Une fermentation d'histamine peut être exclue pour les *B. gracilc* qui sont presque toujours présentes ainsi que pour les longs batônnets et les bactéries acétiques n'apparaissant qu'occasionnellement.

Afin d'éviter la formation d'histamine, on propose un contrôle bactériologique plus intense pendant la rétrogradation malolactique de l'acidité. Il semble particulièrement important de contrôler la flore bactérienne des vins utilisés à des fins d'ensemencement.

Summary

The formation of histamine and the presence of bacteria in 21 wines have been examined during the entire vinification process. Histamine is formed by lactic acid cocci in the course of the malolactic fermentation. In part through laboratory experiments, histamine formation could be excluded in the case of also-present *B. gracile* as well as in the case of occasionally occurring long rods (bacilli) and acetic acid bacteria.

A closer bacteriological surveillance of the wine during the malolactic fermentation is proposed, in order to prevent histamine formation. Control of bacterial flora in wines that are used for inoculation purposes appears especially important.

The determination and occurrence of hexachlorobenzene residues

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1. Introduction

Hexachlorobenzene (HCB), C_6Cl_6, was already discovered by Faraday in 1824, although it was only in 1864 that a detailed description of its composition and properties was given by *Hugo Müller* (1).

In 1945 hexachlorobenzene was introduced as a fungicide and found soon application for seed treatments. HCB proved to be outstanding for destruction of seed- and soil-borne spores of the Bunt fungi. Considering that «Bunt» or «Stinking Smut» is the most important and common fungus disease of wheat, it is not surprising that HCB treatment of seed was adopted as a standard practice. It is usually applied as a dusting powder containing 10—40 % of technical grade HCB. Often a dyestuff is added to ensure uniform application and to distinguish treated seed from other grain. This preventive measure is very important, because treated seed is definitely toxic. It should not even be used for animal feed and it is not allowed to become mixed with commercial grain.

In southeastern Turkey there was an outbreak of cutaneous porphyria in 1955 which could be traced to the consumption of wheat that had been treated with

HCB (2). The intake of this fungicide by the persons affected, mostly children, was up to 200 mg/day during a relatively long period before the disease became apparent.

Upon recognition of HCB as the cause of this minor epidemic (5000 cases), the use of this fungicide was discontinued, whereupon the disease gradually disappeared.

HCB formulations are still used in most countries, because no suitable alternative fungicides are available. Although statistics on production and use of HCB are scarce, there seems to be a very extensive application in Germany and in the mediterranean countries.

It is surprising to note that the residue problem associated with the use of HCB has been somewhat neglected up to now. If we consult the available literature on residue analysis, we are almost tempted to conclude that HCB does not exist!

This impression is enhanced by the fact that total diet studies on pesticide intake (3, 4) never mention it. Moreover, HCB is not yet included in any national residue legislation*, although it has been considered by the FAO/WHO joint committee which has recommended temporary practical residue limits for some raw agricultural commodities (5).

Recently, however, attention on HCB residues was highlighted by two interesting publications: *Taylor* and *Keenan* (6) published a detailed study on the analytical aspects of HCB residue determinations and *Acker* and *Schulte* (7) discovered high HCB levels in human fat and human milk.

As this last finding indicates a biological accumulation of HCB residues, we may expect that the number of publications on this subject will increase in the near future.

The purpose of this paper is to give a contribution to the analytical methodology for the determination of HCB residues and to indicate the levels of this pesticide found in a number of foodstuffs.

2. Analytical methods for the determination of HCB residues

That the use of HCB in agriculture should result in a residue problem is fairly obvious if we consider the physical and chemical properties of this compound.

HCB ist definitely non-polar, it is very much soluble in fats and, unlike other chlorinated hydrocarbons, only slightly soluble in polar solvents such as acetonitrile.

It is not destroyed by treatment with sulphuric acid and studies performed in our laboratory indicated a high stability towards alkaline substances. In fact, we think that its stability may be compared with that of the notorious beta-HCH which is the most persistent of all hexachlorocyclohexane isomers (8, 9). HCB is

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* Pratical residue limits for HCB have meanwhile been issued by the Swiss federal authorities.

probably not broken down to any extent, but constantly recycled in the environment.

There are two reasons why the average residue analyst is less familiar with HCB than with the other chlorinated pesticides:

a) In most laboratories the analysis for residues of organochlorine pesticides is carried out according to the A.O.A.C.-method (10) which is less suitable for the determination of HCB.

b) Most gas chromatographic columns in use are not able to give a complete separation of HCB and HCH isomers and these residues often occur together.

