and in providing guidance on the control of technical grade products and their impurities are as follows:

- The HPLC analyses of pesticides are supported by UV spectra of recorded from 47 active pure substances. This will help laboratories to compare and adjust their analytical conditions according to the data given in the TecDoc. The UV spectra of respective compounds are presented in the Annexes.
- To facilitate the testing of the purity of technical active ingredients, 19 impurities that may occur in 22 different pesticide formulations had been synthesized, purified and identified. The NMR, MS and IR spectra of the respective compounds are presented in the Annexes.
- The majority of the quantitative determination of the pesticides tested could be carried out applying only six internal standards, with occasional need for others: adipic acid, benzyl benzoate, bis-(2-ethylhexyl) adipate, bis-(2-methoxyethyl) phthalate, chlorpyrifos, dibromonaphthalene, dibutyl phthalate, diisobutyl phthalate, diisopentyl phthalate, dimethyl phthalate, di-n-butyl sebacate, dioctyl phthalate, diphenyl phthalate, dipropyl phthalate, docosan, squalane, triphenyl phosphate. The total number could be further reduced in the daily laboratory

practice; in this CRP some laboratories were using other suitable internal standards as available in their stocks.

- The applicability of the MPM concept was demonstrated successfully by method validation data for 53 different formulated products. Many more pesticides showed promising results but the overall time frame of the project did not allow for an extension of the validation studies.
- Certain active ingredients of low volatility (e.g., pyrethroids, phosalone), or formulations that are difficult to homogenize (e.g., suspensible concentrates of atrazine, or granules containing terbufos and kresoxim methyl) turned out to be demanding in their analysis. Suspensible concentrates, granules, controlled-release formulations, baites etc. require special attention and treatment not only for the homogenization of the samples but also for their extraction.

The results of the research work of this CRP provide guidance for developing MPM and give examples for the analysis of particular pesticide products. By describing the underlying principles in detail, this TecDoc also serves as a practical training manual for the scientists and technicians of pesticide formulation laboratories.

The participants agreed that the multi-analyte methods validated within this CRP will greatly enhance national capabilities for pesticide product quality control, and facilitate monitoring of compliance with FAO Specifications and the 'International Code of Conduct on the Distribution and Use of Pesticides' [7].

The particular TecDoc sections "General Guidance Documents", "Experimental results", "Multi-Pesticide Methods", "Synthesis and determination of impurities" describe in greater detail the work done by the participants and the results achieved within the CRP. The Annexes present UV spectra of numerous pure compounds, which were included in the method descriptions. They can be used for further reference to help with the identification of unknown substances. In addition, 16 IR and NMR spectra each of synthesized impurities are given for reference and comparison with own acquired spectra. To support the identification of unknown peaks 11 GC chromatograms plus the respective mass spectra of characteristic impurities of pesticide active ingredients were included. Finally, lists of participants and of abbreviations used conclude the Annex.

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GENERAL GUIDANCE

PRINCIPLES OF SINGLE-LABORATORY VALIDATION OF ANALYTICAL METHODS FOR TESTING THE CHEMICAL COMPOSITION OF PESTICIDES

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Abstract

Underlying theoretical and practical approaches towards pesticide formulation analysis are discussed, i.e. general principles, performance characteristics, applicability of validation data, verification of method performance, and adaptation of validated methods by other laboratories. The principles of single laboratory validation of analytical methods for testing the chemical composition of pesticides are outlined. Also the theoretical background is described for performing pesticide formulation analysis as outlined in ISO, CIPAC/AOAC and IUPAC guidelines, including methodological characteristics such as specificity, selectivity, linearity, accuracy, trueness, precision and bias. Appendices I–III hereof give practical and elaborated examples on how to use the Horwitz approach and formulae for estimating the target standard deviation towards acceptable analytical repeatability. The estimation of trueness and the establishment of typical within-laboratory reproducibility are treated in greater detail by means of worked-out examples.

1. INTRODUCTION

Laboratories performing analytical measurements have to assure that their results are providing true information on the measured parameter. It normally means that the measured values are unbiased, accurate and precise, and the analysts can rely on the results obtained. In order to meet the above requirements the performance characteristics of the analytical methods used should be established through the validation process and recorded for future reference.

Method validation may be described as the set of tests used to establish and document the performance characteristics of a method and thereby demonstrate that the method is fit for a particular analytical purpose.

Application of validated methods is also one of the basic requirements of ISO Standard 17025 [1] and the OECD Principles of GLP [2].

It had been generally agreed in the past that the preferred means to validate a chemical analytical method is a full collaborative study using internationally accepted protocols, in which all participating laboratories operate under internationally accepted principles of quality assurance. AOAC International, IUPAC and ISO have jointly developed detailed protocols for conducting and evaluating collaborative studies [3, 4, 5, 6].

Validation of methods by inter-laboratory study has become impractical in many situations. The AOAC Peer Verified Method Programme [7], which requires the verification of the applicability of the method and comparability of the results in one or two independent laboratories according to a given detailed protocol. It provides a workable and cost effective compromise, which enables the inter-laboratory validation of methods; this would not be practical by means of full collaborative studies.

A collaborative study or verification of the performance of any method within the Peer Verified Method Programme requires that first of all it is validated in the author's laboratory usually. Regardless whether further validation is planned or not, there is a need for the laboratories to validate their own methodology before use. The above mentioned protocols were adapted by a CIPAC

Working Group for pesticide quality control [8] in one laboratory. The term 'single-laboratory validation' had been recommended by an AOAC/FAO/IAEA/IUPAC International Workshop on Principles of Method Validation [9] to describe validation in one laboratory instead of the expression 'in-house validation'. These guidelines had been accepted as a Codex Standard [10].

The laboratories should also demonstrate their competence in performing specified tests through participation in proficiency tests and obtaining accreditation. It is especially important for regulatory and contracting laboratories as the results may be used for governmental and enforcement decisions, or for settling trade disputes.

2. GENERAL PRINCIPLES

Analytical methods may be developed in the laboratory, taken from the literature or otherwise obtained from a third party. The method may then be adapted or modified to match the requirements and capabilities of the laboratory and/or the purpose for which the method will be used.

Typically, validation follows completion of the development of a method. Therefore, it is assumed that some of the method performance requirements such as linearity of calibration, system suitability, analyte stability, etc., have been established satisfactorily, and the analysts are sufficiently experienced in performing the method. When validating and using a method of analysis, measurements must be made within the calibrated range of the detection system used. In general, validation will precede a practical application of the method for the analysis of samples but subsequent internal quality control (IQC, referred to in the present publication as performance verification) remains an important aspect of the process.

Proficiency testing [5, 11], where practicable, provides an important means for verifying the general accuracy of results generated by a method, and provides information on the between-laboratory variability of the results. Successful participation in proficiency test programmes does not replace the establishment of within-laboratory performance of the method. It provides complementary evidence of method and laboratory performance, as does the demonstration of the repeatability of the method in other laboratories by peer verification or collaborative studies.

Where uncertainty data must be reported to the client (the user of results) this information should incorporate performance verification data and not rely solely on method validation data.

Analytical methods, and the validation required for them, must be fit for the purpose for which the results are likely to be used. In general, method performance and validation requirements should be agreed between the analyst and the client (the purchaser/user of results, as appropriate). Method performance requirements have been defined in general by AOAC International [12] and specifically by CIPAC [7] for methods applied in control of pesticide formulations.

Extensions of methods to new analytes, matrices (e.g. additional formulations, or technical active ingredients), lower concentrations, smaller test portions, use of the method in other laboratories, etc., should be validated as indicated in this publication. Minor changes in methods may be validated through performance verification but, where the change leads to unsatisfactory performance, the method may require modification and re-validation.

Inefficiency of extraction can be the major source of bias. Rigorous validation of extraction efficiency of analytes in formulated pesticide products can only be performed with samples containing analyte(s) in exactly known concentration incorporated in the commercial formulation. Recovery of analytes from samples spiked shortly before extraction does not necessarily reveal correct information on the extractability of the analytes from the formulated product (this is the most serious disadvantage of the standard addition method described later). Suitable certified reference materials are rarely available which makes the task of the laboratories more difficult.

Clearly written detailed instructions (preferably standard operating procedures) for the method, including accurate descriptions of equipment and reagents to be used, must be available. These instructions should be closely adhered to during the validation process.

Detailed records on method validation experiments must be maintained where required according to the principles of GLP or ISO 17025.

3. PERFORMANCE CHARACTERISTICS OF METHODS TO BE ESTABLISHED

The development, application and validation of a method are described with different terminology. Table 1 summarizes the expressions used generally in method validation guidelines.

TABLE 1. STAGES OF THE APPLICATION AND USE OF ANALYTICAL METHODS

Stages	Generally used terms	Related expressions
1	Development or adaptation of analytical procedure	Pre-validation
2	Establishment of acceptable performance (validation) in the author's laboratory	Validation proper Single laboratory validation
3a	Demonstration of acceptable performance in a second or a third laboratory	Validation study
3b	Demonstration of acceptable performance in full inter-laboratory collaborative study	Full study
4	Regular use of the method, including quality control	Performance verification

The performance characteristics of the method are the experimentally derived values, for the fundamental parameters of importance in assessing the suitability of the method. The information for the characterization of a method may be gathered during any of the above phases. The parameters to be used for characterization of methods applied for the determination of active ingredients and impurities in pesticide products are listed below, together with the recommended procedure or explanations for their estimation or demonstration, based on the CIPAC Guidelines [7].

(a) Applicability

The scope of application, or applicability, of the method should identify the matrix and analyte being measured, its concentration range and the type of study for which the procedure, as judged from its performance characteristics, is suited. It should also describe the known limitations of the method.

E.g.: This capillary GC method provides for the determination of active ingredient content of X, Y and Z [commercial name of pesticide product].

(b) Specificity

The specificity of the method is a definition of the species giving rise to the signal used for quantitation (CIPAC). It shows that the detected signal is solely due to the analyte, not another compound.

(c) Selectivity (IUPAC)

The selectivity is the ability to discriminate between the analyte(s) to be determined and other materials in the test sample. It describes the separation power of the procedure applied.

Note: The terms specificity and selectivity are often used simultaneously to describe the same phenomenon. However they have specific meaning in this context: specificity describes the performance of detection, while selectivity is used for characterising the chromatographic separation.

Non-analyte interference: This is covered to some extent by the assessment of accuracy, since any interference from excipients will confer a systematic error on the method. However, an analysis should be carried out using an excipient blank, either to demonstrate lack of, or quantify any occurring interference.

Where specific impurities are known to occur in the technical active ingredient, it must be demonstrated that these do not contribute more than 3% to the total peak area measured for the analyte or internal standard under the conditions used for the analysis. If there is such a known bias, it must be indicated whether or not submitted results are corrected.

Notes:

(i) The acceptability criterion of 3% contribution should be applied with great care. If we take, for example, 50% a.i. content, 3% would contribute to 1.5% from the usual ±2.5% tolerance interval. On the other hand, to quantify 3% contribution to the area may be very difficult and sometimes impossible. Ideally there should be no measurable interference with the quantification of the analyte. 3% represent practical reality.

(ii) Manufacturers have blank formulations but they cannot manufacture technical products without impurities and their sources of formulants often change. The problem may be amplified if another manufacturer's product is analyzed with a standard method. Proving selectivity and specificity is likely to require consideration on a case-by-case basis but there are some general points. Manufacturing impurities (including those from formulants) always vary considerably in concentration and there is always a risk that interference will occur. Proving that it has contributed up to +3% to the determination of the active ingredient is likely to be very costly. Proving that it contributes down to -3% through interference with the internal standard is easy (analyze samples without internal standard and look for interfering peaks). Attempts to detect/ determine interference at levels of a few per cent, using different separation systems and/ or different detection systems can be misleading. Therefore, data should be interpreted cautiously.

(iii) Generation of spectral data offers the best chance of success. Attempts to use NMR have not been very encouraging. UV-spectral matching can be useful but impurities can have almost identical spectra to that of the analyte in some cases. GC-MS (or GC-MS^n) using EI in scan mode can be suitable to use for optimising the GC separation system. However, great care is required to avoid distortion of the spectra through overload and inappropriate background subtraction.

(iv) LC-MS (or LC-MS^n) is not widely used for pesticide formulations analysis, partly because of relatively poor precision (in addition to being a more tricky technique than GC-MS in EI mode). However, in principle, low level interferences tend to be suppressed (through competition in accepting or donating protons) in LC-MS. Ironically, suppression of this kind of the matrix from major constituents is a big problem for residues analysts. LC-MS definitely is not a panacea for formulation analysis but it can be useful in certain applications.

(v) The CIPAC guideline only refers to chromatographic methods; it does not help when the only method available is a colorimetric one or using titration. For the future, capillary electrophoresis (CE) may provide a good alternative. The separations obtained in CE can be much better than with HPLC and the potential for interference consequently reduced, but not eliminated, so the requirement for measurement of interferences remains.

(d) Linearity

The linearity of a test procedure is its ability (within a given range) to obtain test results proportional to the concentration (amount) of analyte in the sample.

The linearity of response to the analyte should be demonstrated at least over the range of nominal analyte concentration ±20%. At least three concentrations should be measured in duplicate each. The calibration curve generated should be submitted, together with slope, intercept and correlation coefficient data.

The measured slope should demonstrate a clear correlation between response and analyte concentration. The results should not show a significant deviation from linearity, meaning that the correlation coefficient (r) is > 0.99, over the covered range (nominal concentration ±20%). If this is not the case, the analyst must provide an explanation of how method validity is to be

maintained. In cases where a non-linear response is deliberately used, an explanation must also be provided.

The range of linearity response for a detection system frequently is dependent on a particular instrument. Special attention is required to avoid overloading of narrow bore capillary columns ≤ 0.3 mm internal diameter. If a method is used with a different system, the linearity should be re-checked.

(e) Accuracy

Accuracy is the closeness of agreement between a single test result and the accepted reference or true value of the property being measured (μ-x_i), for example the true content of a specific analyte in a sample.

The accuracy of a method is the degree to which the observed results correspond to the true value of the analyte in the sample.

Note: The procedure and statistical tests recommended by CIPAC for testing accuracy do not correspond with the above definitions. Therefore it is included under Trueness, with the terminology used by ISO and IUPAC.

(f) Trueness (ISO, IUPAC)

The closeness of agreement between the average value, obtained from a large series of test results, and an accepted reference value. The measure of trueness is usually expressed in terms of bias. The true value is very rarely known or can be determined in chemical analysis, therefore it has been replaced by an ”accepted reference value”.

The accuracy of the procedure should be assessed by the preparation and analysis of at least four samples of laboratory-prepared 'synthetic' formulation containing known weights of the analyte. The results may be assessed using the Students t-statistics or other acceptable approaches.

Notes:

(i) The statistical test recommended by CIPAC is shown hereunder as it characterises trueness and not the accuracy.

(ii) Synthetic formulation is rarely available for a regulatory laboratory. Therefore alternative methods have to be chosen for assessing the trueness of the results. One possibility is the comparative analysis of a number of samples (minimum 5) taken from different batches of the pesticide product with the method to be validated and with an official or standard method (CIPAC or AOAC applied exactly as described). Note that the number of samples to be analyzed depends on the difference in the mean values which will have to be quantified and the precision of the methods used.

(iii) Another practical option would be the addition of analytical standard (this not the same as ”standard addition calibration method”).

Standard addition procedures involve the addition of known quantities of pure substance to portions of previously analyzed sample, and repeating the analysis using the same reagents, instruments, and technique. The sample of interest is analyzed in duplicate to ensure that results from various portions of the sample composite will have less error. Then, another portion of the sample composite is taken and spiked with an accurately measured amount of the analyte equivalent to about 20–30% of that found in the original analysis. The recovery (Q) for the method is then calculated as:

$$Q = \frac{C_{m1} - C_{m0}}{C_1}$$

Where C_{m0} is the analyte concentration measured in the sample, C_{m1} is the analyte concentration measured in the spiked sample and C_1 is the analyte added to the sample.

The standard addition procedure is not as rigorous as using a good synthetic formulation. However, the method is especially useful in situations where there is a constant

background of interference from matrix elements and it is not possible to suppress them. Furthermore, spiking with two or more concentrations and comparing the signal of the analyte measured in spiked samples and in pure standard solutions will reveal the matrix effect. It is important that the standard addition or spike be made early in the analysis, and not in the last step. If addition is made in the last step, the validation only extends to the addition of the spike and the subsequent measurement step. Another disadvantage of standard addition sample is that it is frequently easier to recover the pure substance that is added to a prepared extract than it is to extract the compound from the original sample material. In other words, spiking the pesticide product before the analysis may give too high recoveries.

(iv) Theoretically, the analysis of certified reference materials would also be suitable for validating analytical procedures but in practice it is not applicable due to the lack of appropriate materials.

(g) Bias $|\mu - m|$
is the difference between the expectation of the test results (m) and an accepted reference value (μ).

Note: Bias is the total systematic error as contrasted to random error. There may be one or more systematic error components contributing to the bias. A larger systematic difference from the accepted reference value is reflected by a larger bias value.

(h) Precision
Precision is the closeness of agreement between independent test results obtained under stipulated conditions.

Precision is a measure of random errors, and may be expressed as repeatability and reproducibility. These terms are defined in ISO 5725:1986E:

Repeatability is the closeness of agreement between mutually independent test results obtained with the same method on identical test material in the same laboratory by the same operator using the same equipment within short intervals of time.

Reproducibility is the closeness of agreement between test results obtained with the same method on identical test material in different laboratories with different operators using different equipment.

A simple assessment of repeatability will be acceptable. A minimum of five replicate sample determinations should be made together with a simple statistical assessment of the results including the per cent RSD.

If considered appropriate, a suitable test for outliers (e.g. Dixons or Grubbs Test) may be applied to the results. However, it should be clearly indicated if results have been discarded and some attempt made to explain why the outlier may have occurred.

The acceptability of the results should be based on the modified Horwitz equation;

$RSDr < 0.67 \times 2^{(1 - 0.5 \log C)}$

where C = concentration of the analyte in the sample as a decimal fraction.

The derivation and worked examples of the Horwitz equation are in Appendix 1.

Note: Horwitz suggested a simplified formula [13] for the calculation of the expectable relative standard deviation: $RSD = 2 \times C^{(-0.1505)}$

3.1 Terms not used by CIPAC Guidelines but generally required for method validation

(a) Calibration
The calibration or standard curve is a graphic representation of the measuring signal (the response variable) as a function of the quantity of analyte or measurand.

(b) Range
The interval of concentrations within which the analytical procedure demonstrates a suitable level of precision and accuracy.

(c) Limit of detection
The limit of detection is the smallest amount or concentration of analyte in the test sample that can be reliably distinguished, with stated significance, from the background or blank level. This parameter should only be tested for methods intended for measuring trace amount of impurities, i.e. < 100 mg/kg (< 0.01%).

(d) Limit of quantification
The limit of quantification of an analytical procedure is the lowest amount or concentration of analyte in a sample which can be quantitatively determined with an acceptable level of precision and accuracy. This parameter should only be tested for methods intended for measuring trace amount of impurities, i.e. < 100 mg/kg (< 0.01%).

(e) Sensitivity
The sensitivity of a method is a measure of the magnitude of the response caused by a certain amount of analyte.

(f) Ruggedness
The ruggedness of an analytical method is the resistance to change of an analytical result when minor deviations are made in the experimental conditions of the procedure.

(g) Practicability
The ease of operation, in terms of sample throughput and costs, to achieve the required performance criteria and thereby meet the specified purpose.

4. APPLICABILITY OF VALIDATION DATA TO MORE THAN ONE FORMULATION (ACCORDING TO THE CIPAC GUIDELINES)

In general, validation data should be considered formulation specific. However, it is recognized that manufacturers may produce a number of very similar formulations and it may be possible to use a single method for these. The criteria for cross-applicability are:

(a) The formulations should contain the same (or very similar) co-formulants. Any qualitative change in co-formulants should be checked for potential interference.

(b) The formulations should not differ markedly in physico-chemical properties (e.g. pH).

(c) The concentrations of active ingredients in the analytical solutions must remain within the demonstrated linearity ranges.

(d) Any changes in relative co-formulant concentrations should not yield significant interference.

The selectivity and specificity of the detection of the analyte shall be verified before a method is used for a similar type of formulation, which is not an easy task. See notes under the terms.

5. VERIFICATION OF THE PERFORMANCE OF THE METHOD

The tests performed during method validation to determine the trueness and precision of the procedure are very limited due to time and financial restrictions. It is very important that the performance characteristics of the method are verified during its regular use. Based on the performance verification results, the performance characteristics of the method should be refined. The refined performance characteristics should be used further on as reference for demonstrating that the method behaved as expected, that is it was applied under statistical control.

The establishment of typical standard deviations from the results of replicate analysis of samples is demonstrated in Appendix III.

5.1. Quality control of samples analyzed at irregular intervals

When samples are analyzed irregularly control charts cannot be used for demonstrating the performance of the method. In such a case analyze the samples in triplicate. Compare the results to the critical ranges calculated from the standard deviation obtained during method validation, sr' (or the repeatability, s_r, given for CIPAC or standard methods) taking the test quotient of 1.693 for 3 replicate measurements.

The results are acceptable if their range is within the critical range or if their standard deviation (calculated as: $s_r' = (C_{max} - C_{min})/1.693$) is not significantly different from s_r based on F-test. See example in Appendix IV.

6. ADAPTATION OF A VALIDATED METHOD IN ANOTHER LABORATORY

6.1. The method is performed without any change

Check system suitability, selectivity and specificity of detection. Practice the method performing it exactly as described. When the method is "in hand" perform a minimum of five replicate analyses and determine the repeatability standard deviation.

The method is considered adapted if s_r' obtained is not different significantly (2-tailed F test at $P = 0.95$) from the reference value, s_r.

6.2. The method needs to be modified

Adapt the method for your laboratory conditions. Test selectivity and specificity of detection. Verify trueness of results with comparative analysis or standard addition as described above. Continue according to 6.1.

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APPENDIX I

The Horwitz equation for acceptable repeatability

The Horwitz equation was derived from the statistical analysis of a number of collaborative studies done by AOAC over many years.

In the equation, C is the concentration of analyte in the sample as a decimal fraction and RSD_R is the between-laboratory reproducibility coefficient of variation.

So, for a 100% pure sample, C = 1, logC = 0, and

$$RSD_R = 2^{(1 - 0.5 \log C)} = 2^1 = 2\%$$

For a 50% sample (e.g. a 500 g/kg WP), C = 0.5 LogC = -0.3010, and

$$RSD_R = 2^{(1-(0.5 \times (-0.3010)))} = 2^{1.1505} = 2.22\%$$

Other RSD_R values are:

Concentration in %	Fraction	RSD_R
20	0.2	2.55
10	0.1	2.83
5	0.05	3.14
2	0.02	3.60
1	0.01	4.00
0.25	0.0025	4.93
0.10	0.001	5.66
0.01	0.0001	8.00
0.001	0.00001	11.31
0.0001	0.000001	16.00

Horwitz noted that values for RSD_r (the repeatability CV) were usually between half and two thirds that of RSD_R. For this reason, the acceptability limit for repeatability was proposed as the Horwitz values for $RSD_R \times 0.67$. It corresponds very well with the theoretically expected S_R based on the performance of the method in two laboratories, each with S_r ($S_R = 1.41 \times S_r$, or $S_r = 0.709 \times S_R$).

The Horwitz equation is currently used as a criterion of acceptability of the precision of methods collaboratively tested by CIPAC.

APPENDIX II

Estimation of trueness[1]

The following procedures illustrate various approaches to the estimation of the trueness of a procedure.

The accuracy of a procedure may be determined by the examination of a number of 'samples' containing a known quantity of the analyte. These should be laboratory-prepared co-formulant mixes to which a known quantity of analyte (corresponding to the concentration in the targeted formulation) is added. The analyte added should be a technical active ingredient of known purity. The whole sample should be analyzed to eliminate sampling error. At least four recovery experiments should be done, following exactly the proposed procedure. The results should be treated as follows:

(a) Calculate the mean recovery and relative standard deviation (CV_A) of the recoveries.

(b) Apply the F-test to the standard deviation obtained and calculated with Horwitz equation to confirm that the recovery results do not show a significantly different SD to the expected precision.

(c) If (b) is satisfactory, apply Student t-test to the recovery results, i.e. attempt to prove that the observed recovery (mean) differs from the concentration added only by random errors (no evidence of systematic error). (μ = added concentration, $\bar{x}$ = mean value of replicate measurements).

$$t = \frac{\left|\bar{x} - \mu\right|}{s/\sqrt{n}}$$

When the calculated t value is smaller than the tabulated critical value (the tabulated values for 2-tailed test at $\alpha = 0.05$ or P = 0.95 is 3.182), then the mean recovery is not significantly different from 100%.

The MS Excel program has the critical t-values as a built in function. It can be obtained in the English version as '= TINV (probability; deg_freedom) → enter'.

Note that:

- The '$\underline{2\alpha}$ = 1-P' value should always be inserted for probability in the command. The Excel formula provides the critical values for two sided (tailed) t-test. If one-sided test is performed, then the usually applied 95% probability criterion corresponds to P = 0.9, thus 2α is equal to 0.1.
- The degree of freedom shall be selected according to the test. In this example df = n-1, where n is the number of replicate tests.

Some examples for the t_{crit} values are given in Table AII.1. It is good practice to compare the values obtained with Excel with the tabulated ones to verify that the Excel function was correctly used.

The mean percent recovery should be within the following ranges:

Active substance (nominal) %	Mean % recovery
>10	98.0 – 102.0
1-10	97.0 – 103.0
<1	95.0 – 105.0

[1]According to CIPAC GLs which originally used the term accuracy

Active substance (nominal) %	Mean % recovery
> 10	98.0 – 102.0
1-10	97.0 – 103.0
< 1	95.0 – 105.0

Notes:

1. The same procedures may also be applied to sub-samples of a mixture of known composition, but it must be noted that this will tend to give a larger value for the CV of the determination (and consequently to the confidence interval of the mean) for formulations which are not homogeneous at the time of removing the sub-samples. The CV obtained from sub-samples taken from a commercial formulation is actually the CV_L that includes the CV of sample processing (CV_{Sp}) and analysis.
2. Where it is very difficult to prepare the artificial formulation to be analyzed similar to the commercial formulation (e.g. for a pellet or block bait type) the accuracy may be estimated through a standard additions procedure. In this case, full details of how the standard additions were done should be submitted.

TABLE AII.1. CRITICAL VALUES FOR T-TESTS

2-sided test	P = 0.95; α = 0.05	P = 0.90; α = 0.1
1-sided test	P = 0.975; α = 0.025	P = 0.95; α = 0.05
1	12.70620	6.31375
2	4.30265	2.91999
3	3.18245	2.35336
4	2.77645	2.13185
5	2.57058	2.01505
6	2.44691	1.94318
7	2.36462	1.89458
8	2.30600	1.85955
9	2.26216	1.83311
10	2.22814	1.81246
11	2.20099	1.79588
12	2.17881	1.78229
13	2.16037	1.77093
14	2.14479	1.76131
15	2.13145	1.75305
25	2.05954	1.70814
100	1.98397	1.66023
1000	1.96234	1.64638

APPENDIX III

Establishment of typical within-laboratory reproducibility of a method

1. Calculation of within-laboratory reproducibility

The procedure is illustrated with a practical example.

The performance parameters of a method for the analysis of 500 g/l EC formulation were established by CIPAC: r = 8 g/l, R = 15 g/l.

The method was adapted in a laboratory and used for the determination of the active ingredient content of various batches. The results of duplicate analysis of different samples are given in Table AIII/1.

TABLE AIII.1 RESULTS OF DUPLICATE ANALYSES OF BATCHES OF A PESTICIDE

Test 1	Test 2	Mean	Abs range
497	512	504.5	15
510	521	515.5	11
515	525	520	10
500	501	500.5	1
471	486	478.5	15
452	466	459	14
532	522	527	10
471	463	467	8
474	481	477.5	7
521	515	518	6
517	528	522.5	11
521	532	526.5	11
515	512	513.5	3
499	492	495.5	7
515	526	520.5	11
502	493	497.5	9
481	476	478.5	5
482	476	479	6
475	483	479	8
483	489	486	6
Average		498.3	8.7

Evaluate the results and estimate the within-laboratory reproducibility of the method.

Explanation:

The differences between the test results (range) are often higher than the established r value, but they are ≤ R. The CV_{Wr} is 1.54% (see calculation below) well between the RSD_r 1.49% and RSD_R 2.22% calculated from the Horwitz equation (Appendix I).

As the within-laboratory reproducibility can be expected to be somewhere within r and R, the results of the tests can be accepted. Note that the average range (8.7 g/l) is close to the estimated r.

The within-laboratory reproducibility SD, $S_{wR,}$ can be calculated from the average range and factor d2 (see Table AIII.1) as:

$$S_{wR} = 8.7/1.128 = 7.71 \text{ g/l.}$$

$$CV_R = 100 \times 7.71/500 = 1.54\%$$

Alternatively, the following equation may also be used:

$$s = \frac{1}{\sqrt{2}}\sqrt{\frac{\sum d^2}{n}} = \sqrt{\frac{\sum d^2}{2n}} \qquad (1)$$

Where d is the difference between the results of duplicate measurements. In this particular example it would give 6.67 g/l as an estimate for the reproducibility standard deviation. The difference between the two estimates is statistically not different. It is quite common that somewhat different results are obtained where a parameter is estimated with different methods.

2. Control chart for ranges

Calculate the control chart for the range based on the reproducibility value obtained for the method with the constants given in Table AIII.2:

TABLE AIII.2. FACTORS FOR 95% WARNING AND 99.8% ACTION LIMITS

n	w_1	w_2	D_3	D_4	d_2
2	0.039	2.809	0.002	4.124	1.128
3	0.179	2.176	0.036	2.992	1.693
4	0.289	1.935	0.098	2.579	2.059
5	0.365	1.804	0.158	2.358	2.326

Note: n is the number of measurement pairs used to estimate the average reproducibility range, $\overline{R}$.

Upper action limit:	$D_4 * \overline{R}$	36	(35.8788)
Upper warning limit:	$w_2 * \overline{R}$	24	(24.438)
Central value:	$\overline{R}$	8.7	From Table AIII.1
Lower warning limit:	$w_1 * \overline{R}$	0.34	0.339
Lower action limit:	$D_3 * \overline{R}$	0.17	0.0174

Note that for establishing control charts for within-laboratory reproducibility:

1. The samples analyzed in duplicate preferably (but not necessarily) should comply with the specification in order to take the results of their replicate analysis into account.
2. In order to claim that the method is applied under statistical control, during the analyses of samples taken from the same formulation, the difference of replicate measurements should be within the warning limits. Once in every 20 pairs the result may be within the action limits.

STEPWISE PROCEDURE FOR DEVELOPMENT AND VALIDATION OF A MULTI-PESTICIDE METHOD

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Abstract

The stepwise procedure for development and the validation of so called multi-pesticide methods are described. Principles, preliminary actions, criteria for the selection of chromatographic separation, detection and performance verification of multi-pesticide methods are outlined. Also the long term repeatability and reproducibility, as well as the necessity for the documentation of laboratory work are highlighted. Appendix I hereof describes in detail the calculation of calibration parameters, whereas Appendix II focuses on the calculation of the significance of differences of concentrations obtained on two different separation columns.

1. PRINCIPLE OF MULTI-PESTICIDE METHODS

The multi-pesticide analytical method is based on the sample preparation and extraction procedures applied in the reference methods (CIPAC or AOAC) which had been validated. The detection of the active ingredient contents may be carried out applying the same GLC or HPLC columns for various pesticide products included in the method, provided that the elution conditions can be selected to insure the specificity and selectivity of the detection and separation (see Chapter 5.1), respectively.

There are two principal differences between the multi-residue methods widely used in food and environmental control analyses and the multi-pesticide method: currently there is a limited number of active substances (maximum 3) in a single pesticide formulation, and their identity is normally known, in contrast to pesticide residue analysis where a single sample may contain over 10 to 15 chemically different residues of unknown origin. Furthermore, pesticide residues are normally present in the concentration range of 0.001–100 mg/kg, while the pesticide products contain the active substance (AS) in concentrations > 1% (10^4 mg/kg).

Explanatory notes:

(a) Applying the same extracting solvent and sample preparation method for the multi-pesticide method as those of the standard method is important to assure that the efficiency of the extraction is the same, and the results will be comparable. For instance, analysis data showed that using acetone (which is a widely applied in multi-residue analysis with proven efficiency) for extracting Parashoot CS resulted low concentration of parathion methyl. For this reason the acetone extracting solvent had to be replaced by tetrahydrofurane + water as recommended by the manufacturer [1].

(b) The test portions taken from the well homogenized laboratory samples may be reduced if the uncertainty of sample processing remains within acceptable limits (ssp ≤ 0.3sr). However, the sample/extracting solvent proportion must not be changed.

(c) The actual GLC operating conditions (injector and column temperature) may be different for various pesticides to provide optimum conditions for the separation and detection. The detector temperature can be kept at an optimum level according to the type of detector used. The injection conditions should be optimized for the given construction, and adjusted according to the pesticide analyzed. The injected amount of the sample should be adjusted to the capacity of the GC column (to avoid overloading the column), which is critical for narrow bore capillary columns and mainly depends on the internal diameter and film thickness of the liquid phase.

(d) The HPLC elution system (column and components of mobile phase) should be the same in a multi-pesticide method, but the proportion of the components of the mobile phase can vary to provide the optimum conditions for the determination of individual pesticides.

(e) The number of internal standards used should be limited to the minimum necessary for reducing the stock of chemicals and thus the operation cost of pesticide quality control (see chapter 5.3).

(f) The analytical standards are expensive and to prepare large amount of stock and diluted solutions (e.g. ~ 100 ml) is not necessary where a balance with 0.01 mg measuring capability is available. In order to obtain analytical standard solutions for calibration with a precision of ≤ 0.3%, a minimum of 20 mg standard must be weighed in with 0.01 mg accuracy, and the solutions must be prepared based on mass measurement and using a calibrated volumetric flask for the final dilution (see chapter 4.5). Details for the calculation of uncertainty of standard solutions can be found in the EURACHEM/CITAC Guide CG 4 [2].

2. PRELIMINARY ACTIONS

(a) Request formulators of the targeted pesticides to provide blank formulations, technical materials and analytical standards.

(b) Validation with blank formulation and technical material represents the ideal case. The validation may also be performed without these materials, but more work and attention are required. The recommended procedures are described in the following sections.

(c) Check your stock of chemicals, analytical standards and internal standards, and order the chemicals needed for the analysis of selected pesticides.

(d) Collect samples from different batches (preferable 5) of selected pesticides from distribution points. If 5 different batches cannot be found from a pesticide product take altogether 5 independent samples from the available batches.

3. SELECTION OF CHROMATOGRAPHIC SEPARATION AND DETECTION CONDITIONS AND VERIFICATION OF THEIR PERFORMANCE

3.1. Gas chromatographic determination

3.1.1 Determine the “initial optimum” chromatographic conditions for the analysis of selected pesticide product and internal standard according to the multi-pesticide procedure (Chapter 4.3)

(a) Use two capillary (wide bore or 0.32 mm) columns initially:

Column A: 5% phenyl, 95% methylpolysiloxane, chemically bonded (e.g. CP-SIL 8CB or equivalent) liquid phase;

Column B: 14% cyanopropylphenyl, + 86% dimethylpolysiloxane, chemically bonded (e.g. CP-SIL 19CB or equivalent) liquid phase.

The above liquid phases proved to be generally applicable. Certain pesticides may require specific liquid phases for accurate and reproducible analyses. Such pesticides may not fit in the multi-pesticide analysis concept.

(b) Apply the most suitable injection method and elution conditions for your instrument configuration (e.g. split injection and hot column or combination of injection to cold column and rapid temperature rise to the selected isotherm temperature).

(i) The “optimum” condition is relative and depends on the instrument configuration available. It cannot be defined precisely. One should possibly find elution conditions resulting in an elution time around 5–8 min (including analyte and internal standard), symmetrical peaks, and different relative retention (RRT) on the two columns (the RRT

depends on the temperature). The final optimization of elution conditions shall be made taking into account the possible interfering peaks from the technical or formulated products.

(ii) The optimum elution temperature on the two columns will probably be different.

(c) Inject the analytical standard of the AS and the selected internal standard (IS) and the extract of selected pesticide formulation separately.

(d) Test the repeatability of GC injections.

(i) Stabilize the system, run and evaluate the system suitability (SST) test [3].

(ii) If the system performance is acceptable proceed with (e), if not, identify and rectify the problem and run the test again.

(e) Applying the mixture of AS of selected compound with appropriate internal standard make five replicate injections with each pesticide into the stable system, and calculate the standard deviation and CV_{GC} of repeated injections. Use record sheet copied to the CD (File: Calculation sheet d2a.xls contains a worked example. Rename it and fill in the actual experimental data.)

The repeatability is acceptable if CV of individual injections of the pesticide and internal standard is for:

(i) On-column injection: preferably ≤ 1% (otherwise ≤ 2%) for ratios of peaks (with internal standard), and ≤ 5% for individual peaks (exceptionally ≤ 10% for difficult peaks).

(ii) Split injection: for peak ratios is ≤ 2% (preferably ≤ 1%, exceptionally < 10% for compounds difficult to analyze and individual peaks (see note below).

(iii) Retention time CV % ≤ 0.5%.

Note: Chapter 5.1 contains the repeatability of injection obtained under practical conditions. The values reported there can be used for initial guidance, but they do not represent the best performance as they were not particularly optimised.

(f) Proceed with sample analysis if repeatability is acceptable. Otherwise maintain the system and optimise the conditions to obtain acceptable repeatability.

3.1.2 Verification of GC conditions for multi-pesticide methods where blank formulation is available

When blank formulation is not available proceed with step 3.1.3.

(a) Checking for potential interference.

(i) Extract the blank formulation exactly according to the reference procedure, but without internal standard. Analyze the extracts of blank formulations under the selected chromatographic conditions.

(ii) Concentrate carefully a 4 ml portion of the extract obtained in the previous step to 2 ml. Inject a portion of concentrated extract into the GC as above. Inject the AS separately.

(iii) Increase the sensitivity of detection to enable detection of peaks equivalent to 0.01 × signal of AS, and inject the concentrated extract again.

(iv) Check and record the retention time and response of peaks present in the blank extract. Express their retention relative to the AS.

(v) Verify that there are *no interfering peaks* at or in close vicinity to the AS(s), the significant impurities (if known) and the targeted IS.

(vi) If any interference occurs modify the temperature programme to eliminate interference or select other IS which do not interfere.

(b) Checking the repeatability of the procedure.

(i) Apply the sample preparation, sample processing and extraction procedure of the reference method (CIPAC or AOAC) exactly up to the point of instrumental analysis and use the instrumental analytical conditions developed in step 3.1.2.

(ii) Verify the repeatability of the analytical procedure under the actual conditions of the laboratory by performing the analysis of a minimum of three analytical portions of one well-homogenised pesticide sample. Use single-point calibration with AS at the nominal concentration of the AS in the pesticide formulation.

Compare the max − min concentration range obtained for the AS content of the pesticide with the critical difference calculated from the repeatability range (r) of the reference standard method:

If $x_{max}\text{-}x_{min} \leq \boldsymbol{f} \times r/2.8$

the repeatability performance of the method is considered acceptable: go to next step. (For 2, 3, 4, 5, 6 and 7 replicates the $\boldsymbol{f}$ factor is 2.8 (2.77), 3.31, 3.63, 3.86, 4.03 and 4.17, respectively.

If the s_{ra} obtained from the measurements ($s_{ra} = (x_{max}\text{-}x_{min})/\boldsymbol{f}$; in our case with 3 replicate tests $\boldsymbol{f}$ = 3.31) is larger than s_r of the reference method (s_r = r/2.8), identify sources of variability, make appropriate action to reduce it (if possible), and repeat the above steps to verify repeatability of the method.

Note: The chromatographic conditions resulting in acceptable repeatability and at no interfering peaks from the blank formulation, in combination with the extraction procedure used in step 3.1.2. (a)(i), are considered to be the draft MPA procedure for the given pesticide.

3.1.3. Verification of GC conditions for multi-pesticide method without blank formulation

(a) Checking for potential interferences:

(i) Extract a sample of the selected pesticide exactly according to the reference procedure, but without internal standard. Analyze the extract under the selected chromatographic conditions (step 3.1.1).

(ii) Concentrate carefully a 4 ml portion of the extract obtained in the previous step to 2 ml. Inject a portion of concentrated extract into the GC as above. Inject the AS separately.

(iii) Compare the chromatograms of the normal and concentrated pesticide extracts, and the chromatograms of the analytical standards of AS and IS.

(iv) Verify that peak symmetry and shape of the peaks of analytical standard and the AS are the same, and there is no interference with the potential IS.

(v) Adjust the chromatographic conditions to eliminate interferences with IS or change IS if necessary.

(vi) Repeat steps (i) to (v) applying column B and establish the optimum chromatographic conditions.

(vii) Select standard concentrations at about 0.8, 1 and 1.2 times the nominal concentration of a.i. in the formulated product. Prepare independently the standard solutions including the IS for multi-point calibration.

Note: Prepare sufficient IS solution for diluting all standards and samples for the determination of active ingredient content of samples. Internal standard solution of the same concentration should be used to make up the samples and standards.

Use weighing to assure best precision of standard solutions of the AS. Select the amount weighed depending on the available analytical balance taking into account that the target precision of the standard solutions should be: CV ≤ 0.3%.

(viii) Perform multi-point calibration (minimum 3 × 2 injections) under conditions finalized in step 3.2.3., applying liquid phase A under stable GC conditions.

(ix) Determine the critical parameters of calibration (linearity, correlation coefficient, slope and intercept with confidence interval, standard deviation of relative residuals).

(x) Accept calibration if the regression coefficient is ≥0.997, and the standard deviation of relative residuals ≤ 0.01, (exceptionally 0.02). Preferably, the intercept should not be significantly different from 0 (see Appendix I for calculations).

Note: If the intercept is significantly different from 0, single-point calibration should not be used later on for determination of the a.i. content of samples. Therefore the cause should preferably be identified and eliminated.

(xi) Analyze the AS content of samples taken under task 2(c) in duplicate with the draft MP procedure applying liquid phase **A**.

(xii) Place remaining portion of extract into deep-freezer for further analysis in step xvii.

(xiii) Check if replicate injections are within the expected range based on the repeatability tests ($C_{m-x} - C_{min} \leq 2.8 \times s_{ra}$), where s_{ra} is the repeatability standard deviation of the method determined under 3.1.2 (b) (ii).

(xiv) Determine the AS content of the samples based on multi-point calibration.

(xv) Compare chromatograms of samples taken from different batches or different containers, and identify extra peaks which may occur in any of the samples. Calculate their retention relative to the active ingredient and the IS.

(xvi) Analyze another portions of samples in duplicate with either the reference procedure (R) including the exact chromatographic conditions described in the method*, or proceed with step xvii.*

(xvii) Repeat steps (vii) and (xvi) applying GC columns B under conditions optimized in step (vi).

(xviii) Compare the results obtained with the two methods with paired t-test (see example in Appendix II).

(xix) If the results obtained with the two methods are not significantly different, the MP method, including chromatographic analysis on two columns is validated for the tested pesticide.

(xx) If the results obtained are significantly different, it may indicate that the analyte is eluting together with some impurity or component of the formulation. Try to identify the sources of error and rectify it. Then repeat the comparability test.

Note: Changing (usually reducing) the elution temperature or applying longer columns normally improves the separation. Comparing the chromatograms obtained under various conditions may help to eliminate interferences.

3.2. HPLC determination

The HPLC separation in combination with UV or diode array detection has been increasingly used in pesticide formulation analysis due to its usually better reproducibility than GC. The optimum separation conditions are achieved by appropriate selection of the composition of the mobile phase described in chapter 4.4. Optimization of the composition of the mobile phase may also be facilitated with specific software.

The steps of verification of the specificity and selectivity of separation and repeatability of detection is very similar to those of GC, therefore they are not repeated.

3.3. Extension of the method for other pesticides

The potential interfering materials may derive from the materials used for preparing the formulation of the pesticide product or from the technical active substance. Consequently, the selectivity and specificity of the determination must be checked and verified for each individual product, regardless of the type of their formulation. This is a distinct and principal difference from the widely used multi-residue methods.

4. DETERMINATION OF THE LONG TERM REPEATABILITY AND REPRODUCIBILITY OF THE MULTI-PESTICIDE PROCEDURE

Apply the method for regular analysis of the pesticide, as part of the monitoring programme, to establish the long term within-laboratory repeatability and reproducibility according to the procedures described in Appendix III of Chapter 4.1.

In order to obtain reliable estimates for the within-laboratory reproducibility, a minimum of 15 samples shall be analyzed in duplicates on different days.

5. DOCUMENTATION OF LABORATORY WORK

The laboratory work should be documented and the results including all manual calculations should be stored as raw data. All system suitability tests, chromatograms, calibration files, weighing records and analytical record sheets constitute the raw data, which need to be organised systematically and archived.

The use of spreadsheet templates prepared for performing the calculation for various tests greatly facilitate the work. Once the correctness of the calculations performed by them is verified, only the accuracy of the data entry should be checked later on. The calculations will be performed automatically in order to avoid input errors.

A spreadsheet template developed for facilitation of the performance of the CRP work programme can be downloaded from http://www-infocris.iaea.org/Download/Calculation_sheet.xls.

REFERENCES

[1] LANTOS, J., VIRTICS, I., Quality Control of Pesticide Products Progress report, Contract No. 11482/RO (2002).

[2] EURACHEM, EURACHEM/CITAC Guide CG 4, Quantifying Uncertainty in Analytical Measurements 2nd ed. (2000), http://www.measurementuncertainty.org

[3] SOBOLEVA, E., AMBRUS, A. J., Application of a system suitability test for quality assurance and performance optimization of a gas chromatographic system for pesticide residue analysis. J. Chromatography A, **1027** (2004) 55-65.

APPENDIX I

Calculation of Calibration Parameters

As the concentration range is usually narrow ($0.8 – 1.2 \times C_{nom}$), ordinary linear regression (OLR) may be performed (assuming constant residual standard deviation).

Assuming 3-level (n) calibration with duplicate (k) injections the calibration data are given in Table AI.1.

TABLE AI.1. RESULTS OF MULTI-POINT CALIBRATION

Raw data file:		pac21104	Evaluation based on:		peak area	Sample concentration ratio based on:		
Run No	Standard solution ID	f_a C_{ai}/C_{IS}	A_{ai} Malathion	A_{IS} BB	$Y_i = A_{ai}/A_{is}$	Y_{icalc}	Y_{reli}	Response factor
AS1	AS2/st1	1.3328	900000	1405422	0.6404	0.6264	-0.0223	10.88462
AS1	AS2/st1	1.3328	858787	1388377	0.6186	0.6264	0.0126	11.26863
AS2	AS2st2	2.1514	1346702	1370838	0.9824	0.9826	0.0002	11.45272
AS2	AS2st2	2.1514	1355548	1402537	0.9665	0.9826	0.0164	11.64108
AS3	AS3/st3	2.6322	1588844	1339686	1.1860	1.1919	0.0050	11.60711
AS3	AS3/st3	2.6322	1729184	1431306	1.2081	1.1919	-0.0136	11.39445
						SD =	0.0151	

Explanation:

C_{ai}/C_{IS}: concentration ratio of AS and IS in the standard solutions made with independent dilutions;

IS : BB (benzyl benzoate); Y_{icalc} = calculated response from the regression line; $Y_{reli} = (Y_{icalc}-Y_i)/Y_{icalc})$.

The relative residual standard deviation (s_{rr}) can be calculated from the standard deviation of the relative residuals (SD) calculated automatically with nk-1 degrees of freedom as:

$$S_{rr} = \sqrt{\frac{nk-1}{nk-2}}SD = \sqrt{\frac{\sum\left(Y_{reli}-\overline{Y}_{reli}\right)^2}{nk-2}}$$

The OLR can be calculated with the given Excel spreadsheet (Tools → Data analysis → Regression) by entering Yi values into input Y range and f_a values into X input range, and selecting the appropriate cell for the output.

The part of the calculation output is shown in Table AI.2.

TABLE AI.2. OUTPUT OF THE EXCEL LINEAR REGRESSION CALCULATION

SUMMARY OUTPUT

Regression Statistics

Multiple R	0.99876
R Square	0.99751
Adjusted R Square	0.99689
Standard Error	0.01428
Observations	6

	Coefficients	*Standard Error*	*t Stat*	*P-value*	*Lower 95%*	*Upper 95%*
Intercept	0.04644	0.02291	2.02751	0.11253	-0.0172	0.11004
X Variable 1	0.43517	0.01087	40.0502	2.3E-06	0.405	0.46533

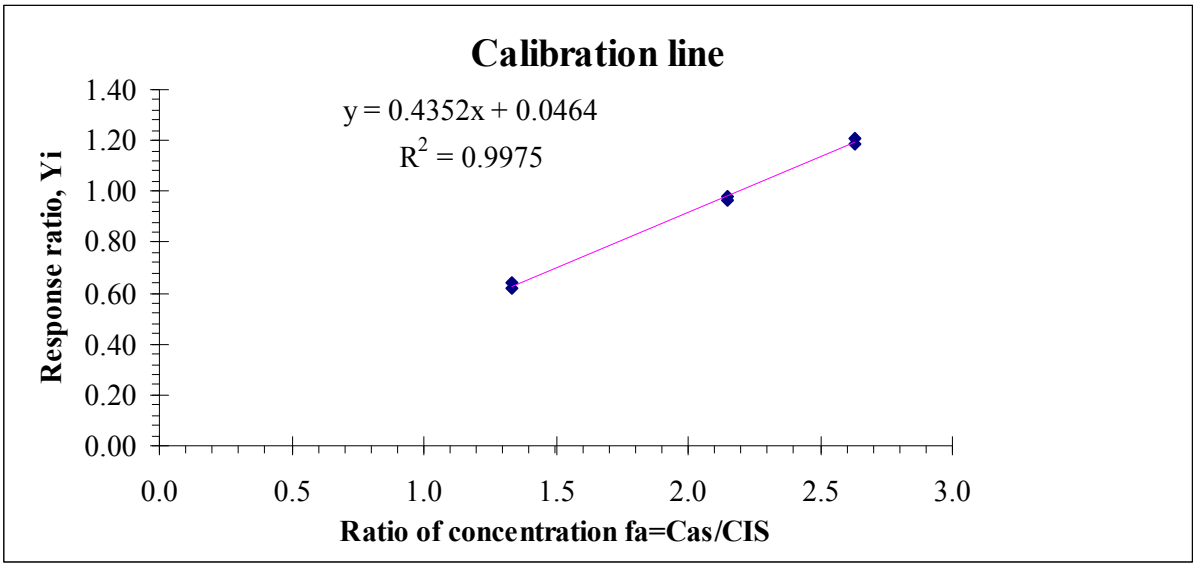
Explanation:

Multiple R 0.99876 → regression coefficient, r
R Square 0.99751 → coefficient of determination, R^2
Adjusted R Square 0.99689 → adjusted coefficient of determination, R'^2
Standard Error 0.01428 → residual standard deviation
Observations 6 number of injections (n × k)

Intercept: 0.046, if the lower and upper 95% confidence limits are encompassing 0, then the intercept is not significantly different from 0.

X variable: 0.43517: the slope of the regression line.

The calibration plot can be easily constructed with Excel chart drawing function. It is always advisable to visually observe the calibration line in proper size. Naturally it can be done by most of the modern chromatographic data processing software too, but its size may be too small for visual observation.



APPENDIX II

Calculation of the Significance of Difference of Concentrations Obtained on Two Columns

A pesticide EC formulation with a nominal AS concentration of 500 g/l was analyzed on column A and column B. The results and the corresponding calculations are shown in Table AII.1.

TABLE AII.1. CALCULATION OF SIGNIFICANCE OF DIFFERENCE OF AS CONTENTS OBTAINED ON TWO COLUMNS

		AS conc.				
	Sample identification	Col. A	Col. B	Average A	Average B	Difference
S1	F119/2-1	543.67	532	518.205	521.365	-3.16
S1	F119/2-1	492.74	510			
S2	F119/2-2/2	496.85	500	498.72	498.45	0.27
S2	F119/2-2/2	500.59	496			
S3	F119/2-3/2	526.42	512	514.07	508.855	5.215
S3	F119/2-3/2	501.72	505			
S4	F119/2-4/1	498.06	490	500.045	493.025	7.02
S4	F119/2-4/1	502.03	495			
S5	F119/3-1/1	492.01	495	492.94	494.475	-1.535
S5	F119/3-1/1	493.87	493			
				SD of differences		4.3787
				Average difference		1.562

The significance of the difference should be calculated with t-test applying the test statistics:

$$t = \frac{\overline{x_d}}{s_d / \sqrt{n}} = 0.1595$$

The tabulated t-value for df = 5-1 = 4 and $\alpha = 0.05$ is 2.77. (The explanation for obtaining critical values for t is given in chapter 4.1 Appendix II.) The calculated t value is much smaller than the tabulated one; consequently the difference between the results obtained on two columns is not significant. The results does not indicate any interference with the AS peak.

PRINCIPLES OF DEVELOPING MULTI-PESTICIDE METHODS BASED ON GC DETERMINATION

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Abstract

Principles for the development of multi-pesticide methods based on GC determination are outlined. A flow chart gives guidance on how to proceed stepwise in the set-up of analytical methods.

1. SUMMARY

The aim of this investigation was to elaborate a procedure for developing and optimising simple and rapid chromatographic separation for pesticides having different chemical structures.

The measurements were made with the classical hot split/ splitless injector system and wide bore capillary columns containing an apolar and a medium polar liquid phase. As more modern and flexible instruments are available in many laboratories, the selection of the operation mode of injector and the corresponding temperature should be made for each instrument to obtain the optimum performance for the given instrument configuration.

Where no information is available for the isothermal elution temperature of a pesticide, an initial multi-ramp temperature programme with injection into cold column can be performed. The elution temperature during the multi-rise programme can be used as a starting point for selection of the column temperature for isothermal elution of the compound. Once the elution temperature has been selected and optimised, the compounds can be determined at isothermal column temperature making the analysis of many compounds much faster than in temperature programme mode.

Altogether five internal standards were applied which sufficiently cover the retention range of compounds of interest.

System suitability test mixture is recommended to monitor the performance of the whole chromatographic system.

Thirty pesticides representing different chemical classes were tested to illustrate the applicability of the method. The details are given in Chapter 6.1.

The flow chart of the multi-pesticide GC method development is shown in Figure 1.

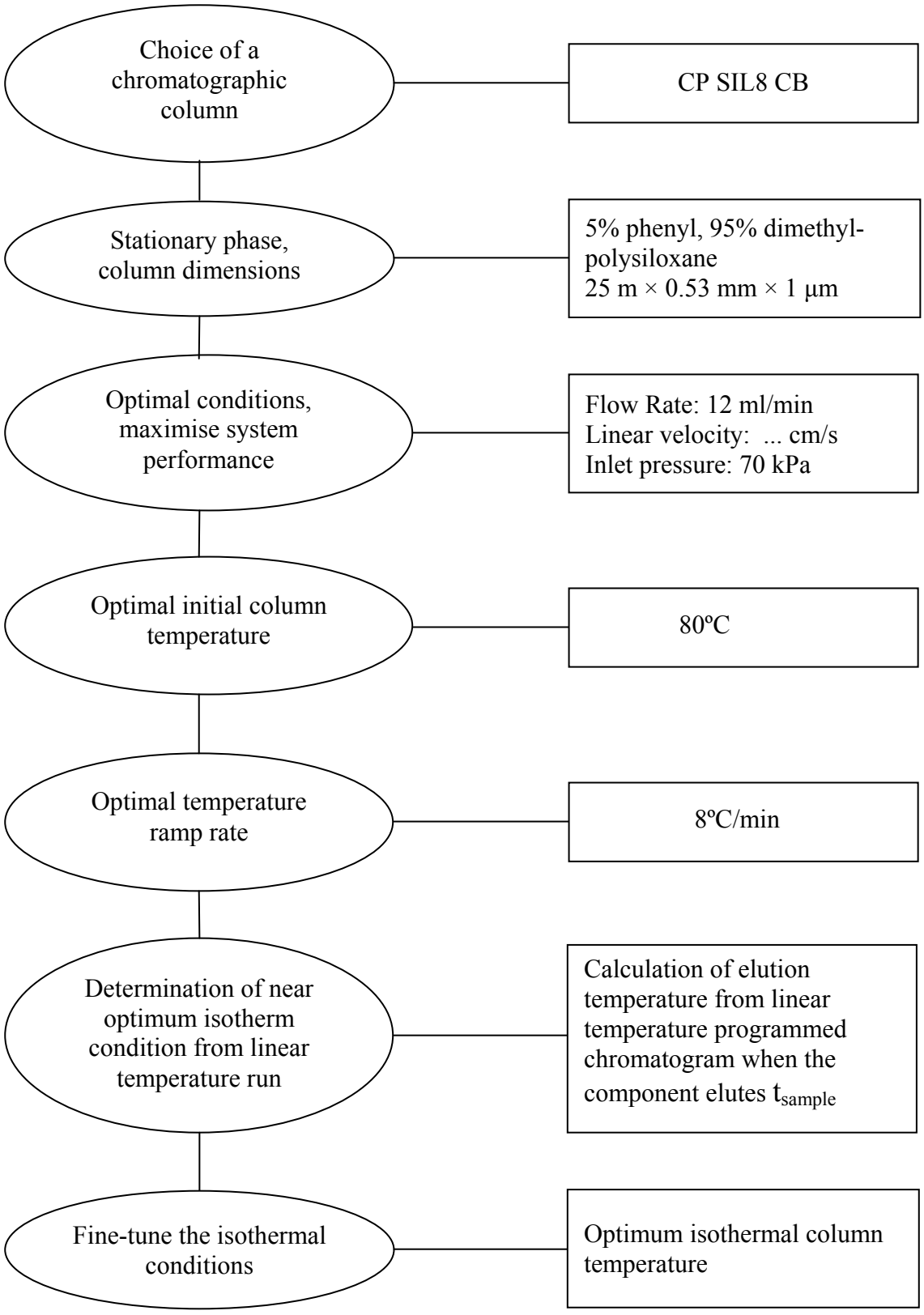

FIG. 1. Block diagram of 'Multi-Analyte' GLC Method Development.

PRINCIPLES OF DEVELOPING MULTI-PESTICIDE METHODS BASED ON HPLC DETERMINATION

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Abstract

Principles for the development of multi-pesticide methods based on HPLC determination are outlined. Flow charts and block diagrams give guidance on how to proceed stepwise in the set-up of respective analytical methods. Detailed information is provided on what to take into consideration for setting up a pesticide formulation analysis method. HPLC variables like the types of column, solvents and their strength, pH value, eluent modifiers, column temperature, etc, and the influence on the separation and resolution of chromatographic peaks are discussed as well as the necessity and benefits of internal standardization. Examples of system suitability testing experiments are given for illustration.

1. SUMMARY

The method development starts with a slow scouting gradient run with the extract of the pesticide. For most purposes scouting gradient in the range of 10–100% is applicable. The scouting gradient will quickly give essential information on the complexity of a sample and the likely difficulty of the separation. Scouting gradients will also allow analysts to determine whether a final isocratic method will be sufficient and estimate the isocratic mobile phase composition or the gradient range where gradient elution is deemed necessary.

The initial identification of the components in the sample may be achieved by their retention time and the identity can be confirmed by the comparison of the UV-VIS spectra of unknown peak with the reference spectra when it is available and applicable. Peak spectrum of the sample can be compared with spectra from a library produced either from analytical standards or previous samples. The Diode Array Detector has a great advantage in the identification of peaks and determination the peak purity.

To assist identification of AS in unknown samples a spectrum library of pure active ingredients and potential impurities, interfering materials should be built up.

The preferred starting conditions are a 15 cm × 4.6 mm, 5 μm particle size, C18 column, 1 ml/min flow rate, and acetonitrile/water or acetonitrile/buffer as the mobile phase.

2. BASIC CONSIDERATIONS FOR HPLC METHOD DEVELOPMENT

The flow chart of the multi-pesticide HPLC method development is shown in Figure 1.

2.1. Selection of chromatographic column

Reversed phase columns are widely used in high performance liquid chromatography applications. Their properties play key roles in achieving a successful separation. Though their specification is apparently the same (e.g. 150 mm × 4 mm, C18), they may differ significantly in chromatographic behaviour due to the qualitative and quantitative differences between the surface structural elements of silica, such as residual silanols, and the nature of the ligand.

Most practical separations will require 8000–10000 effective plates, which can be obtained with 12.5–15 cm long columns packed with 4–5 μm particle size, or 7.5–10 cm long columns with 3 μm particle size.

Guard columns are essential to extend column life and to achieve reproducibility on the main analytical column. The types giving the lowest possible dead volume, not affecting the peak shape and resolution of the high quality analytical columns are preferred.

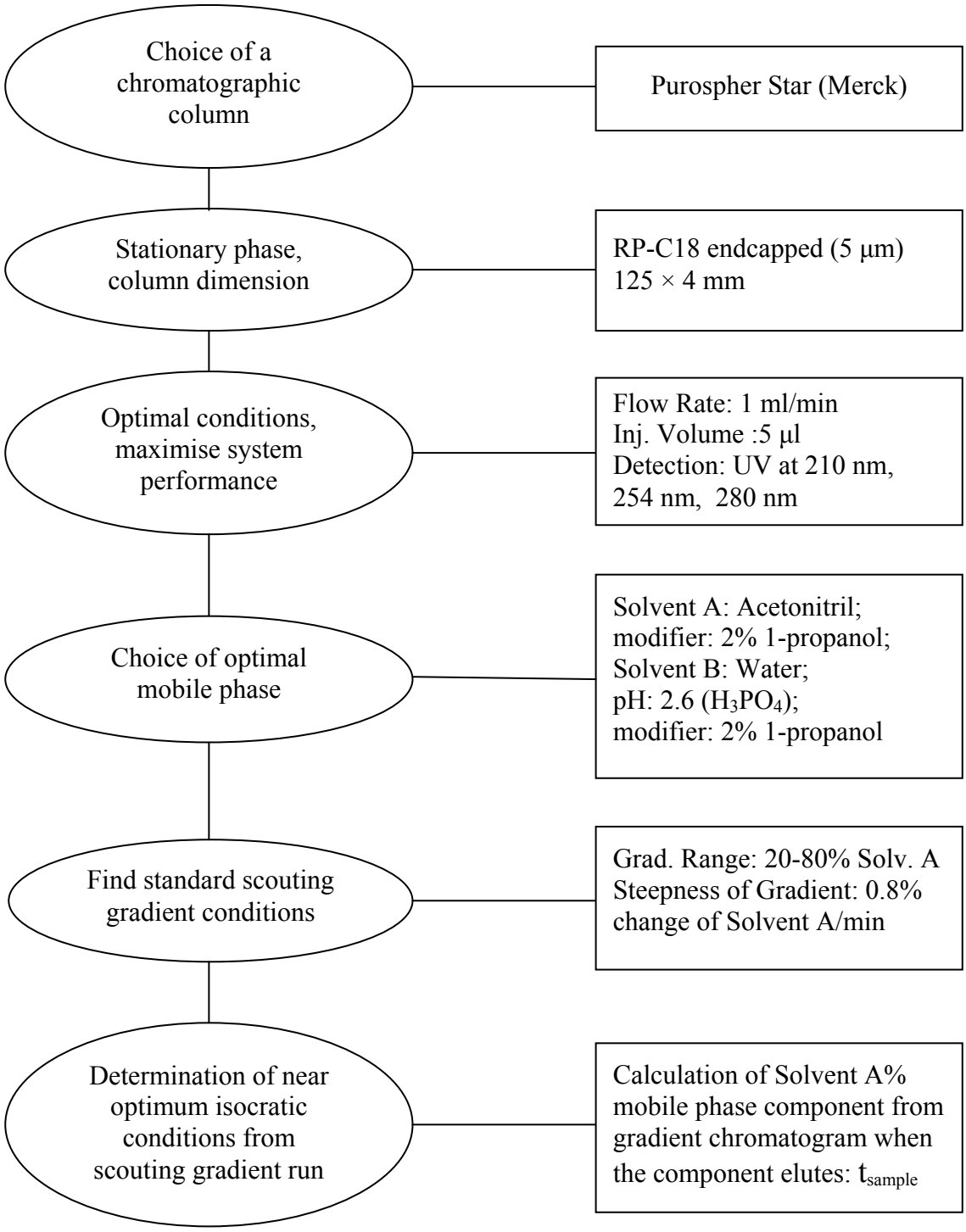

FIG. 1. Flow chart of the multi-pesticide HPLC method development.

2.2. Selection of mobile phase

Solvent Type

The solvent should be:

- suitable for the chosen analytical separation,
- fully miscible with water,
- non-reactive with the analytes and the column, and should have low viscosity.

The three most widely used solvents are acetonitrile, methanol and tetrahydrofuran.

Tetrahydrofuran is generally the worst choice — it is unpleasant to work with, chemically unstable (forms peroxides over time) and slow to equilibrate.

Methanol is relatively non-toxic and is a good choice for use at detection wavelengths higher than 220 nm.

The first choice is acetonitrile, because there are many pesticides that require low-wavelength detection.

Adjustment of solvent strength

The chromatographic separation will be better if the retention factor ($k = [t_R - t_0]/t_0$) is higher. The first peak in the chromatogram is usually the solvent front or garbage peak. This peak is eluted close to the column dead time (t_0), which represents the time for an unretained material to pass through the column. Peaks that elute close to the dead time have little opportunity to interact with the stationary phase and tend to be poorly separated from the junk at the solvent front and other compounds. To obtain k greater than 1, the compound of interest must have a retention time of more than twice the dead time (t_0). The retention can be increased by using a weaker solvent. The general pattern of longer retention and better resolution with weaker mobile phases holds for many compounds, however, it is quite common that resolution decreases under the same circumstances for other compounds.

In reversed phase liquid chromatography (RPLC) the gradient elution technique involves increasing the percentage of an organic solvent in the mobile phase as the chromatographic run progresses. This time-dependent increase of mobile phase strength provides greater retention of early eluting compounds and decreases retention of late eluting compounds with respect to isocratic conditions, thus improving the limits of detection and peak shapes for later eluting compounds.

The strength and type of solvent are two powerful variables that affect chromatographic selectivity but they are by no means the only variables available.

Instead of trying to find a generally applicable mobile phase composition, the solvent strength should be adjusted for optimising separation conditions for the various samples.

The pH of the mobile phase

Aqueous/organic solvent mobile phases are generally used in reverse phase liquid chromatography. The aqueous portion is water for neutral samples or low pH buffer for ionic samples. Using low-pH mobile phases (pH: 2–3) is a good starting point because these conditions suppress ionization of both acidic compounds and the residual silanol groups on the silica surface of the stationary phase, which helps to reduce peak tailing.

When ionic compounds are present, the pH can have a marked influence on retention and selectivity. If the initial low-pH mobile phase is unsatisfactory, the exploration of pH effects is a natural next step in method development. The acid type analyte will be protonated, becoming hydrophobic and thus more strongly retained at low pH than at high pH. On the contrary, bases will be neutral and well retained at high pH, whereas low pH results in ionization and poor retention in a reversed-phase system. Neutral compounds are unaffected by pH, so the retention will vary little, if at all when the mobile phase pH is changed.

It is best to work at a pH at which the compound or compounds of interest are either fully ionised or their ionization is suppressed.

In the case of organic acids, low pH will reduce the degree of ionization making the components more hydrophobic and thus more strongly retained. The compounds would behave as a neutral molecule with predictable retention and acceptable peak shape.

When using pH to control an LC separation, it is especially important to obey the fundamental rules of buffer usage.

A buffer is effective in the range of ±1 pH unit from the pK_a of the buffer. Outside this range, buffering effect is marginal, meaning that methods using those conditions will be less robust.

To work in an ion-suppression mode, the mobile phase should be 1–1.5 pH units below the pKa of the acid.

Organic modifier in the mobile phase

Because of the high percentage of water in the initial mobile phase, a condition referred to as hydrophobic collapse of the C18 phase occurs. This results in poor re-equilibration and irreproducible retention times and peak shapes for early-eluting analytes.

One problem commonly associated with gradient elution is the time required for re-equilibration of the system to the starting gradient conditions following elution of the last compound in the sample. As the mobile phase composition is varied during the course of the gradient elution, the stationary phase composition changes due to varying solvation of the bonded alkyl chains. Thus, it is necessary to flush the column with a large volume of the initial mobile phase to restore the original conditions of the stationary phase. Generally, the equivalent of 15–20 column volumes of the starting mobile phase must be passed through the column to achieve column re-equilibration. Therefore, it is necessary to control the solvation of the bonded alkyl chains.

A good wetting substance, such as a short-chain alkanol, being present in the mobile phase has been shown to effectively alter the chromatographic properties of the stationary phase. A few percent of 1-propanol in the mobile phase provides nearly monolayer coverage of the reverse phase stationary phase. The addition of 2% 1-propanol to both mobile phase A and B had been reported to reduce the time required for re-equilibration at the end of the run and to improve column performance. We found that this consistent solvation provides a robust stationary phase structure.

Tailing peaks

Tailing peaks may create several problems. First, the integration of tailing peaks is more difficult. Data systems determine the start and end of a peak by monitoring the change in the slope of the baseline. With a tailing peak, the point at which the peak returns to the baseline is less clear than for a symmetric peak, which may result in increased uncertainty of the quantitation of the peak. It is indicated by the varying positions of the print tick marks at the start and end of a peaks obtained with replicate injections.

A second concern relates to the limit of detection. The peak height of tailing or broad peaks will be smaller. At the limit of detection, the peak height — not the area — is the limiting parameter, so tailing peaks will have poorer detection limits than the symmetrical ones.

A third problem is the detection of minor peaks in the run, if the method is used for testing stability, analysis of degradation products or contaminants at the concentration range of $\geq 0.1\%$ of the primary peak area. These small peaks can easily hide under the tail of a large peak, and they will be missed. Well-shaped peaks are much less likely to cover minor peaks in the chromatogram.

Finally, separation of tailing peaks requires longer run times for the same resolution.

2.3. Column temperature

The temperature may or may not be a problem. At higher temperatures the solvent viscosity will be lower, so the column pressure will be also lower. At higher temperatures the column will generate higher plate numbers and thus, narrower and taller peaks, which will translate into better resolution and detection limits.

However, high temperatures can cause several problems: the solvent must be preheated before it reaches the column because the temperature gradient can distort peaks and at higher temperatures the column life is shorter.

2.4. Flow rate

The flow rate should be adequate to yield short retention time and at the same time do not increase the backpressure above 200 bar. The high pressure increases mechanical wear of the system.

A flow rate of 1 ml/min, in a column of 4.6 mm diameter and packing of 5 μm particle size, is a satisfactory choice.

2.5. Injection volume and solvent

If the solvent used to dissolve the sample is stronger than the mobile phase, the sample components will migrate initially faster as if it were in a stronger mobile phase and results in peak distortion especially of the early eluting compounds.

The problems caused by the injection solvent can be minimised by either injection of small volume of solvent (the dilution takes place very quickly in this case) or using a solvent for analyte dissolution that is not stronger than the mobile phase. Weaker solvents concentrate the sample at the column entrance producing, in some cases, narrower peaks than they would be if they were injected in a stronger solvent.

Large volumes of too strong solvents can distort peaks, especially at the beginning of the chromatogram. As a general rule, if a sample is dissolved in a solvent which is stronger than the mobile phase, the injection volume should be less than 25 μl.

3. INTERNAL VERSUS EXTERNAL STANDARD METHOD

A suitable internal standard (IS) should have similar elution and physicochemical properties as the analyte(s) of interest (but with distinctly different retention time) in order to enable correction for the potential losses during the extraction and sample processing steps, and for the errors in the injected volume. The behaviour of the internal standard needs to be closely examined during method development and its importance should not be overlooked.

Using an internal standard increases the measurement errors at various extents. Most of the additional uncertainties (due to weighing, diluting, and dispensing of the internal standard) associated with the use of an IS can be eliminated if the extraction of the sample is carried out with a solvent containing the IS. The quantification of its chromatographic peak is an inevitable source of additional random error, which is much smaller in case of a symmetric peak than the error of injection with a syringe. Where a loop is used for injection, the injection error is minimal, and the use of IS may be omitted from the procedure, if the sample processing and extraction is a one step procedure.

The advantages of the use of an internal standard can be utilised only if the error and uncertainty associated with its use is much smaller than the combined uncertainty of the determination of the analyte(s). However, the results can be less accurate and precise if the internal standard is chosen or applied incorrectly.

Another factor to consider is the increased demand concerning the resolution of the internal standard, which requires more extensive method validation compared to external standard methods.

As a result of the more precise HPLC quantitation compared to GC, the internal standards are less frequently used with the HPLC method.

4. SYSTEM SUITABILITY TEST

The purpose of a system-suitability test (SST) is to assess the suitability of a chromatographic system for a particular analysis, and it should indicate when a component, such as the column, the mobile phase, or even the detector lamp should be replaced before samples are analyzed.

The reproducibility of replicate injections, peak tailing or asymmetry, and resolution of peaks can be checked with a single injection of a properly selected SST solution. The results may verify that the chromatographic system performance is suitable for the analyses before the chromatographic run starts, and it also provides the confidence that the analyses have been performed consistently over the time if the SST is repeated at the end of the run.

In its simplest form, this may just involve injecting a mixture of the analytes to be separated and observing the results. Alternately, a specifically selected set of compounds is used to prepare the SST mixture. Based on experience one can tell if:

- the peaks are adequately resolved,
- the shapes of the peaks are acceptable (symmetry factors are within 0.8–1.2),
- the retention times are within the ranges expected (1–2%),
- the baseline noise is acceptable for analysis to be performed.

An SST for a chromatographic separation can involve evaluating a number of parameters before processing samples.

Resolution: will indicate the ability of the column to resolve two closely eluting analytes from each other. The resolution power of the system is considered appropriate if the resolution of critical peaks is ≥ 1.5. Where the resolution is lower, special attention is needed for the accurate determination of the amount/concentration of the analyte. It is advisable to select substances for testing the resolution, the separation of which is critical for the quantitative determination.

Plate number: During method development, one of the parameters to be established is the minimum number of effective plates required for the separation. In combination with the resolution it provides a good indication for the need of replacing the old column.

Reproducibility: Compare the peak area of analytes to those obtained previously.

Peak tailing and asymmetry factor: Depending on the physicochemical properties of the analyte, the column, and the sample matrix, peak symmetry may vary over the lifetime of a column. A quantitative measurement of the tailing or asymmetry factors is a useful indicator of the inertness of the system and condition of the column. The acceptable asymmetry factors are preferably within 0.8–1.2.

Retention time: Retention time tends to vary over time due to a number of factors such as differences between batches of mobile phase, column performance. Ambient temperatures of laboratories can be a major cause of retention time variation during a day or from season to season where the column is not thermostated. As the primary information for peak identification is the retention time, its repeatability must be within 0.5%.

4.1. Performance of SST

A three-component pesticide mixture (Table 1) was used to evaluate the column performance by injecting 5 µl of test mixture and running the isocratic program (Figure 2). It should be injected after the installation of column to verify not only the column condition, but also the proper installation and overall system performance.

The system performance should be checked by injecting the SST mixture daily. The chromatograms have to be compared with the reference chromatogram. All test chromatograms have to be kept in a folder for future reference.

TABLE 1. COMPOSITION AND CHROMATOGRAPHIC PEAK PARAMETERS OF SST FOR ISOCRATIC CONDITION (SEE FIG. 1)

Compound name	t_R (minute)	Peak-width (minute)	Symmetry factor	Resolution	Plate number
Molinate	6.52	0.180	0.792		
Malathion	7.63	0.209	0.832	3.03	
Fenitrothion	8.28	0.223	0.830		7637

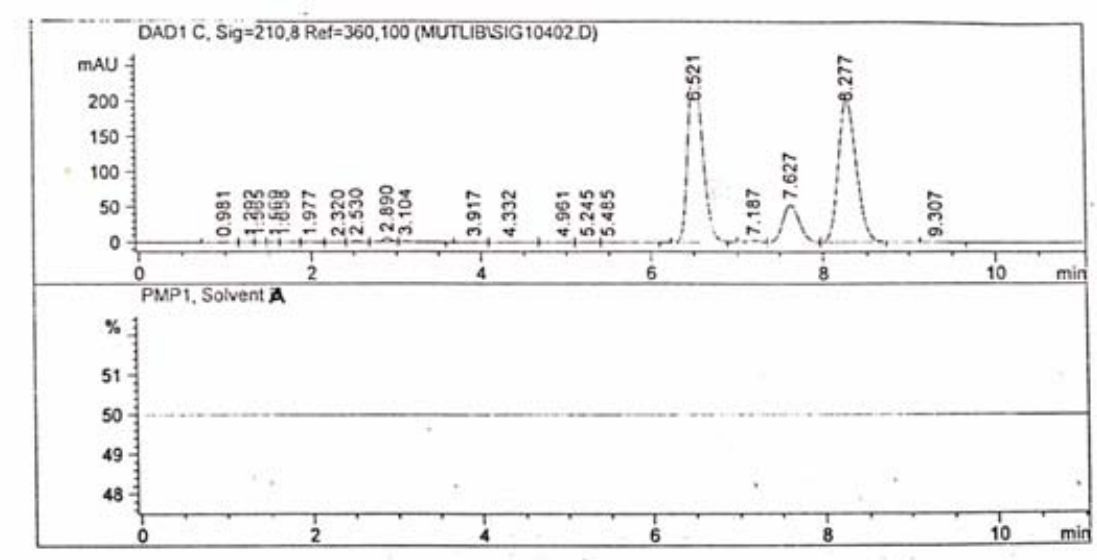

FIG. 2. Chromatogram of SST mixture obtained under isocratic conditions (Solvent A: 50% acetonitrile +2% 1-propanol); Solvent B: water (pH adjusted to 2.6 with H_3PO_4) + 2% 1-propanol).

THE UNCERTAINTY OF MEASUREMENT RESULTS

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Abstract

Factors affecting the uncertainty of measurement are explained, basic statistical formulae given, and the theoretical concept explained in the context of pesticide formulation analysis. Practical guidance is provided on how to determine individual uncertainty components within an analytical procedure. An extended and comprehensive table containing the relevant mathematical/statistical expressions elucidates the relevant underlying principles. Appendix I provides a practical elaborated example on measurement uncertainty estimation, above all utilizing experimental repeatability and reproducibility laboratory data.

1. FACTORS AFFECTING THE PRECISION (UNCERTAINTY) OF THE MEASUREMENT RESULTS

The determination of the active substance (AS) content of a pesticide formulation includes the following main steps: homogenization of the formulated product (sample processing, Sp) preparation of sample extract including weighing of test portion and dilution of the extract (Extr), preparation of analytical standard solutions, chromatographic (or applying another technique) determination (Ch) of the AS in the sample extract.

