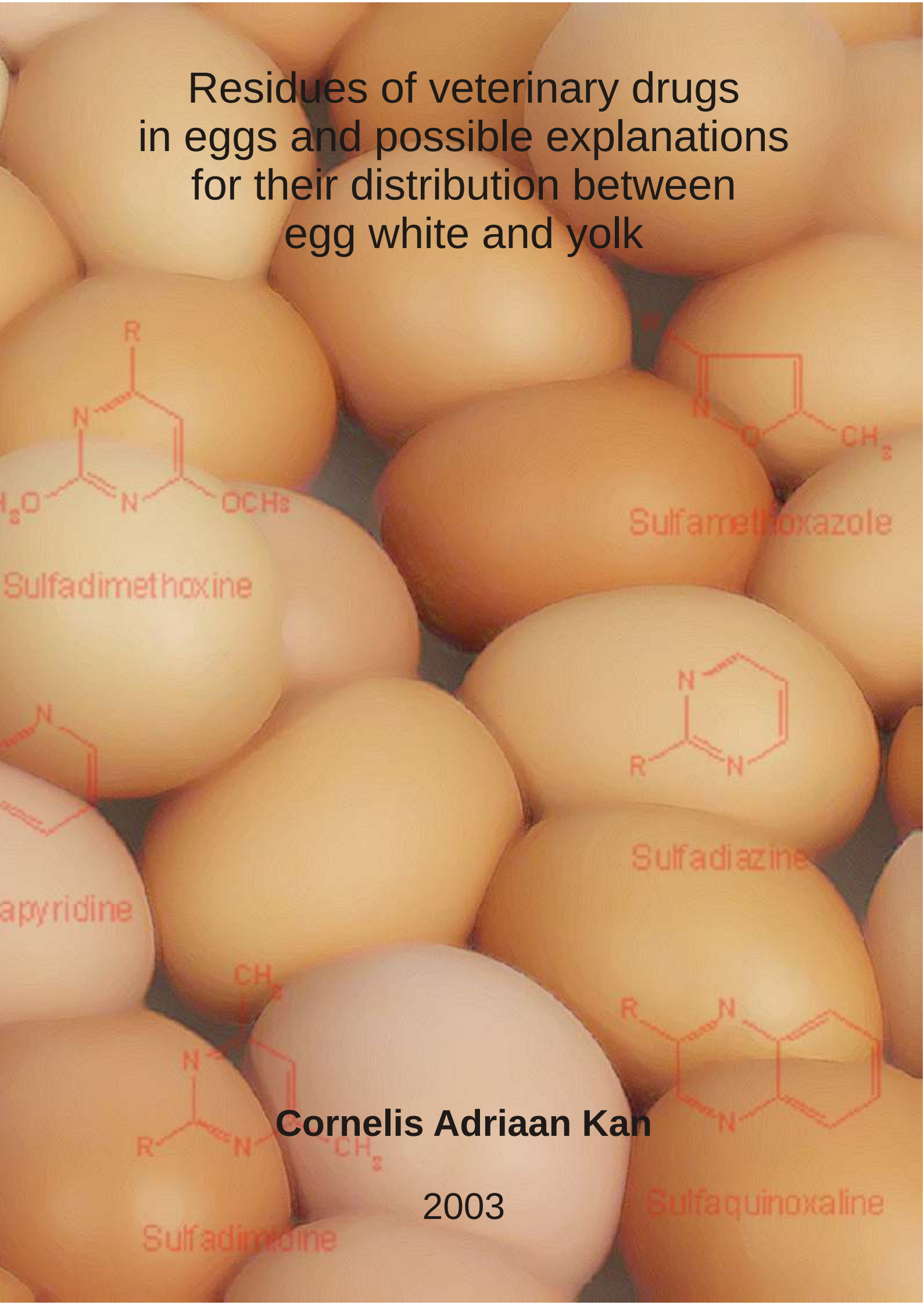
Residues of veterinary drugs in eggs and possible explanations for their distribution between egg white and yolk



Cornelis Adriaan Kan

2003

Residues of veterinary drugs in eggs and possible explanations for their distribution between egg white and yolk

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Cornelis Adriaan Kan

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Professor Dr. Michael Petz, Faculty of Chemistry, Department of Food Chemistry, Bergische Universität Wuppertal, Germany, provided the scientific guidance to this project.

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0. ABSTRACT

Veterinary drugs are therapeutically used for laying hens but may also reach them unintentionally via the feed e.g. as a result of cross-contamination during premix manufacture, feed preparation in the feed mill or during feed transport.
When these compounds reach the bloodstream, they will occur in the ovary with growing follicles and the oviduct, where the egg white is formed and secreted. The deposition of drugs in either yolk or white or both phases determines where one should look for residues.
Three reasons might rule the distribution of drugs between egg yolk and egg white: lipid solubility as fat soluble compounds generally occur in yolk, pK_a value as ionised molecules will distribute in a certain way between phases with different pH values such as yolk and white or protein binding to egg white proteins.
An extensive survey of available literature data on residues in egg yolk and white after administration to laying hens was made. The data on the distribution of residues between yolk and white differ considerably between drugs, but show remarkable resemblance for data on a given drug. All data on sulfonamides as well as on tetracyclines were considered carefully for any relationship between physicochemical characteristics and residue data in yolk and white. The lipid solubility hypothesis was certainly not supported by the available data including own previous experiments.
All three explanations have also been tested in two animal experiments in which several sulfonamides differing in lipid solubility, pK_a value and protein binding were administered to laying hens via the feed during 14 days and the levels in both yolk and egg white measured.
None of the three reasons could satisfactorily explain the ratio of the sulfonamide residues in egg white and yolk found in these experiments.
The conclusion therefore is that – at least for the sulfonamides tested – egg yolk and egg white are not two phases separated by a semi-permeable membrane and in some way in equilibrium with each other.
Rather, two independent physiological processes in the laying hen govern the deposition of residues of the sulfonamides in egg yolk and egg white.

1. INTRODUCTION

Veterinary drugs are therapeutically used for laying hens, generally by mass application via water or feed in order to combat occurring diseases. Drugs or coccidiostats may also reach them unintentionally via the feed e.g. as a result of cross-contamination during premix manufacture, feed preparation in the feed mill or during feed transport.

Some of the drugs are designed to work systemically; thus they must cross the intestinal wall in order to exert their function. Other drugs – and certainly those that combat endoparasites such as coccidiostats – should exert their action within the gastro-intestinal tract but they are nevertheless (partly) absorbed. This absorption is quite logical as both veterinary drugs and coccidiostats possess certain lipophilic properties in order to interact with and pass through membranes. These lipophilic properties are a prerequisite to reach target organs or cells and to fulfil their task of eliminating micro-organisms, coccidia or other endoparasites.

When these compounds reach the bloodstream, they will be distributed over the whole body. In the laying hen this includes also the ovary with growing follicles and the oviduct, where the egg white is formed and secreted. The amount of the compounds or its metabolites in each tissue depends on their characteristics, such as differences in rate of metabolism or lipid solubility.

Knowledge about the deposition of drugs in either yolk or white or both is required for at least three reasons:

1. To know whether either the white or the yolk might still be safe for human consumption in case residues are found in whole egg
2. To know whether yolk or white or both should be sampled to correctly assess the residue content of whole eggs
3. To develop drugs with special properties to target specifically either yolk or white if required.

1.1 Formation and composition of the egg

The main components of the avian egg are: egg shell, white and yolk. Figure 1 gives a schematic drawing of the egg and its components.
The follicles – later becoming the yolks - grow on the ovary and after ovulation the free ovum is picked up by the infundibulum (Figure 2) of the oviduct and then transported towards the albumen secreting region or magnum (Figures 2 and 3).

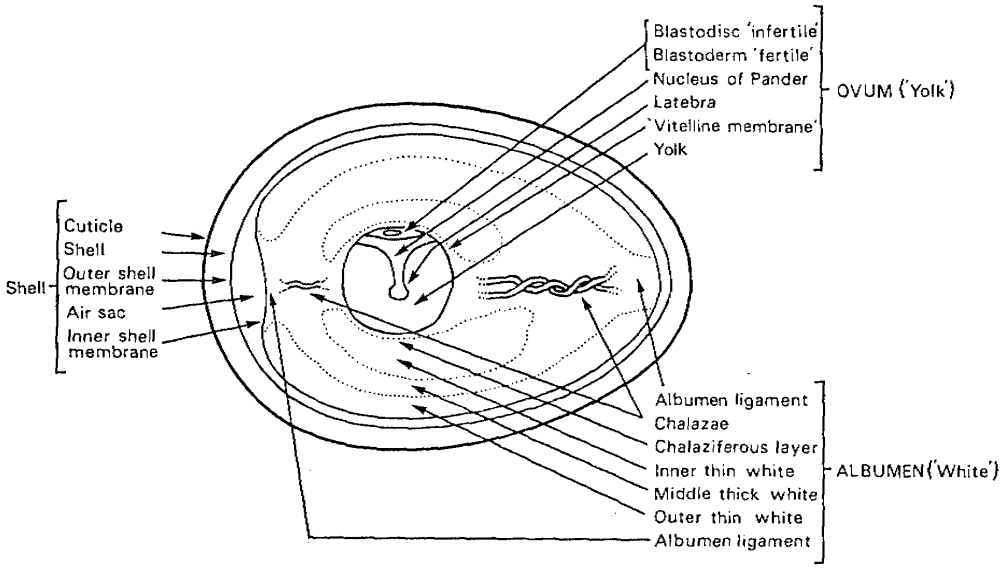


Figure 1: Components of the egg (Gilbert 1971a)

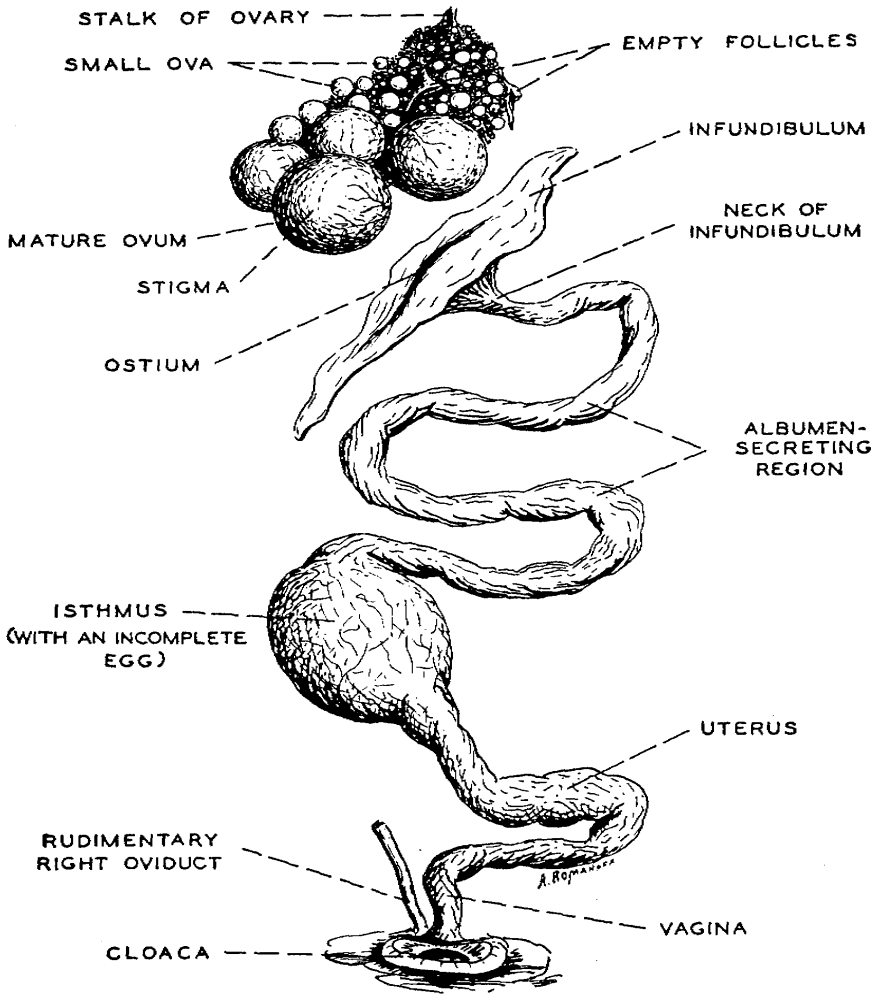


Figure 2: The reproductive tract of the hen (Nesheim et al., 1971)

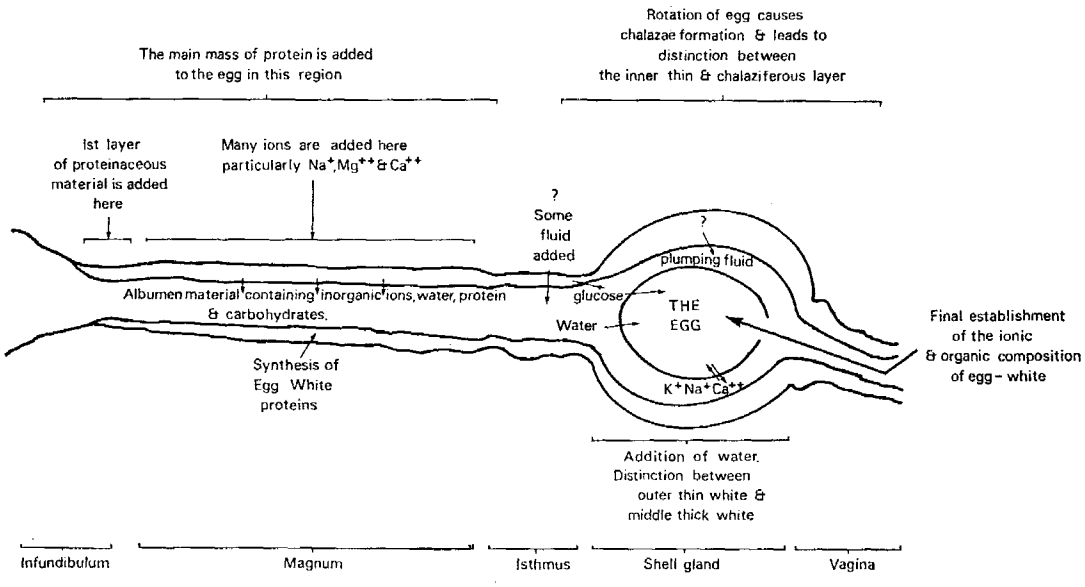


Figure 3: Processes occurring during egg formation (Gilbert 1971b)

Via the isthmus, where the membranes are formed, the “egg” then enters the shell gland or “uterus”. There, fluid and minerals are added during the plumping process to the egg white and the shell is formed. Then the “ready-to-lay” egg is transported via the vagina and through the cloaca (Figure 2), and the egg is laid. The time frame of the whole process is outlined in Figure 4.

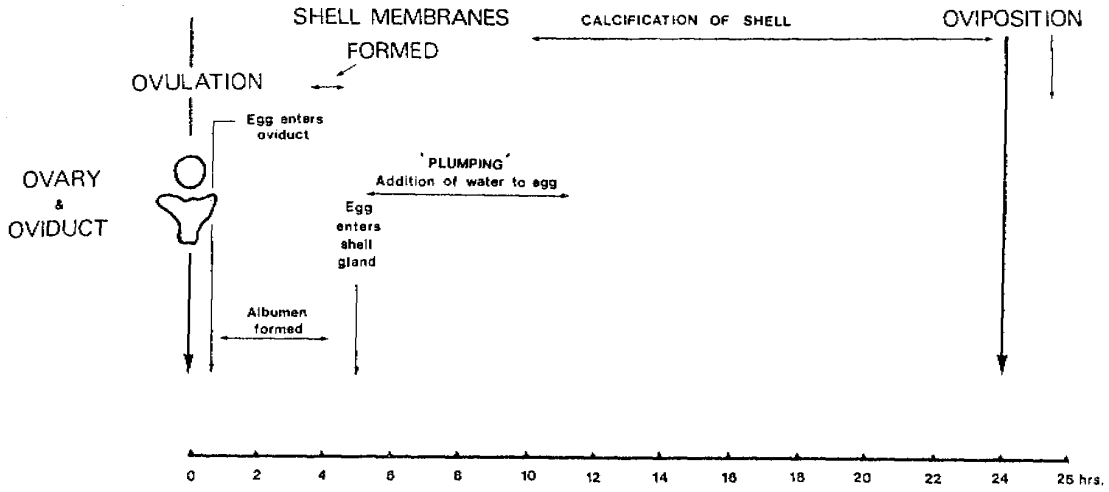


Figure 4: Time schedule of egg formation (Gilbert 1971a)

1.2 Formation of

1.2.1 *Follicles or Yolk*

Yolk components (pre-dominantly lipoproteins) are formed in the liver and transported via the blood to the ovary. The ovary of hens in active production (Figure 2 and 8) contains three types of follicles where the yolk can be deposited:

1. Very small ones, in the slow phase of development which can take months or even years. These are also called the white follicles as no (coloured) carotenoids are deposited there.
2. Those in the intermediate phase of growth (lasting some 60 days)
3. Follicles in the rapid growth phase which lasts approximately 10 days. The follicle weight increases during this time from some 1 gram to about 20 grams and deposition occurs in concentric layers one after each other (Figures 5, 6 and 7).