3. Clean-up procedures

Taylor and *Keenan* (6) have already drawn attention to the fact that HCB is exceptionally non-polar. For this reason it cannot be isolated by methods based on the acetonitrile-pentane partition of *Mills* (11). If a fat solution in pentane is shaken with an equal volume of acetonitrile, the HCB present is distributed preferentially into the pentane phase. An experiment carried out in our laboratory with butter fat fortified with 1 ppm of HCB gave a recovery of not more than 20 percent.

It is obvious that the partition step should be omitted and this can only be done by using a direct extraction with absorption clean-up as has already been pointed out by the afore mentioned authors.

In our laboratory we use the one step direct elution method according to *Langlois* et al. (12) for the determination of all chlorinated pesticides in fatty foods. This procedure has proved to be also outstanding for the quantitative recovery of HCB from milk, cheese, butter and animal fats. It should be pointed out that this method uses 250 ml pentane-methylene chloride 4 : 1, V/V for the complete elution of HCB from the florisil column. The eluate is free from fatty substances and can, after suitable concentration, immediately be used for gas- and thin-layer chromatographic analyses.

The determination of HCB in non-fatty foods such as wheat and cereals proved to be more difficult. The above described direct elution method gave low recoveries and so did ordinary extraction with acetonitrile.

Following the suggestions of *Taylor* and *Keenan* (6) we found that exhaustive extraction with pentane or hexane gave optimal results, especially if the clean-up step was modified. The extraction was carried out in a soxhlet apparatus during 4 hours on 10—25 g of the finely divided material. The extract, thus obtained, was concentrated to 10 ml and passed over a 15 g's column of aluminium oxide (AL$_2$O$_3$ according to Brockmann, activity II—III). Subsequently, HCB was eluted with 100 ml of pentane. The cleaned up extract was found to be sufficiently pure for GLC- and in most cases also for TLC-analyses. However, confirmatory analysis by TLC at levels under 25 ppb could only be carried out by modifying the TLC-procedure. See under 5. Thin-layer chromatography.

An alternative method for the isolation of HCB residues is steam distillation. *Taylor* and *Keenan* (6) cleaned up wheat extracts by distilling from a twenty-fold excess of water.

They let the vapour rise through a Vigreux column and collected the distillate under hexane. The hexane extract of the aqueous distillate produced clear spots during TLC-analysis, even at very low levels.

We tried to recover HCB residues by direct steam distillation of the product to be analysed. The considerable volume of water, necessary for the quantitative entrainment of HCB, forced us to use prolonged distillation with constant extraction of the distillate. It was found that this procedure could be carried out in the classic apparatus as designed by *Dean* and *Stark* (13) for the determination of water by entrainment distillation with xylene.

10—20 grams of the finely divided sample were weighed in a 1 l round-bottom flask, 750 ml of distilled water and 3 ml of pure benzene were added and the flask was connected to the *Dean* and *Stark* apparatus.

When heating the flask in a 150-W heating mantle, the water started to boil, the vapour entraining the benzene. On condensation the distillate flowed into the receiver and the benzene separated from the water as a 3 cm upper layer. Via the system of communicating vessels, the water returned to the boiling flask at the same speed at which the distillate condensed into the receiver.

In this way a prolonged distillation with continuous extraction of the distillate was achieved, because all droplets of condensed water passed through the benzene layer before flowing back into the boiling flask.

A determination of HCB in wheat or cereals required 4 hours distillation. Animal fats, however, at a sample size of 3—5 g, took at least 6 hours distillation for a 80—90 percent recovery. The benzene extract thus obtained was generally pure enough for direct analysis (after drying with anhydrous sodium sulfate), but chicken fat frequently gave rise to Langmuir type peaks, during electron capture GLC, probably representing volatile aldehydes. These interferences could be readily eliminated by clean-up over a small aluminium oxide column as described before.

It should be pointed out that this distillation method is also suitable for the isolation of other volatile insecticides such as alpha- and gamma-HCH, which we frequently found together with HCB.

4. Gas chromatography

The stationary phase commonly used in the determination of multiple chlorinated pesticide residues is the so-called «Burke column» consisting of a mixture of DC-200 silicone and QF-1 fluorinated silicone (14). Lately, OV-17, a phenyl substituted polysiloxane, has also gained some popularity. These columns have the disadvantage that they do not separate HCB and the alpha isomer of HCH under normal operating conditions. This is regrettable, because especially OV-17 is a column suitable for separating most of the chlorinated pesticides for which

goods are at present analysed. This separation is normally carried out at about 200 ° C.

It is not generally known that lowering the column temperature to 150 ° C results in a complete separation of HCB and all HCH isomers but under these conditions the less volatile pesticides such as DDT are not eluted. It is, therefore, better to use a column which separates all chlorinated hydrocarbons commonly sought for at about 200 ° C in a reasonable time. We studied several single and mixed polarity column packings and found that the mixed liquid phase loading of 1,5 % OV-17 + 1,95 % QF-1 as described by *Thompson* et al. (15) performed very well. Although these authors did not report the behaviour of HCB on this column, we found that it yields a good separation of HCB and the HCH isomers as is illustrated in table I.