ISO Standard 17025:2005 [1] requires the estimation of the combined expanded uncertainty of the results taking into account the uncertainty of sampling when appropriate and required. In case of the quality control of pesticide products, according to the FAO Guidelines [2] several units are tested from a batch and each packing unit must comply with the specification. Consequently, there is principally no sampling error in the measurement results on the packing unit scale.

However, where the laboratory sample is withdrawn from a large packing unit, the sampling error can be significant due to the inhomogeneity of the material in the package (e.g. one bag of 50 kg of a fertilizer containing an insecticide incorporated in the formulation or mixed with the fertilizer, or just a granular pesticide). Special attention is required for sampling granular or dust formulations, and liquids which tend to segregate. The sampling error, and the efficiency of the pre-sampling mixing operation should be tested and the uncertainty of sampling should be established for each type of formulation under different ambient storage temperature where the seasonal differences are substantial. The fully nested or staggered nested experimental design (described in section 2.2.1.) can be used to estimate the uncertainty of individual steps of the determination of the quality of a pesticide.

As the AS content of a pesticide formulation was determined by the CRP participants either with the GC or HPLC method, the estimation of the uncertainty of the measurement results is demonstrated for chromatographic measurements in this publication.

The uncertainty of the measurements is mainly due to some random and systematic effects. The separate contributors to uncertainty are called uncertainty components. There are two basically different approaches for estimating the uncertainty of analytical measurements: the “top-down” introduced by the Analytical Methods Committee [3] and recommended for practical use by FAO and CIPAC [4], and the “bottom-up” or error budget introduced by ISO [5] and elaborated by EURACHEM [6].

The top-down method is primarily based on the results of collaborative trials and internal quality control data, and thus it may take into account the between-laboratories variability of the results. It provides the most reliable estimate of the expectable performance of a method and/or a laboratory, and provides the ground for judging the equivalency of results obtained in different laboratories [7].

The bottom-up approach also has its own merit, especially in identifying and quantifying the uncertainty of individual components or steps of the determination where the combined uncertainty is higher than that would be acceptable. Based on the contribution of the individual procedures to the overall uncertainty of the results, the analytical procedures can be optimised to fit for the purpose of the analysis.

1.1. Basic relationships of uncertainty components

If the relative uncertainties of individual steps [sampling (S), sample preparation (Sp), extraction (Extr), chromatographic determination (Ch)] are quantified the combined relative uncertainty of the analytical result can be expressed as:

$$CV_A = \sqrt{CV_S^2 + CV_{Sp}^2 + CV_{Extr}^2 + \ldots + CV_{Ch}^2} \quad (1)$$

The equation can be expanded if more steps are involved in the method.

The uncertainty of the predicted analyte concentration (S_{Ch}) is calculated according to [8] as:

$$S_{Ch} = \sqrt{S_{xo}^2 + S_{AS}^2} \quad (2)$$

where S_{x0} is the standard deviation of the analyte concentration calculated from the calibration data, see Eq. (3), and S_{AS} is the combined uncertainty of the analyte concentrations in the standard solutions.

The standard deviation of x_0 can be obtained [8] either from ordinary linear regression (OLR) or with weighted linear regression (WLR). In pesticide quality control, the concentration range is usually narrow around the nominal concentration of the active substance (X) in the formulated or technical product (0.8 X – 1.2 X). Therefore, the ORL can be applied with about the same precision as the WLR, which makes the calculation much simpler. The OLR can be performed with the Excel programme calculating the R^2 and the confidence limits for the intercept and slope automatically (see Appendix 1 of chapter 4.2). The uncertainty of the predicted concentration of the AS, S_{x0}, based on the calibration is calculated using Eq. (3):

$$s_{x_0} = \frac{s_{y/x}}{b}\left\{\frac{1}{m} + \frac{1}{nk} + \frac{(y_0 - \bar{y})^2}{b^2 \sum_i (x_i - \bar{x})^2}\right\}^{1/2} \quad (3)$$

where $S_{y/x}$ is the residual standard deviation (indicated as standard error on the Excel output table); b is the slope; m and k are the number of replicate sample and standard injections, respectively; n is number of calibration points, y_0 is the signal of the sample, $\bar{y}$ is the average signal obtained from all calibration points; x_i is the concentration of the AS in the standard solutions, and $\bar{x}$ is the average concentration of the AS in the n standard solutions used for the calibration. S_{x0} has nk-2 degrees of freedom.

Where the sample extract is injected m times the y_0 is calculated as:

$$y_0 = \frac{\sum_j^m y_{sj}}{m} \quad (4)$$

The relative uncertainty of the predicted concentration is:

$$CV_{x0} = \frac{S_{x0}}{x_0} \quad (5)$$

For preparing a working standard solution in 2 steps the analyte concentration, C_{As}, is calculated as:

$$c_{As} = \frac{w * P * V_{p1}}{V_{f1} * V_{f2}} \quad (6)$$

where w is the mass of analytical standard, P is its purity and V_{f1}, V_{f2} are the volumes of the volumetric flasks and V_{p1} is the volume of transferred to the second flask. The combined uncertainty is obtained from the relative uncertainties of the steps involved:

$$CV_{AS} = \sqrt{CV_{wht}^2 + CV_P^2 + CV_{p1}^2 + \ldots + CV_{f1}^2 + CV_{f2}^2} \quad (7)$$

2. ESTIMATION OF THE UNCERTAINTY OF THE MEASUREMENT RESULTS

2.1. Estimation of the repeatability and within-laboratory reproducibility of the results

2.1.1. Repeatability of the measurements

The repeatability of the measurements can be determined from the results obtained under the same condition (same operator, instrument, sample) within a short period of time. The standard deviation of the results obtained should be compared to the CIPAC repeatability standard deviation derived from the repeatability range (***r***): $s_r = r/2.8$.

The calculated standard deviation will provide information for that part of the procedure which is repeated. Normally, a minimum of 5 measurements must be performed to get meaningful initial results. More precise estimates would require more than 15 data points, which may be obtained from the results of replicate measurements of samples during the regular use of the method.

Where the actual AS contents of the samples are within or close to the tolerance limits specified for the formulation, the repeatability standard deviation may be calculated with Eq. (8):

$$s = \frac{1}{\sqrt{2}}\sqrt{\frac{\sum d^2}{n}} = \sqrt{\frac{\sum d^2}{2n}} \quad (8)$$

where ***d*** is the difference between the results of duplicate measurements carried out from the same sample.

However, where the actual AS content of the formulations tested are outside the tolerance limits, the different concentrations may affect the calculated standard deviation. Under such conditions it is more appropriate to calculate the relative standard deviation as:

$$CV_r = \sqrt{\frac{\sum_{i=1}^{n} X_{\Delta i}^2}{2n}} \quad (9)$$

where the relative difference of the concentrations measured in replicate test portions is:

$$X_{\Delta S} = 2(X_{i1} - X_{i2})/(X_{i1} + X_{i2}).$$

The s_r of the method may be calculated from the nominal concentration (X_{AS}) of the AS:

$$s_r = X_{AS} \times CV_r \quad (10)$$

The information on within-laboratory reproducibility can be obtained by this method without any extra work as the samples are normally analyzed in duplicate, and those results can be used for the calculation.

2.1.2. Within-laboratory reproducibility of the measurements

The procedure is principally the same as that applied for the estimation of the repeatability, but the procedures should be carried out on different days by different analysts on all equipment used for the determination process.

Where the Eqs (8) or (9) are applied, the analyses should be performed on different days from newly prepared test portions of the retained samples. This is the most powerful internal quality control check, which is also recommended by ISO 17025 Standard. The analysis of only one additional sample portion is required on a given day for obtaining the information on within-laboratory reproducibility of the whole procedure including sample processing and analysis.

The within-laboratory reproducibility value is normally between the within-laboratory repeatability and the CIPAC between-laboratory reproducibility values (R/2.8). It is an intermediate precision parameter.

Examples for the statistical evaluation of the precision tests are given in Appendix I.

2.1.3. The uncertainty of replicate measurements

As the tolerance limits are relatively narrow the expanded uncertainty of the measurement results should preferably not be larger than 1/3 of the tolerance limit.

For a homogeneous liquid formulation (e.g. EC, SC, SL) containing 25 g AS/kg, according to the FAO pesticide specification [2], the tolerance is ± 15% of the declared AS content. The 95% tolerance limits are ±3.75 g/kg which corresponds to 1.9 g/kg standard deviation. As the measured concentration should be within the tolerance limit, the reproducibility standard deviation, s_R, of the analytical method should not be larger than 0.6 g/kg for testing such a formulation for compliance. It corresponds to 0.06% relative standard deviation. For a 500 g AS/kg formulation the tolerance limit is 25 g. The corresponding maximum SD of the analytical procedure is 25/1.96/3 = 4.25 g/kg (0.4%).

The typical repeatability CV values, reported by the CRP participants for the determination of various formulations, range from 0.08% to 1.2% (see chapter 5.1). In order to satisfy the stringent requirement of certifying AS content of formulations, the laboratories should perform the determinations with replicate measurements.

The standard deviation of the average values ($S_{\bar{x}}$) obtained from n replicate measurements relates to the standard deviation of the results obtained with one determination (S_i) as:

$$S_{\bar{x}} = \frac{S_i}{\sqrt{n}} \qquad (11)$$

The number of replicate measurements should be decided taking into account the required precision (uncertainty) of the reported average value and the within-laboratory reproducibility of the measurements. The improvement of the precision is substantial where 2 or 3 replicate measurements are made instead of one, but larger number of replicate measurements usually increases the cost and time requirements of the analysis disproportionally compared to the gain in precision. In such cases the possibility of improving the method performance should be investigated, and if possible the method should be refined and revalidated to obtain lower S_R values.

2.2. Estimation of the uncertainty of individual steps of the method

If the laboratory reproducibility ($S_{R(lab)}$) or repeatability ($S_{r(lab)}$) value is larger than the corresponding CIPAC value or the value calculated with the Horwitz formulae (see Appendix 1 of chapter 4.1), the significance of their difference can be checked with the F-test.

$$F_{calc} = \frac{S^2_{r(lab)}}{S^2_r} \text{ or } F_{calc} = \frac{S^2_{R(lab)}}{S^2_R} \qquad (12)$$

Where the calculated F value is larger than the critical tabulated one $F(\alpha,\nu_{lab},\nu_\infty)$, taking into account the degrees of freedom (νlab) of the estimated value, at 95% probability level (P = 0.95 or $\alpha = 0.05$ depending on the statistical table available), then the difference is significant, and the sources of error should be identified and possibly eliminated. The F critical value can be obtained from Excel as well entering into the English version the following syntax: '=FINV(0.05, ν_{lab},1000000)'. The ν_∞, which cannot be entered, can be replaced with a large number e.g. 1 000 000. For 7 replicate measurements

ν_{lab},= 6 , thus we should enter: =FINV(0.05,6,1000000), and we will get Fcrit=2.098607~ 2.10 which is usually given in F tables.

Where the overall repeatability or reproducibility of the method is significantly larger than the expectable precision (see chapter 4.1), the individual steps should be checked to identify the sources of error and possibly reduce it.

2.2.1. Uncertainty of sample processing and extraction

The sample processing includes the preparation and homogenization of the sample and withdrawal of the test portion which is extracted. The extracts are analyzed.

The testing of the reproducibility of sample processing is especially important in case of solid formulations. The reproducibility of the extraction may be a problem for instance in case of controlled released formulations, granules or some bites.

The random error of the analysis can be determined with the repeated analysis of portions of the same sample extract. However, the separation of the variation of the results derived from the inhomogeneity of the sample and from the performance of the extraction requires more complex experimental design and calculation, which is illustrated in Figure 1.

To obtain reliable estimate of the reproducibility of the procedures a minimum of 15, but preferably 30 different batches should be sampled or duplicate laboratory samples should be taken independently from as many batches as available. The amount of material (packing units) to be withdrawn from the specified primary sampling positions should be decided based on the official sampling protocol or in its absence the Sampling Procedure described in the FAO Pesticide Specifications [2].

Where the contribution of the extraction and sample processing need not be separated the 3rd level of the nested experimental designs can be omitted, and the calculation can be performed with one way ANOVA. The procedure is illustrated with a practical example in Appendix I.

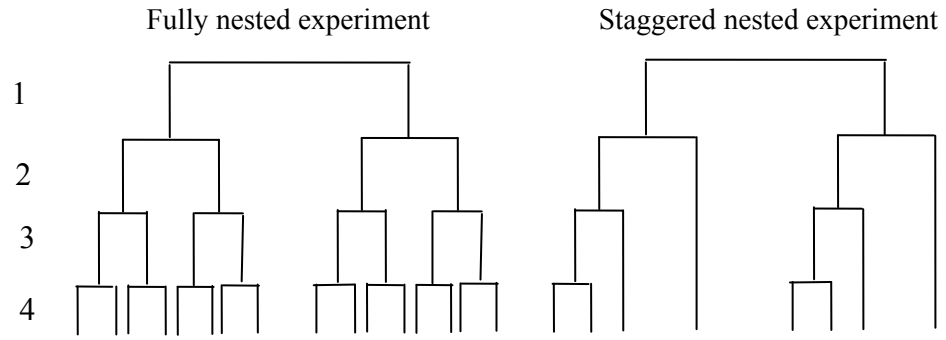

FIG. 1. Experimental designs for estimation of the uncertainty of sampling steps.

1: samples taken preferably from different batches of formulations;
2: subdivision of the sample;
3: test portions removed after homogenization of the sample and extracted separately;
4: replicate injections of extracts.

2.2.2. Uncertainty of chromatographic determination

Eq. (3) provides the best estimate for the uncertainty of the predicted concentration based on multi-point calibration. The calculation is complicated and time consuming and can be best performed with appropriate statistical programme or with an Excel template.

We can confirm the goodness of the calibration much easier with the standard deviation of relative residuals (S_{rr}) calculated with nk – 2 degrees of freedom from the relative difference of the residuals (residual: $\Delta y = y_i - \hat{y}$; $Y_{rel} = \Delta y / \hat{y}$, y_i is the measured response and $\hat{y}$ is the value read from the regression line):

$$S_{rr} = \sqrt{\frac{\sum \left(Y_{rel,i} - \overline{Y}_{rel} \right)^2}{n-2}} \tag{13}$$

It should not be confused with the residual standard deviation $S_{y/x}$ used in Eq. (3). The standard deviation of the residuals is not constant but generally proportional to the response of the instrument, while the standard deviation of the relative residuals reflects the average variability of the calibration points independently from the calibrated range or instrument response. Therefore this makes it possible to compare and characterize various calibration data with a single parameter.

It was found that the S_{rr} much more sensitive indicator for the goodness of the calibration than the regression coefficient (R^2). Table 1 shows the S_{rr} and R^2 values obtained with external standard calibration and nitrogen phosphor selective detector.

TABLE 1. COMPARISON OF S_{RR} AND R^2 VALUES OBTAINED FOR THE SAME CALIBRATION DATA SETS

S_{rr}	R^2
0.042	0.9937
0.061	0.9976
0.085	0.9988

The data indicate that there is no correlation between the two parameters, and the S_{rr} was the best with lowest R^2 values. Applying capillary columns and specific detectors the S_{rr} values should be lower than 0.08 with external standard calibration and ≤ 0.02 with FID and internal standard calibration.

If the S_{rr} is ≤ 0.02 with internal standard calibration, then the analyst can be confident that the calibration solutions were accurately prepared and the instrument is working precisely.

Eq. (3) indicates that the uncertainty of the concentration of the analyte predicted based on multi-point calibration is influenced by several interrelated factors. Despite its complexity, Eq. (3) deserves careful studying, because it shows how best to conduct a calibration experiment to ensure the smallest, i.e. best, possible value of s_{x_0} [8].

Increasing the number of injections (n × k) will improve substantially the precision of the prediction of the analyte concentration of the sample. The number of calibration points, n × k, must not be too small for two separate reasons. Firstly, as n × k decreases, 1/(n × k) increases so s_{x_0} also increases. Secondly, if n × k is small, the number of degrees of freedom, (n × k) – 2, is very small indeed, so the *t*-values will be large resulting in undesirably wide confidence intervals for s_{x_0} .

The equation also contains the term *m*, which is the number of times y_0, the instrument signal for the test sample is measured (note that *m* is not the same as *k*, the number of times each standard is injected in preparing the calibration graph). If *m* is only one, the first term inside the bracket is almost invariably the largest of the three, so it is good practice to make several measurements of y_0: 2 to 4 replicates are often measured, provided that an auto-sampler is available and the instrument can work for 24 hours.

The last term in the brackets shows that s_{x_0} is minimised when y_0 is as close as possible to $\bar{y}$, the centroid of the points.

The above considerations illustrate that no general guidance can be given for the calibration strategy. It depends on the analysis time, the number of samples to be analyzed and the random variation of the analyte response. In addition, it strongly depends on whether manual injections are made or an auto-sampler is used. Taking into account the above factors the analysts must decide on the strategy on a case-by-case basis.

2.2.3. Single-point calibration

In the interests of speed and convenience, analysts sometimes wish to carry out a single-point or one-point calibration. There are several pre-conditions of the relatively accurate application of the single-point calibration:

(a) The multi-point calibration should indicate that the calibration relationship is linear and the intercept of the regression line is close to 0 (statistically not different from 0).

(b) Blank sample should give 0 reading.

(c) The analytical standard concentration should be at (e.g. at the upper tolerance limit) or slightly above the expected concentration of the AS in the sample extract.

(d) Minimum 2 preferably three injections (k) should be made from the analytical standard and two injections from the sample extracts.

The resulting calibration curve is then forced through the origin (0, 0), and its slope, b, is thus $\bar{y}/x$, where $\bar{y}$ is the mean value of the k measurements of the analytical standard with a concentration x. Since only one RM has been studied the only available measure of $s_{y/x}$ is the standard deviation of the k responses. This method cannot be recommended for rigorous analyses, where the results would be the basis of legal action.

2.2.4. Uncertainty of the analytical standard solutions

One of the basic conditions for the application of the linear regression is that the error in the reference materials used for calibration should be zero or negligible compared to that of the response, S_y. Therefore, uncertainty of the preparation of the standard solutions should be estimated and the assumption of $S_y >> S_{AS}$ should be verified.

Considering the repeatability of injections with modern auto-samplers (< 2%), the uncertainty of analytical standards used for calibration should be around 0.3–0.5% to satisfy the preconditions of linear regression. Therefore the standard solution should be prepared based on weight measurements. Where the intermediate solutions of the components of the mixtures of analytical standards are prepared according to method (a), described hereunder, and the mixture is prepared in the last step, the number of compounds being in the mixture does not affect the combined uncertainty of the mixture.

The effect of various external factors on the uncertainty of the diluted standard solution is illustrated with the following example [9]. The standard solutions were prepared by different ways for assessing the error in their concentration:

(a) 25.4 mg of analytical standard (99.9 ± 0.1% = 0.999 ± 0.001) was weighed with a 5-digit analytical balance (linearity ±0.03 mg, repeatability SD = ±0.02 mg). The stock solution was prepared by dissolving the standard in an A-grade 25 ml (tolerance limit ±0.04 ml) volumetric flask. 100 µl of stock solution was transferred with Hamilton syringe (precision ±1 µl) to 25 ml volumetric flask in order to obtain the intermediate solution. The working solution was made by taking 100 µl of intermediate solution with a Hamilton syringe and diluted to 25 ml in a volumetric flask.

(b) 25.4 mg of analytical standard was weighed with a 4-digit analytical balance (linearity: ±0.2 mg, repeatability SD: ±0.03 mg) and diluted to 25 ml. One ml of stock solution was pipetted (tolerance: ±0.007 ml) to 25 ml volumetric flask, made up to mark, then 10 µl of intermediate solution (±0.1 µl) was taken with a Hamilton syringe and diluted to 25 ml.

The uncertainties were calculated following the procedures described in the EURACHEM Guide [6] assuming ±7°C change of temperature change during the day, as the worst case, which can occur if the laboratory is not air-conditioned, and ±2°C for an ideally air-conditioned laboratory. In addition, the uncertainties were calculated for that case where the standard solution was prepared based on weighing except the last dilution to 25 ml.

The results are summarized in Table 2.

The combined uncertainty of the diluted solution made by volumetric dilution is about 0.96% with method *a,* and 1.4% with method *b* (the limiting factor (1%) is pipetting of 1 ml). The larger temperature range had only a marginal effect on the uncertainty. Preparing the solutions based on weighing with 5-digit balance significantly improved the precision of the standard solution (0.2%), but 4-digit balance provides only a slight improvement (0.7%) because weighing of 25 mg materials has an uncertainty of about 0.6%.

Another notable finding is the 7.5% difference, which is > 7.5 times higher than the estimated uncertainty of the solution, in the concentrations calculated from the nominal volumes of the A-grade glassware and from the results of weighing. Since the series of standard solutions for the calibration are prepared with different glassware, the deviation from the nominal value may be positive or negative and may significantly affect the accuracy and the uncertainty of the calibration solutions.

TABLE 2. ILLUSTRATION OF THE UNCERTAINTY COMPONENTS OF A DILUTED ANALYTICAL STANDARD SOLUTION

	Weight [g]	Volumetric	Method (b) 4-digit balance	Method (a) 5-digit balance
			Relative uncertainty	
Purity of analytical standard		0.00058	0.00058	0.00058
Weighting of analytical standard	0.0254	0.00124	0.00664	0.00124
Dilution to 25 ml in A grade volumetric flask	17.21334	0.00121	9.798E-06	1.837E-06
Taking (a) 100 µl, (b) 1 ml	0.07199	0.00657	0.0023	0.00044
Dilution to 25 ml in A grade volumetric flask	17.20969	0.00121	9.798E-06	1.837E-06
Taking (a) 100 µl, (b) 10 µl	0.07125	0.00657	0.0023	0.00044
Dilution to 25 ml in A grade volumetric flask	17.19284	0.00121	0.00121	0.00121
Temperature effect (± 7°C temperature range)		0.00963	0.00744	0.00193
Temperature effect (± 2°C temperature range)		0.00942	0.00738	0.001709
Combined uncertainty[a]		0.01418	0.02561	0.004978
Calculated concentration		1.624E-08[b]		1.757E-08[c]

Notes:
(a) Calculated with ±7°C temperature range for method (b) (dilutions of 1 ml and 10 µl aliquots instead of 2 × 100 µl).
(b) Concentration of standard solution calculated with the nominal volume of volumetric glassware.
(c) Concentration of standard solution calculated with the weights except the last dilution.

3. PREPARATION OF SOLUTIONS WITH PRECISE CONCENTRATION OF ANALYTES

As it was shown in the previous sections, the accurate and precise preparation of analytical standards, RM, and sample extracts is crucial for obtaining unbiased results with low uncertainty, which are required for testing the compliance of pesticide formulations with relatively narrow tolerance limits.

The concentration of internal standard, IS, may change significantly during the dilution procedure. For this reason we have to calculate the precise concentrations for both IS and RM. A stepwise procedure had been developed for the preparation of analytical standard and sample extract solutions applying internal standards. The calculation of the concentrations is facilitated by an Excel spreadsheet [10]. It can be downloaded from http://www-infocris.iaea.org/Download/Calculation_sheet.xls. The calculations programmed in the spreadsheets are detailed in Table 3. The file should be renamed indicating the task for which the calculations are preformed. The new raw data should be entered into the corresponding cells marked with yellow colour. The data in the green cells will be automatically calculated. Data of concentration ratios of analyte/internal standard will be transferred automatically to the calibration sheet.

The solutions are prepared based on weighing and expressed as mass/mass (m/m). Assuming that equal volume is injected into the chromatographs from the standard solutions of different RM concentrations and from the samples, the calibration graph can be constructed based on the m/m of standard solutions. The AS concentration in the sample shall be obtained in m/m. Where the AS content is specified in mass/volume (m/v) unit, then for reporting the results the m/m values shall be converted to m/v taking into account the density of the formulation.

All weighing should be carried out using properly maintained and calibrated balances checked with certified calibration masses. For preparing analytical standard solutions 5-digit balance should be used as far as possible. Otherwise, larger amount of RM shall be weighed in, to keep the relative weighing uncertainty ≤ 0.002, which makes the analysis more expensive.

TABLE 3. STEPWISE PROCEDURE FOR PRECISE CALCULATION OF CONCENTRATIONS OF THE COMPONENTS OF ANALYTICAL STANDARD AND SAMPLE SOLUTIONS

	Step	Calculation
1	**Standard solutions containing one active ingredient and the internal standard.**	
1.1	**Internal standard solution IS**	
1.1.1	Mass of internal standard:	W_{is} [g]
1.1.2	Total mass of IS plus solvent:	W_{Tis} [g]
1.1.3	Concentration of IS solution:	$C_i = W_{is}/W_{Tis}$ [mg/g]
1.2	**Stock solution RM^o**	
1.2.1	Purity of analytical standard:	P [g/g] (e.g. if purity is 95% P = 0.95)
1.2.2	Mass of analytical standard weighed in:	w_A^o
1.2.3	Total mass of weighed analytical standard and internal standard solution used to make the stock solution:	w_{TA}^o
1.2.4	Concentration of RM in stock solution:	$c_A^o = \frac{P w_A^o}{w_{TA}^o}$
1.2.5	Concentration of IS in stock solution:	$c_i^o = \frac{c_i (w_{TA}^o - w_A^o)}{w_{TA}^o}$

	Step	Calculation
1.2.6	RM/IS ratio in stock solution:	$f^{o}_{A} = \frac{c^{o}_{A}}{c^{o}_{i}}$
1.3	**1st dilution of stock analytical standard**	
1.3.1	Mass of stock analytical standard solution weighed in:	w^{I}_{A}
1.3.2	Total mass of weighed stock analytical standard solution and internal standard solution used to make the 1st diluted analytical standard solution:	w^{I}_{TA}
1.3.3	Concentration of RM in the 1st diluted analytical standard solution:	$c^{I}_{A} = \frac{c^{o}_{A} w^{I}_{A}}{w^{I}_{TA}}$
1.3.4	Concentration of IS in the 1st diluted analytical standard solution:	$c^{I}_{i} = \frac{c^{o}_{i} w^{I}_{A} + c_{i}(w^{I}_{TA} - w^{I}_{A})}{w^{I}_{TA}}$
1.3.5	RM/IS ratio in the 1st diluted analytical standard solution:	$f^{I}_{A} = \frac{c^{I}_{A}}{c^{I}_{i}}$
1.4	**2nd dilution of stock analytical solution**	
1.4.1	Mass of stock analytical standard solution weighed in:	w^{II}_{A}
1.4.2	Total mass of weighed stock analytical standard solution and internal standard solution used to make the 2nd diluted standard solution:	w^{II}_{TA}
1.4.3	Concentration of RM in the 2nd diluted standard solution:	$c^{II}_{A} = \frac{c^{o}_{A} w^{II}_{A}}{w^{II}_{TA}}$
1.4.4	Concentration of IS in the 2nd diluted standard solution:	$c^{II}_{i} = \frac{c^{o}_{i} w^{II}_{A} + c_{i}(w^{II}_{TA} - w^{II}_{A})}{w^{II}_{TA}}$
1.4.5	AS/IS ratio in the 2nd diluted standard solution:	$f^{II}_{A} = \frac{c^{II}_{A}}{c^{II}_{i}}$
1.5	Further dilutions are similar to 1.3 and 1.4. Indicate values with superscript III …	
2.	**Sample extract and dilutions**	
2.1	**Sample extract (replicate a1)**	
2.1.1	Active ingredient content of the formulated pesticide:	Fp [g/g][1]
2.1.2	Mass of formulated product weighed in:	w^{a1}_{S}
2.1.3	Total mass of weighed pesticide product and internal standard solution used to make the sample extract:	w^{a1}_{TS}
2.1.4	Theoretical concentration of active ingredient in sample extract (replicate: a1):	$c^{a1}_{S} = \frac{Fp\, w^{a1}_{S}}{w^{a1}_{TS}}$
2.1.5	Concentration of IS in sample extract a1:	$c^{a1}_{Si} = \frac{c_{i}(w^{a1}_{TS} - w^{a1}_{S})}{w^{a1}_{TS}}$

	Step	Calculation
2.1.6	Theoretical AI/IS ratio in sample extract:	$f_S^{a1} = \frac{c_S^{Ia1}}{c_{Si}^{a1}}$
2.2	**Dilution of sample extract a1**	
2.2.1	Mass of pesticide extract weighed in:	w_{Sa1}^{I}
2.2.2	Total mass of weighed pesticide extract and internal standard solution used to make the stock solution:	w_{TSa1}^{I}
2.2.3	Theoretical concentration of AS in diluted pesticide extract:	$c_{Sa1}^{I} = \frac{c_S^{a1} w_{Sa1}^{I}}{w_{TSa1}^{I}}$
2.2.4	Concentration of IS in diluted pesticide extract:	$c_{ia1}^{I} = \frac{c_{Si}^{a1} w_{Sa1}^{I} + c_i \left(w_{TSa1}^{I} - w_{Sa1}^{I} \right)}{w_{TSa1}^{o}}$
2.2.5	Theoretical AS/IS ratio in diluted pesticide extract:	$f_{Sa1}^{I} = \frac{c_{Sa1}^{I}}{c_{ia1}^{I}}$
3.	**Preparation of analytical standard solutions containing more than one active ingredient**	
	Mass of stock analytical standard solution weighed in for RM1:	w_{A1}^{1}
	Mass of stock analytical standard solution weighed in for RM2:	w_{A2}^{1}
	Mass of stock analytical standard solution weighed in for RMj:	w_{Aj}^{1}
	Total mass of weighed stock analytical standard solution and internal standard solution used to make the diluted standard mixture solution:	w_{TM}^{1}
	Concentration of RM1 in the diluted standard mixture solution: The other RM-s concentration is calculated similarly.	$c_{A1}^{M1} = \frac{c_{A1}^{o} w_{A1}^{1}}{w_{TM1}^{1}}$
	Concentration of IS in the diluted standard mixture solution containing ***j*** RMs:	$c_{iM}^{1} = \frac{\sum_{k=1}^{j} c_{Ak}^{o} w_{Ak}^{1} + c_i \left(w_{TM}^{1} - \sum_{k=1}^{j} w_{Ak}^{1} \right)}{w_{TM}^{1}}$

[1]Normally it is expressed in g/kg or g/l or %. Be careful, as here we have to convert the dimension to g/g unit!

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APPENDIX I

Estimation of within-laboratory repeatability and reproducibility of the procedure

As part of the validation of the method, a 0.5% dust formulation of a pesticide was analyzed by three analysts (A, B, C) of a laboratory in five replicates in one day to determine the within-laboratory repeatability and reproducibility of the method. The test portions analyzed by one analyst on one day were prepared independently from separate sub-samples. The results [%] obtained within a day and different days (d1-d3) are given in Table AI.1. Previous measurements indicated that the distribution of measurement results is normal.

TABLE AI.1. THE ACTIVE SUBSTANCE CONCENTRATION OF 0.5% DUST FORMULATION

A d1	B d1	C d1	A d2	B d2	C d2	A d3	C d3
0.43	0.51	0.5	0.51	0.49	0.47	0.49	0.52
0.52	0.5	0.48	0.5	0.48	0.49	0.55	0.52
0.5	0.53	0.48	0.48	0.51	0.51	0.54	0.5
0.49	0.5	0.52	0.49	0.47	0.54	0.44	0.49
0.51	0.52	0.46	0.56	0.49	0.5	0.51	0.5

We can get answers from the results to a number of questions, which are partly interrelated.

(a) What is the average repeatability achieved by the analysts?
(b) Is the performance of the analysts during the 8 tests different?
(c) What is the within-laboratory repeatability (S_r) of the method?
(d) What is the within-laboratory reproducibility (S_R) of the method?
(e) Are the S_r and S_R values significantly different?
(f) Is the processed sample "homogeneous" (well mixed at analytical portion level)? Or alternately we can ask: are the mean values obtained significantly different?

Solution:

1. Calculate the mean, standard deviation, variance (V) and relative standard deviation (coefficient of variation, CV) of the measurements:

	A d1	B d1	C d1	A d2	B d2	C d2	A d3	C d3
Ave	0.49	0.512	0.488	0.508	0.488	0.502	0.506	0.506
SD	0.03536	0.01304	0.02280	0.03114	0.01483	0.02588	0.04393	0.01342
V	0.00125	0.00017	0.00052	0.00097	0.00022	0.00067	0.00193	0.00018
CV	0.072	0.025	0.047	0.061	0.030	0.052	0.087	0.027

2. Observe the result obtained, and note the possible outliers, marked in Table 1.

3. Check if there is any outlier among the measurements.

The suspect populations are the Ad3 and Ad1 measurements:

Dixon test for Ad3 data set:

Arrange the data in rank order: 0.55 0.54 0.51 0.49 0.44

Calculate the test statistics:

$$r_{10} = (x_n - x_{n-1})/(x_n - x_1) \quad \text{or} \quad (x_2 - x_1)/(x_n - x_1) \tag{1}$$

$r_{10} = 0.454545$

The critical values for the Dixon test can be found in basic statistical handbooks (note that the formula for calculation changes is depending on the number of data points.):

$r_{10.\,0.05} = 0.642$, $r_{10.\,0.02} = 0.729$ and $r_{10,\,0.01} = 0.78$

$r_{10} < r_{10,\,0.05}$ critical

The 0.44% value is not an outlier.

Calculation for Ad1 (note that the order of measured values is not important)

0.43 0.49 0.5 0.51 0.52

$r_{10} = 0.6666$, it is between the critical values of $r_{10.\,0.05} = 0.642$, $r_{10.\,0.02} = 0.729$, so it may be considered as a suspect value.

Grubbs test:

$$G'_{lowest} = (\bar{x} - x_1)/s \quad \text{or} \quad C'_{highest} = (x_n - \bar{x})/s \tag{2}$$

- If the test statistics G is $\leq G_{crit,0.05}$ (5% critical value) the item tested is accepted as correct.
- If $G_{crit,0.05} < G \leq G_{crit,0.01}$ the item is a straggler.
- If $G > G_{crit,0.01}$ the item is a statistical outlier.

Some critical values for the Grubb's test are given below. Detailed information on the application of Grubb's test may be found elsewhere. [1], [2]

n[1]	95% G'	99% G'
4	1.463	1.492
5	1.672	1.749
6	1.822	1.944
7	1.938	2.097

Notes: 1. Number of measurement points in the data set.

Calculate ***s*** with all data points, see Annex of ISO 5725 for critical values.

G'_{lowest} = for Ad3: 1.502, for Ad1: 1.697

$\mathbf{G'_{0.05}} = 1.672$; $\mathbf{G'_{0.01}} = 1.749$

The value of 0.43 is an outlier but the 0.44% value is not an outlier.

[1] T. Farrant, *Practical Statistics for the Analytical Scientist*, Royal Society of Chemistry, London, 1997.

[2] ISO 5725, parts 1-6, *Accuracy (Trueness and Precision) of Measurement Methods and Results*. (1994).

The H_0 is rejected at $\alpha = 0.05$ level with both tests for Ad1 data set, but the test statistics is between $\alpha = 0.02$ and 0.05 for Dixon test and between 0.01 and 0.05 for Grubb's test. Taking into account that the data sets include only 5 measurements, and the standard deviation of the 5 measurements results is not the largest in the 8 sets of measurement, the value of 0.43 is retained, and it is not considered to be an outlier.

Statistical outlier tests should be interpreted with great care where small number of measurements are available, as they may lead to artificially trimmed data set and apparently good precision, which cannot be obtained during the regular routine use of the method.

However, note below that the precision of analyst A is much lower than that of analysts B and C (though the difference is statistically not significant!), analyst A should practice the procedure further on to verify that it can be performed with required precision.

(a) What is the average repeatability achieved by the individual analysts?

It can be calculated from the average of the variances obtained by each analyst. The pooled standard deviation is calculated as:

$$s_p = \sqrt{\frac{(s_1^2 x df_1) + (s_2^2 x df_2) + ... + (s_n^2 x df_n)}{df_1 + df_2 + ... + df_n}} \quad (3)$$

The average repeatability standard deviations and corresponding coefficient of variations of the individual analysts (calculated from their measurements made on different days) are:

$S_{A,v=12}$	0.037193%	CV = 0.074189
$S_{B,v=8}$	0.013964%	CV = 0.027928
$S_{C,v=12}$	0.021370%	CV = 0.042854

(b) Is the performance of the analysts different?

Apply Cochran test to verify that the eight sets of measurements may come from the same population:

$$g = \frac{s_{max}^2}{\sum_{i=1}^{p} s_i^2} \quad (4)$$

g = 0.00193/0.00591 = 0.326565

The critical value for 8 data sets, and n = 5 number of replicate measurements: $g_{0.05} = 0.391$.

The critical value is larger than the calculated one (0.391 > 0.32), thus there is no significant difference between the daily performances of analysts.

Note: for comparing the results of a series of replicate measurements, the F-test cannot be applied!

Some critical values for the Cochran test are given in the following table:

Number of data sets	Number of replicate measurements			
	2	3	4	5
2	0.999	0.975	0.939	0.906
3	0.967	0.871	0.798	0.746
4	0.906	0.768	0.684	0.629
5	0.841	0.684	0.598	0.544
6	0.781	0.616	0.532	0.480
7	0.727	0.561	0.480	0.431
8	0.680	0.516	0.438	0.391

The table gives critical values of g at 5% level for the Cochrane test for homogeneity of variance. The values depend on the number of data sets, p, and the number of replicate measurements on each sample, n. The number of measurements should be the same in each data set.

(c) What is the within-laboratory repeatability (S_r) of the method?

It is the average of the variations obtained by all analysts, and calculated with Eq. (3). The degree of freedom for the pooled standard deviation is

$$df_p = df_1 + df_2 + \ldots + df_n \quad (5)$$

The $df = \nu$ of each set of measurement in this case is 5 – 1 = 4. The $\nu_p = 8 * 4 = 32$.

$S_p = S_r = 0.027$

(d) What is the within-laboratory reproducibility (S_R) of the method?

The within-laboratory reproducibility of the method is the SD of all measurements calculated with the usual formulae: $S_R = 0.0263117$

Note: this result is in some way surprising; one would expect that the $S_r \leq S_R$, but we should not forget that S_r and S_R are estimated values, so it may be possible to get such results. Their difference is statistically not significant. Test it with F test to verify.

(e) Are the S_r and S_R values significantly different?

Apply F-test to decide (if it is not obvious):

$F = s_r^2/s_R^2 = 1.0672$ (the larger variance is always in the nominator, F > 1!)

Apply two sided test at P = 0.95, read F_{crit} from Excel entering the following command: =FINV(α, ν_1,ν_2) (ν_1 is always the degree of freedom of the nominator, α = 1-P = 0.05):

$F_{0.05,\ 32,\ 39} = 1.739263$.

The difference is not significant.

Note that the probability is indicated in a different way in the statistical table and in the Excel sheet.

(f) Is the processed sample “homogeneous” (well mixed at analytical portion level)? Or alternately we can ask: are the mean values obtained significantly different?

Where more than two mean values have to be compared apply analysis of variance, ANOVA.

The calculation of the one way ANOVA can be conveniently performed with Excel. Enter the values of Table I.1 starting in cell A1. Go to Tools → Data analysis → ANOVA single factor → OK, click on input range and highlight cells A1:H6, select Grouped by columns, Labels in First Row, accept alpha 0.05, select cell A8 for output range and click OK.

The result of the calculation is given in Table AI.2.

TABLE AI.2. OUTPUT OF EXCEL SINGLE FACTOR ANOVA CALCULATION

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
A d1	5	2.45	0.49	0.00125
B d1	5	2.56	0.512	0.00017
C d1	5	2.44	0.488	0.00052
A d2	5	2.54	0.508	0.00097
B d2	5	2.44	0.488	0.00022
C d2	5	2.51	0.502	0.00067
A d3	5	2.53	0.506	0.00193
C d3	5	2.53	0.506	0.00018

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	0.00336	7	0.00048	0.6497	0.711786	2.312741
Within Groups	0.02364	32	0.000739			
Total	0.027	39				

If the F value (0.6497) is smaller than the F_{crit} (2.313), the difference between groups (the mean values) is not significant.

In our example, it means that at the given precision of the replicate analysis (s_r), the difference between the mean values can be attributed to the variation of the analysis of the AS in the extracts. With other words, the combined uncertainty of sample processing and extraction is insignificant compared to the uncertainty of analysis, and the sample preparation produced a homogeneous material at the given mass of the test portions.

If the material is statistically well mixed, the product of the mass of test portion and the square of the relative standard deviation of the sample processing error gives a constant value which is described by the so called sampling constant [1].

$$K_s = m \times CV_{Sp}^2 \quad (6)$$

If we would like to reduce the test portion size, and determine the uncertainty of sample processing for various test portions sizes, we have to estimate the sampling constant and then the CV_{Sp} can be calculated for any test portion size. The procedure had been described in detail [2, 3] elsewhere.

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EXPERIMENTAL RESULTS

SUMMARY OF VALIDATION OF MULTI-PESTICIDE METHODS FOR VARIOUS PESTICIDE FORMULATIONS

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Abstract

The validation of multi-pesticide methods applicable for various types of pesticide formulations is treated. In a worked-out practical example, i.e. lambda cyhalothrin, the theoretical considerations outlined in the General Guidance section are put into practice. GC conditions, selection of an internal standard and criteria for an acceptable repeatability of injections are outlined, followed by sample preparation, calibration, batch analysis and confirmation of results through comparison using different separation columns. Complete sets of data are displayed in tabular form for other pesticide active ingredients and real formulations.

1. INTRODUCTION

The CRP participants followed the stepwise procedure described in Chapter 4.2, following the general principles provided in Chapters 4.1, 4.3 and 4.4 for optimizing the chromatographic conditions according to the configuration of their instruments.

A worked-out example, based on the report of Esperanza Uy, is given in the following section [1] to illustrate the procedure, followed by the results reported by the other participants of the CRP.

2. WORKED-OUT EXAMPLE FOR THE VALIDATION OF A MULTI-PESTICIDE METHOD

2.1. Gas chromatographic conditions for lambda cyhalothrin

Taking into account the prior test runs the following chromatographic conditions were chosen for the draft multi-pesticide method:

Instrument:	HP 6890 GC with HP 6973 Autosampler, split inlet	
Column:	DB-5, 30 m × 0.32 mm × 0.25 µm	
Temperature:		
Oven:	150°C	for 1 minute
Rise at 35°C/min to:	270°C	keep for 8 minutes
Inlet:	290°C	
Detector:	300°C	
Helium carrier gas flow rate:	2.5 ml/min	
Linear velocity:	46 cm/sec	
Split Ratio:	50:1	
Hydrogen:	40 ml/min	
Air:	400 ml/min	

2.1.1. Selection of internal standard

Applying the multi-rise temperature programme described in chapter 6.1 section 3.1 (initial time 1 min at 80°C, rise I. (fast) 35°C/min to 150°C, rise II. (slow) 8°C/min to 300°C, final time 15 min) three internal standards (IS) were tested from the recommended compounds namely diethyl phthalate

(DEP), dibutyl phthalate (DBP), and diphenyl phthalate (DPP). The elution times of lambda cyhalothrin, DEP, DBP and DPP were 17.381, 6.996, 10.781 and 16.839 minutes, respectively.

Based on its elution time DPP would be the proper IS, but under the GC conditions given in section 2.1 there was an interference with a compound from the formulated product (Figure 1). Therefore two additional IS's were considered which eluted with multi-ramp temperature programme at 17.255 min (hexacosane) and 15.375 min (adipic acid).

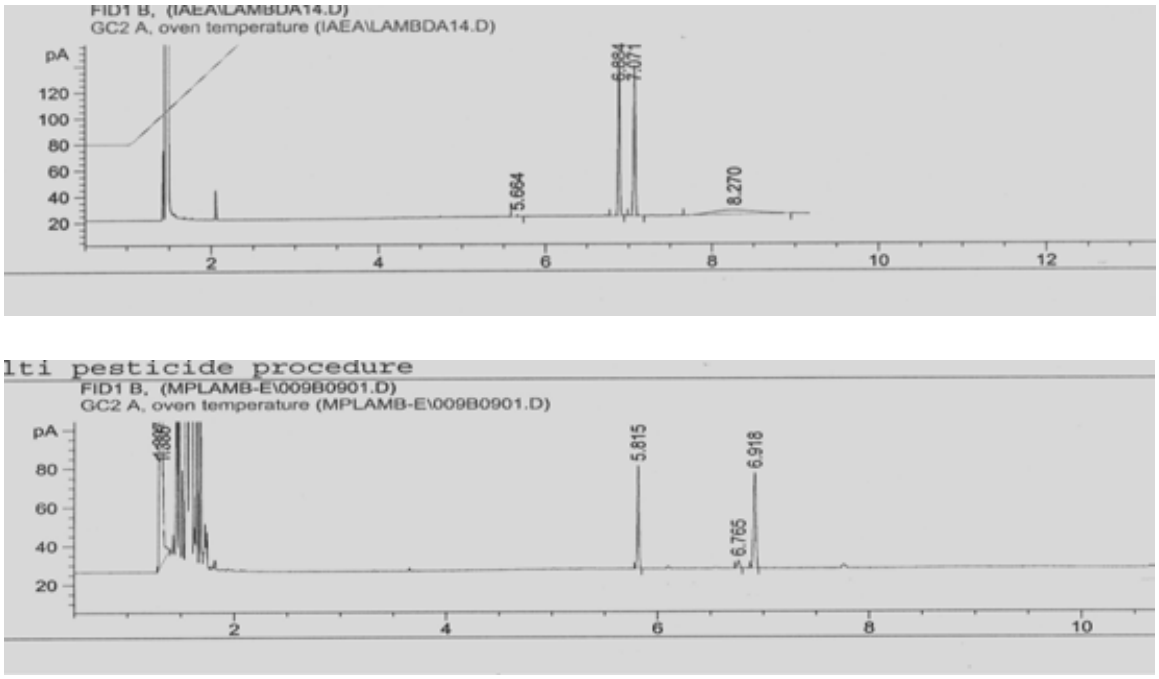
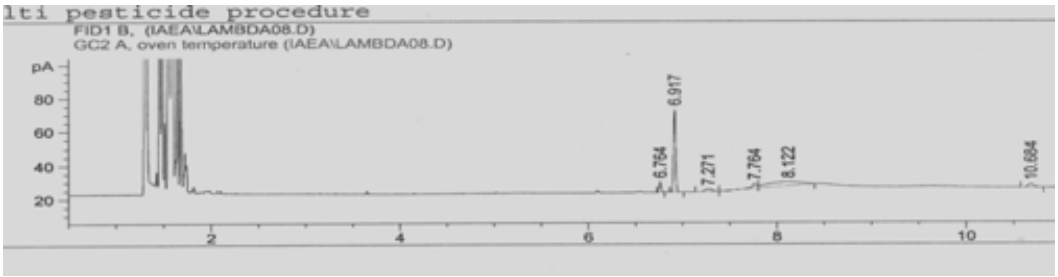


FIG. 1. Chromatograms of Karate 2.5 EC extract and DPP (upper) and adipic acid (lower) under selected chromatographic conditions.

Under the GC conditions given in section 2.1 adipic acid was chosen as the IS because it eluted (5.815 min) near to lambda cyhalothrin (6.918 min) with no observed interferences. DEP and DBP eluted very early, whereas the formulated product (Karate 2.5 EC) resulted in many peaks (Figure 2).

Normal



Concentrated

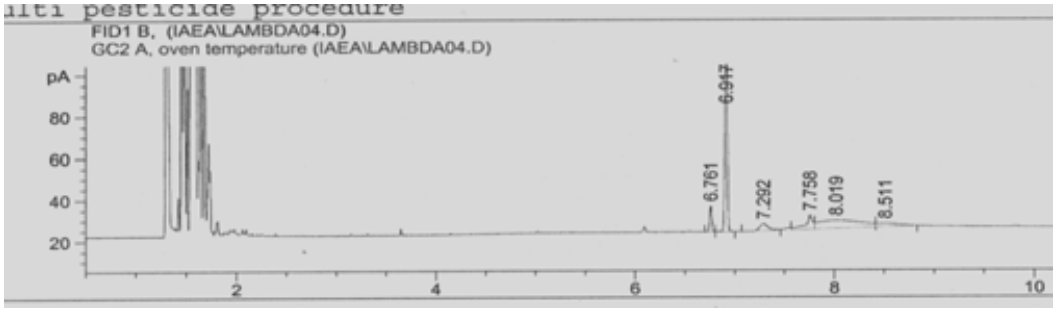


FIG. 2. Chromatograms of Karate 2.5 EC extract under GC conditions given in section 2.1.

2.1.2. Repeatability of injections

The repeatability of injection was tested with the selected internal standard and the active substance. The results are given in Table 1.

TABLE 1. REPEATABILITY OF INJECTION UNDER SELECTED CHROMATOGRAPHIC CONDITIONS

Injection	Retention time (IS)	Area(IS)	Retention time (AS)	Area (AS)	Area Ratio
1	5.814	59.4418	6.918	77.8443	1.309589
2	5.812	58.5028	6.914	77.0021	1.316213
3	5.812	59.0859	6.915	77.4855	1.311405
4	5.812	59.045	6.915	77.4778	1.312183
5	5.811	58.2548	6.914	76.3545	1.310699
6	5.812	58.5356	6.914	76.8313	1.312557
Average	5.8122	58.8110	6.915	77.1659	1.312108
Std. Dev.	0.001	0.44909	0.001549	0.53978	0.002275
CV	0.0002	0.00764	0.000224	0.007	0.001734

The results indicate that the repeatability of the retention times (0.02%) as well as the area ratio of the response (0.1%) were very good, indicating that the chromatographic system was stable and properly set.

2.2. Sample preparation

The preparation and extraction of the test portion were carried out following the CIPAC sample preparation method. Acetone was used as a solvent. The samples were analyzed using the method described in section 2.1.

The CIPAC method gave a repeatability range of 0.02. The corresponding range for 3 replicate measurements is 0.0236 (3.31 × r/2.8). The difference between the maximum and minimum concentration of the AS in sample portions analyzed was 0.0134 (Table 2) indicating that the repeatability of the method was good. The repeatability CV of 0.00138, calculated from the duplicate

analysis results was in good agreement with that obtained from the repeatability test of injections (0.0017) given in Table 1.

No blank formulation was available, therefore the formulated product was extracted without IS and the extract and its concentrated portion (4 ml to 2 ml) were injected into the GC. No interference was observed that might affect result of analysis as shown in Figure 2.

2.2.1. Analysis of five batches

Five different batches of the pesticide product were collected and analyzed. Standard concentrations of 0.8, 1.0, and 1.2 times the nominal concentration of active ingredient in the formulated product were prepared by independent weighing of the analytical standard. Multi-point calibration with 6 injections of each standard solution was performed. The results are given in Table 3.

The linear regression equation obtained was $y = 3.234x - 0.029$ with $R^2 = 0.9961$, and $S_{rr} = 0.01$ (see chapter 4.5, Eq. (12)). The calibration chart is shown in Figure 3.

The results of the analysis of the 5 batches are summarized in Table 4. The CV of the repeatability of the injections of test portion extracts, calculated as the average of 5 times 4 injections, was 0.004 (0.4%). The average CV of repeatability of test portion analysis was 0.0059 (0.59%), which indicates very good repeatability of the procedure.

TABLE 2. RESULTS OF THE ANALYSIS OF THREE TEST PORTIONS

Inj.	Conc (mg/ml)	Area (IS)	Area (AI)	Area Ratio	Conc. (AI)	Average
Std	0.4076	58.2548	76.354	1.3107		
Std	0.4076	58.5356	76.831	1.31256		
Trial 11	15.855	54.4859	80.321	1.47418	2.853	2.852256
Trial 12	15.855	55.3556	81.532	1.47288	2.851	
Std	0.4076	58.832	76.786	1.30519		
Std	0.4076	58.6823	76.548	1.30446		
Trial 21	16.092	54.5723	81.153	1.48708	2.843	2.838902
Trial 22	16.092	56.3933	83.598	1.48242	2.8344	
Std	0.4076	60.0134	79.823	1.33009		
Std	0.4076	60.0026	77.828	1.29709		
Trial 31	16.105	55.8584	83.213	1.48973	2.8445	2.845919
Trial 32	16.105	54.5603	81.360	1.4912	2.8473	
Std	0.4076	59.1471	76.875	1.29973		
Std	0.4076	59.7367	77.395	1.29562		
Mean						2.84569
Std. Dev.					0.00395[a]	0.006680
CV					0.00138[b]	0.00235
Cmax-Cmin						0.01335

Notes:
(a) repeatability standard deviation of replicate injections;
(b) coefficient of variation of replicate injections calculated with Eqs (8) and (10) given in chapter 4.5.

TABLE 3. CALIBRATION DATA ON A DB-5 COLUMN

	Injection	Area(IS)	Area(AS)	Area Ratio	RM (mg/ml)
Std 1	1	64.6309	102.425	1.58477	0.4938
	2	64.8819	102.332	1.5772	0.4938
Std 1	3	63.883	99.977	1.565	0.4938
	4	66.3739	103.696	1.5623	0.4938
Std 1	5	66.845	102.147	1.52811	0.4938
	6	59.906	94.9967	1.58576	0.4938
Std 2	1	64.0195	84.2447	1.31592	0.4132
	2	65.9893	86.6518	1.31312	0.4132
Std 2	3	66.6514	87.6481	1.31502	0.4132
	4	68.0068	88.3574	1.29924	0.4132
Std 2	5	60.5426	79.8826	1.31944	0.4132
	6	59.6119	79.0418	1.32594	0.4132
Std 3	1	65.9223	68.1516	1.03382	0.3296
	2	66.2507	68.4513	1.03322	0.3296
Std 3	3	65.9457	67.9517	1.03042	0.3296
	4	63.8107	65.2408	1.02241	0.3296
Std 3	5	60.994	63.414	1.03968	0.3296
	6	60.925	63.3915	1.04048	0.3296

2.2.2. Quantitative confirmation of the results obtained with the draft procedure

As the chromatographic column specified in the CIPAC method was not available the reference method could not be followed. Therefore a second column of different polarity was used for quantitative confirmation of the results.

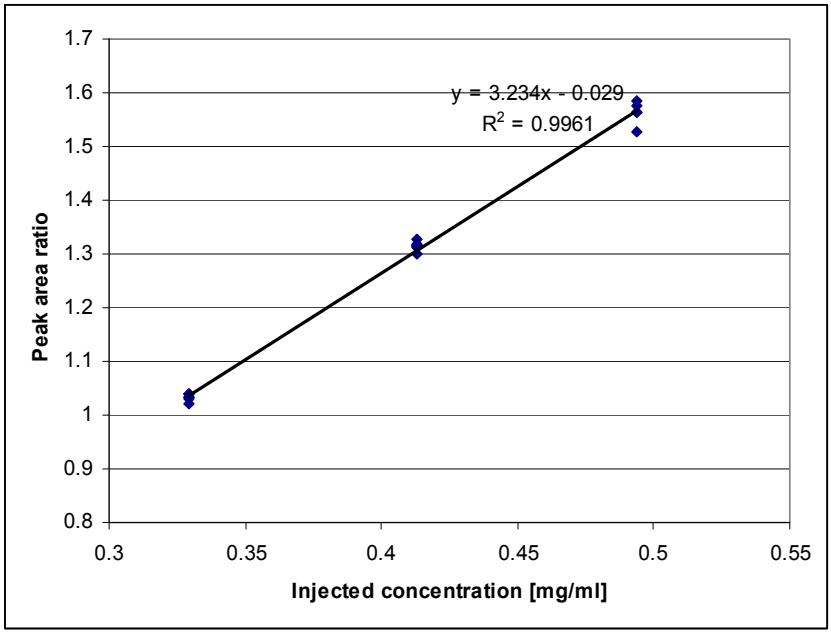


FIG. 3. Calibration chart of lambda-cyhalothrin (S_{rr} = 0.010) obtained on a DB-5 column.

TABLE 4. RESULT OF THE ANALYSIS OF FIVE BATCHES

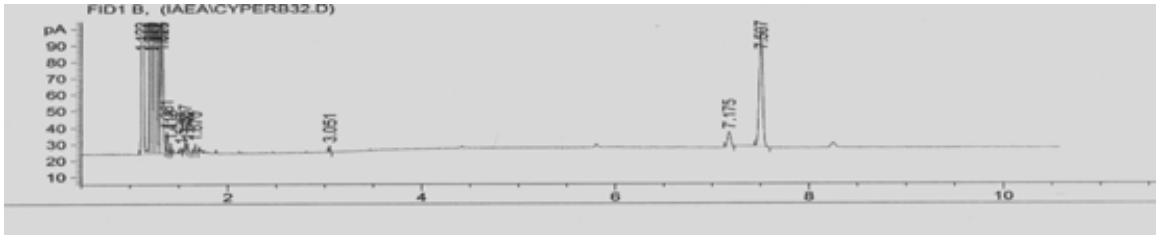
Sample	Area (IS)	Area (AS)	Area Ratio	Conc. (mg/ml)	% AI	Average	
MPF-057	58.666	80.636	1.3745	0.434	2.696	2.685	2.688
	58.626	79.918	1.3632	0.430	2.674		
	57.289	78.727	1.3742	0.434	2.700	2.692	
	58.975	80.518	1.3653	0.431	2.683		
MPF-058	59.636	81.626	1.3688	0.432	2.672	2.657	2.677
	60.686	82.115	1.3531	0.427	2.642		
	58.445	80.236	1.3728	0.433	2.701	2.697	
	60.019	82.152	1.3688	0.432	2.693		
MPF-059	60.581	67.506	1.1143	0.354	2.206	2.206	2.195
	61.102	68.031	1.1134	0.353	2.205		
	60.100	66.574	1.1077	0.352	2.186	2.184	
	61.482	67.994	1.1059	0.351	2.182		
MPF-060	54.413	78.816	1.4485	0.456	2.846	2.859	2.856
	53.147	77.683	1.4617	0.460	2.871		
	55.474	80.452	1.4503	0.457	2.853	2.853	
	55.247	80.155	1.4508	0.457	2.854		
MPF-108	56.111	80.988	1.4434	0.455	2.813	2.814	2.820
	56.022	80.933	1.4447	0.455	2.815		
	65.771	96.065	1.4606	0.460	2.831	2.825	
	65.072	94.613	1.4540	0.458	2.819		

The following chromatographic conditions were used with the second column:

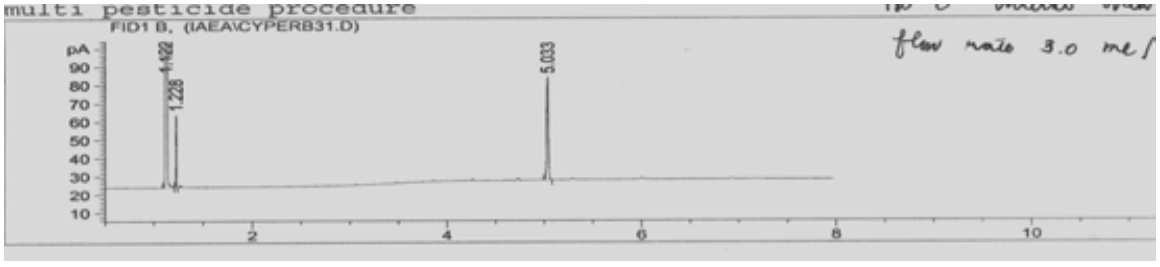
Column:	CP Sil 19CB, 30 m × 0.32 mm × 0.25 µm
Oven:	180°C, keep for 1 minute
	rise at 35°C/min to:
	270°C, keep for 7 minutes
Inlet:	280°C
Detector:	300°C
Split ratio:	50:1
Carrier gas:	helium 3.0 ml/minute, average velocity: 54 cm/sec

A 4-ml aliquot of the sample extract without IS was taken and concentrated to 2 ml to check for interferences. No interference was observed (Figure 4).

Concentrated



Adipic Acid



Normal sample + adipic acid

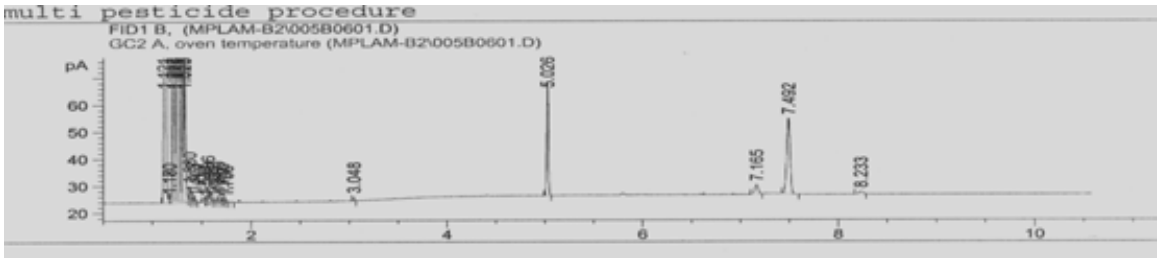


FIG.4. Chromatogram of Karate 2.5 EC and the internal standard on CP-SIL 19CB column.

The checks of the repeatability of injection and the analysis of replicate test portions were carried out as in the case of the DB-5 column. The results are summarized in Table 5.

TABLE 5. REPEATABILITY OF LAMBDA CYHALOTHRIN INJECTIONS

	IS t_R	Area IS	AS t_R	Area AS	Area Ratio
Mean	5.025	55.850	7.488	66.835	1.197
SD	0.0010	0.4333	0.0019	0.8053	0.0060
CV	0.0002	0.0078	0.0003	0.0120	0.0050

The CIPAC method gave a repeatability range of 0.02. The corresponding range for 3 replicate measurements is 0.0236 (3.31 × r/2.8). The difference between the maximum and minimum concentration of the AS in sample portions analyzed was 0.0273. The corresponding s_{wr} is not significantly different from the CIPAC s_r taking into account the limited number of measurements made. It should be pointed out that the tests performed on the second column indicated somewhat lower repeatability of the analysis (CV = 0.005) than it was in the other tests.

The multi-point calibration was performed with 6 injections of each standard solution. The linear regression equation was: $y = 3.35x – 0.005$, with $R^2 = 0.9958$ and $S_{rr} = 0.012$.

The results of the analysis of the 5 batches are summarized in Table 6.

TABLE 6. RESULTS OF ANALYSIS OF FIVE BATCHES ON A CP-SIL 19CB COLUMN

	Area (IS)	Area (AS)	Area Ratio	Conc. (mg/ml)	% AI	Average	
MPF-057	53.149	69.980	1.317	0.4519	2.809	2.805	2.790
	50.016	65.651	1.313	0.4505	2.801		
	52.430	68.219	1.301	0.4466	2.781	2.775	
	53.079	68.719	1.295	0.4444	2.768		
MPF-058	52.952	68.960	1.302	0.4470	2.766	2.791	2.801
	51.895	68.845	1.327	0.4552	2.816		
	53.219	69.485	1.306	0.4481	2.795	2.810	
	51.381	67.832	1.320	0.4530	2.825		
MPF-059	52.928	55.350	1.046	0.3605	2.249	2.297	2.295
	52.155	56.904	1.091	0.3758	2.345		
	52.332	56.186	1.074	0.3699	2.299	2.293	
	52.896	56.450	1.067	0.3678	2.286		
MPF-060	52.033	71.374	1.372	0.4704	2.934	2.928	2.933
	51.437	70.295	1.367	0.4687	2.923		
	51.836	70.638	1.363	0.4674	2.918	2.938	
	51.288	70.896	1.382	0.4740	2.959		
MPF-108	52.427	71.227	1.359	0.4660	2.882	2.885	2.873
	53.280	72.549	1.362	0.4670	2.889		
	62.597	84.730	1.354	0.4643	2.857	2.860	
	62.893	85.330	1.357	0.4654	2.864		

The CV of the repeatability of the injections of test portion extracts, calculated as the average of 5 times 4 injections, was 0.005. The average CV of repeatability of test portion analysis was 0.009 (0.9%), which indicates good repeatability of the procedure.

2.3. Comparison of results obtained on two columns

As described above the test portions of the Karate 2.5 EC were prepared and extracted according to the CIPAC method, but the analysis of the AS was performed on two different columns.

The results are summarized in Table 7 to facilitate comparison and evaluation.

The paired t-test (t_{calc} = 7.635, $t_{0.05,4}$ = 2.776) indicates that the average AS values obtained on the two columns are significantly different, though both results are within the tolerance limits.

Furthermore, the difference 0.09 is much larger than the CIPAC r or the laboratory's repeatability range.

TABLE 7. COMPARISON OF THE RESULTS OF LAMBDA CYHALOTHRIN ANALYSIS ON TWO COLUMNS

Sample No	Method A			Method B			
	Replicate 1	Replicate 2	Average	Replicate 1	Replicate 2	Average	Difference
MPF-057	2.68	2.69	2.69	2.80	2.77	2.79	-0.10
MPF-058	2.66	2.70	2.68	2.79	2.81	2.80	-0.12
MPF-059	2.21	2.18	2.19	2.30	2.29	2.29	-0.10
MPF-060	2.86	2.85	2.86	2.93	2.94	2.93	-0.08
MPF-108	2.81	2.82	2.82	2.89	2.86	2.87	-0.06
Average			2.647			2.738	-0.09111
SD							0.026682

Further tests are required to identify the sources of the deviation, and after that the method should be validated.

3. SUMMARY OF REPEATABILITY OF GC INJECTIONS

The repeatability of injection depends primarily on the inertness of the injector and column, the selected mode of injection and the physico-chemical properties of the compounds analyzed.

The repeatability of injection and the retention times obtained under practical conditions are shown in Tables 8 and 9. The chromatographic conditions for the analysis are summarized in Table 10. The repeatabilities of injections reported in Chapter 6 are not included in the tables.

The values reported can be used for initial guidance, but they do not represent the best performance as they were not particularly optimised.

Concerning the precision of injection, the major criterion is the CV of the peak ratios of AS and IS as it will determine the precision of the analysis. If the amount of injected material is not constant, but the system is stable, the CV of the ratios can be very small regardless the CV of individual injections (e.g. Refs 4, 12, and 14). However, if the system is not stable the random variation of the signals will cause larger variation in their ratios (e.g. 21, 23) resulting in unacceptable conditions for precise quantitative measurements. Refs 20, 22, 26 may be examples for "difficult to analyze compounds".

The injections with references from 1 to 26 in Table 8 were performed with manual injection, while the rest was performed with auto-sampler. The difference in the repeatability of the injection is obvious.

Further on, note the significant difference of repeatability of individual compounds with split and on column injection (Table 9). Unfortunately, the data base for on-column injection is very limited so the conclusion is only preliminary.

The multi-point calibration gave varying results (Table 11). In several cases the criterion of $S_{rr} \leq 0.02$ could be met, in other cases, especially on more polar column the S_{rr} values were larger which coincided with larger variability of the results (CV_r).

The results confirmed that the S_{rr} is a very sensitive indicator of the goodness of the calibration and the stability of the system. Where multi-point calibration is used and the injection of reference material and the sample extracts are alternately made, if the calculated S_{rr} is ≤ 0.02 it provides firm assurance for the stability of the chromatographic system and goodness of the calibration. It does not provide, however, any information on the potential systematic error derived from interference with the AS and erroneous calibration solutions.

TABLE 8. EXAMPLES FOR THE REPEATABILITY OF INJECTIONS[A]

Ref	No of inj.	AS	IS	AS		IS		Sign. AS/IS
				Sign. CV	t_R CV	Sig. CV	t_R CV	CV
1	5	Pendimethalin	Dibutyl phthalate	8.74	0.13	8.82	0.30	0.22
1		Atrazine	Dibutyl phthalate	9.04	0.31			0.24
2	6	Chlorpyrifos	Diphenyl phthalate	4.42	0.14	5.19	0.10	0.85
2		Cypermethrin	Diphenyl phthalate	4.69				1.39
3	5	Alphamethrin	Diphenyl phthalate	4.99	0.64	4.24	0.57	1.43
4	5	Dimethoate	Dibutyl phthalate	15.28	0.80	14.89	0.57	0.85
5	5	Fenarimol	Dibutyl phthalate	3.92	0.35	4.07	0.26	0.26
6	5	Penconazole	Dibutyl phthalate	5.30	0.23	5.43	0.21	0.36
7	3	Cypermethrin	Diphenyl phthalate	3.20	0.42	1.32	0.25	2.35
8	3	Difenoconazole	Diphenyl phthalate	9.37	0.22	9.31	0.30	0.36
9	3	Triadimefon	Diphenyl phthalate	6.96	0.34	6.19	0.17	1.03
10	4	Iprodion	Dibutyl phthalate	10.36	0.11	10.83	0.08	1.36
11	2	Fenpropathrin	Dibutyl phthalate	4.24	0.13	5.03	0.35	0.79
12	4	Captan	Dibutyl phthalate	22.77	0.47	23.68	0.46	1.24
13	5	Bromopropylate	Dibutyl phthalate	9.62	0.10	8.98	0.15	0.83
14	8	Chlorothalonil	Dibutyl phthalate	17.59	0.21	18.77	0.20	1.42
15	5	Phosalone	Dibutyl phthalate	4.57	0.27	4.82	0.03	0.92
16	5	Pendimethalin	Dibutyl phthalate	15.59	0.37	15.41	0.29	0.33
17	5	Diazinon	Dibutyl phthalate	25.23	0.31	27.33	0.26	1.95
18	5	Captan	Dibutyl phthalate	17.17	0.22	17.28	0.24	0.68
19	5	Folpet	Diphenyl phthalate	14.25	0.28	16.40	0.27	3.45
20	5	Dichlobutanil	Diphenyl phthalate	33.10	0.31	36.04	0.17	2.48
21	5	Tebuconazole	Dibutyl phthalate	33.97	0.27	27.74		1.81
21		Triadimefon	Dibutyl phthalate	28.07	0.45			8.10
22	5	Phosalone	Dibutyl phthalate	20.15	0.36	18.63	0.76	2.21
23	4	Fenpropathrin	Dibutyl phthalate	29.86	0.47	23.63	0.77	6.98
24	3	Propiconazole	Dibutyl phthalate	15.36	0.08	14.03	0.04	1.51
25	3	Pendimethalin	Dibutyl phthalate	12.64	0.45	13.21	0.45	0.66
26	3	Captan	Dibutyl phthalate	19.48	0.11	21.42	0.07	2.35
27	5	Butachlor	Dibutyl phthalate	0.453	0.014	0.443	0.027	0.000
28	5	Propoxur	Chlorpyrifos	4.800	0.000	3.600	0.000	0.022
28	5	Parathion	Benzyl benzoate		0.390		0.400	0.020
29	6	Butachlor	Dibutyl phthalate		2.100		2.000	0.003
29	6	Butachlor	Dibutyl phthalate	1.300		1.100		0.006
30	5	Butachlor	Dibutyl phthalate	0.837	0.023	0.779	0.008	0.001
31	5	Carbaryl	Dimethyl phthalate	0.895	0.416	0.975	0.499	0.003
31	5	Cypermethrin	Adipic acid	0.700	0.022	0.020	0.764	0.002
32	5	Lambda cyhalothrin	Adipic acid	0.700	0.022	0.764	0.020	0.002
33	5	Cypermethrin	Triphenyl phosphate	1.274	0.012	1.156	0.025	0.003
34	5	Cypermethrin	Triphenyl phosphate	2.197	0.033	2.481	0.029	0.010
35	5	Diazinon	Dibutyl phthalate	4.200	1.000	5.790	0.840	0.006
37	5	Propiconazole	Dibutyl phthalate	3.900	0.246	2.200	0.170	0.045
38	5	Lindane	Dipropyl phthalate	5.410	0.140	6.052		0.009
39	5	Lindane	Dipropyl phthalate	3.415	1.750	3.532		0.006

Ref	No of inj.	AS	IS	AS		IS		Sign. AS/IS
				Sign. CV	t_R CV	Sig. CV	t_R CV	CV
40	5	Parathion me	Bis-(2 methoxyethyl) phthalate	3.415		3.532		0.006
41	5	Cypermethrin	Dioctyl phthalate	1.200		1.000		0.010
42	5	Cypermethrin	Dioctyl phthalate	6.400		6.500		0.045
42	5	Trifluralin	Diisopentyl phthalate	1.000	0.200	1.000	0.100	0.006
43	6	Fenthion	Diisopentyl phthalate	0.200	0.900	0.200	0.700	0.004
44	6	Fenthion	Diisopentyl phthalate	1.000	0.100	1.000	0.100	0.001
44	5	Trifluralin	Diisopentyl phthalate	0.900	0.100	0.700	0.200	0.002
48	5	Chlorpyrifos	Dibromonaphthalene	1.600		1.600		0.001
50	5	Deltamethrin	External	1.005	0.883			
51	5	Chlorpyrifos	Dibromonaphthalene	1.900		1.840		0.002

(a) The actual chromatographic conditions applied are listed in Table 10 under the corresponding reference.

TABLE 9. SUMMARY OF REPEATABILTY OF ON-COLUMN INJECTION

Ref	Compound, inj. amount [g]	No.	Area CV %	Height CV %	t_R CV %
27	Lindane, 10 E-12	5	1.808	1.987	0.069
	Aldrin, 10 E-12	5	2.621	3.275	0.121
	Dieldrin, 10E-12	5	2.557	2.149	0.065
	Lindane, 25 E-12	5	1.243	2.506	0.010
	Aldrin, 25 E-12	5	4.836	3.687	0.040
	Dieldrin, 25E-12	5	3.983	3.508	0.010
	Lindane, 50 E-12	5	2.216	2.164	0.004
	Aldrin, 50 E-12	5	2.065	4.080	0.018
	Dieldrin, 50E-12	5	2.528	3.225	0.004

TABLE 10. CHROMATOGRAPHIC CONDITIONS FOR THE ANALYSIS OF VARIOUS PESTICIDES

Ref.	**Pesticide**	**Chromatographic conditions**
1	Standard mixture	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min
2	Standard mixture	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C /3min 15°C /min, 240°C/4min, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
3	Fendona 2 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 220°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F: N_2, make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
4	BI 58 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
5	Rubigan 12 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C , I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
6	Topas 100 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min,

Ref.	Pesticide	Chromatographic conditions
		F H_2 = 30 ml/min, F air = 250 ml/min
7	Sherpa EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 220°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
8	Dividend 030 FS	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 220°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
9	Bayleton 25 WP	CP Sil 5CB, 10 m × 0.25 mm × 0.12 µm; C = 200°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up= 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
10	Rovral 50 WP	CP Sil 5CB, 10 m × 0.25 mm, 0.12µm; C = 200°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2= 30 ml/min, F air = 250 ml/min
11	Danitol 10 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12µm; C = 200°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
12	Buvicid K	CP Sil 5CB, 10 m × 0,25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
13	Neoron 500 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
14	Bravo 500	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 160°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up= 31 ml/min, F H_2= 30 ml/min, F air = 250 ml/min
15	Zolone 35 EC	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
16	Stomp 330	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min
17	Basudin 5 G	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 160°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min
18	Orthocid 50WP	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
19	Buvicid F	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C, I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
20	Systhane 12 E	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C , I = 230°C; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
21	Folicur top	CP Sil 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C C, I = 230°C ; head pressure 50 kPa, split flow 100 ml/min, F N_2 make up = 31 ml/min, F H_2 = 30 ml/min, F air = 250 ml/min
22	Zolone 30 WP	CP-SIL 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C, I = 230°C; split flow 100 ml/min 50 kPa
23	Danitol 10 EC	CP-SIL 5CB, 10 m × 0.25 mm, 0.12 µm; C = 200°C, D = 250°C, I = 230°C; split flow 100 ml/min 50 kPa,
24	Tilt 250	CP-SIL 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; split flow 100 ml/min 50 kPa,

Ref.	Pesticide	Chromatographic conditions
25	Stomp 330	CP-SIL 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C; split flow 100 ml/min 50 kPa
26	Buvicid K	CP-SIL 5CB, 10 m × 0.25 mm, 0.12 µm; C = 180°C, D = 250°C, I = 230°C split flow 100 ml/min 50 kPa,
	Standard mixture	CP-Sil 5CB, 25 m × 0.32 mm, 0.25 µm, He 2 ml/min; 70°C, 1 min, 20°C/min, 160°C, 4°C, 270°C, ECD
27	Machete	DB-5, 0.53 × 25 m, 1 µm; C = 210°C, D = 240°C, I = 230°C; He flow rate 5.0 ml/min
28	Parashoot CS	K47, CP Sil 8 CB, 25 m × 0.53 mm × 1 µm; C = 80°C, 1 min 35°C/min to 290°C, D = 250°C, I = 270°C; Detector temperature: 270°C, Injector temperature 220°C
29	Butachlor	5% phenyl methyl siloxane, 0.53 mm × 15 m, 1.5 µm, 180, 10, 280
30	Butachlor	DB-5 column, 15 m × 0.53 mm, 1.5 µm; DB-1701- 15 m × 0.53 mm, 1.0 µm; C = Oven temp 80°C, 1 minute, 35°C/min to 235°C, 8 minutes, D = 270°C, I = 260°C
31	Cypermethrin	DB-5 column , 15 m × 0.53 mm, 1.5 µm; C = Oven temp: 150°C, 1 minute, 35°C/min to 270°C, 8 minutes, D = 300°C, I = 290°C
32	Lambda-cyhalothrin	DB-5 column, 30 m × 0.32 mm, 0.25 µm; C = Oven temp 150°C, 1 minute, 35°C/min to 270°C, 8 minutes, D = 300°C, I = 290°C, Carrier gas helium
33	Cypermethrin	DB-5 column, 30 m × 0.32 mm, 0.25 µm; C = 240°C, 22 minutes, D = 280°C, I = 270°C, split ratio 100:1, carrier gas helium, flow rate 1.5 ml/minute
34	Cypermethrin	CP Sil 19 CB, 30 m × 0.32 mm, 0.25 µm; C = 260°C, 22 minutes, D = 280°C, I = 270°C, split ratio- 100:1, carrier gas helium
35	Diazinon	CP SIL 8 CB; C = 214°C, D = 260°C, I = 250°C, nitrogen
36	Diazinon	CP SIL 19 CB; C = 214°C, D = 260°C, I = 250°C
37	Propiconazole	CP SIL 19 CB; C = 290°C, D = 300°C, I = 290°C
38	Lindane	CP SIL 8 CB; C = 200°C, D = 250°C, I = 220°C
39	Lindane	CP-SIL 19 CB; C = 200°C, D = 250°C, I = 200°C
40	Parathion methyl	CP SIL 8 CB; C = 220°C, D = 300°C, I = 220°C
41	Cypermethrin	CP SIL 8 CB; C = 275°C, D = 300°C, I = 275°C
42	Cypermethrin	CP-SIL 19 CB; C = 250°C, D = 275°C, I = 250°C
43	Trifluralin Fenthion	CP-SIL 8CB, 25 m × 0.53 mm, 1.0 µm; C = 80°C, 35°C/min to 230°C , 1 min; isothermal at 80°C, 35°C/min to 230°C, 12 min, D = 300°C, I = 250°C
44	Deltamethrin	DB-1701, 15 m × 0.53 mm, 1.0 µm; C = 80°C, 35°C/min to 230°C , 1 min; isothermal at 80°C, 35°C/min to 230°C, 10 min, D = 300°C, I = 250°C
45	Standard mix	CP Sil 8 CB, 25 m × 0.53 mm, 1 µm; C = 150°C, 25°C, 90°C, CP-SIL 19CB, 25 m × 0.53 mm, 1 µm; D = 270°C, I = 220°C
46	Carbaryl	Purospher Star Merck endcapped (5µm); acetonitrile:water adjusted to pH 2.6 with H_3PO_4 (40:60); 1 ml/min

Ref.	Pesticide	Chromatographic conditions
47	Deltamethrin	Purospher Star Merck RP-18 endcapped (5 µm); acetonitrile:water (80:20) adjusted to pH 2.6 with H_3PO_4; Chromolite Performance RP-18e, 100 × 4.6 mm; acetonitrile:water (70:30) adjusted to pH 2.6 with H_3PO_4
48	Chlorpyrifos	Purospher Star Merck RP-18 endcapped (5 µm); Chromolite Performance RP-18e, 100 × 4.6 mm; acetonitrile:water (70:30)
49	Carbaryl	Chromolith Performance RP 18e, 100 × 4,6 mm; acetonitrile:water (30:70) adjusted to pH 2.6 with H_3PO_4
50	Deltamethrin	Chromolite Performance RP-18e, 100 × 4.6 mm; acetonitrile:water (70:30) adjusted to pH 2.6 with H_3PO_4
51	Standard mixture	CP SIL 8 CB; C = 250°C, D = 300°C, I = 250°C

C: Column; D: detector; I: injector

TABLE 11. CHARACTERISTICS OF MULTI-POINT CALIBRATIONS

Ref	Comp	IS	r	Srr	Level & injection
28	Malathion	Benzyl benzoate	0.995	0.0312	3 × 2
28	Parathion	Docosan	0.9997	0.0115	3 × 2
29	Isoprothiolane	Di-n-butyl phthalate	1		3 × 2
29	Iprobenfos	Di-n-butyl phthalate	0.9993		3 × 2
30	Butachlor	Di-n-butyl phthalate	0.9998	0.004	3 × 2
32	Lambda cyhalothrin	Adipic acid	0.9966	0.01292	3 × 2
33	Cypermethrin	Triphenyl phosphate	0.998	0.0437	IS far from AS
35	Diazinon[a]	Dibutyl phthalate	0.9995	0.024	3 × 1
35	Diazinon	Dibutyl phthalate	0.9988	0.039	3 × 1
35	Diazinon	Dibutyl phthalate	0.999	0.039	3 × 1
35	Diazinon	Dibutyl phthalate	0.9997	0.017	3 × 1
36	Diazinon	Dibutyl phthalate	0.9992	0.045	3 × 1
36	Diazinon	Dibutyl phthalate	0.9992	0.032	3 × 1
36	Diazinon	Dibutyl phthalate	0.9994	0.031	3 × 1
43	Trifluralin	Diisopentyl phthalate	0.998	0.007	3 × 2
44	Trifluralin	Diisopentyl phthalate	0.9994	0.004	3 × 2
43	Fenthion	Diisopentyl phthalate	0.9999	0.001	3 × 2
44	Fenthion	Diisopentyl phthalate	1	0.001	3 × 2
45	Parathion	Benzyl benzoate	0.999	0.0115	3 × 2

(a) Calibration was performed on different days

4. ANALYSIS OF PESTICIDE FORMULATIONS

The multi-pesticide procedures were applied for a number of various formulations used in the countries of the participants. The applicability of the procedures was confirmed based on the protocol described in chapter 4.2, and demonstrated in section 2 of this chapter.