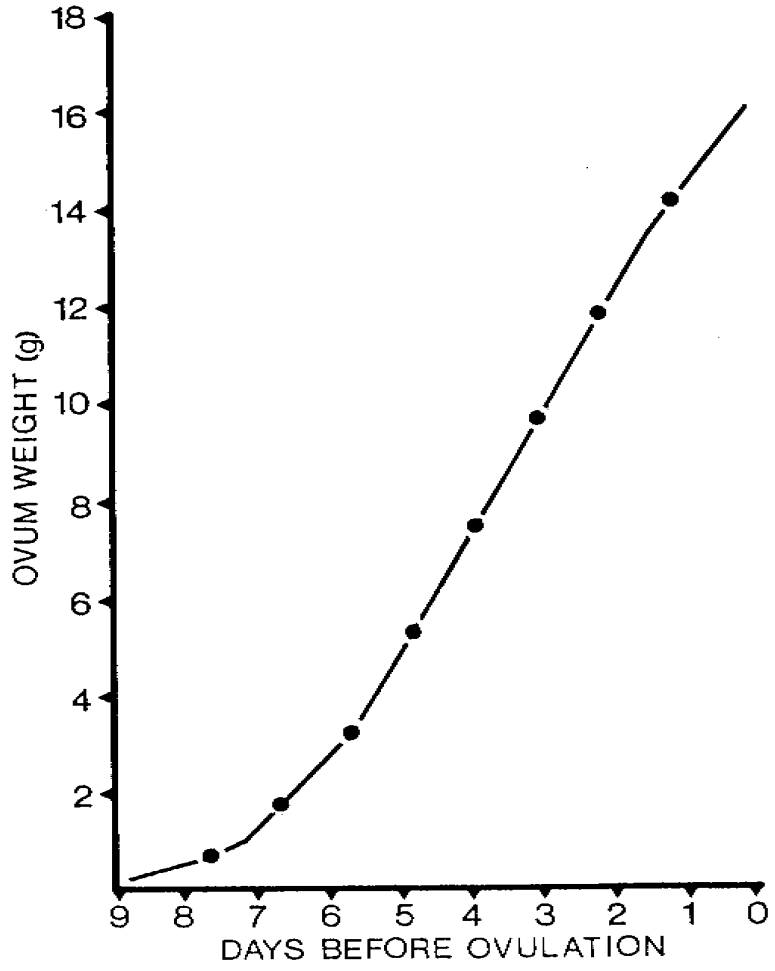


Figure 5: Growth curve of the follicles (Gilbert 1972)

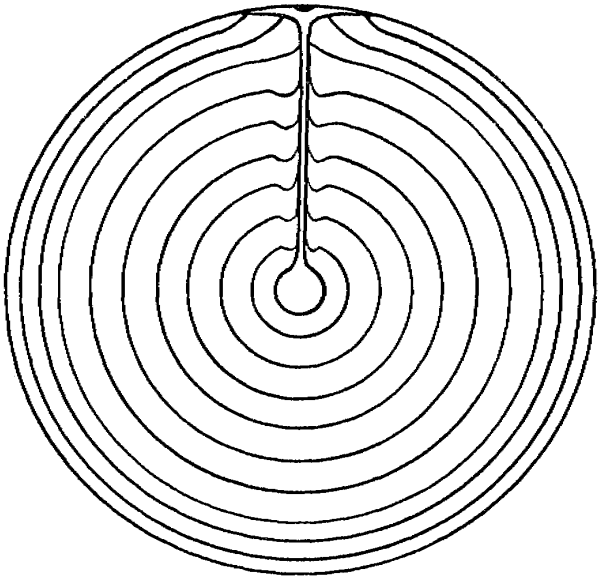


Figure 64. Diagram illustrating the growth and development of egg yolk.

The concentric rings represent daily growth of yolk, and their positions were determined by injecting into the hens a dye, Sudan III, which stains fat. (From Warren and Conrad, *J. Agric. Res.*, 1939).

Figure 6: Yolk deposition schematically

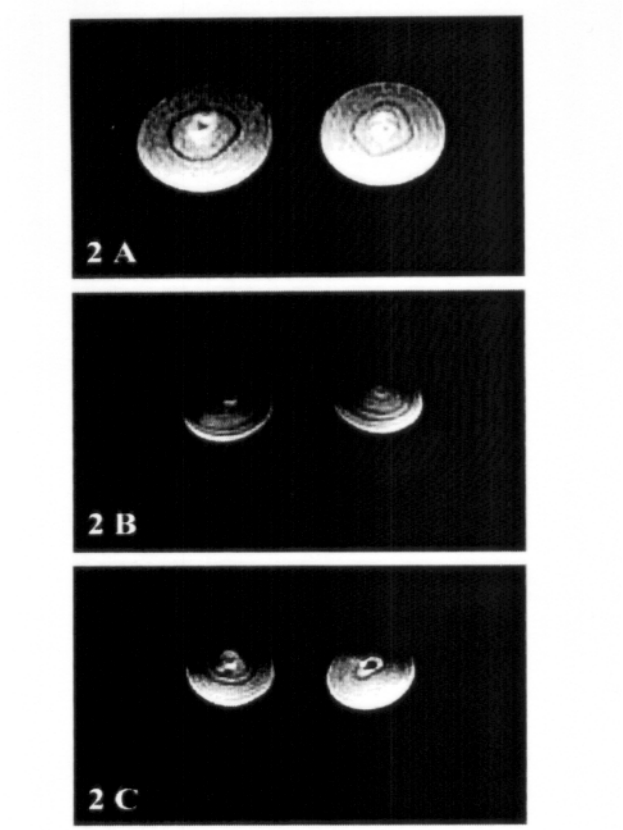


Figure 2. (A) Eggs collected 5 days from two different hens (left or right image, respectively) after a single injection of magnevist. Notice a single ring incorporates drug residues. (B) Eggs collected 3 or 4 days (left or right image, respectively) after single injections of magnevist on two consecutive days. Notice two separate rings incorporate drug residues. (C) Eggs collected 5 or 6 days (left or right image, respectively) after single injections of magnevist on two consecutive days. Notice two separate rings incorporate drug residues.

Figure 7: Yolk deposition as pictured by MRI (Donoghue and Myers, 2000)

As one follicle ovulates approximately every 24 hours, roughly ten follicles are present in different stages of the rapid growth. Figure 8 (Donoghue and Myers, 2000) shows the ovary with follicles as can be found in hens in active production and the separate yolks.

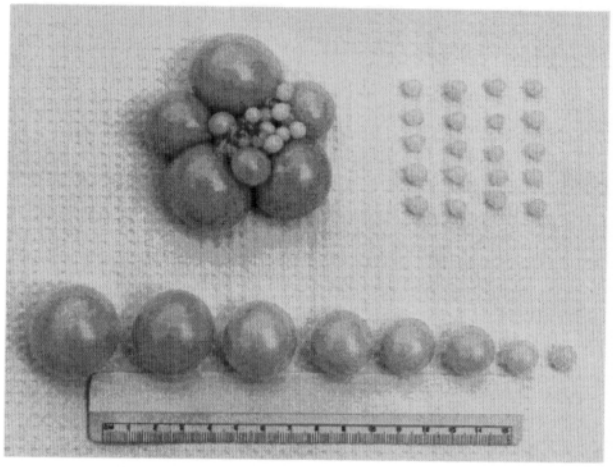


Figure 1. Photograph of an intact hen ovary (upper center) and dissected yolks from the large (lower) or small (upper right) phase of yellow yolk formation. Large preovulatory yolks (>0.2 g) are within 2 weeks of ovulation and arranged within a follicular hierarchy. The largest, heaviest yolk usually ovulates within 24 h, the second largest yolk usually ovulates 24 h after the largest yolk, the third largest yolk usually ovulates 24 h after the second largest yolk, etc. Small preovulatory yolks (<0.2 g) are within 2–6 weeks of ovulation.

Figure 8: Ovary and separate yolks (Donoghue and Myers, 2000)

It is not well known, whether yolk material deposition carries on right until the moment of ovulation or that one day elapses between last deposition of yolk material and ovulation.
The physiological and endocrinological processes probably taking place before ovulation are schematically shown in Figure 9.

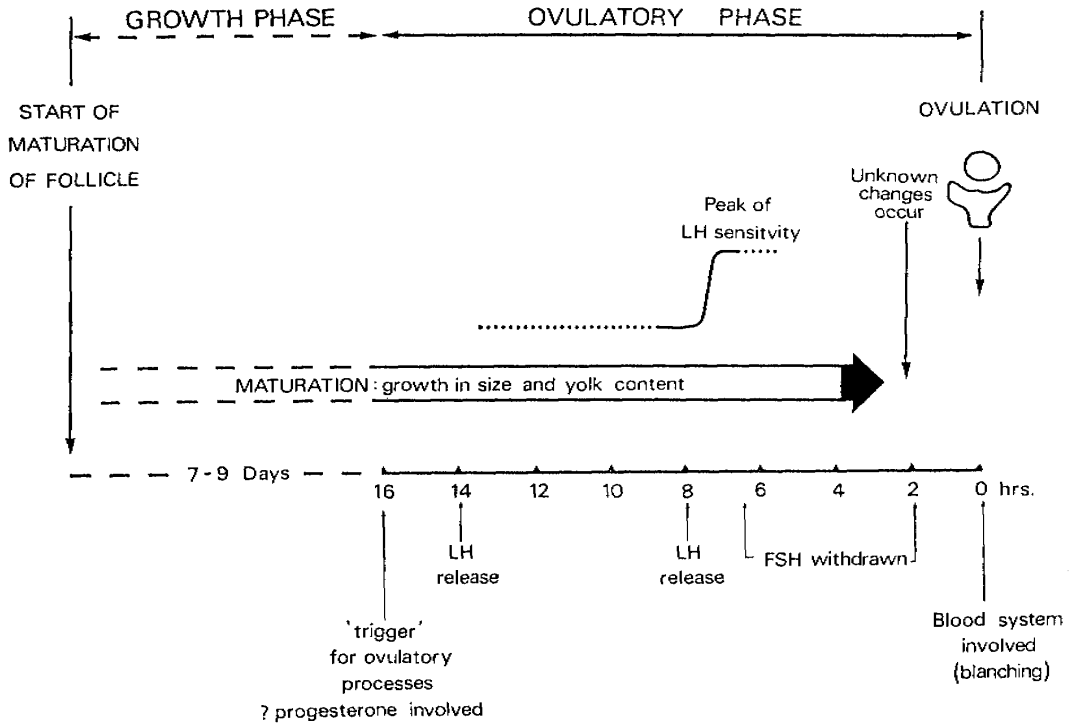


Figure 9: Time schedule of ovulation (Gilbert 1971a)

LH = Luteinizing Hormone; FSH = Follicle Stimulating Hormone

A more detailed description of the total physiology of egg formation and laying can be found in various textbooks on poultry physiology e.g. Bell & Freeman (1971)

1.2.2 *Egg white*

The (water-soluble) proteins are formed in and secreted by one part of the oviduct called the magnum. Formation of the proteins takes 1-2 days and deposition of egg white around the yolk at some 2-3 hours after ovulation (Figure 3)

1.2.3 *Shell membrane and shell*

Shell membranes are formed at some 3-4 hours after ovulation (Figure 4) and finally addition of water and salts ("plumping") (Figures 2 and 3) and the deposition of the (calciumcarbonate) eggshell (Figures 1 and 4) takes place in about 18-20 hours (Bell & Freeman, 1971).

The time schedule as outlined in Figure 4 has been deducted many years ago when egg production was much lower than presently achieved by high productive

hens. Therefore, it is not certain that at present this time schedule is still totally accurate.

1.3 Residues in egg white and yolk

Due to the physiological processes described above, the pharmacokinetics of drug residues in yolk and egg white show the following features:

1. Residues of drugs appear first in egg white, at least when the drug is distributed towards that compartment (Donoghue et al., 2000).
2. Residues in egg white are a reflection of drug plasma levels and will therefore show a constant level over time when plasma levels do. The time needed to achieve a constant drug level in egg white is generally 2-3 days.
3. Residues in yolk reflect the plasma drug levels during the ten days of their rapid growth. Thus depending on the length and time of exposure to the drug relative to yolk growth, drug levels in yolk – when measured during a number of consecutive days -can increase, be constant or decrease.
4. Residues of drugs in yolk generally require exposure for about 8 - 10 days to reach a constant level.
5. A single exposure towards a drug might be sufficient to detect the drug in either egg white or yolk (Donoghue et al., 1998), depending on the characteristics of the drug and the sensitivity of the analytical method used.
6. Disappearance of drugs from white and yolk depends heavily on the plasma levels of the drug tested. Drugs that clear rapidly from the body also disappear from egg white in about 2-3 days after cessation of exposure. Disappearance of drugs from yolk generally takes about 10 days. By that time, drug-containing lipoproteins deposited during the rapid growth phase of the yolk have been excreted with the eggs.
7. However, if the exposure level is very high and the detection limit for the drug involved is very low, then residues deposited in the yolks, which are in the intermediate stage of growth, will also be detectable. This can explain the observation, that Arnold & Somogyi (1986) found chloramphenicol residues in eggs until 70 days after administration.

8. On the other hand, if the limit of detection of the method is similar to the drug residue levels in the egg, residues may not be detected at all or only during a very short time period.

   Microbiological methods, which measure the unbound fraction of antimicrobial drugs only, may for that reason indicate lower residue levels than methods – such as those using HPLC - which measure also the amount absorbed to protein as the organic solvent used to extract the drug, generally breaks up this type of binding.

Both Anhalt (1977) and Hafez (1991) consider the egg yolk to be the main compartment of eggs to be taken into account when considering drug residues. This in contrast to the observations of Blom (1975) – quoted by both authors – who reported much higher residues of some sulfonamides in egg white than in egg yolk. Recently Donoghue et al. (2000) showed that transfer of drugs – or at least oxytetracycline – into egg white also occurs during the plumping phase. This had been anticipated to be found, as we observed several times, that drugs administered to the hens in the afternoon led to residues in the eggs laid the next day. This could only occur if transfer of the drug into the white during the plumping phase happened. In addition Furusawa (1999) detected the presence of spiramycine, oxytetracycline and sulfamonomethoxine in both the isthmus and magnum part of the oviduct.

1.4 Available data on drug levels in egg white and yolk

The data found in the literature from experiments where exposure was sufficiently long to expect a constant residue level in egg white and yolk and from own studies that have not been published are summarised in Table 1. They sometimes are (educated) guesses from either graphs or tables made on steady state levels and may deviate from the data in the original papers.

1.5 General observations on the distribution data

Sulfonamides show appreciable levels in both egg white and yolk and levels in egg white are at least equal to those in yolk, but often they are (much) higher.

Tetracyclines as a group show a more divergent picture. Remarkably some very lipophilic ones (doxycycline and minocycline) show higher levels in egg white than in (the fat rich) yolk. On the contrary the more water soluble oxytetracycline and chlortetracycline show similar levels in white and yolk.
The quinolones flumequine, oxolinic acid and enrofloxacine also show much higher residues in egg white than in yolk.
Many other substances such as macrolides and nitrofurans show diverging patterns of distribution, but in all instances, levels in egg white are substantial.
Some compounds like trimethoprim, pyrimethamine, amprolium, nicarbazine, decoquinate, dinitolmide, and ivermectin occur almost exclusively in yolk and show very low levels in egg white.

1.6 Possible explanations

The physicochemical properties of drugs largely determine their pharmacokinetics. Martinez (1998) in a review mentions the following factors influencing drug kinetics in animals:

- molecular weight: too bulky molecules not being able to cross membranes,
- lipid solubility as measured by the octanol/water partition coefficient
- pKa value, which determines whether a molecule is ionised at a certain pH, as according to some theories only unionised compounds would penetrate biological membranes.
- protein binding to plasma proteins as it determines availability to other compartments,

Hafez (1991) in his review makes a distinction between drug factors, bird factors and analytical method factors. Drug factors deal with metabolism that includes absorption, distribution, biotransformation and excretion. The bird factor focuses on the yolk as the drug-containing compartment of eggs and pays little attention to residues in egg white. Regarding analytical factors he emphasises variation in sensitivity within substances between methods and between substances within methods. The possibility of false positive results, both in microbiological and chemical methods, due to natural substances or contamination after sampling is also mentioned.

Anhalt (1977) largely concentrates on yolk deposition processes in relation to drug residues in eggs and treats the possibility of residues in egg white as a minor issue.

1.6.1 *Lipid solubility*

Lipid solubility of the drug certainly influences its deposition in the (fat rich) yolk (Blom, 1975) but higher residues of (the lipophilic) doxycycline in egg white than in yolk (Table 1) can not be explained in this way. Nevertheless Furusawa (1999) states " The drugs having the property of lipid-solubility are found in much higher levels in egg yolk than in albumen, whereas those having water-solubility are found in higher concentrations in albumen than in egg yolk". However, he also admits (Furusawa personal communication, 2000) that it can not be the only determining factor.

Gorla et al. (1997) consider the possibility that differences in lipid solubility due to a different chemical structure may alter intracellular penetration and thus explain the patterns of distribution they observed for enrofloxacine end ciprofloxacine between yolk and egg white. As ciprofloxacine is a metabolite of enrofloxacine (and thus generally better water-soluble) their observation that ciprofloxacine is predominantly present in yolk, does not concur with this explanation.