Table I

Relative retention times of HCB and HCH isomers on a 1,5 % OV-17 + 1,95 % QF-1 column at a temperature of 200 ° C and a N_2 carrier gas flow of 60 ml/minute.

Aldrin	1.00	(reference compound)
HCB	0.47	
alpha-HCH	0.53	
gamma-HCH	0.67	
beta-HCH	0.80	
delta-HCH	0.92	
epsilon-HCH	0.98	

As this column is also capable of giving a complete separation of the cyclodiene pesticides and of the DDT analogues, we feel that at present it is the best column available for multi-residue analyses.

5. Thin-layer chromatography

In the normal procedure recommended for the TLC of chlorinated pesticides (10, 16), HCB is sharply separated from the other chlorinated hydrocarbons. Being an extremely unpolar compound, it runs close to the front in a mobile phase such as heptane (R_f ca. 0,90).

Unfortunately, certain impurities which are often present in the extract also have a tendency of moving close to the front. Low levels of HCB are, therefore, not seldom masked. For this reason, we have looked for an alternative chromatographic system in which HCB should be more easily detectable. Most suitable chromatography proved to be «reversed phase» which separated not only the chlorinated insecticides, but several chlorobenzenes as well.

In order to have an unpolar adsorbent layer, we impregnated aluminium oxide with 5 % liquid paraffin. As a polar mobile phase we chose a mixture of acetonitrile-methanol-water-acetone 4 : 3 : 2 : 1, V/V.

The R_f-values obtained under conditions of saturation at room temperature are given in table II. Using photochemical revelation with silver nitrate (16) we achieved a limit of detection of about 25 nanograms of HCB.

Table II

R_f-values of chlorinated insecticides and chlorobenzenes in the system $AL_2O_3 + 5\%$ paraffin/CH_3CN—CH_3OH—H_2O—acetone $4 : 3 : 2 : 1$, V/V.

DDE	0.27	HCB	0.16
Heptachlor	0.37	Pentachlorobenzene	0.23
DDT	0.42	1,2,3,4 tetrachlorobenzene	0.41
Heptachlorepoxide	0.55	1,2,4,5 tetrachlorobenzene	0.34
TDE	0.62	1,3,5 trichlorobenzene	0.38
Dieldrin	0.68	1,2,4 trichlorobenzene	0.50
Lindane	0.72	1,2,3 trichlorobenzene	0.57
alpha-HCH	0.76		
beta-HCH	0.84		

6. Occurrence of HCB residues

As small survey carried out on various raw materials from different parts of the world indicated that HCB residues were present in a significant proportion (28 %) of the samples analysed (See table III).

But the number of samples being small, it is still too early to draw any conclusion.

The appreciable amounts of this fungicide in animal fats are no doubt due to uptake from contaminated feed.

The practical residue limits for Hexachlorobenzene submitted to the Codex Alimentarius Commission (17) are as follows:

Animal fats	1	ppm
Milk products	0.3	ppm on a fat basis
Milk (whole)	0.01	ppm
Cereal products (from wheat)	0.01	ppm

Although the greater part of the samples analysed was found to contain HCB residues below these permissible levels, there were a few, especially among the cereals in which the practical residue limit was exceeded. It is, therefore, desirable that the analyses for HCB residues should be included in the pesticide residue monitoring program of industry and government food inspection laboratories.

NB.: When this paper went into print, our attention was drawn by the fact that technical grade pentachloronitrobenzene, a widely used fungicide, contains 2—3 percent of HCB as an impurity. This could be an additional source of HCB contamination.

Table III
HCB residues in various raw materials from different parts of the world.

Commodity	Number of samples	Number with HCB	Less than 0.01 ppm	0.01 to 0.10	0.10 to 0.25	0.25 to 0.50	0.50 to 1.0	Over 1.0
Cereals and flours	52	7	3	3	1	0	0	0
Milk	93	29	26	2	0	1	0	0
Peanut oil, mais oil	10	0	0	0	0	0	0	0
Pork fat	3	2	0	2	0	0	0	0
Beef fat	3	2	0	1	0	1	0	0
Chicken fat	10	8	0	2	4	0	1	1
Total	171	48=28 %						

Total elimination of HCB from food will be very difficult to achieve, but the residue levels can be reduced by a more careful application of this fungicide. For this purpose, all contact of commercial grain with HCB treated seed, contaminated machinery, grain sacks and storage premises should be avoided.

7. Metabolism of HCB

According to *Parkes* and *Williams* (18) hexachlorobenzene does not appear to be metabolized in mammals. They found that HCB did not form any conjugated glucuronic acids, ethereal sulphates or mercapturic acids. Rabbits, receiving 100 mg of HCB/kg subcutaneously, did not excrete chlorinated benzenes in the feces.