Examples for the repeatability of the analysis of test portions of pesticide products carried out with the elaborated procedures are given in Table 12.

Table 13 indicates those pesticide products for which a multi/pesticide method was successfully validated. The formulated products were extracted according to relevant standard methods, and the chromatographic conditions applied are listed in Table 10.

There are a number of formulations which were amenable to multi-pesticide methods, but the full validation could not be performed within the time frame of the project.

TABLE 12. REPEATABILITY OF ANALYSIS OF REPLICATE TEST PORTIONS OF PESTICIDES

Ref.	No. of sample	No. of injection	Pesticide[a]	AS	AS Conc. CV %
27	5	4	Machete	Butachlor	0.20
28	5	5	Parashoot CS	Parathion-methyl	2.04
28	5	5	Fayfon	Malathion	2.00
29	10	5		Iprobenfos	0.76
29	10	5		Iprobenfos	0.59
30	5	4		Butachlor	0.15
30	5	4		Butachlor	0.35
32	5	4	Karate 2.5 EC	lambda cyhalothrin	0.40
33	5	4	Cymbush 5 EC	Cypermethrin	0.69
34	5	4	Cymbush 5 EC	Cypermethrin	1.05
38	5	2	Lindane 0.65%	Lindane	0.80
38	5	2	Lindane 6.5%	Lindane	0.53
39	5	2	Lindane 0.65%	Lindane	0.73
39	5	2	Lindane 6.5%	Lindane	0.26
35			Diazol 40	Diazinon	0.73
40	5	2	Parathion me 50%	Parathion-methyl	1.30
	5	2	Parathion me 50%	Parathion-methyl	0.65
41	5	2		Cypermethrin	4.00
42	5	2		Cypermethrin	1.10
48	5	2		Chlorpyrifos	2.90
51	5	2		Chlorpyrifos	2.40
43	5	2	Trifluralin 48EC	Trifluralin	0.32
44	5	2	Trifluralin 48EC	Trifluralin	0.23
43	5	2	Fenthion 50 EC	Fenthion	0.09
44	5	2	Fenthion 50 EC	Fenthion	0.09
45	5	2	Dantox	Parathion-methyl	0.85
45	5	2	Parashoot	Parathion-methyl	0.38
45	5	2	Fyfanon	Malathion	1.02
46	5	4		Carbaryl	0.70
49	5	4		Carbaryl	0.53
33	5	4	Decis 2.5 EC	Deltamethrin	1.19
50	5	4	Decis 2.5 EC	Deltamethrin	0.46

Note: (a) Where not indicated the commercial name of the formulation was not reported.

TABLE 13. PESTICIDE PRODUCTS WITH VALIDATED MULTI-PESTICIDE METHOD

Active substance	**Pesticide product**
Acetamiprid	Mospilan 20 SP
Acetochlor	Guardian Extra
Acetochlor	Guardian Max SC
Acetochlor	Trophy EC
Alachlor	Lasso 48EC
Alpha cypermethrin	Bestseller 100 EC
Amitraz	Mitac 20 EC
Atrazine	Guardian Extra
Atrazine	Tazastomp SC
Benefin	Benefex EC
Benefin	Flubalex
Beta-cyfluthrin	Enduro 258 EC
Boscalid	Pictor SC
Butachlor	Butachlor 600 EC
Butachlor	Machete 60 EC
Captan	Buvicid K 370 SC
Carbaryl	Sevin 85 wp,
Chlorpyrifos	Cyren EC
Chlorpyrifos	Nurelle D50/500
Chlorpyrifos-methyl	Reldan 2E
Cypermethrin	Force 10 EC
Cypermethrin	Cypermethrin 10% ai
Cypermethrin	Sherpa EC
DDVP	F94_Unifosz_EC
DDVP	Unifosz 50 EC
Deltamethrin	Decis WST
Deltamethrin	Splendour EC
Diazinon	Basudin 5G
Diazinon	Basudin 600 EW
Diazinon	Diazinon 5 G
Diazinon	DIAZOL 40 PM
Dimethoate	Bi-58 EC
Dimethoate	Dimethoate 59/EC
Endosulfan	Thionex 35 EC
Fenitrothion	Buvatox 5 G
Fenitrothion	Galition 5 G
Fenthion	Lebaycid 50EC
Fluquinconazole	Clarinet SC
Hexaconazole	Amistar Ter SC
Hexaconazole	Contaf SC
Isoprothiolane	Isoprotiolan 12 GR
Kresoxim-methyl	Discus DF
Lambda cyhalothrin	Karate 2.5 EC
Lambda-cyhalothrin	Karate 2.5 WG
Malathion	Fyfanon EW
Metolachlor	Igran Combi Gold EC
Miclobutanil	Systhane 12 E
Miclobutanil	Systhane 12 E EC
Parathion-methyl	Parashoot CS
Parathion-methyl	Parathion me 50WP
Parathion-methyl	Danatox 50 EC
Penconazole	Topas 100 EC
Phosalone	Zolone 30 WP
Phosalone	Zolone 35 EC
Propiconazole	Tilt 250
Propisochlor	Proponit 720 EC

Active substance	Pesticide product
Tebuconazole	Folicur Solo EC
Terbufos	Counter 5 G
Triadimefon	Bayleton 25 WP
Trifluralin	Olitref 480 EC
Trifluralin	Treflan 48EC

The performance characteristics of the methods for pesticides printed in italics did not meet the criteria. They have to be further examined, and the sources of uncertainty eliminated.

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MULTI-PESTICIDE METHODS

GC 'MULTI-ANALYTE' DETECTION METHOD

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Abstract

Elaborated methodologies for GC multi-analyte detection are presented, comprising the steps of method development, chromatographic conditions and procedures including the determination of relative retention times and summary results tables.

1. INTRODUCTION

The aim of this investigation was to elaborate a procedure for developing and optimising a simple and rapid chromatographic separation for pesticides having different chemical structure.

Thirty pesticides representing different chemical classes were selected to illustrate the applicability of the method. Altogether 5 internal standards were applied which sufficiently cover the retention range of compounds of interest.

The measurements were made with the classical hot split / splitless injector system and wide bore capillary columns containing an apolar and a medium polar liquid phase.

Once the elution temperature has been selected and optimised, many compounds can be determined at isothermal column temperature making the analysis much faster than with the temperature programme mode.

System suitability test mixture is recommended to monitor the performance of the whole chromatographic system.

The instrumentation of laboratories is different. Some GCs are equipped with flexible injection systems improving the separation of compounds. As the GC system configuration significantly affects the separation and detection conditions, the 'Multi-Analyte' method will have to be optimised for the particular instrument and should not be copied automatically.

2. STEPS OF METHOD DEVELOPMENT

- The GC 'Multi-Analyte' method was elaborated as preliminary method for 30 pesticide active ingredients. These pesticides were measured separately with the linear column temperature program under the chromatographic condition described in Section 3.
- The retention times of these pesticide active ingredients were measured during the chromatographic run and their relative retentions were calculated. *Chlorpyriphos* was chosen as a reference compound.
- The retention time during the linear column temperature program was converted to the column temperature, see Section 4.1. These temperatures were applied for the starting isothermal conditions.
- The retention times, peak width, and peak symmetry were determined applying the selected isotherm column temperature.
- In the final step, the isotherm column temperatures were fine-tuned to get shorter retention and analysis times together with good separation of compounds of interest.

3. CHROMATOGRAPHIC PROCEDURE

3.1 Apparatus and operating conditions

- Chrompack 9000 GC, equipped with all-glass injection system (classical hot split/ splitless injector with 4 mm i.d. inlet liner), and flame ionization detector.
- Column: 25 m, 0.53 mm i.d.
- Stationary phases:
  - CP-SIL 8 CB (5% phenyl, 95% dimethylpolysiloxane, chemically bonded)
  - CP-SIL 19 CB (14% cyanopropylphenyl, 86% dimethylpolysiloxane, chemically bonded
- Film Thickness: 1.0 µm
- Detector temperature: 300 °C
- Injector temperature: 270 °C
- Injector liner: silanised pyrex glass
- Injection volume: 1 µl
- Carrier gas: Helium
- Flow rate: 12 ml/min

Notes:

(a) Other columns e.g. DB-5 (or HP-5) 0.53 mm, 15 m, 1.5 µm, or DB-1701 (or HP-1701) 0.53 mm, 15 m, 1.0 µm can also be used.

(b) The injector and column connection should be inert; preferably it should be an "all glass system" applying silanised quartz or deactivated Pyrex glass insert and silanised glass wool or use on-column injection if possible.

(c) The operating conditions of the GC and isothermal temperature described in the method should only be used as a starting point. The appropriate resolution of adjacent peaks and the peak purity of the active ingredient have to be assured and verified according to the procedures described in Chapters 4.1 and 4.2.

(d) The isothermal temperatures and flow rates described below for each compound may have to be adjusted to provide the shortest possible retention time with good resolution and flow rate according to local conditions.

Temperature program:

In order to get sharp solvent peak with minimal tailing, the standards and samples are injected into cold column (80°C) and multi-rise program is used to reduce the run time.

The solvent elute at 80°C, pesticide compounds elute above 150 °C. Therefore, between 80–150°C there is a fast temperature rise to reduce the run time.

Column temperature	80°C – 300°C
Initial time	1 min at 80°C
Rise I (fast)	35°C/min to 150°C
Rise II (slow)	8°C/min to 300°C
total run time	15 min

The elution temperature is calculated from the retention time of component X eluted, for instance, at 5.43 min:

$$T_X\,(°C) = T_I + (t_{RX} - t_I) \times r_{II} \qquad (1)$$

$$T_X = 150 + (5.43\text{-}3) \times 8 = 169.44°C$$

$$t = \Delta t + t = t = 2+1 = 3 \text{ minutes} \qquad (2)$$

$$\Delta t = \frac{T_I - T_0}{r_I} \text{ (minutes)} \qquad (3)$$

$$\Delta t = \frac{150 - 80}{35} = 2 \text{ minutes}$$

Where

t_0 = initial time	1 min
t_I = first ramp time	2 min (total time: 2 + 1 min)
t_{RX} = retention time of X component	5.43 min
r_I = first ramp rise	35°C/min
r_{II} = second ramp rise	8°C/min
T_0 = initial temperature	80°C
T_I = first ramp temperature	150°C
T_X = elution temperature of component X	

The temperature converted from retention time during the column temperature programmed run may be used as the initial isotherm column temperature for the selected active ingredient.

When the GC cannot operate with the multi-ramp program, use a linear temperature program, e.g.:

Initial oven temperature: 80°C
Final oven temperature: 300°C
Oven rise: 8°C /min

4. DETERMINATION OF RELATIVE RETENTION TIMES

The elution behaviour of the selected pesticides was studied with wide bore capillary columns containing the two basic liquid phases. The results are summarized in Tables 1 and 2.

Where only packed columns are available, all glass system must be used paying special attention to the inertness of the injector and the column. Isotherm conditions usually provide acceptable solutions. Most pesticides elute at or above 180°C.

The 1.5% SP2250 + 1.95% SP2401 packed column can only be operated on a long run at a maximum of 210°C isothermal temperature. Most of pesticides elute above 190°C, limited number of pesticide active ingredients can be analyzed on it. The results are shown in Table 3.

It was found that the retention range can be covered with five internal standards. Their retention times at various elution temperatures are summarized in Tables 4 and 5.

4.1. Control of system suitability

The chromatographic system may be contaminated with non-volatile compounds (e.g. surfactants, adjuvants) derived from the formulated products. These compounds may be deposited on the wall of the injector or at the initial part of the column, and may affect the stability of labile compounds, cause irreversible adsorption or distort the peak shape by other mechanisms. Continuous system contamination, caused by the accumulation of matrix components in the inlet and front section of the capillary column, might lead to curvatures in the calibration line during a long series of analysis.

At the time of the installation of the column the lack of gas tight fitting or remaining dead volumes can seriously affect the efficiency of the separation and performance of the system.

Therefore, the proper operation of the system should be checked after each maintenance and before each analysis sequence. This can be most conveniently done by injecting 1 µl of pesticide test mixture

with temperature the program given in section 3.1, but applying 200°C injector temperature. The composition of the pesticide test mixture and the chromatographic parameters are summarized in Table 6.

TABLE 1. ELUTION PARAMETERS OF PESTICIDE ACTIVE INGREDIENTS ON CP SIL8 CB 25 M × 0.53 MM × 1 μM COLUMN

Compound name	Temperature program		Isothermal condition			
	Elution temp. [°C]	t_R [min]	Column temp [°C]	t_R [min]	Internal standard (IS)	IS t_R [min]
Alachlor	198	9.00	200	6.4	Benzyl benzoate	5.3
Amitraz	254	15.95	260	8.06	Bis (2-ethylhexyl) adipat	6.87
Chlorothalonil	190	8.03	200	5.72	Dibutyl phthalate	6.86
Chlorpyriphos-ethyl	205	9.88	210	6.52	Benzyl benzoate	5.12
Chlorpyriphos-methyl	196	8.8	200	6.28	Benzyl benzoate	5.3
alpha Cypermethrin	270	18.03	280	8.51	Squalane	7.47
Cypermethrin	270	18.02 18.15 18.26	280	8.53	Squalane	7.47
Diazinon	188	7.82	190	5.96	Dibutyl phthalate	8.06
Dimethoate	182	7.05	190	5.4	Dibutyl phthalate	8.04
Dichlorvos	151	3.18	130-160; 5°C/min		Diethyl phthalate	
Fenitrothion	201	9.411	200	6.8	Benzyl benzoate	5.3
Fenthion	205	9.843	200	7.26	Benzyl benzoate	5.3
			220	5.99	Di-n-butyl sebacate	7.24
Fenvalerate	279	19.11	290	8.62 8.77	Squalane	7.32
	280	19.35	280	9.26 9.48	Diphenyl phthalate	7.22
Esfenvalerate	281	19.36	290	8.76	Squalane	7.31
			280	9.48	Diphenyl phthalate	7.22
Isoprothiolane	220	11.73	220	7.35	Dibutyl phthalate	5.8
			230	6.73		
Iprobenfos	192	8.185	200	5.73		
			180	7.36	Diethyl phthalate	4.7
Lambda Cyhalothrin	255	16.12	260	8.08	Diphenyl phthalate	7.8
			250	8.92	Squalane	9.39
			250	8.92	Ethylhexyl adipate	7.2
Malathion	203	9.67	200	7.01	Benzyl benzoate	5.3
Metalaxyl	199	9.1	200	6.49	Benzyl benzoate	5.3
Parathion-ethyl	205	9.88	200	7.34	Benzyl benzoate	5.3
Parathion-methyl	196	8.79	200	6.89	Benzyl benzoate	5.3
Propiconazole	235	13.56; 13.71	260	6.84	Diphenyl phthalate	7.8
			250	7.13	"	8.46
Trifluralin			190	4.95	Dibutyl phthalate	8.04
			170	6.089	Diethyl phthalate	5.14

Note: Isothermal conditions highlighted are preferable.

TABLE 2. ELUTION PROPERTIES OF PESTICIDE ACTIVE INGREDIENTS ON CP-SIL19 CB; 25 m × 0.53 mm × 1 µm

Compound name	Temperature program		Isothermal condition			
	Elution temp. [°C]	t_R [min]	Column temp. [°C]	t_R [min]	Internal standard (IS)	IS t_R [min]
Alachlor	209.8	10.48	200	7.9	Benzyl benzoate	5.85
			210	6.89	Benzyl benzoate	5.5
Amitraz	261.8	16.98	260	8.77	Squalane	7.56
			270	8.021	Squalane	7.03
			280	7.638	Squalane	7.18
Butachlor			220	8.28	Dibutyl phthalate	6.36
			230	7.32	Dibutyl phthalate	6
Chlorothalonil	210.6	10.57	200	8.05	Benzyl benzoate	5.85
			210	7.03	Benzyl benzoate	5.5
Chlorpyrifos-ethyl	213.6	10.95	210	7.35	Benzyl benzoate	5.5
Chlorpyrifos-methyl	206	10.02	210	6.54	Benzyl benzoate	5.5
alfa Cypermethrin	283	19.64	280	9.58	Squalane	7.06
	285	19.91		9.86		
Cypermethrin	283	19.64	280	9.59	Squalane	7.06
	285	19.87		9.81		
	286	20.02		9.99		
Diazinon	195	8.68	200	5.94	Dibutyl phthalate	8.21
Dimethoate	205	9.92	200	7.22	Dibutyl phthalate	8.2
			210	6.38	Dibutyl phthalate	6.97
Dichlorvos	157	3.81	160	3.65	Benzyl benzoate	7.98
Fenitrothion	218	11.47	210	7.95	Benzyl benzoate	5.5
			220	6.97	Dibutyl phthalate	6.325
Fenthion	217	11.32	210	7.75	Benzyl benzoate	5.5
Fenvalerate	292	20.76	280	10.93	Squalane	7.06
	295	21.07		11.32		
Esfenvalerate	295	21.07	280	10.99	Squalane	7.06
Isoprothiolane			220	9.92	Dibutyl phthalate	6.39
			230	8.38	Dibutyl phthalate	6
Iprobenfos			180	9.86	Diethyl phthalate	5.59
			190	7.94	Diethyl phthalate	5.11
Lambda Cyhalothrin	269	17.83	270	8.42 8.67	Squalane	7.21
Malathion			210	7.79	Benzyl benzoate	5.5
			220	6.89	Dibutyl phthalate	6.318
Metalaxyl	217	11.37	210	7.21	Benzyl benzoate	5.5
Parathion-ethyl	221	11.89	210	7.06	Benzyl benzoate	5.5
Parathion-methyl	214	10.99	210	7.43	Benzyl benzoate	5.5
Propiconazole	249	15.31 15.42	250	8.17 8.26	Diphenyl phthalate	10.61
Trifluralin	187	7.64	190	5.66	Diethyl phthalate	5.12
			180	6.364	Benzyl benzoate	7.65

Note: Isothermal conditions highlighted are preferable.

TABLE 3. ELUTION PROPERTIES OF PESTICIDE ACTIVE INGREDIENTS ON 1.5% SP2250 + 1.95% SP2401 90 cm × 3 mm PACKED PYREX GLASS COLUMN

Compound name	Isothermal condition			
	Column temp. [°C]	t_R [min]	Internal standard (IS)	IS t_R [min]
Alachlor	200	9.45	Benzyl benzoate	6.88
Chlorothalonil	200	8.62	Benzyl benzoate	6.83
Chlorpyrifos ethyl	200	9.2	Benzyl benzoate	6.88
Chlorpyrifos methyl	200	8.18	Benzyl benzoate	6.86
Diazinon	200	6.955	Dibutyl phthalate	8.9
Dimethoate	200	7.87	Dibutyl phthalate	8.93
	190	8.7	Dibutyl phthalate	10.3
Dichlorvos	190	4.324	Dibutyl phthalate	10.3
	170	4.379		
	150	4.64	Diethyl phthalate	
Fenitrothion	200	10.04	Benzyl benzoate	6.897
Fenthion	200	9.87	Benzyl benzoate	6.87
			Diethyl phthalate	5.943
Malathion	200	10	Benzyl benzoate	6.87
Metalaxyl	200	8.93	Benzyl benzoate	6.9
Parathion ethyl	200	8.88	Benzyl benzoate	6.86
Parathion methyl	200	9.3	Benzyl benzoate	6.88
Trifluralin	200	6.2	Dibutyl phthalate	8.9

TABLE 4. RETENTION TIMES OF INTERNAL STANDARDS ON CP-SIL 5 CB COLUMN

Column temp. [°C]	Retention time, t_R , [min]						
	Benzyl benzoate	Diethyl phthalate	Dibutyl phthalate	Diphenyl phthalate	Di-isobutyl phthalate	Di-n-butyl sebacate	Squalane
170		5.14					
180		4.7					
190		4.52	8.06		6.65		
200	5.3		6.84				
210	5.12		6.15				
220			5.8			7.24	
230						6.62	
240						6.29	
250				8.46			9.39
260				7.8			
280				7.22			7.45
290							7.32

The system performance is evaluated by calculation of:

- the signal ratio of carbaryl/propoxur, metobromuron/propoxur; dimethoate/chlorpyrifos;
- the resolution for the adjacent peaks: metobromuron/carbaryl.

With the mixture designed for pesticide analysis additional useful information can be obtained about the performance of column.

Some compounds are difficult to analyze, and it is almost impossible to achieve perfect peak shape. The tailing peaks are caused by non-volatile contaminants deposited from the extract. The poor peak shape does not influence the linearity but may seriously affect the precision of quantitation and

therefore the sources of tailing should be eliminated as far as possible. The dimethoate is a typical example of these "difficult" compounds. If its peak shape is within the acceptable limits, practically all compounds will give quantifiable peaks.

TABLE 5. RETENTION TIMES OF INTERNAL STANDARDS ON CP-SIL 19 CB COLUMN

Column temp. [°C]	Retention time, t_R, [min]						
	Benzyl benzoate	Diethyl phthalate	Dibutyl phthalate	Diphenyl phthalate	Di-isobutyl phthalate	Di-n-butyl sebacate	Squalane
170	7.53	6.56					
180		5.65					
190	6.57	5.16	10.26		8.11		
200	5.9		8.28		6.89		
210	5.54		7.13		6.2		
220			6.45		5.83	7.9	
230						7.05	
240						6.58	
250				10.71			8.38
260				9.35			7.62
280				8.02			7.09
290				7.74			

TABLE 6. COMPOSITION AND CHROMATOGRAPHIC DATA OF SST MIXTURE

Pesticide compound	Concentration mg/ml	t_R, [min]	α Eq. (4)	Peak Width [sec]	Signal ratio[2] Eq. (6)	R_S[3] Eq. (7)
α-naphthol		4.90				
Propoxur	0.498	5.83	0.570	3.20		
Dimethoate	0.508	7.02	0.699	3,90	0. 6265	
Metobromuron	0.518	8.34	0.840	3. 60	0. 5219	
Carbaryl	0.52	8.90	0.899	3.80	0.8191	5.133
Chlorpyrifos	0.526	9.9	1	4.0		

Carbaryl and metobromuron can be used to check the inertness of the chromatographic system. Carbaryl is very sensitive to surface effects, decomposing to alpha-naphthol and some volatile compounds. The decomposition of carbaryl may also occur in the injector port resulting in alpha-naphtol peak on the chromatogram that can be detected with FID. The degradation can be characterised by the signal ratios of labile carbaryl and stable propoxur or metobromuron/ propoxur. Therefore, the signal ratio is a good indicator of the inertness of the column.

Equations used for calculation of system performance:

Relative retention, α:

$$\alpha = \frac{k_{R'}}{k_{rel}} = \frac{t'_x}{t'_{ref}} \quad (4)$$

Capacity factor, $k_{R'}$:

$$k_{R'} = \frac{t_{R1} - t_0}{t_0} \quad (5)$$

Where:

t_R = solute retention time

$t_R' = t_R - t_0$

t_0 = retention time of a non-retained peak (methane)

Signal ratio:

Signal ratio = (Signal / mass of carbaryl) / (Signal / mass of propoxur) (6)

Resolution is calculated for the adjacent peaks: metobromuron/carbaryl and carbaryl/chlorpyrifos

$$R_S = \frac{1.18\,(t_{R2} - t_{R1})}{W_{h1} + W_{h2}} \quad (7)$$

Further details on the application of the SST for controlling the chromatographic conditions are given in Reference 1.

REFERENCE

[1] SOBOLEVA, E., AMBRUS, A., Application of a system suitability test for quality assurance and performance optimization of a gas chromatographic system for pesticide residue analysis. J. Chromatography A. **1027** (2004) 55-65.

QUALITY CONTROL OF SELECTED PESTICIDES WITH GC[1]

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Abstract

The practical quality control of selected pesticides with GC is treated. Detailed descriptions are given on materials and methods used, including sample preparation and GC operating conditions. The systematic validation of multi methods is described, comprising performance characteristics in routine analysis, like selectivity, specificity etc. This is illustrated by chromatograms, calibration curves and tables derived from real laboratory data.

1. INTRODUCTION

The aim of the present work was the development and validation of a 'Multi-Pesticide' (MP) gas chromatographic method (GC-FID) for the quantitative determination of four active ingredients in their commercially available EC formulations widely used in Greece: Lasso 48EC (alachlor), Reldan 2E (chlorpyrifos-methyl), Lebaycid 50EC (fenthion), and Treflan 48EC (trifluralin).

Validation refers to an 'analytical system' rather than an 'analytical method'. The term "analytical system" includes a defined method protocol, a defined concentration range for the analyte and a specified type of test material [1]. Method validation may be described as a set of tests used to establish and document the performance characteristics of a method and thereby demonstrate that the method is fit for a particular analytical purpose [1, 2]. In the case of pesticide formulation analysis, the required performance characteristics have been defined in general by AOAC International [3], and specifically by CIPAC [4]. There are a number of other requirements prescribed by international legislation, such as the Directive 91/414/EEC [5] in the case of the European Union (EU).

According to CIPAC [4] and EU [5] guidelines, validation studies for quantitative analytical methods for the determination of the active ingredient in pesticide formulations are required to be "robust, accurate and precise", and must address the following issues: specificity, repeatability, selectivity and linearity of response to the analyte in the method [6]. The linearity should be demonstrated at least over the range of a nominal concentration of ±20% [6, 7]. At least three concentrations should be measured with duplicate measurements for each concentration [8, 9].

The method was applied for regular analysis of the commercially available EC formulations of pesticides widely used in Greece, within the annual testing program of the laboratory, to establish the long term within-laboratory repeatability and reproducibility based on the results of duplicate measurements. For each active ingredient ten different samples were analyzed based on two separate analytical portions per sample.

The summary of the results of method development and validation of the MP method is presented in this report.

[1] This report incorporates the results published by:
Karasali, H., George Balayannis, B., Hourdakis A., Ambrus A.: Novel "Multi-Pesticide" Method for Commercial EC (Emulsifiable Concentrate) Formulations: Development and "Single laboratory". J. Chromatography A, **1129** (2006) 300-303.

MATERIALS AND METHODS

2.1. Materials

2.1.1. Analytical standards (reference materials, RM)

The analytical standards were donated by the manufacturers: trifluralin (99.4%) and chlorpyrifos-methyl (99.8%), Dow Agrosciences Ltd; fenthion (96.2%), Bayer Crop Sciences; and alachlor (99.8%), Monsanto.

Stock solutions for each active ingredient (AS) were prepared by adding the appropriate amount of the respective analytical standard in a 100 ml volumetric flask, diluting to the volume with the internal standard solution and kept refrigerated at < 5°C. The final concentration of the AS of these solutions was about 0.8 mg ml^{-1}. Three working solutions for each AS [low calibrated level (LCL), medium calibrated level (MCL), and high calibrated level (HCL)] were prepared by three independent dilutions of the stock solution with internal standard solution at concentration of about 0.8, 1.0 and 1.2 times the nominal concentration of AS in the formulated product (Table 1). Working solutions were made with independent dilutions in order to avoid the propagation of errors and kept refrigerated at < 5°C. The freshly prepared working solutions were used for establishing the chromatographic system's precision through repeatability testing and for defining the linearity of response for each individual component.

TABLE 1. COMPOSITION OF STOCK AND WORKING SOLUTIONS FOR TESTING PESTICIDES

Active Ingredient	Stock Solution (mg ml^{-1})	Working Solution (mg ml^{-1})
Trifluralin	0.810	0.197 0.341 0.465
Chlorpyrifos-methyl	0.808	0.247 0.330 0.411
Fenthion	0.822	0.206 0.349 0.411
Alachlor	0.802	0.201 0.322 0.403

Dipentyl phthalate (99% pure, purchased from Noechema) was used as internal standard (IS). An internal standard solution of 0.166785 mg/ml was prepared. This solution was used throughout the whole procedure of all standard (stock and working) and sample preparation.

Each of the analytical standards as well as the internal standard was supplied with a Certificate of Analysis (CoA) stating their concentration, determined by the manufacturer.

2.1.2. Pesticide products

For each active ingredient five different batches of commercially available EC (Emulsifiable Concentrate) formulations together with their blank formulations were given by the suppliers. These batches were accompanied with their CoA and were: Treflan 480EC (trifluralin 48% w/v), Reldan 2E (chlorpyrifos-methyl 22% w/v), Lebaycid 500EC (fenthion 50% w/v) and Lasso EC (alachlor 48% w/v).

2.2. Sample Preparation

For the preparation of the sample solution, the appropriate quantity of commercial EC formulation, containing approximately 80 mg (±5%) of active ingredient, was added in a 100 ml volumetric flask, followed by dilution to the volume with internal standard solution (concentrated sample extracts).

Sufficient quantity of this solution was diluted with internal standard solution, so that the final concentration fell within the concentration range of the respective reference material (RM) working solution (diluted sample extracts).

For each active ingredient, a concentrated blank solution was prepared, by diluting with acetone to 50 ml to obtain double concentration of that of the commercial formulation used for the preparation of the respective commercial sample solution. The concentrated blank solutions were used for the evaluation of the specificity of the method.

The FID detector of the chromatographic system used is sensitive enough to operate reliably with concentrations much lower than the ones applied by the CIPAC sample preparation procedures. Furthermore, the low concentrations do not overload the capillary column. Consequently, the CIPAC sample preparation procedures were not followed concerning the analyte concentration, and the actual AS concentration was much lower than used by the CIPAC methods. As the CIPAC Handbooks describe sample preparation for gas chromatographic analysis only for Alachlor [10] and trifluralin [11] and not for chlorpyrifos-methyl or fenthion, a single sample preparation procedure, based on extraction of AS with acetone was validated for all four formulations.

2.3. Gas chromatographic system and analysis conditions

2.3.1. Chromatographic conditions

Gas chromatograph: Thermo Finnigan Trace GC, consisting of a Programmable Temperature Vaporizing (PTV) split injector, a Flame Ionization Detector (FID) and an autosampler (Thermo Finnigan AS 2000).

Columns:
(A) CP-Sil 8CB, 25 m × 0.53 mm internal diameter, 1 µm film thickness (apolar) applied with split flow of 95 ml min^{-1} and split ratio of 13, injection volume 0.5 µl.

(B) DB 1701, 15 m × 0.53 mm internal diameter, 1µm film thickness (medium polar) applied with split flow of 45 ml min^{-1} and split ratio of 13, injection volume 0.5 µl.

Temperature for column A:

Column:	80°C for 1 min, heated to 200°C at 35°C min^{-1}, kept for 19 minutes
Detector:	250°C

Temperature for column B:

Column:	80°C for 1 min, heated to 220°C at 35°C min^{-1}, kept for 10 minutes
Detector:	250°C

Temperature program for PTV split injector for both columns:

Injection phase:	60°C for 0.05 minutes
Evaporation phase:	from 60°C to 150°C at a heating rate of 14.5°C sec^{-1}, kept at 150°C for 0.5 minutes
Transfer phase:	from 150°C to 250°C at a heating rate of 14.5°C/sec, kept at 250°C for 0.5 minutes
Cleaning phase:	1 minute

2.3.2. System suitability tests

Before method development and performing regular analysis, the suitability of the chromatographic system should be verified. That can be most efficiently performed with the system suitability test. If

the test indicates appropriate column performance and stability of instruments, it means that the results will be reliable and good [12].

The following compounds were used for the evaluation of column performance:

- CP-Sil 8CB: 2-chlorophenol, undecane, 2,4 dimethylaniline, 1-undecanol, tetradecane, acenaphthylene and pentadecane;
- DB-1701: undecane, 2,4 dimethylphenol, 2,6 dimethylaniline, tetradecane, 1-undecanole, 1-methylnaphthalene and hexadecane.

The test mixture contained 250 μg/ml of each component in hexane. The chromatographic conditions of the system suitability tests as well as the mixture of the test compounds were provided by the manufacturers of the columns. The test was performed with CP-Sil 8CB column at 140°C isotherm temperature. The other parameters were the same as given in 2.3.1.

The repeatability of injections was tested with 5 replicate injections of the test mixture. The results are given in Table 2.

TABLE 2. STATISTICAL EVALUATION OF FIVE REPLICATE INJECTIONS OF TEST MIXTURE IN COLUMN CP-SIL 8CB

	2-chph	Udec	2,4 dim	1-udol	Tdec	Anph	Pdec
Retention time							
Average	2.98	3.74	4.92	10.38	11.77	15.59	18.57
CV %	0.0466	0.037	0.037	0.057	0.066	0.072	0.075
Peak area							
CV %	0.558	0.746	0.996	1.032	0.727	0.923	0.390
Peak width at 50%							
Average	0.0300	0.037	0.0503	0.1170	0.1277	0.1650	0.2007
CV %	0.000	0.000	1.5103	0.6115	0.6865	0.0000	0.4640
Tailing factor							
Average	1.0111	1.027	1.0232	1.1551	1.0064	1.0051	1.0024
CV %	2.4592	1.853	1.4616	0.8132	0.9992	1.5124	0.8404
Asymmetry							
Average	1.0625	1.042	1.0450	1.2313	1.0177	1.0071	1.0018
CV %	0.000	5.534	1.7076	2.1522	2.5725	3.2212	1.1978

2-chph: 2-chlorophenol; Udec: undecan; 2-4dim: 2,4 dimethylaniline; 1-udol: 1-undecanol; Tdec: tetradecane; Acnph: acenaphthylene; Pdec: pentadecane

The performance of the column was evaluated by injection of methane for determination of t_0 (97.2 sec) and calculation of retention factor (k). The number of effective theoretical plates per meter (N_{eff}/m), resolution (R_s), peak asymmetry (A_s); and tailing factor (T) and Trennzahl separation number (Tz) were calculated from the chromatograms. The results (Table 3) obtained were compared with those of the test reports of the ISO certified companies.

It was concluded that the performance of the column was satisfactory as the values for the measured parameters were in accordance with the values given by the manufacturers in the column specifications.

TABLE. 3. RESULTS OF GC SYSTEM SUITABILITY TEST

	2-chph	Udec	2,4 dim	1-udol	Tdec	Anph	Pdec
t_R	178.58		295.32	623.63	706.22	935.53	1114.22
t_R' (sec)	81.4	127.7	198.1	526.4	609	838.3	1017
k (sec)	0.8	1.3	2	5.4	6.3	8.6	10.5
N_{eff}/m	453	746	956	1247	1401	1590	1582
W_h (sec)	1.8	2.2	3.02	7.02	7.66	9.9	12.04
T	1.01	1.03	1.02	1.16	1.01	1.01	1
Rs	117.1	13.7	15.9	38.6	6.6	15.4	9.6
As	1.0625	1.0421	1.0450	1.2313	1.0177	1.0071	1.0018

2.3.3. "Initial Optimum" Chromatographic Conditions

The most significant factors for optimization are carrier gas flow rate, column temperature, phase ratio (diameter and film thickness for capillary columns), and stationary phase polarity. The choice of the stationary phase, column diameter, and film thickness is usually limited, and should be based on prior experience and published results. Once the column is selected the carrier gas flow rate and column temperature can be optimized. Optimization aims at providing the desired resolution within minimum elution time. The procedure followed involved the analysis of the mixture of the analytical standards of the selected pesticides and the internal standard, applying a temperature programme. The initial optimum temperature, T_i, was determined from the elution temperature during the temperature program. The final optimization was made taking into account the possible interfering peaks from the technical or formulated products. The pesticides of interest were analyzed under isothermal conditions at around the selected temperature. The results were compared, and the temperature with the best performance characteristics (retention time, resolution and peak asymmetry) was applied for further analysis. Where the temperature did not affect the stability of analytes, the highest temperature providing good resolution was selected in order to reduce the analysis time. The parameters obtained for the compounds of interest at different temperatures are summarized in Table 4.

TABLE 4. EFFECT OF TEMPERATURE ON THE RETENTION TIME (T_R), ASYMMETRY FACTOR (A_S) AND RESOLUTION (R_S), OF THE TESTED PESTICIDES ON COLUMN CP-SIL 8CB

Temperature	200°C			210°C			220°C		
	t_R (min)	A_s (10%)	R_s	t_R (min)	A_s (10%)	R_s	t_R (min)	A_s (10%)	R_s
Trifluralin	6.39	1.00	-	6.05	1.06	-	5.86	1.08	-
Lindane	7.65	1.04	17.46	7.05	1.00	16.94	6.68	1.06	17.08
Chlorpyrifos-methyl	9.45	0.98	18.00	8.35	1.00	16.13	7.64	0.92	15.21
Alachlor	9.72	1.00	2.13	8.53	0.94	1.88	7.77	0.96	1.68
Fenthion	11.38	0.96	11.33	9.70	1.00	10.76	8.62	1.03	9.99
I.s.[a]	16.61	0.96	22.59	13.20	1.00	21.74	11.02	0.98	20.01

Based on the optimization process the chromatographic conditions given in section 2.3.1 were selected as optimal for initial tests.

2. VALIDATION OF THE MP METHOD

3.1. Performance characteristics of GC determination

Repeatability of injections was tested with five replicate injections for each active ingredient separately, using the medium calibrated level working standard solution, following the selected chromatographic conditions. The mean value and the relative standard deviation (% RSD) of the peak

area of the working standards, the peak area of the internal standard, the peak area ratios, as well as the retention times, for all active ingredients are presented in Table 5.

TABLE 5. REPEATABILITY OF INJECTIONS OF ANALYTICAL STANDARDS AND DIPHENYL PHTHALATE AS INTERNAL STANDARD

		RM area	IS area	RM/IS area	RM t_R	ISt_R
Alachlor	Average	972721	616365	1.57814	9.490	16.139
	CV %	1.4	1.2	0.5	0.02	0.03
Chlorpyrifos methyl	Average	372189	618365	0.60190	9.271	16.219
	CV %	2.0	2.1	0.2	0.12	0.08
Fenthion	Average	712777	583175	1.22221	11.128	16.179
	CV %	1.8	1.6	0.3	0.04	0.04
Trifluralin	Average	649265	582811	1.11402	6.287	16.103
	CV %	1.0	1.0	0.3	0.0000	0.02

The repeatability of the individual injections was ≤ 2% in all cases, which is in accordance with the precision specification of the auto-sampler. The CV of the RM/IS area ratio was 3 to 5 times smaller than that of the corresponding individual injections, indicating the advantage of using the internal standard method.

The linearity of the response was determined by analysing in duplicate three working solutions of different concentrations for each of the tested active ingredients.

After having performed the 'multi-point' (3 × 2) calibration, correlation coefficient (r), standard deviation of relative residuals (S_{rr}), slope and intercept, with confidence limits at 95% level were calculated (Table 6). Figure 1 illustrates one of the calibration charts.

TABLE 6. PARAMETERS OF LINEAR REGRESSION OF CALIBRATION

Compound	b	a	$CL_{0.95}$ of a	r	S_{rr}
Alachlor	0.8553	0.031	-0.0055 – 0.057	0.9999	0.0055
Chlorpyrifos methyl	0.3054	0.0087	-0.006 – 0.024	0.9998	0.0058
Fenthion	0.6519	-0.029	-0.043 – 0.015	0.9999	0.0024
Trifluralin	0.5753	-0.0099	-0.039 – 0.0198	0.9998	0.0063

3.2. Testing the specificity of the method

The ability of the analytical method to distinguish the analyte to be determined from degradation products, metabolites or known additives was investigated. Blank formulations were available for checking the potential interfering peaks in the vicinity of the active ingredient. Concentrated extracts were prepared which contained double amount of pesticide product or blank formulation compared to the samples prepared for determination of the AS content. The concentrated sample extracts were prepared without IS to verify that there was no interference with the IS either.

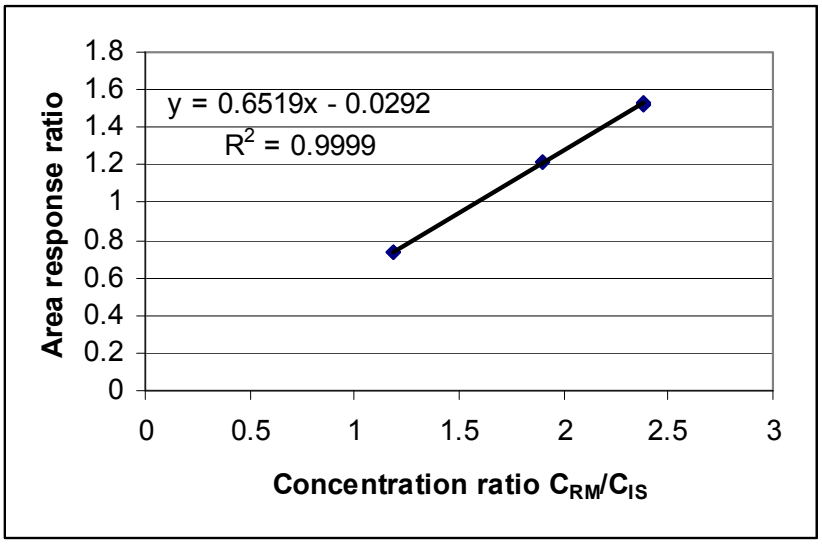

FIG. 1. Calibration chart of fenthion.

The lack of interference was demonstrated by the analysis of the concentrated blank formulations and concentrated sample extracts (Figures 2–4). The use of a second column of different polarity also confirmed interference-free separation.

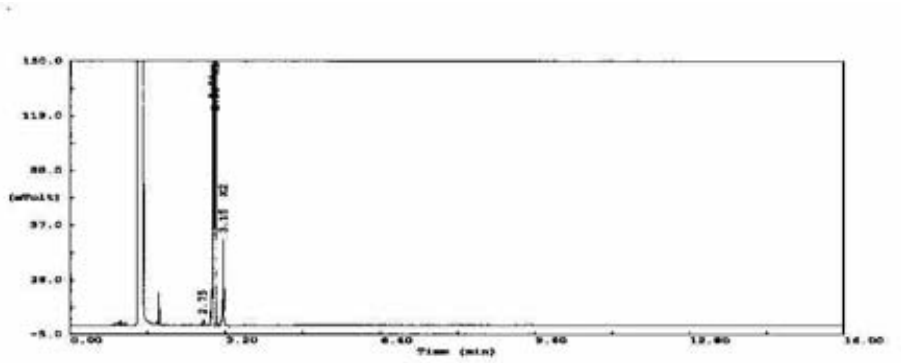

FIG. 2. Chromatogram of concentrated blank formulation of fenthion on CP-SIL 8CB column.

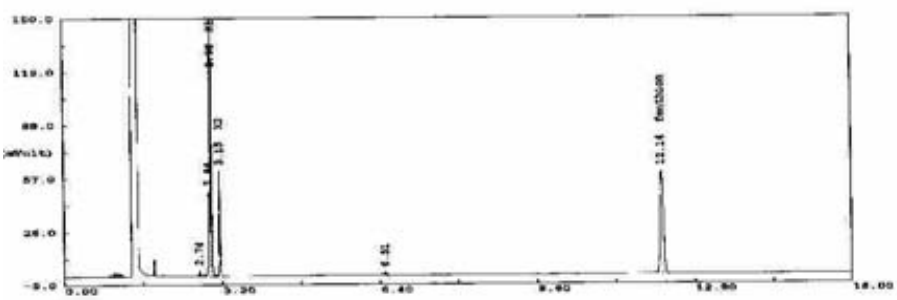

FIG. 3. Chromatogram of concentrated sample extract of fenthion on CP-SIL 8CB column.

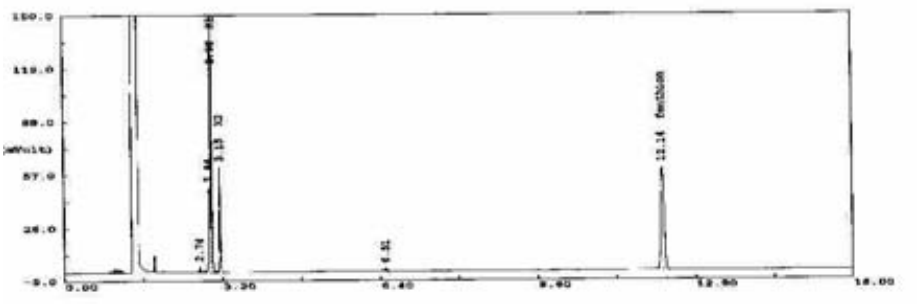

FIG. 4. Chromatogram of fenthion sample extract on CP-SIL 8CB column.

3.3. Batch Analysis

Batches were analyzed in duplicate according to the draft MP procedure. Results are presented in Table 7.

The differences between the measured concentrations and the reference values given by the manufacturer were not significantly different (t_{crit} = 2.776). The reproducibility of the MP procedure was good for chlorpyrifos-methyl, fention and trifluralin ($F_{calc} < F_{crit}$). In the case of alachlor the within-laboratory reproducibility was about 10 times higher than the corresponding value based on the analyses performed by the manufacturing company.

As the results obtained with the MP procedure and with the validated method of the company did not show any statistically significant differences, and its repeatability and reproducibility was generally good, it was concluded that the MP method was validated. The decision was made based on the acceptable results of the analysis of additional Lasso 48 EC formulations as part of the long term repeatability and reproducibility tests (see section 3.3).

TABLE. 7. RESULTS OF REPLICATE ANALYSIS OF AS IN FIVE DIFFERENT BATCHES

	CP-SIL 8CB				Statistical evaluation	
Pesticide	1st	2nd	Average	Ref.conc.		
Alachlor	473.06	474.03	473.55	481	Bias [%]	-0.972
	493.74	494.38	494.06	482	t_{calc}	-0.250
	472.45	471.02	471.84	482	CV_{Rlab}	0.018
	480.88	484.47	482.67	483	CV_{Rref}	0.0017
	483.38	480.65	482.02	481	F_{calc}	**112**
	Grand average		**480.8**	**481.8**	$F_{(0.05,4,4)}$	6.38
Chlorpyrifos methyl	230.55	232.38	231.46	228	Bias [%]	-0.855
	232.80	232.82	232.81	231	t_{calc}	-0.460
	218.65	217.65	218.15	230	CV_{Rlab}	0.0425
	210.67	211.57	211.12	223	CV_{Rref}	0.0194
	229.43	230.11	229.77	221	F_{calc}	4.72
	Grand average		**224.6**	**226.6**	$F_{(0.05,4,4)}$	6.38
Fenthion	514.86	511.59	513.23	510.0	Bias [%]	1.175
	520.92	519.71	520.32	511.0	t_{calc}	1.925
	517.49	515.45	516.47	502.0	CV_{Rlab}	0.0136
	509.23	512.27	510.75	504.0	CV_{Rref}	0.0076
	502.86	500.72	501.79	505.8	F_{calc}	3.28
	Grand average		**512.5**	**506.7**	$F_{(0.05,4,4)}$	6.38
Trifluralin	480.54	485.80	483.17	478.00	Bias [%]	0.852
	485.60	487.98	486.79	480.0	t_{calc}	2.60
	487.74	486.08	486.91	480.0	CV_{Rlab}	0.0082
	488.48	487.90	488.19	485.0	CV_{Rref}	0.0054
	479.79	477.05	478.42	480.0	F_{calc}	2.32
	Grand average		**484.7**	**480.6**	$F_{(0.05,4,4)}$	6.38

3.4. Method performance during routine analysis

After the validation of the methods, it was applied for routine analysis of pesticides of interest. For each active ingredient at least ten samples were analyzed on three separate days with two analytical portions of each.

3.4.1. Reproducibility of instrument operation conditions

The instrument was calibrated on each day of analysis with the same calibration solutions prepared initially. The instrument performance was very stable as it is indicated by the regression parameters for calibrations obtained during two-year period of the method development and routine use (Table 8).

The results indicate the stability of the GC only, but do not provide any information for the uncertainty of the calibration derived from the preparation of standard solutions.

3.4.2. Analysis of samples on different days

The samples were prepared and analyzed according to MP procedures. Results are presented in the following tables (Table 9).

TABLE 8. REGRESSION PARAMETERS OBTAINED DURING A PERIOD OF TWO YEARS

	Time	**Regression equation**	**R**	**S_{rr}**
Alachlor	1st yr	y = 0.8553x + 0.0314	0.9999	0.0055
	2nd yr Day 1	y = 0.851x + 0,0361	0.9999	0.0037
	2nd yr Day 2	y = 0.8486x + 0.035	0.9999	0.0044
	2nd yr Day 3	y = 0.8594x + 0,0199	1.000	0.0031
	2nd yr Day 4	y = 0.8505x + 0.0283	0.9998	0.0059
Chlorpyrifos methyl	1st yr	y = 0.3497x-+ 0.015	0.9998	0.0058
	2nd yr Day 1	y = 0.3223x-+ 0.0093	0.9997	0.0077
	2nd yr Day 2	y = 0.3233x-+ 0.0121	1.000	0.0018
	2nd yr Day 3	y = 0.3237x-+ 0.0118	1.000	0.0028
Fenthion	1st yr	y = 0.3497x-+ 0.015	0.9999	0.0024
	2nd yr Day 1	y = 0.6418x-+ 0.0146	1.000	0.029
	2nd yr Day 2	y = 0.6519x-+ 0.0292	0.9998	0.0067
	2nd yr Day 3	y = 0.6477x-+ 0.0208	0.9998	0.0065
Trifluralin	1st yr	y = 0.5755x-+ 0.0101	0.9998	0.0063
	2nd yr Day 1	y = 0.5788x-+ 0.0139	0.9998	0.0066
	2nd yr Day 2	y = 0.5788x-+ 0.0147	0.9996	0.0096
	2nd yr Day 3	y = 0.5833x-+ 0.0215	0.9996	0.0097
	2nd yr Day 4	y = 0.5846x-+ 0.0184	0.9997	0.0085

TABLE 9. RESULTS OF THE DUPLICATE ANALYSIS OF DIFFERENT COMMERCIAL FORMULATIONS

	AS in replicate test portions			Statistical evaluation
Pesticide	1st	2nd	Average	
	498.01	498.43	498.22	
	476.38	475.73	476.05	
	495.96	493.28	494.62	
	484.44	485.49	484.97	
	492.21	493.74	492.98	
Alachlor	478.09	477.75	477.92	$CV_r = 0.0040$
[g/l]	464.53	464.53	464.53	
	470.55	467.36	468.95	
	461.69	467.32	464.51	
	473.18	474.02	473.60	
	471.58	466.09	468.83	
	499.87	498.10	498.99	**Bias [%]**
Average	**480.54**	**480.15**		**0.080**
	224.64	224.27	224.45	
	220.25	219.84	220.05	
	221.66	219.93	220.79	
	208.33	208.83	208.58	
	204.97	206.99	205.98	
Chlorpyrifos methyl	209.56	208.85	209.20	$CV_r = 0.0030$
[g/l]	211.51	211.57	211.54	
	204.63	204.50	204.56	
	209.49	209.75	209.62	
	205.47	205.36	205.42	
	199.73	199.41	199.57	**Bias [%]**
Average	**210.93**	**210.85**		**0.041**
	489.65	489.96	489.81	
	515.30	513.82	514.56	
	517.78	517.60	517.69	
	500.03	499.40	499.72	
Fenthion	499.09	497.16	498.12	
[g/l]	517.37	516.20	516.78	
	517.88	517.09	517.49	$CV_r = 0.0030$
	508.26	511.68	509.97	
	482.49	482.66	482.57	
	501.94	507.13	504.53	**Bias [%]**
Average	**504.979**	**505.27**		**-0.058**
	480.53	480.37	480.45	
	483.24	485.42	484.33	
	489.29	490.47	489.88	
	496.59	499.23	497.91	
Trifluralin	491.57	489.03	490.30	
[g/l]	485.27	487.49	486.38	$CV_r = 0.00370$
	489.27	482.98	486.13	
	490.75	491.70	491.23	
	497.75	497.27	497.51	
	486.28	486.29	486.28	**Bias [%]**
Average	**489.054**	**489.025**		**0.006**

The repeatability of the method on a long run was very good (< 0.08%) and there was practically negligible difference between the duplicate measurements. The CV of the AS content of 12 commercial formulations was 2.73%. The minimum-maximum deviation of the measured AS content from the nominal concentration is within 480 ±19 g/l, which is much smaller than the permissible variation of the AS content of the formulated product 480 ±72 g/l according to the FAO specification.

The results indicate that the MP method can be applied reproducibly and accurately for determination of AS content of the four formulations.

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APPLICATION OF MULTI-ANALYTE METHODS FOR PESTICIDE FORMULATIONS

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Abstract

The application of multi-analyte methods for pesticide formulations by GC analysis is discussed. HPLC was used to determine active ingredients. HPLC elution sequences were related to individual n-octanol/water partition coefficients. Real laboratory data are presented and evaluated with regard to validation requirements. The retention time data of pesticides on different HPLC columns under gradient and isocratic conditions are compared to illustrate the applicability of the methodologies.

1. INTRODUCTION

The applicability of gas chromatographic multi-analyte methods has been tested with four replicates for 44 different pesticides containing 31 active substances. All analyses were carried out according to the standardised protocols. The results were compared to the performance parameters specified in the relevant CIPAC method, or to the within-laboratory reproducibility calculated with the Horwitz equation.

The repeatability of the method (CV) was better or equal to that of the reference values in 34 cases. The other pesticides gave higher CV values as the stability of the GC system or the configuration of the injector was not suitable for the analysis which was also indicated by intercepts statistically different from zero, or shifting response during the repeated calibration.

The examples for the separation of substances, the analytical conditions and the results are summarized in this report.

2. MATERIALS AND METHODS

2.1. Conditions of GC analysis

Gas chromatograph: Varian 3900 equipped with FID detector, auto sampler model CP-8400 and Star Workstation 6.00 operation software
Capillary column: 25 m × 0.53 mm × 1.0 µm BPX-5 (95% methyl + 5% phenyl silicone phase)
Temperatures

Injector temperature	220°C
Detector temperature	270°C
Column temperature	80°C 1 min, 39.9°C/min to 140°C, 15°C/min to 290°C, hold for 5 min

Flow rates

Carrier gas (nitrogen):	10 ml/min
Hydrogen:	25 ml/min
Air:	300 ml/min
Split ratio:	3
Run time:	31 min
Injected volume:	2 µl

2.2. Reagents

Internal standards: analytical grade.
Pesticide analytical standards: known purity with quality certificate.

2.3. Preparation of samples and standard solutions

The following internal standards (IS) were selected:
Diethyl phthalate (DEF)
Benzyl benzoate (BB)
Dibutyl phthalate (DBF)
Diphenyl phthalate (DFF)
Docosan (DOC)
Squalane

*Preparation of internal standard solution **(IS):***
Weigh into a 1 litre volumetric flask 0.2 g (±0.02 mg/ml) internal standard. Dissolve in acetone and dilute to volume with acetone.

*Preparation of standard solutions (**RM**)*
Measure approximately 25 mg pure certified standard into a 50 ml volumetric flask and weigh the mass. Add 25 ml of properly selected internal standard solution, shake the flask to dissolve or put into ultrasonic bath. Fill-up to mark with internal standard solution and weigh this solution.

*Preparations of working standard solutions (**SH1**)*
Weigh an empty 5 ml screw capped vial. Pipette 1.0 ml of standard stock solution (**RM**) and weigh again. Dilute to 5 ml with internal standard solution and weigh this solution.

*Preparations of working standard solutions (**SH2**)*
Weigh empty 10 ml screw capped vial. Pipette 2.0 ml of standard stock solution (**RM**) and weigh again. Dilute to 6 ml with internal standard solution and weigh this solution.

*Preparations of working standard solutions (**SH3**)*
Weigh empty 5 ml screw capped vial. Pipette 2.0 ml of standard stock solution (**RM**) and weigh again. Dilute to 5 ml with internal standard solution and weigh this solution.

*Preparation of sample (**F**)*
Weigh an empty volumetric flask and weigh in it approximately x mg homogenised sample portion specified in Table 1 to get approximately 0.2 mg ai/ml solution and weigh the mass. Add 25 ml of internal standard solution, shake the flask to dissolve the sample material or ultrasonicate it for few seconds. Fill-up to mark with internal standard solution and weigh this solution.

*Preparation of working sample solutions (**FH**)*
Weigh empty 10 ml screw capped vial. Pipette 2.0 ml of sample solution (**F**) and weigh again. Dilute with 4 ml internal standard solution, weigh the diluted solution.

Use the standardised Excel file for calculation of standard, internal standard and sample concentration and ratios of concentrations.
Inject the diluted standards and samples within one day after dilution.

2.4. Gas chromatographic analysis

2.4.1. System suitability tests

Before starting the actual GC analyses, use three injections of acetone to clean the injector and column. Check the condition of gas chromatographic system with suitability tests mixtures. Mixture A contains pesticides: propoxur, carbaryl, metobromuron, chlorpyrifos, and dimethoate) and Mixture B contains internal standards: diethyl phthalate, benzyl benzoate, dibutyl phthalate, diphenyl phthalate and squalane).

Observe the chromatograms, and calculate the method performance parameters described in detail in Chapter 6.1.

2.4.2. Calibration, evaluation of AS concentration in the sample

Inject all standards and samples at least twice. Use standardised Excel sheets to calculate the parameters of calibration line and the active ingredient content of pesticide.

An example for the evaluation of Basudin 600 EW (diazinon) is given as Figures 1–5 including: chromatogram of standard + IS; chromatogram of Basudin + IS; chromatogram of IS-mix; calculation of within-laboratory reproducibility from duplicate analytical portions of different samples and a copy of the Excel worksheet for calibration and calculation of sample concentration.

3. RESULTS

Detailed data on active ingredients and pesticides measured as well as calibrations obtained are given in Table 1.

The repeatability of the procedure was determined normally in four replicates. The standard deviation (SD) and the relative standard deviation of the results (CV_r) were calculated. The repeatability data of the multi-analyte method for pesticides tested are given in Table 2.

Repeatability results were compared to those of CIPAC methods. When data were not available for the CIPAC method, repeatability data were compared to the modified Horwitz equation.

$$\% \, RSD_r = 2^{(1-0.5\log C)} \times 0.67 \qquad (1)$$

In the equation the C is the analyte concentration expressed as m/m fraction (see details in Chapter 4.1., Appendix I).

3.1. Evaluation of results

The results were considered acceptable where the CV_r of the method was ≤ RSD_r of the reference method, the intercept was ≤ 0.02 and the relative residuals standard deviation S_{rr} was ≤ 2%.

In view of the very few measurements, no final conclusion can be drawn on the repeatability of the determination of the active substance content of pesticides. Further tests, including reproducibility and selectivity, would be required to confirm the performance of the method and its applicability.

It should be pointed out that the CV_r and the S_{rr} values are independent from the conditions of the actual analysis, and thus they are suitable for comparison with other results obtained in different laboratories. On the other hand, the numerical value of the intercept depends on the data processing software and can only be used to compare results obtained under the same conditions.

Calibration data

In most cases the parameters of calibration lines fulfilled the requirement: SD of relative residuals ≤ 0.01; intercept < 0.02; correlation coefficient > 0.995.

Detailed information for different pesticides is given in Table 1.

Repeatability of method

Altogether 44 different pesticide formulations containing 31 active substances were investigated.

The repeatability of the method was acceptable in case of 34 products containing acetamiprid, acetochlor, amitraz, atrazine, benefin, boscalid, captan, chlorpyrifos, cypermethrin, dichlorvos, deltamethrin, diazinon, dimethoate, endosulfan, fenitrothion, fluquinconazole, fenitrothion, hexaconazole, lambda cyhalothrin, malathion, metolachlor, miclobutanil, parathion-methyl, penconazole, propisochlor, tebuconazole, and trifluralin.

Some other pesticides gave higher CV values than reported for the reference method. In certain cases the stability of the GC system or the configuration of the injector was not suitable for the analysis which was indicated by intercepts statistically different from 0, or shifting response during the repeated calibration.

The problematic analytes belong to the low volatility compounds (e.g. pyrethroids, phosalone), or to those formulations that are difficult to homogenize (suspensible concentrate of atrazine, granules of terbufos and kresoxim methyl).

The suspensible concentrates, granules, controlled release formulations, baites, etc. require especial attention not only for the homogenization of the sample but also for the extraction procedure. Their extraction may be a complex, time dependent equilibrium between different phases, and its efficiency depends very much on the solvent used, the solvent/solid material ratio, temperature, equipment, etc. Consequently it is mandatory to follow exactly the validated procedure described in the reference method, or the new extraction procedure must be thoroughly validated which cannot be done with spiking of the samples. The situation is distinctly different from EC formulations where strictly speaking not extraction but only dilution is taking place and a different suitable solvent would give comparable results.

TABLE 1. DATA OF PESTICIDES AND CALIBRATIONS MEASURED WITH MULTI-ANALYTE METHOD

Active ingredient	Formulated pesticide	Formu-lation	AS [%]	Anal. method	Sample portion [mg]	IS	t_R of AS	t_R of IS	n	Slope	Intercept	S_{rr}	Srr [%]
Acetamiprid	Mospilan 20 SP	SP	20	GC	125	DFF	16.12	16.81	4	-0.0042	0.9999	**0.40**	0.65
Acetochlor	Trophy	EC	70	GC	100	BB	9.587	8.552	4	0.718	-0.00025	0.9997	0.72
Acetochlor	Guardian Extra	EC	33	GC	150	DBF	9.643	10.513	4	0.903	0.00455	1	0.19
Acetochlor	Guardian Max	SC	75	GC	100	DBF	9.643	10.513	4	0.903	0.00455	1	0.19
Alpha cypermethrin	Bestseller 100 EC	EC	10	GC	250	DFF	19.35	16.8	4	0.695	0.00926	1	2.09
Amitraz	Mitac 20 EC	EC	20	GC	200	DBF	17.07	10.46	4	1.2951	0.01909	0.9977	2.15
Benefin	Flubalex	EC	19	GC	120	BB	6.949	8.451	4	0.534	-0.0049	1	0.22
Atrazine	Tazastomp SC	SC		GC					4	0.4722	0.0151	0.9999	0.00345
Atrazine	Guardian extra	EC	16	GC	160	DBF	8.24	10.459	4	0.5440	-0.0008	0.9999	0.42
Beta cyfluthrin	Enduro 258 EC	EC	0.75	GC	1000	DOC	18.9	12.814	4	0.3239	0.0249	0.9996	1.04
Boscalid	Pictor	SC	17.9	GC	130	DFF	19.33	16.77	3	0.7748	-0.014	0.9994	
DDVP	Unifosz 50 EC	EC	48	GC	110	BB	3.405	8.447	4			0.9997	

Active ingredient	Formulated pesticide	Formu-lation	AS [%]	Anal. method	Sample portion [mg]	IS	t_R of AS	t_R of IS	n	Slope	Intercept	S_{rr}	Srr [%]
DDVP	Unifosz 50 EC	EC	48	GC	106	BB	3.45	8.45	2	0.1981	-0.0072	0.9999	0.46
Deltamethrin	Decis WST	WST	25	GC	200	DFF	21.12	16.39	4	0.5267	0.0029	0.9993	1.83
Deltamethrin	Splendour	EC	2.5	GC	1000	DFF	21.273	16.636	2	0.4803	-0.0074	0.9996	0.81
Diazinon	Basudin 600 EW	EW	57	GC	170	DBF	8.54	10.504	4	0.6596	-0.0264	1	0.32
Diazinon	Basudin 600 EW	EW	57	GC	170	DBF	8.4754	10.504	3	0.6830	-0.0131	1	0.44
Diazinon	Diazinon 5 G	G	5	GC	1000	DBF	8.54	10.504	4	0.6596	-0.0264	0.9999	1.74
Diazinon	Basudin 5 G	G	4.7	GC	1000	DBF	9.587	10.503	4	0.6574	0.0086	0.9999	0.35
Dimethoate	Bi-58 EC	EC	38	GC	130	DBF	8.02	10.41	4	0.2982	-0.01157	0.9999	0.88
Dimethoate	Bi-58 EC	EC	38	GC	135	DBF	7.95	10.34	2	0.3344	-0.0256	0.9999	0.53
Endosulfan	Thionex 35 EC	EC	35	GC	80	DBF	I.	10.48	4	0.375	-0.00457	1	0.88
Endosulfan	Thionex 35 Ec	EC	35	GC	150	DBF		10.34	2	0.4295	-0.0023	0.9999	0.55
Fenitrothion	Galition 5 G	G	4.5	GC	1100	BB	10.44	8.55	4	0.4478	-0.0001	0.9997	1.07
Fenitrothion	*Buvatox 5 G*	G	4.7	GC	1150	BB	10.407	8.507	4	0.4597	-0.04041	0.9999	1.18

Active ingredient	Formulated pesticide	Formu-lation	AS [%]	Anal. method	Sample portion [mg]	IS	t_R of AS	t_R of IS	n	Slope	Intercept	S_{rr}	Srr [%]
Fluquinconazole	Clarinet	SC	4	GC	250	DBF	18.35	10.503	4	0.7752	0.00735	0.9999	0.82
Phosalone	Zolone 30 WP	WP	30	GC	100	DBF	16.635	10.35	4	0.4002	-0.0393	0.9999	1
Phosalone	Zolone 35 EC	EC	35	GC	100	DBF	16.635	10.33	2	0.4002	-0.0393	0.9999	1
Hexaconazole	Amistar Ter	SC	6.5	GC	400	DBF	12.85	10.47	4	0.7189	-0.01324	0.9997	0.91
Hexaconazole	Contaf	SC	5	GC	500	DBF	12.67	10.297	2	0.6386	-0.0052	0.9995	0.97
Captan	Buvicid K 370 SC	SC	31	GC	160	DBF	12.084	10.406	4	0.4147	-0.0194	1	0.57
Chlorpyrifos	Nurelle D50/500	EC	50	GC	100	DBF	10.73	10.51	4	0.4184	0.0079	1	1.07
Chlorpyrifos	Nurelle D50/500	EC	50	GC	100	DBF	10.54	10.31	2	0.3814	-0.0141	0.9999	0.35
Chlorpyrifos	Cyren	EC	44	GC	115	BB	10.668	8.536	4	0.3534	-0.00002	0.9999	0.32
Cypermethrin	Sherpa	EC	26	GC	200	DFF	19.272	16.726	4	0.8583	-0.00543	0.9999	0.43
Kresoxim-methyl	Discus DF	DF	50	GC	50	DBF	13.27	10.5	4	1.0400	0.00329	0.9999	1.3
Lambda-cyhalotrin	Karate 2.5 WG	WG	2.5	GC	2.5				4				
Malathion	Fyfanon EW	EW	40	GC	120	BB	10.62	8.552	4			0.9999	0.96

Active ingredient	Formulated pesticide	Formu-lation	AS [%]	Anal. method	Sample portion [mg]	IS	t_R of AS	t_R of IS	n	Slope	Intercept	S_{rr}	Srr [%]
Parathion-methyl	Danatox 50 EC	EC	2.5	GC					4	0.4766	-0.0114	1	0.22
Metolachlor	Igran Combi Gold	EC	25	GC	100	BB	10.72	8.552	4	0.7133	-0.004	1	0.25
Miclobutanil	Systhane 12 E	EC	12	GC	175	BB	13.21	8.552	4	0.7033	-0.0229	0.9993	0.96
Miclobutanil	Systhane 12 E	EC	12	GC	175	BB	13.466	8.552	2	0.7409	0.0268	0.9996	0.69
Penconazol	Topas 100 EC	EC		GC					4				
Propizochlor	Proponit 720 EC	EC	68	GC	110	BB	9.705	8.412	4	0.7447	-0.0173	0.9997	0.73
Tebuconazol	Folicur Solo	EC	25	GC	200	DOC	15.24	12.81	4	0.5346	0.0012	1	0.56
Terbufos	Counter 5 G	G	4	GC	1000	DBF	8.45	10.41	4	0.5654	0.0037	1	1.41
Triadimefon	Bayleton 25 WP	WP	22.5	GC	220	BB	10.99	8.552	4	0.6336	0.00031	1	1.37
Trifluralin	Olitref 480 EC	EC	45	GC	105	BB	6.7	8.552	4	0.5324	0.00473	1	0.48

Performance criteria were not complied with in cases printed in italic fonts.

TABLE 2. REPEATABILITY DATA OF MULTI-ANALYTE GAS CHROMATOGRAPHIC METHOD

Formulated pesticide	Active ingredient	Anal. method	n	Average AS [%]	SD	CVr [%]	RSDr	CIPAC Ref.	CIPAC method	r CIPAC method	RSD_R [%] CIPAC method (calculated)	Quality of analysis based on CV % or CD
Mospilan 20 SP	Acetamiprid	GC	4	20.34	0.13	0.65	1.71	-		-		A
Trophy EC	Acetochlor	GC	4	69.3	0.23	0.33	1.41	-				A
Guardian Extra EC	Acetochlor	GC	4	32.4	0.12	0.37	1.61					A
Guardian Max SC	Acetochlor	Gc	4	71.8	0.41	0.57	1.41					A
Bestseller 100 EC	Alpha cypermethrin	GC	4	11.02	0.23	2.09	1.9	H. p14	GC	r = 2.9 g/kg at 95 g/kg	1.09	N
Mitac 20 EC	Amitraz	GC	4	20.96	0.14	0.66	1.71	G. p5	GC	r = 8.1 g/kg at 200 g/kg	1.46	A
Benefex EC	Benefin	GC	4	20.01	0.15	0.76	1.71					A
Tazastomp SC	Atrazine	GC	4	X[1]				H. p37	GC	-		N
Guardian Extra	Atrazine	GC	4	16.4	0.11	0.66	1.71	H. p37	GC			A
Enduro 258 EC	Beta-cyfluthrin	GC	4	0.8	0.32	2.96	2.68-3.3			-		N

Formulated pesticide	Active ingredient	Anal. method	n	Average AS [%]	SD	CVr [%]	RSDr	CIPAC Ref.	CIPAC method	r CIPAC method	RSD_R [%] CIPAC method (calculated)	Quality of analysis based on CV % or CD
Pictor SC	Boscalid	GC	3	20.518	0.77	0.38	1.71	-		-		A
Unifosz 50 EC	DDVP	GC	4	47.59	0.22	0.46	1.49	H. p135	GC	-		A
F94_Unifosz_EC	DDVP	GC	3	52.8		0.63		H. p135	GC	-		CV % from different samples
Decis WST	Deltamethrin	GC	4	25.18	0.46	1.83	1.71	D. p59-63	HPLC	-		N
Splendour EC	Deltamethrin	GC	3	2.53		1.05				-		CV % from different samples
Basudin 600 EW	Diazinon	GC	4	55.33	0.17	0.32	1.49	H. p126	GC	-		A
Basudin 600 EW	Diazinon	GC	3	55.33		0.24	1.49	H. p126	GC	-		A
Diazinon 5 G	Diazinon	GC	4	5.15	0.09	1.74	2.1	H. p126	GC	-		?
Basudin 5G	Diazinon	GC	4	4.95	0.07	1.39	2.68	H. p126	GC			A
Bi-58 EC	Dimethoate	GC	4	37.47	0.33	0.88	1.49	H. p159	GC	r = 14-15 g/kg at 368 g/kg	1.41	A
Bi-58 EC	Dimethoate	GC	3	38.41		0.7		H. p159	GC			CV % from different samples

Formulated pesticide	Active ingredient	Anal. method	n	Average AS [%]	SD	CVr [%]	RSDr	CIPAC Ref.	CIPAC method	r CIPAC method	RSD_R [%] CIPAC method (calculated)	Quality of analysis based on CV % or CD
Thionex 35 EC	Endosulfan	GC	4	34.96	0.31	0.88	1.49	C. p 2111	GC	r = 0.72 at 34.0 m/m	0.76	A
Thionex 35 EC	Endosulfan	GC	3	33.2		0.55			GC			CV % from different samples
Galition 5 G	Fenitrothion	GC	4	4.3	0.05	1.07	2.1	C. p 2117	GC	-		A
Nurelle D50/500	Chlorpyrifos	GC	4	47.36	0.51	1.07	1.49	C. p 2028	HPLC	-		A
Nurelle D50/500	Chlorpyrifos	GC	1	47.01								
Cyren EC	Chlorpyrifos	GC	4	44.34	0.16	0.37	1.54					A
Sherpa EC	Cypermethrin	GC	4	24.7	0.27	1	1.65					A
Discus DF	Kresoxim-methyl	GC	4	45.46	0.59	1.3	1.49	K. p 77	HPLC	r = 16 at 490 g/kg	1.17	N
Karate 2.5 WG	Lambda cyhalotrin	GC	4	2.45	0.11	2	2.41					?
Fyfanon EW	Malathion	GC	4	41.66	0.4	0.96	1.49	K. p 88	GC	r = 13 at 406 g/kg	1.14	A

Formulated pesticide	Active ingredient	Anal. method	n	Average AS [%]	SD	CVr [%]	RSDr	CIPAC Ref.	CIPAC method	r CIPAC method	RSD_R [%] CIPAC method (calculated)	Quality of analysis based on CV % or CD
Danatox 50 EC	Parathion-methyl	GC	4	48.79	0.8	1.64	1.49			-		?
Igran Combi Gold EC	Metolachlor	GC	4	25.9	0.07	0.25	1.71	D. p 135	GC	-		A
Systhane 12 E EC	Miclobutanil	GC	4	13.8	0.13	0.96	1.9			-		A
Systhane 12 E EC	Miclobutanil	GC	1	14.3								A
Topas 100 EC	Penconazole	GC	4	10.5	0.06	0.58	1.9			-		A
Proponit 720 EC	Propizachlor	GC	4	67.9	0.61	0.9	1.41					A
Folicur Solo EC	Tebuconazole	GC	4	26.13	0.15	0.56	1.71	H. p 261	GC	r = 4 at 262 g/kg	0.55	A
Counter 5 G	Terbufos	GC	4	5.05	0.07	1.41	2.1	H. p 269	GC	r = 2.6 at 150 g/kg	0.62	N
Bayleton 25 WP	Triadimefon	GC	4	25.52	0.35	1.37	1.71	C. p 2241	HPLC	r = 0.55% at 25% a.i.	0.79	N
Olitref 480 EC	Trifluralin	GC	4	46.92	0.22	0.48	1.51	H.p292	GC	-		A

Notes: n = Number of repeatability studies; x^1 = one result is an outlier; RSDr calculated from Horwitz equation; CV % from different samples; Performance criteria were not complied with in the underlined cases.

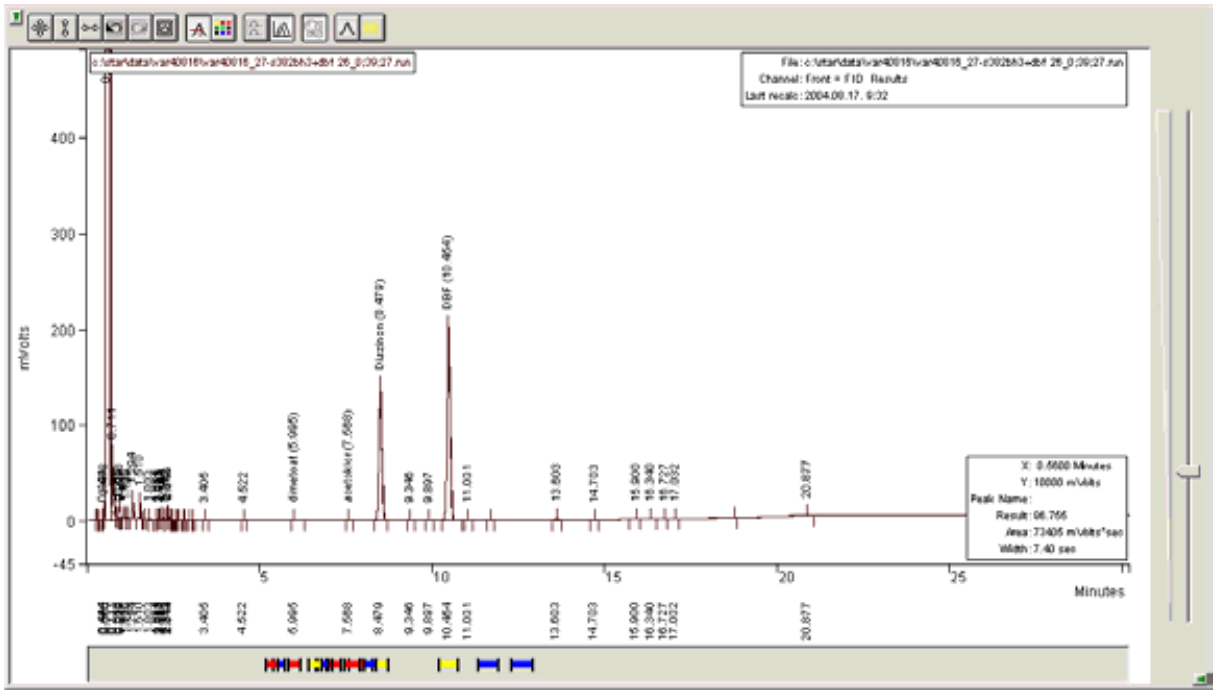


FIG. 1. Diazinon standard + Dibutyl phthalate IS.