1.6.2 *Partitioning between phases with different pH*

The pH of yolk is around 6.0 when the egg is just laid and that of egg white at that time is 7.6. Distribution of drugs between compartments with different pH values according to their pKa values has been established for a number of different combinations of compartments. Distribution of drugs between plasma and gastric juice was e.g. explained by Shore et al. (1957), distribution between plasma and cerebrospinal fluid by Rall et al. (1959) and intestinal absorption of drugs by Hogben et al. (1959). Schanker et al. (1964) established it for passage of drugs into red cells and Atkinson and Begg (1990) predicted in this way the distribution of drugs between plasma and milk. Blom (1975) compared distribution of three sulfonamides between plasma and egg white with the pKa values of those drugs and could not draw an unambiguous conclusion whether or not this hypothesis should be accepted.

1.6.3 *Protein binding*

Roudaut (1998) considered protein binding to be a possible explanation for the observed distribution of oxolinic acid between egg white and yolk. Gorla et al.(1997) considered the same possibility for the related compounds enrofloxacine and ciprofloxacine.

Blom (1975) measured protein binding of three sulfonamides and pyrimethamine in plasma and egg white both *in vitro* and *in vivo*. Sulfadimidine had the lowest binding percentage (some 10 %) and sulfaquinoxaline the highest (some 50 % in egg white). The ratio of residues in egg white and yolk did however not differ in the same way in that study (Table 1). Furusawa (Personal communication, 2000) considers protein binding and especially the rate of binding and the amount of binding material of major importance for distribution of a drug within body or egg.

As to binding of drugs to yolk or to yolk macromolecules, Blom (1975) testing sulfonamides and Kan and Rump (unpublished observations on tetracyclines) were not able to find a satisfactory methodology.

1.6.4 *Other*

Riberzani et al. (1993) suggested that the much higher levels of flumequine in egg white than in yolk might be caused by the high solubility of the “acid” drug flumequine in the basic matrix egg white. Roudaut (1998) studying the related compound oxolinic acid also considered this a possibility to explain higher levels of both oxolinic acid and sulfadimidine in egg white than in yolk.

Diffusion from yolk to white during storage as suggested by Geertsma et al. (1987), was ruled out by Roudaut (1998) as eggs where separated immediately after laying. We also compared (Kan and Rump, unpublished observations) the distribution of doxycycline in eggs in experiments with direct separation of egg white and yolk to experiments with prolonged storage of whole eggs and found no differences in distribution patterns. Exchange of drugs between yolk and egg white during egg formation – especially during the 18 hours of shell deposition at a body temperature of 41 ^{0}C – can however not be ruled out. In line with this option, Gorla et al. (1997) consider diffusion a possibility to explain the observed distribution of enrofloxacine between yolk and egg white

Botsoglou et al. (1989) ascribe the different distribution of furazolidone between yolk and egg white to the dissimilarity in their mode of formation in respect to the time frame: this time of formation being much longer for yolk than for egg white. This explanation might be true for drugs like furazolidone showing higher levels in yolk, but it can not explain how levels of certain drugs in egg white can be higher than their levels in yolk.

1.6.5 *Other remarkable points*

Roudaut and Boisseau (1990) could detect residues of oxolinic acid in egg white much longer than in plasma and somewhat longer than in yolk. This is not compatible with the theory mentioned above (Paragraph 1.3) that the levels in egg white are a reflection of levels in plasma. Also the general rule that residues are detectable longer in yolk than in white is not followed here. Van Leeuwen and van Gend (1989) observed that levels of the related compound flumequine also persisted longer in egg white than in yolk. The results of these two studies suggest that quinolones might have special characteristics in combination with egg white (proteins).

Furusawa (1999) observed that levels of spiramycine are quite high in the oviduct and suggests that this “storage” might be the reason for the longer presence of spiramycine in egg white than in yolk (Yoshida et al., 1971; Roudaut and Moretain, 1990). He also found that levels of spiramycine, oxytetracycline and sulfamonomethoxine in albumen and oviduct tissue were higher than those in blood.

The difference in distribution ratios between the ionophore type coccidiostats narasin and salinomycine is also striking. These two compounds differ only by one - uncharged - methyl group in a rather large and complicated molecule but evidently this difference is sufficient to result in a substantial difference in distribution (Table 1) between egg white and yolk.

1.7 Conclusions

Drug residues will appear in both egg white and yolk after intentional or unintentional administration to laying hens. Bacitracine (Furusawa 2001) might be an exception to this rule. Intestinal absorption of the drug is a pre-requisite for residue formation, as transport via blood (plasma) is responsible for deposition of drugs in follicles on the ovary or in egg white in the oviduct. Physicochemical properties of the drugs and the physiology of the hen and physiology of egg formation will determine how much drug will be deposited and at what place. At present we cannot explain or predict from variables measurable *in vitro* what will happen *in vivo.*

1.8 Table 1: Literature data on drug residues in white and yolk

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
Sulfonamides						
Sulfanilamide	10.5	35	43	0.8	1000 mg/l in water 8 days	Blom (1975)
Sulfadiazine	6.5	0.22	0.14	1.6	200 mg/l in water 5 days	Atta et al. (2001)
		0.32	0.18	1.8	400 mg/l in water 5 days	Atta et al. (2001)
		0.015	< 0.008	> 1.8	1.3 mg/kg in feed 21 days	Tomassen et al. (1996b)
		0.04	< 0.008	> 5	3.8 mg/kg in feed 21 days	Tomassen et al. (1996b)
		0.10	0.022	4.5	8.1 mg/kg in feed 21 days	Tomassen et al. (1996b)
Sulfadimethoxine	6.3	4.6	1.7	2.7	400 mg/kg feed 5 days	Furusawa et al. (1994)
		8.2	1.6	5	500 mg/kg feed 14 days	Furusawa (2001)
		3.0	1.5	2	0.02 % in rofenaid feed C 14, 14 days	Laurencot et al. (1972)
		0.15	0.05	3	25 mg/kg in feed 21 days	Nagata et al. (1989)
		0.25	0.1	2.5	50 mg/kg in feed 21 days	Nagata et al. (1989)
		0.5	0.2	2.5	100 mg/kg in feed 21 days	Nagata et al. (1989)
		0.04	0.02	2	10 mg/kg in feed 14 days	Nagata et al. (1992)
		0.04	0.01	4	10 mg/kg in feed 14 days	Nagata et al. (1992)
		35	12	2.9	2000 mg/kg in feed 25 days	Onodera et al. (1970)
		35	9	3.9	500 mg/l in water 5 days	Roudaut (1993)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
Sulfadimidine	7.5	56	45	1.2	1000 mg/l in water 8 days	Blom (1975)
		97	83	1.2	2000 mg/l in water 8 days	Blom (1975)
		20	5	4	5 x 100 mg/kg BW in 5 days	Geertsma et al. (1987)
		19	19	10	500 mg/kg feed 14 days	Furusawa (2001)
		70	30	2.3	1000 mg/l in water 5 days	Krieg (1966)
		51	34	1.5	1000 mg/l in water 5 days	Roudaut (1993)
		50	34	1.5	1000 mg/l in water 5 days	Roudaut and Garnier (2002)
		70	45	1.6	2000 mg/l in water 5 days	Roudaut and Garnier (2002)
		38	23	1.7	1500 mg/kg in feed 5 days	Seib (1991)
Sulfamerazine	7.0	23	6	3.8	2000 mg/kg in feed 25 days	Onodera et al. (1970)
Sulfamethoxazole		20	1.8	11	2000 mg/kg in feed 5 days	Oikawa et al. (1977)
		39	2.9	14	4000 mg/kg in feed 5 days	Oikawa et al. (1977)
Sulfamonomethoxine	6.0	6.8	1.5	4.5	400 mg/kg in feed 5 days	Furusawa and Mukai (1995)
		5.2	1.1	4.9	400 mg/kg in feed 7 days	Furusawa (1999)
		10.5	2.8	3.8	500 mg/kg feed 14 days	Furusawa (2001)
		0.20	0.04	5	25 mg/kg in feed 21 days	Nagata et al. (1989)
		0.35	0.07	5	50 mg/kg in feed 21 days	Nagata et al. (1989)
		1.0	0.2	5	100 mg/kg in feed 21 days	Nagata et al. (1989)
		21	5	4	2000 mg/kg in feed 25 days	Onodera et al. (1970)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
Sulfaquinoxaline	5.5	50	36	1.4	400 mg/l in water 8 days	Blom (1975)
		2.3	2.0	1.2	200 mg/kg in feed 7 days	Furusawa et al. (1998)
		0.95	1.6	0.6	60 mg/kg in feed 14 days	Nose et al. (1982)
		3.7	1.4	2.6	100 mg/kg in feed 7 days	Petz (1993)
		80	20	4	350 mg/l water 3 days	Rana et al. (1993)
		3.4	2.9	1.2	500 mg/kg in feed 12 days (intermittent)	Righter et al. (1970)
		8	2	4	400 mg/l water 3 days	Romvary and Simon (1992)
		8.3	2	4.2	6000 mg/l in water	Sakano et al. (1981)
Folic acid antagonists						
Ormetoprim		0.44	5.5	0.08	500 mg/kg feed 14 days	Furusawa (2001)
		0.25	3.5	0.07	0.02 % in feed C14, 14 days	Laurencot et al. (1972)
Trimethoprim	6.6	0.24	0.43	0.6	200 mg/l in water 5 days	Atta et al. (2001)
		0.43	0.8	0.5	400 mg/l in water 5 days	Atta et al. (2001
		<0.02	0.05	< 0.4	4 mg/kg in feed 19 days	Nagata et al. (1991)
		0.02	0.25	0.08	16 mg/kg in feed 19 days	Nagata et al. (1991)
		0.07	0.9	0.08	56 mg/kg in feed 19 days	Nagata et al. (1991)
Pyrimethamine	7.0	2	88	0.02	100 mg/l in water 8 days	Blom (1975)
		<0.01	0.03	< 0.3	0.1 mg/kg in feed 21 days	Nagata et al. (1990)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
		0.04	0.7	0.06	1 mg/kg in feed 21 days	Nagata et al. (1990)
		0.13	3.3	0.04	10 mg/kg in feed 21 days	Nagata et al. (1990)
		<0.02	0.25	< 0.08	1 mg/kg in feed 14 days	Nagata et al. (1992)
Coccidiostats						
Amprolium		0.4	5	0.08	500 mg/kg feed 14 days	Furusawa (2001)
		0.006	0.2	0.03	5 mg/kg in feed 10 days	Kan et al. (1989)
		0.05	1.7	0.03	250 mg/kg in feed 10 days	Kan et al. (1989)
		0.05	0.6	0.08	100 mg/kg in feed 14 days	Nose et al. (1982)
Decoquinate		0.02	0.6	0.03	40 mg/kg in feed 14 days	Nose et al. (1982)
Dinitolmide		0.15	1.6	0.09	125 mg/kg in feed 14 days	Nose et al. (1982)
Meticlorpindol		5	2.5	2	110 mg/kg in feed 10 days	Mattern et al. (1990)
Nicarbazine		0.3	13	0.02	500 mg/kg feed 14 days	Furusawa (2001)
Other feed additives						
Dimetridazole		0.002	0.002	1	0.95 mg/kg in feed 21 days	Kan et al. (1995)
		0.01	0.01	1	4.7 mg/kg in feed 21 days	Kan et al. (1995)
		0.25	0.30	0.8	3 x 50 mg/kg BW 3 days	Posyniak et al. (1996)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
		1.2	1.4	0.9	3 x 250 mg/kg BW 3 days	Posyniak et al. (1996)
Olaquindox		0.002	<0.001	> 2	0.39 mg/kg in feed 21 days	Keukens et al. (1996)
		0.007	0.003	2.3	1.7 mg/kg in feed 21 days	Keukens et al. (1996)
		0.02	0.008	2.5	5.4 mg/kg in feed 21 days	Keukens et al. (1996)
Tetracyclines						
Chlortetracycline	3.4; 7.4; 9.3	0.1	0.4	0.25	600 mg/kg in feed 5 days	Roudaut et al. (1989)
		0.25	0.25	1	8000 mg/kg in feed 7 days	Yoshida et al. (1973a)
Doxycycline	3.5; 7.7; 9,5	0.015	<0.01	> 1.5	1.1 mg/kg in feed 21 days	Tomassen et al. (1996a)
		0.08	0.04	2	6.7 mg/kg in feed 21 days	Tomassen et al. (1996a)
		0.15	0.07	2	11.5 mg/kg in feed 21 days	Tomassen et al. (1996a)
		11	3.5	3	0.5 g/l water 7 days	Yoshimura et al. (1991)
Minocycline	2.8; 5.0; 7.8; 9.5	0.7	0.1	7	90 mg/l water 4 days	Kan and Rump unpublished results (1989)
Oxytetracycline	3.3; 7.3; 9.1	0.10	0.06	1.6	400 mg/kg in feed 7 days	Furusawa (1999)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
		0.21	0.25	0.8	500 mg/kg feed 14 days	Furusawa (2001)
		1.9	2.9	0.7	2 g/l water 7 days	Nagy et al. (1997)
		<0.05	0.2	< 0.2	400 mg/l 7 days	Omija et al. (1994)
		0.05	0.5	0.1	600 mg/l 7 days	Omija et al. (1994)
		0.25	0.6	0.4	800 mg/l 7 days	Omija et al. (1994)
		0.13	0.3	0.4	0.25 g/l water 5 days	Roudaut et al. (1987a)
		0.15	0.3	0.5	0.5 g /l water 5 days	Roudaut et al. (1987a)
		0.08	<0.20	> 0.4	300 mg/kg in feed 7 days	Roudaut et al. (1987a)
		0.17	0.5	0.3	600 mg/kg in feed 7 days	Roudaut et al. (1987a)
		0.6	0.5	1.2	2000 mg/kg in feed 7 days	Yoshida et al. (1973b)
		0.8	1.1	0.7	4000 mg/kg in feed 7 days	Yoshida et al. (1973b)
		0.7	1.2	0.6	0.5 g/l water 5 days	Yoshimura et al. (1991)
Tetracycline	8.3; 10.2	0.11	0.5	0.2	0.25 g/l water 5 days	Roudaut et al. (1989)
		0.2	0.9	0.2	0.5 g/l water 5 days	Roudaut et al. (1989)
		0.17	0.9	0.2	300 mg/kg in feed 7 days	Roudaut et al. (1989)
		0.3	1.5	0.2	600 mg/kg in feed 7 days	Roudaut et al. (1989)
Nitrofurans						
Furazolidone		0.05	0.07	0.7	100 mg/kg in feed 28 days	Botsoglou et al. ((1989)
		0.1	0.15	0.7	200 mg/kg in feed 14 days	Botsoglou et al. ((1989)
		0.2	0.25	0.8	400 mg/kg in feed 14 days	Botsoglou et al. ((1989)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
		0.22	0.37	0.6	500 mg/kg feed 14 days	Furusawa (2001)
		0.015	0.010	1.5	400 mg/kg in feed 7 days	Krieg (1972)
		0.48	0.17	2.8	400 mg/kg in feed 8 days	McCracken et al. (2001)
		0.4	0.5	0.8	400 mg/kg in feed 14 days	Petz (1984)
Furaltadon		0.1	0.2	0.5	100 mg/kg in feed 7 days	Petz (1993)
Nitrofurazone	9.28	0.25	0.4	0.6	100 mg/kg in feed 7 days	Petz (1993)
Nitrofurantoine	7.2	0.1	<0.001	> 100	100 mg/kg in feed 7 days	Petz (1993)
Quinolones						
Flumequine	6.25	2.1	0.3	7	90 mg/l water 10 days	van Leeuwen and van Gend (1989)
		9	1.7	5.3	200 mg/l water 5 days	Riberzani et al. (1993)
		2	0.3	6.7	5 x 12 mg/kg BW 5 days oral	Samaha et al. (1991)
Oxolinic acid	6.3	1.5	0.2	7.5	0.15 (0.5??) g/l water 5 days	Roudaut and Boisseau (1990)
		11.5	1.2	9.6	300 mg/kg in feed 5 days	Roudaut (1998)
Enrofloxacine	6.2	1.1	0.3	3.7	5 mg/kg/day in water 5 days	Gorla et al. (1997)
Ciprofloxacine	6.3	< 0.15	0.18	< 0.8	5 mg/kg/day in water 5 days	Gorla et al. (1997)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
Macrolides						
Erythromycine	8.7	0.04 IU/g	0.12 IU/g	0.3	0.22 g/l water 5 days	Roudaut and Moretain (1990)
		0.1 IU/g	0.3 IU/g	0.3	0.5 g/l water 5 days	Roudaut and Moretain (1990)
		0.03 IU/g	0.12 IU/g	0.3	400 mg/kg in feed 7 days	Roudaut and Moretain (1990)
		0.47	1.54	0.3	0.5 g/l water 7 days	Yoshimura et al. (1978)
Kitasamycine	6.7	0.35	0.27	1.3	0.5 g/l water 7 days	Yoshimura et al. (1978)
Oleandomycine		4.6	11.4	0.4	0.5 g/l water 7 days	Yoshimura et al. (1978)
Spiramycine	8.0	0.40	0.32	1.3	400 mg/kg in feed 7 days	Furusawa (1999)
		2.1 IU/g	4.5 IU/g	0.5	0.4 g/l water 5 days	Roudaut and Moretain (1990)
		0.9 IU/g	1.3 IU/g	0.7	400 mg/kg in feed 7 days	Roudaut and Moretain (1990)
		3	2.2	1.4	1000 mg/kg in feed 7 days	Yoshida et al. (1971)
		2.7	4.2	0.6	0.5 g/l water 7 days	Yoshimura et al. (1978)
Tylosin	7.1	0.05	0.05	1	500 mg/kg feed 14 days	Furusawa (2001)
		0. 25 IU/g	0.6 IU/g	0.4	1 g/l water 5 days	Roudaut and Moretain (1990)
		5	5	1	8000 mg/kg in feed 7 days	Yoshida et al. (1973c)
		1	1	1	0.5 g/l water 7 days	Yoshimura et al. (1978)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
Ionophores						
Monensin		0.1	0.08	1.3	110 mg/kg in feed 7 days	Keukens, Aerts and Kan unpublished results (1987)
Narasin		0.25	0.8	0.3	70 mg/kg in feed 7 days	Keukens, Aerts and Kan unpublished results (1987)
Salinomycine		< 0.01	1.4	< 0.007	30 mg/kg in feed 14 days	Akhtar et al. (1996)
		0.08	2	0.04	60 mg/kg in feed 14 days	Akhtar et al. (1996)
		0.1	2.8	0.04	90 mg/kg in feed 14 days	Akhtar et al. (1996)
		0.2	3.7	0.05	150 mg/kg in feed 14 days	Akhtar et al. (1996)
		0.05	1.5	0.03	60 mg/kg in feed 7 days	Keukens, Aerts and Kan unpublished results (1987)
		<0.01	0.22	< 0.05	66 mg/kg in feed days	Sambeth et al. (1985)
		<0.01	0.4	< 0.02	60 mg/kg in feed 5 days	Sinigoj-Gacnik (1996)
Anthelmintics						
Flubendazole		<0.02	0.04	< 0.5	2.6 mg/kg in feed 21 days	Kan et al. (1998)
		0.02	0.1	0.2	9.4 mg/kg in feed 21 days	Kan et al. (1998)
		0.03	0.3	0.1	27.0 mg/kg in feed 21 days	Kan et al. (1998)
Ivermectine		<0.0005	0.001	< 0.5	0.11 mg/kg in feed 21 days	Van Dijk et al. (1997)