In our laboratory we have found some evidence concerning a partial breakdown of HCB in the chicken. During gas chromatographic analyses of chicken fat samples, we were sometimes puzzled by the observation of a peak with a very short retention time which was eluted well before HCB. This peak was first thought to be a breakdown product of (often simultaneously present) hexachlorocyclohexane isomers, but the retention times of trichlorobenzenes, tetrachlorobenzenes and pentachlorocyclohexenes were found to be different.

Finally we noticed that a slight impurity in our HCB reference substance had the same retention time as the unknown peak which was subsequently identified as pentachlorobenzene. It is already known that technical grade HCB used in agriculture contains 1,8 % of pentachlorobenzene. We found, however, that the amount of this substance in the chicken fat samples varied between 7 and 15

percent of the HCB content. As it is not likely that pentachlorobenzene is more soluble in fat than its higher chlorinated homologue, we cannot altogether exclude a partial breakdown of HCB.

Summary

Hexachlorobenzene (HCB), C_6Cl_6, is a potent fungicide which is extensively used to protect wheat against Bunt fungi. Being an exceptionally stable compound which is very soluble in fat, it provokes residue problems quite similar to those associated with the use of chlorinated insecticides. HCB, however, is often not recognized during conventional residue analysis, because it is not sufficiently separated from the frequently simultaneously present hexachlorocyclohexane isomers on many commonly used GLC columns.

In this paper detailed information is given about analytical methods for the determination of HCB in cereals, fats and dairy products. Complete gaschromatographic separation of HCB and the hexachlorocyclohexane isomers is achieved on a mixed liquid phase loading of 1,5 % OV-17 and 1,95 % QF-1. Confirmation of identity is performed in a special reversed phase thin-layer chromatographic system which is also suitable for the separation of other chlorobenzenes.

A small survey carried out on various raw materials from different parts of the world indicated that HCB was present in a significant proportion (28 %) of the samples analysed.

The presence of pentachlorobenzene as a possible breakdown product of HCB in chicken fat is also reported.

Zusammenfassung

Hexachlorbenzol (HCB) C_6Cl_6, ein sehr stabiles, fettlösliches, starkes Fungizid, findet weitverbreitete Anwendung zum Schutz von Weizen gegen Bunt-Schimmel.

HCB-Rückstände können jedoch mit den üblichen Methoden zur Bestimmung der Pestizid-Rückstände oft nicht erkannt werden, da es nicht genügend von den häufig auch anwesenden Hexachlorcyclohexan-Isomeren getrennt wird. Die vorliegende Arbeit enthält detaillierte Angaben über Bestimmungsmethoden für HCB in Getreide, Fetten und Milchprodukten. Dabei wird komplette gaschromatographische Trennung von HCB und Hexachlorcyclohexan-Isomeren erreicht, mittels einer gemischten flüssigen Phase von 1,5 % OV-17 und 1,95 % QF-1. «Reversed phase» — Dünnschichtchromatographie ermöglicht Bestätigung des Befundes, sowie Trennung anderer Chlorbenzole.

Die Anwesenheit von HCB wurde in einer bestimmten Anzahl (28 %) verschiedener Rohstoffe der Weltproduktion festgestellt.

In Hühnerfett wurde Pentachlorobenzol als ein mögliches Abbauprodukt von HCB nachgewiesen.

Résumé

L'hexachlorobenzène (HCB), C_6Cl_6, est un fongicide puissant employé de façon intensive pour la protection des céréales contre les moisissures du type Bunt. Etant lipo-

soluble et exceptionnellement stable, il pose un problème de résidus semblable à celui des insecticides organochlorés. Toutefois les résidus de HCB sont souvent difficiles à détecter, car les méthodes habituelles d'analyse ne permettent pas de le séparer de façon suffisante d'avec les isomères de l'hexachlorocyclohexane (HCH), fréquemment présents simultanément.

La présente publication décrit en détail les méthodes de dosage des résidus de HCB dans des céréales, des graisses et des produits laitiers. C'est ainsi que la séparation complète, par chromatographie de phase gazeuse, du HCB d'avec les isomères du HCH est obtenue sur une colonne imprégnée d'une phase liquide mixte composée de 1,5 % OV-17 et 1,95 % QF-1. L'identité des résidus est confirmée par chromatographie sur couche mince en phase renversée, système qui permet de séparer aussi d'autres chloro-benzènes.

L'analyse d'un certain nombre de matières premières d'origines très diverses a montré que le HCB était présent dans un nombre appréciable (28 %) d'échantillons.

La présence dans la graisse de volaille de pentachlorobenzène pourrait être expliquée comme provenant de la dégradation du HCB.

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