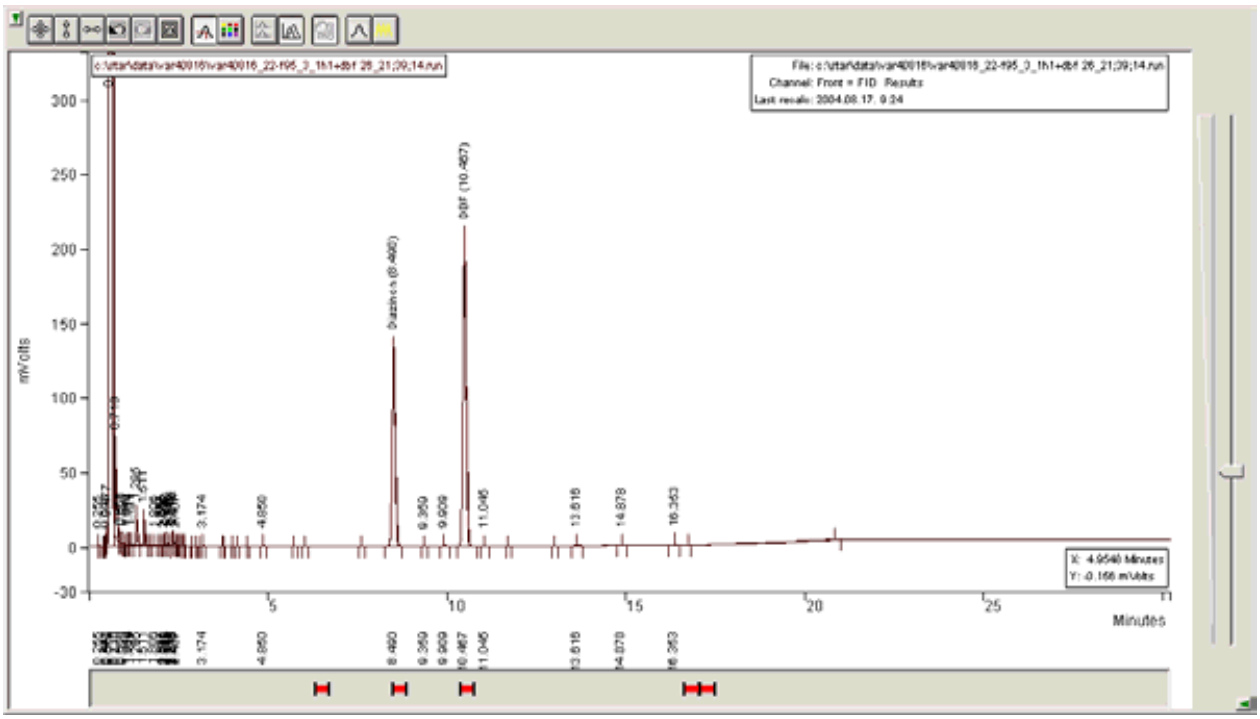


FIG. 2. Basudin 600 EW + Dibutyl phthalate IS.

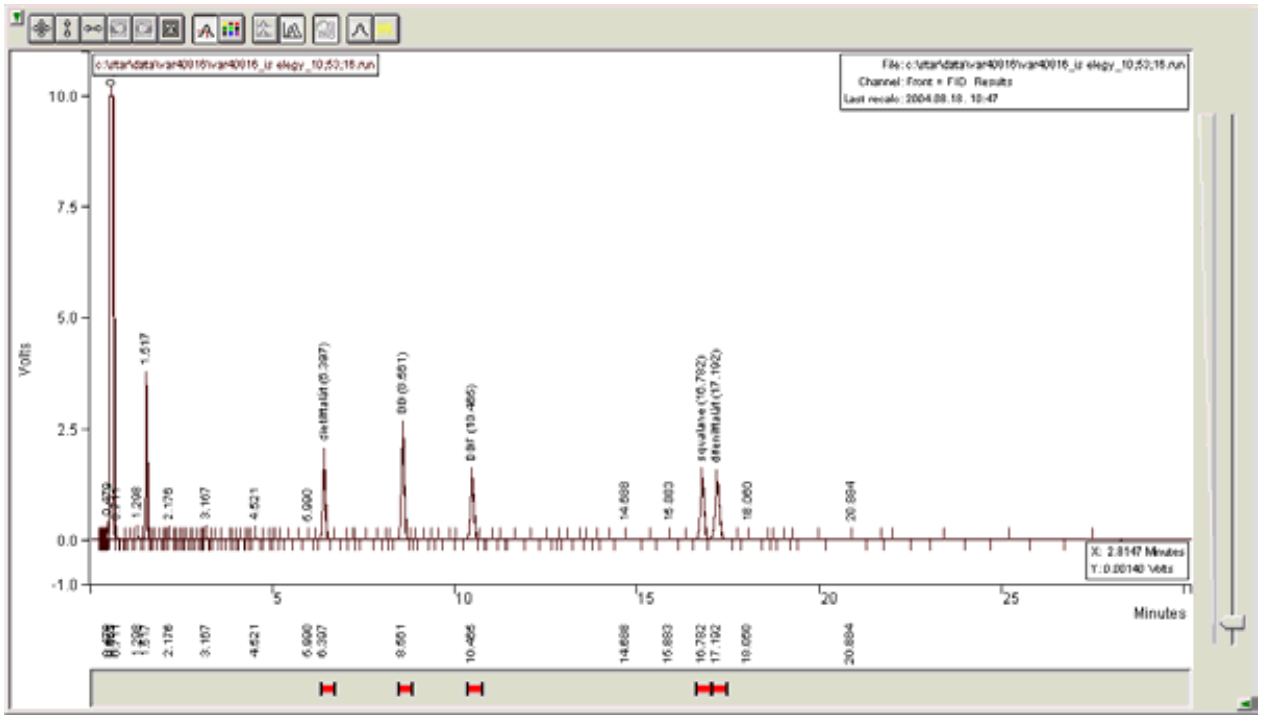
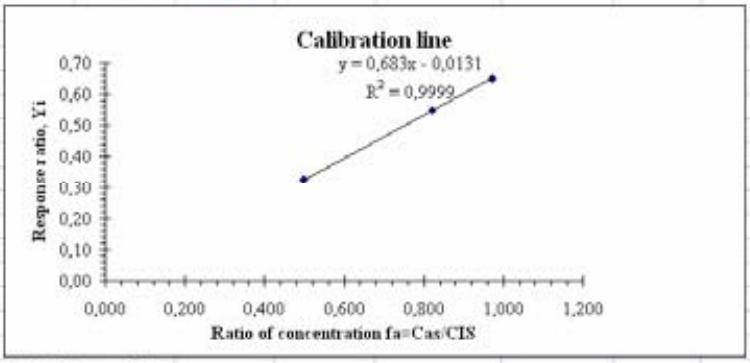


FIG. 3. Chromatogram of internal standards used.

Calculation of within-laboratory reproducibility from replicate analytical portions								
Diazinon-Basudin 600 EW								
Plant Protection and Soil Conservation Service of Szabolcs-Szatmár-Bereg County								
Lab sample code 1	**ai content 1 m/m %**	**Date of analysis**	**Lab code 2**	**ai content 1 m/m %**	**Date of analysis**	**ai content mean m/m %**	**Relative difference**	**Rel.dif^2**
F95/1-1H1	55.64		F95/1-2H1	55.37		55.51	0.004864	2.36626E-05
F95/2-1H1	55.32		F95/2-2H1	55.30		55.31	0.000444	1.96903E-07
F95/3-1H1	55.30		=varYYMMDD_GC!B57	55.12		55.21	0.003276	1.07334E-05
$CV_{L\%}$ =	**0.24**							

FIG. 4. Calculation of within-laboratory reproducibility from replicate analytical portions.

Laboratory/Vizsgáló laboratórium			Szabolcs-Szatmár-Bereg m. NTSz								
Pesticide/Készítmény			Basudin 600 EW			Active ingredient/h.a.		Diazinon			
Nominal A.i. content/névleges ha. tartalom			57	tolerance: (+)		2,5	Conc unit:	m/m %			
mean density of pesticide for g/l; m/V% ai. content unit						(g/cm^3)					
Extraction method identity/Extrakciós módszer			aceton+IS	IS azonosító:		DBF		Extrakciót végzi:			
Standard törzsoldat bemérés		25	mg	50		ml-re	conc. mg/ml	0,166			
Közép standard hígítás	2	ml -->	6	ml		Mintabemérés (kb, mg):		170,0	Mintatérf térfoga	100	
Instrument and type/GC készülék			GC, Varian 3900								
Column applied/GC oszlop			K60, BPX5, 25m x 0.53mm x 1um								
Detector (wavelength)/Detektor tipus, hőmérséklet			FID, 270 °C								
Column temperature/Solvent composition programme			M2 K60 method 80C 1min 39.9°C/min 150C 8C/p 290°C								
Retention time (min)/Retenciós idő		Diazinon	8.470	DBF		10.448					
Calibration data/Kalibráció		based on duplicate injection of independent standard dilutions; for calculation: mg/g unit ratio									
Raw data file:		var40816	Evaluation based on:			area		sample concentration ratio based on:		weighed mass	
Run N<	1	f_s	A_{ai}	A_{IS}		Yi=Aai/Ais		Yi calc	Yrel i	calculation of	
	solution ID	C_{ai}/C_{IS} (mg/g)	Diazinon	DBF 26						response factor	
25	S382BH1	0,49663477	406119	1238590		0,3279		0,3260	-0,0057	1,51465	
8	S382BH1	0,49663477	408954	1262280		0,3240		0,3260	0,0063	1,53292	
13	S382BH2	0,82078092	683295	1248287		0,5474		0,5474	0,0001	1,49946	
14	S382BH2	0,82078092	686177	1251796		0,5482		0,5474	-0,0013	1,49735	
19	S382BH3	0,97213969	826525	1272481		0,6495		0,6508	0,0019	1,49666	
20	S382BH3	0,97213969	821466	1260718		0,6516		0,6508	-0,0012	1,49196	
			CV%=	0,95				SD=	0,0040	0,0155	SD of response factor
	Slope a:	Meredekség	0,68296501							1,03	CV% response factor
	Intercept b:	Metszet	-0,0131399	Calibration equation/Kalibrációs egyenlet:							
	r:	Korr koeff	1,0000	$Y_{i\,calc} = a*f_s + b$				$Yrel = (Y_{i\,calc} - Y_i)/Y_{mak}$			
	sYrel:	Relativ szórás	0,0044								



Sample analysis/Minta analizis

raw data file:		var40816	Evaluation based on		area	duplicate injection of same sample portion				
Run N<	Sample	$f_s=C_a/C_{IS}$	$A_{ai,s}$	A_{IS}	$Yi=A_{a,i,s}/A_{IS}$	C_{ai}/C_{IS}	ai content	ai content in unit	mean	bizonyt
	solution id	ratio of conc (mg/g)	Diazinon	DBF 26	calculated	calculated	calculated (g/kg)	m/m *'s	m/m %	alanság %
9	F95/1-1H1	1,452261	677353	1257726	0,53855	0,807792	556,2	55,62	55,64	0,34
10	F95/1-1H1	1,452261	679920	1261734	0,53888	0,808266	556,6	55,66		
11	F95/1-2H1	1,425218	671421	1277496	0,52558	0,788790	553,5	55,35	55,37	0,34
12	F95/1-2H1	1,425218	680058	1292694	0,52608	0,789525	554,0	55,40		
15	F95/2-1H1	1,476494	707108	1299339	0,54421	0,816068	552,7	55,27	55,32	0,34
16	F95/2-1H1	1,476494	703431	1290069	0,54527	0,817620	553,8	55,38		
17	F95/2-2H1	1,512864	721207	1292384	0,55804	0,836330	552,8	55,28	55,30	0,34
18	F95/2-2H1	1,512864	727681	1303143	0,55840	0,836858	553,2	55,32		
21	F95/3-1H1	1,636704	795366	1315070	0,60481	0,904803	552,8	55,28	55,23	0,34
22	F95/3-1H1	1,636704	787631	1304612	0,60373	0,903221	551,9	55,19		
23	F95/3-2H1	1,429707	690456	1316093	0,52463	0,787398	550,7	55,07	55,12	0,34
24	F95/3-2H1	1,429707	692470	1317788	0,52548	0,788648	551,6	55,16		
13	S382BH2	0,82078	683295	1248287	0,54739	0,820724	999,9	999,93		

cella tart	Minta	megegyzés	Átlag	SD	CV%	Bizonytalansági tartomány		Bizonytalansági határ képlete	
47-54	F95	3*2 ismétlés	55,33	0,17	0,32	±	0,35	D61-2*E61	D61+2*E61
47-50	F95/1	2 ismétlés	55,51			±	0,35		
51-54	F95/2	2 ismétlés	55,31			±	0,35		
55-58	F95/3	2 ismétlés	55,33			±	0,35		

Comments on the results/megjegyzés: vizsgálat befejezve, megfelel

Date: 2004.08.18 Responsible analyst/analitikus: Lantos

FIG. 5. Copy of Excel worksheet for calibration + calculation of sample concentration.

HPLC 'MULTI-ANALYTE' DETECTION METHOD

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Abstract

The application of multi-analyte methods for pesticides carrying chromophoric structures by HPLC is described. Details are given on the materials and methods used. Recorded UV spectra of active substances are presented for allowing the verification of purity and the confirmation of substances eluting from the HPLC column.

1. INTRODUCTION

Applicability: This method may be applied for the determination of a broad range pesticides having UV chromophores, e.g., acetanilides, aryloxyalkanoic acids, cabamates, azoles, organophosphates, phenols, pyrethroids, sulfonyl ureas, sulfonamides, triazines, urea and uracil pesticides. The list of pesticide active ingredients included in 'Multi-Analyte' method development is given in Table 1.

Interferences: Because of the broad response of the UV detector at shorter wavelengths, there are many potential interferences.

Matrix effect: Many compounds may come from formulation, formulation additives, pesticide hydrolysis products and pesticide impurities, which are potentially interfering with the target analyte.

Identification: Relative retention time (retention indices for a particular set of conditions) may be used for the initial identification of unknown analytes.

Confirmation: May be achieved through comparison of unknown spectra with reference spectra where available. Whenever the identity of an analyte is uncertain, conformation may be achieved by analysis on an alternate column (e.g. cyano type column) or by changing to a water/methanol mobile phase.

2. STEPS OF METHOD DEVELOPMENT

- The pesticide active ingredients were measured separately with a linear gradient (scouting) program under the standardised chromatographic condition, which is described in Section 3.
- The retention times of these pesticide active ingredients were measured during the chromatographic run and their relative retentions were calculated. *Chlorpyrifos* was chosen as a reference compound.
- The retention time during linear gradient run was converted to the percentage of acetonitrile in the eluent. See Section 4.1.
- These converted eluent compositions were chosen for the starting isocratic conditions.
- The retention times and chromatographic peak parameters e.g. peak width, and peak symmetry were determined under these starting isocratic runs.
- Finally, the isocratic eluent compositions were fine-tuned to get shorter retention and analysis times. The criterion of acceptable peak symmetry was between 0.8 and 1.2.

3. CHROMATOGRAPHIC PROCEDURE

3.1 Apparatus and operating conditions

- High performance liquid chromatograph equipped with constant-flow pumps, constant-temperature column compartment, sample injector capable of injecting 5 µl aliquots, variable-wavelength UV detector and data-handling capability. The instrument used to develop this method was a Hewlett Packard Model 1100 Liquid Chromatograph with Variable-Wavelength UV, Diode Array Detector (DAD) and Chemstation Software.
- Analytical column: LichroCart 125-4 HPLC cartridge, Merck.
  Column dimension: 125 × 4 mm, 5 µm.
  Stationary phase: Purospher Star RP-18 end-capped.
- Filtering apparatus for sample and standards solutions.
- Ultrasonic bath.
- Water purification system (Milli-Q-System).
- pH meter.

3.2 Reagents

1. Pesticide standard mix
2. Analytical standards
3. Acetonitrile
4. Phosphoric acid 85%
5. Water (HPLC quality)

3.3 Recommended liquid chromatographic conditions for scouting run

Column:	125 × 4 mm, 5 µm column packed with Purospher Star Merck RP-18 endcapped (5 µm)
Mobile Phase:	
Solvent A:	Acetonitrile + 2% 1-propanol
Solvent B:	pH: 2.6 Water (pH adjusted with H_3PO_4) + 2% 1-propanol
Solvent C:	Water
Gradient range:	20-80% solvent A
Steepness of the gradient:	0.80% A solvent/min
Gradient time:	75 min

$$t = \frac{Gradient\ range\ (A\%)}{steepness\ (0.8\%/\min)} = \frac{80-20}{0.8} = \frac{60}{0.8} = 75\ minutes$$

Post run: 5 min	100% A
Pre run: 15 min	20% A
Flow rate:	1 ml/min
Injection volume:	5 µl
Column temperature:	30 °C
Detection:	UV 210, 254, 280 nm
Reference Wavelength:	400 nm
Dead time:	t_0 = 1.16 min

Under these conditions one should allow at least 15 min to equilibrate the column under the initial solvent condition (20% A) between each run and place a 5 min hold at 100% A at the end of the gradient. The equilibration time can be decreased by increasing the flow rate to 2 ml/min.

The use of 1-propanol in both eluents (A and B) helps the equilibration and stability of mobile phase, retention time will be very stable and reproducible.

4. DETERMINATION OF RELATIVE RETENTION TIMES FROM LINEAR GRADIENT RUN

Using linear 20–100% full range gradient run (for solvent A) under the conditions described in section 3.3 the retention times of 30 pesticide active ingredient were measured.

During the linear gradient run the chromatogram of the active ingredient is recorded. The retention time of the active ingredient can be read on the chromatogram, the relative retention times of active ingredients were calculated. The results are summarized in Table 1.

TABLE 1. RETENTION DATA OF PESTICIDE ACTIVE INGREDIENT ON PUROSPHER COLUMN

Compound name	Linear gradient programme			Isocratic condition			
	t_R [min]	A % at t_R [min]	t_R'	t_R [min]	A % at t_R [min]	Peak asym	Peak width
Methomyl	2.500	22	0.025	2.31	22	0.807	0.106
				2.485	20	0.832	0.115
Imidacloprid	4.230	23.40	0.057	3.36	24	0.833	0.13
Dimethoate	5.062	24	0.072	4.00	24	0.865	0.143
Cymoxanil	6.580	25.26	0.100	4.97	25	0.844	0.157
				7.111	20	0.852	0.209
Dichlorvos	11.530	29.20	0.192	6.42	30	0.800	0.178
Carbofuran	13.090	30.47	0.231	7.930	30	0.834	0.221
				5.955	34	0.814	0.174
Atrazine	15.644	32.50	0.273	8.083	33	0.829	0.224
				5.159	40	0.785	0.154
Carbaryl	15.900	32.72	0.310	7.372	34	0.852	0.207
Metalaxyl	17.890	34.30	0.310	7.890	34	0.851	0.173
Diuron	18.880	35.100	0.328	8.38	35	0.832	0.223
Triadimenol	25.37 26.8 28.07	41	0.449	7.81 8.82	41	0.825 0.867	0.218 0.241
				6.18 6.9	44	0.801 0.837	0.177 0.195
Molinate	27.900	42.30	0.496	11.230	42	0.842	0.278
				9.200	45	0.842	0.233
				6.515	50	0.806	0.227
				5.140	55	0.797	0.138
Parathion	31.33	45.00	0.559	9.89	45	0.866	0.268
				6.37	50	0.820	0.179
Parathion-methyl	31.37	45.00	0.560	9.89	45	0.845	0.271
				6.36	50	0.826	0.180
Isoprothiolane	33.62	46.90	0.602	10.130	47	0.852	0.254
				7.60	50	0.826	0.202
				5.58	55	0.824	0.150
Iprobenfos	34.20	47.40	0.612	9.053	48	0.811	0.239
				8.390	49	0.813	0.222
				7.250	50	0.824	0.215
				5.100	51	0.777	0.188
Malathion	34.75	47.80	0.623	10.500	47		
				7.45	50	0.800	0.219
				5.44	55	0.803	0.151
Fenitrothion	35.900	48.720	0.644	9.54	49	0.875	0.252
				7.710	51	0.827	0.206
Propiconazole	36.13 36.47	49.20	0.648	8.65	50	0.592	0.351
				6.200	55	0.702	0.244
				5.500	57	0.721	0.218
Diazinon	40.41	52.65	0.728	10.40	53	0.841	0.281
				6.66	60	0.827	0.188
Fenthion	41.57	53.26	0.749	9.99	53	0.830	0.269
				6.080	60	0.808	0.173
Amitraz	43.460	55.00	0.784	11.083	55	1.61	0.311

Compound name	Linear gradient programme			Isocratic condition			
	t_R [min]	A % at t_R [min]	t_R'	t_R [min]	A % at t_R [min]	Peak asym	Peak width
				6.433	65	1.97	0.231
Chlorpyrifos-methyl	45.71	57.00	0.826	9.99	57	0.842	0.267
				8.093	60	0.814	0.220
Butachlor	52.700	62.120	0.955	10.250	63	1.196	0.588
				8.95	65	1.205	0.530
				8.35	66	1.224	0.509
Pendimethalin	54.740	63.80	0.972	10.84	64	0.843	0.280
Chlorpyrifos	55.087	64.00	1.00	10.81	64	0.817	0.289
				7.110	70	0.808	0.200
Trifluralin	57.690	66.00	1.048	9.91	66	0.816	0.260
Cypermethrin	65.0 65.59		1.184	6.246 6.56	77	1.059	
Deltamethrin	65.82	72.660	1.199	9.526	73	0.830	0.253
	65.85 65.82			12.45	70	0.866	0.339
				5.368	80	0.8	0.311
Fenvalerate	67	73.60	1.221	9.140	74	0.934	0.306
				5.580	80	0.864	0.179
Lambda cyhalothrin	67.0	73.60	1.221	8.330	74	0.864	0.219
				4.962	80	0.783	0.213
Ethofenprox	69.68	75.70	1.270	10.63	76	0.869	0.267
				7.507	80	0.783	0.213
					85		
Carbosulfan	71.200	77.00	1.298	10.90	77	0.803	0.289
				8.57	80	0.856	0.241

The 'Multi-Analyte' method was also used for NovaPack RP 18, 150 × 3.9 chromatographic column. The difference between the two measurements was only 10°C in column temperature. The results can be seen in Table 2. The applicability of method can be extended for further pesticide active ingredients.

TABLE 2. RETENTION DATA OF PESTICIDE ACTIVE INGREDIENTS ON NOVAPACK COLUMN

Compound name	Linear gradient programme			Isocratic condition			
	t_R (min)	A % at t_R [min]	t_R'	t_R [min]	A % at t_R [min]	Peak asym	Peak width
Dimethoate	3.218	22.5	0.0380				
Cymoxanil	4.11	23.30	0.054				
Nicosulfuron	6.204	24.96	0.093	4.03	25	1.029	0.161
Dicamba	7.092	25.67	0.11	4.23	29	1.351	0.129
Thifensulfuron	8.0260	26.42	0.127	5.26	26		
Carbofuran	9.218	27.37	0.149	5.62	28	0.907	0.192
Pyrimethanil	9.32	27.40	0.151				
Bentazone	10.615	28.50	0.175	6.20	29	0.907	0.199
Amidosulfuron	10.989	28.80	0.182	6.06	29	0.915	0.179
Chlortoluron	11.086	28.87	0.184	6.34	29	0.897	0.173
Chlorsulfuron	11.086	28.9					
Carbaryl	11.270	29.00	0.187	6.46	29	0.822	0.202
				5.07	32		
Rimsulfuron	11.375	29.10	0.189	5.87	29		
Atrazine	11.424	29.00	0.190	6.91	29		
				5.52	32		
Isoproturon	13.543	30.83	0.229	7.51	30		
			0.237	4.85	35	0.937	0.163
2,4-D	13.505	30.80	0.228	7.55	30		
			0.238	4.86	35		
Metobromuron	13.930	31.11	0.237	8.37	30		
			0.329	5.56	35	0.940	0.181
MCPA	14.022	31.20	0.238	7.88	30		

Compound name	Linear gradient programme			Isocratic condition			
	t_R (min)	A % at t_R [min]	t_R'	t_R [min]	A % at t_R [min]	Peak asym	Peak width
				4.98	35		
Dichlorprop	18.894	35.12	0.329	7.84	35	0.781	0.251
				5.06	40		
Captan	20.359	36.29	0.356	9.03	36		
				6.52	40		
Linuron	20.454	36.36	0.358	8.85	36	0.871	0.284
				6.35	40		
Chlorbromuron	20.090	37.60	0.351	8.43	38	0.909	0.259
				7.02	40		
Flupyrsulfuron	22.32	37.86	0.392				
Cyproconazole	22.753	37.68	0.400	7.78	38	0.925	0.328
	22.753	38.20		8.15		0.881	0.357
Prosulfuron	23.062	38.45	0.406	7.79	38	0.899	0.255
				4.87	43		
Fluquinconazole	25.95	40.76	0.46	8.26	41		
				5.88	45	0.874	0.171
Folpet	26.452	41.16	0.469	6.73	45	0.934	0.218
Metolachlor	28.088	42.47	0.499	9.00	43		
				7.58	45		
Tebuconazole	28.435	42.75		8.18	43		
			0.505	6.83	45		
Primisulfuron	28.457	42.76		7.45	43	0.923	0.207
Propiconazole	31.688	45.36	0.506	9.24	45		
	32.16	45.76		9.30			
			0.566	15.1	40		
				15.72			
Metconazole	30.67	44.80	0.55	8.44	45		
	31.00		0.55				
Cypermethrin	59.2	67.45	1.077	8.92	67		
	59.4			9.03			
				18.37	60		
				18.71			
Chlorpyrifos	55.10		1.00				

4.1 Determination of the isocratic conditions

The linear gradient separation run is suitable to determine the isocratic mobile phase composition. The active ingredient elutes during the linear gradient run at a certain time, which can be converted to the eluent composition expressed as the percentage of the more polar solvent in the eluent. For example parathion elutes at 31.33 minutes in the linear gradient run. At that time the percentage of the component with higher elution strength (in this case acetonitrile + 2% 1-propanol modifier) can be calculated by Eqs (1) and (2).

Determination of the percent of solvent A at the retention time of the analyte:

$$A\ (t_R)\ \% = t_R * g + A_0 \qquad (1)$$

where A_o: initial solvent A % (in this case 20%), t_R: retention time of the analyte

$$\text{g: steepness of gradient (A \% /minute)} = \frac{A_t\%(final) - A_0\%(initial)}{\Delta t\ (gradient\ \ program\ time)} \qquad (2)$$

The calculation resulted in 45% of component A and it is also indicated by the software as shown on Figure 1.

Setting 45% of solvent A, parathion is eluted under isocratic conditions. See the chromatogram Figure 2.

The mobile phase strength where the compound elutes obtained during the scouting run is appropriate for setting the initial isocratic condition. Some refinement may be necessary to fine-tune the separation by means of varying the percent organic solvent within a few percent. Resolutions are generally improving with weaker mobile phases.

Table 2 contains the retention times and chromatographic peak parameters for isocratic measurement conditions too. For the compounds, the first isocratic mobile phase composition is converted from linear gradient run. The other isocratic conditions are fine-tuned by variation of the percent organic solvent.

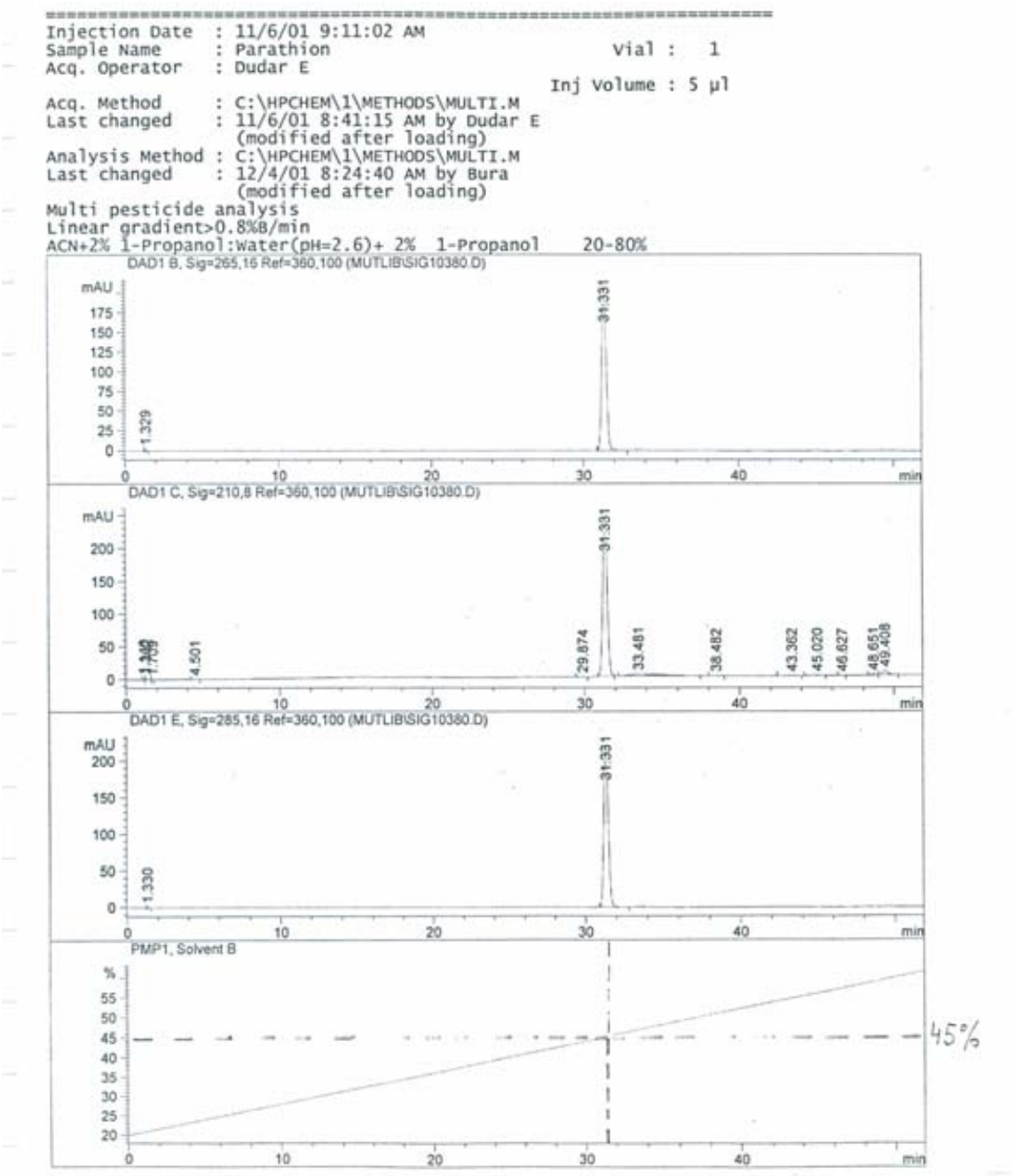

FIG. 1. Calculation of eluent composition from the HPLC chromatogram.

With the stronger mobile phase the retention times and analysis times decrease. Peak parameters also change. In most cases peak width and peak asymmetry is better under the isocratic condition calculated from linear gradient run but the retention time is longer. With the stronger mobile phase the peak asymmetry may increase, and the peak width will decrease.

Cypermethrin, deltamethrin, propiconazole and triadimenol are mixtures of isomers. Triadimenol isomers can be separated from baseline to baselines under isocratic conditions. Cypermethrin cannot be separated from baseline to baseline. Deltamethrin and propiconazole isomers can not be separated in isocratic condition in our standardised set of conditions.

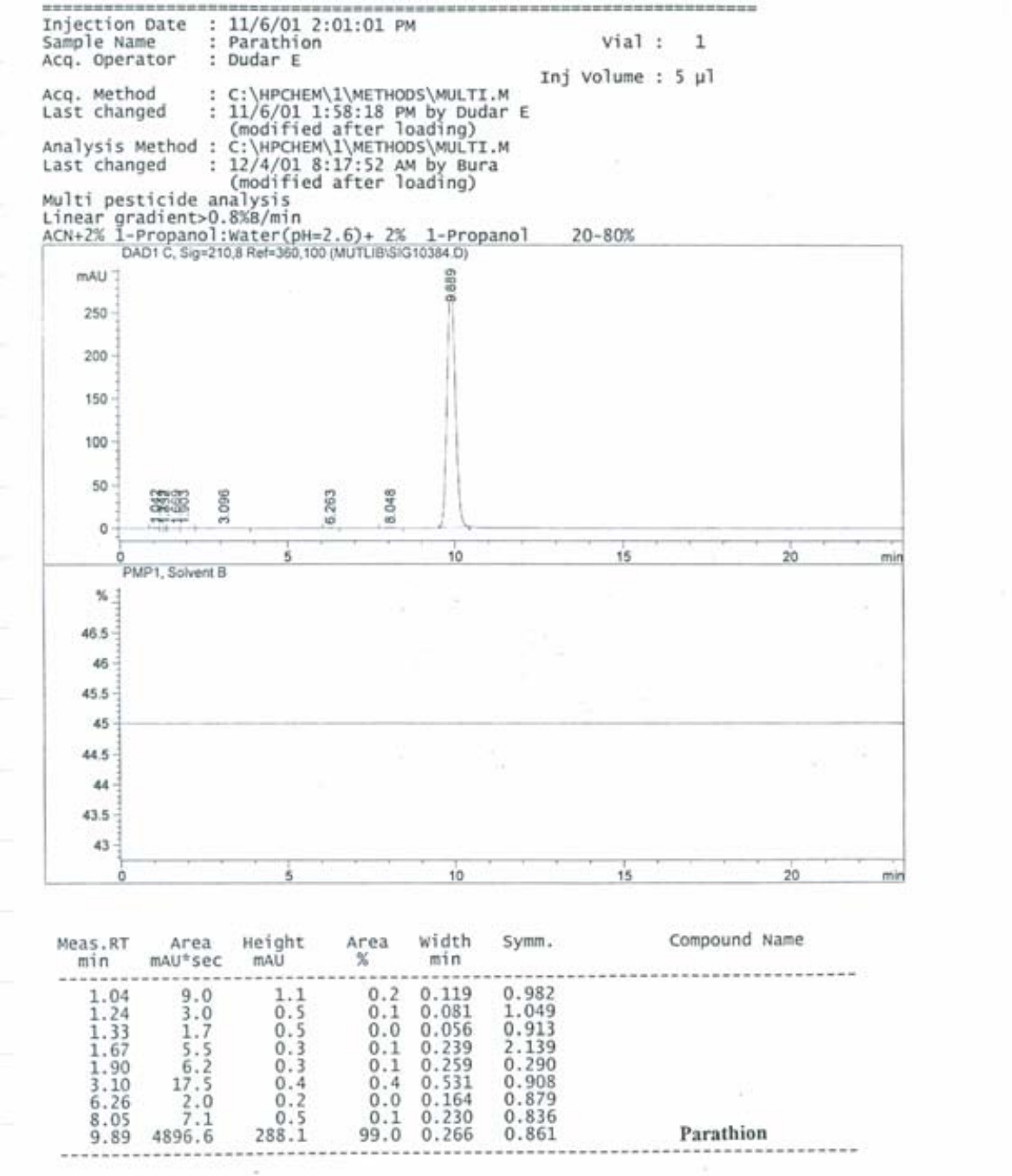

FIG. 2. Elution pattern of parathion under the isocratic condition determined from the scouting run (see FIG. 1.).

4.2. Spectral Analysis

The UV-Visible diode-array detector allows continuously acquiring spectra in the UV-VIS region of the spectrum, because the diode-array detector acquires all wavelengths simultaneously.

The analysis of spectral data adds a third dimension to our analytical data when using it with chromatographic data.

Figure 3 shows the structure of spectral information of a chromatogram. The three-dimensional plot of the five components SST pesticide mixture is shown in Figure 4. The ChemStation system enabled extracting spectral data from chromatographic signals to determine the optimum detection wavelength for each peak.

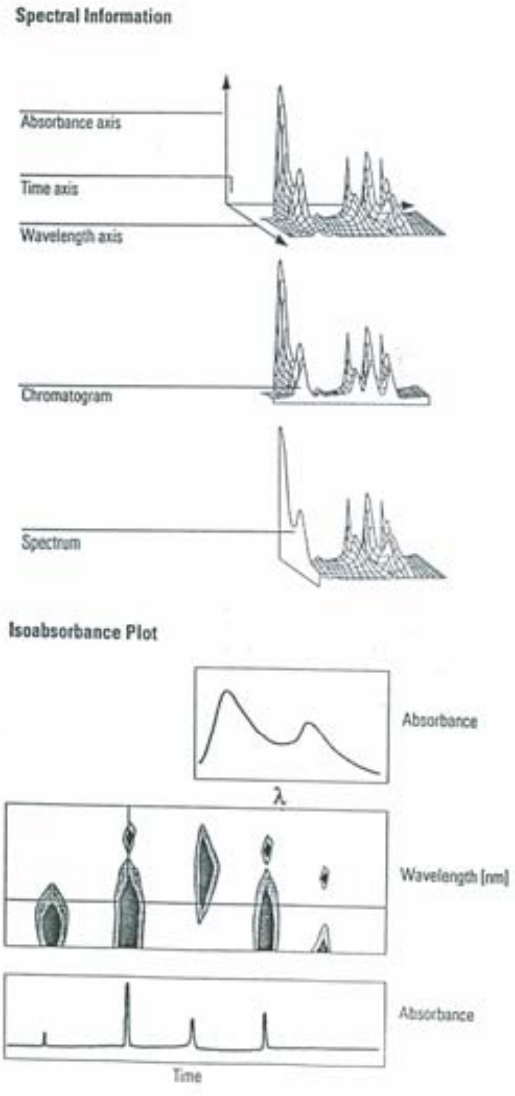

FIG. 3. Spectral information of a chromatogram.

4.2.1. Determining the Optimum Detection Wavelength

After suitable conditions have been developed for peak separation, the next step in method development is to determine the optimum detection wavelength for each peak. One technique is to present the peak intensity (absorbance), wavelength and time as a contour map called an isoabsorbance plot (Figure 3). This technique plots the spectral information as a series of isoabsorptive, concentric lines in the wavelength and time plane. This allows for all spectral information to be presented and inspected simultaneously. This technique is useful in the method development to find the optimum detection wavelength for each separated peak in a multi-component analyte.

On Figure 3 the wavelength corresponds to the horizontal cursor position in the isoabsorbance plot. When moving the cursor along the wavelength axis, the chromatogram is reconstructed in the lower window. The three dimensional plot of a 5-component mixture is shown on Figure 4. Figures 5 and 6 illustrate the isoabsorbance bit map of a 3-component test sample and the corresponding isoabsorbance contour lines.

4.2.2. Building a Spectrum Library

Absorption spectra can be acquired during the chromatographic run. Standard spectra was acquired from reference samples under well-defined chromatographic conditions and stored in a database (spectral library).

The collection of these UV spectra is attached in the Annex 1.

Beside the UV spectra there is a little table containing the characteristic minimum and maximum wavelengths and the actual absorbance values. This collection of UV spectra can be useful as a database to choose the right wavelength during method development.

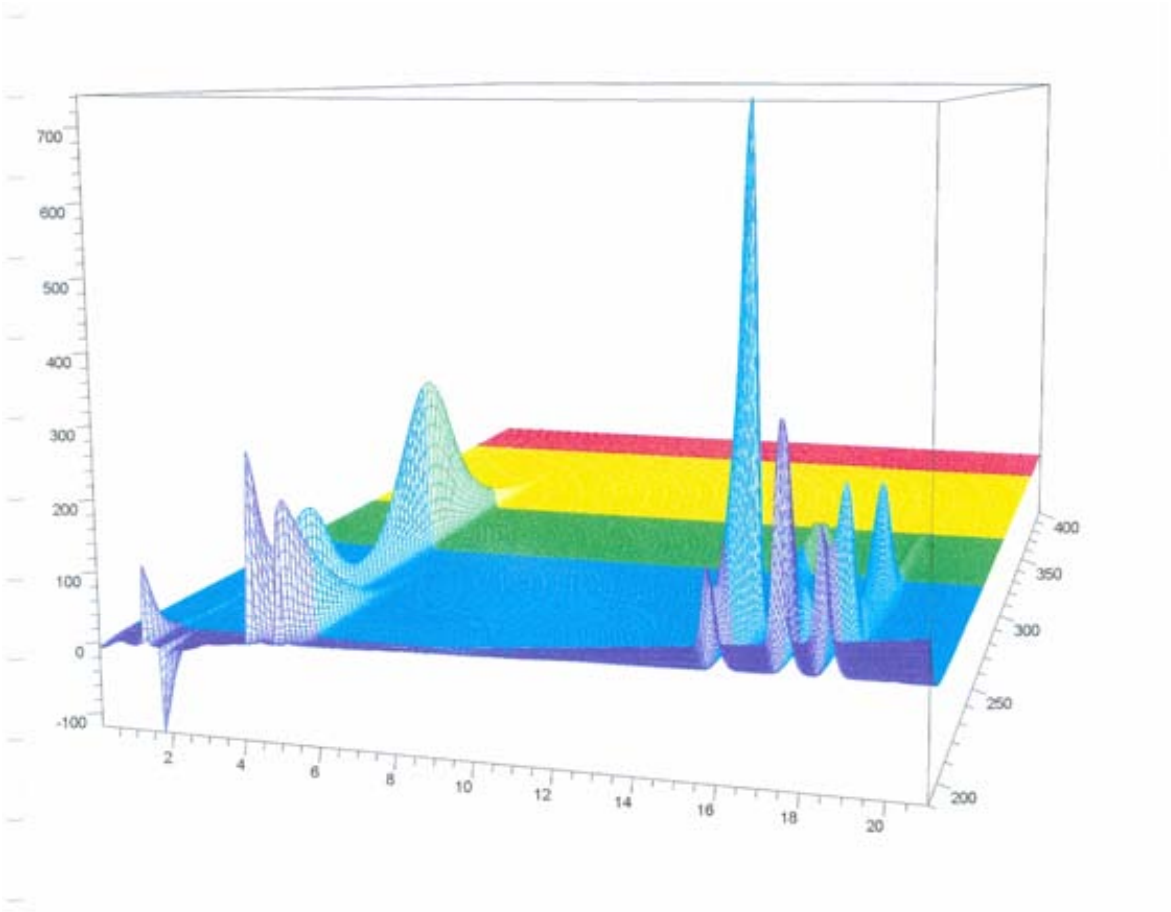

FIG. 4. Three dimensional plot of a five-component mixture.

The peak spectra of an unknown sample can be compared with those stored ones in spectral library database. Spectra can be overlaid for visual comparison and the similarity of standard and sample spectra can be calculated. The software can automate this process for all peaks within a chromatogram. In addition to such peak confirmation, the applied software can also perform a peak purity check. There are several data processing softwares produced by instrument manufacturers[1].

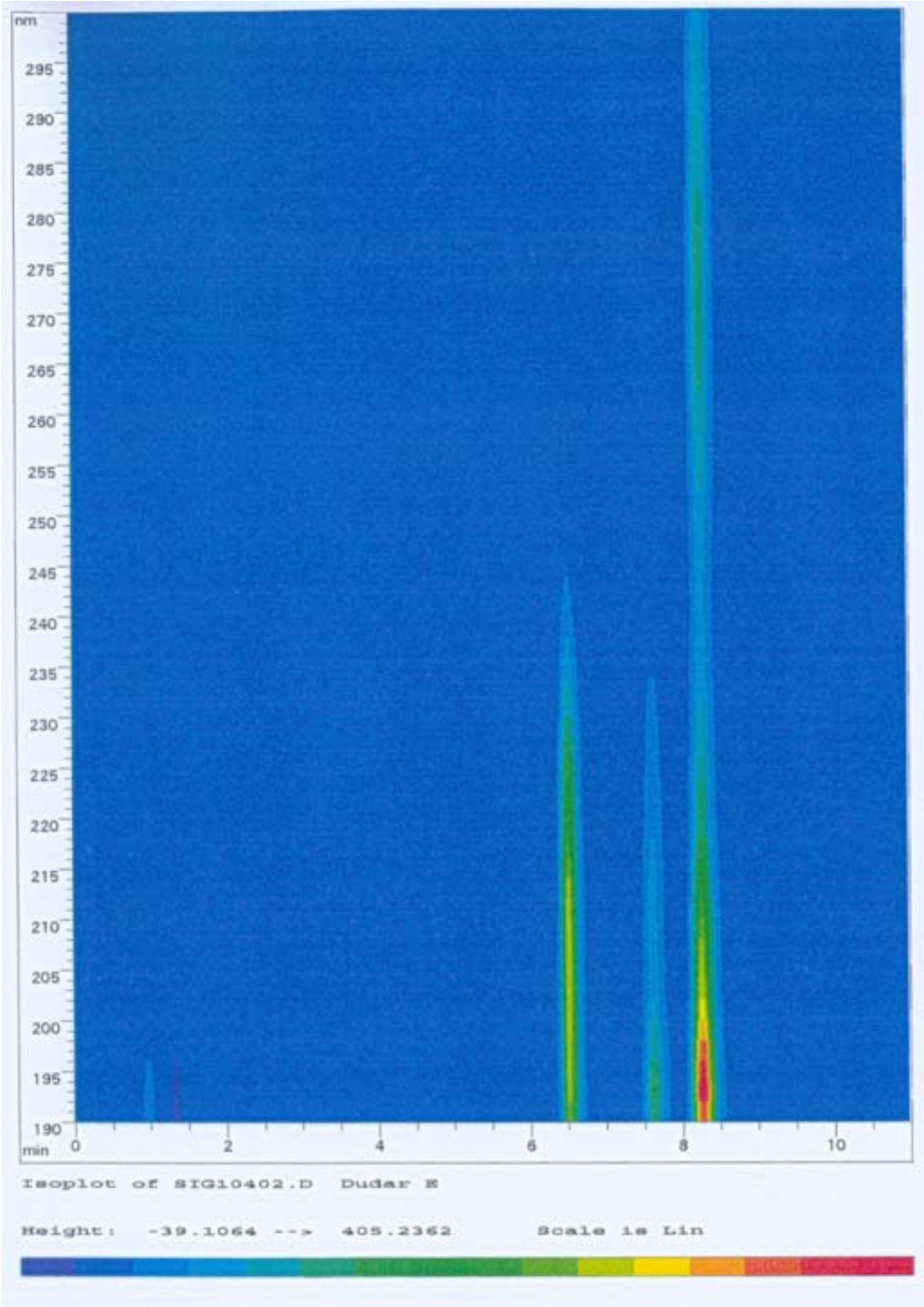

FIG. 5. The isoabsorbance bit map of a three-component mixture.

[1] The advanced ones have similar capabilities, which should be verified and possibly tested before a new instrument.

4.2.3. Peak purity

Peak purity testing involves the determination whether the peak is spectrally homogeneous from baseline lift-off to touchdown. For this purpose the applied software evaluated the peak purities automatically during the method development.

The most common data-analysis methods include normalization of spectra from different peak sections, spectra are selected from the up slope, apex, and down slope of the chromatographic peak, then normalised and overlaid. Differences in curve shape indicate a hidden impurity (normalization compensates for the changing concentration of the component as the peak elutes).

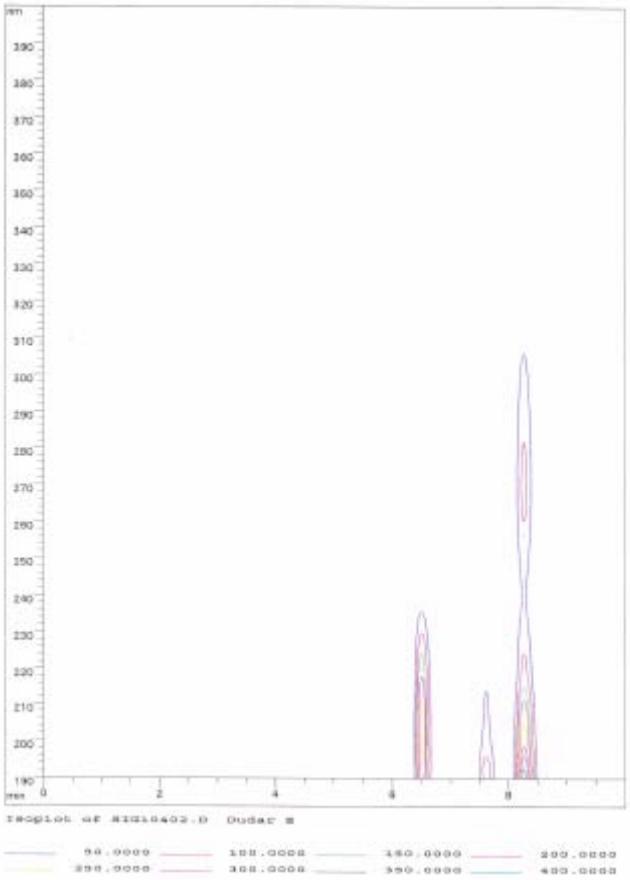

FIG. 6. The isoabsorbance contour lines of a three-component mixture.

Peak spectra can be overlaid with spectra from a library to compare the sample with either standards or previous samples.

Similarity /threshold is a comparison between the results for each spectrum across the peak. For a pure peak each point in the similarity ratio is below 1, the spectra overlay nicely, and differences between the spectra do not show a systematic pattern. The purity factor is within the calculated threshold limit.

If the peak spectra are not identical, the peak theoretically contains a spectral impurity. The spectral impurity can consist of one or more components. There are several causes of this, e.g., non-baseline separated peaks, or even background absorption.

Another useful feature of the software is its ability to compare first and second derivatives of spectral scans, allowing closer comparisons of peak minima and maxima (although increased noise can be a problem).

Two common problems interfere with purity testing:

1. Purity evaluation using non-linear data.
2. Purity evaluation with high background absorbance.

The non-linearity is caused by a too high sample concentration. This is easy to spot since the apex spectrum is a mismatch with all other spectra in the peak. The second problem occurs when regions of the spectrum with high background absorbance are included in the purity evaluation. Then the similarity ratio becomes very noisy and the spectral overlay is quite poor.

The purity factor is not an absolute measure of the peak purity. It is a function of the parameters used in the calculations especially the purity threshold.

A threshold curve shows the effect of noise on a given similarity curve. The effect increases rapidly toward the start and end of a peak. In essence, a threshold curve is a similarity curve of a pure peak with a background noise component. Therefore, the results need to be interpreted together with the set threshold. For pure peaks comparable results are generated for different parameter settings. For impure peaks a worst case analysis is done. Therefore a change in the threshold value can drastically influence the purity factor for an impure peak. If the purity factor is within the threshold value, (set manually, or calculated from threshold curve), the peak is classified as pure. If the purity factor exceeds the threshold value, the peak is classed as impure.

Note: The detected impurity is a spectral impurity, which does not necessarily mean a compound impurity. Spectral impurities can be caused by changes in solvent composition (gradients) or occur in peaks that are not baseline separated.

The peak-purity technique alters slightly in different softwares.

QUALITY CONTROL OF SELECTED PESTICIDES WITH HPLC

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Abstract

Laboratory data obtained on two different HPLC separation columns and detection by UV and DAD under repeatability conditions are presented and discussed. The behaviour of pesticides on different HPLC columns under gradient and isocratic conditions is evaluated concerning the applicability of respective methodologies. Representative chromatograms of real formulations and "empty" formlants are given for illustration.

1. INTRODUCTION

The rapid expansion of the use of chromatographic methods for pesticide products analysis has led to a wide choice of chromatography columns and variation in experimental parameters, along with increased demands on instrumentation. Whilst this approach suits agrochemical manufacturers and suppliers of a limited range of specific products, its inherent complexity can cause problems when implementing on a global basis in laboratories where many different pesticide products have to be analyzed. The need for the development and validation of new methods with higher sample output and lower cost of analysis has become imperative. An answer to this need is the use of 'multi-pesticide' methods.

The aim of the present work was the development and validation of a 'multi-pesticide' (MP) liquid chromatographic method with UV detection (HPLC-DAD), for the quantitative determination of two active ingredients (famaxadone and cymoxanil) of Equation Pro pesticide product.

We applied the principles described in Chapter 4.4 and used a scouting gradient to determine the initial elution conditions of the sample.

2. MATERIALS AND METHODS

2.1. HPLC system

A Shimadzu HPLC was used, which consisted of a quaternary gradient pump (LC-10ADVP), a vacuum degasser (DGU-14A), a diode array detector (SPD-M10ADVP), an oven (CTO-10AS), an auto sampler (SIL-10ADVP), and a data acquisition and processing computer software (Class-VP).

Two different columns were used:

i) Chromolith RP-18e, 100 mm length and 4.6mm internal diameter.

ii) LichroCART 150 mm length and 4.6mm internal diameter. With stationary phase Purospher STAR RP-18 endcapped (5μm)

2.2. Use of Chromolith RP-18e column

2.2.1. Initial 'optimum' chromatographic conditions

The optimum chromatographic conditions for the analysis of famaxadone and cymoxanil were the following:

Mobile phase (eluent):

Solvent A: Acetonitrile + 2% 1-propanol
Solvent B: pH = 2.6 Water (pH adjusted to 2.6 with H_3PO_4) + 2% 1-propanol

Gradient of mobile phase:

Time (min)	Solvent A	Solvent B	Flow rate (ml/min)
0	20	80	1
4	20	80	1
6	50	50	2
12	50	50	2

Injection volume: 5 µl
Detection: UV 230 nm
Column Temperature: 40°C
Solvent: Acetonitrile

The system was allowed to equilibrate under the initial solvent condition (20% A) for at least 5 min between each run, and the gradient run was completed with a 5 min hold at 100% A.

The use of 1-propanol in both solvents (A and B) helped the equilibration and stability of mobile phase resulting in very stable and reproducible retention time.

2.2.2. System suitability test with Chromolith RP-18e

After determining the 'initial optimum' chromatographic conditions, system suitability test was performed under the following conditions:

Mobile phase (eluent): Acetonitrile/Water (50/50, v/v)
Flow rate: 1 ml/min
Injection Volume: 1.0 µl
Detection: UV 254 nm
Column Temperature: 40°C
Solvent: Methanol
Isocratic Analysis

The test compounds for system suitability test in Chromolith RP-18e were: thiourea, nitroaniline and nitrobenzene.

2.2.2.1. Preparation of system suitability test (SST) solutions

Thiourea (99% pure), nitroaniline (99% pure) and nitrobenzene (99% pure, all purchased from Merck) were used for the preparation of the system suitability test solution.

Stock solutions for thiourea, nitro aniline and nitrobenzene were prepared by adding the appropriate amount of the respective substance in a 100 ml volumetric flask, diluting to the volume with methanol and kept refrigerated at -18°C. The final concentration of each compound was about 1 mg/ml.

The *working solution* was prepared by adding the appropriate volume from the stock solutions and diluting to volume with methanol. The final concentration of this solution was 0.3 mg/ml for each compound.

2.2.2.2. Results of the SST

The results of the tests are summarized in Tables 1 and 2. Thiourea was used as unretained compound (t_0 = 87.06 sec) in the calculations.

TABLE 1. STATISTICAL EVALUATION OF SEVEN REPLICATE INJECTIONS OF TEST MIXTURE IN CHROMOLITH RP-18E COLUMN [a]

		Thiourea	Nitroaniline	Nitrobenzene
Peak area	CV %	0.507	0.427	0.474
Retention time	Average	1.451	1.952	2.475
	CV %	0	0	0
Asymmetry factor (at 10%)	Average	0.996	1.266	1.226
	CV %	2.648	4.693	4.514
Peak width (at 505)	Average	0.09	0.07	0.08
	CV %	0	0	0

(a) Where CV = 0 reported, no difference could be observed in the measured values.

TABLE 2: PERFORMANCE CHARACTERISTICS OF CHROMOLITH RP-18E COLUMN

Peak No.	Compound	t_R (sec)	t_R' (sec)	k (sec)	N_{eff}/m (plates/m)	W_h (sec)	Rs
1.	Thiurea	87.06	0	0.0	37484	0.09	-
2.	Nitroaniline	117.12	30.06	0.35	28985	0.07	221.7
3.	Nitrobenzene	148.5	61.44	0.71	100873	0.08	246.9

2.2.3. Method development

The chromatographic conditions described in section 2.2.1. were applied in the elaboration of the method.

2.2.3.1. Preparation of stock and working solutions

Analytical standards of famoxadone (99.4%) and cymoxanil (99.6%) were provided by commercial suppliers. Each of the compounds was supplied with a certificate of analysis stating the exact purity.

Stock solutions for each active ingredient were prepared by weighing the appropriate amount of the respective analytical standard in a 50 ml volumetric flask and diluting to the volume with acetonitrile. The final concentration of these solutions was 2.432 mg/ml for cymoxanil and 1.996 mg/ml for famoxadone. The five working solutions for each active ingredient were prepared by five independent dilutions of the stock solution with acetonitrile (Table 3). The same working solutions were used with both HPLC columns.

2.2.3.2. Sample preparation

For sample preparation, the appropriate quantity of formulation, containing ~266 mg (±5%) of active ingredient, was weighed in a 100 ml volumetric flask, followed by addition of ~75ml acetonitrile. This solution was sonicated for ½ hour. Then the mixture was left to reach room temperature and diluted to the volume by acetonitrile (concentrated sample extracts). The solution was filtered through 45 μm silica filters. Sufficient quantity of this solution was diluted in 10 ml volumetric flasks with acetonitrile so that the final concentration fell within the calibration curve limits (diluted sample extracts).

2.2.3.3. Performance characteristics of the method

The performance of the method were characterised by the repeatability, linearity, specificity and precision.

TABLE 3. ANALYTE CONCENTRATION IN INDIVIDUAL WORKING SOLUTIONS

Active ingredient	Stock solution concentration (mg/ml)	Working solution concentration (mg/ml)
Cymoxanil	2.432	0.730 0.976 1.219 1.464 1.716
Famoxadone	1.996	0.599 0.798 1.002 1.203 1.404

Repeatability of injections was tested for each active ingredient separately, using the medium calibrated level working standard solution, under the selected chromatographic conditions. Five replicate injections of medium calibrated level working solution were made. The mean value and the relative standard deviation (CV %) of the peak area of the working standards of cymoxanil and famoxadone and the retention times on Chromolith RP-18e column are presented in Table 4.

TABLE 4. REPEATABILITY TEST OF INJECTIONS

	Cymoxanil		Famoxadone	
	Peak Area	t_R	Peak Area	t_R
Mean	12263229	3.3834	7000652	10.560
CV %	0.6674	0.2615	0.8087	0.09

The *linearity of response* was determined by analysing in duplicate five working solutions of different concentrations for each of the tested active ingredients. The selected concentrations were at about 0.6, 0.8, 1, 1.2 and 1.4 times the nominal concentration of a.i. in formulated products. The standard working solutions were prepared independently from the stock solutions for multi-point calibration.

After having performed the multi-point calibration (5 × 2), the critical parameters (correlation coefficient, slope, intercept and standard deviation of relative residuals) were determined (Table 5). The calibration chart is shown in Figure 1.

The calibration parameters were acceptable, except the relatively large intercept. The linear regression of the calibration data for cymoxanil gave 95% confidence limits of -1115413 and -686836, and for famoxadone -958408 and -619200, which indicated that the intercepts were significantly different from zero. Consequently, single-point calibration should not be used under such conditions.

2.2.3.4. Specificity of the separation on Chromolith RP-18e column

The ability of the analytical method to distinguish the analyte to be determined from degradation products, metabolites or known additives was investigated.

Lack of interference on Chromolith RP-18e column is demonstrated by the analysis of the concentrated blank formulations and the concentrated sample extracts (Figure 2).

TABLE 5. CALIBRATION DATA OF CYMOXANIL AND FAMOXADONE USING CHROMOLITH RP-18E COLUMN

	CYMOXANIL	FAMOXADONE
Concentration of calibration solutions [g/g]	0.000944469	0.000775811
	0.001260209	0.0010326
	0.001580933	0.001298605
	0.001893909	0.001561003
	0.002223733	0.001822545
Slope a:	7661116183	5588946337
Intercept	- 901126.7191	- 745949.7445
Correlation coefficient	0.9998	0.9998
S_{rr}	0.0092	0.0073

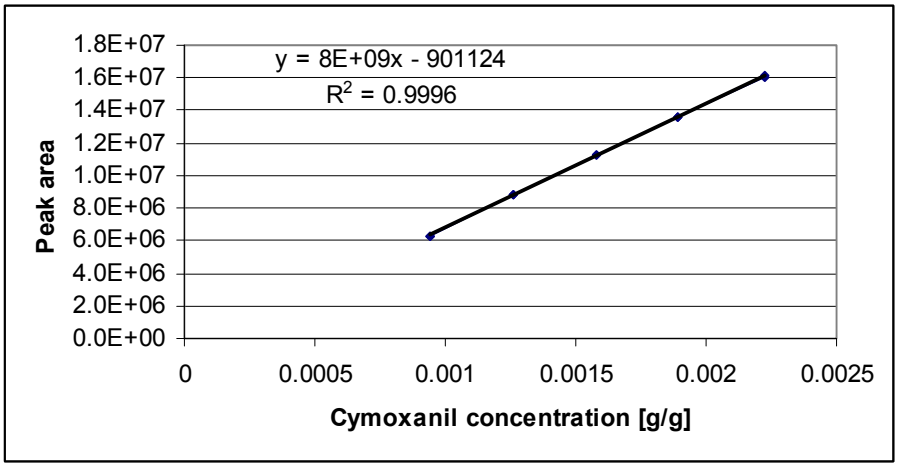

FIG. 1. Regression line and regression equation for cymoxanil using Chromolith RP-18e column.

2.2.4. Analysis of different batches of commercial products

Samples taken from different batches of Equation Pro (nominal active substance content: cymoxanyl 30.0% and famoxadone 22.5%) were analyzed in duplicate according to the draft MP procedures. The results are presented in Table 6 and on Figure 3.

The results indicate that the grand average of cymoxanil content is significantly lower from the grand average of the reference value. The variance of the measured AS content is larger than the reference values (but not significantly) due to one large measured value (Figure 3).

Comparing the difference of the measured concentrations of cymoxanil and famoxadone in sample 4 (Figure 3) it is clear that the large value is partly due to sample processing error as higher concentrations were measured for both active substances, but the cymoxanil result was also affected by the error in chromatographic determination.

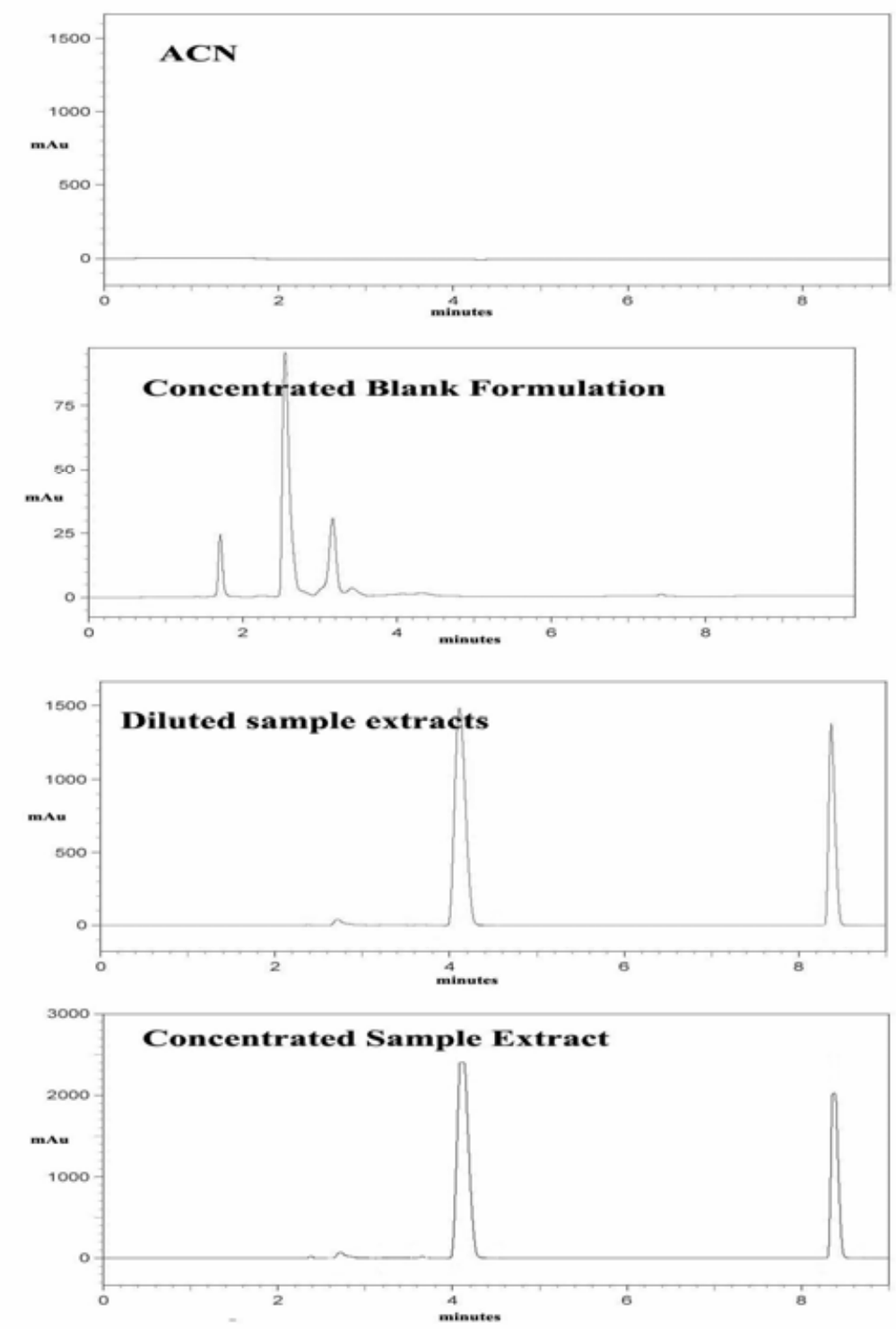

FIG. 2. Specificity of the separation on Chromolith RP-18e column.

TABLE 6. RESULTS OF THE ANALYSIS OF EQUATION PRO WITH CHROMOLITH RP-18E COLUMN

	Cymoxanil [g/kg]			Famoxadone [g/kg]		
Sample	Calculated	Average	Reference	Calculated	Average	Reference
1	310.608	310.986	296	224.814	224.932	229
	311.364			225.049		
2	309.824	311.506	298	217.387	218.075	226
	313.189			218.763		
3	304.903	305.219	286	219.753	220.075	232
	305.535			220.397		
4	342.123	343.084	302	246.417	246.851	233
	344.046			247.285		
5	309.738	309.567	303	222.555	222.553	226
	309.396			222.551		
Average		297	316.1		229.2	226.5
CV %		2.28	4.84		1.43	5.15

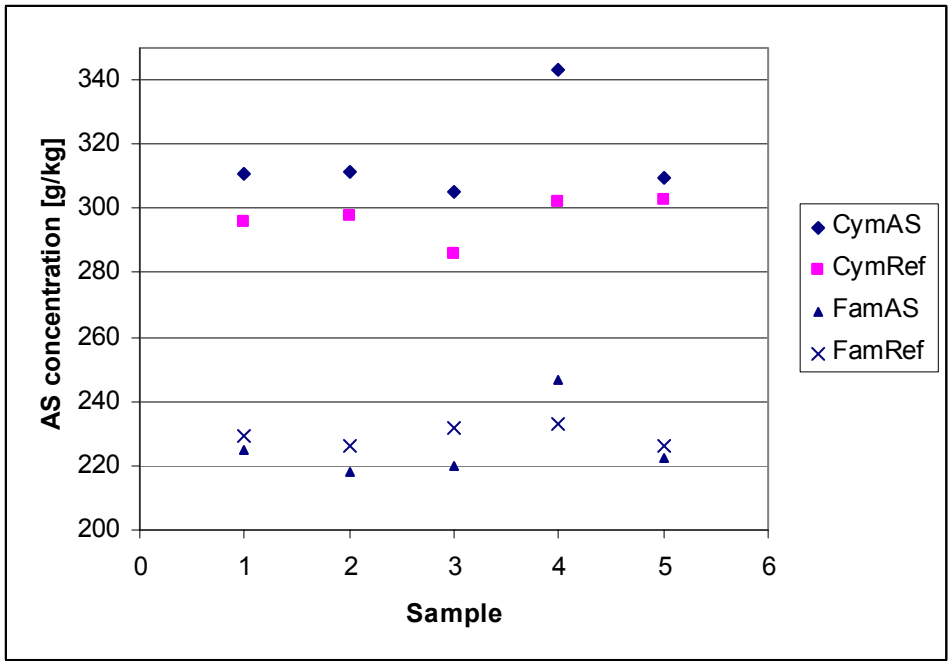

FIG. 3. Graphical presentation of the results of the analysis of 5 batches of Equation Pro.

2.3. Use of Purosphere STAR RP-18 column

2.3.1. 'Initial optimum' chromatographic conditions

The optimum chromatographic conditions for the analysis of famoxadone and cymoxanil were the following:

Column: LichroCART
Column length: 150 mm
Internal diameter: 4.6 mm
Stationary phase: Purosphere Star RP
Mobile phase (eluent):
Solvent A: Acetonitrile + 2% 1-propanol
Solvent B: pH = 2.6 Water (adjust pH with H_3PO_4) + 2% 1-propanol

TABLE 7. GRADIENT OF SOLVENTS FOR THE ANALYSIS OF FAMAXADONE, CYMOXANIL

Time (min)	Solvent A	Solvent B	Flow rate (ml/min)
0	35	65	1
4.5	70	30	2
8.5	35	65	1
10	35	65	1

Injection Volume: 5 µl
Detection: UV 230 nm
Column Temperature: 40°C
Solvent: Acetonitrile

The system was allowed to equilibrate under the initial solvent condition (35% A) for at least 5 min between each run, and the gradient run was completed with a 5 min hold at 100% A.

2.3.2. System suitability test

After determining the 'initial optimum' chromatographic conditions, system suitability test was performed under the following conditions:

Mobile phase (eluent): Acetonitrile/Water (50/50, v/v)
Flow rate: 1 ml/min
Injection Volume: 1.0 µl
Detection: UV 254 nm
Column Temperature: 40°C
Solvent: Acetonitrile/Water (75/25, v/v)
Isocratic Analysis

Test compounds for system suitability test in LichroCART/Puroshpere column were: uracil, benzamide, benzene, benzophenone and biphenyl. The test substances were purchased from Phenomenex and accompanied with its certificate of analysis.

Uracil was used as an unretained compound (t_0 = 88.98 sec). The results of the tests obtained from six replicate injections of the text mixture are summarized in Tables 8 and 9.

2.3.3. Method development

The chromatographic conditions described in section 2.3.1. were applied for the elaboration of the method.

The sample preparation and preparation of analytical standard and sample extract solutions were the same what was described under section 2.2.

TABLE 8. STATISTICAL EVALUATION OF SIX REPLICATE INJECTIONS OF TEST MIXTURE

		Uracil	Benzamide	Benzene	Benzophenone	Biphenyl
Peak area	CV %	0.677	0.303	0.638	0.239	0.516
Retention time	Average	1.483	1.707	3.085	3.447	5.042
	CV %	0.634	0.550	0.402	0.362	0.256
Asymmetry at 10%	Average	1.26	1.20	1.40	1.30	1.42
	CV %	7.68	4.80	2.23	2.17	2.71
Peak width at 50%	Average[a]	0.06	0.11	0.12	0.14	0.24

(a): No difference in measured values was found, the CV = 0 is not reported.

TABLE 9. PERFORMANCE CHARACTERISTICS OF PUROSPHERE STAR RP COLUMN

Peak No.	**Compound**	**t_R (sec)**	**t_R' (sec)**	**k (sec)**	**N_{eff}/m**	**W (sec)**	**Rs**
1.	Uracil	88.98	0	0.0	4473	0.06	
2.	Benzamide	102.42	13.44	0.15	3037	0.11	93.3
3.	Benzene	185.1	96.12	1.08	120223	0.12	424.2
4.	Benzophenone	206.82	117.84	1.32	132422	0.14	98.6
5.	Biphenyl	302.52	213.54	2.4	149297	0.23833	298.5

2.3.3.1. Performance characteristics of the method

The *repeatability of injections* was determined for each active ingredient separately injecting 5 times the medium calibrated level working solution (Table 10).

TABLE 10. REPEATABILITY TEST OF INJECTIONS

	Cymoxanil		Famoxadone	
	Peak Area	t_R	Peak Area	t_R
Mean	10129990	4.117	6055212	8.376
CV %	0.67	0.00	0.51	0.14

The *linearity of response* was determined by analysing in duplicate five working solutions of different concentrations for each of the tested active ingredients (Table 5).

After having performed the multi-point calibration (5 × 2), the critical parameters (correlation coefficient, slope, intercept and standard deviation of relative residuals) were determined (Table 11).

TABLE 11. CALIBRATION DATA OF CYMOXANIL AND FAMOXADONE ON PUROSPHERE STAR RP COLUMN

	CYMOXANIL	FAMOXADONE
Slope a:	8092797845	5842870566
Intercept	-1570283.2	-618107
Correlation coefficient	0.9998	0.9999
S_{rr}	0.0076	0.0066

As it was the case for the Chromolith column, the calibration parameters were acceptable, except the relatively large intercept. The linear regression of the calibration data for cymoxanil gave 95% confidence limits of – 1783107 and – 1357454 and for famoxadone – 765678 and – 470535, which indicated that the intercepts were significantly different from zero. Consequently, single-point calibration should not be used under such conditions. As similar results were obtained on both columns, the injected volume or the accuracy of standard solutions could be the cause of error.

2.3.3.2. Specificity of the separation on Purosphere column:

Figure 4 indicates that there was no observable interference from the blank formulation. Thus it was concluded that the separation power and the selectivity of the elution system is sufficient for the determination of the AS content of the formulated product.

2.4. Analysis of different batches of commercial products

Five different batches were analyzed in duplicate as described previously. The results are presented in Table 12.

There was no statistically significant difference between the average concentrations measured with the MP method and obtained with the reference method, or between the reproducibility of the two procedures.

TABLE 12. RESULTS OF THE ANALYSIS OF EQUATION PRO ON LICHROCHART/PUROSPHERE COLUMN

Sample	**Cymoxanil content [g/kg]**			**Famoxadone content [g/kg]**		
	Calculated	**Average**	**Reference**	**Calculated**	**Average**	**Reference**
1	315.171	315.293	296	239.229	239.296	229
	315.415			239.364		
2	304.222	304.289	298	220.514	220.776	226
	304.356			221.038		
3	301.168	300.460	286	225.269	224.947	232
	299.751			224.625		
4	300.292	299.919	302	225.289	222.612	233
	299.545			219.935		
5	290.951	286.833	303	219.042	215.970	226
	282.715			212.898		
Ave		301.36	297.0		224.72	229.2
CV %		3.39	2.28		3.91	1.43
CVr %	0.92			1.18		

3. COMPARISON OF THE RESULTS OBTAINED ON TWO COLUMNS

The results obtained with the two columns for both active ingredients were compared with paired t-test. The critical t-value is 2.776, the calculated t-values for cymoxanil was 1.76 and for famoxadone 0.272.

Results obtained with the two columns are not significant different as t_{crit} is < t_{calc}. It can be concluded that the MP method was validated for the tested active ingredients on two columns.

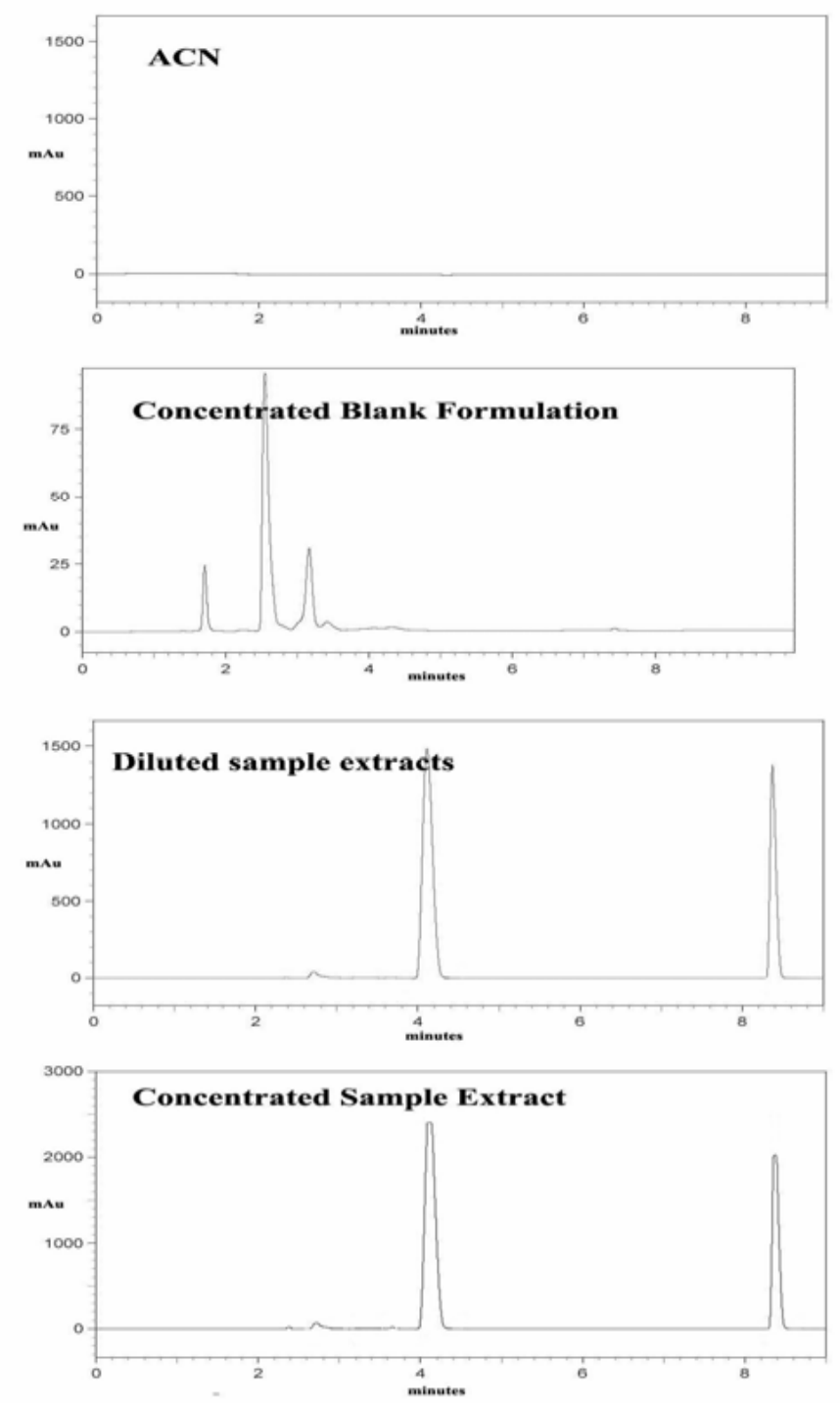

FIG. 4. Illustration of the specificity of the separation on LichroCart/Purosphere column.