Compound name	pKa value	Content in white in mg /kg	Content in yolk in mg/kg	Ratio white/yolk	Exposure way	Author
		<0.0005	0.005	< 0.1	0.36 mg/kg in feed 21 days	Van Dijk et al. (1997)
		<0.0005	0.02	< 0.02	0.76 mg/kg in feed 21 days	Van Dijk et al. (1997)
Various						
Ampicilline	2.5; 7.3	0.008	0.025	0.3	1.5 g/l water 5 days	Roudaut et al. (1987b)
Chloramphenicol	5.5	2	10	0.2	400 mg/l water 10 days	Arnold and Somogyi (1986)
		0.35	2	0.17	500 mg/kg feed 14 days	Furusawa (2001)
		0.5	1.8	0.3	400 mg/kg in feed 14 days	Petz (1984)
		0.05	0.2	0.3	200 mg/kg in feed 5 days	Samouris et al. (1998)
		0.5	1.5	0.3	500 mg/kg in feed 5 days	Samouris et al. (1998)
		0.5	2.5	0.2	800 mg/kg in feed 5 days	Samouris et al. (1998)
		1.2	4	0.3	1000 mg/kg in feed 5 days	Samouris et al. (1998)
		0.15	0.2	0.8	40 mg/l water 5 days	Sisodia and Dunlop (1972)
Kanamycine	7.2	< 0.5	1.5	< 0.3	4.000 mg/kg feed 7 days	Yoshida et al. (1976)
		<0.5	2.2	< 0.2	8.000 mg/kg feed 7 days	Yoshida et al. (1976)
		<0.5	4	< 0.1	16.000 mg/kg feed 7 days	Yoshida et al. (1976)

2 EXPERIMENTAL PART

2.1 Introduction

The available data from the literature - with the exception of the work of Blom (1975) – lack a comparative approach, that should help to elucidate what physicochemical factors determine the partitioning of drugs between egg white and yolk.
The following factors were considered of possible importance:

- Lipid solubility
- Distribution between the aqueous and organic phase (log p value)
- pKa value
- Protein binding

Therefore we ran a number of tests with a group of sulfonamides, as a possible relationship between physicochemical characteristics and distribution within the egg was considered more likely to be found within a group of related compounds. A further advantage of choosing sulfonamides as model substances was their relative stability in different matrices and easiness to carry out residue determinations. The different sulfonamides and their structural formulas are given in Figure 10.

The following experiments were therefore planned:

1. Determination of log p values of 11 sulfonamides (Figure 10), with pKa values ranging from 4.7 – 11.3, with both dichloromethane and cyclohexane as organic phase against phosphate buffers of pH 6.0 and 7.6 respectively. The pH of yolk is around 6.0 when the egg is just laid and that of egg white at that time is 7.6.
2. Administration of feeds with the 11 different sulfonamides to groups of 3 laying hens during 3 weeks and assessment of the residues in mixed samples per group of both yolk and egg white after about 2 weeks of administration.
3. Administration of feeds with 5 sulfonamides, diverging in physicochemical characteristics, to groups of 5 laying hens during 3 weeks. Assessment of the residues in mixed samples per hen of both yolk and egg white and the amount of protein or macromolecular binding *in vivo* in both matrices.

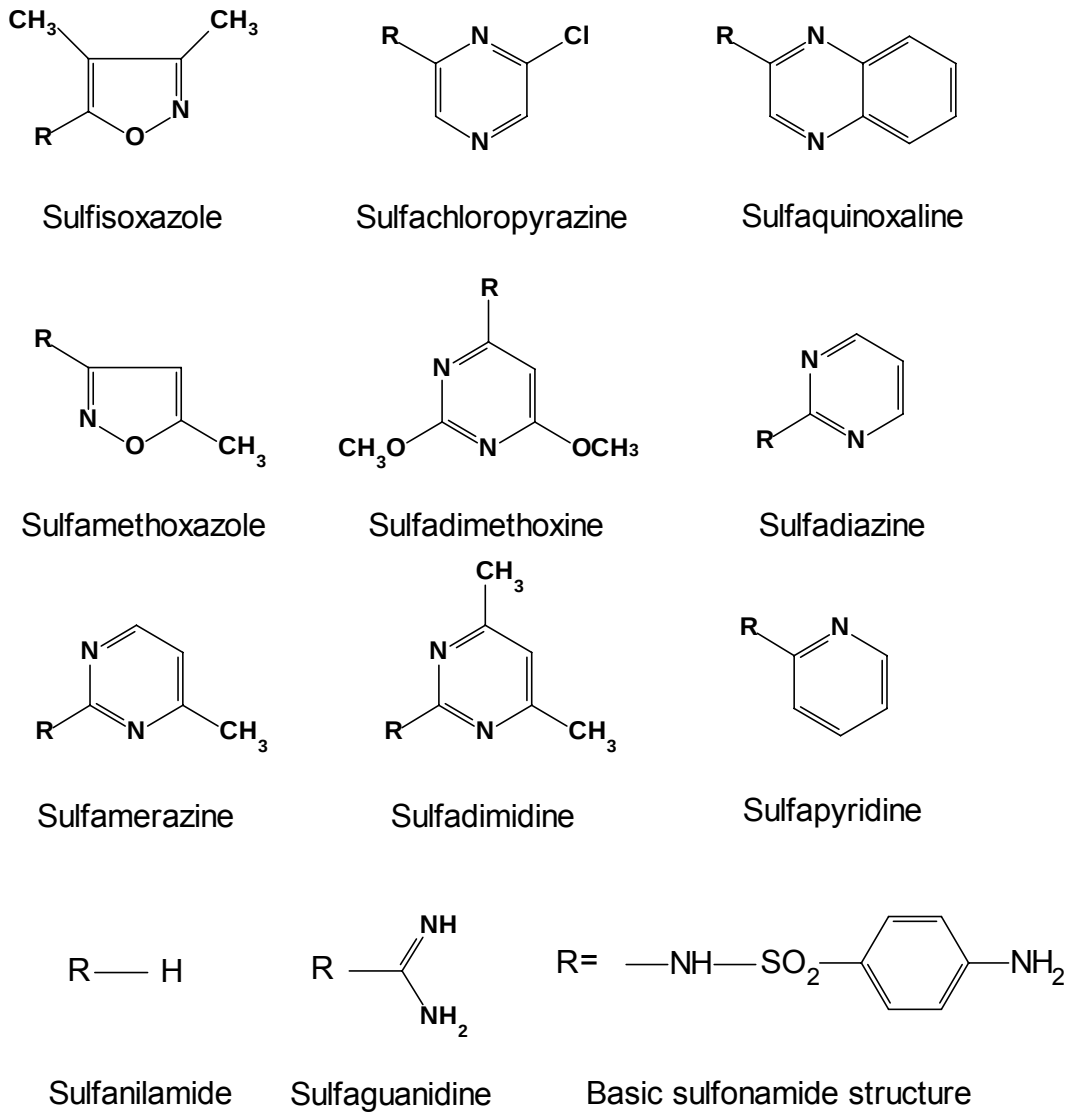


Figure 10: Structural formulas of the sulfonamides examined

2.2 Materials and methods

2.2.1 *Determination of partitioning coefficients*

The OECD guideline 107 was used as starting point for the determination of the partitioning coefficients. They lead to the following requirements for the testing:

1. Testing of one substance (as pure as possible) at the time with a not too high concentration.
2. The substance must be water soluble and stable in aqueous solutions; the substances must not associate or dissociate or behave as a surfactant.
3. Three different concentrations of the substance or of the aqueous/organic phase should be tested in duplicate and the equilibrium should be reached at one constant temperature and pressure.

The partitioning coefficients were determined at both pH 6.0 (to mimic yolk pH) and 7.6 (to mimic egg white pH) of the aqueous phase and dichloromethane and cyclohexane as the organic phase. Octanol, which is often used in these kinds of measurements, was avoided, as the formation of hydrogen bonds between solvent and sulfonamides, which could lead to aberrant values, was feared.
The sulfonamide buffer solutions should have had an strength of 0.3-0.4 mM, but this could not always be achieved due to the limited solubility of the sulfonamides in aqueous media. The solutions were prepared in a phosphate buffer saturated with dichloromethane.

The partitioning between the aqueous and the organic phase was carried out in 25 ml glass tubes with a screwing cap lined with teflon®. The tubes were rotated at approximately 20 rpm during some 30 minutes. The tubes were then centrifuged at approximately 1000 g for 15 minutes and afterwards left for equilibration for about one hour. All processes took place at room temperature being about 23 ^{0}C.
To achieve three different concentrations of the substance as prescribed by the above mentioned OECD guideline, amounts of 18, 12 and 6 ml aqueous buffer were mixed with 6, 12 and 18 ml of the organic phase. The sparely water-soluble sulfonamides sulfachloropyrazine, sulfaquinoxaline and sulfadimethoxine were mixed in the ratios 21, 22 and 23 ml aqueous buffer and 3, 2 and 1 ml organic phase.

2.3 Analysis of sulfonamides

The HPLC analysis of sulfonamide concentrations in the different samples was carried out on a C 18 column with an ammonium acetate buffer/acetonitrile mixture as eluent and UV/fluorescence detection as outlined below.

2.3.1 *Sulfonamide standards*

Sulfisoxazole; Sigma nr. S 6377
Sulfachloropyrazine-sodium.1 aq; Ciba-Geigy prod.standard. 90
Sulfaquinoxaline-sodium; Sigma nr. S 7382
Sulfamethoxazole; Sigma nr. S 7507
Sulfadimethoxine; Sigma nr. S 7007
Sulfadiazine; Sigma nr. S 8626
Sulfamerazine; Sigma nr. S 8867
Sulfadimidine; Sigma nr. S 6256
Sulfapyridine; Sigma nr. S 6252
Sulfanilamide; Sigma nr. S 9251
Sulfaguanidine; Sigma nr. S 8751

2.3.2 *Chemicals*

Acetonitrile p.a.; Merck nr. 3
Ammoniumacetate p.a.; Merck nr 1116
Acetic acid 99-100% p.a.; Baker nr. 6052
Buffer pH 7.00; Merck nr. 9477
Buffer pH 4.00; Merck nr. 9475
Citric acid monohydrate p.a.; Merck nr. 244
Dichloromethane UVASOL; Merck nr. 6048
Ethylacetate UVASOL; Merck nr.863
Methanol p.a.; Merck nr. 6009
Ortho-phosphoric acid 85 % p.a.; Merck nr. 573
Petroleum-ether boiling point 40-60 ^{o}C p.a.; Merck nr. 1775
di-Potassiumhydrogenphosphate (anhydrous) p.a.; Merck nr 5104
Sodiumdihydrogenphosphate monohydrate p.a.; Merck nr. 6346

di-Sodiumhydrogenphosphate dihydrate p.a.; Merck nr 6580
Sodiumhydroxide pellets p.a.; Merck nr. 6498
Water (milliQ)

2.3.3 *Solutions*

Ammoniumacetate buffer 0.01 M pH 5.3

Dissolve 0.77 g ammoniumacetate in ca. 800 ml water. Adjust to pH 5.3 with approximately 10 drops 100 % acetic acid measured with a pH meter. Adjust volume to1000 ml with water and mix well.

Dichloromethane saturated with sodiumphosphatebuffer 0.10 M pH 6.0 and pH 7.6

Transfer approximately 800 ml dichloromethane into a bottle with screw cap and teflon lining. Add approximately 100 ml sodium phosphate buffer 0.10 M pH 6.0 or pH 7.6 and close the bottle. Mix during 24 hours on a mechanical shaker at room temperature and let the phases separate for 24 hours at 23 ^{0}C. Take the lower layer by pipette. During transfer of the pipette through the upper layer apply some air pressure on it, in order to avoid the upper layer from entering the pipette.

Phosphate buffer 0.05 M pH 10

Dissolve 4.36 g di-potassiumhydrogenphosphate in approximately 400 ml water. Adjust pH to10.0 with approximately 13 drops of 1 M sodiumhydroxide measured with a pH meter. Adjust the volume to 500 ml with water and mix well.

Ortho-phosphoric acid 0.5 M

Add 3.4 ml ortho-phosphoric acid 85 % to approximately 50 ml water. Add water till 100 ml and mix well.

Sodiumcitratebuffer 2 M pH 4.2

Dissolve 21 g citric acid monohydrate with approximately 25 ml water and approximately 10 ml 10 M sodiumhydroxide. Adjust pH to 4.2 with a pH meter by adding approximately 5 ml 10 M sodiumhydroxide; make sure that the solution is at room temperature. Add water till a volume of 50 ml and mix well.

Sodium-di-hydrogenphosphate solution 0.10 M

Take 13.8 g sodium-di-hydrogenphosphate monohydrate. Dissolve and adjust the volume with water till 1000 ml and mix well.

di-Sodiumhydrogenphosphate solution 0.10 M

Take 8.8 g di-sodiumhydrogenphosphate dihydrate. Dissolve and adjust the volume with water till 1000 ml and mix well.

Sodiumphosphatebuffer 0.10 M pH 6.0 and pH 7.6

Mix the sodium-di-hydrogenphosphate solution 0.10 M and the di-sodiumhydrogenphosphate solution 0.10 M till the desired end-pH is obtained, by measuring with a calibrated pH-meter. To obtain pH 6.0 or pH 7.6 respectively the mixing ratios will be approximately 85:15 and 15:85. Mix and measure pH after each addition and add smaller amounts when the pH reaches it desired value.

Sodiumphosphatebuffer 0.10 M pH 6.0 and pH 7.6 saturated with dichloromethane

Transfer approximately 800 ml sodiumphosphatebuffer 0.10 M pH 6.0 or pH 7.6 into a bottle with screw cap lined with teflon. Add approximately 100 ml dichloromethane and close the bottle. Mix during 24 hours on a mechanical shaker at room temperature and let the phases separate for 24 hours at 23 ^{0}C. Use the upper layer.

Sodiumphosphatebuffer approximately 0.01 M and pH 7.6

Dilute sodiumphosphatebuffer 0.10 M pH 7.6 in a ratio1:10 with water.

Sodiumhydroxide 10 M

Dissolve 40 g sodiumhydroxide pellets in 100 ml water.

Sodiumhydroxide 1 M

Dilute10 ml 10 M sodiumhydroxide with water until 100 ml and mix well.

Sulfonamide spike-solutions

Accurately weigh about 2.5 mg sulfonamide in a 100 ml bottle. Dissolve it in sodiumphosphatebuffer ca. 0.01 M and about pH 7.6 . Add water till 100 ml and mix well.

2.3.4 *Apparatus*

A/D Converter 18652A ; Hewlett-Packard
Analytical Systems 3359AX (HP 1000); Hewlett-Packard
Balance (0.01 mg accuracy), AT 250; Mettler
Centrifuge, IECCR-6000; IEC
Desiccator; glass
Dispenser, Multipette; Eppendorf
Evaporator, Reacti-vap&therm ; Pierce
Fluorescence HPLC Monitor RF-530 ; Shimadzu
Guard Column bestelnr.28141, 1 cm length 2 mm ID stainless steel column packed with Reversed Phase R2; Chrompack
Horizontal mechanical mixer; Edmund Bühler
HPLC Column Chromsep ordernr. 28267 2*10 cm length 3 mm ID glass column packed with Chromspher C18, 5 μm; Chrompack
Liquid Chromatograph + LC Terminal, Series 4 ; Perkin-Elmer
Membrane pump Divac 2.4L; Leybolt
pH meter (2 decimals accuracy), SA 720; Orion
Pipettes fixed volume 10 μl-1 ml; Eppendorf
Programmable Multiwavelength Detector Type 490 ; Waters
Recorder BD 111/112 ; Kipp & Zonen
Rotation apparatus for 24 tubes; GFL 3025
Sample Injector Model 230 + 401 Dilutor + 20μl loop; Gilson
Tube shaker, Vortex-Evaporator; Buchler
Ultrasonic bath, T 480/H-2; ELMA
Ultra-turrax T25; IKA
Vial-crimper for 12 mm cap's; Phase Sep

2.3.5 *Usables*

Glass round bottom tubes 15 ml 16*100 mm; Renes
Glass culture tubes with screwing caps and teflon® lining 25 ml, 18*145 mm; Elgebe
Glass flask with screwing cap and teflon® lining
Pasteur pipette (glass) 23 cm long

Polypropylene pointed tube with screwing cap 50 ml nr. 227261; Greiner
Polypropylene round bottom tube
Sep-Pak Silica Plus column nr 20520; Waters
Nitrogen gas, purity 2.8 or better
Vial (glass) 1-2 ml 12*32 mm with crimp-cap; Phase Sep
Disposable syringe 50 ml, Plastipak; Becton Dickinson

2.3.6 *Sample preparation*

Aqueous samples

The dilution of the sample with 1.00 ml eluent is aimed at reaching a concentration of about 2 μg/ml sulfonamide in the vial.

Samples from the organic phase

Transfer sample into polypropylene tube and transfer from here the required amount into a vial. Evaporate under nitrogen and dissolve the residue in 0.250 ml acetonitrile; Add 1.00 ml 0.01 M ammoniumacetate buffer pH 5.2 (1.5 % volume contraction). Sulfaguanidine had such a low partitioning coefficient, that a larger amount of dichloromethane had to be evaporated first.

Yolk/Egg white

Pure yolk or white is homogenised with an ultra-turrax (30 seconds at about 10.000 rpm) and 5.0 ± 0.05 g is weighed into a 50 ml polypropylene pointed tube. Add 0.5 ml of a 2 M sodium citrate buffer pH 4.2 and mix immediately; add then 40 ± 0.5 ml dichloromethane and mix shortly at once. Let the air escape and shake the tube horizontally for 30 minutes at 200 rpm.
Yolk samples, centrifuge for 5 minutes at about 1000 g. White samples, break the gel by shaking.
Penetrate the clean lower surface of the tube with a sharp needle and collect the extract in a 100 ml Erlenmeyer; Yolk samples will yield 38 ± 0.5 ml extract and egg white samples 33 ± 2 ml.
Connect the "barrel" of a 50 ml disposable syringe to a Sep-Pak Silica Plus column (stored in a desiccator) and fill the "barrel" with 10.0 ml petroleum-ether 40-60 first and with 10.0 ml extract thereafter. Suck the fluid at a 10-20 ml/min rate through the column,

rinse with 5 ml 1:1 petroleum-ether 40-60/dichloromethane and dry at least 20 minutes with 100 ml/min nitrogen gas.
If no further concentration is needed (content > 0.25 μg/g): elute with 5.0 ml 0.05 M phosphate buffer pH 10 at a rate of about 10 ml/minute. Weigh the collected amount (approximately 4 ml) together with the glass tube and adjust the pH of the eluate to 5.5-4.0 with 250 μl 0.5 M ortho-phosphoric acid. Transfer an aliquot part to a 11 x 32 mm vial for HPLC analysis.
Concentration can be achieved by collecting the eluate, adjusting pH to 6.0-5.5 with 200 μl 0.5 M ortho-phosphoric acid and shake 5 minutes with 5 ml ethylacetate. Centrifuge 5 minutes at approximately 1000 g, transfer as much ethylacetate as possible to a glass tube and extract the aqueous phase again with 5 ml ethylacetate. Evaporate the combined extracts under nitrogen gas at about 40 ^{o}C. The residue is dissolved in 20 μl acetonitrile and 500 μl 0.01 M ammonium acetate buffer pH 5.3 is added. After an ultrasonic treatment during 10 minutes the solution is transferred for the HPLC analysis into a 11 x 32 mm vial.

2.3.7 *Recovery measurements*

Linearity of the clean-up method has not been checked. Therefore recovery measurements were made at expected concentrations in the samples.
Blank eggs were treated in the same way as residue-containing eggs. After weighing the required amount into a 50 ml polypropylene tube, 20 μl of the spike solution of sulfisoxazole, sulfadiazine, sulfamerazine, sulfadimidine and sulfapyridine was added to the yolk to obtain a spike level of 100 ppb to measure recovery. All other recovery measurements in yolk and those in white were done at 500 ppb after a spike of 100 μl. Then the samples were homogenised with the aid of a Vortex mixer and they were incubated for 1.5 hours at room temperature. Then citrate buffer was added and the clean-up proceeded as described above for yolk and egg white samples.
At the same time duplicate 100 μl portions of the spike solutions were pipetted into the vials and after addition of 1.25 ml eluent these samples were analysed by HPLC to determine the real concentration.

2.3.8 *HPLC-conditions*

Isocratic

Eluent: 78% 0.01 M ammoniumacetate pH 5.2 + 22% acetonitrile

Gradient

Starting eluent: 0.01 M ammoniumacetate buffer pH 5.3 with 2.5 % acetonitrile
Final eluent: 0.01 M ammoniumacetate buffer pH 5.3 with 22 % acetonitrile
Equilibrate during 20 minutes with 100 % starting eluent, then injection, then in 1 minute linear to 100 % final eluent. Elute during 15 minutes with final eluent and finally switch back to starting eluent.

Dwell volume of pump + pulse equaliser: 3.5 ml
Flow: 0.6 ml/min
Pressure: ca. 10 Mpa
Column temperature: ca. 26 °C
Solvent for injection in isocratic run: eluent
Solvent for injection meant for gradient elution: ca 0.05 M phosphate buffer with pH lower or equal to pH (if no concentration was needed) or starting eluent (if concentration was carried out)
Injection: 100 μl sample in 20 μl loop

UV-detector: Channel	1	2	3	4
Mode	A	A	A	A
AUFS	1.000	0.100	1.000	0.100
Wavelength	280 nm	280 nm	254 nm	254 nm
Second List	Yes	Yes	Yes	Yes
Time Const.	1.0	1.0	1.0	1.0
Threshold	0% FS	0% FS	0% FS	0% FS
Auto Zero	Yes	Yes	Yes	Yes
Chart Mark	No	Yes	No	Yes
Auto Range	No	No	No	No
Polarity	+	+	+	+
Chart Zero	0% FS	0% FS	0% FS	0% FS
Connected to	A/D 4	Rec.blue	A/D_1	

Fluorescence detector:	Excitation	275 nm
	Emission	340 nm
	Sensitivity	High
	Range	16
	Connected to	A/D_7 and Recorder red

Recorder: Chart speed 5 mm/min.

Data acquisition and management: Data acquisition lasted 20 minutes. When running the gradient programme it thus extends into the equilibration phase. On applying the gradient conditions a chromatogram with the following peaks and retention times was obtained:

Name	Approximate retention time in minutes
Dead volume (potassium nitrate)	1.1
Sulfaguanidine	3.3
Sulfanilamide	3.9
Sulfadiazine	8.8
Sulfapyridine	9.8
Sulfamerazine	10.1
Sulfisoxazole	10.6
Sulfadimidine	11.1
Sulfachloropyrazine	12.8
Sulfamethoxazole	13.1
Sulfaquinoxaline	17.2
Sulfadimethoxine	18.0

2.3.9 *Ultrafiltration*

Two ml egg white or yolk was transferred into a disposable ultrafiltration cartridge (Ultrafree-CL Polysulfone 30000 NWML, Millipore Cat.No. UFC4TTK25) and centrifuged for 2 hours at 3000 rpm (1000 g) in a Sorvall RC-5B centrifuge with SA-600 rotor. The sample size of the ultrafiltrate for HPLC was 0.3 ml.

2.4 Animal experiments

Trial 1:
Groups of 3 laying hens received during 3 weeks feeds with the following amounts of sulfonamides added (Table 2)

Table 2: Concentrations in the feed

Group	Sulfonamide	mg/kg feed
1:	Sulfisoxazole	100
2:	Sulfachloropyrazine	50
3:	Sulfaquinoxaline	20
4:	Sulfamethoxazole	50
5:	Sulfadimethoxine	100
6:	Sulfadiazine	20
7:	Sulfamerazine	100
8:	Sulfadimidine	20
9:	Sulfapyridine	50
10:	Sulfanilamide	20
11:	Sulfaguanidine	100

The eggs were stored at 4 ^{0}C until analysis and mixed samples per group of both yolk and egg white laid on days 14/15 and 16/17 have been analysed.

Trial 2:
Groups of five hens received during three weeks feed with 50 mg/kg of the following sulfonamides

Group 1: Sulfachloropyrazine
Group 2: Sulfadimethoxine
Group 3: Sulfadiazine
Group 4: Sulfadimidine
Group 5: Sulfaguanidine

The eggs laid were stored at 4 ^{0}C until analysis. A mixed sample per animal of eggs laid from day 15-20 for both yolk and egg white was analysed. Protein binding was assessed by filtration through an ultrafiltration cartridge as described above.

2.5 Technical results

2.5.1 Chemical analysis

2.5.1.1 Chromatographic conditions

The separation of the sulfonamides on the analytical column is influenced by many factors like pH, ionic strength and temperature of the column. Figure 11 illustrates the influence of pH on the separation.

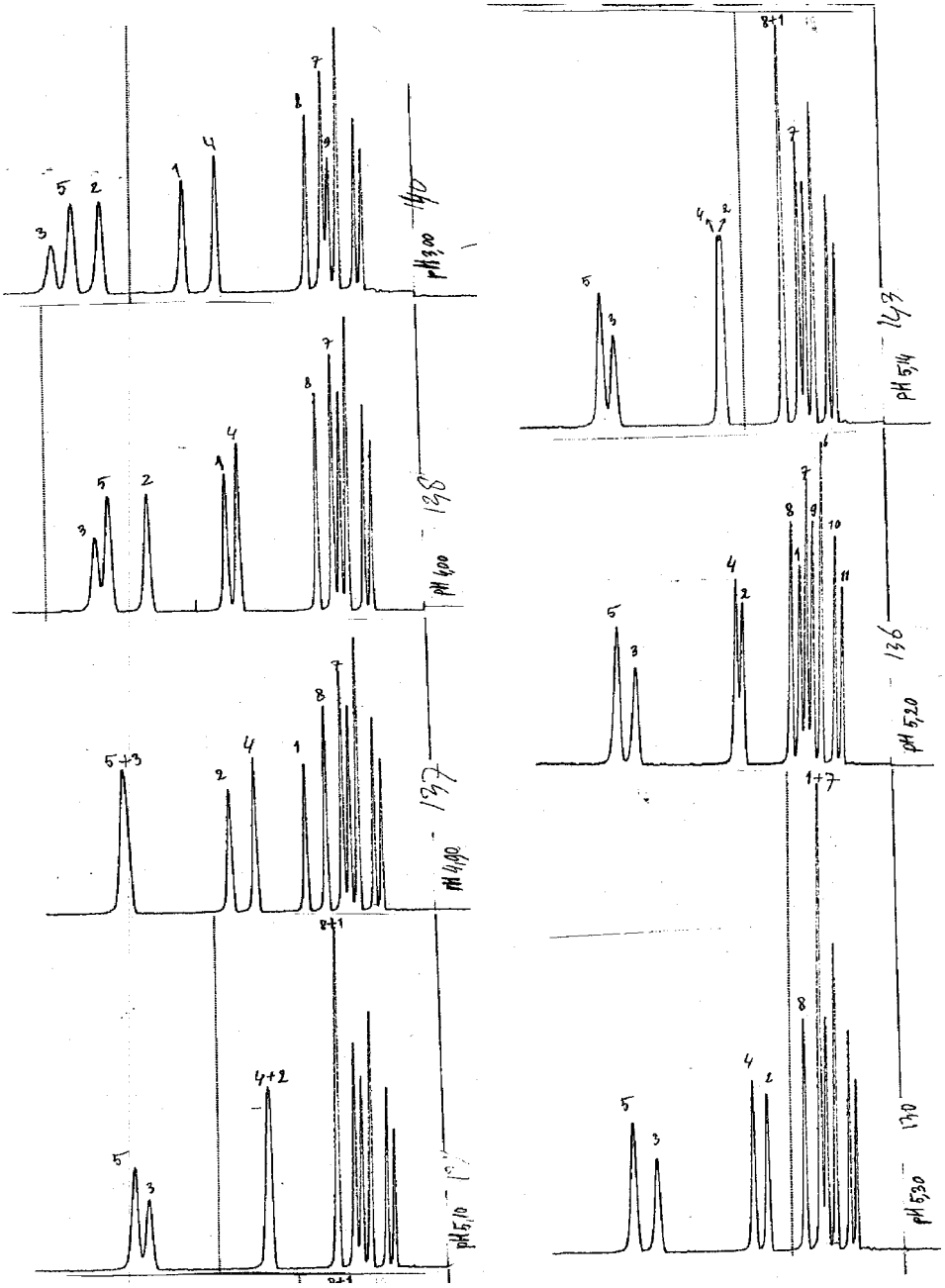


Figure 11: Influence of pH on separation of the sulfonamides (Top to bottom pH values of 3.00, 4.00, 4.90, 5.10, 5.14, 5.20 and 5.30) T = 26 ^{0}C, 0.01 M eluent

It is quite clear from these graphs that the optimal pH for separation of the different sulfonamides lies around pH 5.2-5.3.

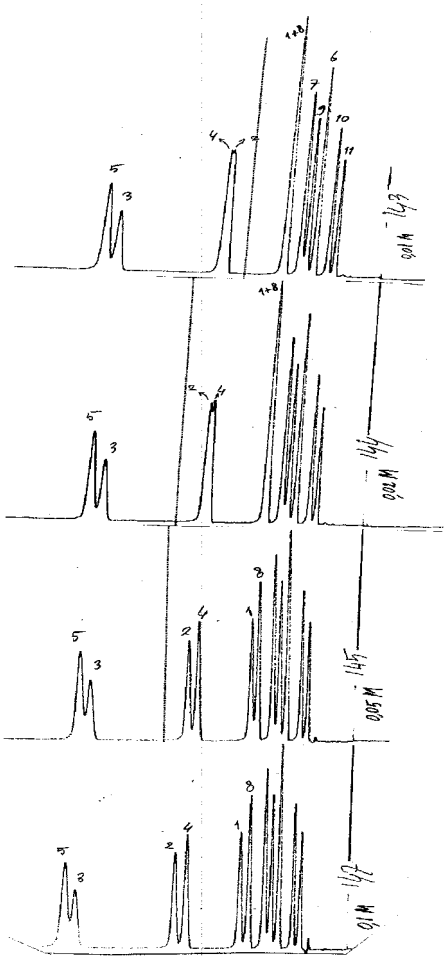


Figure 12: Influence of molarity on separation of the sulfonamides (Top to bottom molarity of 0.01, 0.02, 0.05 and 0.1 M) T = 26 ^{0}C, pH = 5.14

Figure 12 shows the influence of molarity on the separation. Similarly Figure 13 shows the influence of temperature. The different sulfonamides used in the laboratory and animal experiments were always tested one at the time. Therefore a method which always separated all eleven sulfonamides was not an absolute prerequisite. However for the sake of simplicity and avoidance of confusion, we have chosen to use the one set of conditions described above for all determinations in the study.

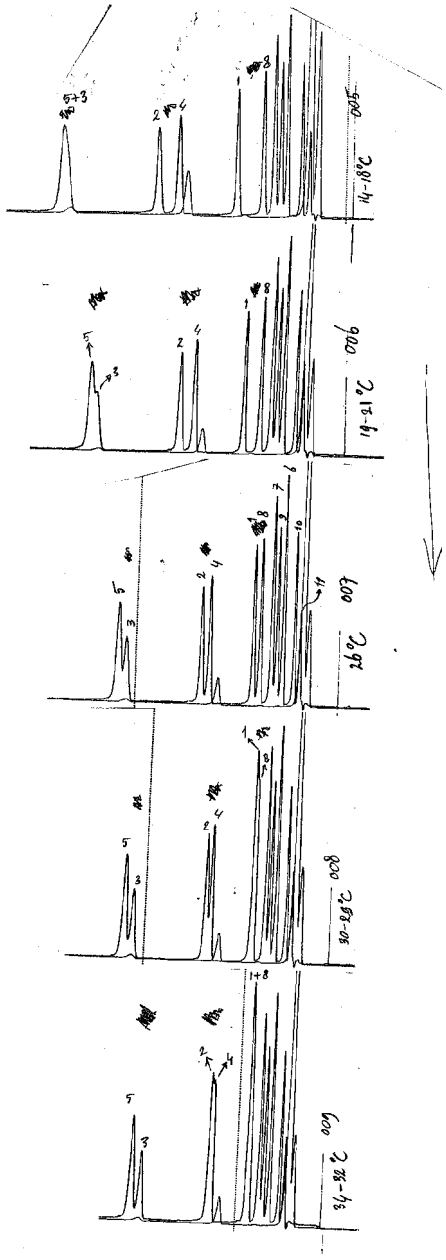
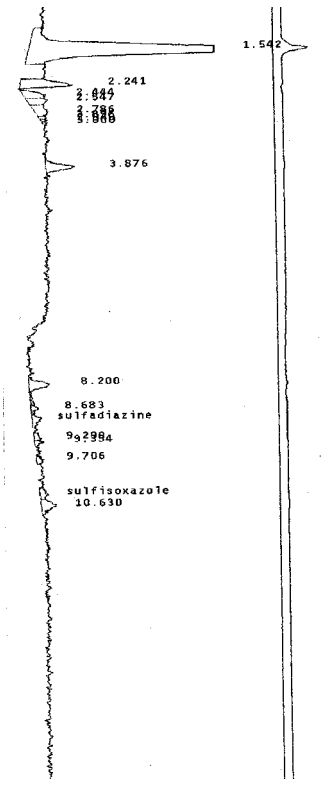


Figure 13: Influence of temperature on separation of the sulfonamides (Top to bottom temperature increase from 14 till 34 ^{o}C) pH = 5, 0.01 M eluent

To illustrate the applicability of the conditions chosen, figures 14 and 15 show respectively a chromatogram of a blank sample to prove the absence of interfering peaks and a chromatogram after injection of a standard solution of all eleven sulfonamides.