MULTI-ANALYTE SEPARATION METHODS FOR HPLC DETERMINATION OF THE ACTIVE INGREDIENTS OF PESTICIDES

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Abstract

The practical quality control of selected pesticides, such as carbamates, organophosphorous compounds, phthalimides, pyrethroids, with HPLC is described. Detailed descriptions are given of materials and methods used, including sample preparation and HPLC operating conditions. The relationship between pH value of the HPLC eluent and the $\log P_{ow}$ is discussed, illustrated by chromatograms, graphics and tables. The results are also compared with those elaborated by. E. Dudar and presented above.

1. INTRODUCTION

In many cases the HPLC analysis seems to be the most appropriate technique for the qualitative identification and quantitative evaluation either for quality control of pesticides or for determination of pesticide residues. The aim of this study was to develop an HPLC separation system that enables the identification of active ingredients present in a pesticide formulation or in a sample based on the separation of active ingredients by a linear gradient programme of water-acetonitrile mobile phase using C-18 column and UV detection.

The time requirement of a single run is 36 minutes including stabilization time. The retention times of 53 active ingredients were determined. The reproducibility of retention time is excellent. The system is applicable to separate wide range of pesticides. The separation sequence mainly follows the n-octanole/water partition coefficient. A significant change in retention times occurs for acidic (e.g. phenoxy acetic acids, retention time increases) or basic (e.g. carbendazim, retention time decreases) analytes when acidified eluent is used instead of neutral one. Significant asymmetry of peak was observed in a few cases (e.g. thiabendazole).

The retention times given in this report are valid for the combination of the specified column and gradient programme. The method is applicable for screening purposes to separate and identify the potential active substances present either in a pesticide product of unknown identity or pesticide residues in a sample. The gradient system itself is not recommended for the evaluation of active ingredient content of pesticide products as the analysis time is longer than with properly selected isocratic elution, but may be appropriate for the analysis of a complex formulation.

2. MATERIALS AND METHODS

2.1. HPLC system

- Pump HP 1100 (binary).
- Injector with 20 µl loop.
- Analytical column (LiChrospher 100 RP-18 endcapped, 250 × 4 mm, 5 µm, Merck, Cat. No. 50995) thermostated at 30°C.
- UV detector (Waters 745B, operated at 238 nm and at 260 nm wavelength simultaneously); AUFS = 0.1 range.
- Chromatographic evaluation system, Mosaic.

2.2. Reagents

- Water, HPLC grade (Merck, LiChrosolv).
- Water, HPLC grade, acidified with 85% phosphoric acid to pH = 2.6.
- Acetonitrile, HPLC grade (Merck, LiChrosolv, Cat No. 1.00030.2500).
- Ortho-Phosphoric acid, 85% GR for analysis.
- Pesticide analytical standards: certified purity from different manufacturers.
- Stock solutions of analytical standards: prepare approximately 0.5 mg/ml analytical standard solutions in methanol.
- Working solutions: prepare approximately 0.05 mg/ml analytical standard solutions in water:methanol = 1:1 solvent (v/v). These solutions should be used for the preliminary determination of retention times of individual pesticides. Pesticides with isomer composition (e.g., dimethomorph, cypermethrin) may result two or more peaks.
- Pesticide mixtures: Prepare mixed stock solutions of pesticides from the individual stock solutions in methanol. These mixtures may be used to check the stability of the chromatographic system (retention time and detector sensitivity). Components of the mixture should be selected based on the retention times determined earlier for the individual pesticides. Prepare working dilutions daily with water:methanol = 1:1 (v/v) to get approximately 0.01 mg/ml (20 ng injected).

2.3. Method

2.3.1. Determination of retention times of individual analytical standards

Inject 20 µl working solution equivalent to approximately 100 ng analytical standard. Determine the retention time of each analytical standard at both wavelengths.

Gradient programme

Solvent A: water, HPLC grade (or acidified water, HPLC grade)
Solvent B: acetonitrile, HPLC grade

Note: in the majority of measurement the water:acetonitrile mixture was used for the gradient programme. In certain measurements HPLC grade water, acidified with phosphoric acid to pH 2.6, was used to establish data for ionisable compounds too.

Program of the solvent composition for HPLC pump is according to Table 1 (see also Figure 1).

TABLE 1. GRADIENT PROGRAMME FOR SEPARATION OF SUBSTANCES

Time	B % setting
0	20
5	20
16	100
22	100
29	20
36	20

Method 1: apply the gradient programme with HPLC grade water

Method 2: apply the gradient programme with HPLC grade acidified water

Adjust the flow rate to 1.2 ml/min.

The gradient programme is abbreviated as H43Gr2 (for column ID. H43) or H44Gr2 (for column ID. H44) in the figures.

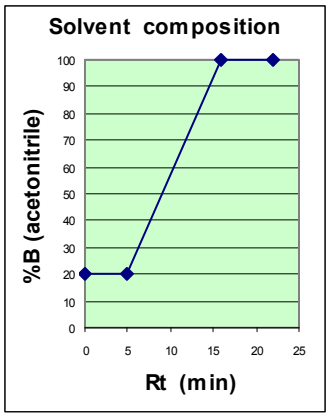

FIG. 1. Increase of organic solvent in the gradient programme.

3. EVALUATION OF RESULTS

The retention times of 53 individually injected active ingredients were determined with the gradient programme using water: acetonitrile mixture. Retention times of active ingredients are given in Table 2. This table also contains the logarithm of n-octanol – water distribution coefficient ($logP_{OW}$) values taken from the Pesticide Manual.

The compound tested covers wide range of chemical classes e.g., carbamates, organophosphorus compounds, phthalimides, pyrethroids, etc. In many cases the HPLC analysis seems to be the most appropriate technique for the qualitative identification and quantitative evaluation either for quality control of pesticides or for determination of pesticide residues. Figures 2–5 show part of the chromatograms of pesticides mixtures and several other active ingredients (e.g. captan, tetraconazole on Figure 2 and chlorothalonil, chlorpyrifos-methyl on Figure 3) using the gradient programme with Method 1. Furthermore, the positions of retention times are given for additional pesticides.

TABLE 2. $LOGP_{OW}$ AND RETENTION TIME VALUES OF 53 PESTICIDES MEASURED WITH GRADIENT PROGRAMME

Active substance	**logP (log Kow)**	**Rt (min)**
Methomyl	0.093	4.65
Thiamethoxam	-0.13	5.74
Imidacloprid	0.57	8.81
Dimethoate	0.704	9.37
Acetamiprid	0.8	9.63
Cymoxanil	0.67	10.31
Aldicarb	2.00	10.9
Thiophanate methyl	1.5	11.79

Active substance	logP (log Kow)	Rt (min)
Carbaryl	1.59	12.6
Metalaxyl	1.71	12.831
Dimethomorph	2.63	13.41
Fludioxonyl	4.12	14.04
Azinphos-methyl	2.96	14.07
Methidathion	2.2	14.12
Captan	2.8	14.2
Azoxistrobin	2.5	14.24
Tetraconazole	3.56	14.4
Fenhexamid	3.51	14.47
Propyzamide	3.15	14.67
Diflubenzuron	3.89	14.84
Malathion	2.75	14.92
Triazophos	3.34	15.0
Folpet	3.11	15.04
Chlorothalonil	2.92	15.05
Procimidon	3.14	15.18
Vinclozolin	3	15.44
Bupirimate	3.90	15.5
Dichlofluanid	3.7	15.5
Kresoxim-methyl	3.4	15.51
Triflumuron	4.91	15.52
Cyprodinil	3.9	15.79
Indoxacarb	4.65	16.2
Diazinon	3.3	16.23
Phosalone	4.01	16.3
Teflubenzuron	4.3	16.33
Trifloxystrobin	4.5	16.38
Chlorpyrifos-methyl	4.24	16.41
Pirimiphos-methyl	4.2	16.61
Lufenuron	5.12	16.68
Flufenoxuron	4	16.94
Imazalil	3.82	16.942
Trifluralin	4.83	17.27
Bromopropylate	5.4	17.3
Chlorpyrifos	4.7	17.36
Endosulfan	4.74	17.43
Beta-cyfluthrin	5.94	17.62
Fenpyroximate	5.01	17.69
Lambda-cyhalothrin	7	17.75
Cypermethrin	6.6	17.81

Active substance	logP (log Kow)	Rt (min)
Deltametrin	4.6	17.98
Fenasaquin	5.51	18.31
Permetrin	6.1	18.5
Bifenthrin	8.15	18.7

The chromatographic conditions selected provides a reasonable analysis time for a single run (36 minutes including equilibration). Due to the fast change in solvent composition, the system cannot be in equilibration during the run. Nevertheless the reproducibility of retention times is good, better than 0.1 min within one month time for compounds tested in a mixture (e.g. Hmix 3 in Figure 3). Between-column reproducibility is also acceptable; the gradient programme can be used without modification with a new column.

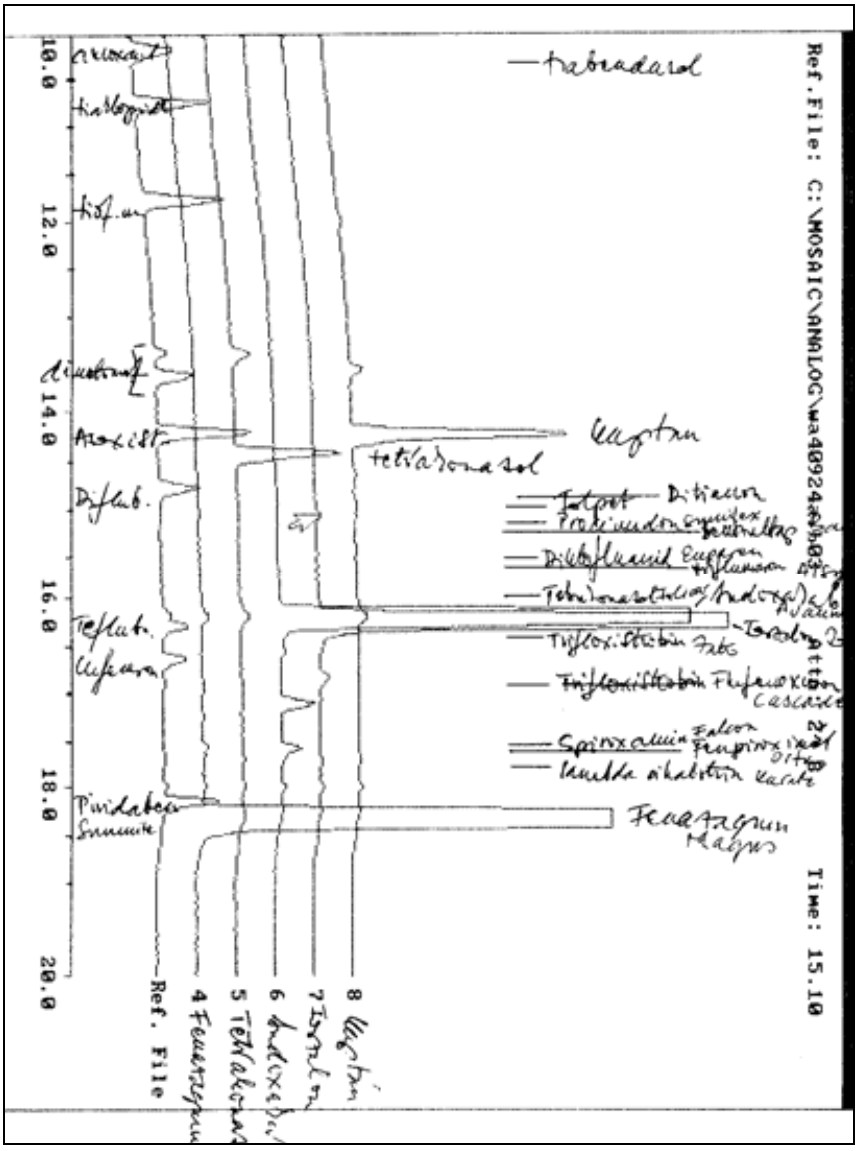

FIG.2. Chromatogram of mixture of pesticides and several other active ingredients using the gradient programme.

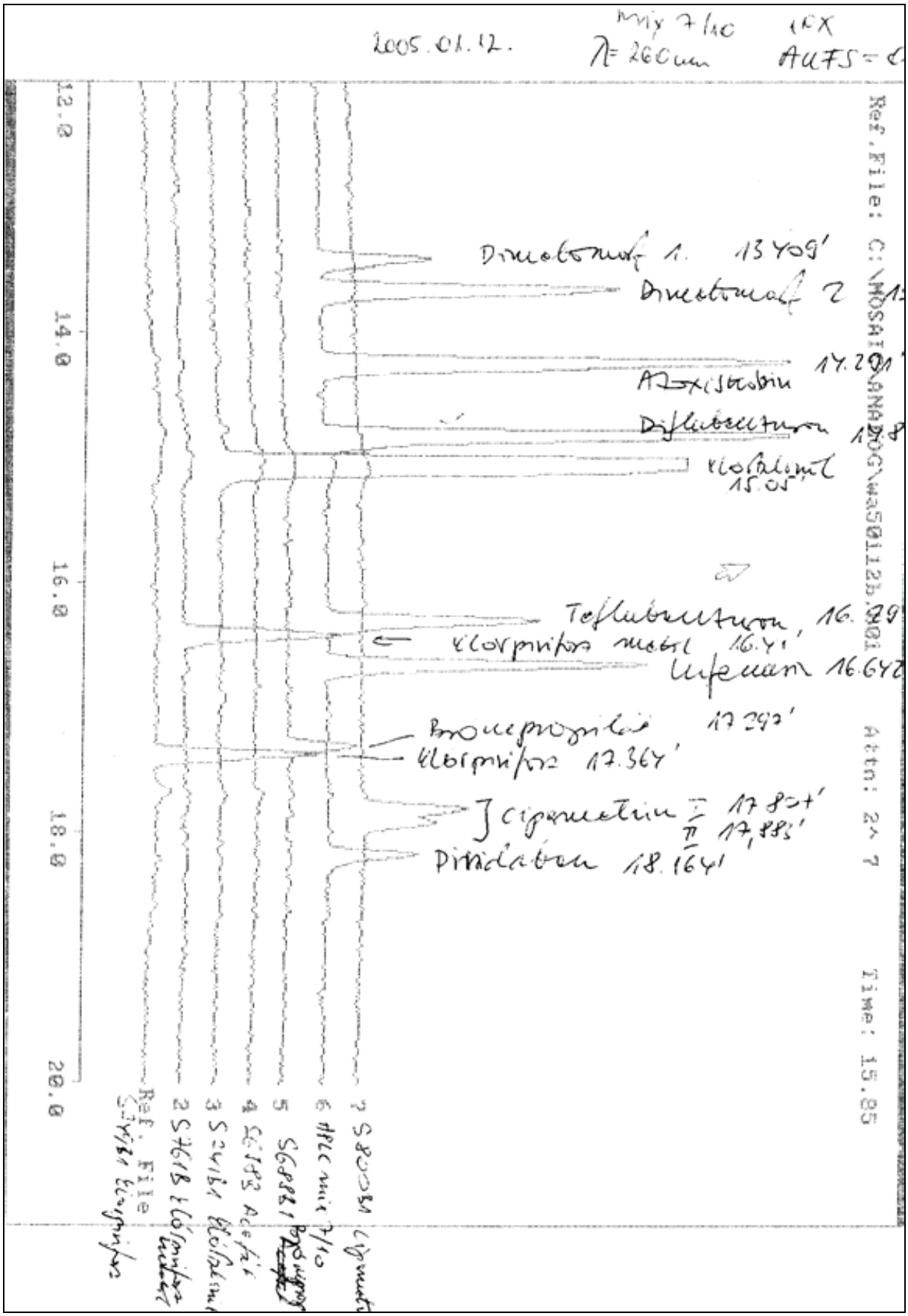

FIG. 3. Chromatogram of Hmix and other standard solutions using the gradient programme.

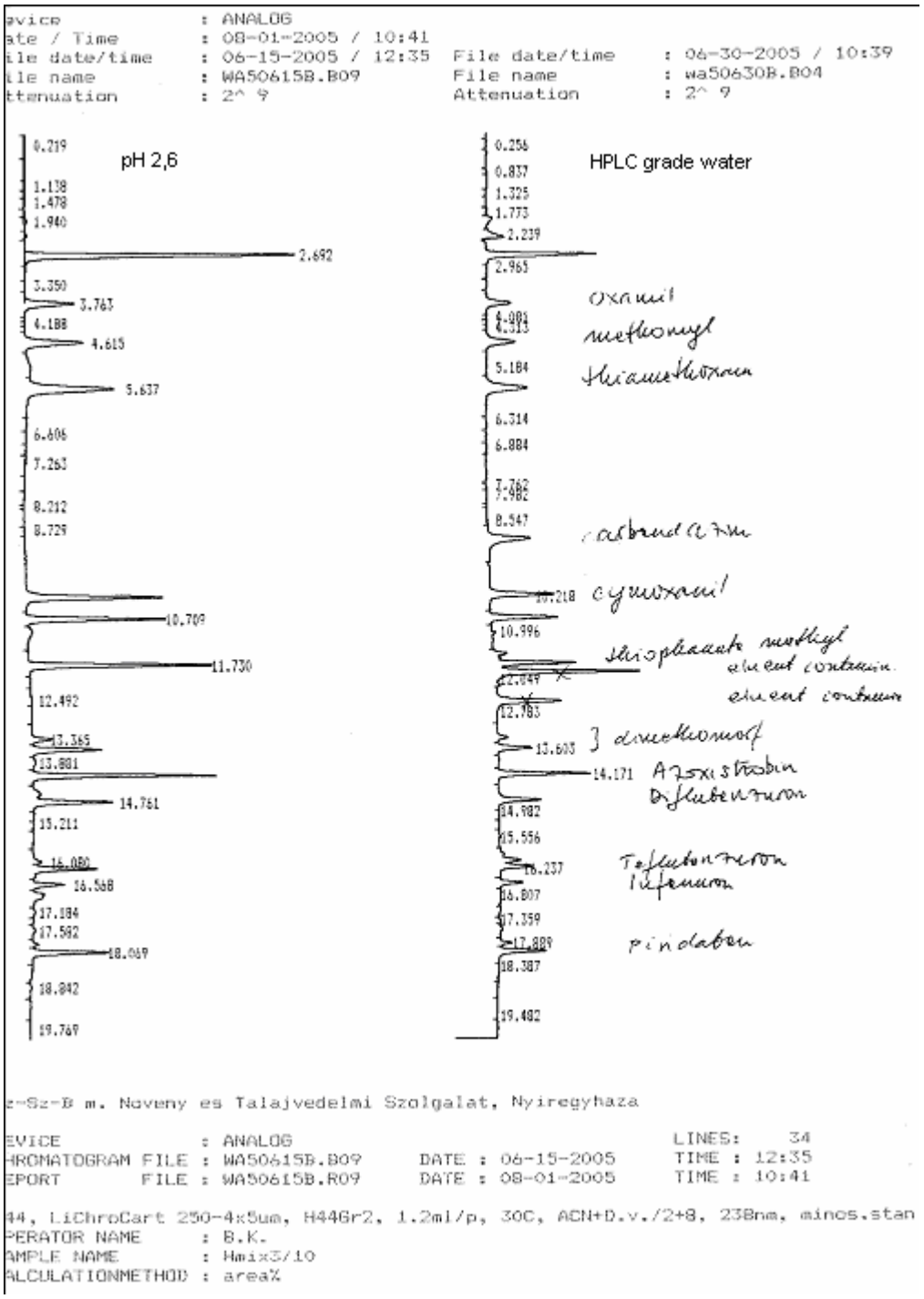

FIG. 4. Effect of the pH value of the mobile phase on retention times of carbendazim: chromatograms of Hmix3 (approximately 20 ng injected for each component) using the gradient programme with HPLC grade water (right side) and gradient programme with pH 2,6 acidified water instead of distilled water (left side); Column: Lichrospher 100 RP-18, end-capped, 250 mm × 4 mm, 5 µm.

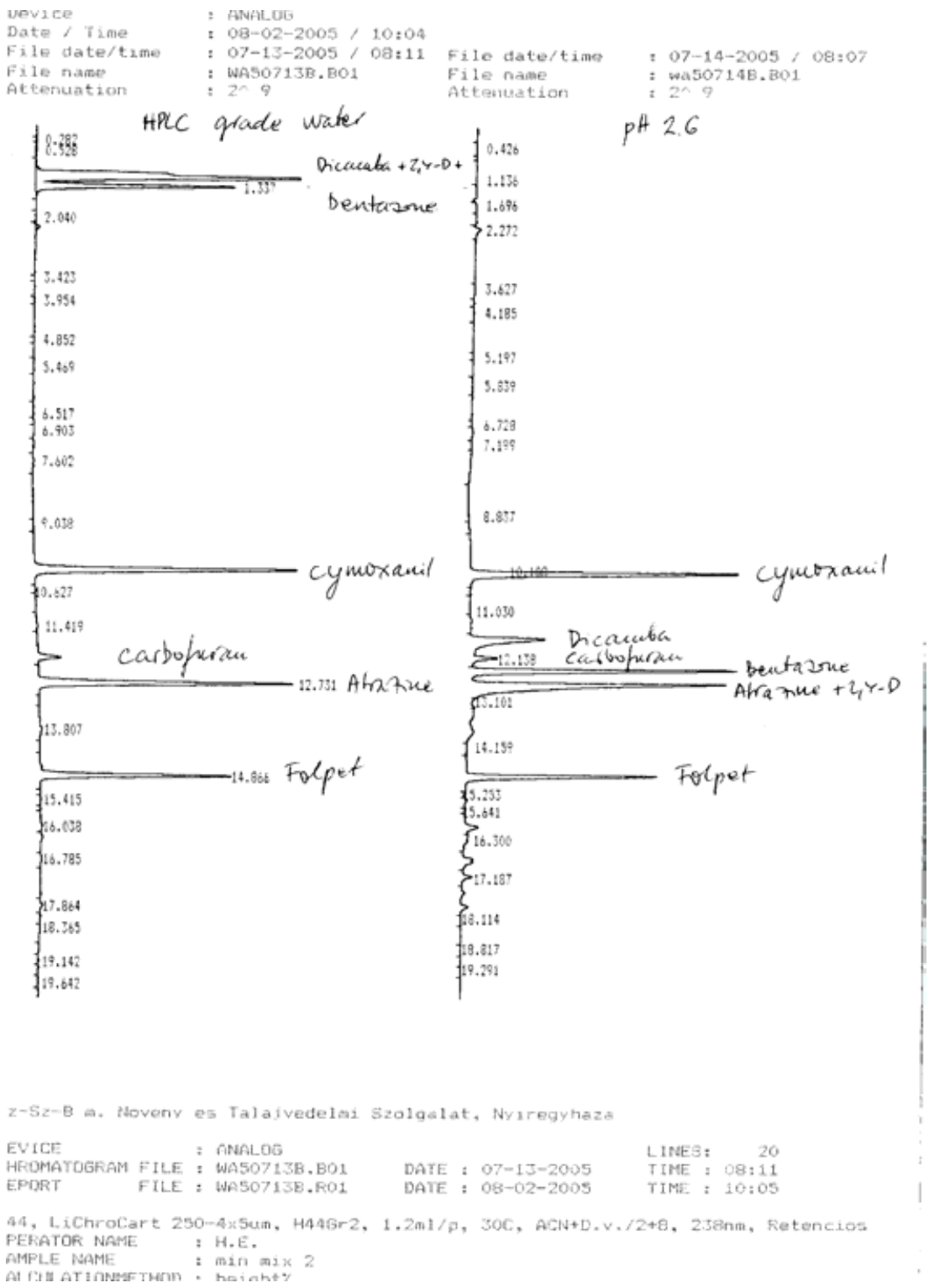

FIG.5. Effect of the pH value of the mobile phase on retention times of dicamba and 2,4-D: chromatograms of Minmix2 using the gradient programme with HPLC grade water (left side) and gradient programme with pH 2.6 acidified water (right side); Column: Lichrospher 100 RP-18, endcapped, 250 mm × 4 mm, 5µm; Detector: 238 nm.

3.1. Characterization of chromatographic separation of several active ingredients

The chromatographic behaviour of analytes are characterised by the retention time (Rt), peak width at 50% peak height ($w_{h50\%}$), capacity factor (k), effective plate number (N_{eff}), asymmetry at 5% peak height ($As_{5\%}$), and resolution of adjacent active ingredients (R_s). Representative data for pesticide

mixture of Hmix, given in Table 4 and Table 5, show sharp peaks ($3.7 > w_{h50} > 8.6$ sec) and good resolution of the components. This mixture contains pesticides of high water solubility (e.g., oxamyl, methomyl) and of low solubility (e.g. pyridaben). Peak asymmetry is also acceptable, only the carbendazim resulted in an asymmetry factor of As = 1.42. Significant asymmetry of peak was observed in a few cases (e.g. thiabendazole). The effective theoretical plate numbers were in the range of 3494-5495 for pesticides eluting in the isocratic solvent composition, all other components resulted in $N_{eff} > 16000$.

TABLE 4. CHARACTERISTIC CHROMATOGRAPHIC DATA OF SEVERAL PESTICIDES USING GRADIENT PROGRAMME (METHOD 1)

Pesticide	Rt***	$w_{h50\%}$ (sec)	N_{eff}	k	$As_{5\%}$	R_s
Oxamyl	3.755	6.343	3494	2.4	1.02	
Methomyl	4.65	6.956	5195	3.2	0.95	7.74
Thiamethoxam	5.644	8.657	5495	4.1	0.93	8.072
Carbendazim	8.945	8.663	16355	7.1	1.42	12.5
Cymoxanil	10.16	5.239	59645	8.2	0.93	10.2
Tiacloprid	10.692	4.713	82611	8.7	0.96	6.27
Thiophanate methyl	11.694	3.746	159513	9.6	1.03	12.8
Dimethomorph I	13.358	4.823	128830	11.1	1	17
Dimethomorph II	13.562	4.8	134433	11.3	1	3.1
Azoxistrobin	14.139	4.608	159689	11.9	0.92	6.75
Diflubenzuron	14.713	4.483	183900	12.4	1.29	3.39
Teflubenzuron	16.17	6.514	106744	13.7		18.92
Lufenuron	16.529	4.574	226932	14.0	1.1	5.35
Pyridaben	18.033	5.513	188150	15.4	1.1	22

*** for separation see Figure 3.

TABLE 5. CHARACTERISTIC CHROMATOGRAPHIC DATA OF SEVERAL PESTICIDES USING GRADIENT PROGRAMME WITH ACIDIFIED SOLVENT COMPOSITION (METHOD 2)

Pesticide	Rt***	$wh_{50\%}$ (sec)	N	k	$As_{5\%}$	R_s
Oxamyl	3.763	6.343	3515	2.42	1.12	
Methomyl	4.65	6.956	5195	3.23	1.07	7.74
Thiamethoxam	5.644	8.657	5495	4.13	1.06	7.86
Carbendazim	-					
Cymoxanil	10.16	5.239	59645	8.24	1.08	38.175
Tiacloprid	10.692	4.713	82611	8.72	1.1	5.25
Thiophanate methyl	11.694	3.746	159513	9.63	1.05	11.67
Dimethomorph I	13.365	4.29	163017	11.15	0.95	20.44
Dimethomorf II	13.603	4.8	135319	11.37	1.2	2.88
Azoxistrobin	14.139	4.608	159689	11.85	1.17	6.43
Diflubenzuron	14.713	4.483	183900	12.38	1.09	6.68
Teflubenzuron	16.17	6.514	106744	13.70	1.06	17.36
Lufenuron	16.529	4.574	226932	14.03	1.18	4.27
Pyridaben	18.033	5.513	188150	15.39	1.18	18.76

*** for separation see Figure 4.

3.2. Comparison of the gradient programme to Dudar's programme

Principally the system worked out by Dudar similar to this one in terms of type of stationary phase (C18). Data established by Dudar (Ref 1) also uses the HPLC gradient programme, that applies a very slow increase of organic solvent in order to reach an equilibrated system and thus to provide information for solvent composition for isocratic analytical conditions. For this reason the typical retention time in Dudar's method is > 20 minutes, in many cases > 40 minutes. The system given in this report uses a fast gradient programme in order to analyze pesticides belonging to different classes of compounds including water soluble and fat soluble analytes. Components of HPLC test mixture worked out by Dudar was also tested with the method given above. Except for phenoxy acetic acids, the other components elute in the middle time-range of chromatographic run (from 10.1 min to 14.87 min). Figure 4 shows that the retention times of 2,4-D, bentazone and dicamba significantly change in acidic eluents.

3.3. Elution sequence of pesticides based on their partition coefficient between n-octanol and water

The n-octanol/water partition coefficient (P_{OW}) of pesticides is used to predict the behaviour of active substance in the environment, e.g., accumulation in fat. As the stationary phase used is composed of C-18 chain, it was assumed that the retention time measured should correlate with the partition coefficient. Because this parameter covers very wide range, the logarithm of this value (log P_{OW}) was used to establish a correlation with retention time. Data of Table 2 were evaluated in this respect. It can be concluded that the retention times of pesticides mainly follow the n-octanol/water partition coefficients. The polynomial equation gives very good correlation, $R^2 = 0.963$ as shown in Figure 6. This provides the possibility to predict the retention time of an analyte for the given gradient programme, provided that the log P value is known from literature (e.g., Pesticide Manual) and the analyte does not tend to dissociate. A significant change in retention times occurs for acidic (e.g. phenoxy acetic acids, retention time increases) or basic (e.g. carbendazim, retention time decreases) analytes when acidified eluent is used instead of neutral one. This phenomenon is demonstrated in Figures 3–4. A different gradient programme will result in a different equation and correlation coefficient.

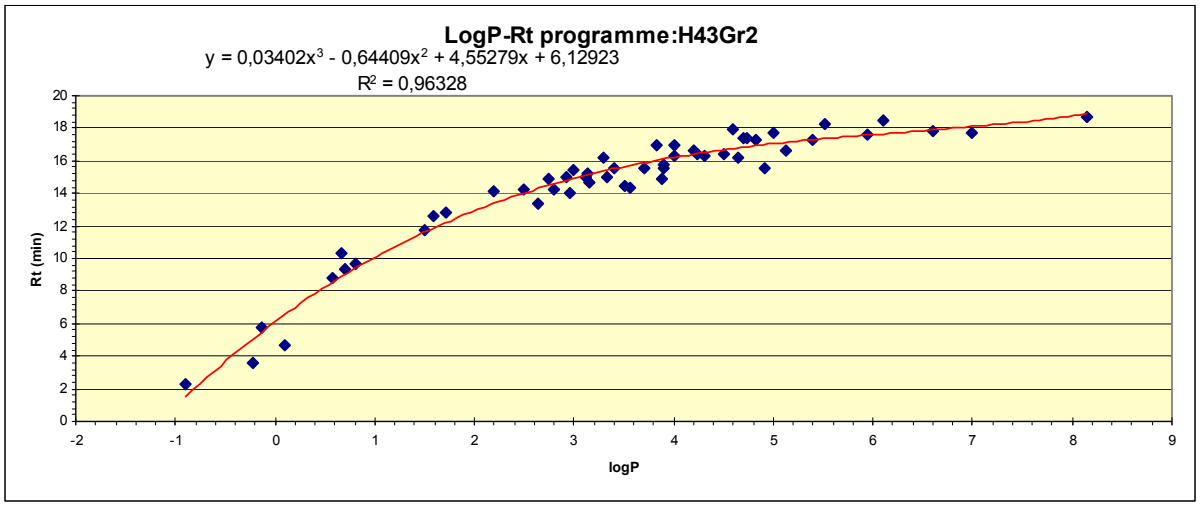

FIG. 6. Correlation of log P and retention time measured for HPLC separation of pesticides with method 1.

3.4. Selection of wavelength and AUFS

Simultaneous data acquisition was carried out at 2 wavelengths (238 and 260 nm) for each compound tested. These wavelengths are not necessarily optimal for a single analyte, but provide good compromise for screening purposes of a number of pesticides. The retention time of an analyte and the different response factors at the two wavelengths provides some certainty for the qualitative identification. For individual active ingredients of pesticides alternative wavelengths and AUFS scales of the detector may be selected according to the absorbance maximum of the analyte, for the purpose of optimizing the analysis and the sensitivity of the detector.

3.5. Applicability of the separation system

The retention times given in this report are valid for the combination of specified types of columns and gradient programmes. Any changes in these two parameters will change the absolute retention times of pesticides. The method is applicable for screening purposes to separate and identify the potential active substances present either in a pesticide product or in a sample for pesticide residues.

Additional peaks marked as “eluent contam.” on Figure 4 aroused from the solvent mixture in several cases. The gradient system itself is not recommended for the evaluation of active ingredient content of pesticide product as the analysis time is longer than with properly selected isocratic solvent composition, but may be appropriate for the analysis of a complex formulation.

REFERENCES

[1] BRITISH CROP PROTECTION COUNCIL, The Pesticide Manual, 10th Edition, The Royal Society of Chemistry (1994).
[2] DUDAR, E., HPLC ‘Multi-analyte’ detection method, in this publication.

SEPARATION AND SIMULTANEOUS DETERMINATION OF 14 FUNGICIDES WITH THE COMBINATION OF MULTI-ANALYTE METHODS AND HPLC DETECTION

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Abstract

The separation and simultaneous HPLC-MS determination for a series of fungicide products is reported. Multi-analyte methods were applied on a Chromolith RP-18e monolithic column having low resistance and enabling high flow rates and short analysis time at very good separation power. Details and analytical conditions are described with chromatograms illustrating the results and work done.

1. INTRODUCTION

This report presents a method for the separation of 14 fungicides with HPLC. The detection was carried out with MS. The MS detection has not been optimized yet at the time of preparation of the report. This work has been published in a scientific journal [1].

The fungicides selected for analysis were: carboxin, cymoxanil, dazomet, diethofencarb, hexaconazole, thiophanate-methyl, flutriafol, iprodione, metalaxyl, prochloraz, procymidone, tebuconazole, triadimefon, triadimenol.

2. EQUIPMENT AND MATERIALS

2.1. HPLC/MS system

Agilent HP1100 Series LC/MS equipped with a quaternary pump, vacuum degassing equipment and an auto-sampler.

Waters liquid chromatograph instrument with a 600E pump, a 717 plus auto-sampler and a 2996 ultraviolet diode array detector. Helium degassing was used.

Columns: 150 × 4.6 mm column packed with 5 μm XBP-C18 particles (Agilent, USA); Chromolith RP-18e monolithic column 100 × 4.6 mm (Merck)
Mobile Phase: 1.00 ml/min
A: water + 20 mM/l CH_3COONH_4
B: acetonitrile

Solvent gradient: 0-8 min, 40% – 45% B; 8-40 min, 45% B
Temperature: 25˚C
Wave length: 225 nm
Injected amount: 20 μL

MS conditions
ion polarity: positive; ion source type: ESI; scan:150-400 m/z
dry gas: 6.5 L/min; nebulizer: 40.00 psi; dry temp.: 350°C

2.2. Materials

Pesticide standards of analytical grade were purchased from Institute for Control of Agrichemicals Ministry of Agriculture (China). Purity was > 94%. HPLC-grade methanol purchased from Merck (Darmstadt, Germany); deionised water (>18 MΩ/cm resistivity) obtained from Milli-Q SP Reagent Water system; ammonium acetate; acetic acid, analytical grade.

3. RESULTS

The elution pattern of the standard mixture of the 14 pesticides in five replicate injections (overlaid) is shown in Figure 1.

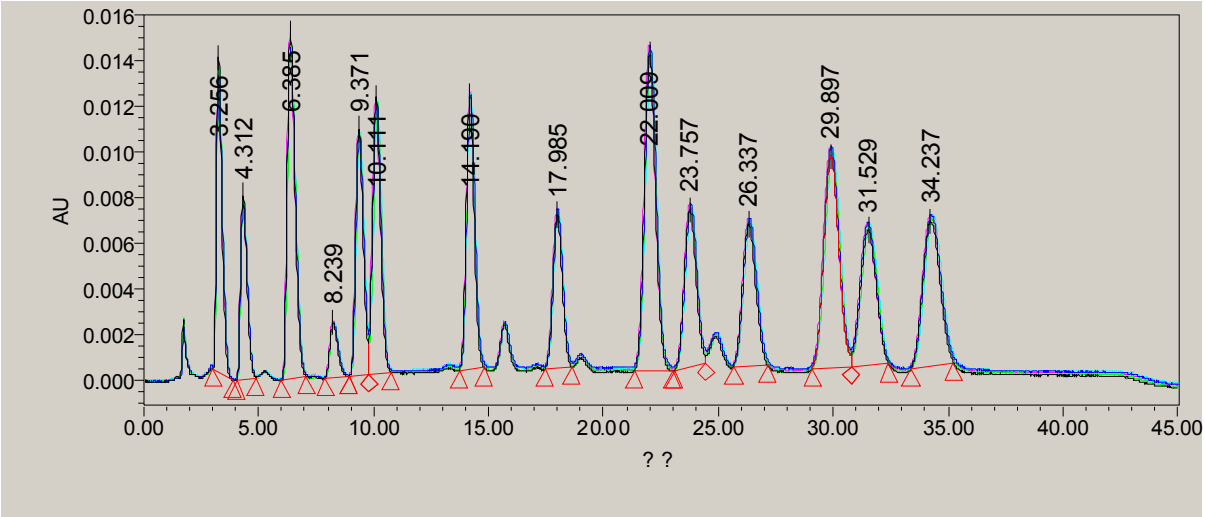

FIG. 1. Repeatability of 5 injections of the mixed standard on XBP-C18 column (8 ng/µl of each compound).

The pesticide mixture was also separated on the Chromolith RP-18e column applying acetonitrile: water 40/60 mobile phase and programmed flow rate operation mode:

Time [min]	0-5	5.1-7	7.1-11	11.1-20
Flow [ml/min]	0.5	1.0	2.0	3.0

The elution pattern of the components of the pesticide mixture is shown on Figure 2.

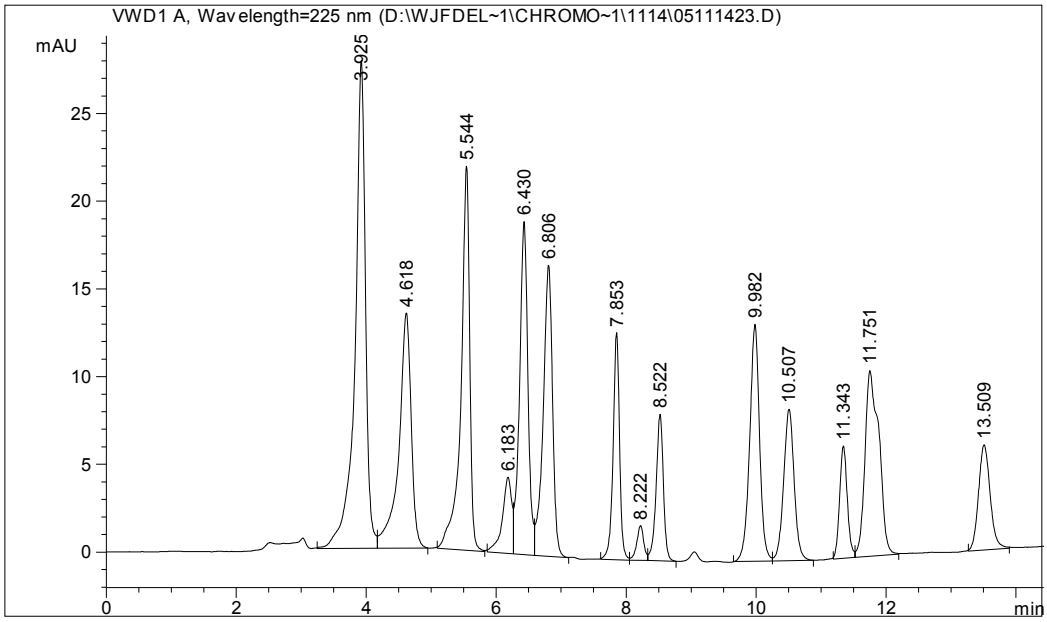

FIG.2. Elution pattern of 14 fungicides on Chromolith column applying acetonitrile: water 40/60 mobile phase and programmed flow rate.

Comparing the two chromatograms the advantage of Chromolith column is obvious. Its low resistance enables to apply a higher flow rate without extra large head-pressure, and a much faster analysis time with very good separation power.

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SYNTHESIS AND DETERMINATION OF IMPURITIES

SYNTHESIS AND IDENTIFICATION OF SELECTED IMPURITIES

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Abstract

Non-active substances undesired, but often unavoidable compounds accompanying target active ingredients in various pesticide formulations have been synthesized for supporting product registration and evaluation of the total toxicological and physicochemical properties of formulated products. The synthesis and structural characterization of various impurities of pesticide active ingredients are described and illustrated by IR, NMR, GC and GC/MS data.

1. INTRODUCTION

The quality control of pesticides is of great importance for assuring that the products marketed are effective against the targeted pests and diseases, do not adversely affect the health of people handling or applying them and do not pose any risk to the environment. The impurities play a special role in the toxicity of the technical and formulated product and may alter their physical properties [1].

The impurities, being present usually in the range from a few percent to 0.1%, but in special cases at or below 0.01 mg/kg must be determined. The analysis and identification of the impurities may require the combined application of several instrumental analytical techniques such as GC-MS, LC-MS, GC-FTIR and high resolution NMR. In GC or HPLC quantitative analysis to separate the active ingredient from the impurities is of vital importance.

Even where GC/MS and LC/MS instruments are available for the quality control of the technical active substances, analytical standards are required for the unambiguous identification of the compounds and for the validation of the analytical procedures.

The impurity profile with full identification must be provided with the petition for registration, and the significant impurities are also listed in the FAO Specification for pesticides [2]. However, the impurities are not readily available for laboratories carrying out quality control of pesticide products.

The aim of this work was to elaborate possible methods for the synthesis and identification of the selected impurities, and provide them as example for laboratories where suitable facilities are available for organic synthesis and structural identification. Within this project 16 impurities were synthesised, purified and identified. The procedures applied are described in this report.

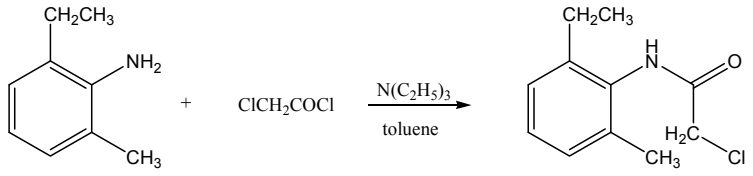
2. SYNTHESIS AND STRUCTURAL IDENTIFICATION OF IMPURITIES

The impurities included in the programme and the corresponding pesticides in which they may be present are given in Table 1.

TABLE 1. THE IMPURITIES OF PESTICIDES SYNTHESIZED

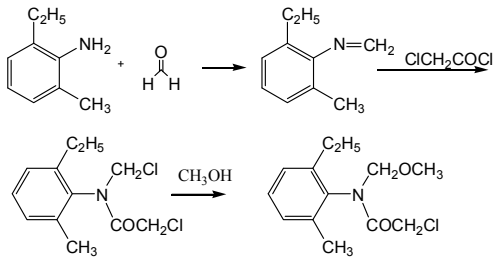
Pesticides	Impurities
Acetochlor; Butachlor	2-ethyl-6-methyl-N-chloroacetanilide 2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide 2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide
Oxadiazon	2,4-dichloroisopropoxybenzene, Trimethylacetamide
Propiconazole	2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan
Benalaxyl, Metalaxyl, Furalaxyl	2,6-dimethylaniline
Chlorpyrifos	TCP (3,5,6-trichoropyridin)-2-ol
Butachlor	Butyl chloroacetate
Diazinon	$(C_2H_5O)_3PS$
Dimethoate, Fenitrothion, Malathion, Methamidophos, Fensulfothion, Parathion, Parathion-methyl	$(CH_3O)_3PS$
Parathion-methyl, Phorate	$C_6H_4(NO_2)OH$
Trifluralin	$C_6H_2(NO_2)(CF_3)Cl_2$,$C_6H_2(NO_2)_2(CF_3)Cl$
Fenitrothion	3-methyl-4-nitrophenol 3-methyl-6-nitrophenol

2.1. 2-ethyl-6-methyl-N-chloroacetanilide



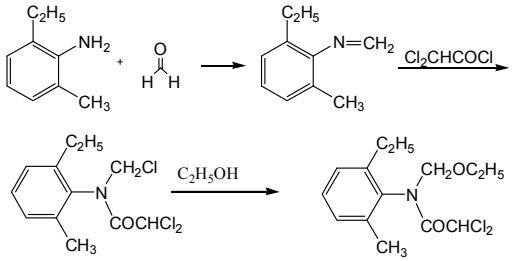
Stir the solution of 6.8 g 2-chloroacetyl chloride (0.055 mol) and 30ml anhydrous toluene and add 6 g 2-ethyl-6-methylbenzenamine (0.05 mol) and 5.1 g triethylamine (0.05 mol) dissolved in 30 ml anhydrous toluene. Stir the mixture at 80°C for an hour and pour it into 50ml ice-water. Wash the separated organic layer with 20 ml saturated $NaHCO_3$ aqueous solution, 20ml water, and finally dry it by anhydrous sodium sulphate. Evaporate the solvent. The yield is 10.5 g.

2. 2. 2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide



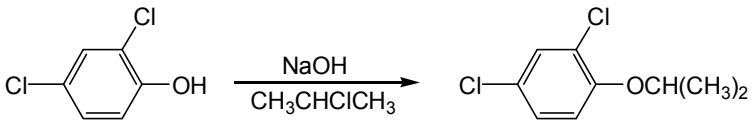
Place 13.5 g 2-ethyl-6-methylbenzenamine (0.1 mol) and 4.5 g (0.15 mol) paraformaldehyde into a 100ml flask and stir the mixture for an hour then cool it to room temperature, and add ethanol to the cool mixture. Remove ethanol and water using a rotary evaporator. Add 12 g 2-chloroacetyl chloride in 15 ml toluene to the residual oily material and stir it for 30 minutes. Warm the mixture to 60°C, and add drop wise the mixture of 30 ml methanol and 11 g triethylamine during 10 minutes. Stir the mixture at 80°C for 30 minutes. Cool the reaction mixture to room temperature, and transfer it to 100 ml water, extract with toluene (3 × 50 ml). Dry and evaporate the solvent. The raw material is about 20 g. Purify 5 g of the raw product on flash column chromatography (eluent: EtOAc-hexane: 1:4, v/v). The yield is 3.5 g (purity > 99%).

2.3. 2-ethyl-6-methyl-N- (ethoxy-methyl)-dichloroacetanilide



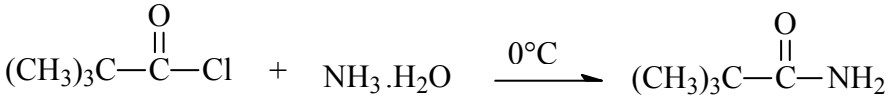
Place 13.5 g 2-ethyl-6-methylbenzenamine (0.1 mol) and 4.5 g (0.15 mol) paraformaldehyde into a 100 ml flask, and stir the mixture for an hour before being cooled to room temperature. Add ethanol to the cool mixture. Remove ethanol and water using a rotary evaporator leaving behind stiff oil. Add 15 g 2,2-dichloroacetyl chloride in 15 ml toluene to the residual oily material and stir it for 30 minutes. Warm the mixture to 60°C and add drop wise the mixture of 30 ml ethanol and 11 g triethylamine during 10 minutes. Stir the mixture at 80°C for 30 minutes and cool it to room temperature. Transfer it to 100 ml water, extract with toluene (3 × 50 ml). Evaporate the solvent to dryness. The yield produced is about 20 g. Purify 5 g coarse product by flash column chromatography (eluent: EtOAc-hexane = 1:4, v/v). The yield of product is about 3.5 g (purity > 99%).

2.4. 2,4-dichloroisopropoxybenzene



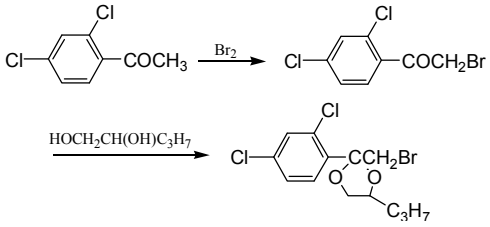
Add 2.4 g sodium hydroxide into the solution of 8.3 g 2,4-dichlorophenol in 50 ml toluene, and then reflux it for 20 minutes. Cool to room temperature. Add 8 g 2-chloropropane (0.1 mol) and stir for 6 hours at room temperature. Wash the reaction mixture with water, separate water layer. The organic layer is dried with sodium sulphate. After filtration and evaporation, the yield of the product is about 8 g.

2.5. Trimethylacetamide



Add drop-wise the solution of 12 g trimethylacetylchloride (0.1 mol) in 20 ml chloroform to ammonia at 0°C. Filter the resultant solid and wash it with water, dry under vacuum. The yield of trimethylacetamide is about 9.3 g.

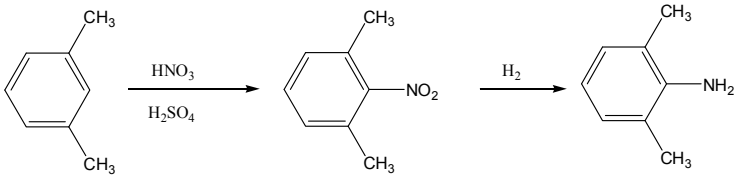
2.6. 2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl-1,3-dioxolan



Place 18.9 g 2,4-dichloroacetophenon and 70 ml methanol into a 250 ml flask equipped with mechanical stirring bar and acidify it with drops of concentrated hydrochloric acid. Add drop wise 30 ml methanol containing some bromine during 2 hours into the stirred solution which is cooled in water bath. Stir the mixture at room temperature for an hour, and then stir it in ice-water bath for 2 hours. Filter the precipitation, wash it with methanol, evaporate remaining methanol. Dry the residue under vacuum at 40°C. The white solid yield is about 20 g.

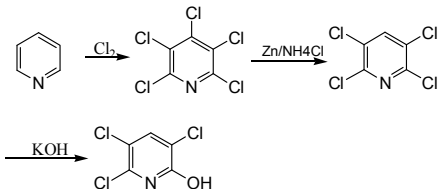
Dissolve the white product in anhydrous toluene and add 15.6 g pentane-1,2-diol (0.15 mol) and 1 g 4-methylbenzenesulfonic acid to the solution. Reflux and dehydrate the mixture for about 20 hours. Re-crystallization from EtAc – hexane yields 18 g end product.

2.7. 2,6-dimethylaniline



Add 7.9 g (0.08 mol) concentrated sulphuric acid into 8.56 g (0.08 mol) m-xylene continuously stirred in a 250 ml three-necked flask. Stir the mixture at 100–105°C for an hour and then cool it with ice-water bath. Add 12 g concentrated (98%) sulphuric acid and 6 g fuming nitric acid to the cool mixture, being vigorously stirred. Stir the resultant mixture at 30~35°C for 20 minutes and dilute it with 50 ml water, while adjusting pH to 4. After wet distillation, the fraction is extracted with 3×100 ml ethyl ether. Wash the extract with sodium hydrogen carbonate solution and evaporate, distil under reduced pressure (100 kPa). Collect the fraction at 224~225°C. The yield is a yellow material (~9.6 g).

2.8. 3,5,6-trichloropyridin-2-ol



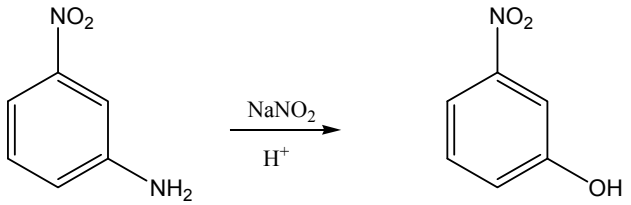
Bubble gaseous Cl_2 at 80°C to the suspension of 16 g pyridine, 20 ml CCl_4 and 1.5 g $CoCl_2$. Upon disappearance of starting materials (monitored by TLC), stop the reaction, evaporate the solvent. The yield is about 50 g perchloropyridine.

Dissolve the perchloropyridine in warm acetonitrile. Stir the solution and add drop-wise 40 ml aqueous solution of 15 g ammonium chloride and 9.6 g zinc powder. Stir the mixture for an hour, then

add 10 ml 5% hydrochloric acid. Distil the resultant mixture under reduced pressure at 77–78°C. The yield is about 21 g 2,3,5,6-tetrachloropyridine.

Stir the mixture of 21 g 2,3,5,6-tetrachloropyridine, 87 g water and 13 g potassium hydroxide at 95–100°C, and filter it while it is hot. Acidify the separated solution to pH 3.5 with concentrated sulphuric acid. After being cooled to 50°C, the resultant mixture is filtered, washed with water and evaporated. The yield of the end product is about 18 g.

2.9. 3-nitrophenol ($C_6H_4(NO_2)OH$)



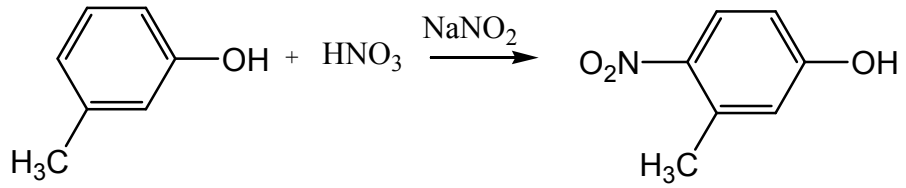
Step 1:

Place 200 g 65% concentrated sulphuric acid into a 250 ml four-necked flask fitted with a condenser, a mechanical stirrer and a thermometer. Heat the mixture to 70°C. Add 9 g 3-nitrobenzenamine and stir it vigorously at 110°C for 30 min, and then cool to 10–15°C before adding drop-wise 5 g sodium nitrite during an hour. The resultant mixture is supposed to be diazonium salt.

Step 2:

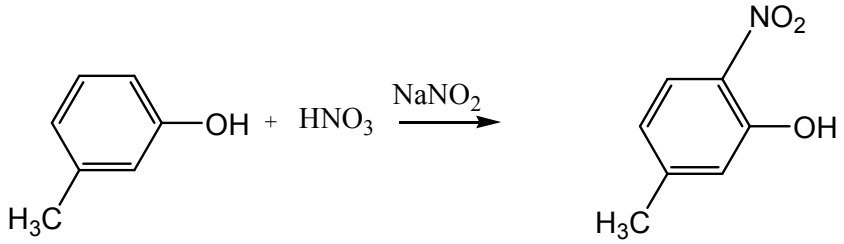
Place 60 g 65% concentrated sulphuric acid and 80ml o-xylene into a 500 ml four-necked flask fitted with a condenser, a mechanical stirrer and a thermometer. After heating the solution to 115–125°C, add diazonium salt in small quantities during an hour. Stir the mixture vigorously at 120–125°C for 2–3 hours, then cool it quickly. Concentrate the separated organic phase using a rotary evaporator. Add aqueous sodium hydroxide, and separate the aqueous layer, acidify it with sulphuric acid, filter, wash with water and finally dry. The yield of yellow crystals is 6 g (66%).

2.10. 3-methyl-4-nitrophenol



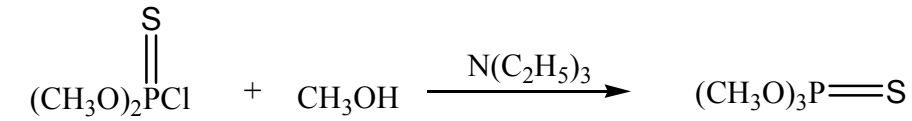
Place 11 g o-cresol, 18 g sodium nitrite, 30 g water into a 250ml flask, add 110 g 80% concentrated sulphuric acid drop by drop at 0°C during 3 hours. The resulting mixture is stirred at 0°C for over 3 hours. After that, the reaction mixture is filtered and the filter cake is washed with water and dried. The yield is about 14 g.

2.11. 5-methyl-2-nitrophenol



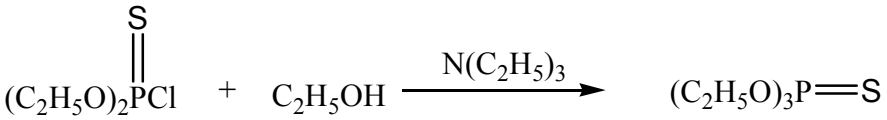
Add 25 g concentrated sulphuric acid with continuous stirring into 50 ml water solution of 20 g sodium nitrate. Stir the solution vigorously at 15°C, while adding 13 g o-cresol through a dropping funnel. Stir the mixture for half an hour and then keep it still. After wet distillation, the fraction is re-crystallized from ethanol and water. The yield is about 7 g.

2.12. *O*,*O*,*O*-trimethyl phosphorothioate ($(CH_3O)_3PS$)



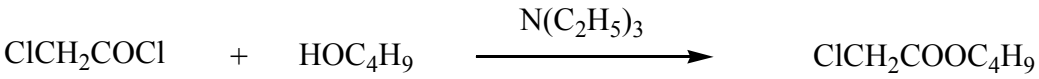
Add 10 g triethylamine in 15 ml methanol to 16 g O,O-diethyl phosphorochloridothioate at room temperature during 20 minutes. Stir the mixture for 2 hours, and distil it under reduced pressure. The yield is about 12 g.

2.13. *O*,*O*,*O*-triethyl phosphorothioate ($(C_2H_5O)_3PS$)



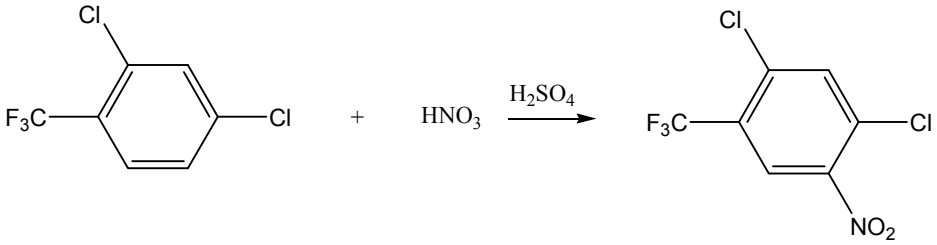
Add 10 g triethylamine in 10 ml ethanol to 19 g O,O-diethyl phosphorochloridothioate at room temperature during 20 minutes. Stir the mixture for 2 hours and distil it under the reduced pressure. The yield is about 18 g.

2.14. Butyl 2-chloroacetate



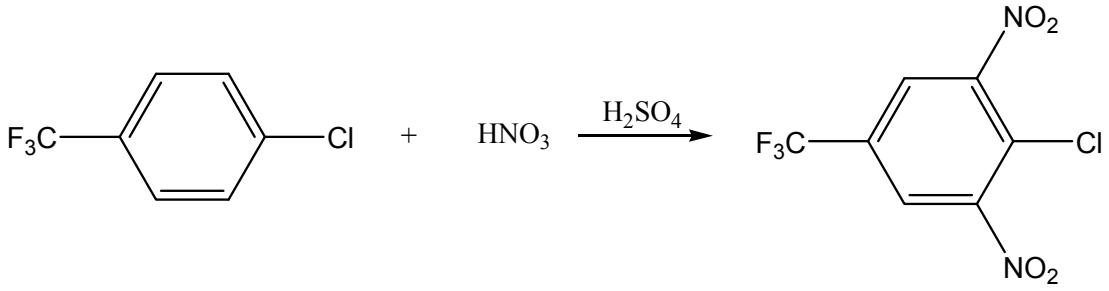
Add 10 g triethylamine in 10 ml butan-1-ol to 11 g 2-chloroacetyl chloride. Stir the mixture for 2 hours and distil it under reduced pressure. The yield is about 13 g.

2.15. 1,5-dichloro-2-(trifluoromethyl)-4-nitrobenzene ($C_6H_2(NO_2)(CF_3)Cl_2$)



Add the mixture of 12 g 98% concentrated sulphuric acid and 6 g fuming nitric acid to 10 g 2,4-dichloro-1-(trifluoromethyl)benzene in a 100 ml flask at room temperature. Stir the mixture for 20 minutes at 30–35°C. The reaction mixture is poured to 100 ml ice-water. The filtered solid phase is washed with water, and dried. The yield of the product is about 11 g.

2.16. 2-chloro-5-(trifluoromethyl)-1,3-dinitrobenzene ($C_6H_2(NO_2)_2(CF_3)Cl$)



Add the mixture of 12 g 98% concentrated sulphuric acid and 6 g fuming nitric acid to 2,4-dichloro-1-(trifluoromethyl)benzene in a 100ml flask at room temperature. Stir the mixture for 20 minutes at 30–35°C. Pour the reaction mixture into 100 ml ice-water. Wash the filtered solid phase with water, and dry. The yield is about 10 g.

3. IDENTIFICATION OF THE COMPOUNDS

TABLE 2. EMPIRICAL FORMULA AND MOLECULAR MASS OF COMPOUNDS

Chemical name	ID No[a]	Empirical formula	Molecular mass
2-ethyl-6-methyl-N-chloroacetanilide	1	$C_{11}H_{14}ClNO$	211.69
2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide	2	$C_{13}H_{18}ClNO_2$	255.74
2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide	3	$C_{14}H_{20}Cl_2NO_2$	304.22
2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan	6	$C_{13}H_{15}BrCl_2O_2$	354.07
2,4-dichloroisopropoxybenzene	7	$C_9H_{10}Cl_2O$	205.08
Trimethylacetamide	4	$C_5H_{11}NO$	101.15
2,6-dimethylaniline	5	$C_8H_{11}N$	121.18
3,5,6-trichoropyridin-2-ol	8	$C_5H_2Cl_3NO$	198.44
3-nitro-phenol	2003/1	$C_6H_5NO_3$	139.11
3-methyl-4-nitrophenol	2003/2	$C_7H_7NO_3$	153.14
3-methyl-6-nitrophenol	2003/3	$C_7H_7NO_3$	153.14
Thiophosphoric acid O,O',O''-trimethylester	2003/4	$C_4H_{11}O_3SP$	170.16
Thiophosphoric acid O,O',O''-triethylester	2003/5	$C_7H_{17}O_3SP$	212.24
Chloro-acetic acid butylester	2003/6	$C_6H_{11}O_2Cl$	150.61
1,5-Dichloro-2-nitro-4-trifluoromethyl-benzene	2003/7	$C_7H_2NO_2Cl_2F_3$	260.00
1-Chloro-2,6-dinitro-4-trifluoromethyl-benzene	2003/8	$C_7H_2N_2O_4F_3Cl$	270.55

[a]: Numbers for the short identification of the compounds

3.1. Identification of impurities by infra red spectroscopy

The IR spectra of the compounds were obtained in KBr disks with Shimadzu IR-435 Spectrometer. The characteristic bands of the compounds are summarized in Table 3. The IR spectra of the compounds are given in Annex 2.

3.2 Identification of Impurities by NMR

A Bruker 300 MHz NMR apparatus was used to determine the number, type and relative positions of proton atoms in impurities molecules based on ^{1}H-NMR or ^{13}C-NMR spectrum.

Impurities 12, 13, 14 and 15 were dissolved in $CDCl_3$ solution, while impurities 9, 10 and 11 were dissolved in CD_3COCD_3. The chemical shifts (δ, ppm) of protons were measured. The NMR spectra of the compounds are given in Annex 3.

TABLE 3. CHARACTERISTIC ABSORPTION BANDS OF INFRA SPECTRA OF THE COMPOUNDS

Chemical name		Absorption bands
2-ethyl-6-methyl-N-chloroacetanilide	1	3200(vs), 3000(vs), 2950(vs), 2860(vs), 1685(w), 1660(m), 1585(m), 1530(m), 1460(m), 1330(vs), 1260(m), 1240(m), 980(vs), 880(vs), 780(s), 710(m), 660(s)
2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide	2	2980(s), 1680(m), 1500(m), 1470(s), 1400(s), 1380(vs), 1320(s), 1240(w), 1200(m), 1120(w), 1100(w), 1070(m), 1010(m), 780(w), 760(s), 640(w)
2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide	3	2950(s), 1690(vs), 1460(w), 1370(m), 1285(s), 1220(s), 1185(s), 1040(w), 1020(w), 890(m), 760(m), 700(m), 580(w)
Trimethylacetamide	4	3250(w), 3200 (s), 2950(s), 1650(m), 1620(w), 1500(s), 1400(w), 1380(m), 1230(s), 1120(m), 860(w), 820(w), 730(m), 610(m)
2,6-dimethylaniline	5	3450(w), 3360(m), 3000(w), 2960(w), 2900(m), 2860(w), 1620(s), 1480(s), 1440(w), 1280(m), 1090(m), 760(m)
2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan	6	3400(w), 2920(w), 2860(w), 1580(s), 1550(m), 1460(s), 1380(s), 1100(w), 1060(w), 1040(w), 870(m), 820(s)
2,4-dichloroisopropoxybenzene	7	2960(s), 2900(m), 1600(w), 1580(s), 1480(m), 1280(m), 1260(m), 1180(m), 1100(s), 1060(s), 960(s), 870(m), 800(m), 730(m)
3,5,6-trichoropyridin-2-ol	8	3300(m), 1510(s), 1460(s), 1320(m), 1190(w), 1180(w), 1060(s), 1000(w), 760(s), 680(w)
3-nitro-phenol	2003/1	3360(s), 1960(s), 1740(vs), 1700(s), 1620(vs), 1520(s), 1350(s), 1300(s), 1210(m), 1080(s), 1000(vs), 940(vs), 875(vs), 815(m), 795(m), 740(vs), 670(s), 600(w)
3-methyl-4-nitrophenol	2003/2	3360(s), 1605(w), 1580(w), 1510(s), 1480(m), 1460(m), 1420(m), 1380(s), 1320(w), 1260(m), 1210(s), 1080(s), 860(s), 760(s), 790(w), 620(m)
3-methyl-6-nitrophenol	2003/3	3300(w), 1620(m), 1580(m), 1525(w), 1480(m), 1320(m), 1280(w) 1230(w), 1180(w), 1140(w),950(w),880(w), 760(m), 660(s)
Thiophosphoric acid O,O',O''-trimethylester	2003/4	2960(vs), 1475(w), 1440(m), 1390(s), 1170(m), 1030(s), 970(s),820(s)
Thiophosphoric acid O,O',O''-triethylester	2003/5	2995(m), 2920(vs), 2820(vs), 1860(s), 1460(w), 1440(w), 1285(s), 1030(s), 820(s),
Chloro-acetic acid butylester	2003/6	2950(vs), 2860(s), 1760(m), 1460(m), 1410(m), 1310(w), 1280(w), 190(m), 1060(w), 1020(w), 780(m)
1,5-Dichloro-2-nitro-4-trifluoromethyl-benzene	2003/7	3800(s),3200(m),1610(s), 1560(m), 1530(m), 1470(s), 1380(w), 1340(s), 1295(m), 1280(m), 1140(w), 1080(s), 900(s), 880(s) 810(w), 660(w), 600(w)
1-Chloro-2,6-dinitro-4-trifluoromethyl-benzene	2003/8	3050(s), 1620(s), 1540(vs), 1350(m), 1310(s), 1180(m), 1150(m), 1120(m), 920(w), 900(w), 760(w), 720(s), 620(m)

Notes: vs: very strong; s: strong; m: medium; w: weak

The spectrum of a molecule in nuclear magnetic field reflects its chemical structure. The characteristic absorption bands are summarized in Table 4.

TABLE 4. NMR SPECTRA OF THE COMPOUNDS

Chemical name		^{1}H-NMR (δ_{ppm}) or ^{13}C-NMR(δ_{ppm})	
2-ethyl-6-methyl-N-chloroacetanilide	1	7.8674(1H, s); 7.2566-7.1051(3H, s); 4.2491(2H, s); 2.6263-2.5507(2H, m); 2.2388(3H, s); 1.2200-1.1696(3H, s)	
2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide	2	7.2773-7.1622(3H, m); 4.9700(2H, s); 3.7114(2H, s); 3.4961(3H, s); 2.5982-2.5415(2H, m); 2.2631(3H, s); 1.2662-1.2159(3H, s)	
2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide	3	7.3972-7.2054(3H, m); 5.7100(1H, s); 5.4937-5.3912(2H, m); 2.6661-2.5464(2H, m); 2.3248(3H, s); 1.3144-1.2643(3H, t)	
Trimethylacetamide	4	5.8937-5.6852(2H, d); 1.4344-1.0112(9H, t)	
2,6-dimethylaniline	5	6.9345-6.9097(2H, d); 6.6495-6.5999(3H, t); 3.4328(2H, s); 2.1468(6H, s)	
2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan	6	7.6833-7.5835(1H, m); 7.4932-7.3774(1H, m); 7.2703-7.2298(1H, m); 4.0361-3.7397(2H, m); 3.4576-3.4041(1H, m); 1.7624-1.3045(6H, m); 0.9897-0.8879(5H, m)	
2,4-dichloroisopropoxybenzene	7	7.3512-7.3427(1H, d); 7.2536-7.1218(1H, m); 4.5348-4.4538(1H, m); 1.3673-1.3471(6H, d)	
3,5,6-trichoropyridin-2-ol	8	7.8158(1H, s); 7.26319(1H, s)	
3-nitro-phenol	2003/1 (9)	9.28668(1H,s);7.73243-7.69487(1H,m); 7.65825-7.64356(1H,t); 7.50570-7.45130(1H,,m); 7.28793-7.24927(1H,m)	
3-methyl-4-nitrophenol	2003/2 (10)	10.4665(1H,s), 7.9712-7.9423(1H,d); 6.9726-6.9653(1H,q); 6.8966-6.8599(1H,m); 2.3827(3H,s)	
3-methyl-6-nitrophenol	2003/3 (11)	3.7992-3.7830, 3.7566-3.7380 (J_{PH})	
Thiophosphoric acid O,O',O''-trimethylester	2003/4 (12)	4.17896-4.07629(6H,m); 1.39465-1.20806(9H,m)	
Thiophosphoric acid O,O',O''-triethylester	2003/5 (13)	4.2214-4.1770(2H,t); 4.0659(2H,s); 1.7058-1.6110(2H,m); 1.4610-1.3371(2H,m); 0.9700-0.9211(3H,t)	
Chloro-acetic acid butylester	2003/6 (14)	4.2214-4.1770(2H,t); 4.0659(2H,s); 1.7058-1.6110(2H,m); 1.4610-1.3371(2H,m); 0.9700-0.9211(3H,t)	
1,5-Dichloro-2-nitro-4-trifluoromethyl-benzene	2003/7 (15)	^{1}H-NMR	8.28568(1H,s); 7.77958(1H,s)
		^{13}C-NMR	145.675(s), 137.317(s), 135.075-134.334(t), 131.992(s), 128.927-127.592(q), 126.762(s), 125.194-124.977(q), 123.133(s)
1-Chloro-2,6-dinitro-4-trifluoromethyl-benzene	2003/8 (16)	^{1}H-NMR	8.2794(1H,s); 7.2690(1H,s)
		^{13}C-NMR	149.929(s), 131.990-130.543 (q), 126.819(s), 124.942-124.698(m), 123.191(s), 115.937(s)

3.3. GC/MS spectra of compounds

The GC/MS identification of the compounds was performed under the following conditions:

GC Column: DB-5MS 30 m × 0.25 mm × 0.25 μm

Column Temperature:

Impurity 1, 5, and 6: 40°C (3min) → 100°C (10°C /min) → 300°C (25°C/min)

Impurity 2, 3, 7 and 8: 60°C (1.5min) → 300°C (15°C/min)

Impurity 4: 35°C (5 min) → 50°C (3°C/min) → 80°C (8°C/min) → 140°C (12°C/min) → 280°C (15°C/min)

Injector temperature: 240°C
Flow rate (He): 1.0 ml/min

MS Conditions:

MS Source temperature: 240°C
MS Scan Range: 40–350 mu

The mass fragments of the substances are given in Table 5. The collection of these mass spectra is attached in the Annex 4.

TABLE 5. MASS FRAGMENTS OF MS SPECTRA OF THE COMPOUNDS

Chemical name		Mass fragments of compounds
2-ethyl-6-methyl-N-chloroacetanilide	1	211, 196, 184, 177, 169, 162, 154, 147, 134, 127, 120, 113, 106, 98, 91, 77, 65, 58, 51, 39, 27, 15
2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide	2	255, 240, 223, 214, 203, 197, 189, 174, 164, 156, 146, 132, 117, 105, 91, 77, 65, 53, 45, 37, 29, 15
2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide	3	306, 258, 244, 222, 210, 194, 174, 160, 146, 132, 117, 103, 91, 77, 65, 51, 39
Trimethylacetamide	4	101, 95, 86, 78, 69, 63, 57, 51, 41, 36, 30
2,6-dimethylaniline	5	121, 106, 91, 77, 65, 51, 39, 30
2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan	6	353, 311, 295, 259, 251, 201, 191, 173, 159, 145, 136, 123, 109, 99, 89, 77, 69, 55, 41
2,4-dichloroisopropoxybenzene	7	207, 202, 197, 171, 161, 144, 134, 122, 107, 98, 91, 84, 77, 72, 63, 53, 47, 36
3,5,6-trichoropyridin-2-ol	8	204, 189, 175, 162, 145, 133, 126, 109, 103, 98, 91, 83, 73, 63, 50, 43, 38
3-nitro-phenol	2003/1 (9)	139, 123, 109, 93,81, 66, 65, 63, 53, 50
3-methyl-4-nitrophenol	2003/2 (10)	153, 136, 123, 109, 108, 107, 81, 80, 77, 68, 67, 63, 62, 55, 53, 51,50, 41
3-methyl-6-nitrophenol	2003/3 (11)	153, 136, 123, 107, 105, 95, 79, 78, 77, 67, 65, 63, 53, 51, 50, 43
Thiophosphoric acid O,O',O''-trimethylester	2003/4 (12)	158, 156, 126, 125, 109, 93, 79, 77, 63, 48, 47
Thiophosphoric acid O,O',O''-triethylester	2003/5 (13)	199, 198, 171, 170, 154, 142, 137
Chloro-acetic acid butylester	2003/6 (14)	121, 109, 107, 95, 79, 77, 71, 57, 56, 49, 41
1,5-Dichloro-2-nitro-4-trifluoromethyl-benzene	2003/7 (15)	263, 259, 231, 215, 213, 201, 180, 178, 166, 159, 143, 123, 111, 109, 99, 93, 75, 74, 69, 61, 50
1-Chloro-2,6-dinitro-4-trifluoromethyl-benzene	2003/8 (16)	272, 270, 251, 240, 189, 178, 166, 159, 143, 131, 123, 99, 97, 93, 75, 74, 69, 62, 46

4. GC ANALYSIS OF SYNTHESIZED COMPOUNDS

The purity of the compounds was quantified by GC analysis applying the following conditions.
Column: HP-5, 15 m × 0.25 mm × 0.25µm
Temperature:
Impurities: 1, 2, 3 and 7:
Inj.: 250°C
Det.: 260°C
Column: 80°C (1 min) → 240°C at 20°C/min, keep for 10 min.
Impurity 4, 5 and impurity 6:
Inj.: 200°C
Det.: 220°C
Column: 80°C (1 min) → 180°C at 10°C /min, keep for 10 min.

The estimated purity of the purified materials is given in Table 6.

TABLE 6. ESTIMATED PURITY OF SUBSTANCES SYNTHESISED

Chemical name	Purity [%]
2-ethyl-6-methyl-N-chloroacetanilide	98.5
2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide	93.3
2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide	93.1
Trimethylacetamide	94.9
2,6-dimethylaniline	96.7
2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan	85.2
2,4-dichloroisopropoxybenzene	97.3
3,5,6-trichoropyridin-2-ol	93.0
3-nitro-phenol	98.5
3-methyl-4-nitrophenol	96.3
3-methyl-6-nitrophenol	99.2
Thiophosphoric acid O,O',O''-trimethylester	98.2
Thiophosphoric acid O,O',O''-triethylester	94.4
Chloro-acetic acid butylester	98.8
1,5-Dichloro-2-nitro-4-trifluoromethyl-benzene	95.4
1-Chloro-2,6-dinitro-4-trifluoromethyl-benzene	97.9

DETERMINATION OF IMPURITIES OF ATRAZINE BY HPLC-MS

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Abstract

The determination of the main impurities of the herbicide atrazine by GC/FID, GC/MS and LC/MS is described. The most relevant technical impurities were synthesized and characterized by IR and UV spectroscopy as well. The impurity profiles of different technical grade formulated products were tested and the typical impurities identified.

1. SUMMARY

The technical grade atrazine was analyzed by LC/MS to identify any impurities which could not be detected by GC/MS. The HPLC/MS analysis revealed that only those three impurities were present in the technical materials above 0.1%, which were found by GC/MS analysis, too.

The postulated impurities were synthesized to confirm the structure and the identity of impurities:

Impurity I: 6-chloro-N^2,N^4-diisopropyl-1,3,5-triazine-2,4-diamine
Impurity II: N^2,N^4-diethyl-N^6-isopropyl-1,3,5-triazine-2,4,6-triamine
Impurity III: 6-chloro-N^2, N^4-diethyl-1,3,5-triazine-2,4-diamine

A GC method was elaborated for the simultaneous detection of atrazine and its impurities. The impurity content of technical grade products was tested.

This report describes the analytical procedures and the results of the analysis of technical grade atrazine.