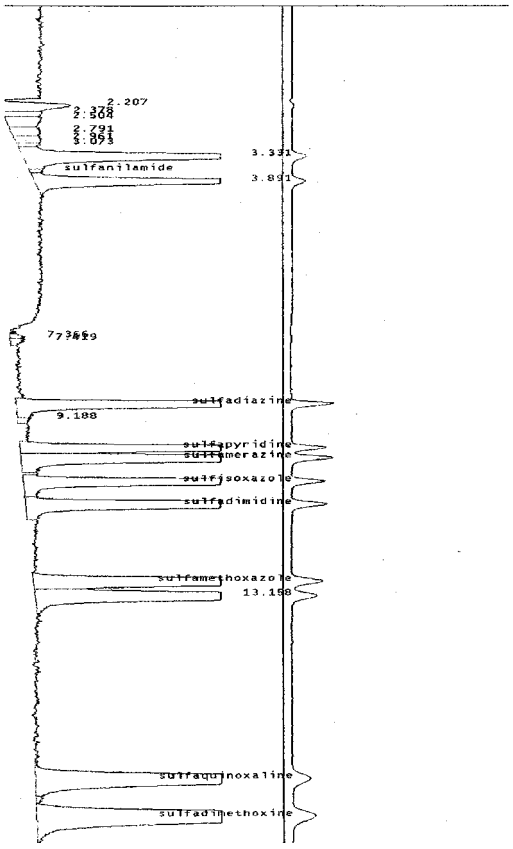


Comment:
Sequence: Subseq/Sample/Bottle: / / 0
Methode: /DATA/JJ4SULFA.MTH Instrument: A/D_4
Meth.Par.File: Inject: Sat Nov 4, 1995 9:19:46
Report File: JORGDUAL.FMT Report: Sat Nov 4, 1995 9:46:35

Peaks Reported	17	Peak Processor	Genie
Threshold	6.0	Multilevel	False
Area Reject	10	Application	Loop
Ident. Level	0	Quantitation	AreaUnits
Suppress Unkn.	False	Calculation	ExternalSTD
Shoulders	False	Run Time	19.52
Extended Peaks	False	Delay	0.00
Peak Width	.100	Dead Time	1.10
Plates	0		
Ref. Wind. Abs.	.100	Sample Amount	1.000000
Ref-RTW	.050	Dilutionfactor	100.000000
Non Ref.Win.Abs	.100	Inj. Vol. Ratio	1.000000
Non Ref-RTW	0.000	Standard Amount	1.000000
Update Times	False	Purity %	100.000000
Update %	25.000		

Pk#	RT	ID-tm	Resp.Fact.	Area Code	ug/ml	Name
12	8.89	8.79	1.0000E+00	2674 VV	267394.	sulfadiazin
16	10.40	10.47	1.0000E+00	2435 VV	243457.	sulfisoxazo
		Totals:		217965	21796528.	ug/ml

Figure 14: Chromatogram of blank egg white sample



bepaling van sulfonamides Report No : 2491.0
Sample: std File: /DATA/JJ4SF192.RES
Comment:
Sequence: Subseq/Sample/Bottle: / / 0
Methode: /DATA/JJ4SULFA.MTH Instrument: A/D_4
Meth.Par.File: Inject: Sat Nov 4, 1995 9:56:14
Report File: JORGDUAL.FMT Report: Sat Nov 4, 1995 10:19:48

Peaks Reported	21	Peak Processor	Genie
Threshold	6.0	Multilevel	False
Area Reject	10	Application	Loop
Ident. Level	0	Quantitation	AreaUnits
Suppress Unkn.	False	Calculation	ExternalSTD
Shoulders	False	Run Time	19.50
Extended Peaks	False	Delay	0.00
Peak Width	.100	Dead Time	1.10
Plates	0		
Ref. Wind. Abs.	.100	Sample Amount	1.000000
Ref-RTW	.050	Dilutionfactor	100.000000
Non Ref.Win.Abs	.100	Inj. Vol. Ratio	1.000000
Non Ref-RTW	0.000	Standard Amount	1.000000
Update Times	False	Purity %	100.000000
Update %	25.000		

Pk#	RT	ID-tm	Resp.Fact.	Area Code	ug/ml	Name
8	3.65	3.75	1.0000E+00	2150 VV	214959.	sulfanilami
12	8.85	8.79	1.0000E+00	186487 VV	$$$$$$$	sulfadiazin
14	9.84	9.81	1.0000E+00	154046 VV	$$$$$$$	sulfapyridi
15	10.07	10.04	1.0000E+00	205498 VV	$$$$$$$	sulfamerazi
16	10.59	10.47	1.0000E+00	158079 VV	$$$$$$$	sulfisoxazo
17	11.10	#11.09	1.0000E+00	188587 VV	$$$$$$$	sulfadimidi
18	12.84	12.99	1.0000E+00	184304 PV	$$$$$$$	sulfamethox
20	17.22	16.82	1.0000E+00	171447 PV	$$$$$$$	sulfaquinox
21	18.05	17.67	1.0000E+00	240593 VV	$$$$$$$	sulfadimeth
		Totals:		1912064	191206432	ug/ml

Figure 15: Chromatogram of standard sulfonamide solution

2.5.1.2 *Linearity of the response*

The linearity of the detector response has also been tested and the figures included in the appendix prove that for all eleven sulfonamides a good linear response was obtained.

2.5.1.3 *Recovery percentages*

The recovery percentages from the aqueous and organic solutions were considered to be around 100 %, as no interfering factors are suspected to be present.
The method used in this study to determine the sulfonamides in yolk and white was necessarily a compromise and for that reason some low recovery percentages occurred. In study 1 the following percentages were found:

Recovery %	Yolk	White
Sulfisoxazole	36	94
Sulfachloropyrazine	81	86
Sulfaquinoxaline	40	70
Sulfamethoxazole	80	92
Sulfadimethoxine	72	94
Sulfadiazine	97	102
Sulfamerazine	84	91
Sulfadimidine	81	82
Sulfapyridine	59	84
Sulfanilamide	44	33
Sulfaguanidine	20	< 3

The low but consistent recovery percentages in some instances were nevertheless accepted as they were considered to have no impact on the outcome of the studies

2.5.2 *Laboratory tests*

2.5.2.1 Partitioning coefficient

The solubility of the sulfonamides in cyclohexane proved to be so low, that the measurements with this solvent have not been completed and the results are not shown here.

The partitioning coefficients obtained with dichloromethane as the organic phase together with the Sd values are given in Table 3.

The variability in the analytical results largely results from variation (about 5%) in the analyses in the dichloromethane layer. Evaporation of the solvent might have played an important role in causing this variability. The variability of the analyses of the aqueous layer was about 3 % and that of the standard solutions and the pipettes about 1 %.

The reliability of the partitioning coefficients was tested according to the OECD guidelines, by comparison of the P value at any given condition to the average P value. This test did not show that a relationship between concentration and P values existed. The calculated log P values were well within the prescribed 0.3 log unit range, to be acceptable according to the guidelines.

The partitioning of the sulfonamides between dichloromethane and an aqueous buffer solution showed large differences with sulfadimethoxine having (on average) the largest affinity for the organic phase and sulfaguanidine the least. The P and log P values – which are also indicator of the solubility – showed no clear relationship with the pKa values of the different sulfonamides (see also figure 16).

Table 3: Sulfonamides with their pKa values and determined partitioning coefficients at pH 6.0 and 7.6 (Sd based on n=6)

Sulfonamide	pKa	P DCM/H_2O at pH 6.0			P DCM/H_2O at pH 7.6		
		P	Sd	Log P	P	Sd	Log P
Sulfisoxazole	4.7	0.92	0.03	-0.037	0.0225	0.0010	-1.648
Sulfachloropyrazine	5.1	4.17	0.18	0.619	0.114	0.003	-0.941
Sulfaquinoxaline	5.5	47	6	1.67	2.16	0.13	0.33
Sulfamethoxazole	5.9	4.7	0.3	0.67	0.190	0.026	-0.72
Sulfadimethoxine	6.3	66	4	1.82	3.8	0.2	0.58
Sulfadiazine	6.5	1.93	0.17	0.28	0.215	0.014	-0.67
Sulfamerazine	7.0	5.1	0.6	0.70	1.228	0.018	0.089
Sulfadimidine	7.5	9.4	0.8	0.97	5.5	0.2	0.738
Sulfapyridine	8.4	2.68	0.14	0.43	2.49	0.11	0.40
Sulfanilamide	10.5	0.092	0.002	-1.038	0.093	0.002	-1.030
Sulfaguanidine	11.3	0.00183	0.00007	-2.738	0.00187	0.00006	-2.728

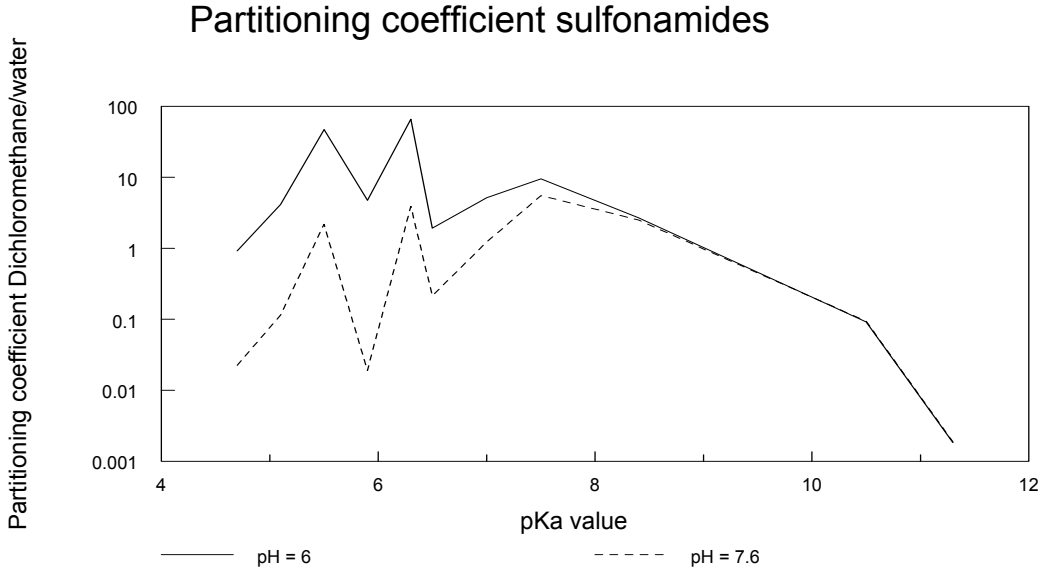


Figure 16: Relationship between partitioning coefficient and pKa value of the sulfonamides

The partitioning at the different pH values of the aqueous buffer solutions did show – as anticipated - a relationship with the pKa values.

Table 4 gives the calculated ratio of the measured partitioning ratios at pH values 6.0 and 7.6 and the calculated distribution of the uncharged molecule over a semi-permeable membrane separating two aqueous phases with pH values 6.0 and 7.6. This calculation was made according to Hogben et al. (1959).

Table 4: Ratio of the partitioning coefficients measured at pH values 6.0 and 7.6 and the calculated distribution.

Sulfonamide	pKa	Ratio of P values measured at pH 6.0 and 7.6	Calculated distribution at pH values 6.0 and 7.6
Sulfisoxazole	4.7	40.9	38.0
Sulfachloropyrazine	5.1	36.6	35.5
Sulfaquinoxaline	5.5	21.8	30.5
Sulfamethoxazole	5.9	24.7	22.6
Sulfadimethoxine	6.3	17.4	14.0
Sulfadiazine	6.5	9.0	10.3
Sulfamerazine	7.0	4.2	4.5
Sulfadimidine	7.5	1.7	2.2
Sulfapyridine	8.4	1.08	1.15
Sulfanilamide	10.5	0.99	1.00
Sulfaguanidine	11.3	0.98	1.00

The ratio of the partitioning at the pH values 6.0 and 7.6 showed a good correlation with the calculated distribution of the uncharged molecule over a semi-permeable membrane separating aqueous phases of these pH values. This strongly suggests that both the pKa values used in the calculations were about right and that in measuring the partitioning coefficients between organic and aqueous phases of different pH values, indeed only the uncharged molecules contribute to the distribution.

2.5.3 *Animal trials*

2.5.3.1 Contents in yolk and egg white (Trial 1)

The average contents in yolk and white measured in the samples after about two weeks of feeding (days 14/15 and 16/17) are given in Table 5.

Table 5: Contents of the sulfonamides in feed, yolk and white.

Sulfonamide	Feed content mg/kg	Yolk content in µg/kg*	White content in µg/kg*	Ratio white/yolk
Sulfisoxazole	100	10 + 32 SA	< 15 + 16 SA	< 1.5 SA 0.48
Sulfachloropyrazine	50	167	523	3.13
Sulfaquinoxaline	20	261	542	2.08
Sulfamethoxazole	50	117	418	3.56
Sulfadimethoxine	100	370	860	2.32
Sulfadiazine	20	15	135	9.27
Sulfamerazine	100	26	226	8.77
Sulfadimidine	20	9	27	2.88
Sulfapyridine	50	< 4.5 + 31 SA	< 100 + 16 SA	? SA 0.51
Sulfanilamide	20	137	149	1.09
Sulfaguanidine	100	336	< 1000	? (<3)

*****The yolk and white data are an average of two measurements in mixed samples; each mixed sample consisting of 5-6 individual egg samples and are corrected for recovery

The recovery of the sulfonamides with high pKa values was quite low (see above), so the results of these compounds must be viewed with caution.
The analyses of yolk and white from hens in the sulfisoxazole and the sulfapyridine groups indicated the possible presence of sulfanilamid. We have not been able to confirm these observations later on.

The concentrations of the different sulfonamides are nearly always higher in egg white than in yolk. The ratio of the contents in white/yolk of the different sulfonamides does

not show a clear correlation with their pKa values (Table 3). The partitioning coefficients at the different pH values (Table 3) and the ratio of the partitioning coefficients at pH values 6.0 (Yolk) and 7.6 (White) given in Table 4 also showed no correlation with the observed white/yolk ratios. Figures 17 and 18 illustrate this.