2. EQUIPMENT AND MATERIALS

2.1. HPLC/MS system

Equipment: LC: Agilent 1100; MSD: Finnigan Xcalibur
HPLC Column: Zorbax SB-C18, 2.1 mm i.d. × 150 mm
Column Temperature: 30°C
Injection volume**:** 5 µl
Flow rate: 0.2 ml/min
Wavelength: 254 nm
Mobile phase: acetonitrile:water:CH_3COOH = 70:30:0.5 (%)
N_2 Flow: 5.0 l/min
Ionization Mode: APCI
Polarity: positive
Drying Gas temperature: 320°C
CID: 60 V
Neb Press: 55 psig
Vaporizer temperature: 350°C
Corona current: 4 µA
Signal l: MSD 1TIC, MS File
Signal 2: DAD 1A, 254 nm
Scan Range: 100–500 amu

2.2. GC/MS system

Equipment: Shimadzu GC-MSD
GC Column: HP-5, 30 m 0.25 mm id × 0.25 µm ft
Column Temperature**:** 100°C (1 min) to 250°C (hold) programmed by rate 5°C/min
Injection temperature**:** 250°C
Injection volume**:** 1 µl
Column flow rate: 1.0 ml/min
Injection mode: split
Split ratio: 10:1

MS Conditions:
Polarity: Positive
MS Source temperature: 230°C
Aux-2 temperature: 250°C
MS Scan Range: 40–550 amu
MS Quad Temperature: 150°C
Energy: 70eV
Solvent delay time: 5.0 min

2.3. GC conditions for analysis of technical product

Equipment: HP4890D GC fitted with a FID detector
GC Column: FFAP 30 m (0.25 mm film)
Column Temperature: 100°C 1min, 20°C/min to 230°C, hold 15 min
Injector temperature: 250°C
Detector temperature: 250°C
Injection volume: 1 µl
N_2 Flow: 1.50 ml/min
Retention times:
Impurity I: 23.9 min; Impurity II: 26.6 min, Impurity III: 33.1 min

2.4. Chemicals

Acetonitrile: HPLC grade
Acetone: A.R
Water: distilled
Atrazine standard (purity: 99.5%)
Atrazine technical (97.0% min)
Impurity I standard: (purity 99.0%)
Impurity II standard: (purity 95.5%)
Impurity III standard (purity 98.0%)

3. HPLC AND GC ANALYSIS OF IMPURITIES

Test solutions were prepared by dissolving 100 mg technical atrazine in the mobile phase and making up the volume to 25 ml. After thorough mixing 5 µl solution was injected into HPLC/MS system.

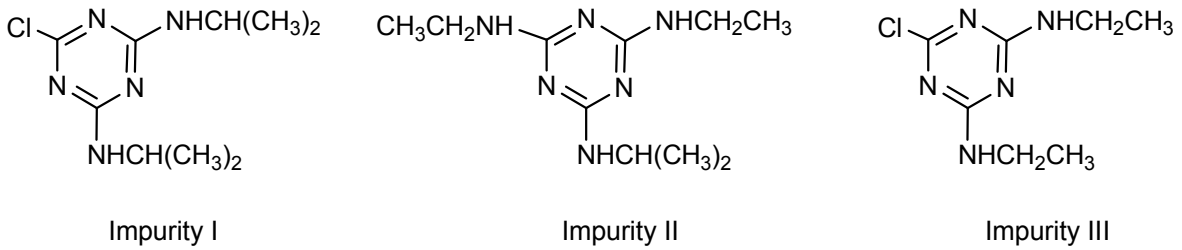
Under the HPLC-MS conditions given in section 2.1, three impurities and the parent compound were well separated and detected (Table 1). The result indicated that the impurities were similar to those detected with GC-MS. No other compound was observed above the concentration equivalent to 0.1% of atrazine in the technical product.

The apolar HP-5 column does not completely separate the four compounds; therefore the chromatographic system described under 2.3 was used for the quantitative determinations.

TABLE 1. RESULTS OF HPLC-MS ANALYSIS

	Rt (Mass detector)	MW	MS detected (M+1 ion)	MS-MS fragmentation analysis of M+1 ion
Impurity I	4.11	229	230	188
Impurity II	2.99	224	225	183
Impurity III	5.13	201	202	160
Atrazine	5.69	215	216	174

The chemical structures of the impurities are shown in Figure 1.



Impurity I Impurity II Impurity III

Impurity I: 6-chloro-N^2,N^4-diisopropyl-1,3,5-triazine-2,4-diamine
Impurity II: N^2,N^4-diethyl-N^6-isopropyl-1,3,5-triazine-2,4,6-triamine
Impurity III: 6-chloro-N^2,N^4-diethyl-1,3,5-triazine-2,4-diamine

FIG. 1. Chemical structures and names of impurities of technical grade atrazine.

4. SYNTHESIS AND IDENTIFICATION OF IMPURITIES

Impurities I, II, III were synthesised through the routes shown in Figure 2. The identities of the impurities were confirmed by their spectra. The characteristic patterns are given in Table 2.

TABLE 2. CHARACTERISTIC WAVELENGTHS OF THE SPECTRA OF THE IMPURITES OF ATRAZINE

Method	**Impurity I**	**Impurity II**	**Impurity III**
IR spectra in KBr disks	3250(m), 2950(w), 1620(m), 1520(s), 1460(w), 1400(w), 1310(w), 1170(w), 1020(m), 800(m)	3250(w), 2950(w), 1560(s), 1510(s), 1370(w), 1330(m), 1200(w), 810(w)	3250(m), 3100(w), 2950(w), 1630(m), 1560(s), 1440(m), 1400(m), 1370(m), 1340(m), 1300(m), 1140(w), 1100(w), 990(w), 800(w)
UV maximum	229 nm, 238 nm, 264 nm	264 nm, 238 nm	238 nm, 231 nm
^{1}H-NMR (δ_{ppm}, $CDCl_3$)	5.243-5.114(2H, d, 2NH); 4.194-4.102(2H,m, 2CH); 1.232-1.176(12H, t, $4CH_3$)	4.856-4.751(3H, d, 3NH); 4.136(1H, S, CH); 3.393-3.551(4H, t, $2CH_2$)	7.820-7.685(2H, m, 2NH); 3.303-3.168(4H, m, $2CH_2$); 1.112-1.036(6H, m, $2CH_3$)
MS Spectrum	43, 58(B), 68, 83, 100, 130, 145, 152, 159, 172, 187, 214, 229(M), 231(M+2)	43, 58, 68, 83, 97, 111, 124, 139, 154, 167, 181, 196, 209, 224(B)	44(B), 55, 68, 86, 96, 104, 123, 138, 145, 158, 173, 186, 201(M), 203(M+2)

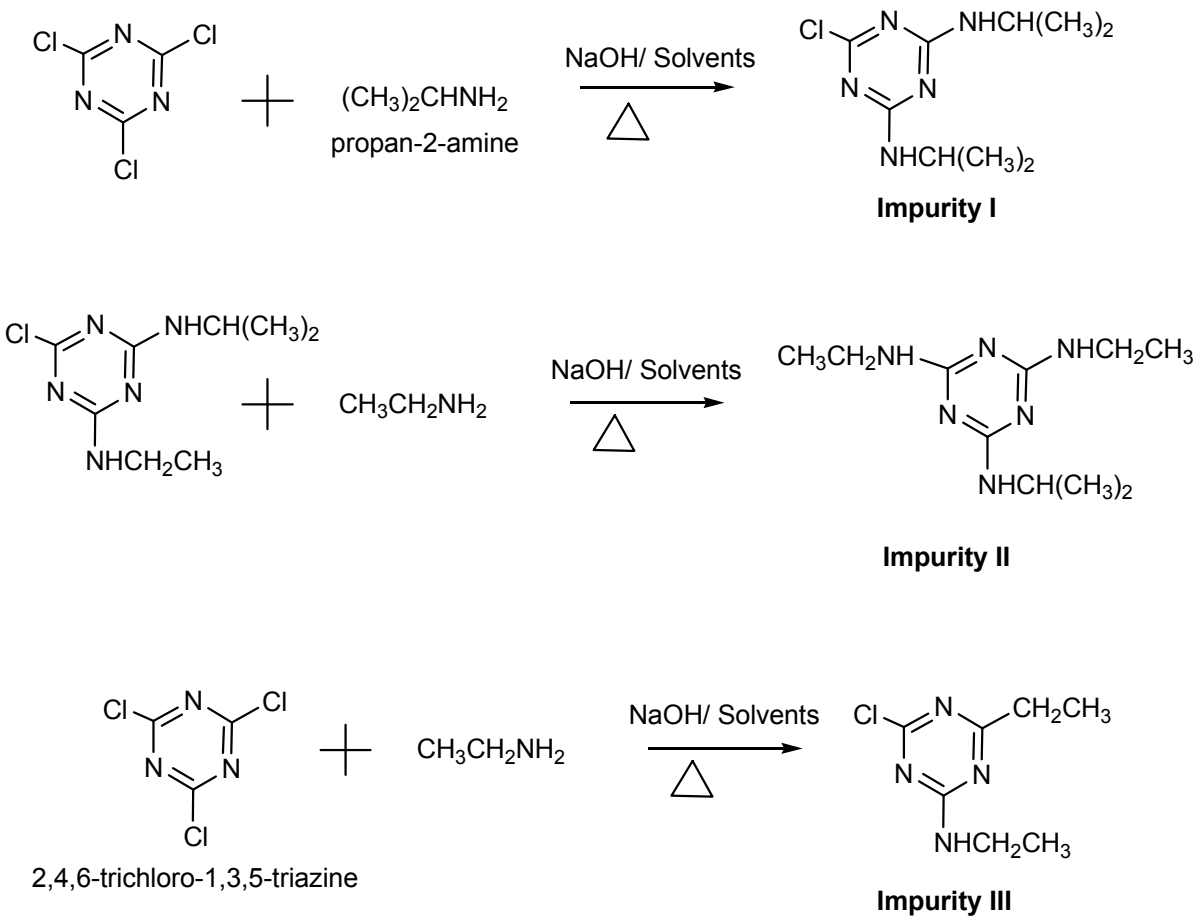


FIG. 2. Routes of synthesis of impurities.

5. QUANTITATIVE ANALYSIS ON THE IMPURITIES OF TECHNICAL ATRAZINE

The gas chromatographic conditions described in section 2.3 were used for the analysis. The characteristic parameters of the calibration were determined with 3 × 2 points calibration (Table 3).

TABLE 3. CALIBRATION DATA OF IMPURITIES

Compound	Regression equation	Linear range µg/ml	R^2	LOQ µg/ml
Impurity I	y = 172.32x – 235.3	20 – 120	0.9946	1
Impurity II	y = 452.55x – 1909.8	30 – 150	0.9998	8.05
Impurity III	526.14x – 1518.3	15 – 60	0.9999	2.62

5.1. Preparation of standard and sample solutions

Weigh about 5 mg of Impurity I, 2 mg of Impurity II and III into 25 ml glass flask, and prepare the standard solution mixture with chloroform. Dilute the above solution to make a 2 and 4 times diluted solutions.

Weigh about 300–400 mg technical atrazine into a glass stoppered bottle (50 ml), dissolve it and make up mark with chloroform. Inject the sample solution and analytical standard solutions into the chromatograph when it reaches stability.

The impurities determined in various technical grade products are summarized in Table 4.

TABLE 4. IMPURITIES IN 5 BATCHES DETERMINED BY GC-FID

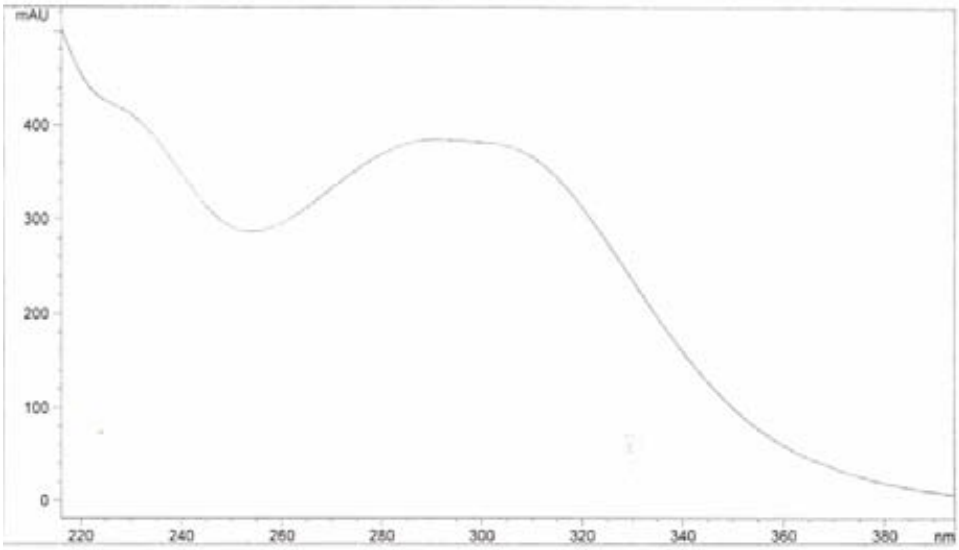
Runs ID	M (mg)	Impurity I (%)	Impurity II (%)	Impurity III (%)	Average I (%)	Average II (%)	Average III (%)
Run 1-1	371.40	0.39	1.14	0.43	0.42	1.25	0.47
Run 1-2	371.40	0.44	1.36	0.50			
Run 2-1	369.80	0.39	1.25	0.45	0.39	1.22	0.45
Run 2-2	369.80	0.39	1.20	0.44			
Run 3-1	326.70	0.30	0.89	0.34	0.34	1.01	0.38
Run 3-2	326.70	0.38	1.13	0.42			
Run 4-1	298.30	0.29	0.83	0.31	0.34	1.00	0.38
Run 4-2	298.30	0.39	1.17	0.44			
Run 5-1	318.60	0.33	0.92	0.34	0.38	1.11	0.42
Run 5-2	318.60	0.43	1.30	0.49			

ANNEXES

Annex 1

UV spectra of compounds included in Section "HPLC 'MULTI-ANALYTE' DETECTION METHOD".

(23) Amitraz

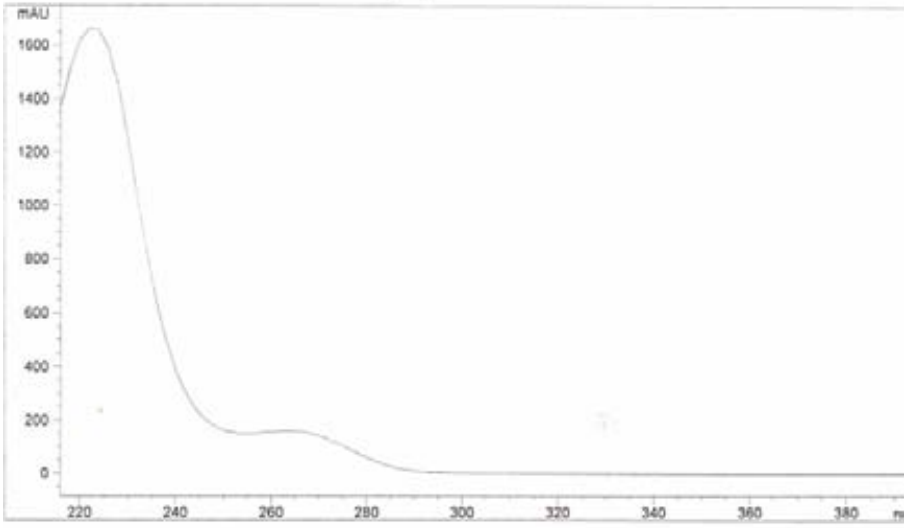



```
      name : Amitraz
Time [min] : 11.09
        ID# : 0
   ID Name :
      Info :  55%ACN(modifier:2% 1-propanol:water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.5mg/ml

   created : 14-NOV-2001          created by : Dudar E
   changed : 14-NOV-2001          changed by :
```

		nm	mAU
File	D:\HPDATA\MUTLIB\SIG10419.D		
Time [min]	11.091	254.0	287.2
scan#	832	290.0	385.5

(24) Atrazin

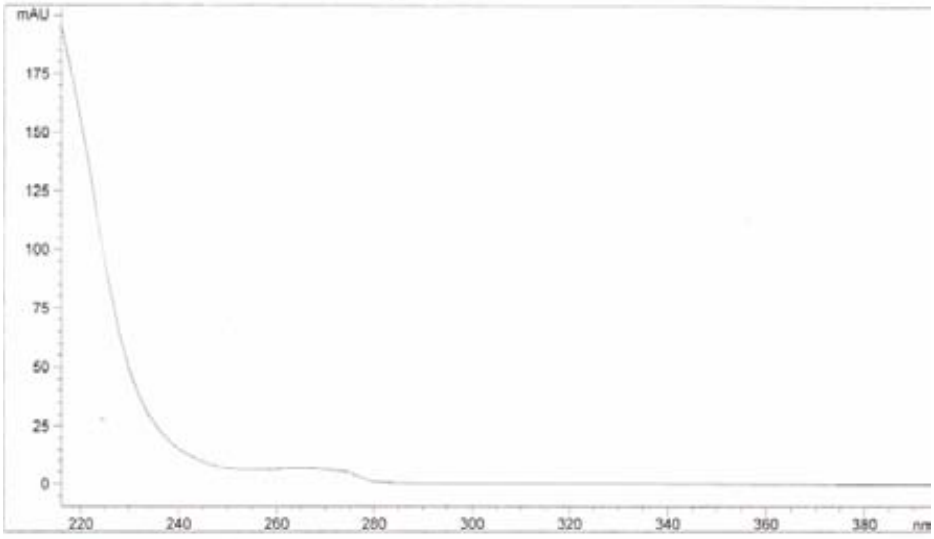



```
      name : Atrazin
Time [min] : 8.09
        ID# : 0
   ID Name :
      Info : 33%ANC(modifier:2% 1-propanol):water pH:2.6(modifier:2%1-propanol
    Sample : Reference
   Solvent : ACN 0.5mg/ml

   created : 14-NOV-2001          created by : Dudar E
   changed : 14-NOV-2001          changed by :
```

		nm	mAU
File	D:\HPDATA\MUTLIB\SIG10308.D		
Time [min]	8.087	222.0	1665.7
scan#	607	254.0	149.5
Range [nm]	216.0 - 394.0 (step : 2.0)	264.0	159.6

(25) Butachlor

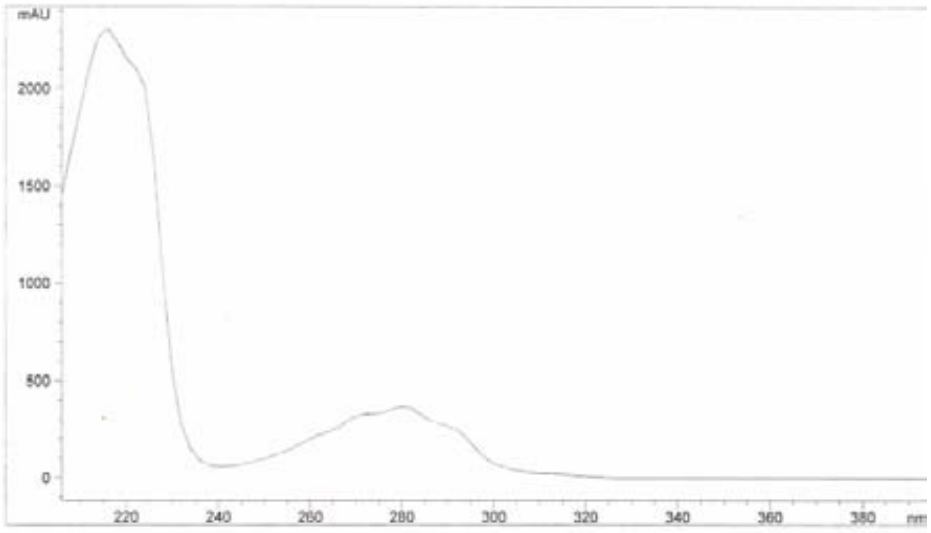



```
      name : Butachlor
Time [min] : 8.95
       ID# : 0
   ID Name :
      Info : 65% ACn(modifier:2% 1-propanol:ware pH:2.6(modifier:2%1-propanol
    Sample : Reference
   Solvent : ACn 0.5 mg/ml

   created : 14-NOV-2001          created by : Dudar E
   changed : 14-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10364.D          nm    mAU
Time [min] : 8.947                              | 216.0 | 194.5 |
      scan# : 672                               | 256.0 |   6.0 |
Range  [nm] : 216.0 - 394.0  (step : 2.0)       | 266.0 |   7.0 |
```

(33) Carbaryl

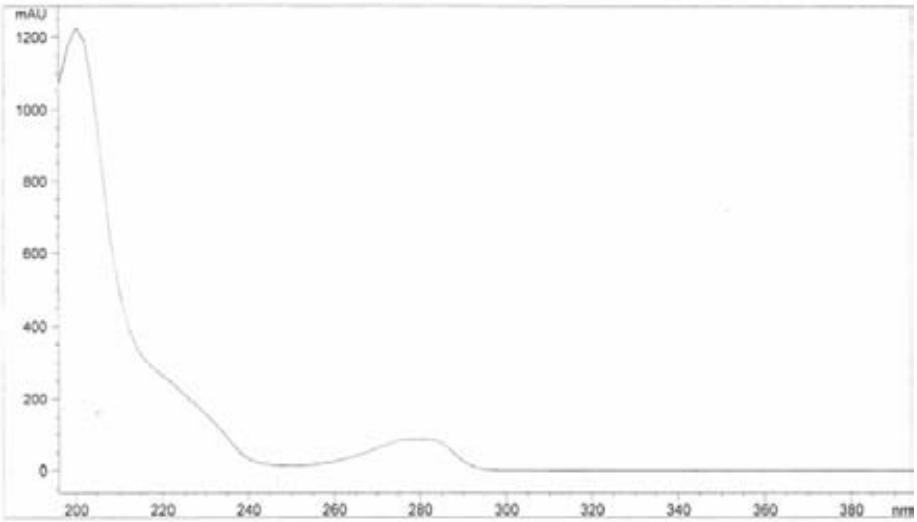



```
      name : Carbaryl
Time [min] : 7.38
       ID# : 0
   ID Name :
      Info : 34%ACN (modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Referece
   Solvent : ACN  0.536 mg/ml

   created : 28-NOV-2001          created by : Dudar E
   changed : 28-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10234.D          nm    mAU
Time [min] : 7.378                              | 216.0 |2310.4 |
      scan# : 554                               | 240.0 |  59.3 |
Range  [nm] : 206.0 - 394.0  (step : 2.0)       | 280.0 | 372.8 |
```

(26) Carbosulfan

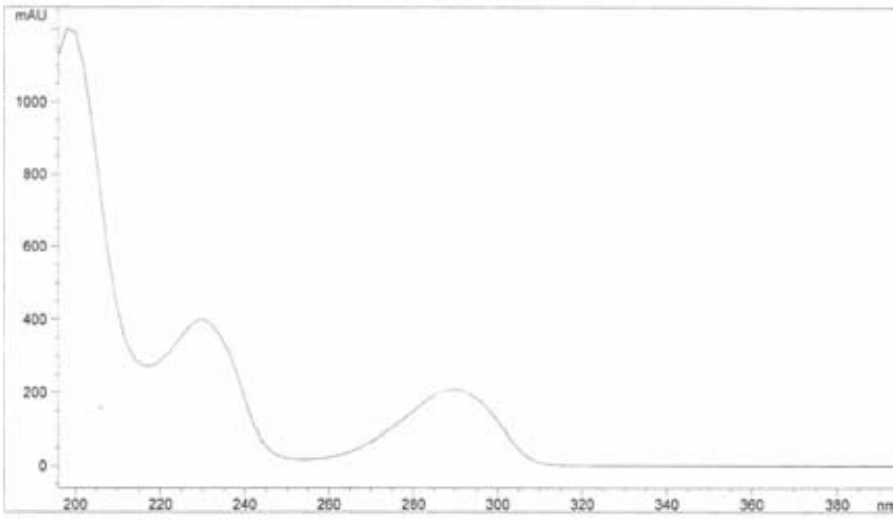



```
      name : Carbosulfan
Time [min] : 8.57
       ID# : 0
   ID Name :
      Info : 80%ACN(modifier:2% 1-propanol):water pH2.6((modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.524 mg/ml

   created : 14-NOV-2001          created by : Dudar E
   changed : 14-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10397.D          nm    mAU
Time [min] : 8.571                              | 202.0 |1188.3 |
      scan# : 643                               | 220.0 | 265.4 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)       | 250.0 |  12.7 |
      [mUA] :   0.1 - 1227.4                    | 280.0 |  87.0 |
```

(35) Chlorpyriphos

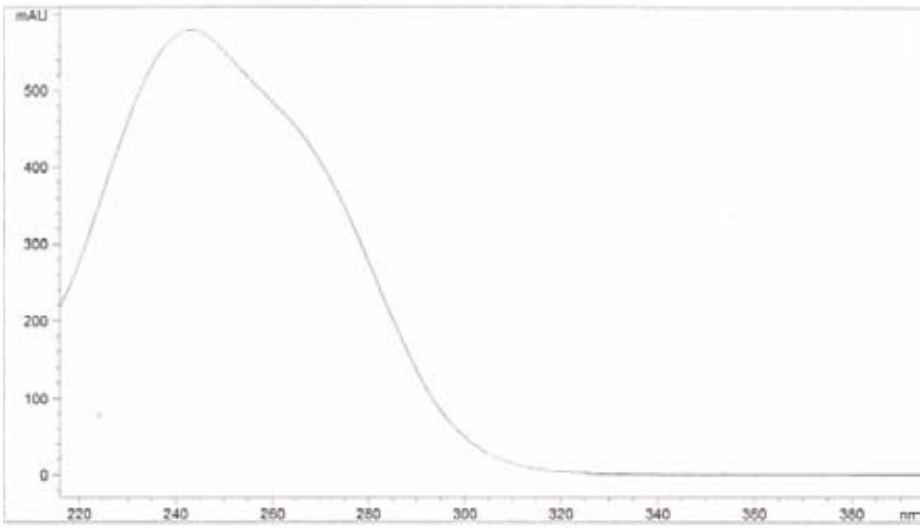



```
      name : Chlorpyriphos
Time [min] : 7.11
       ID# : 0
   ID Name :
      Info : 70%ACN(modifieer:2% 1-propanol):water pH:2.6(modifier:2% 1-propanol)
    Sample : Chlorpyrifos
   Solvent :

   created : 28-NOV-2001          created by : Dudar E
   changed : 28-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10391.D          nm    mAU
Time [min] : 7.108                              | 198.0 |1201.0 |
      scan# : 534                               | 216.0 | 272.8 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)       | 230.0 | 402.2 |
      [mUA] :   0.2 - 1201.0                    | 254.0 |  16.9 |
                                                | 290.0 | 210.0 |
```

(37) Cymoxanyl

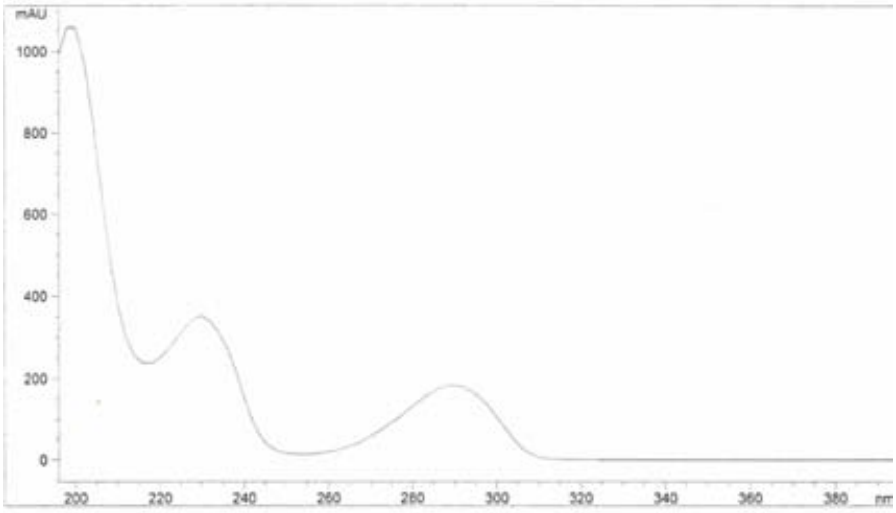



```
      name : Cymoxanyl
Time [min] : 7.11
       ID# : 0
    ID Name :
      Info : 20%ACN(modifier:2% 1-propanol):wate pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN  0.54 mg/ml

   created : 28-NOV-2001          created by : Dudar E
   changed : 28-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10224.D          |   nm  |  mAU  |
Time [min] : 7.114                                | 244.0 | 580.4 |
      scan# : 534                                 | 254.0 | 524.7 |
Range  [nm] : 216.0 - 394.0  (step : 2.0)         | 280.0 | 279.5 |
```

(36) Chlorpyriphos- Me

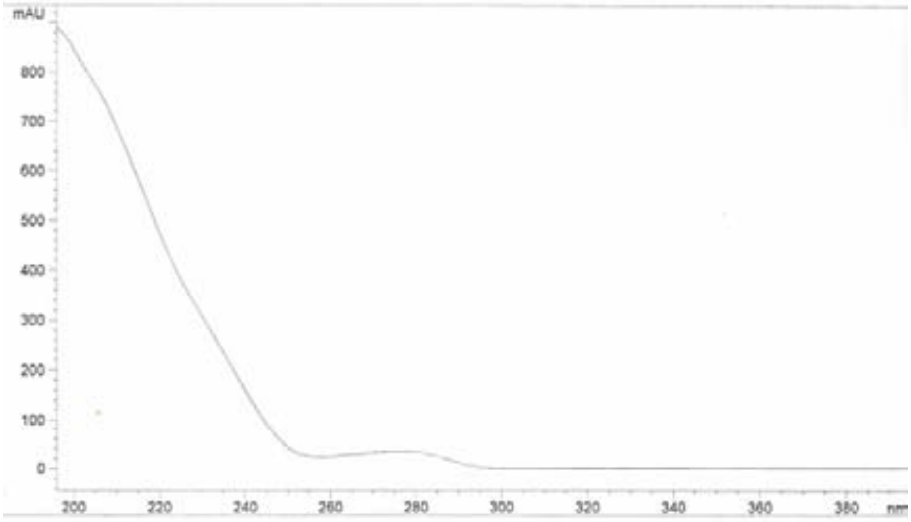



```
      name : Chlorpyriphos- Me
Time [min] : 8.09
       ID# : 0
    ID Name :
      Info : 60%ACN(modifier:2% 1-propanol):waterp:2.6(modifier:2% 1-propanol)
    Sample : Chlorpyrifos Me
   Solvent : ACN  0.48mg/ml

   created : 28-NOV-2001          created by : Dudar E
   changed : 28-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10399.D          |   nm  |  mAU   |
Time [min] : 8.087                                | 198.0 | 1060.5 |
      scan# : 607                                 | 216.0 |  238.3 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)         | 230.0 |  353.0 |
      [mUA] :   0.1 - 1060.5                      | 254.0 |   13.7 |
                                                  | 290.0 |  184.3 |
```

(27) Cypermetrin

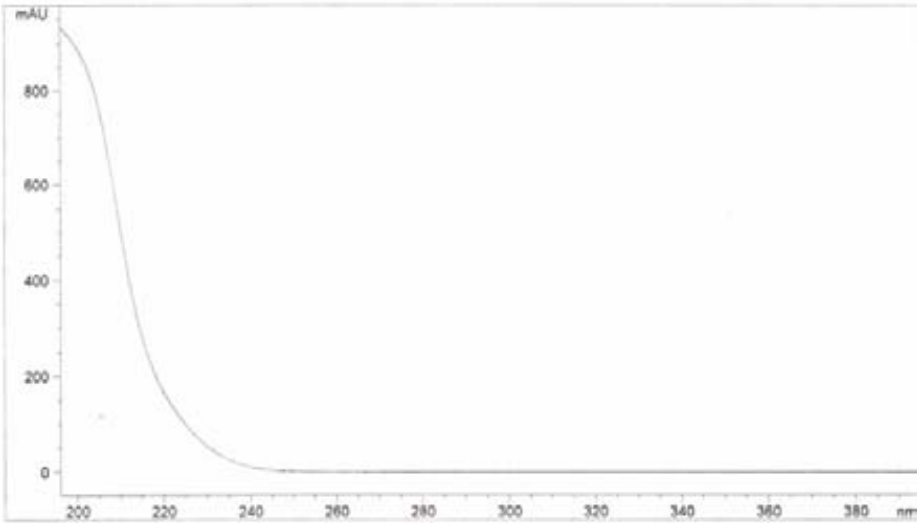



```
     name : Cypermetrin
Time [min] : 6.25
     ID# : 0
   ID Name :
     Info : 77%ACN(modifier:2% 1-prpanol):water pH:2.6(modifier:2% 1-propanol0
   Sample : Reference
  Solvent : ACN 0.5 mg/ml

  created : 14-NOV-2001          created by : Dudar E
  changed : 14-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10372.D          nm    mAU
Time [min] : 6.248                              | 200.0 | 847.7 |
     scan# : 469                                | 210.0 | 689.8 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)       | 254.0 |  26.0 |
      [mUA] :   0.1 - 890.4                     | 276.0 |  34.5 |
```

(41) DDVP

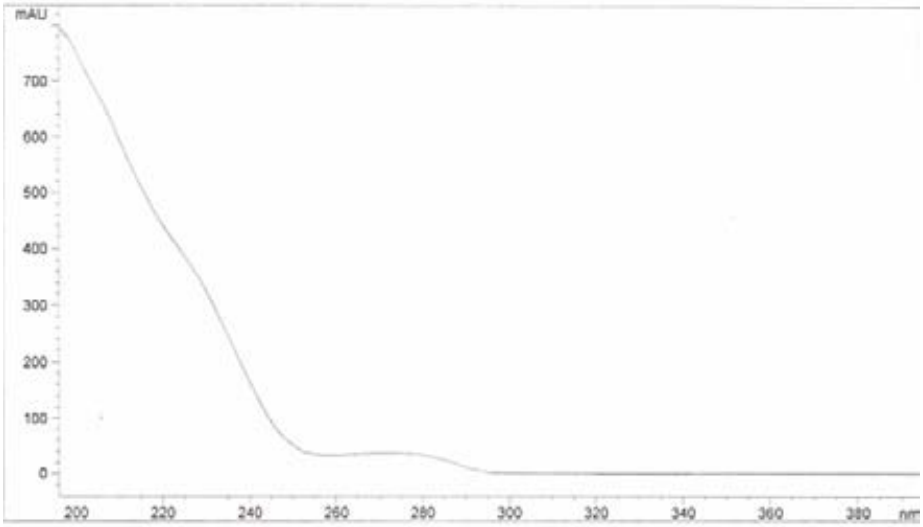



```
     name : DDVP
Time [min] : 4.51
     ID# : 0
   ID Name :
     Info : 30%ACN(modifier:2% 1-propanol):water pH2.6(modifier;2% 1-propanol)
   Sample : Reference
  Solvent : ACN 0.5 mg/ml

  created : 28-NOV-2001          created by : Dudar E
  changed : 28-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10409.D          nm    mAU
Time [min] : 4.511                              | 200.0 | 888.5 |
     scan# : 339                                | 210.0 | 502.6 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)       | 220.0 | 166.6 |
```

(38) Deltametrin



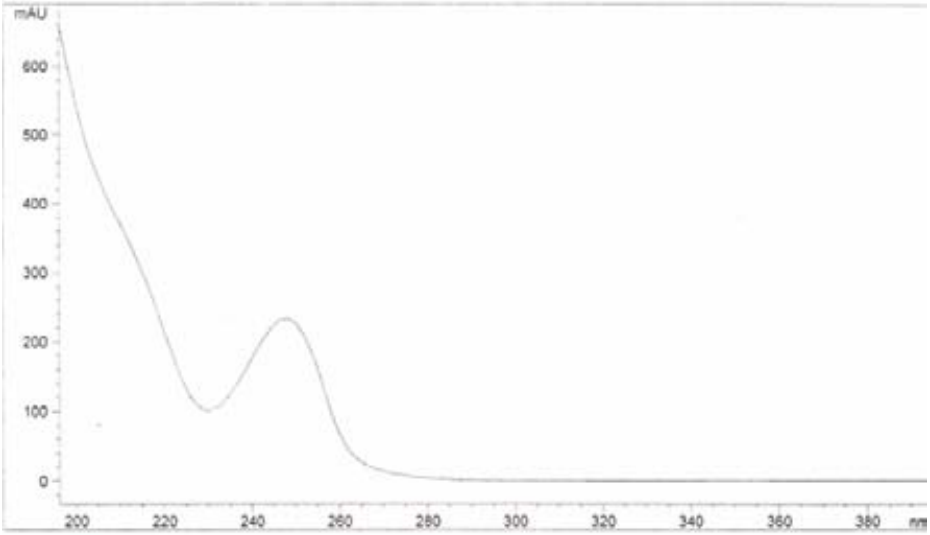

```
      name : Deltametrin
Time [min] : 12.45
        ID# : 0
   ID Name :
      Info : 70%ACN(modifier:2% 1-propaol):water pH2.6(modifier:25 1-propanol)
    Sample : Reference
   Solvent : ACN  0.5 mg/ml

   created : 28-NOV-2001          created by : Dudar E
   changed : 28-NOV-2001          changed by :
```

```
       File : D:\HPDATA\MUTLIB\SIG10392.D
 Time [min] : 12.454
       scan# : 935
Range  [nm] : 196.0 - 394.0  (step : 2.0)
      [mUA] :   0.1 - 794.7
```

nm	mAU
210.0	593.5
220.0	440.6
254.0	35.5
260.0	32.5

(39) Diazinon



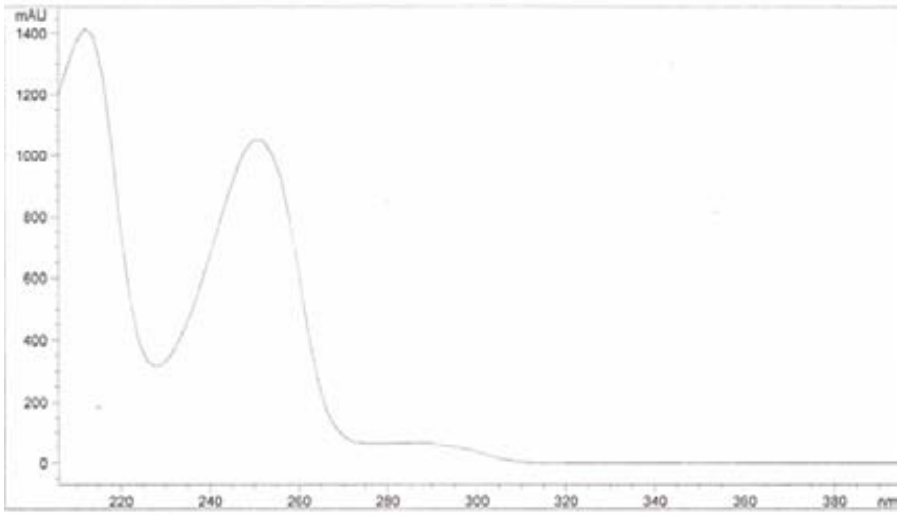

```
      name : Diazinon
Time [min] : 6.66
        ID# : 0
   ID Name :
      Info : 60%ACN(modifier:2% 1-propanol):water pH2.6(modofier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.62 mg/ml

   created : 28-NOV-2001          created by : Dudar E
   changed : 28-NOV-2001          changed by :
```

```
       File : D:\HPDATA\MUTLIB\SIG10408.D
 Time [min] : 6.655
       scan# : 500
Range  [nm] : 196.0 - 394.0  (step : 2.0)
```

nm	mAU
210.0	369.5
230.0	101.3
248.0	234.5

(40) Diuron



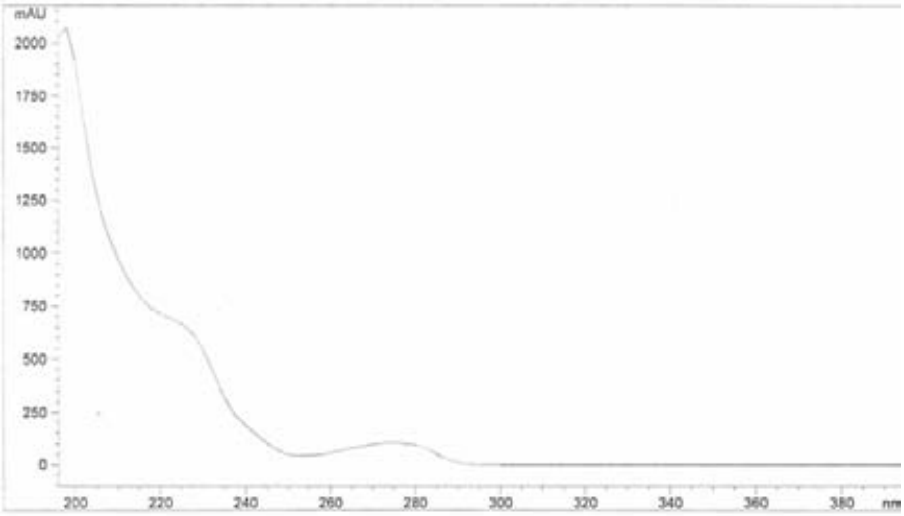

```
     name : Diuron
Time [min] : 8.37
       ID# : 0
   ID Name :
     Info : 35%ACN(modifier:2% 1-propanol):water pH:2.6(modifier:2% 1-propanol)
   Sample : ACN   0.532 mg/ml
  Solvent :

  created : 28-NOV-2001          created by : Dudar E
  changed : 28-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10236.D
Time [min] : 8.374
     scan# : 629
Range  [nm] : 206.0 - 394.0  (step : 2.0)
      [mUA] :   0.3 - 1415.8
```

nm	mAU
212.0	1415.8
228.0	314.1
250.0	1052.1
278.0	61.5
286.0	64.4

(42) Etofenprox



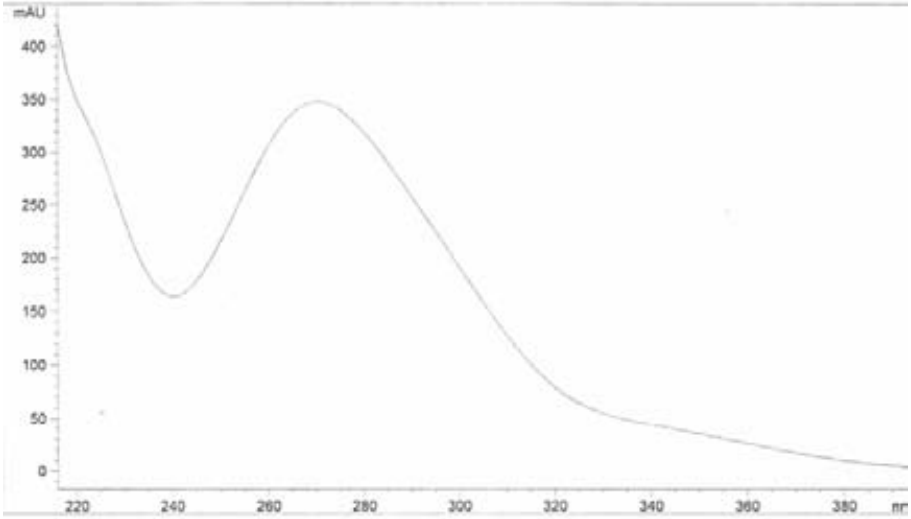

```
     name : Etofenprox
Time [min] : 7.51
       ID# : 0
   ID Name :
     Info : 80%ACN(modofier:2% 1-propanol):water pH2.6(modofier:2% 1-propanol)
   Sample : Reference
  Solvent : ACN 0.528 mg/ml

  created : 29-NOV-2001          created by : Dudar E
  changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10395.D
Time [min] : 7.510
     scan# : 564
Range  [nm] : 196.0 - 394.0  (step : 2.0)
      [mUA] :   0.2 - 2073.6
```

nm	mAU
200.0	1921.4
220.0	713.6
254.0	41.6
274.0	104.0

(43) Fenitrothion

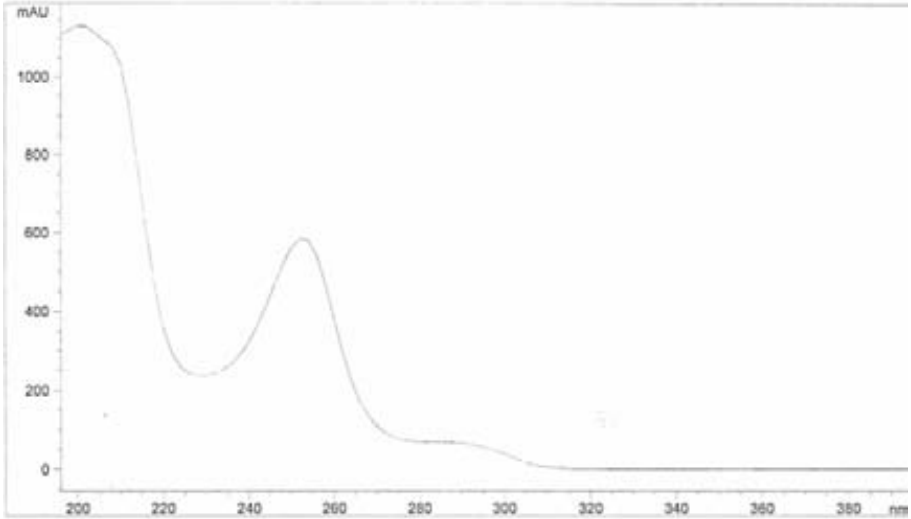



```
      name : Fenitrothion
Time [min] : 7.71
       ID# : 0
   ID Name :
      Info : 51%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.0.66 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10327.D          nm    mAU
Time [min] : 7.705                              | 216.0 | 419.4 |
      scan# : 578                               | 240.0 | 163.7 |
Range  [nm] : 216.0 - 394.0  (step : 2.0)       | 270.0 | 348.1 |
```

(45) Fenthion

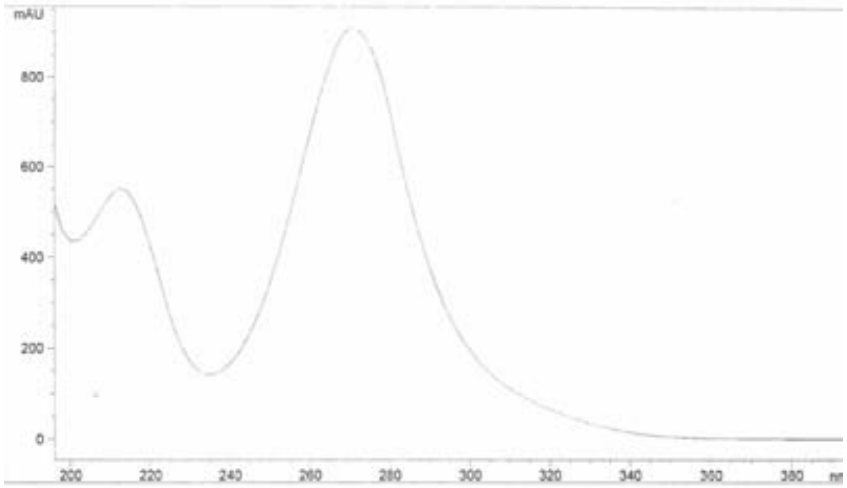



```
      name : Fenthion
Time [min] : 6.08
       ID# : 0
   ID Name :
      Info : 60%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN  0.5 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10407.D          nm    mAU
Time [min] : 6.083                              | 200.0 |1131.9 |
      scan# : 457                               | 230.0 | 236.7 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)       | 252.0 | 585.5 |
      [mUA] :   0.2 - 1131.9                    | 280.0 |  70.3 |
```

(46) Imidacloprid



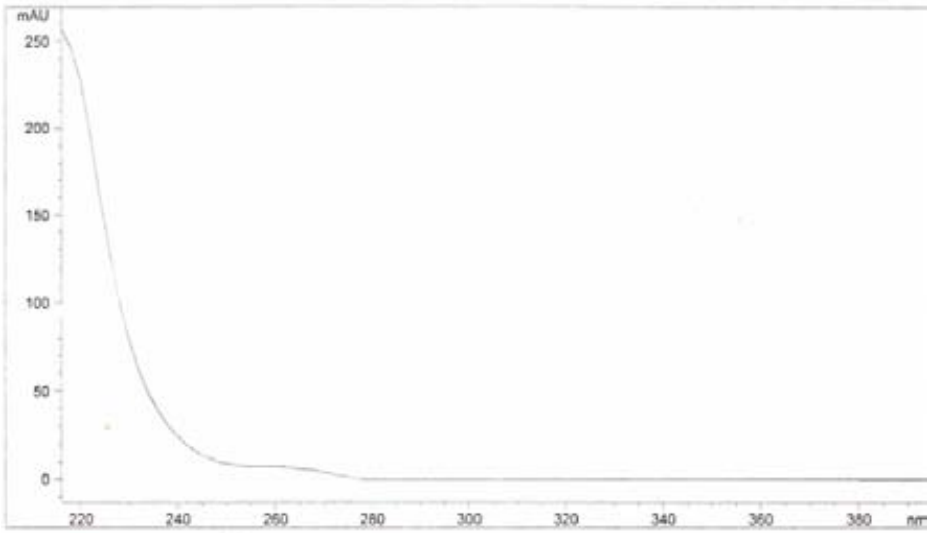
name : Imidacloprid
Time [min] : 6.68
ID# : 0
ID Name :
Info : 15%ACN(modifier:2% 1-propanol):water pH2.69(modifier:2% 1-propanol)
Sample : Reference
Solvent : ACN 0.5 mg/ml

created : 29-NOV-2001 created by : Dudar E
changed : 29-NOV-2001 changed by :

File : D:\HPDATA\MUTLIB\SIG10418.D
Time [min] : 6.677
scan# : 501
Range [nm] : 196.0 - 394.0 (step : 2.0)
[mUA] : 0.4 - 911.5

nm	mAU
200.0	436.2
202.0	437.3
212.0	551.0
234.0	143.2
270.0	911.5
280.0	723.9
298.0	221.0

(47) Iprobenfos



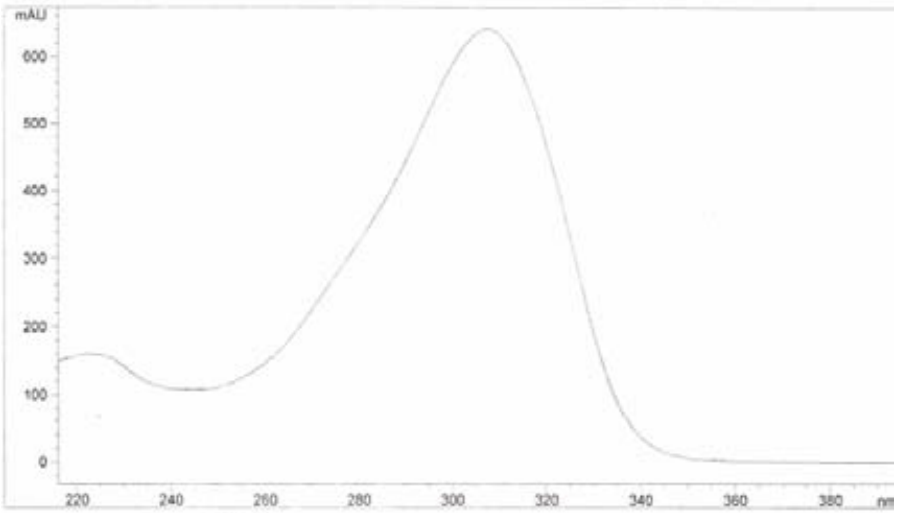
name : Iprobenfos
Time [min] : 8.39
ID# : 0
ID Name :
Info : 49%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
Sample : Reference
Solvent : ACN 0.452 mg/ml

created : 29-NOV-2001 created by : Dudar E
changed : 29-NOV-2001 changed by :

File : D:\HPDATA\MUTLIB\SIG10319.D
Time [min] : 8.392
scan# : 630
Range [nm] : 216.0 - 394.0 (step : 2.0)

nm	mAU
216.0	256.9
220.0	226.4
254.0	7.9

(48) Isoprothiolan



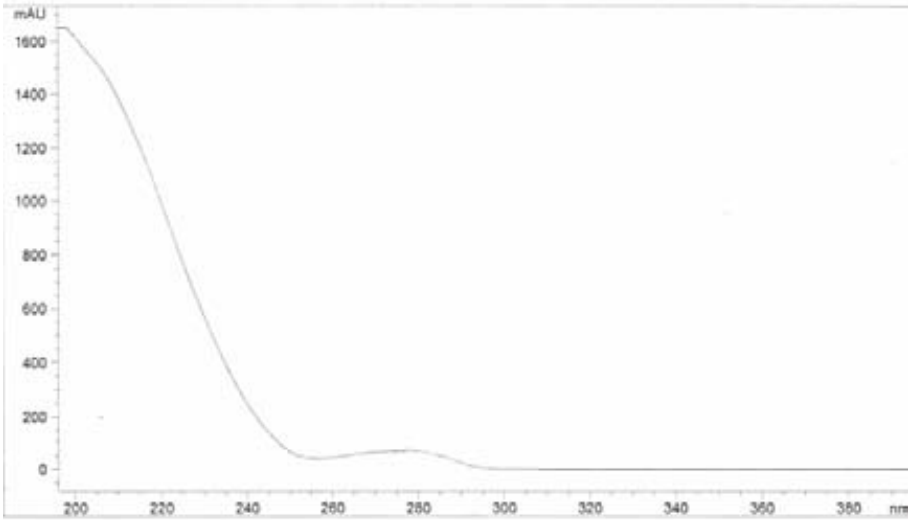

```
      name : Isoprothiolan
Time [min] : 7.60
        ID# : 0
   ID Name :
      Info : 50%ACN(modifier:2% 1-propanol):water pH2.6(modifier:25 1-propanol
    Sample : Reference
   Solvent : ACN  0.476 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10321.D
Time [min] : 7.596
      scan# : 570
Range  [nm] : 216.0 - 394.0  (step : 2.0)
      [mUA] :   0.3 - 640.6
```

nm	mAU
222.0	160.0
246.0	106.9
254.0	120.6
280.0	325.1
308.0	640.6

(49) Lambda-Cyhalothrin



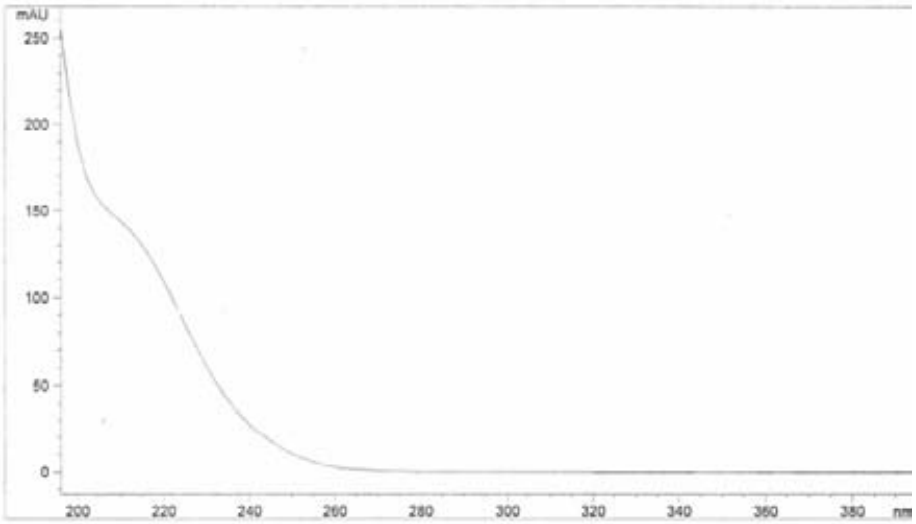

```
      name : Lambda-Cyhalothrin
Time [min] : 8.33
        ID# : 0
   ID Name :
      Info : 74%ACN(modifier:2% 1-propanol):waer pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN0.5 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10396.D
Time [min] : 4.959
      scan# : 372
Range  [nm] : 196.0 - 394.0  (step : 2.0)
      [mUA] :   0.2 - 1651.4
```

nm	mAU
196.0	1651.4
220.0	993.5
254.0	42.1
280.0	67.0

(50) Malathion



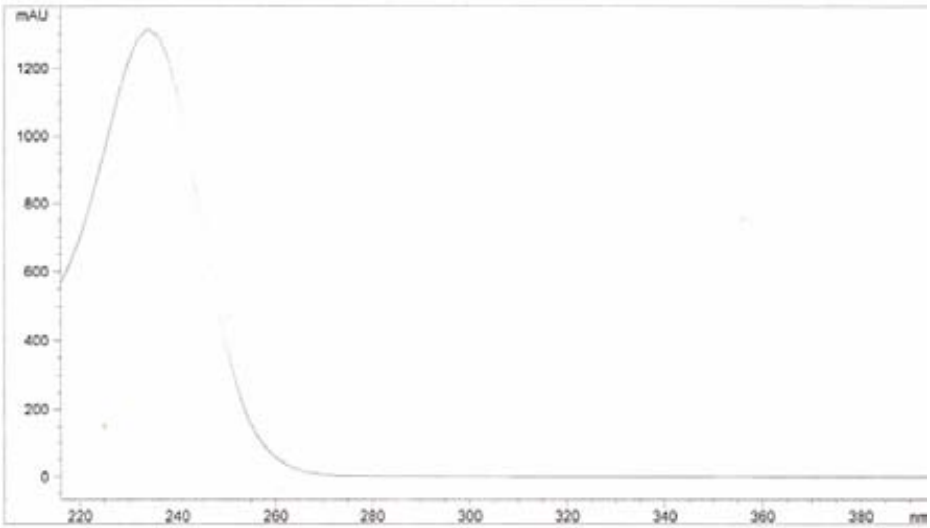

```
      name : Malathion
Time [min] : 7.46
       ID# : 0
   ID Name :
      Info : 50%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN  0.58 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10389.D
Time [min] : 7.456
      scan# : 560
Range  [nm] : 196.0 - 394.0  (step : 2.0)
      [mUA] :   0.0 - 255.5
```

nm	mAU
196.0	255.5
210.0	144.1
222.0	99.7
254.0	6.5

(52) Methomil



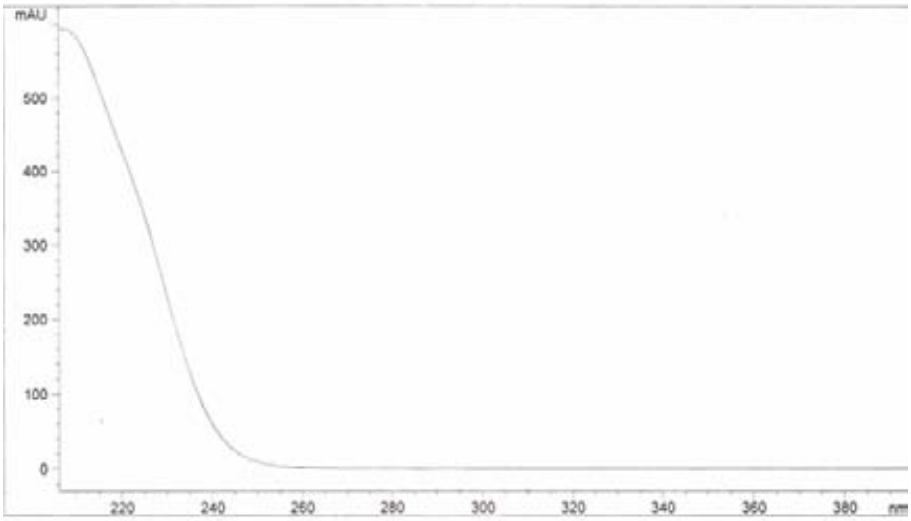

```
      name : Methomil
Time [min] : 2.48
       ID# : 0
   ID Name :
      Info : 20%ACN(modirier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN  0.496 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10223.D
Time [min] : 2.484
      scan# : 187
Range  [nm] : 216.0 - 394.0  (step : 2.0)
```

nm	mAU
216.0	567.0
234.0	1315.1
254.0	187.2

(53) Molinate

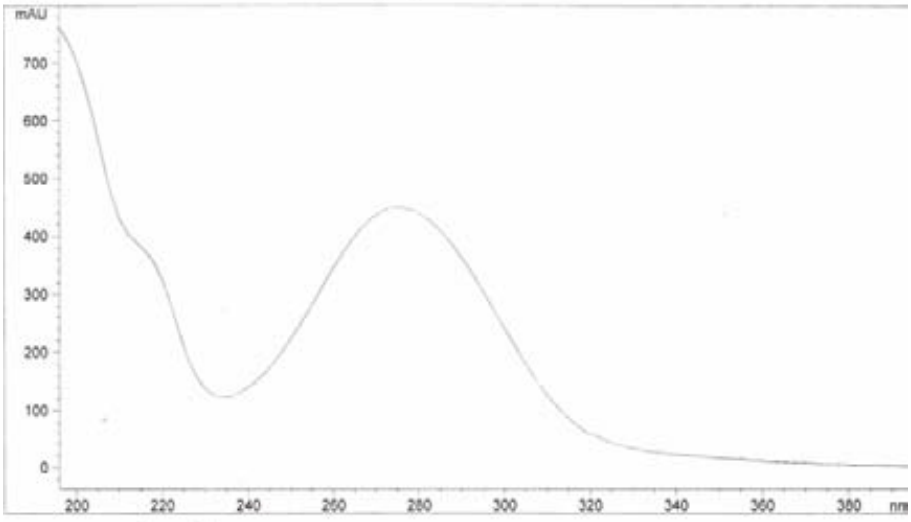



```
      name : Molinate
Time [min] : 6.51
       ID# : 0
   ID Name :
      Info : 50%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.628 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10242.D          nm    mAU
Time [min] : 9.203                              | 210.0 | 582.6 |
      scan# : 691                               | 220.0 | 428.9 |
Range  [nm] : 206.0 - 394.0  (step : 2.0)       | 230.0 | 230.7 |
```

(54) Parathion

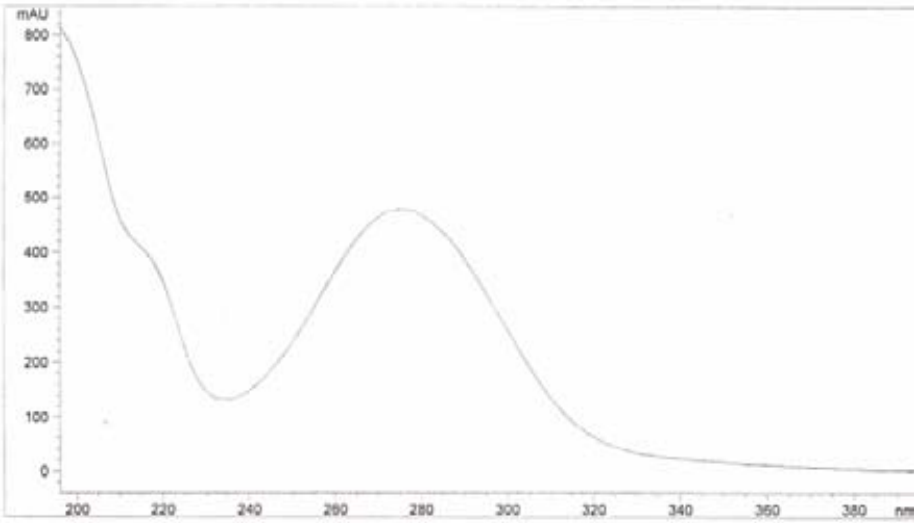



```
      name : Parathion
Time [min] : 6.37
       ID# : 0
   ID Name :
      Info : 50%ACN(modifier:2% 1-propanol0:water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN  0.48 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10387.D          nm    mAU
Time [min] : 6.372                              | 216.0 | 374.4 |
      scan# : 478                               | 234.0 | 123.0 |
Range  [nm] : 196.0 - 394.0  (step : 2.0)       | 276.0 | 449.2 |
```

(55) Parathion-Me



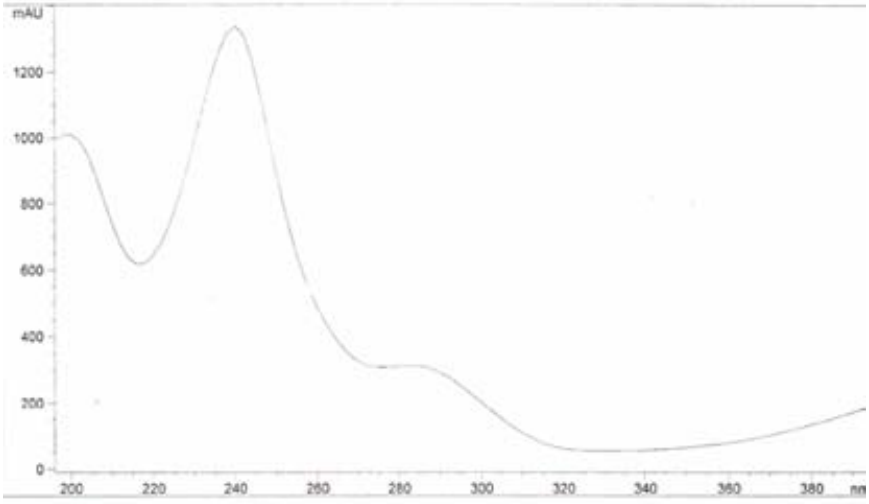

```
      name : Parathion-Me
Time [min] : 6.36
        ID# : 0
   ID Name :
      Info : 50%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.5mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10386.D
Time [min] : 6.363
      scan# : 478
Range  [nm] : 196.0 - 394.0  (step : 2.0)
```

nm	mAU
216.0	399.9
234.0	131.4
276.0	479.9

(58) Pendimethalin




```
      name : Pendimethalin
Time [min] : 5.95
        ID# : 0
   ID Name :
      Info : 73%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.585 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10355.D
Time [min] : 5.952
      scan# : 447
Range  [nm] : 196.0 - 394.0  (step : 2.0)
      [mUA] :  54.4 - 1339.3
```

nm	mAU
200.0	1011.5
216.0	618.0
240.0	1339.3
276.0	306.7
282.0	311.7
330.0	54.4

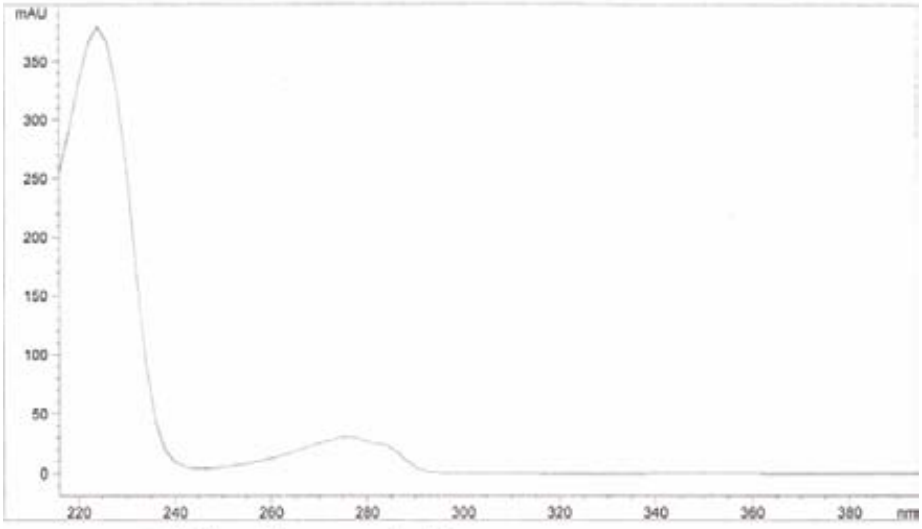
(56) Proiconazole


```
      name : Proiconazole
Time [min] : 8.66
        ID# : 0
   ID Name :
      Info : 50%ACN(modifier:2% 1-propanol):water(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.6mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

		nm	mAU
File	D:\HPDATA\MUTLIB\SIG10324.D		
Time [min]	8.657	220.0	274.2
scan#	650	230.0	185.6
Range [nm]	216.0 - 394.0 (step : 2.0)	270.0	5.8

(57) Triadimenol isomer ratio 7:3

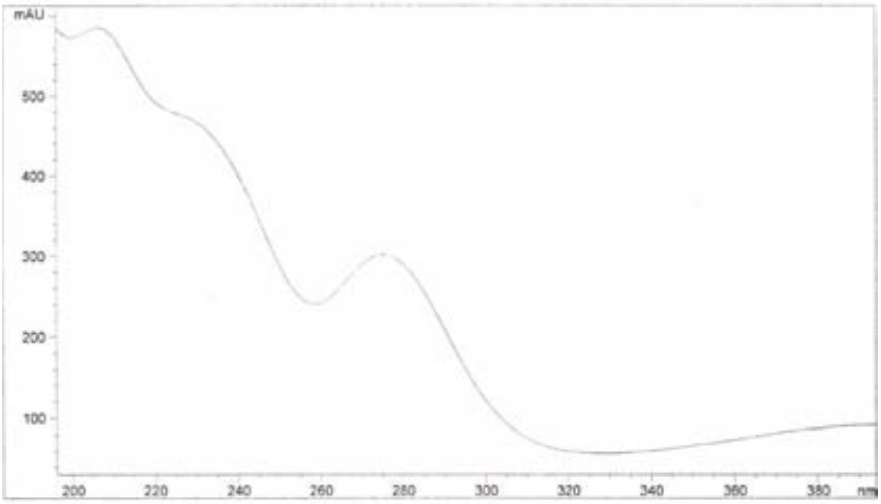



```
      name : Triadimenol isomer ratio 7:3
Time [min] : 7.81
        ID# : 0
   ID Name :
      Info : 41% ACN(modifier:25 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN  0.528 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

		nm	mAU
File	D:\HPDATA\MUTLIB\SIG10313.D		
Time [min]	7.809	224.0	379.6
scan#	586	246.0	4.3
Range [nm]	216.0 - 394.0 (step : 2.0)	276.0	31.4