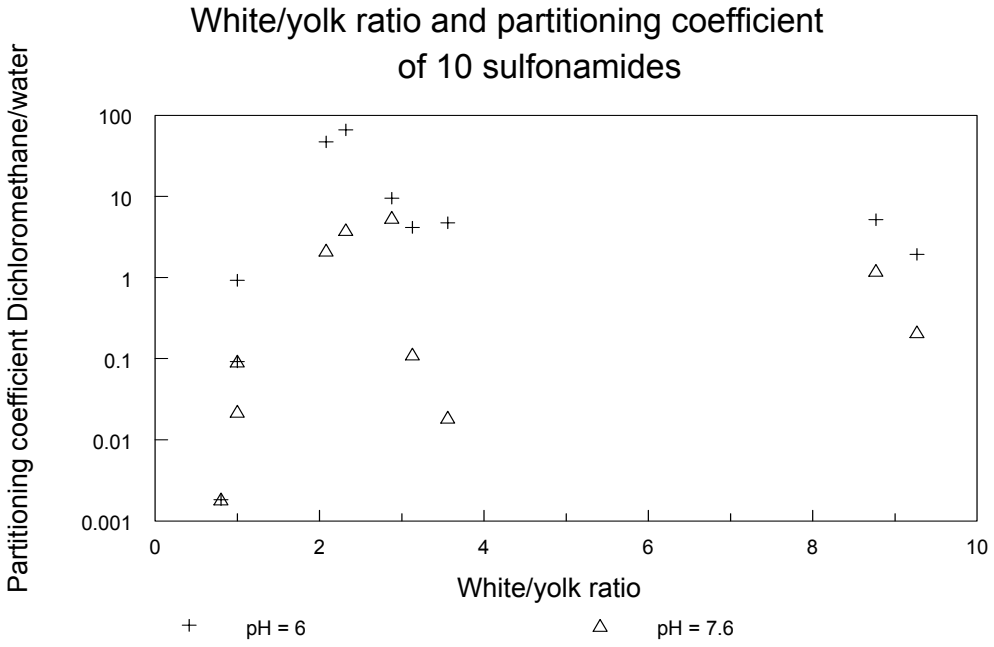


Figure 17: The white/yolk ratio of 10 sulfonamides and the P values at pH 6 and 7.6

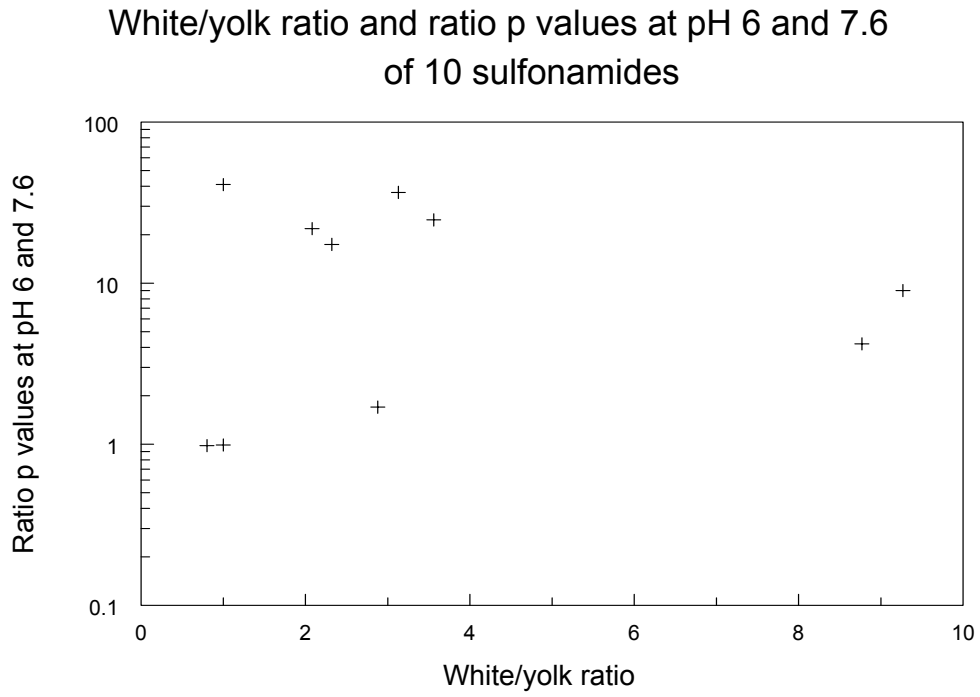


Figure 18: White/yolk ratio and ratio of p values at pH 6 and 7.6 of 10 sulfonamides

2.5.3.2 Contents in white and yolk (Trial 2)

The residue levels reported in Table 6 are the results of a mixed sample of 4-5 eggs per animal laid during days 15-20 of the experiment. The results were corrected for recovery.

The data show that at the same level in the feed (50 mg/kg) the different sulfonamides will give different residue levels in both yolk and white. They also show that although between hens there are considerable differences in absolute amounts, for each substance the ratio of the levels in white and yolk is quite constant.
Furthermore the differences between substances both in absolute amounts and in the white/yolk ratio are also quite consistent. The ratios sometimes agree quite well between the two experiments and sometimes they do not. As the methodology was improved between both experiments (especially for sulfaguanidine) and levels near the detection limit (sulfadiazine, sulfadimidine and sulfaguanidine) were not present in the second trial, these factors may explain some of the differences in the results between the trials.

Table 6: Average contents of sulfonamides in yolk and egg

Hen nr	Sulfonamide	Yolk in µg/kg	White in µg/kg	Ratio white/yolk
1	Sulfachloropyrazine	191	416	2.17
2	Sulfachloropyrazine	234	668	2.85
3	Sulfachloropyrazine	253	705	2.79
4	Sulfachloropyrazine	292	806	2.76
5	Sulfachloropyrazine	401	1108	2.76
Mean	*Sulfachloropyrazine*	*274*	*741*	*2.67*
6	Sulfadimethoxine	170	431	2.53
7	Sulfadimethoxine	177	531	1.98
8	Sulfadimethoxine	176	444	2.52
9	Sulfadimethoxine	161	330	2.05
10	Sulfadimethoxine	215	440	2.04
Mean	*Sulfadimethoxine*	*180*	*399*	*2.23*
11	Sulfadiazine	146	639	4.38
12	Sulfadiazine	141	622	4.41
13	Sulfadiazine	158	642	4.07
14	Sulfadiazine	136	635	4.67
15	Sulfadiazine	167	636	3.81
Mean	*Sulfadiazine*	*149*	*635*	*4.27*
16	Sulfadimidine	23	147	6.55
17	Sulfadimidine	44	229	5.20
18	Sulfadimidine	34	121	3.57
19	Sulfadimidine	34	159	4.71
20	Sulfadimidine	22	121	5.49
Mean	*Sulfadimidine*	*31*	*155*	*5.11*
21	Sulfaguanidine	352	249	0.71
22	Sulfaguanidine	218	170	0.78
23	Sulfaguanidine	236	199	0.84
24	Sulfaguanidine	289	231	0.80
25	Sulfaguanidine	360	273	0.76
Mean	*Sulfaguanidine*	*291*	*224*	*0.78*

2.5.3.3 Protein/macromolecular binding

Ultrafiltration of yolk samples proved not to be possible due to clogging of material on the membrane.

The ultrafiltration of the white samples indicated the following average protein binding;

Sulfachloropyrazine	27 %
Sulfadimethoxine	30 %
Sulfadiazine	- 31 %
Sulfadimidine	5 %
Sulfaguanidine	9 %

The data on sulfadiazine can only be explained if a much lower recovery from the incurred total white samples occurred, than measured in the recovery experiments with added material. Therefore the absolute values of the protein binding experiments must be viewed with caution. Assuming however that in relative terms they are correct, the differences in protein binding percentages can not explain the observed differences in white/yolk ratios. Sulfadimidine and sulfaguanidine have a similar protein binding but differ considerably in white/yolk ratio, whereas sulfadimidine having a lower protein binding than sulfachloropyrazine and sulfadimethoxine has a much higher white/yolk ratio.

3 RESULTS AND DISCUSSION

3.1 Introduction

Furusawa (1999) makes the following statements on the reasons for drug residues in white and yolk and the distribution between of drugs between white and yolk.

" *Therefore, the drug content in egg yolk is a cumulative sum of the drug during the growth of the yolk. On the other hand, albumen synthesized in the cells of the magnum are secreted by this part of the oviduct (concentrated albumen). The albumen is then later diluted with water (plumping water) in the shell gland. Therefore the drugs presence in the albumen reflects closely the blood concentration during the substantial time (~10-13 h) required for albumen formation in the oviduct. This seems to agree with the hypothesis of passive diffusion of the drug across the albumen glandular epithelium and an equilibrium between the drug concentrations in blood and in the albumen in the oviduct. The drugs tested are transported as one soluble compound in blood. The relative contents of a drug in egg yolk or albumen depends on its relative solubility in lipid or water, respectively. The drugs having the property of lipid-solubility are found in much higher levels in egg yolk than in albumen, whereas those having water-solubility are found in higher concentrations in albumen than in yolk."*

Furusawa (2001) later stated shortly *"Large variations in the ratio of the contents of the drugs in egg yolk to that in albumen occur with different drugs. This variation should depend mainly on the relative lipid-solubility and water-solubility of the drug. Drugs that are lipid soluble, such as amprolium or nicarbazin are found in much higher concentrations in egg yolk than in albumen. In contrast, those that are water soluble, such as the sulfonamides, are found in a higher concentration in albumen than in egg yolk."*

These statements nicely summarise the traditional approach to the subject of this study. Literature data and data out of our previous studies and the present one clearly indicate certain inconsistencies or mistakes in this concept.

3.2 Parameters studied

To prove or disprove the concept outlined above, the distribution of sulfonamides between egg white and yolk was studied and the following physicochemical characteristics of the different substances were considered of possible importance for this distribution:

- Lipid solubility
- Distribution between the aqueous and organic phase (log p value)
- pKa value
- Protein binding

3.2.1 *Lipid solubility and partitioning coefficient in relation to white/yolk ratio*

The partitioning coefficients of the different sulfonamides obtained with dichloromethane as the organic phase have been given in Table 3. The white/yolk ratios have been given in Tables 5 and 6. Table 7 shows the direct comparison of P values at pH values 6.0 and 7.6 and the white yolk ratios found in the two trials. The partitioning of the sulfonamides between dichloromethane and an aqueous buffer solution showed large differences between the different substances. Sulfadimethoxine shows (on average) the largest affinity for the organic phase and sulfaguanidine the least. The P or log P values – which are also indicator of the solubility in the organic phase – and the white/yolk ratios of the different sulfonamides given in Table 7 show no obvious relationship. Sulfadimethoxine, which has the highest P values, so the highest affinity for the organic phase and probably the highest lipid solubility, certainly has not the lowest white/yolk ratio, as would follow from the lipid solubility theory. Sulfanilamide having a low P value and lipid solubility should have had a high white to yolk ratio, if lipid solubility would have been the main driving force but it has a white/yolk ratio close to 1. Sulfaguanidine has an extremely low partitioning ratio and solubility in the organic phase. It does not have a correspondingly low white to yolk ratio, which also does not support lipid solubility as being the driving force for distribution of sulfonamides between white and yolk.

Table 7: Sulfonamides and their determined partitioning coefficients at pH 6.0 and 7.6 and white/yolk ratios found in trials 1 and 2

Sulfonamide	P at pH 6.0 DCM/H_2O	P at pH 7.6 DCM/H_2O	Ratio white/yolk trial 1	Ratio white/yolk trial 2
Sulfisoxazole	0.92	0.0225	< 1.5 SA 0.48	
Sulfachloropyrazine	4.17	0.114	3.13	2.67
Sulfaquinoxaline	47	2.16	2.08	
Sulfamethoxazole	4.7	0.190	3.56	
Sulfadimethoxine	66	3.8	2.32	2.23
Sulfadiazine	1.93	0.215	9.27	4.27
Sulfamerazine	5.1	1.228	8.77	
Sulfadimidine	9.4	5.5	2.88	5.11
Sulfapyridine	2.68	2.49	? (SA 0.51)	
Sulfanilamide	0.092	0.093	1.09	
Sulfaguanidine	0.00183	0.00187	? (<3)	0.78

The data in Table 7 thus show no obvious general relationship between P value and white to yolk ratio.

Gorla et al. (1997) and Furusawa (1999, 2001) both state, that lipid soluble drugs will predominantly be present in yolk and water-soluble drugs predominantly in white. The results on the lipid soluble doxycycline in contrast to the less lipid soluble oxytetracycline (see Table 1) as well as the results presented above on the sulfonamides do not fully support this statement.

So - generally spoken - lipid solubility of a drug, when given to laying hens, on its own does not determine the white/yolk ratio of that drug.

3.2.2 *pKa value and white/yolk ratio*

Table 8 shows the calculated ratio of the measured partitioning ratios at pH values 6.0 and 7.6 (Table 4) and for comparison the white/yolk ratios measured in the two trials (Tables 5 and 6). The pH values 6.0 and 7.6 were chosen for this determination as they mimic the pH values of yolk and white in freshly laid eggs.

Table 8: Ratio of the partitioning coefficients measured at pH values 6.0 and 7.6 and the white/yolk ratios.

Sulfonamide	pKa	Ratio of P values measured at pH 6.0 and 7.6	White/yolk ratio trial 1	White/yolk ratio trial 2
Sulfisoxazole	4.7	40.9	< 1.5 SA 0.48	
Sulfachloropyrazine	5.1	36.6	3.13	2.67
Sulfaquinoxaline	5.5	21.8	2.08	
Sulfamethoxazole	5.9	24.7	3.56	
Sulfadimethoxine	6.3	17.4	2.32	2.23
Sulfadiazine	6.5	9.0	9.27	4.27
Sulfamerazine	7.0	4.2	8.77	
Sulfadimidine	7.5	1.7	2.88	5.11
Sulfapyridine	8.4	1.08	? (SA 0.51)	
Sulfanilamide	10.5	0.99	1.09	
Sulfaguanidine	11.3	0.98	? (<3)	0.78

The ratio of the P values at pH 6.0 and 7.6 shows quite a good correlation with the pKa of the different sulfonamides. This indicates that at each pH of the aqueous phase, only the uncharged molecule seems to contribute to the observed distribution between the organic phase and aqueous phase. The ratio of the distributions observed at the two different pH values shows data very similar to those, calculated for a distribution of uncharged molecules between aqueous phases with the different pH values separated by a semi-permeable membrane.

Such a relationship between pKa of various drugs and pH values of different aqueous phases has been proven accurate for the explanation of a number of observed distribution ratios. The successful explanation of distribution of different drugs in different physiological systems, such as plasma/gastric juice, plasma/cerebrospinal fluid, plasma/red cells and plasma/milk has been outlined in the introduction.

The ratio of the P values at pH 6.0 (yolk) and 7.6 (white) and the white/yolk ratio observed for the 11 different sulfonamides tested (Table 8) shows no obvious (linear) relationship. The white/yolk ratio of the 11 sulfonamides seems to be non-related to

their pKa value. The assumption that the egg can be considered as two aqueous phases with different pH values separated by a semi-permeable membrane and distribution of the sulfonamides between white and yolk being only governed by distribution of the uncharged sulfonamide molecule, can thus not be substantiated. Blom (1975) tried to explain the distribution between plasma and egg white of the three sulfonamides studied by him on the basis of their pKa values and could not draw an unambiguous conclusion.
Apparently the distribution of sulfonamides between plasma and white and between white and yolk is not only – or may be not at all - governed by diffusion or passage of the uncharged molecule only.

3.2.3 *Protein/macromolecular binding and white/yolk ratio*

Numerous drugs including sulfonamides have been observed to bind to (plasma) proteins. This binding has both been observed *in vivo* and *in vitro*: thus either after treatment of an individual with a drug or after adding a drug to a blank plasma, the plasma sample contained both bound and free drug. Protein binding measured *in vivo* has the large advantage that it is governed by those process occurring in the living object, where as *in vitro* artefacts not occurring in life might show up.
The results obtained by measuring *in vivo* protein binding of five sulfonamides in egg white and the observed white/yolk ratios are given in Table 9.

Table 9: Comparison of protein binding and white/yolk ratio

Sulfonamide	Protein binding %	White/yolk ratio
Sulfachloropyrazine	27	2.67
Sulfadimethoxine	30	2.23
Sulfadiazine	-31	4.27
Sulfadimidine	5	5.11
Sulfaguanidine	9	0.78

No obvious relationship between measured *in vivo* protein binding in egg white and white/yolk ratio is evident from the data. Blom (1975) measured both *in vivo* and *in vitro* binding of three sulfonamides in egg white. No relationship between protein binding and white to yolk ratio could be deduced from his data. Nevertheless some

kind of active binding must take place in oviduct or albumen as Furusawa (1999) reported lower concentrations of spiramycine, oxytetracycline and sulfamonomethoxine in blood than in oviduct tissue and albumen. He nevertheless assumes that passive diffusion from blood to albumen occurs and that water or lipid solubility is the major determining factor. On the other hand (Furusawa personal communication, 2000) he believes, that protein binding (both rate of binding and amount of binding material) can have a major impact on distribution of the drug in the body and within the egg.

3.3 Other (unexplained) observations

Spiramycine (Yoshida et al., 1971; Roudaut and Moretain, 1990) as well as the quinolones, oxolinic acid (Roudaut and Boisseau, 1990) and flumequin (van Leeuwen and van Gend, 1989) show a longer persistence of residues in egg white than in yolk. McCracken et al. (2001) fed furazolidone to laying hens during 8 days and measured both the parent compound and a major metabolite (AOZ) in egg white as well as in yolk. During treatment they found all compounds in all compartments but at 11 days after withdrawal no parent furazolidone could be detected (as can be anticipated) in either yolk or egg white, but the metabolite was present still in both egg white and yolk. The presence of a drug or metabolite in both white and yolk suggests storage of it at some other place in the body and redistribution via the blood (or lymphatic system). The presence of a compound in white only suggests more specifically a kind of storage in the oviduct tissue (or elsewhere in the body) together with a strong affinity of the egg white for it (or vice versa). Protein binding of some kind is the most likely candidate for this.

Furusawa (1999) observed quite high levels of spiramycine in oviduct tissue –much higher than in blood or albumen -, which he suggested (Furusawa personal communication, 2000) to be caused by some kind of protein binding. This high binding might in turn be responsible for the prolonged half-live in egg white observed by others. Furusawa (1999) also observed high levels of oxytetracycline and sulfamonomethoxine in oviduct tissue, but these were comparable to levels in albumen.

Furusawa and Kishida (2002) fed five different sulfonamides (sulfadiazine, sulfadimidine, sulfamonomethoxine, sulfamethoxazole and sulfaquinoxaline) at 100 mg/kg diet to laying hens for seven days and then measured levels in plasma, liver,

muscle, ovary, the magnum fraction of the oviduct and the shell gland fraction. They found considerable differences in the distribution over the different tissues between the five sulfonamides tested. Plasma levels were (much) higher than levels for all other tissue with the exception of sulfadimidine in which case plasma and liver contents were about equal. Sulfadiazine, which in our experiments had a considerable white to yolk ratio and to a lesser extent sulfaquinoxaline, showed in their experiment a similar content in oviduct (magnum) tissue and ovary. Sulfadimidine and sulfamethoxazole showed a higher content in ovary tissue than in magnum tissue which contradicts our white to yolk ratios of about 3. The results from Furusawa and Kishida (2002) on distribution of sulfonamides within the tissues of the laying hen, thus do not explain the white to yolk ratios found in our experiments.