(59) Triflulalin



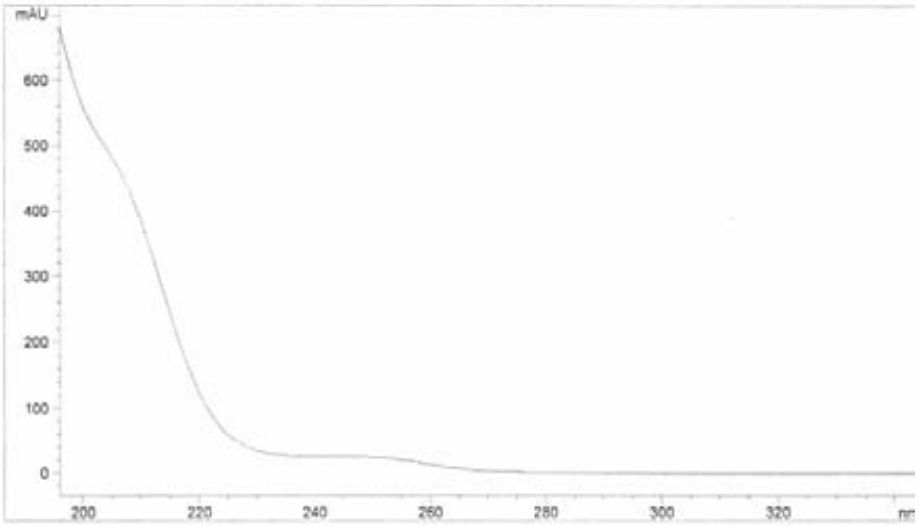

```
      name : Triflulalin
Time [min] : 7.32
       ID# : 0
   ID Name :
      Info : 70%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol
    Sample : Reference
   Solvent : ACN 0.53 mg/ml

   created : 29-NOV-2001          created by : Dudar E
   changed : 29-NOV-2001          changed by :
```

```
      File : D:\HPDATA\MUTLIB\SIG10367.D
Time [min] : 7.324
      scan# : 550
Range  [nm] : 196.0 - 394.0  (step : 2.0)
      [mUA] :  57.3 - 586.4
```

nm	mAU
200.0	573.2
206.0	586.4
230.0	467.1
258.0	240.3
276.0	301.6
328.0	57.3

(79) Captan



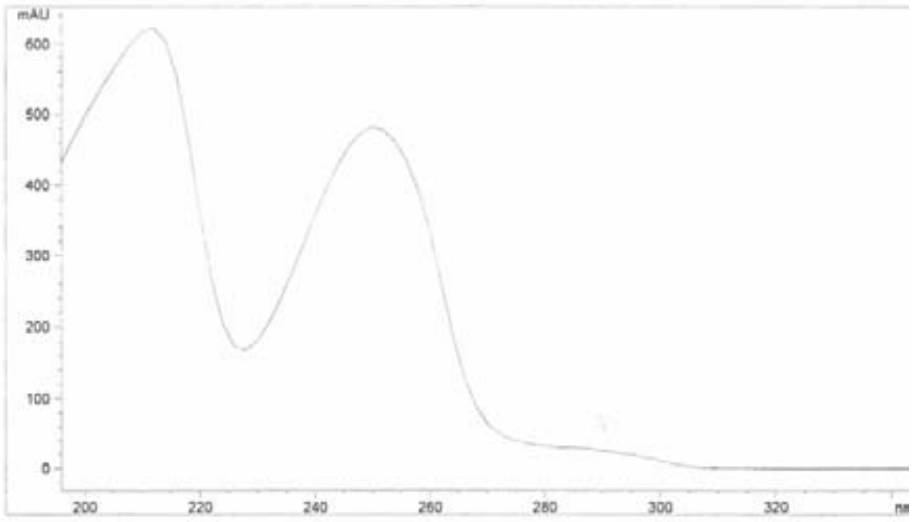

```
      name : Captan
Time [min] : 6.52
       ID# : 0
   ID Name :
      Info : 40%ACN(modifier:2% 1-propanol0;water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN   0.5 mg/ml        Column:NovaPack C18   3.9x150

   created : 07-DEC-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10501.D
Time [min] : 6.524
      scan# : 490
Range  [nm] : 196.0 - 344.0  (step : 2.0)
```

nm	mAU
206.0	469.7
210.0	386.8
254.0	20.8

(71) Chlorbromuron



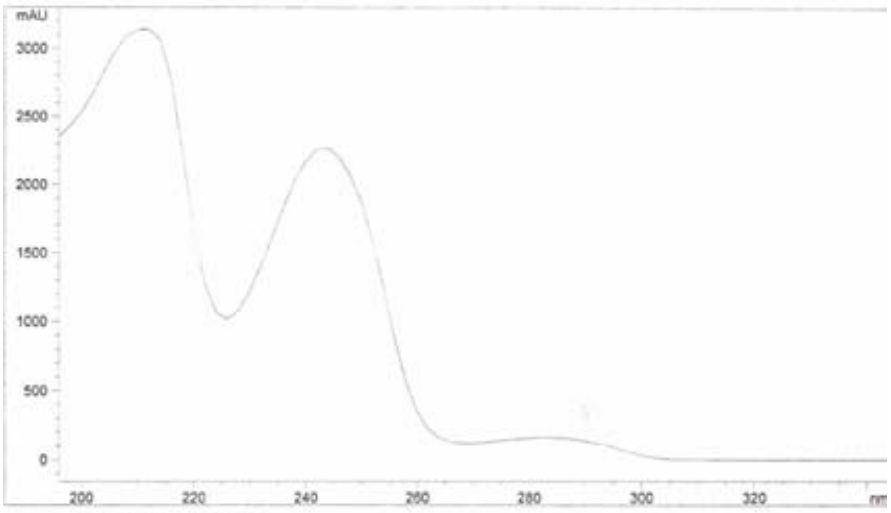

```
     name : Chlorbromuron
Time [min] : 8.43
       ID# : 0
   ID Name :
     Info : 38%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
   Sample : Reference
   Solvent : ACN                Column:NovaPack C18    3.9x150

   created : 07-DEC-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10497.D
 Time [min] : 8.429
      scan# : 633
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.4 - 622.1
```

nm	mAU
212.0	622.1
228.0	168.1
250.0	481.9
280.0	32.3

(69) chlortoluron



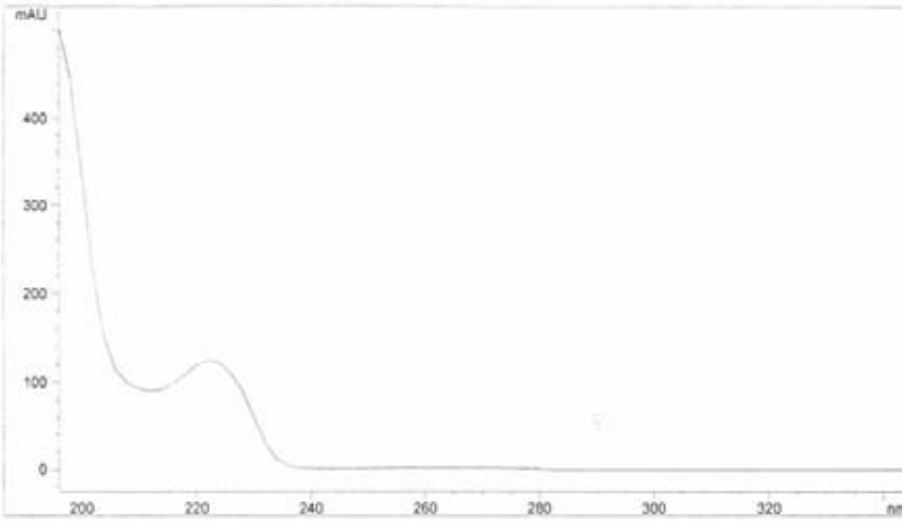

```
     name : chlortoluron
Time [min] : 6.34
       ID# : 0
   ID Name :
     Info : 29%ACN(modifier:2% 1-propanol):water pH22.6(modifier:2% 1-propanol)
   Sample : Reference
   Solvent : ACN  1mg/ml     Column: NovaPack C 18 3.9x150

   created : 07-DEC-2001          created by : Dudar
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10382.D
 Time [min] : 6.343
      scan# : 476
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.6 - 3139.7
```

nm	mAU
212.0	3139.7
226.0	1015.9
244.0	2269.3
268.0	119.6
284.0	160.3

(68) Cyproconazole



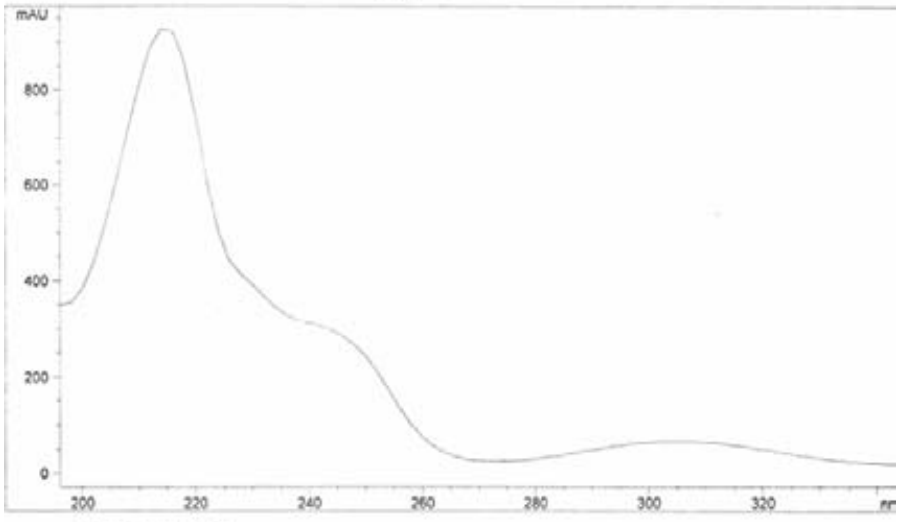

```
     name : Cyproconazole
Time [min] : 10.51
       ID# : 0
   ID Name :
      Info : 35%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN            Column:NovaPack C18  3.9x150

   created : 07-DEC-2001           created by : Dudar E
   changed : 07-DEC-2001           changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10489.D
 Time [min] : 10.509
      scan# : 789
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.1 - 502.2
```

nm	mAU
212.0	90.0
222.0	124.7
244.0	1.2
266.0	2.8

(77) Bentazon



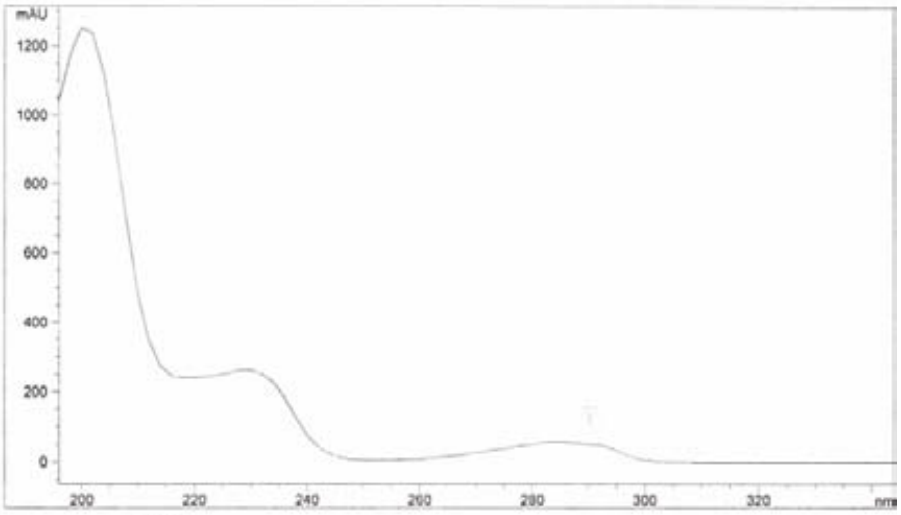

```
     name : Bentazon
Time [min] : 6.21
       ID# : 0
   ID Name :
      Info : 29% ACN(modifier:2% 1-propanol);water pH2.69modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN   0.5mg/ml   Column: NovaPack C18   3.9x150

   created : 07-DEC-2001           created by : Dudar E
   changed : 07-DEC-2001           changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10476.D
 Time [min] : 6.205
      scan# : 466
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :  18.2 - 929.7
```

nm	mAU
214.0	929.7
240.0	312.3
254.0	175.0
272.0	24.9
306.0	67.7

(75) 2,4-D



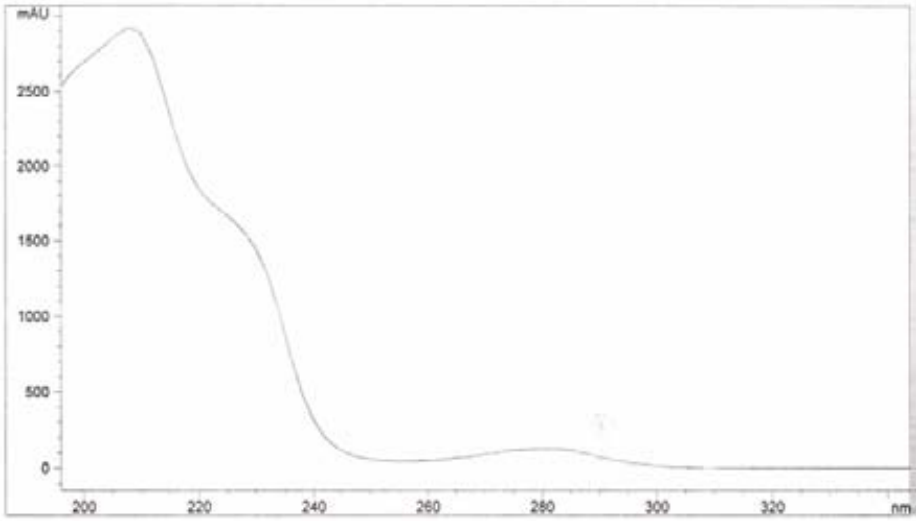

```
      name : 2,4-D
Time [min] : 6.29
        ID# : 0
   ID Name :
      Info : 32%ACN(modifier:2% 1-propanol):ware pH2.69modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.5 mg/ml   Column: NovaPack C18 3.9x150

   created : 07-DEC-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10494.D
Time [min] : 6.293
      scan# : 472
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.2 - 1251.5
```

nm	mAU
200.0	1251.5
218.0	240.1
230.0	263.3
252.0	5.7
284.0	58.0

(73) Dicamba




```
      name : Dicamba
Time [min] : 4.23
        ID# : 0
   ID Name :
      Info : 29%ACN(modifier:2% 1-propanol):water pH2.69(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN            Column: NovaPack C18   3.9x150

   created : 07-DEC-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10340.D
Time [min] : 4.229
      scan# : 318
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.4 - 2924.0
```

nm	mAU
208.0	2924.0
222.0	1754.7
256.0	44.3
282.0	128.9

(74) Dichlorprop

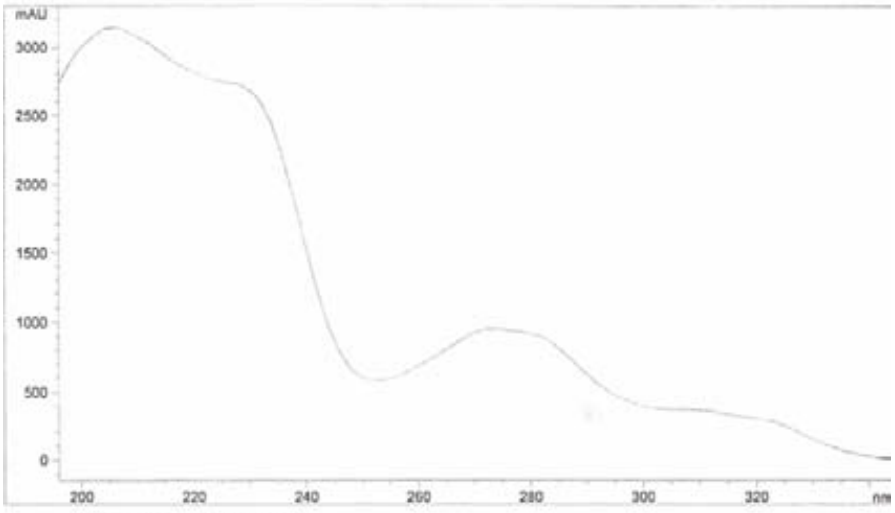

```
     name : Dichlorprop
Time [min] : 7.85
       ID# : 0
   ID Name :
     Info : 35% ACN(modifier:2% 1-propanol):water pH2.69(modifier:2% 1-propanol)
   Sample : Reference
  Solvent : ACN                    Column: NovaPack C18  3.9x150

  created : 07-DEC-2001          created by : Dudar E
  changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10490.D
Time [min] : 7.845
     scan# : 589
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.3 - 1514.6
```

nm	mAU
202.0	1514.6
218.0	308.9
230.0	338.8
254.0	7.6
284.0	70.0

(67) Fluquinconazole



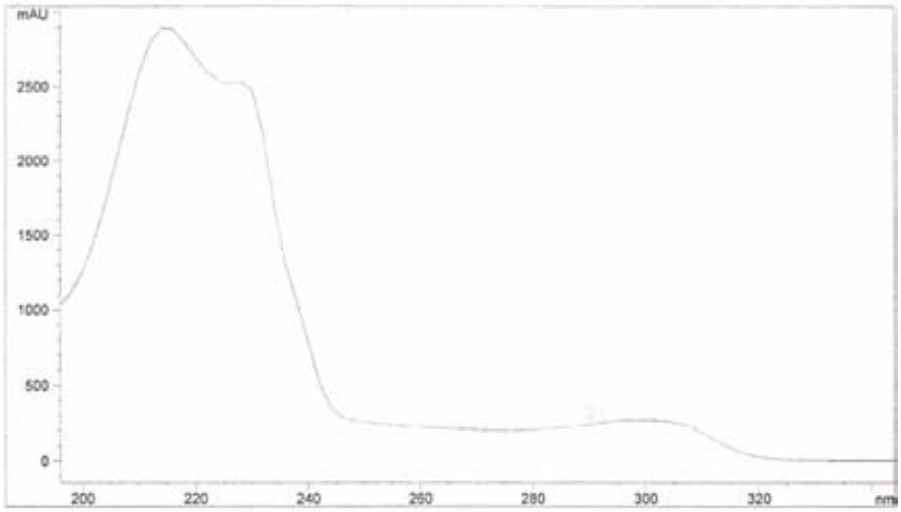

```
     name : Fluquinconazole
Time [min] : 5.89
       ID# : 0
   ID Name :
     Info : 45%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
   Sample : Reference
  Solvent : ACN  1 mg/ml    Column:NovaPack C18 3.9x150

  created : 07-DEC-2001          created by : Dudar
  changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10413.D
Time [min] : 5.892
     scan# : 442
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :  11.9 - 3144.6
```

nm	mAU
206.0	3144.6
230.0	2685.0
252.0	580.9
274.0	954.6
312.0	363.6

(72) Folpet



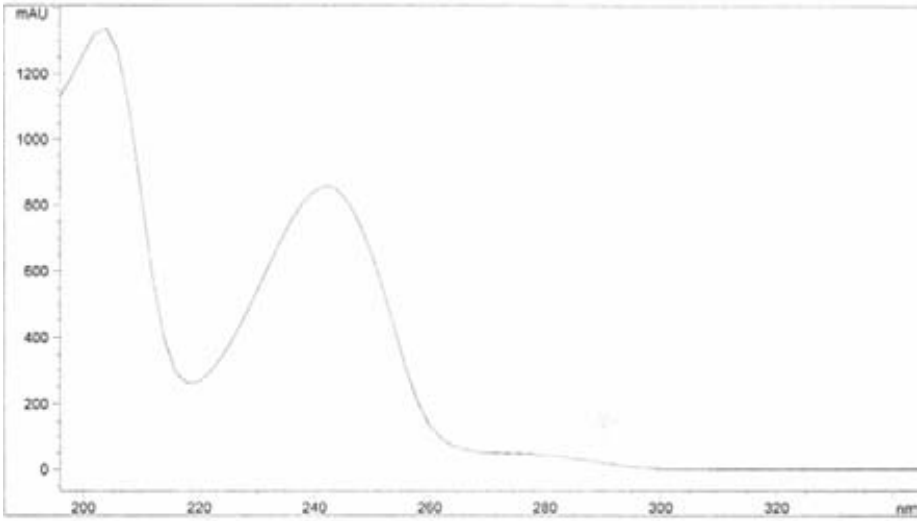

```
      name : Folpet
Time [min] : 6.73
       ID# : 0
   ID Name :
      Info : 45%ACN(modifier:2% 1-propanol):water pH2.69modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN                  Column: NovaPack C18 3.9x150

   created : 07-DEC-2001            created by : Dudar
   changed : 07-DEC-2001            changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10412.D
Time [min] : 6.734
     scan# : 506
Range  [nm] : 196.0 - 344.0  (step : 2.0)
     [mUA] :   0.8 - 2896.8
```

nm	mAU
214.0	2896.8
228.0	2533.2
254.0	243.6
276.0	201.0
298.0	271.4

(70) Isoproturon



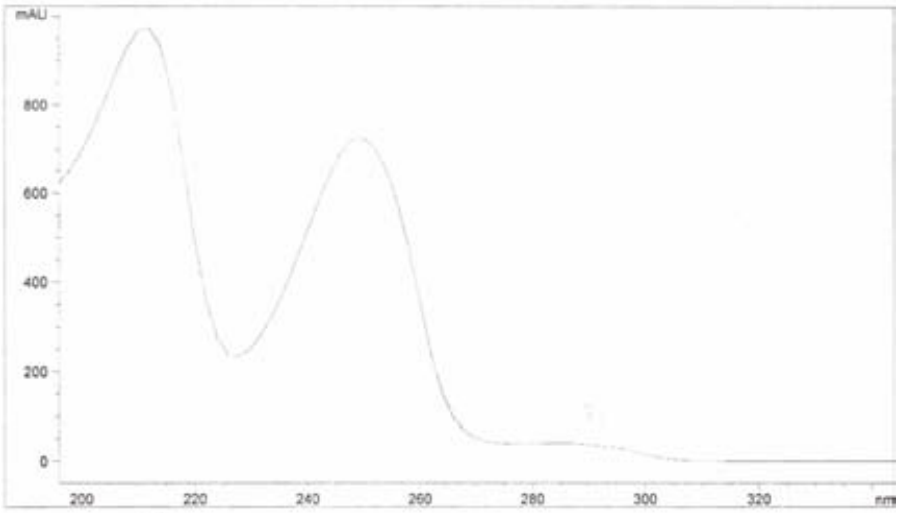

```
      name : Isoproturon
Time [min] : 4.92
       ID# : 0
   ID Name :
      Info : 35%ACN(modifier:2% 1-propaol):water pH2.69modifier;2% 1-propanol)
    Sample : Reference
   Solvent : ACN              Column: NovaPack C18  3.9x150

   created : 07-DEC-2001            created by : Dudar E
   changed : 07-DEC-2001            changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10487.D
Time [min] : 4.915
     scan# : 369
Range  [nm] : 196.0 - 344.0  (step : 2.0)
     [mUA] :   0.4 - 1340.6
```

nm	mAU
204.0	1340.6
218.0	261.7
242.0	859.2
280.0	42.3

(78) Linuron



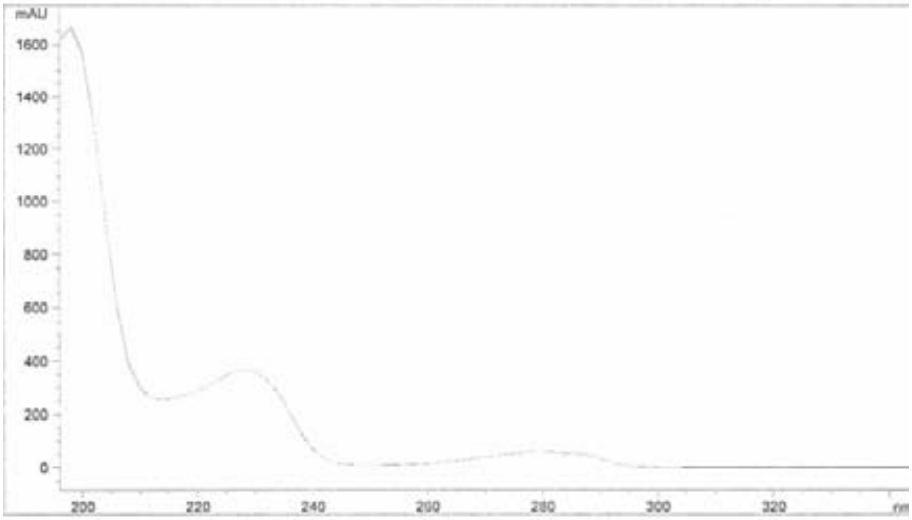

```
      name : Linuron
Time [min] : 6.34
        ID# : 0
   ID Name :
      Info : 40% ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN     0.5 mg/ml   Column: NovaPack C18  3.9x150

   created : 07-DEC-2001           created by : Dudar E
   changed : 07-DEC-2001           changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10503.D
Time [min] : 6.335
      scan# : 476
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.4 - 974.7
```

nm	mAU
212.0	974.7
228.0	234.5
250.0	726.9
278.0	39.8
286.0	41.4

(76) MCPA



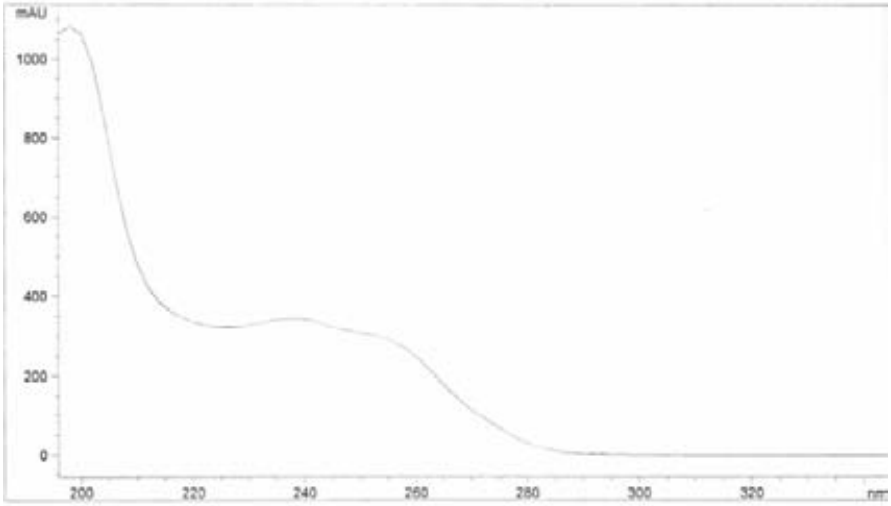

```
      name : MCPA
Time [min] : 6.52
        ID# : 0
   ID Name :
      Info : 32%ACN(modifier:2% 1-propanol):ware pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN          Column:NovaPack C18  3.9x150

   created : 07-DEC-2001           created by : Dudar E
   changed : 07-DEC-2001           changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10492.D
Time [min] : 6.518
      scan# : 489
Range  [nm] : 196.0 - 344.0  (step : 2.0)
      [mUA] :   0.2 - 1665.0
```

nm	mAU
214.0	255.9
228.0	370.4
250.0	8.2
280.0	61.6

(63) Nicosulfuron



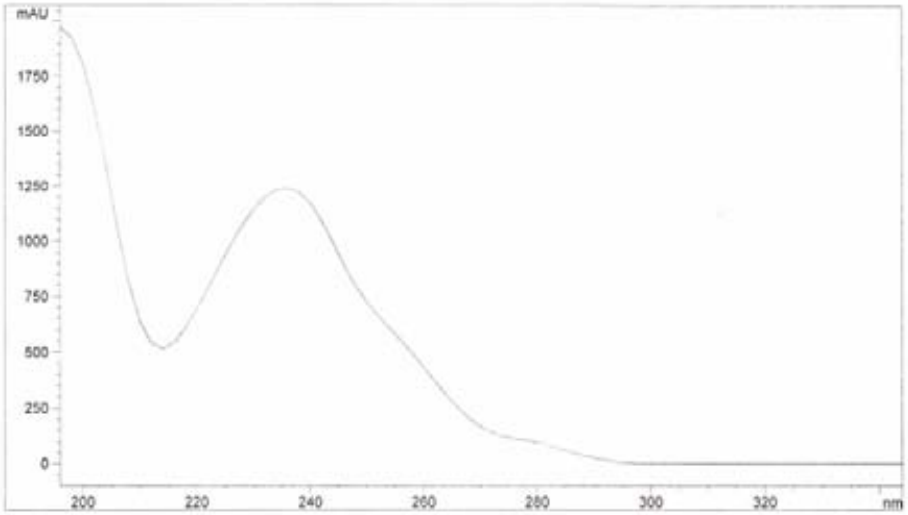

```
      name : Nicosulfuron
Time [min] : 4.01
       ID# : 0
   ID Name :
      Info : 25%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN 0.5 mg/ml   Column:NovaPack C18 3.9x150

   created : 07-DEC-2001          created by : Dudar E
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10473.D
Time [min] : 4.013
      scan# : 301
Range  [nm] : 196.0 - 344.0  (step : 2.0)
     [mUA] :   0.3 - 1083.6
```

nm	mAU
198.0	1083.6
226.0	322.8
238.0	344.7
254.0	298.1
274.0	76.8

(61) Primisulfuron



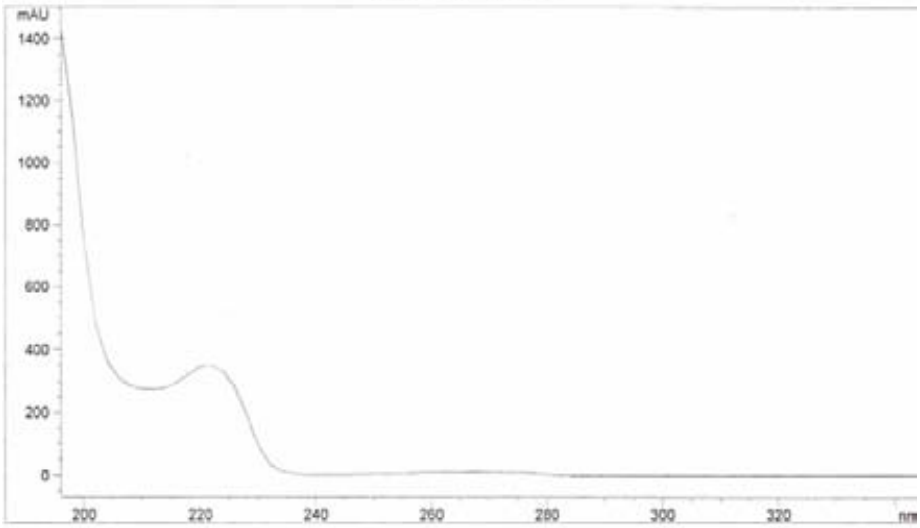

```
      name : Primisulfuron
Time [min] : 7.42
       ID# : 0
   ID Name :
      Info : 43% ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
    Sample : Reference
   Solvent : ACN     Column NovaPack C18 3.9x150

   created : 07-DEC-2001          created by : Dudar
   changed : 07-DEC-2001          changed by :
```

```
      File : D:\HPDATA\MULTILIB\SIG10423.D
Time [min] : 7.419
      scan# : 557
Range  [nm] : 196.0 - 344.0  (step : 2.0)
     [mUA] :   0.5 - 1974.4
```

nm	mAU
214.0	515.7
236.0	1241.8
254.0	609.2
280.0	97.3

(65) Tebuconazole



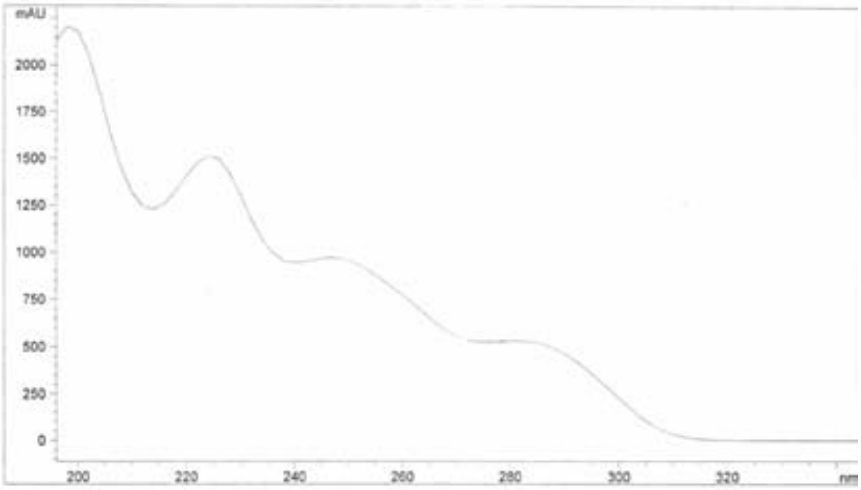

```
     name : Tebuconazole
Time [min] : 4.50
     ID# : 0
  ID Name :
     Info : 50%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
   Sample : Reference
  Solvent : ACN       Column:NovaPack C18 3.9x150

  created : 07-DEC-2001          created by : Dudar E
  changed : 07-DEC-2001          changed by :
```

```
     File : D:\HPDATA\MULTILIB\SIG10507.D
Time [min] : 4.498
     scan# : 338
Range  [nm] : 196.0 - 344.0  (step : 2.0)
     [mUA] :   0.1 - 1433.1
```

nm	mAU
212.0	273.2
222.0	350.8
242.0	2.8
268.0	11.4

(62) Thifensulfuron




```
     name : Thifensulfuron
Time [min] : 5.26
     ID# : 0
  ID Name :
     Info : 26%ACN(modifier:2% 1-propanol):water pH2.6(modifier:2% 1-propanol)
   Sample : Reference
  Solvent : ACN      Column:NovaPack C18  3.9x150

  created : 07-DEC-2001          created by : Dudar
  changed : 07-DEC-2001          changed by :
```

```
     File : D:\HPDATA\MULTILIB\SIG10379.D
Time [min] : 5.260
     scan# : 395
Range  [nm] : 196.0 - 344.0  (step : 2.0)
     [mUA] :   0.8 - 2205.0
```

nm	mAU
198.0	2205.0
214.0	1229.0
224.0	1513.8
240.0	944.4
246.0	970.4
276.0	525.9
282.0	530.6

Annex 2

IR spectra of impurities synthesized

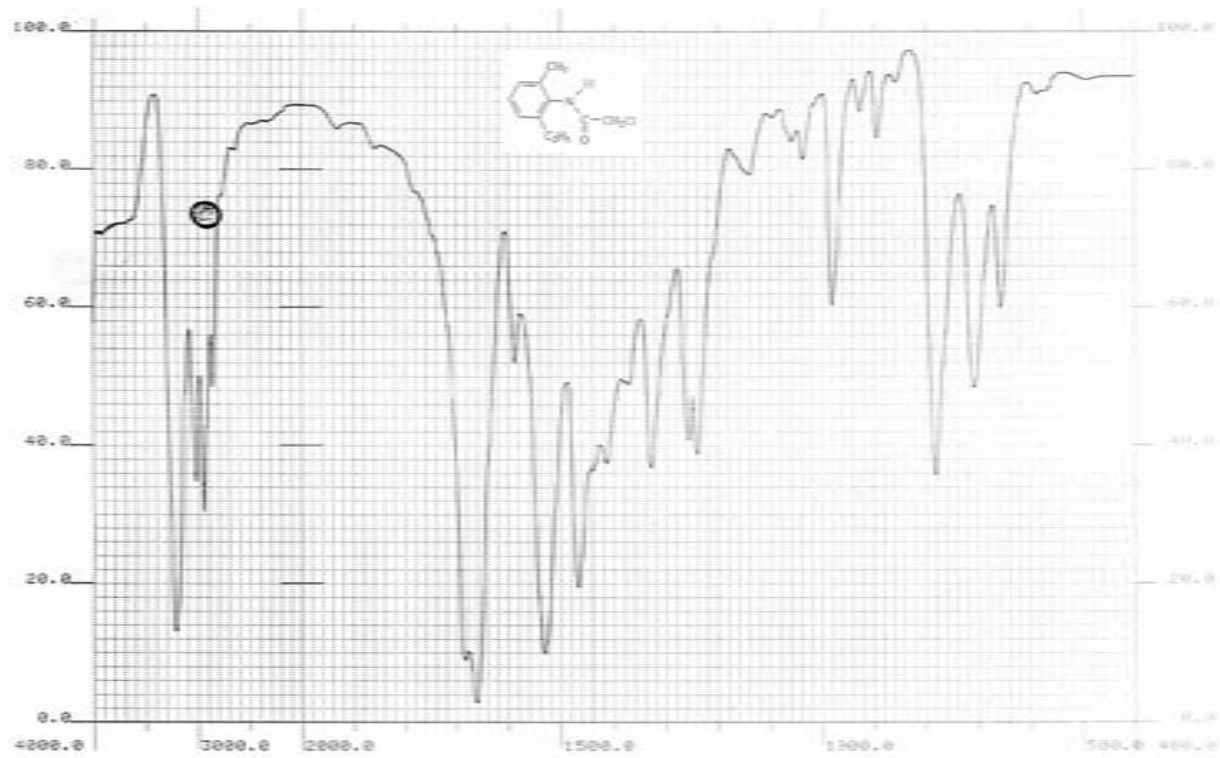


FIG. 1. The IR spectrum of 2-ethyl-6-methyl-N-chloroacetanilide.

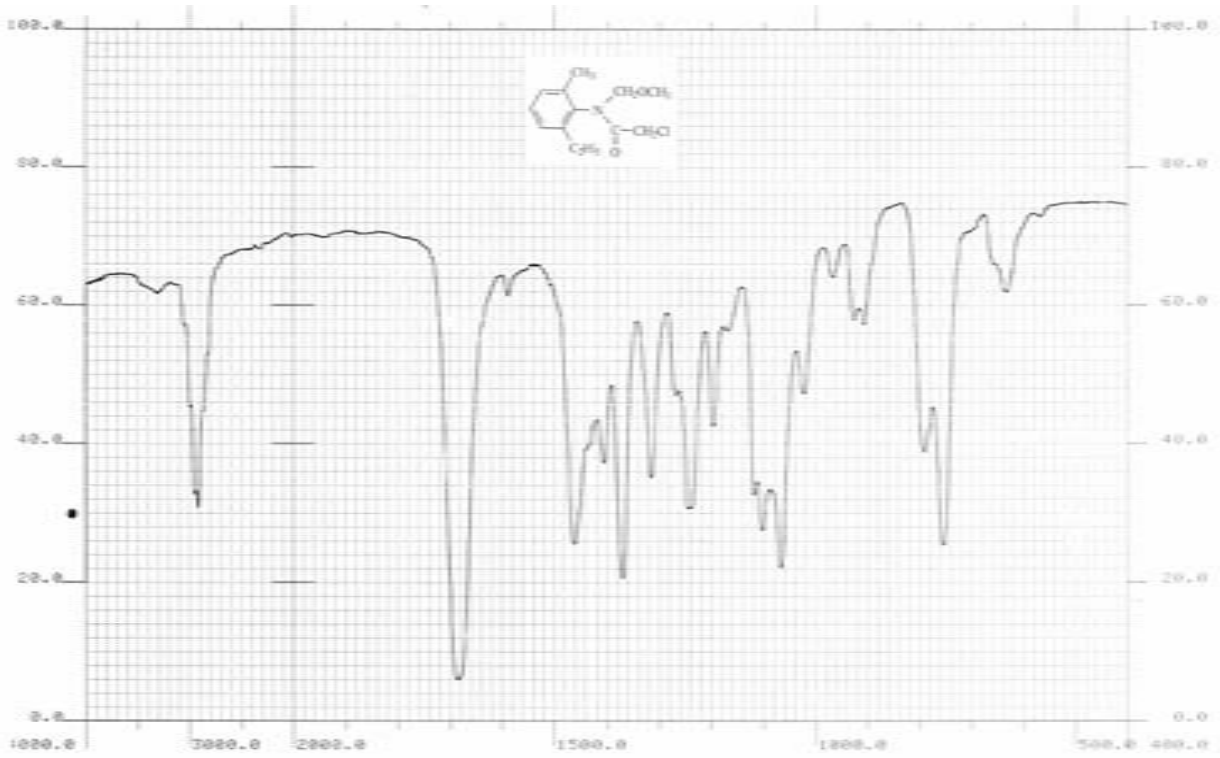


FIG. 2. The IR spectrum of 2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide.

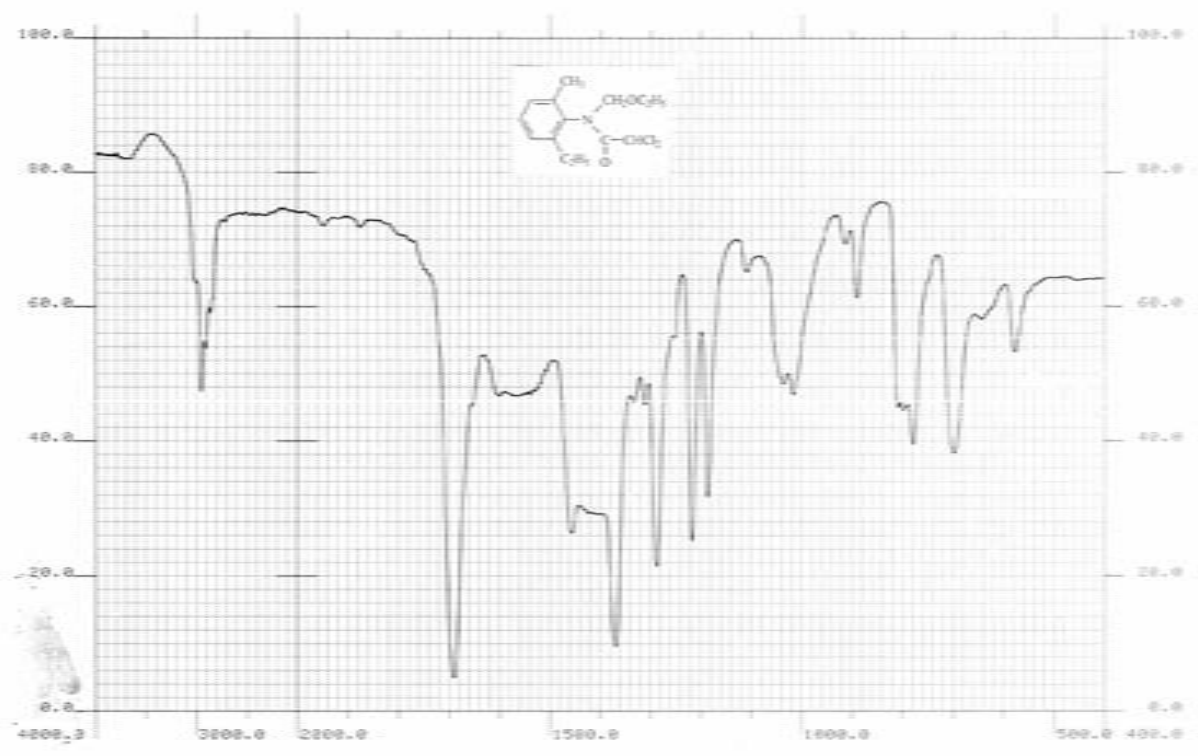


FIG. 3. The IR spectrum of 2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide.

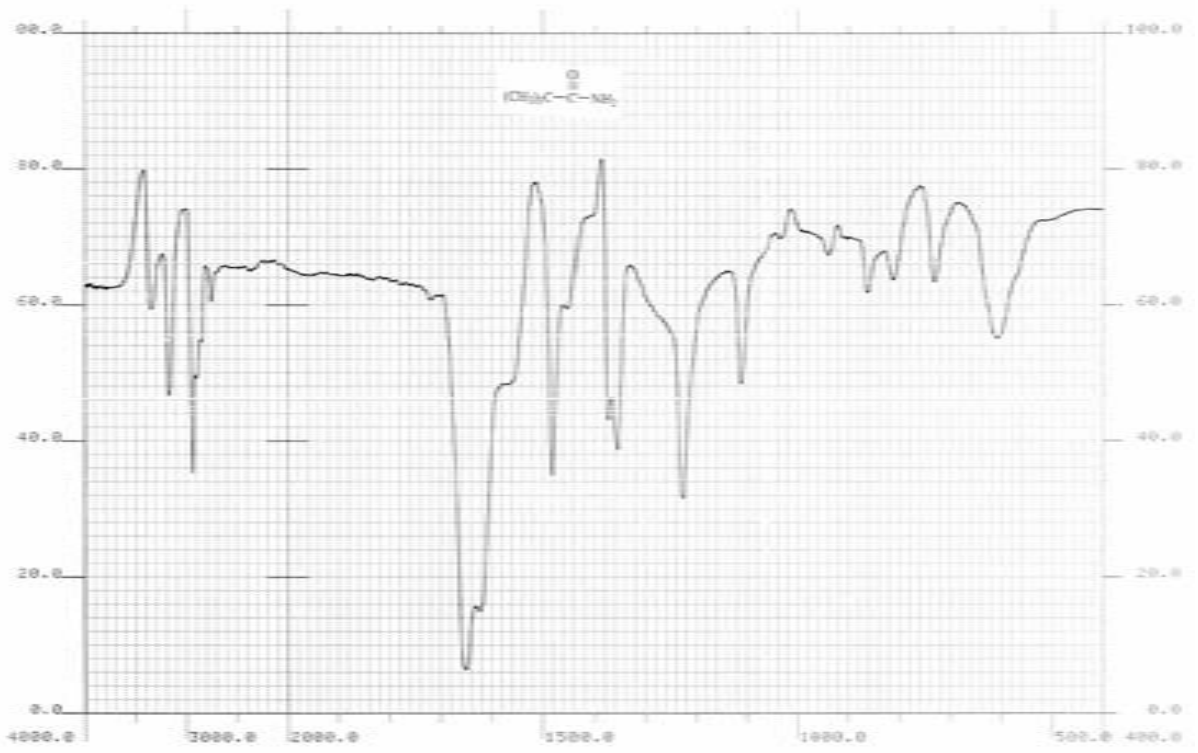


FIG. 4. The IR spectrum of Trimethylacetamide.

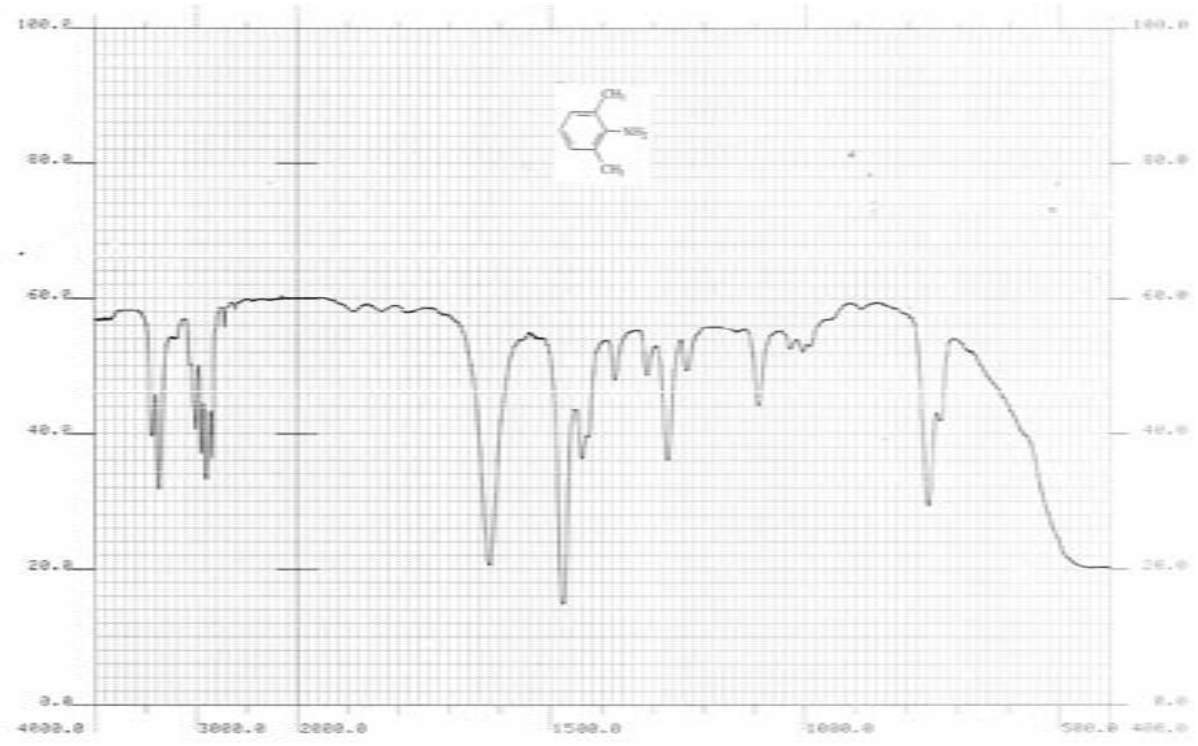


FIG. 5. The IR spectrum of 2,6-dimethylaniline.

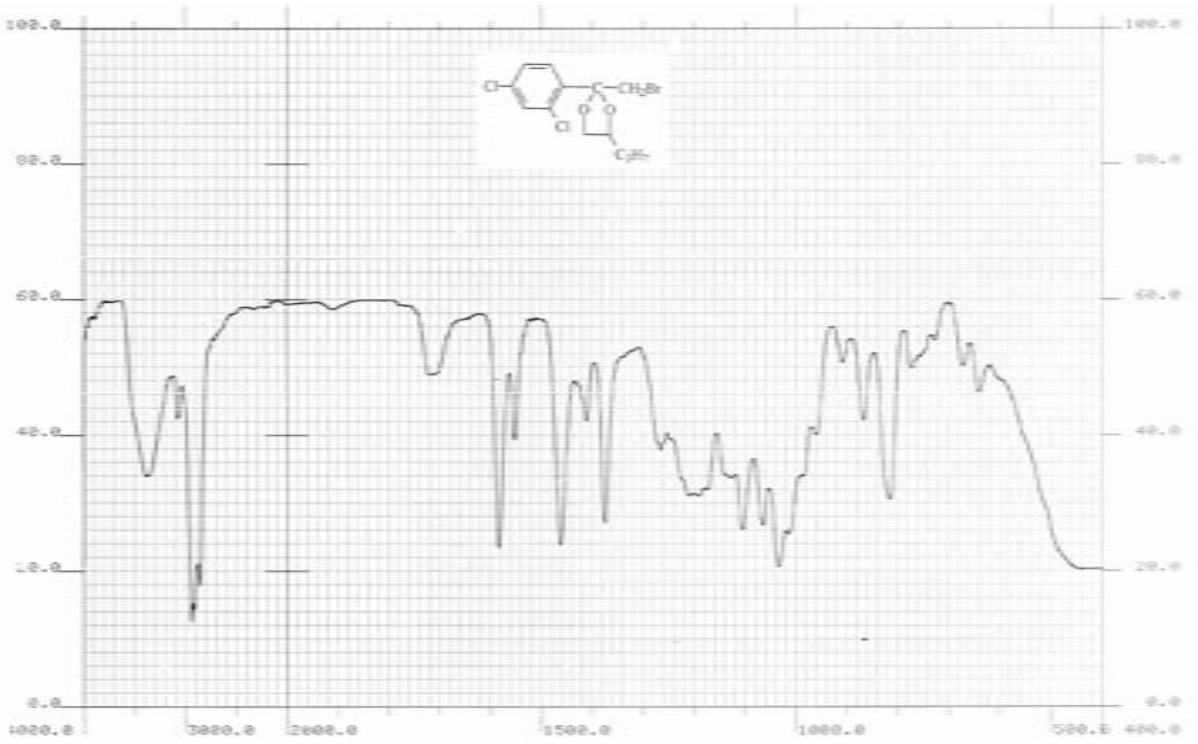


FIG. 6. The IR spectrum of 2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan.

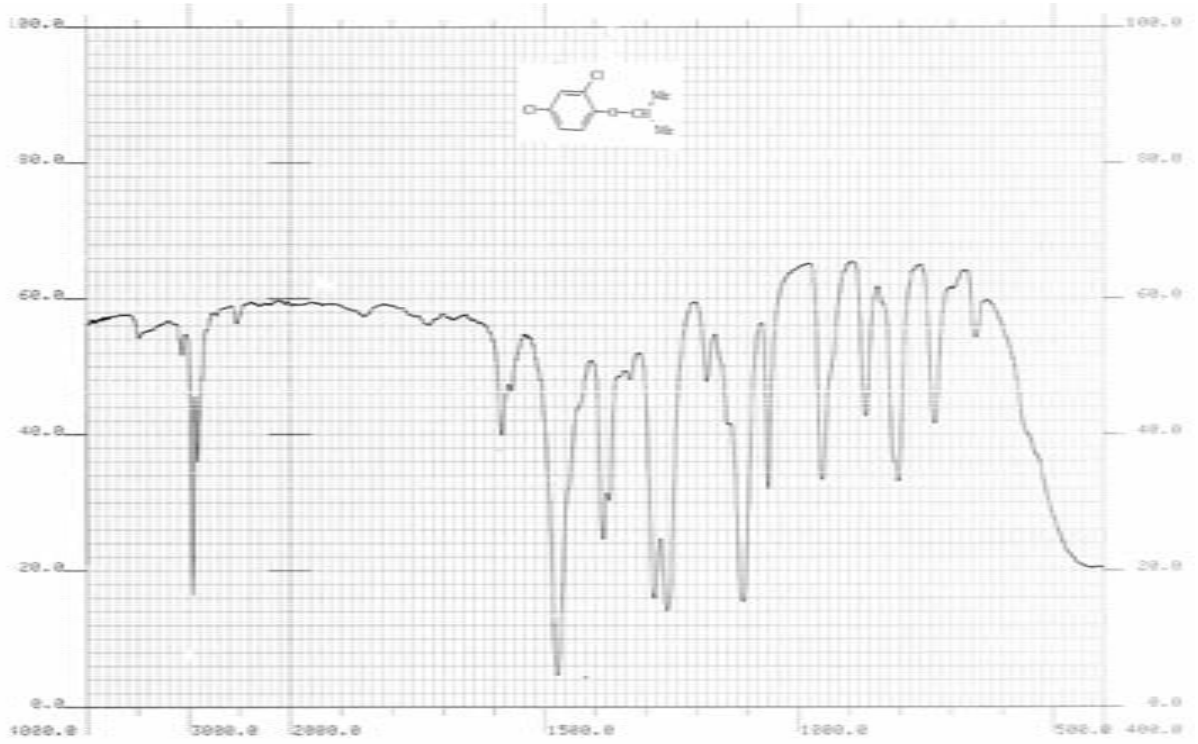


FIG. 7. The IR spectrum of 2,4-dichloroisopropoxybenzene.

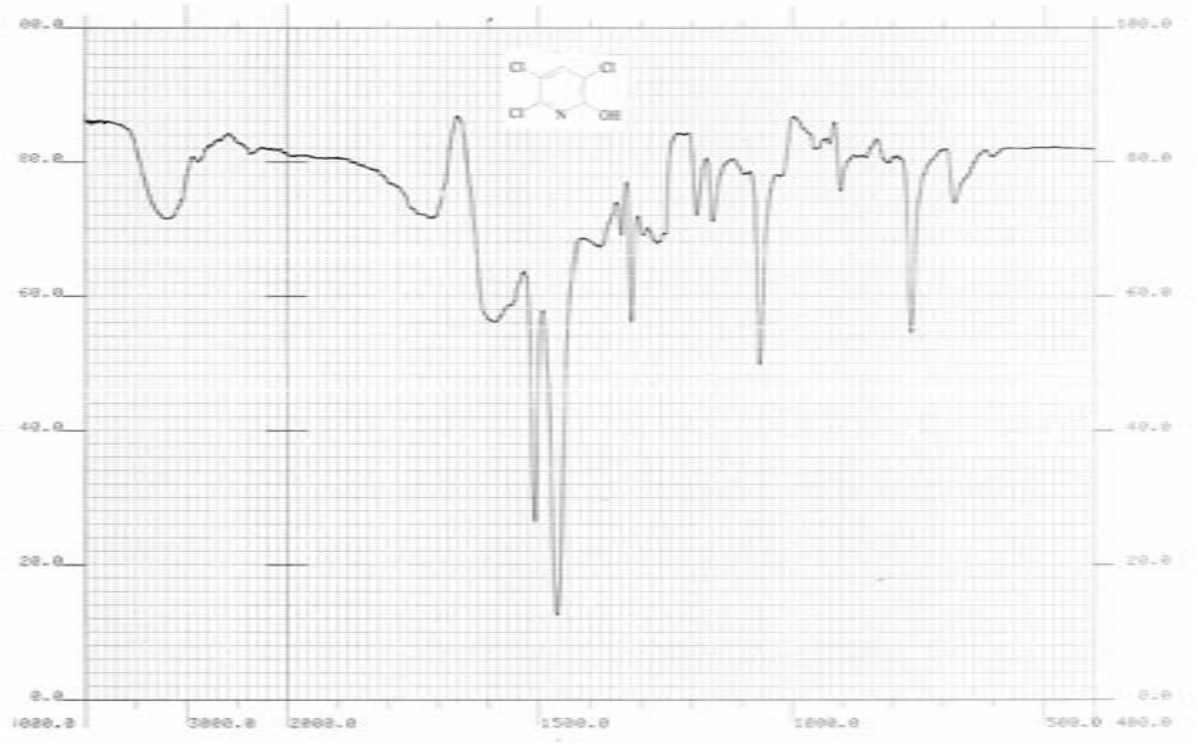


FIG. 8. The IR spectrum of 3,5,6-trichoropyridin-2-ol.

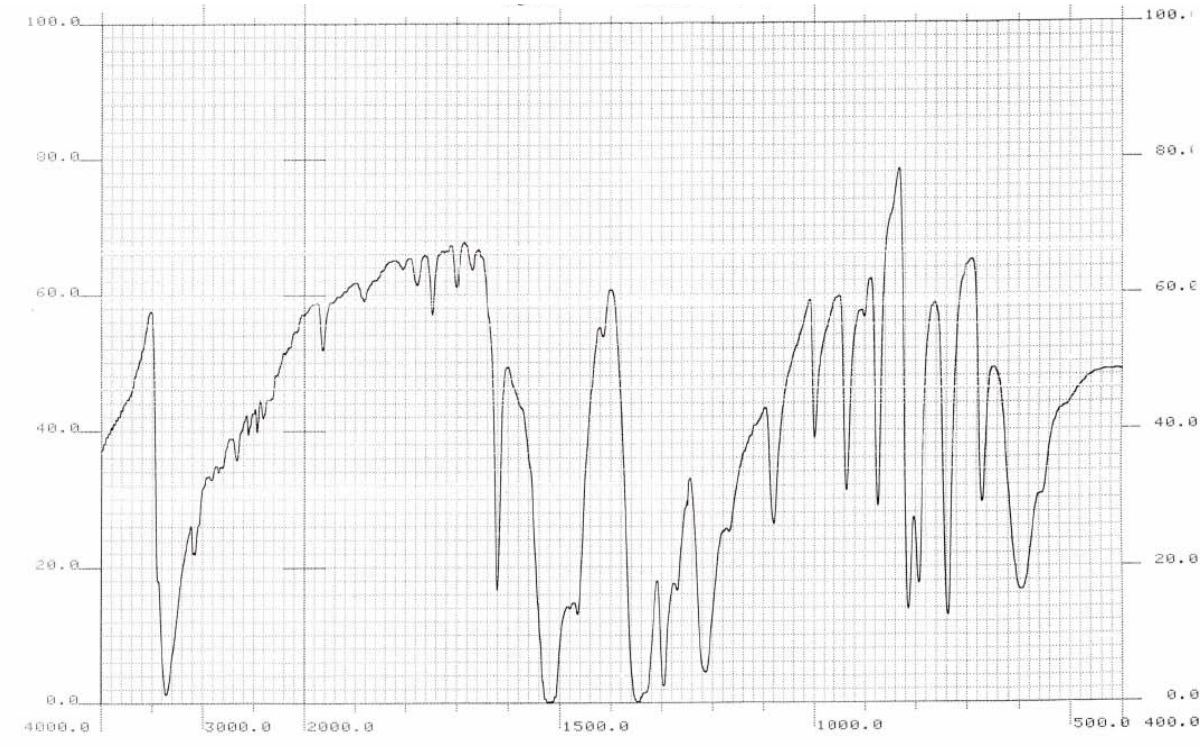


FIG. 9. The IR spectrum of 3-nitro-phenol.

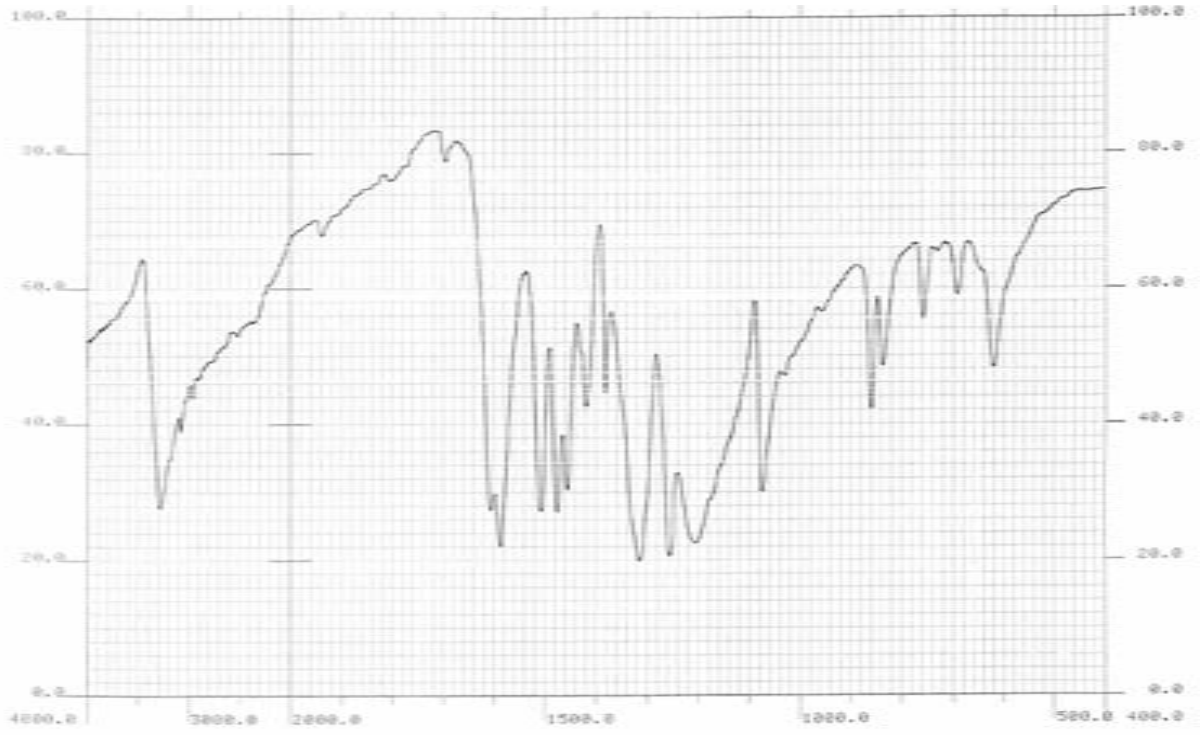


FIG. 10. The IR spectrum of 3-methyl-4-nitrophenol.

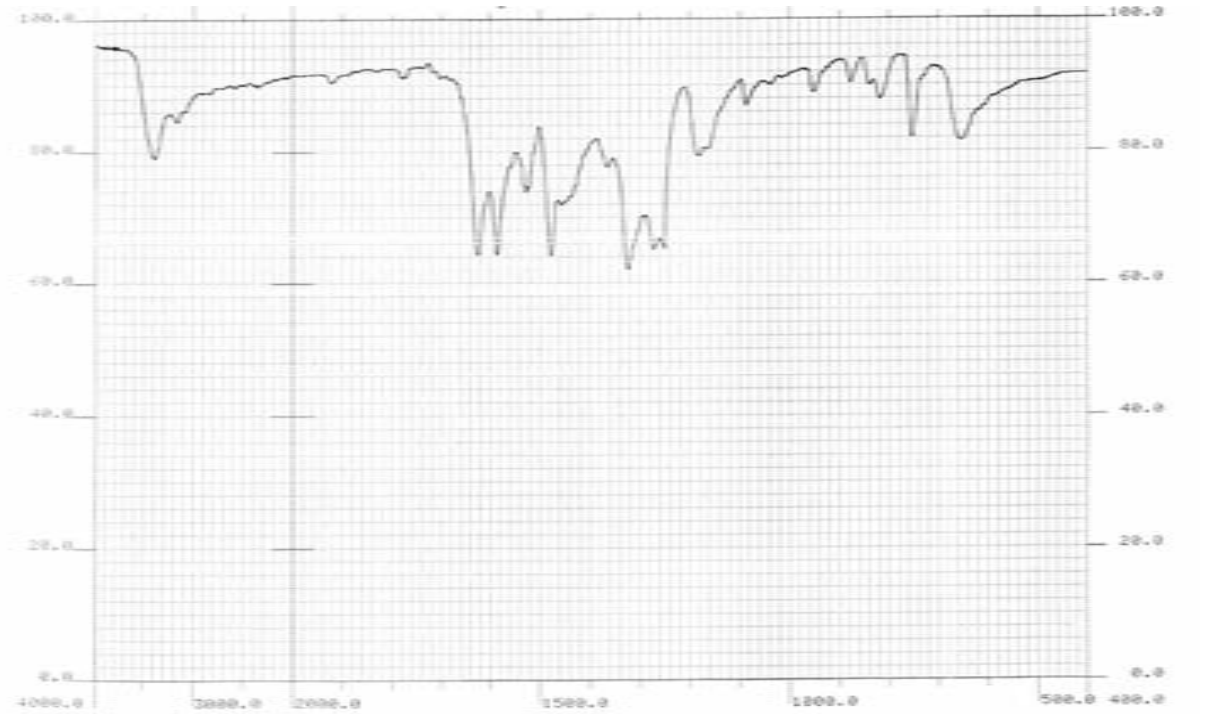


FIG. 11. The IR spectrum of 3-methyl-6-nitrophenol.

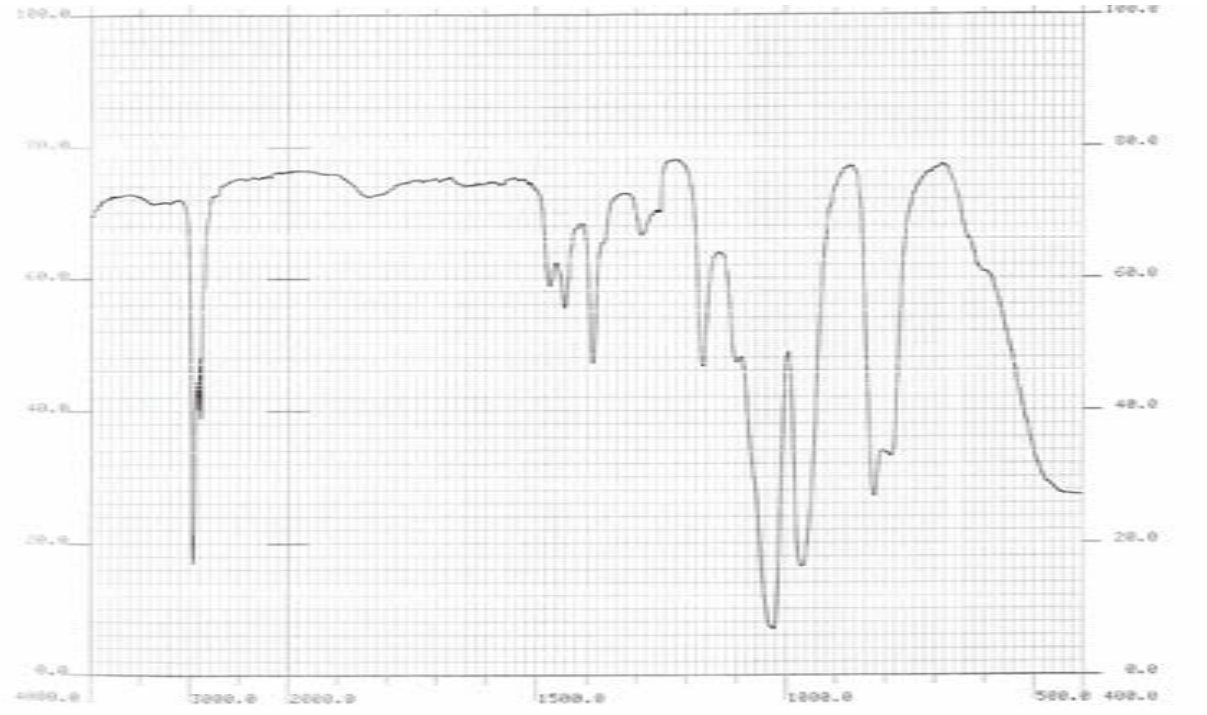


FIG. 12. The IR spectrum of Thiophosphoric acid O,O',O''-trimethylester.

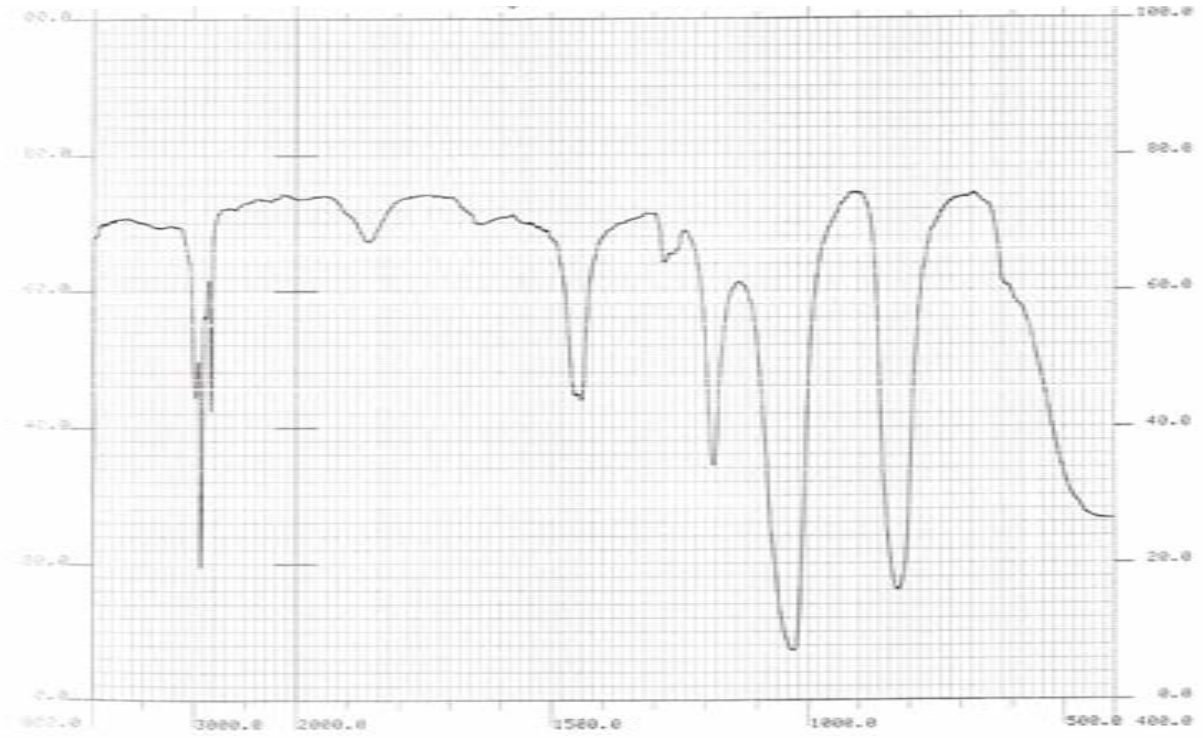


FIG. 13. The IR spectrum of Thiophosphoric acid O,O',O''-triethylester.

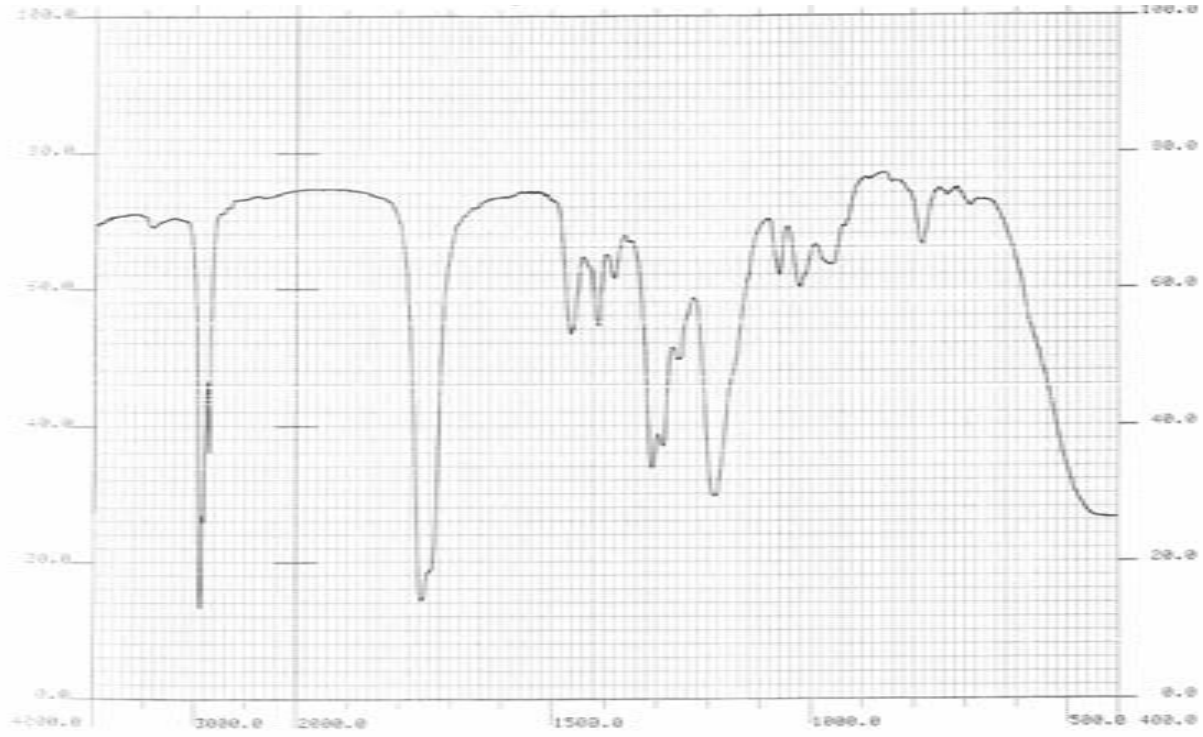


FIG. 14. The IR spectrum of Chloro-acetic acid butylester.

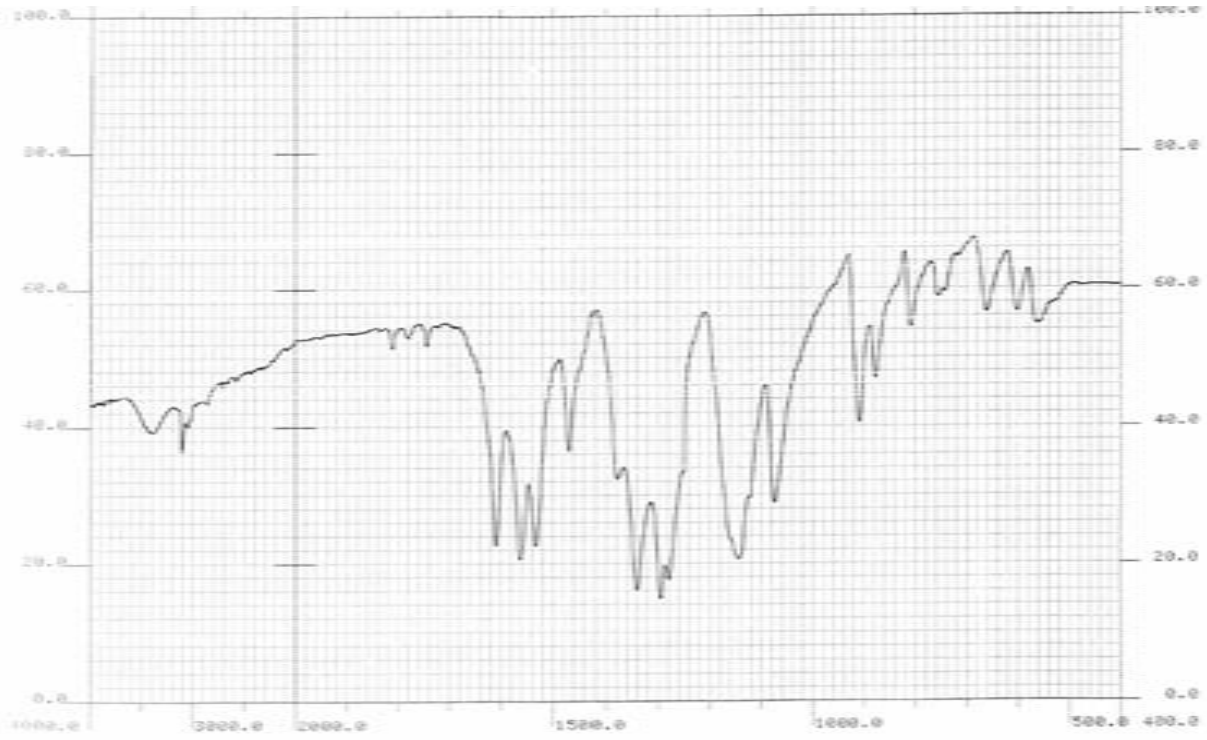


FIG. 15. The IR spectrum of 1,5-Dichloro-2-nitro-4-trifluoromethyl-benzene.

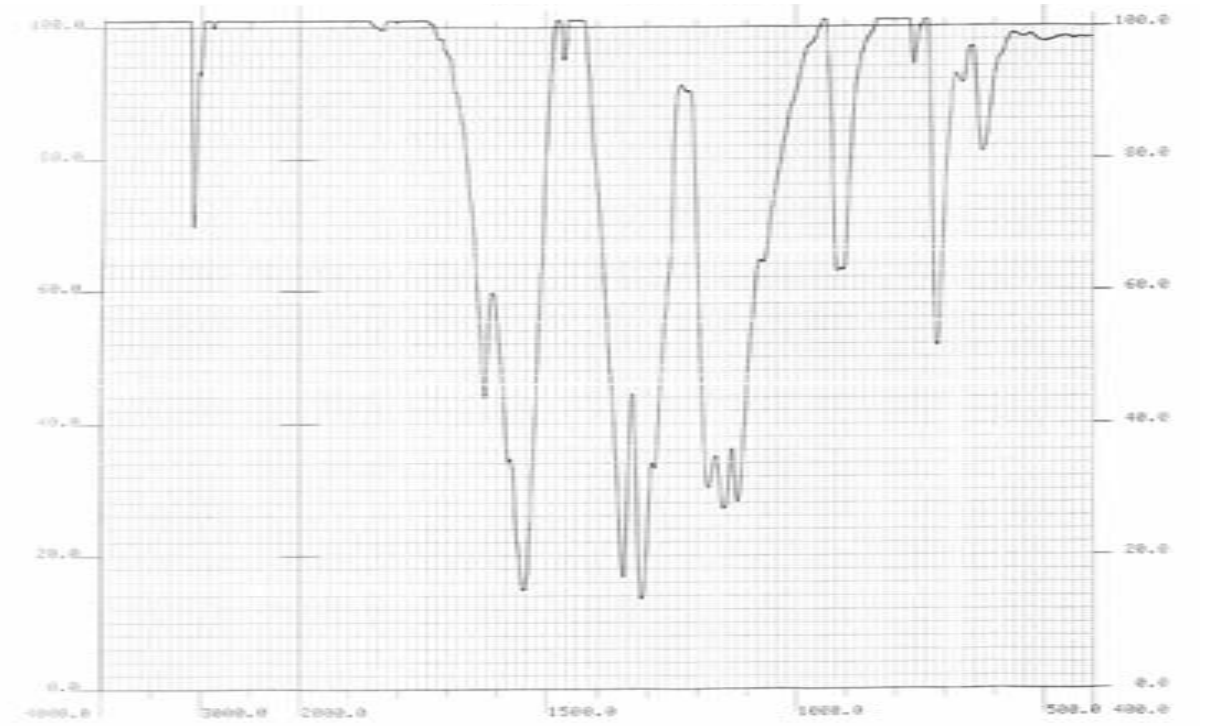


FIG. 16. The IR spectrum of 1-Chloro-2,6-dinitro-4-trifluoromethyl-benzene.

Annex 3

NMR spectra of impurities synthesized

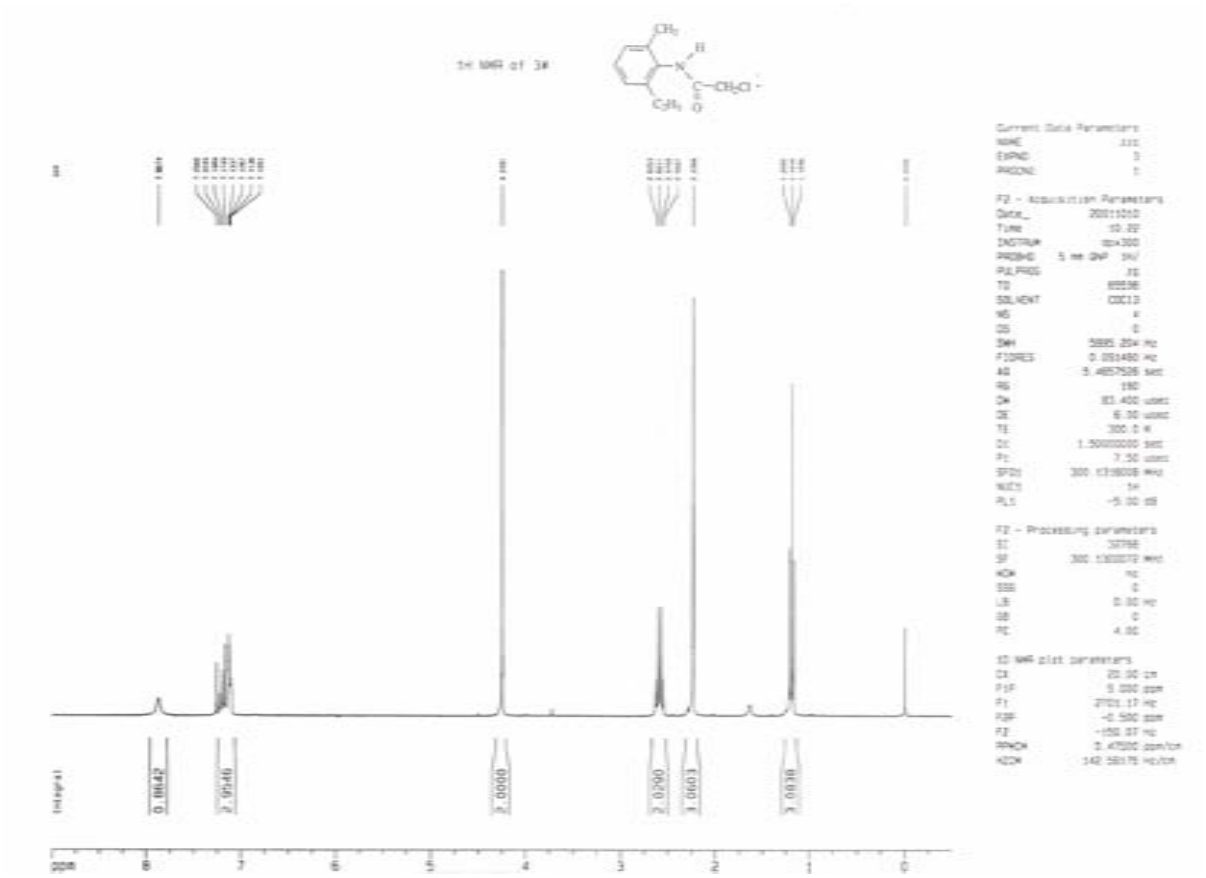


FIG. 1. The NMR spectrum of 2-ethyl-6-methyl-N-chloroacetanilide.

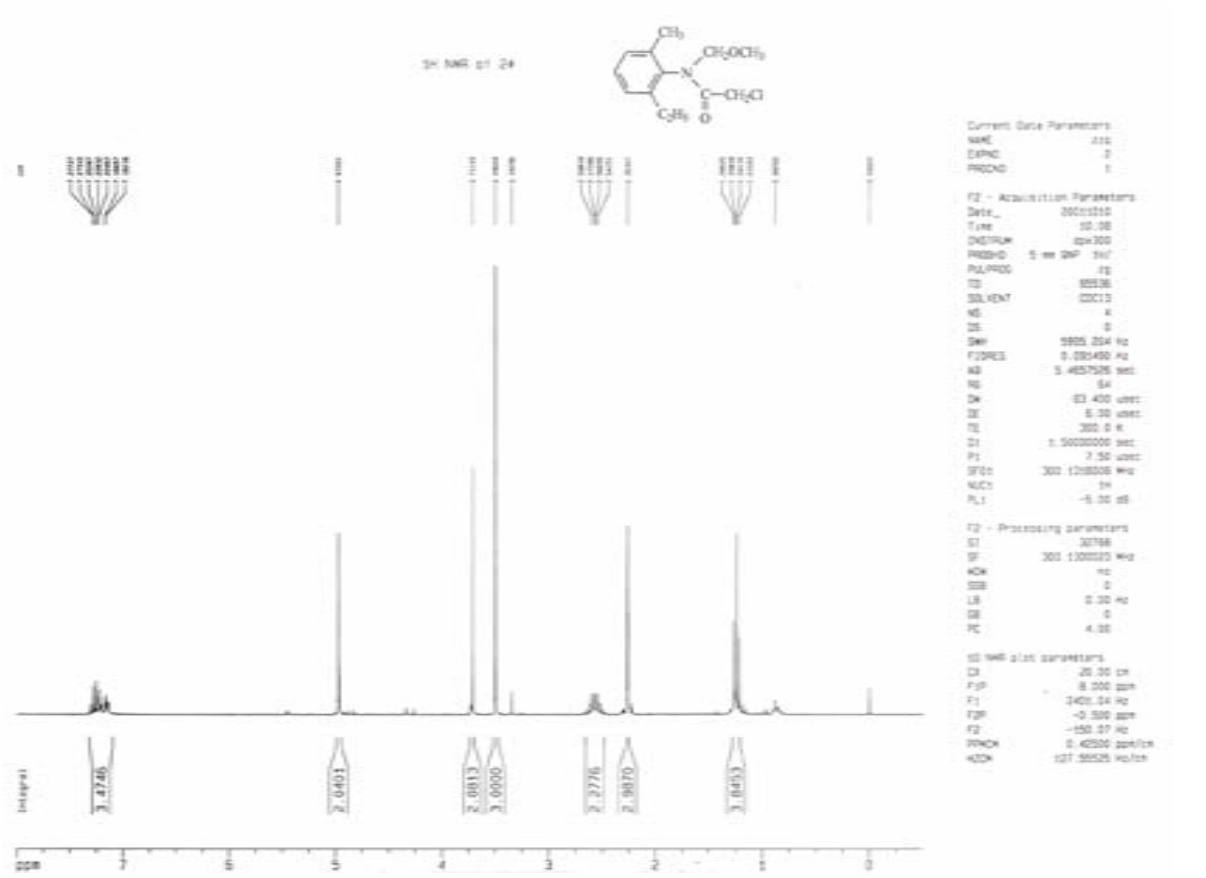


FIG. 2. The NMR spectrum of 2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide.

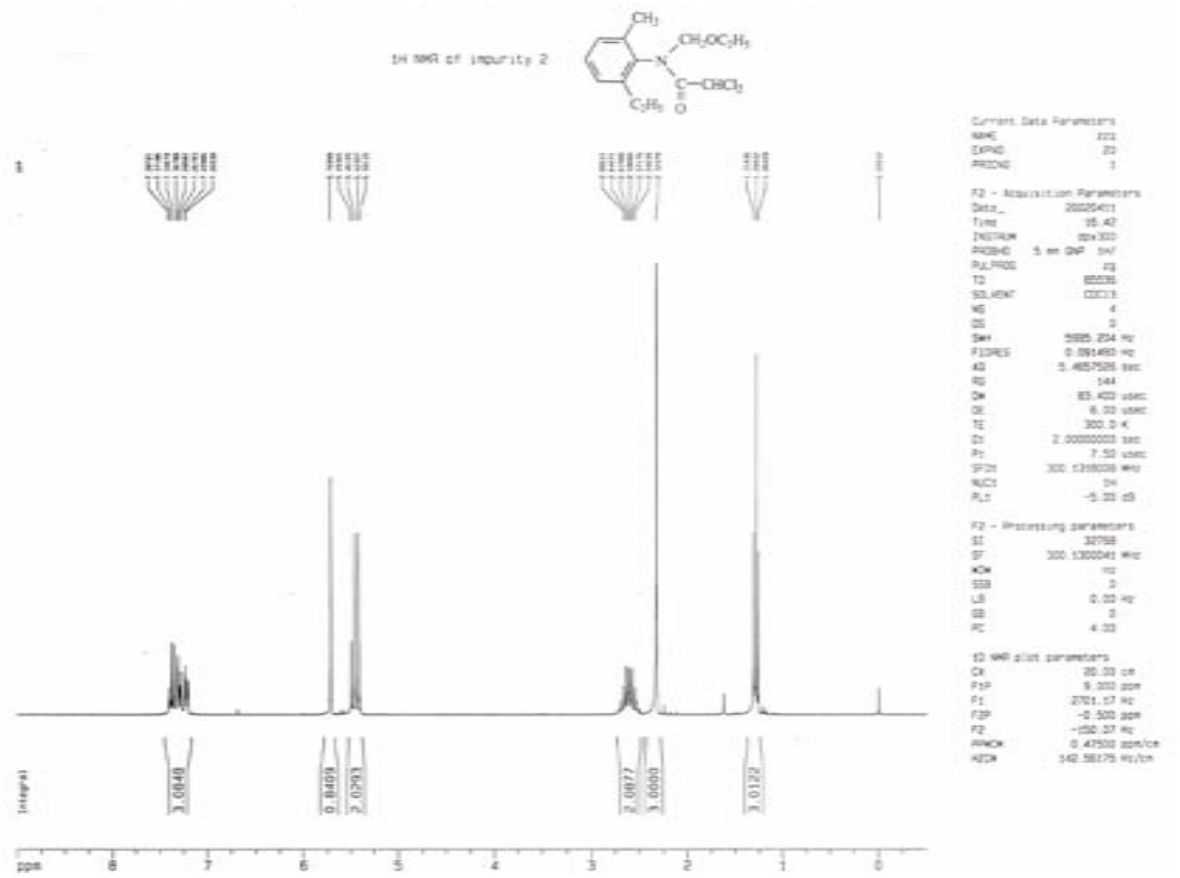


FIG. 3. The NMR spectrum of 2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide.

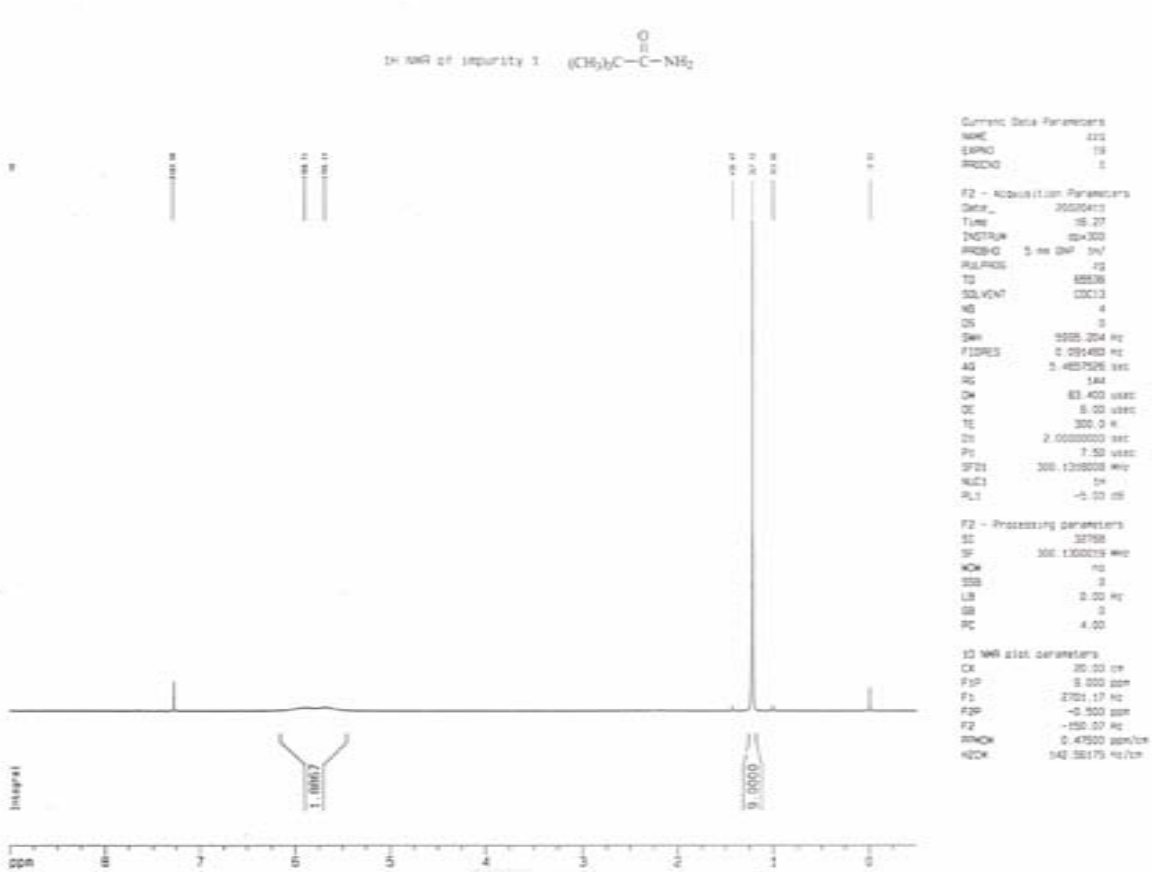


FIG. 4. The NMR spectrum of trimethylacetamide.

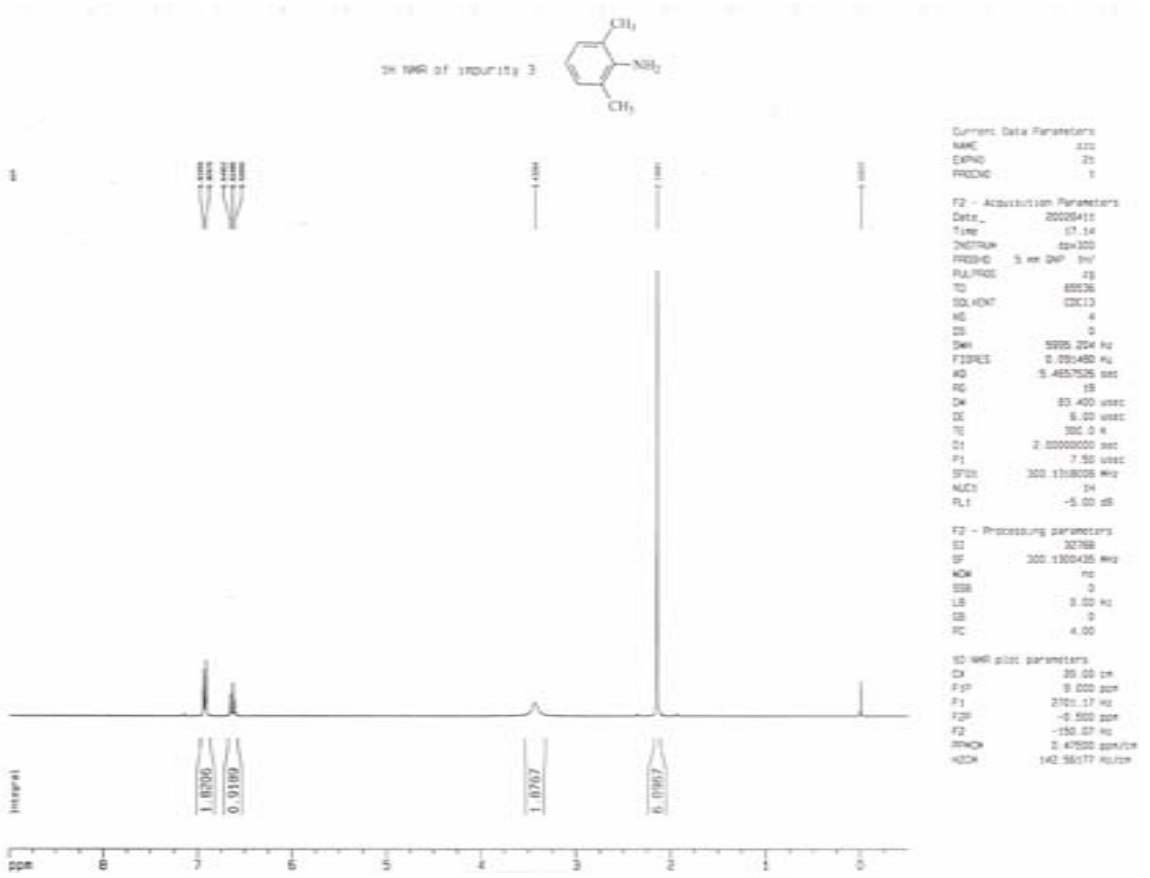


FIG. 5. The NMR spectrum of 2,6-dimethylaniline.

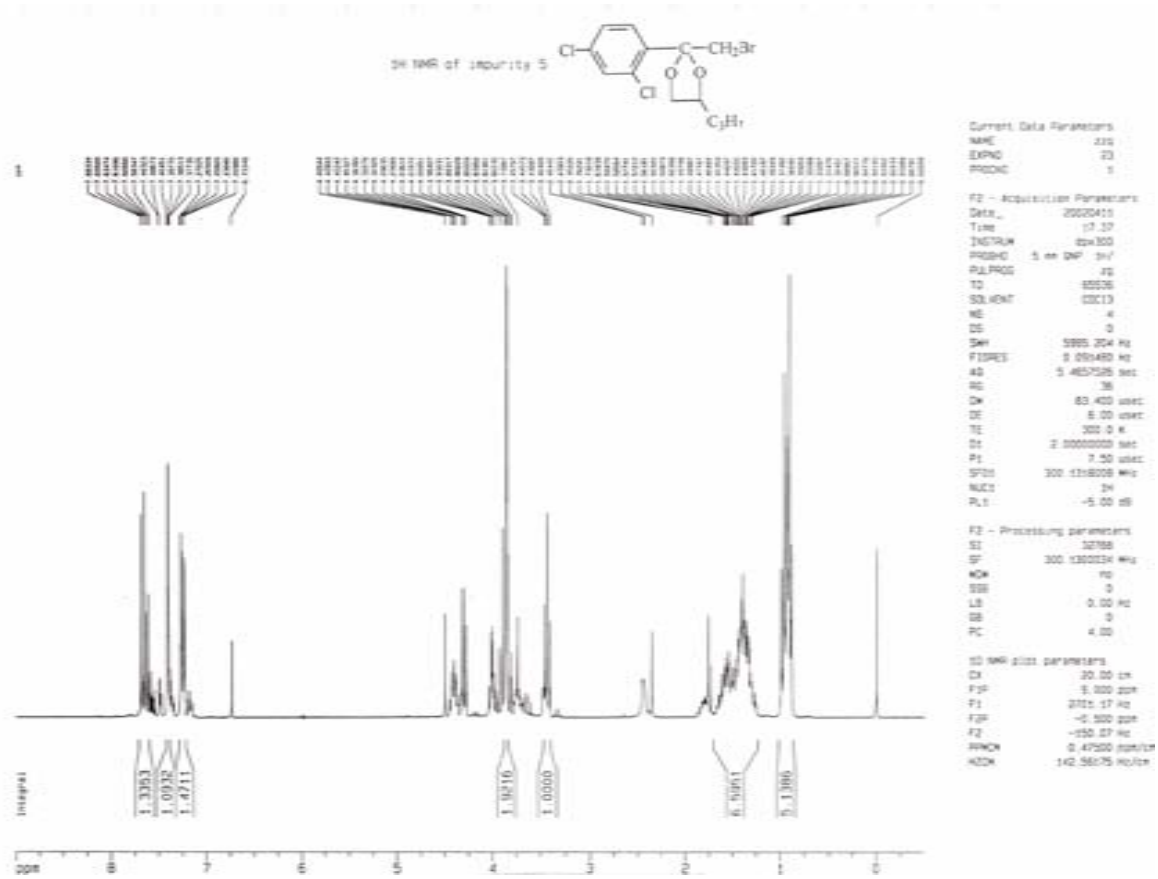


FIG. 6. The NMR spectrum of 2-bromomethyl-2-(2,4-dichlorophenyl-4-propyl)-1,3-dioxolan.

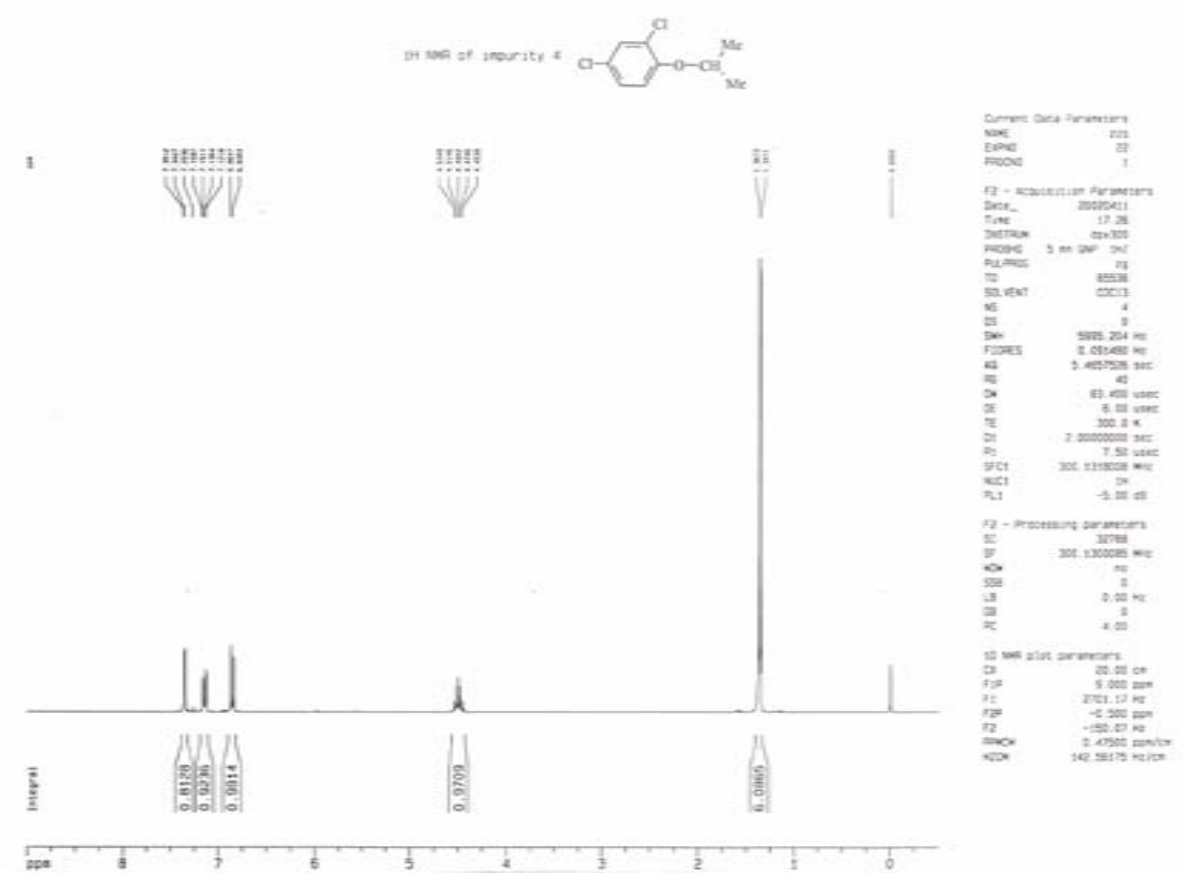


FIG. 7. The NMR spectrum of 2,4-dichloroisopropoxybenzene.

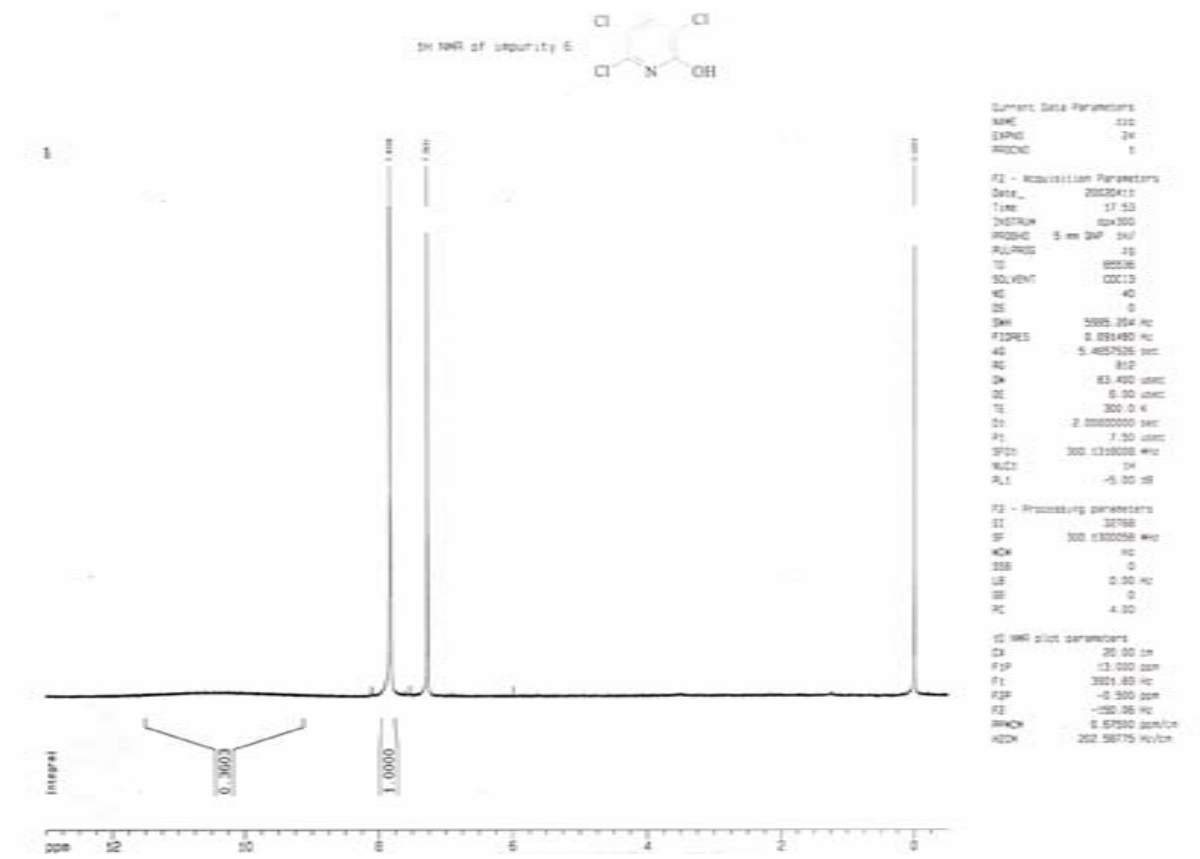


FIG. 8. The NMR spectrum of 3,5,6-trichoropyridin-2-ol.

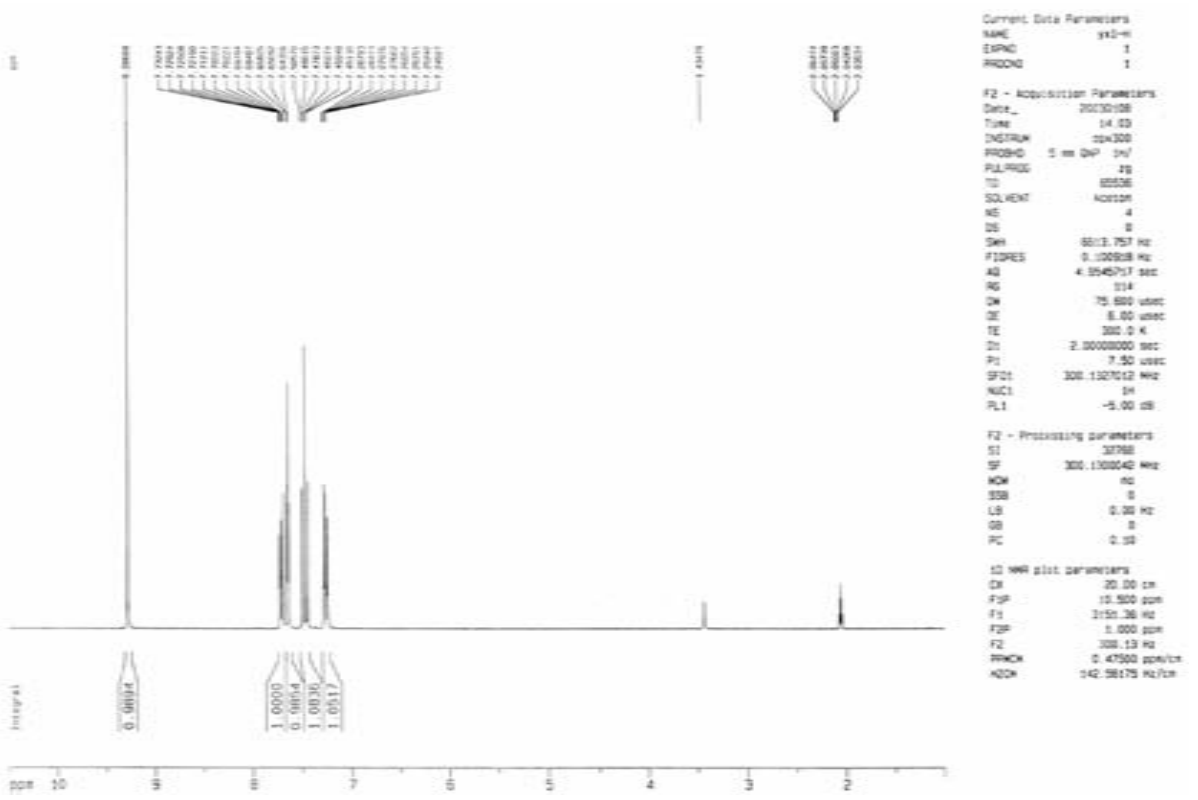


FIG. 9. The NMR spectrum of 3-nitro-phenol.

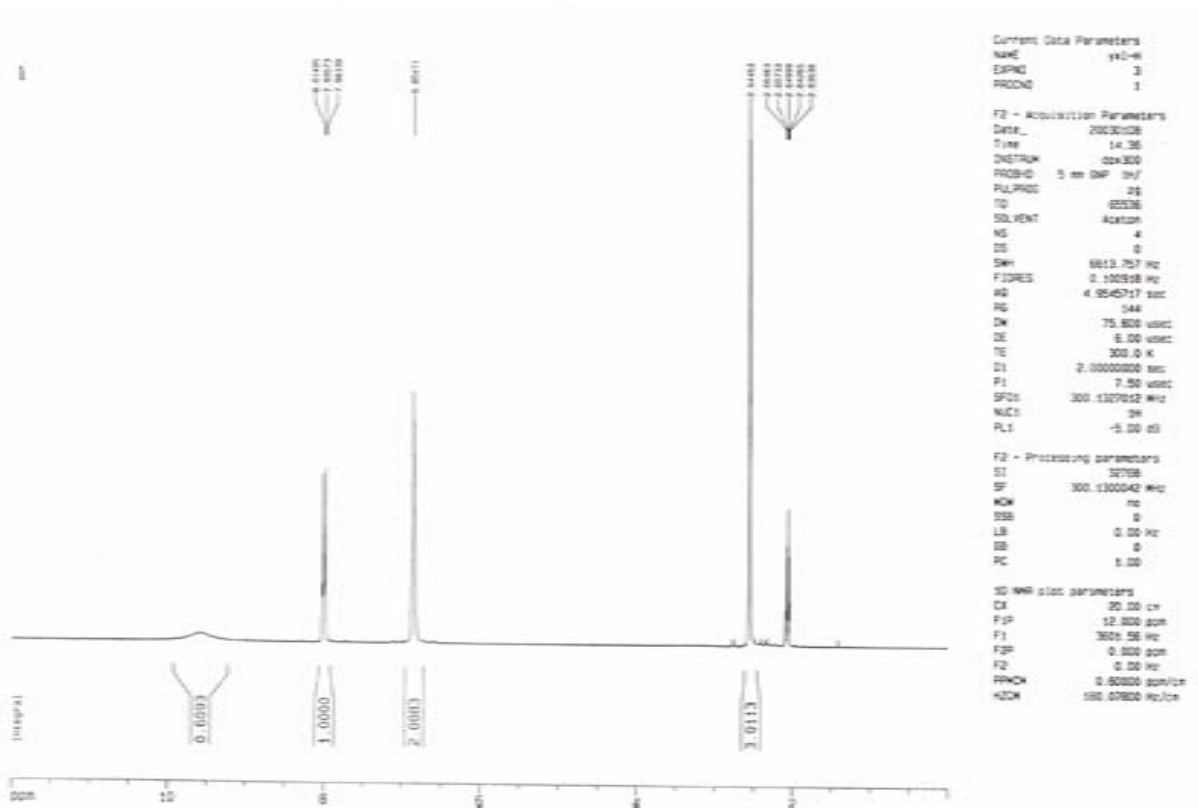


FIG. 10. The NMR spectrum of 3-methyl-4-nitrophenol.

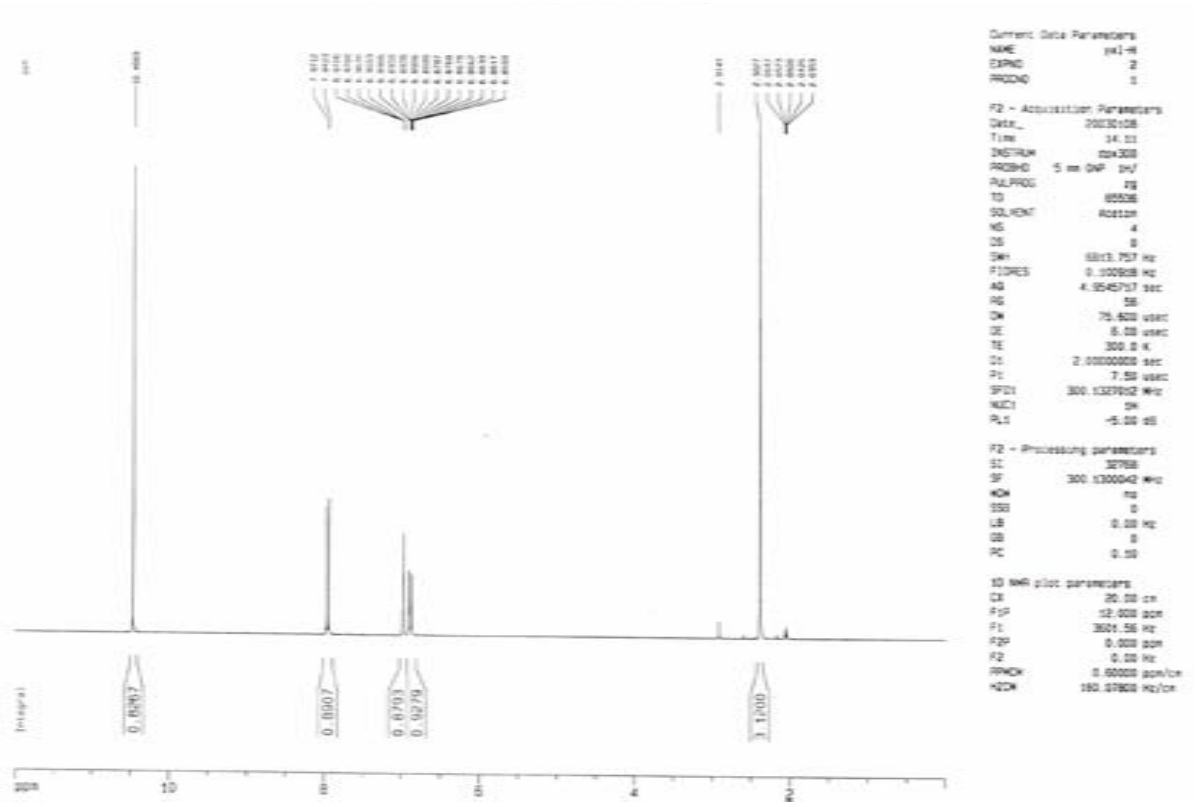


FIG. 11. The NMR spectrum of 3-methyl-6-nitrophenol.

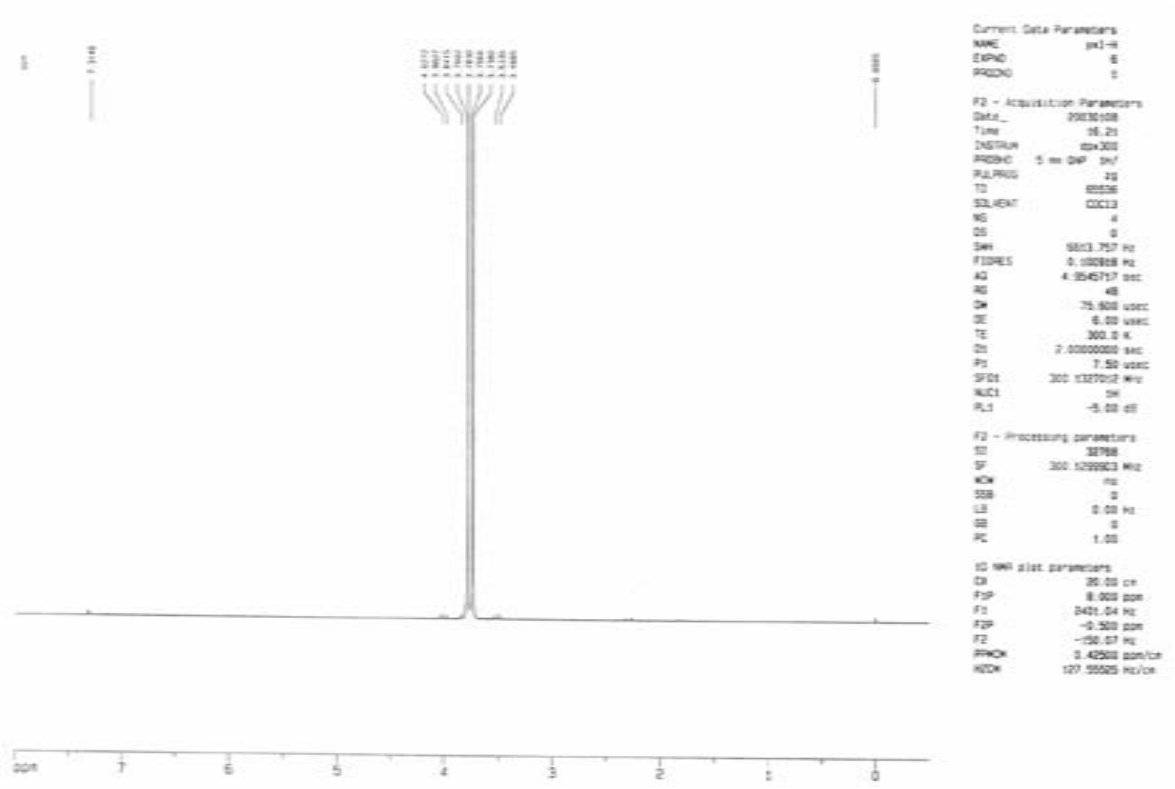


FIG. 12. The NMR spectrum of thiophosphoric acid O,O',O''-trimethylester.

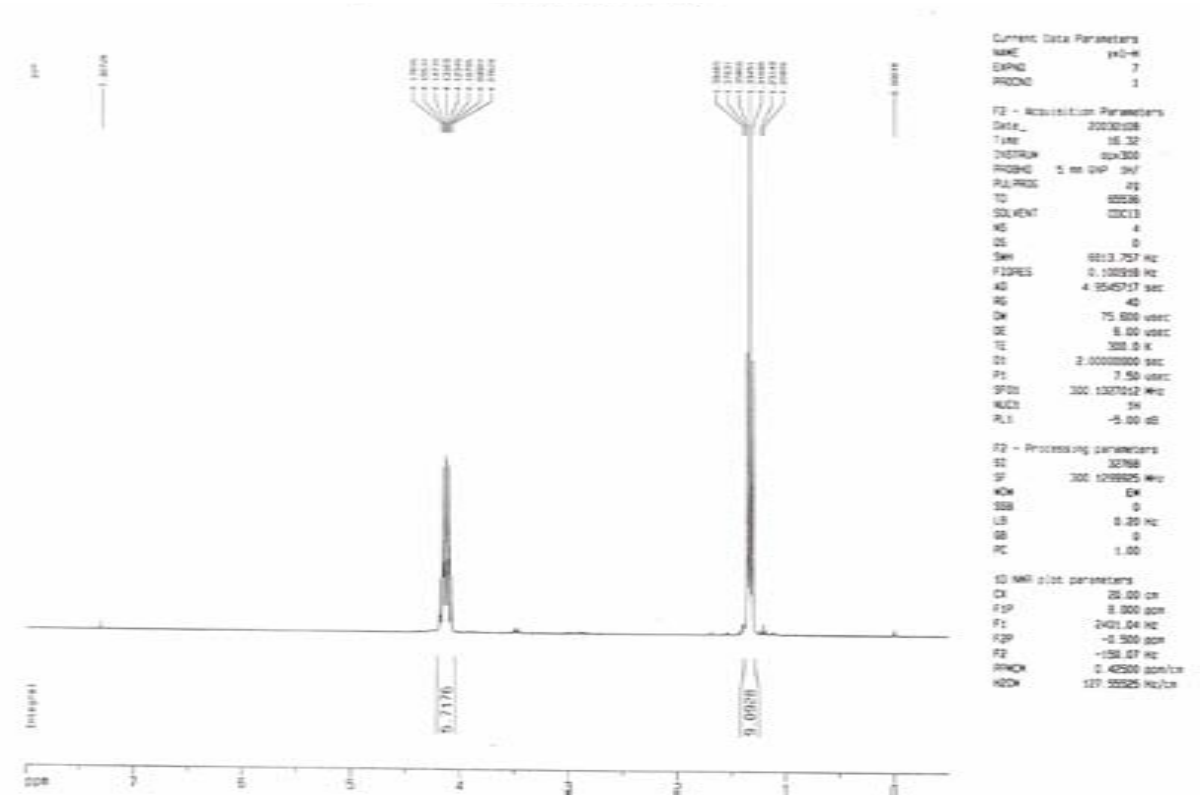


FIG. 13. The NMR spectrum of thiophosphoric acid O,O',O''-triethylester.

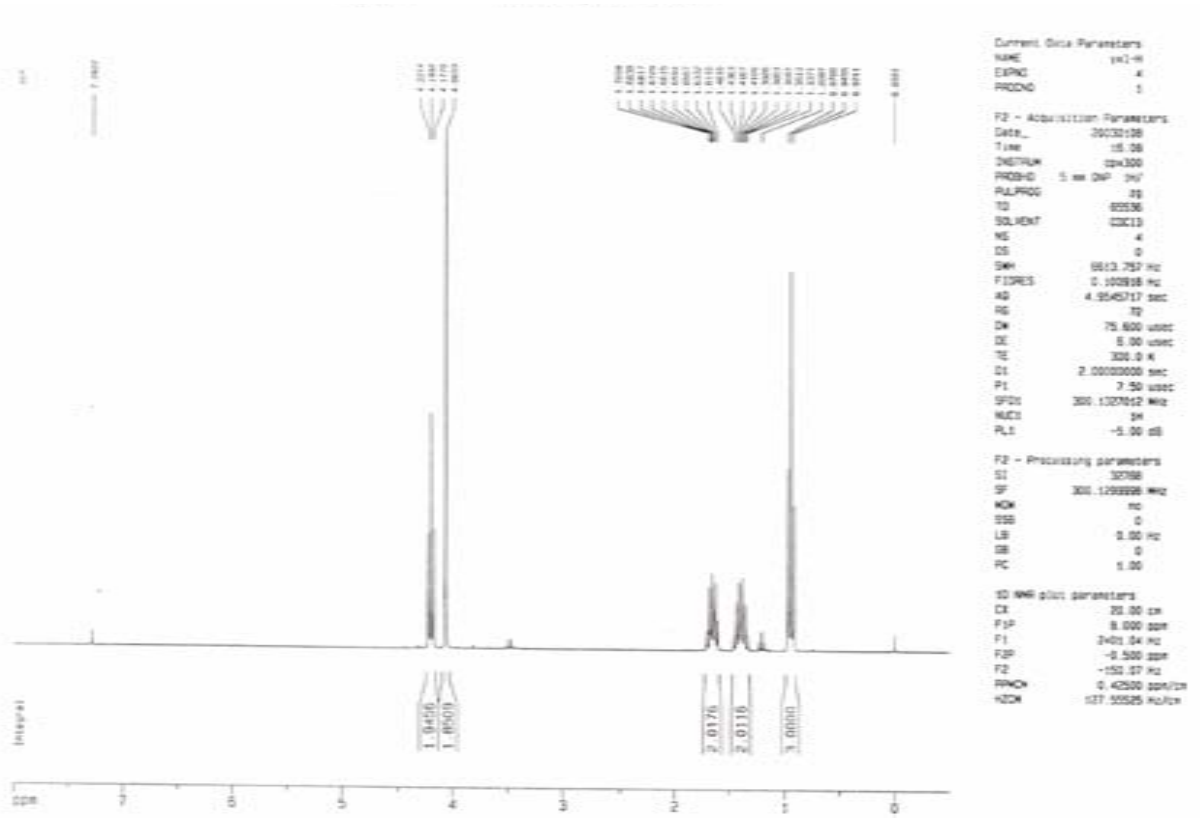


FIG. 14. The NMR spectrum of chloro-acetic acid butylester.

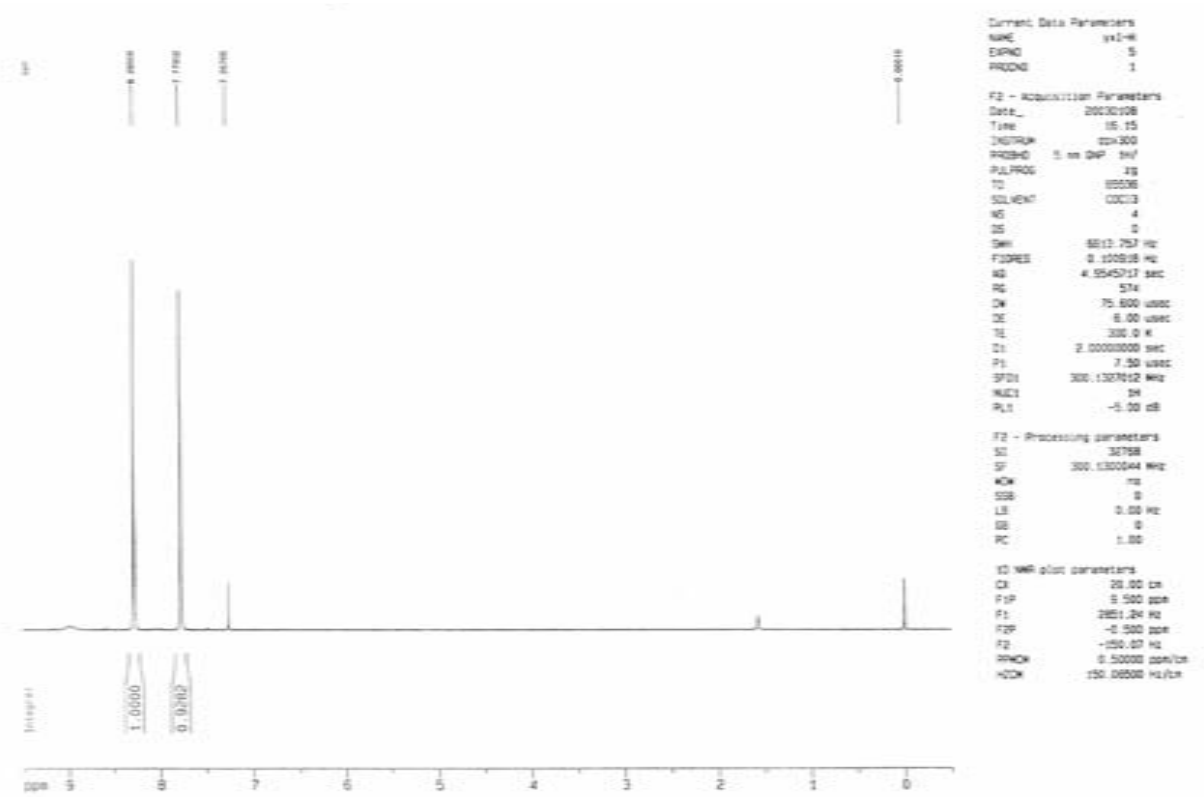


FIG. 15. The NMR spectrum of 1,5-dichloro-2-nitro-4-trifluoromethyl-benzene.

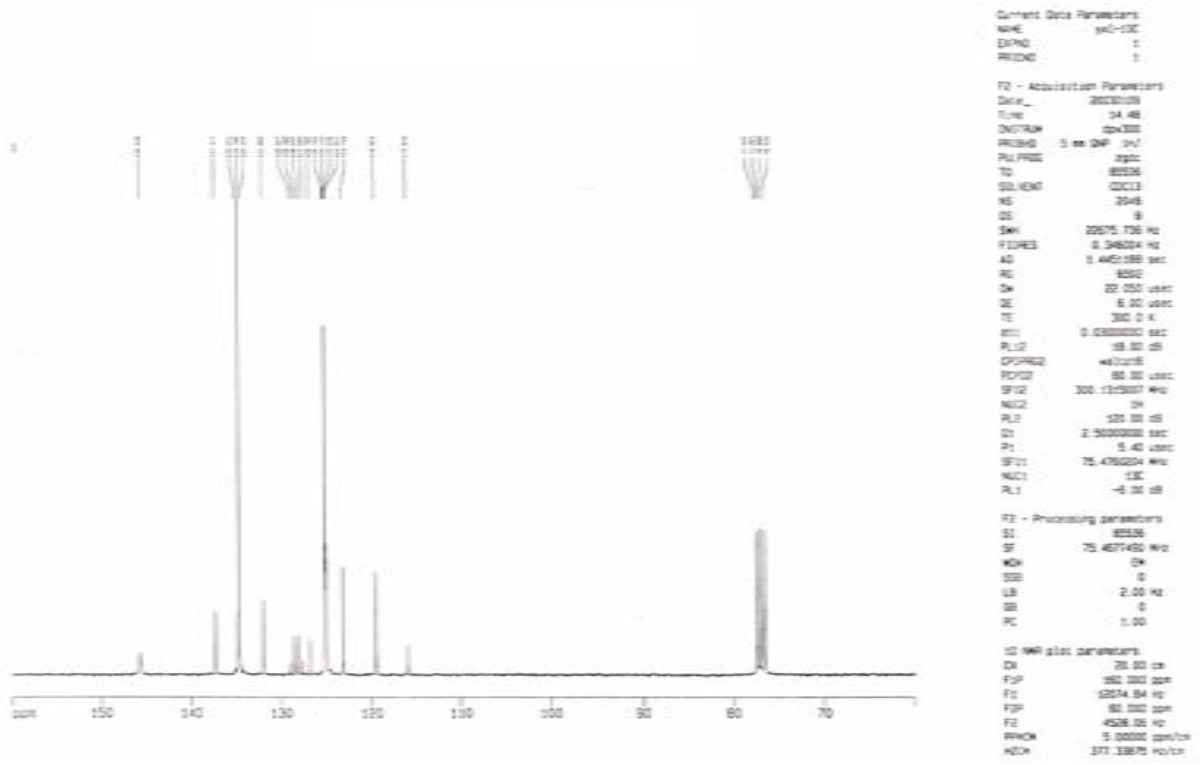


FIG. 16. The NMR spectrum of 1-chloro-2,6-dinitro-4-trifluoromethyl-benzene.

Annex 4

GC/MS chromatograms and spectra of impurities synthesized

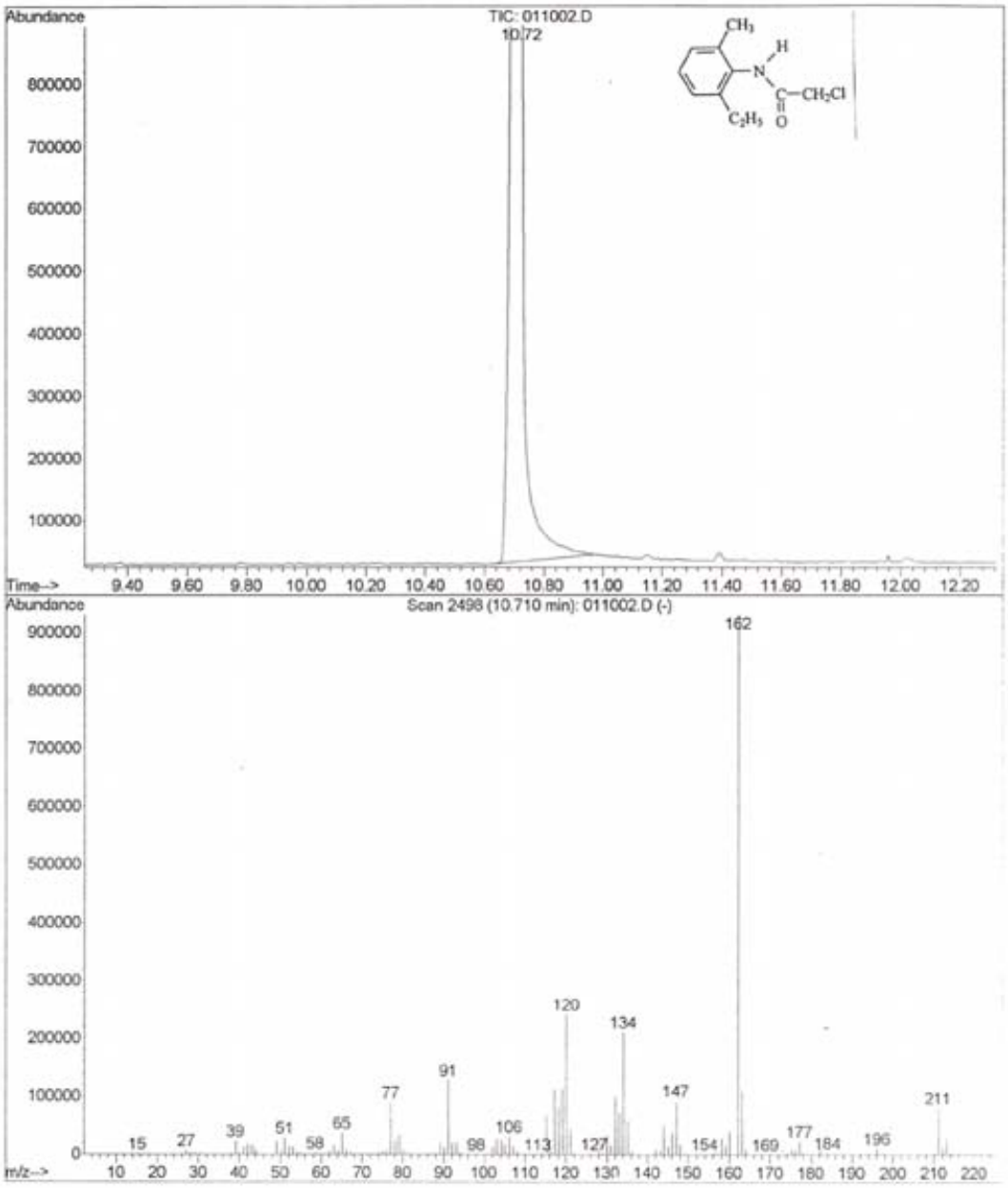


FIG. 1. The GC chromatogram and GC/MS spectrum of 2-ethyl-6-methyl-N-chloroacetanilide.

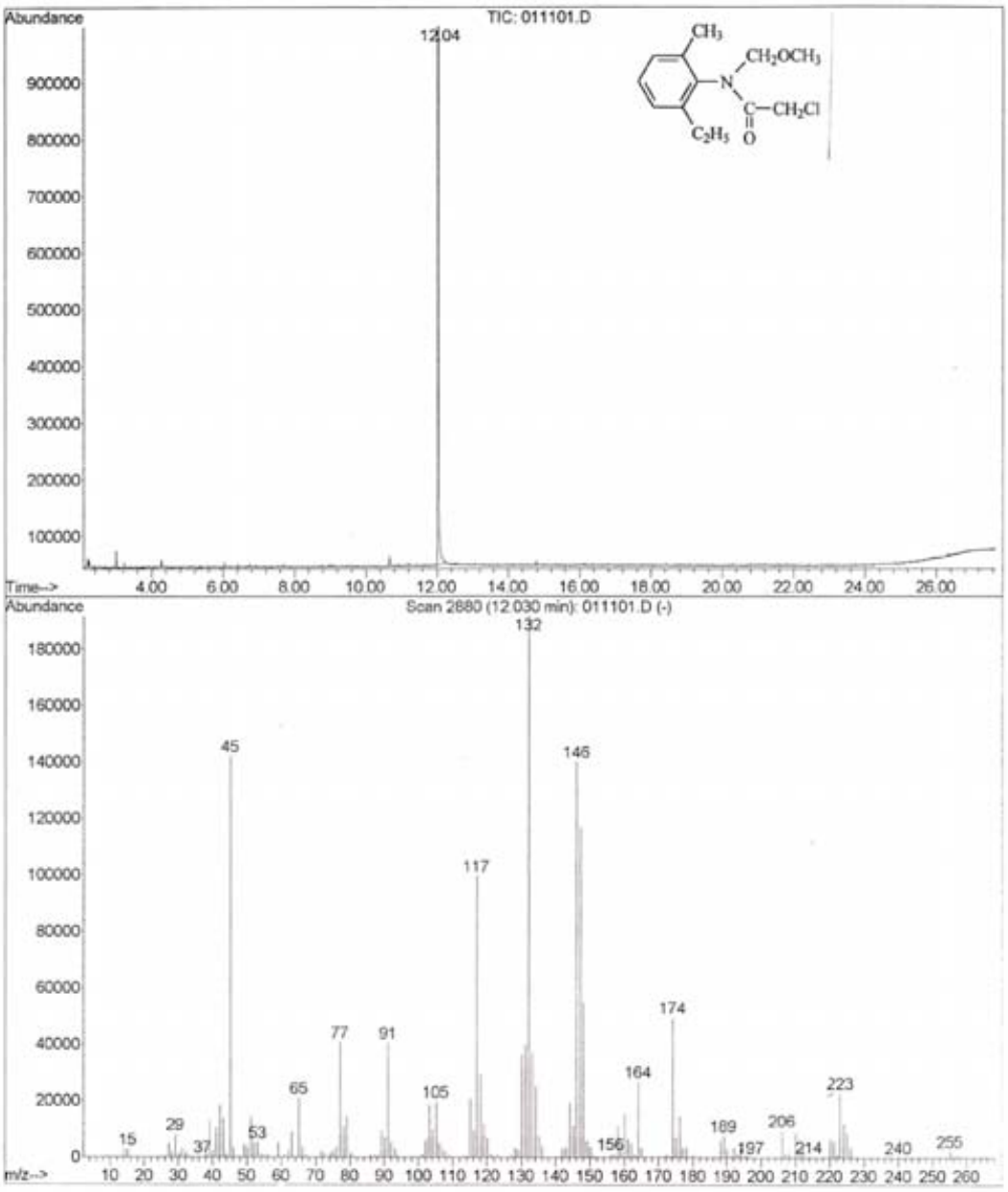


FIG. 2. The GC chromatogram and GC/MS spectrum of 2-ethyl-6-methyl-N-(methoxy-methyl)-chloroacetanilide.

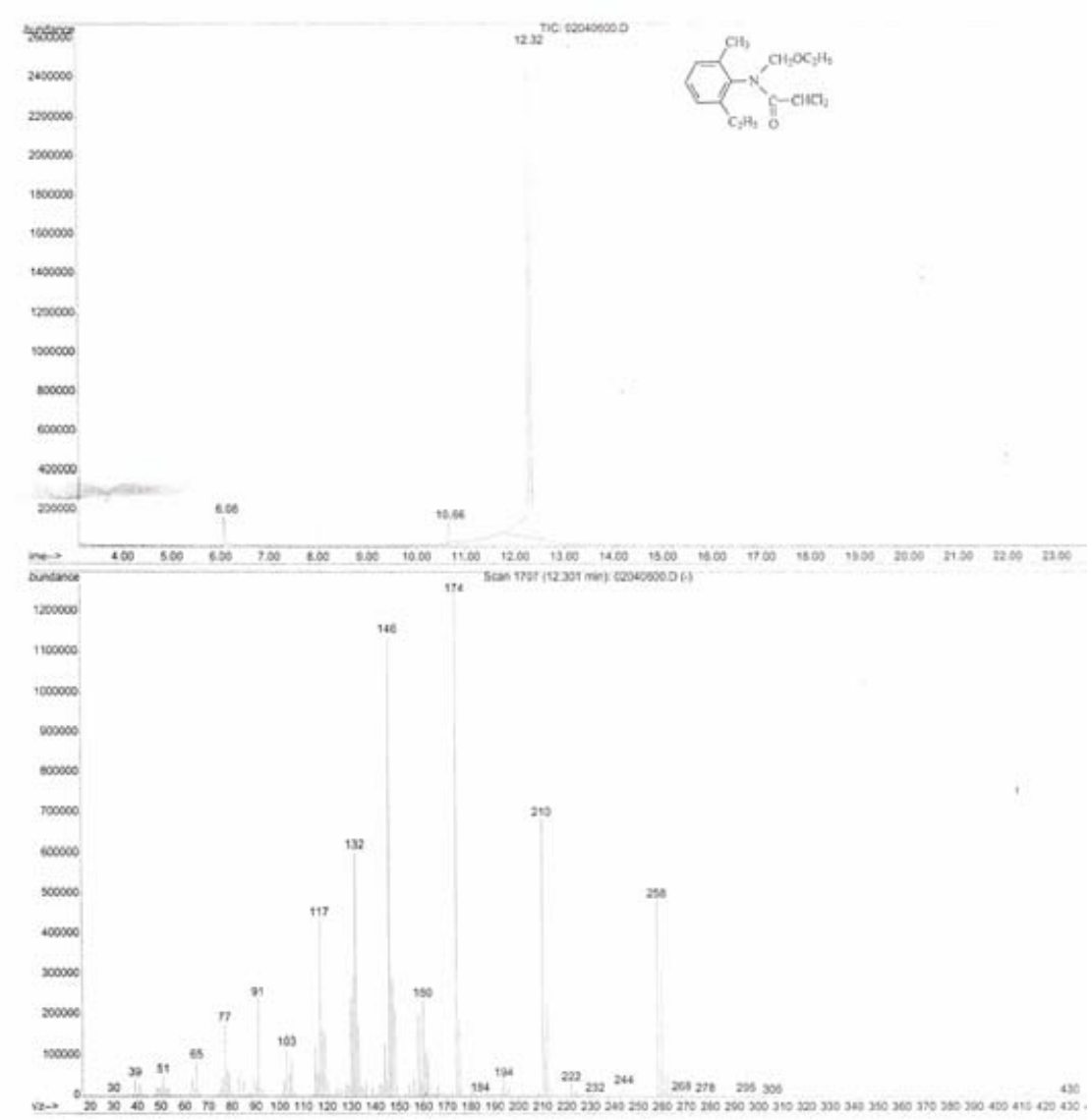


FIG. 3. The GC chromatogram and GC/MS spectrum of 2-ethyl-6-methyl-N-(ethoxy-methyl)-dichloroacetanilide.

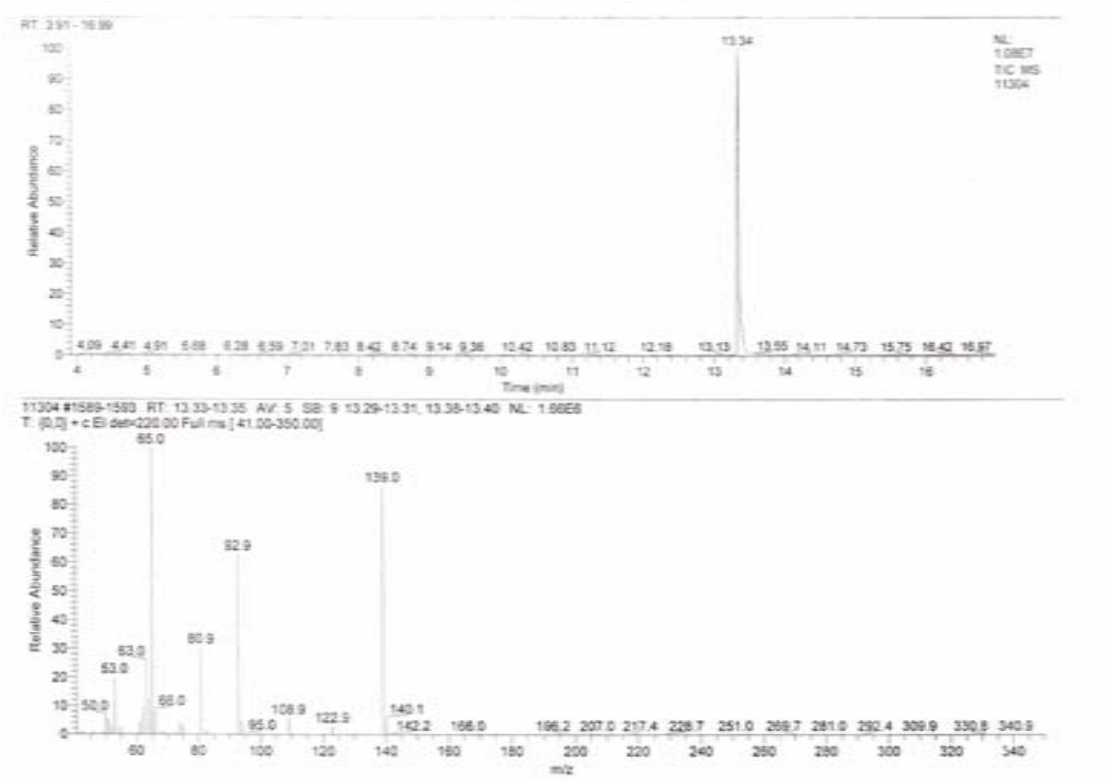


FIG. 4. The GC chromatogram and GC/MS spectrum of 3-nitro-phenol.

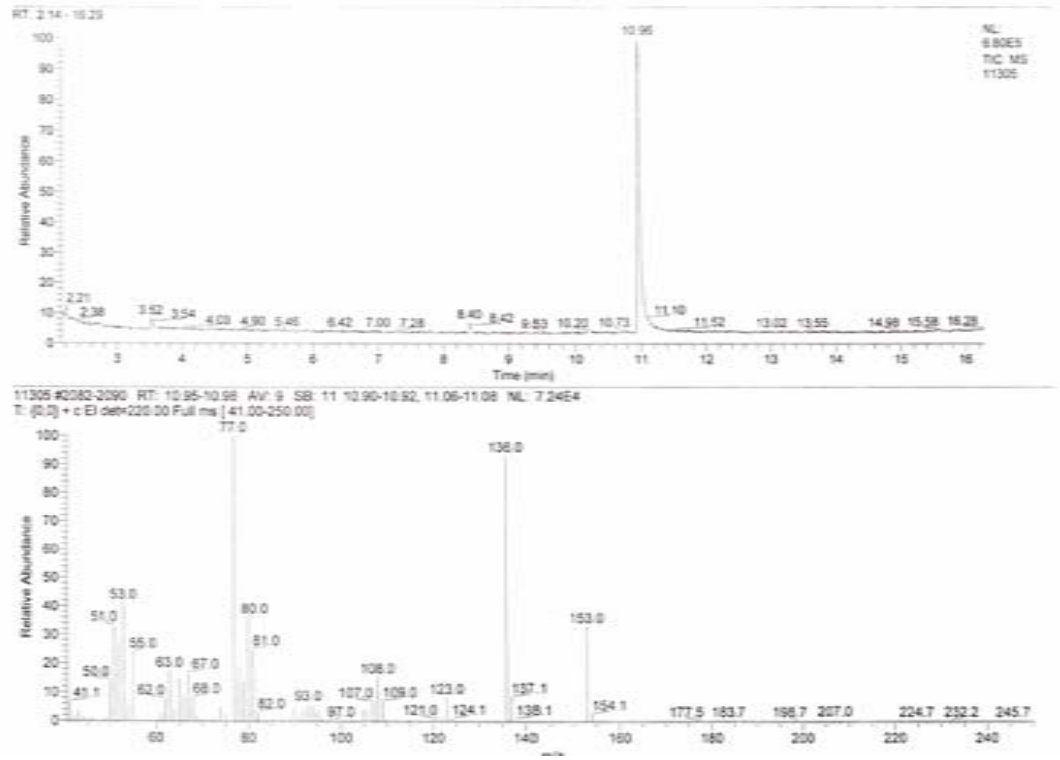


FIG. 5. The GC chromatogram and GC/MS spectrum of 3-methyl-4-nitrophenol.

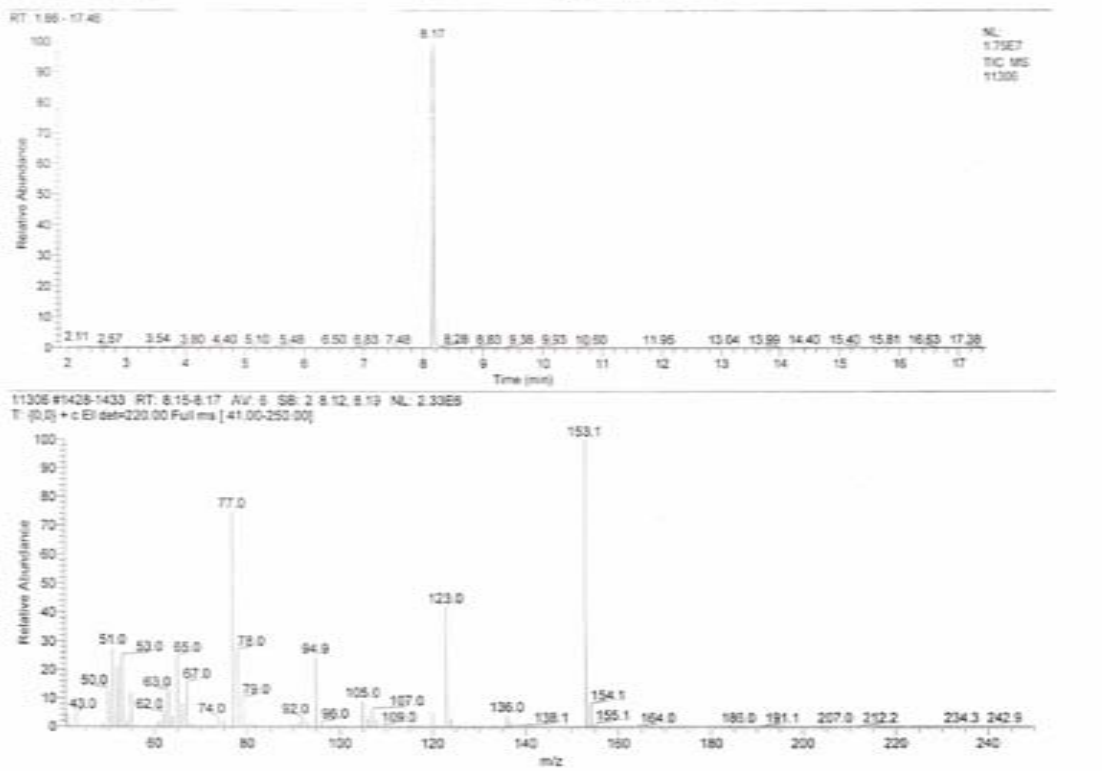


FIG. 6. The GC chromatogram and GC/MS spectrum of 3-methyl-6-nitrophenol.

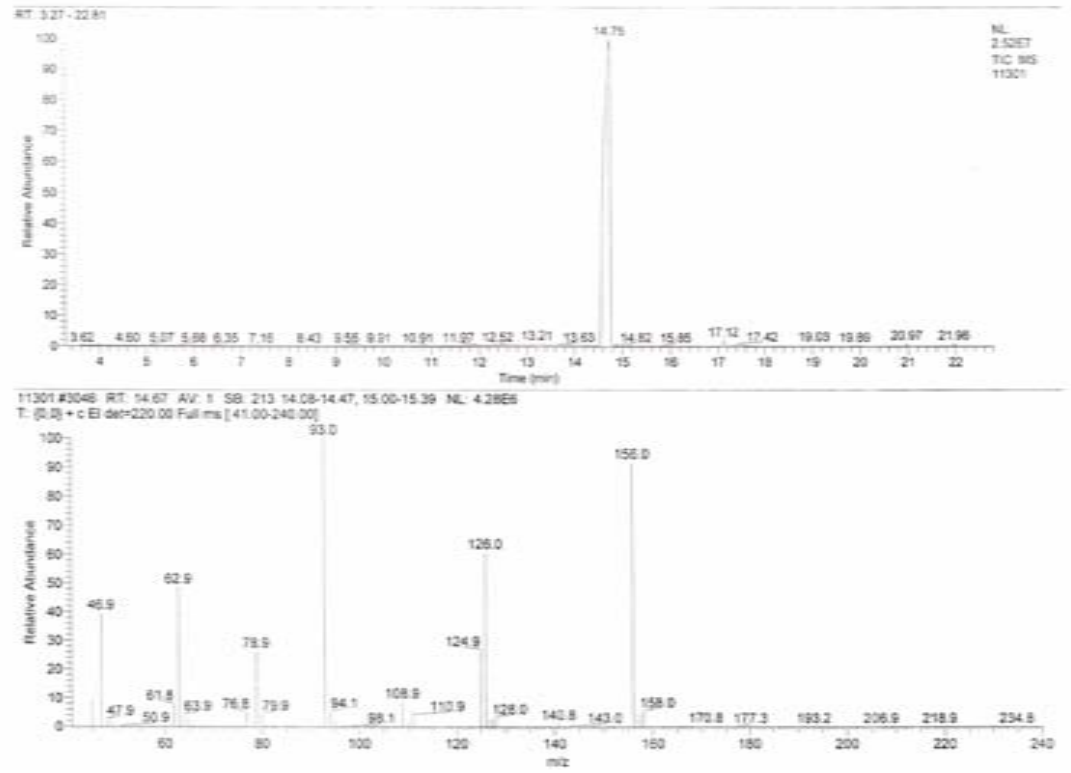


FIG. 7. The GC chromatogram and GC/MS spectrum of thiophosphoric acid O,O',O''-trimethylester.

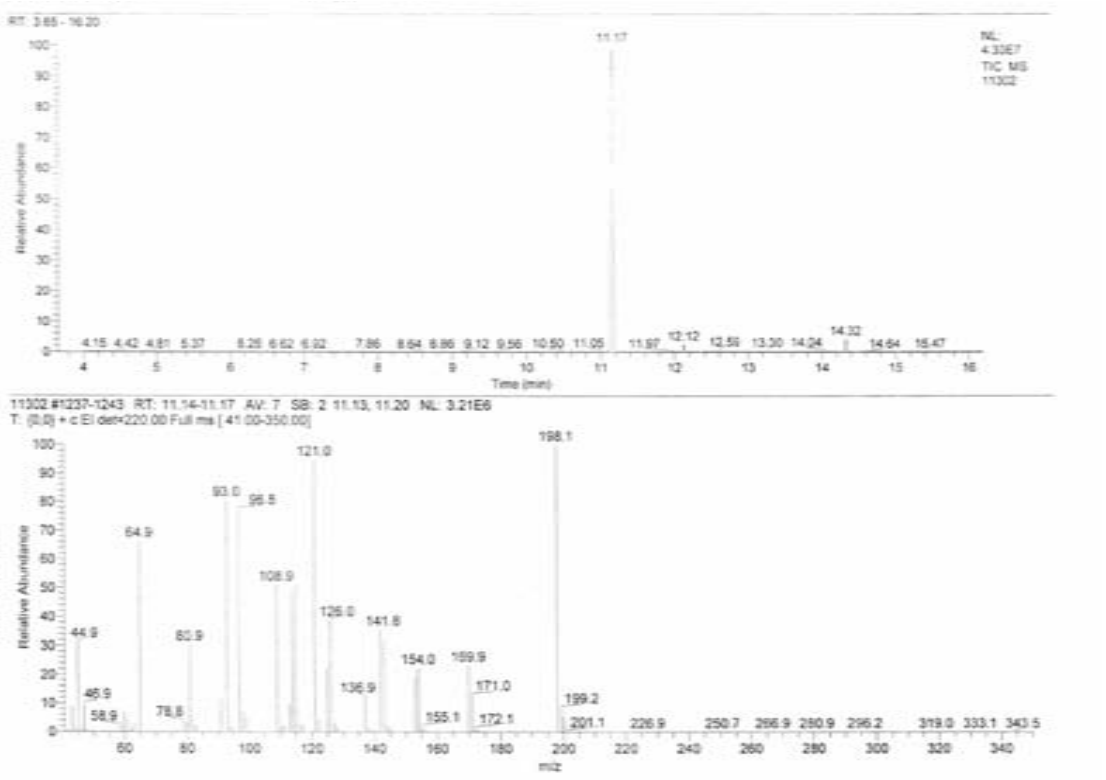


FIG. 8. The GC chromatogram and GC/MS spectrum of thiophosphoric acid O,O',O''-triethylester.

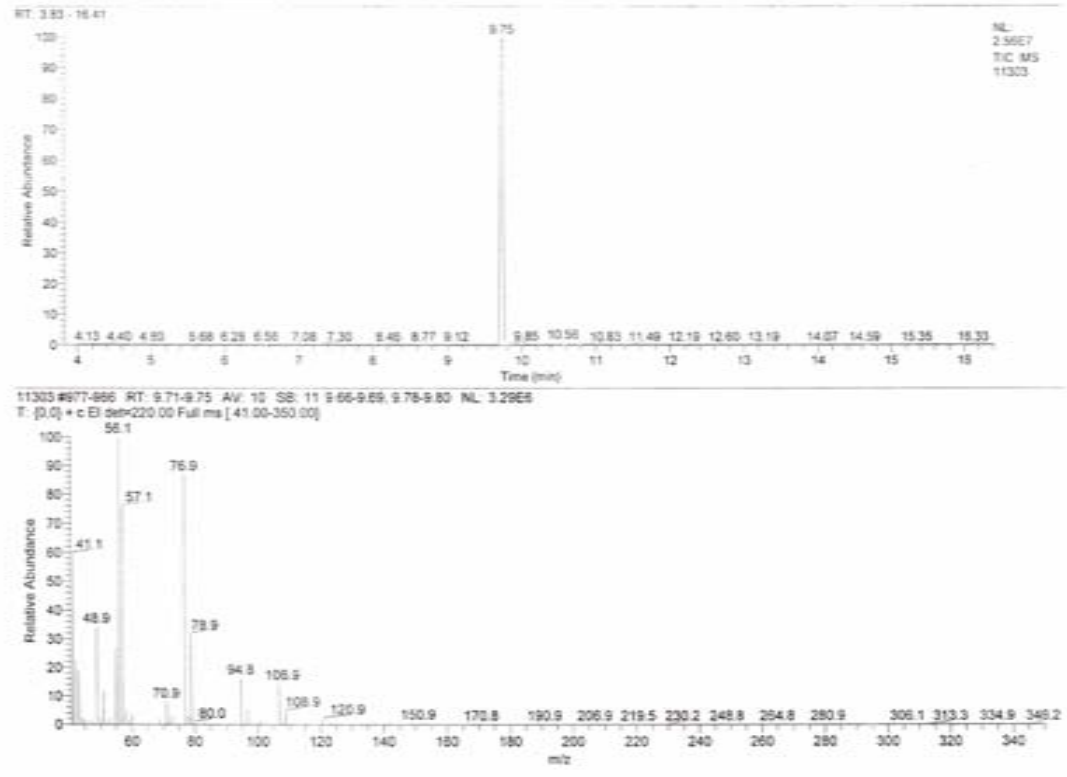


FIG. 9. The GC chromatogram and GC/MS spectrum of chloro-acetic acid butylester.

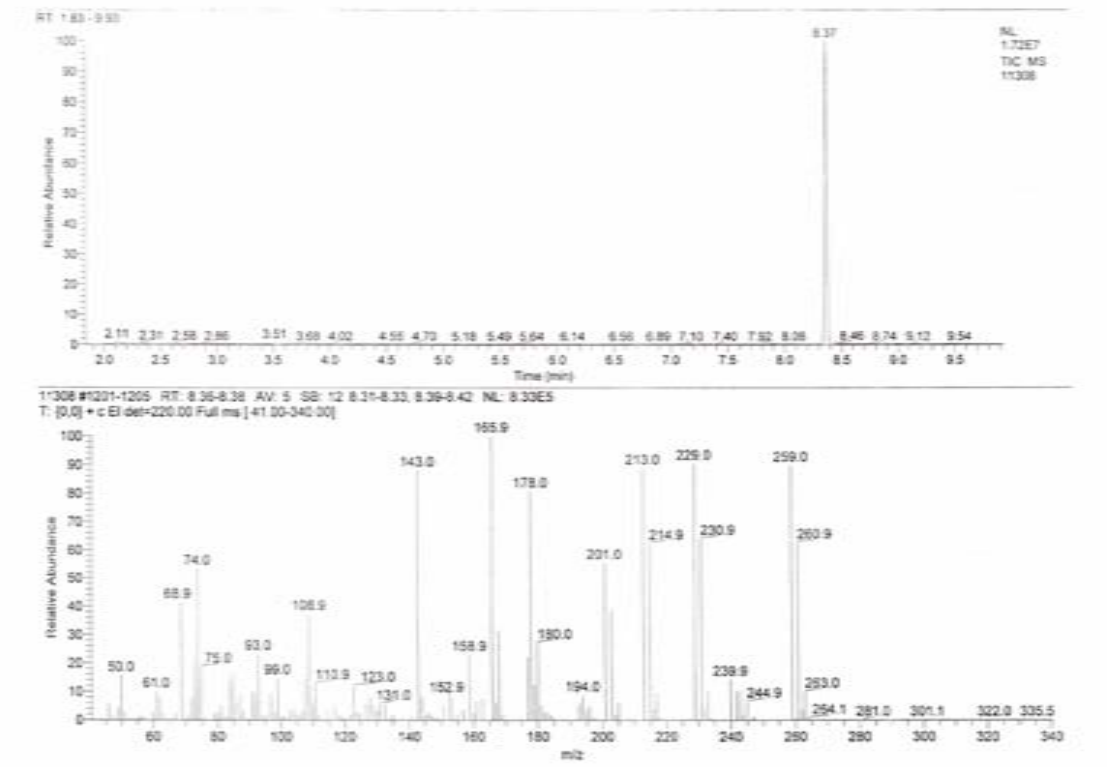


FIG. 10. The GC chromatogram and GC/MS spectrum of 1,5-dichloro-2-nitro-4-trifluoromethyl-benzene.

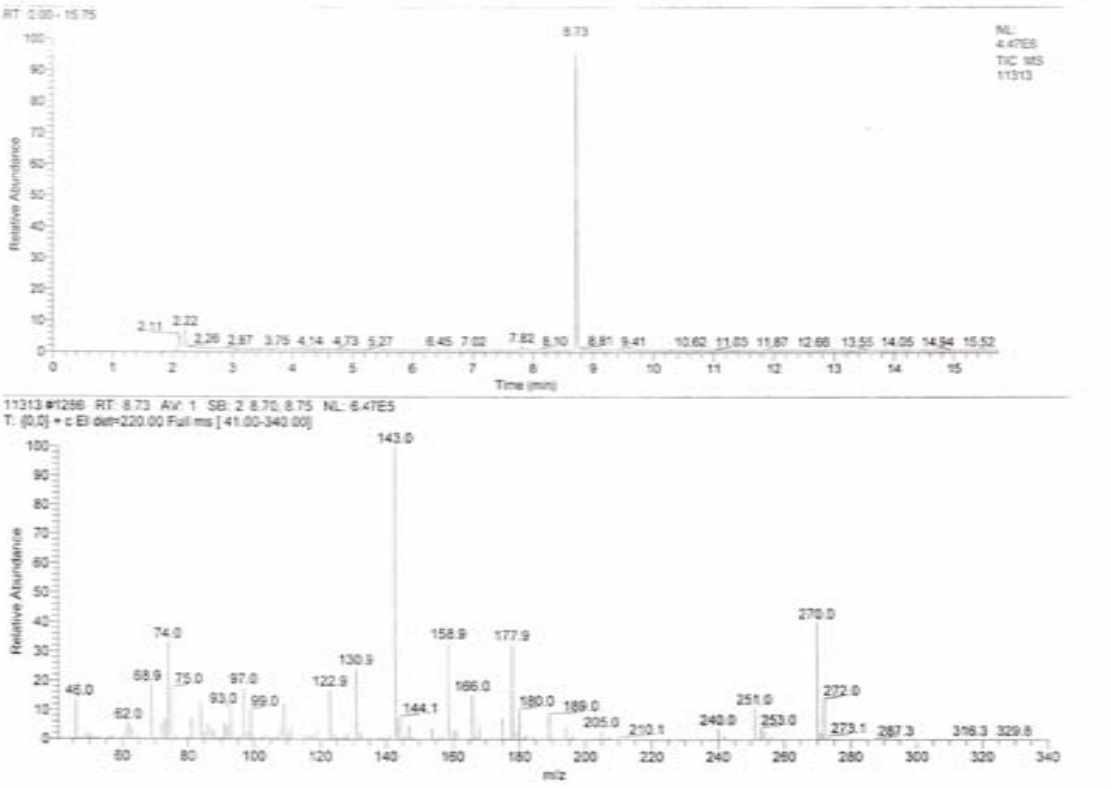


FIG. 11. The GC chromatogram and GC/MS spectrum of 1-chloro-2,6-dinitro-4-trifluoromethyl-benzene.

LIST OF ABBREVIATIONS

AOAC	Association of Official Analytical Chemists
CIPAC	Collaborative International Pesticide Analytical Council
CRP	Coordinated Research Project
CV	Coefficient of Variation
DAD	Diode Array Detector
f.t.	Film thickness
FTIR	Fourier Transform Infrared spectroscopy
GLC, GC	Gas Liquid Chromatography, Gas Chromatography
GLP	Good Laboratory Practice
HPLC	High Performance Liquid Chromatography
IPM	Integrated Pest Management
i.d.	Internal diameter
MPM	Multi-Pesticide Method
MRL	Maximum Residue Limit
MS	Mass spectrometry
NMR	Nuclear Magnetic Resonance
RCM	Research Coordination Meeting
RPLC	Reverse Phase Liquid Chromatography
RRT	Relative Retention Time
RSD	Relative Standard Deviation
SST	System Suitability Test
TLC	Thin Layer Chromatography
UV/Vis	Ultra Violet/ Visible light

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