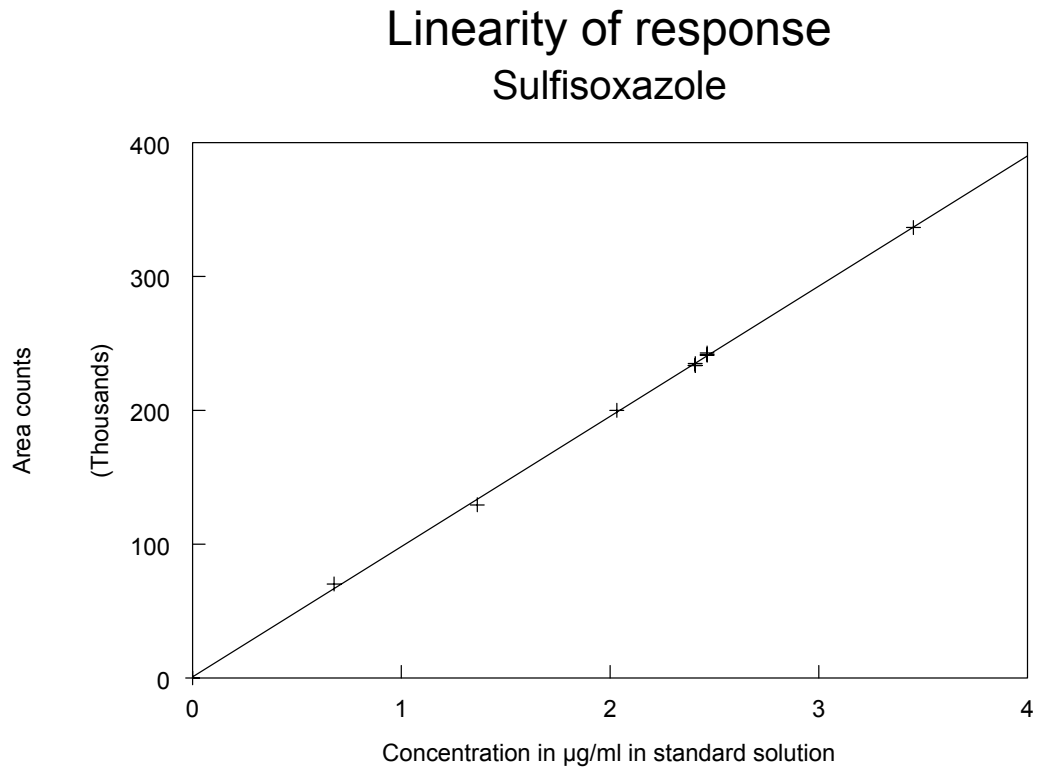
3.4 Some points still to be tackled

1. Determination of the complex or form in which drugs are transported (via the blood) to and deposited in the ovary.
2. Determination how and at what time and place drugs enter the egg white: during deposition of egg white or (also) during plumping or even during calcification of the egg. Donoghue et al. (2000) have proven the transfer of oxytetracycline during plumping, but more data should be gathered.
3. Determination of pH values at the micro-scale at those places where processes really occur and not only in the bulk of the phase. This would help to ascertain whether indeed only the non-ionised form of the molecules is involved in the different processes.
4. Study of possible (re) distribution processes of drugs between egg white and yolk during egg- and shell-formation.

3.5 Overall conclusions

1. The processes of yolk formation and deposition and the processes of egg white formation and deposition govern the shape of the residue curves in eggs (white and yolk) when drugs are given to or withdrawn from laying hens.
2. The eleven tested sulfonamides all show considerable levels in egg white despite their often high affinity for the organic phase, which – according to the lipid solubility concept - would suggest the predominant deposition in yolk.
3. The physiochemical characteristics measured: log P (at two different pH values), pKa and protein binding fail to predict or explain the observed distribution of the eleven sulfonamides between egg white and yolk when administered to laying hens.
4. The concept that concentrations of sulfonamides in egg white and yolk are directly related to and can be explained or predicted by one or two physicochemical measurements or characteristics is too simplistic. In other words, egg white and yolk are not two liquid phases separated by a semi-permeable membrane exhibiting equilibrium in contents of sulfonamides between them.
5. The complicated reality is that levels in yolk depend on both yolk deposition and solubility or affinity of the drug for the lipid phase. Levels in egg white probably depend on circulating plasma levels and specific binding of the drug to protein(s) in plasma and egg white and the affinity constants of that binding. Thus two non-directly related processes are taking place.

4.ANNEXES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3
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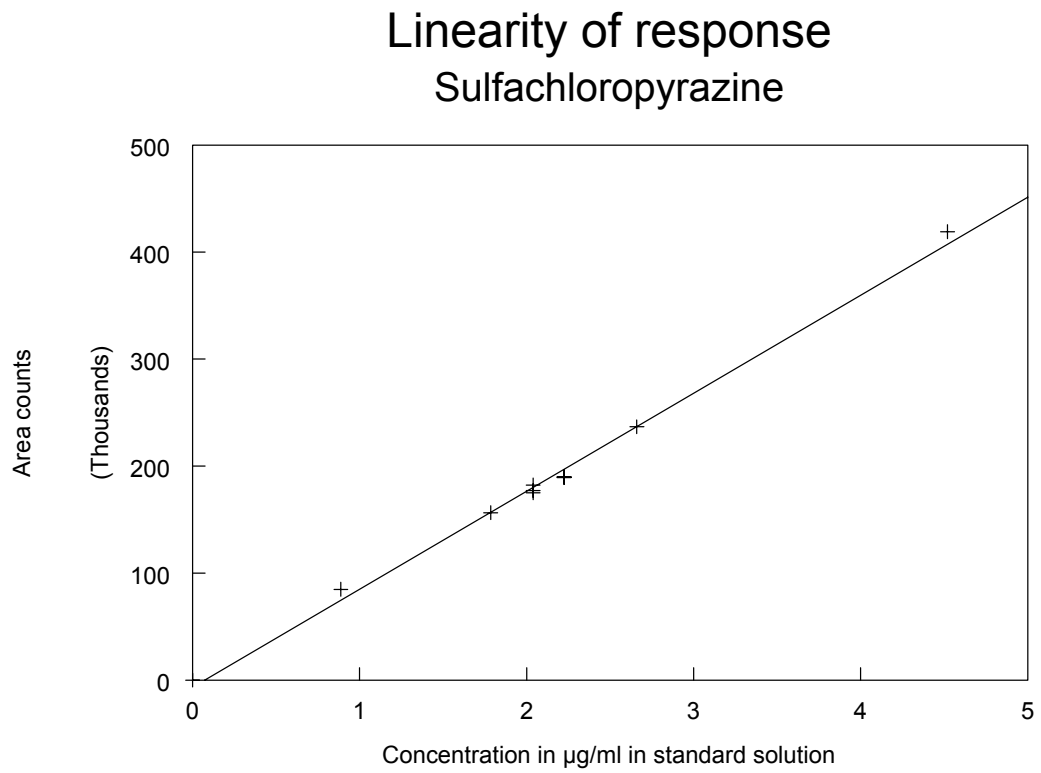
Concentration in µg/ml in standard solution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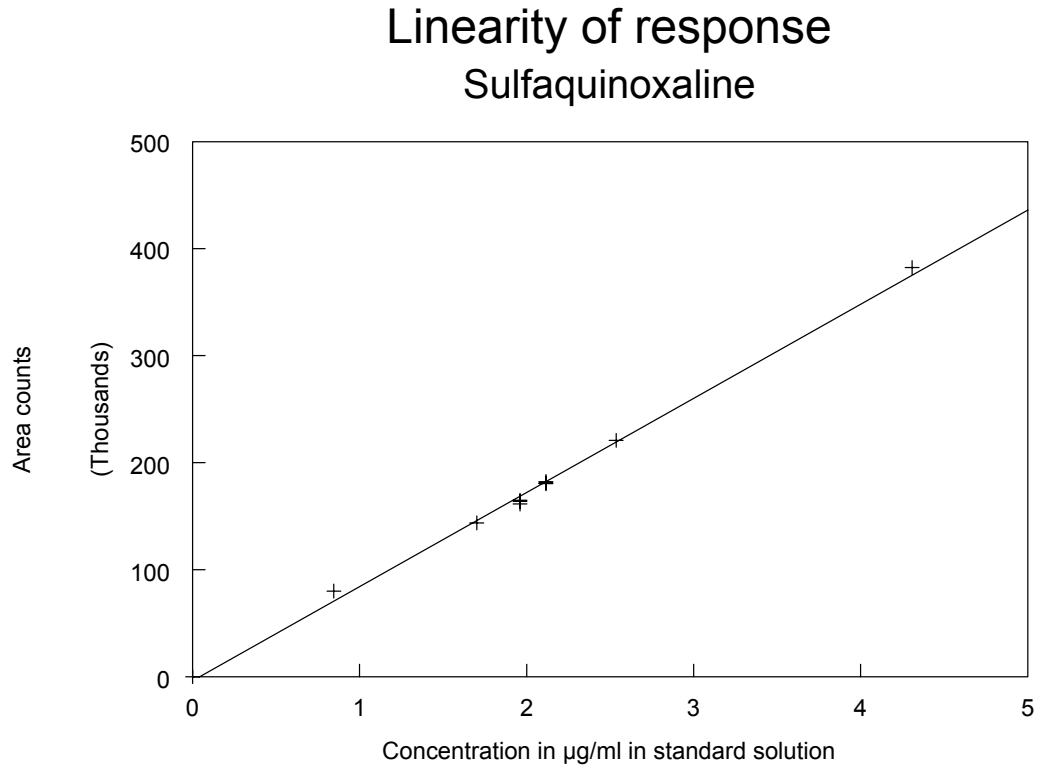
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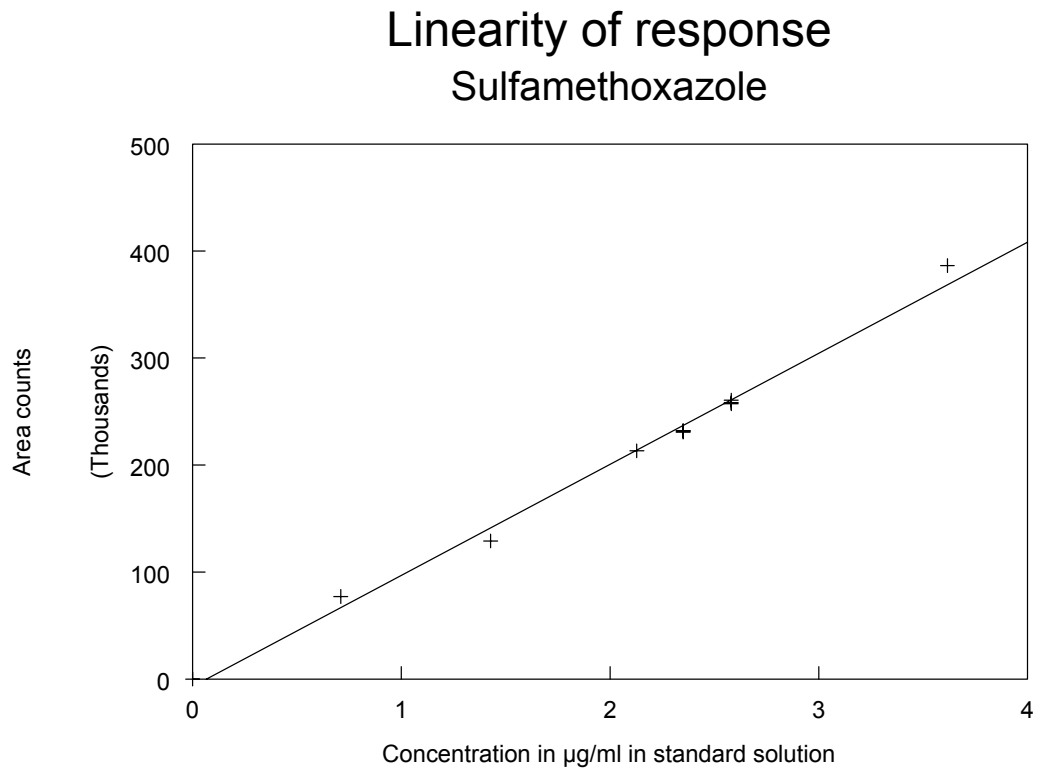
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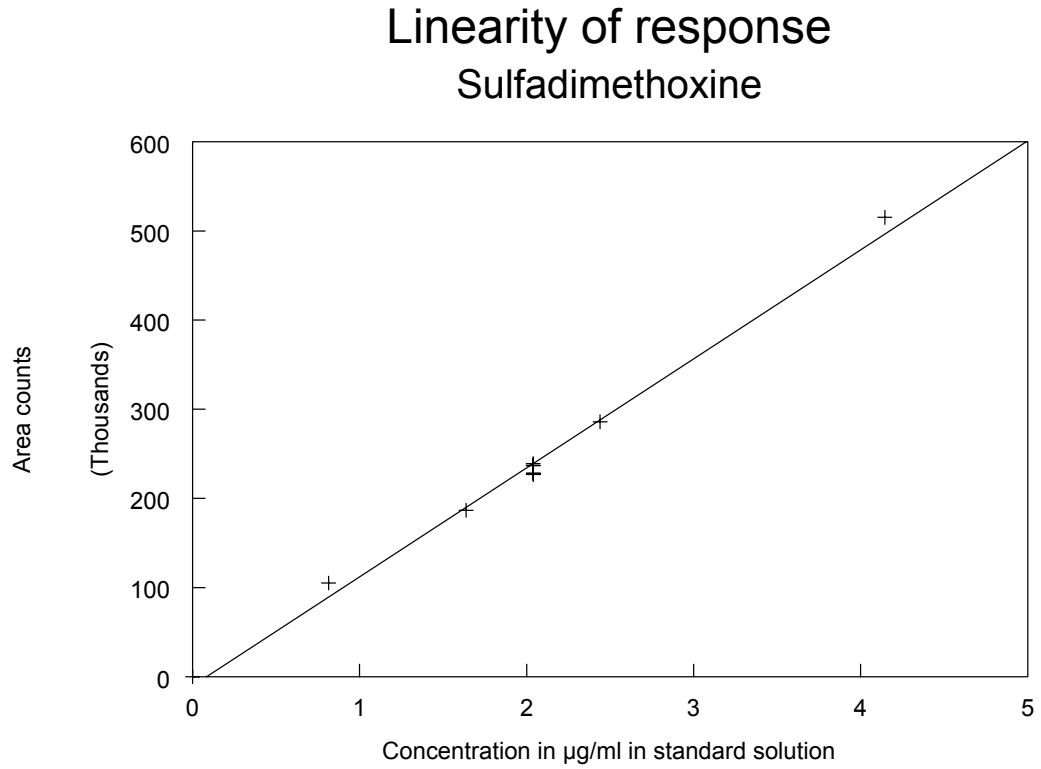
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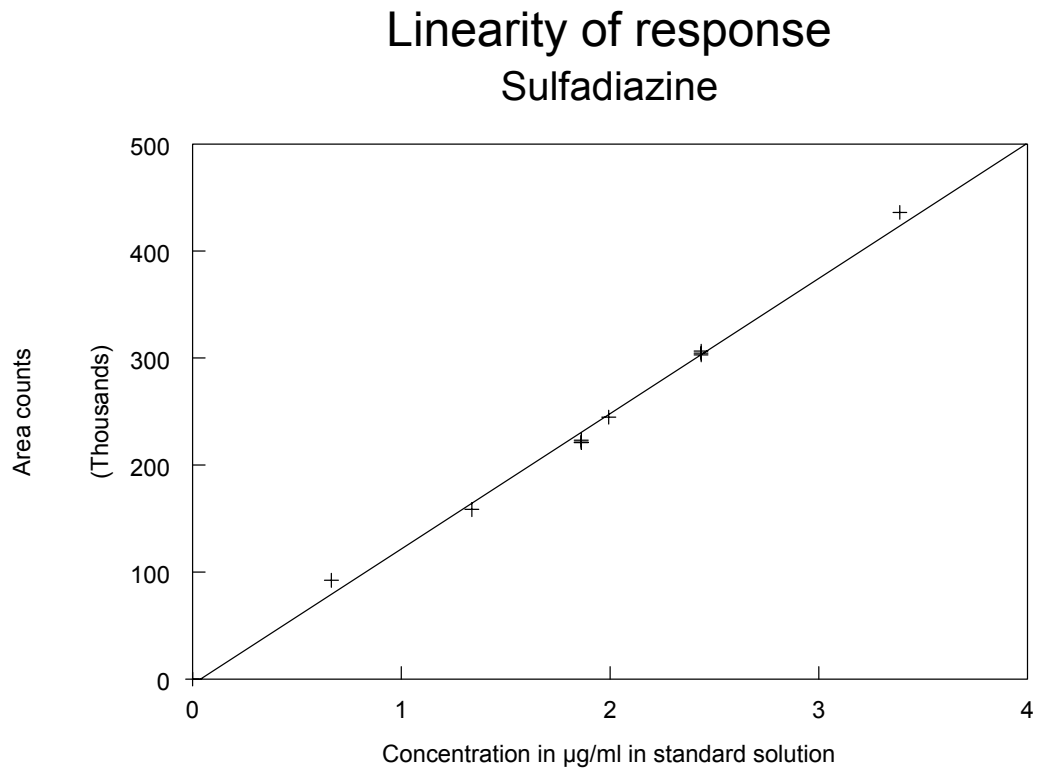
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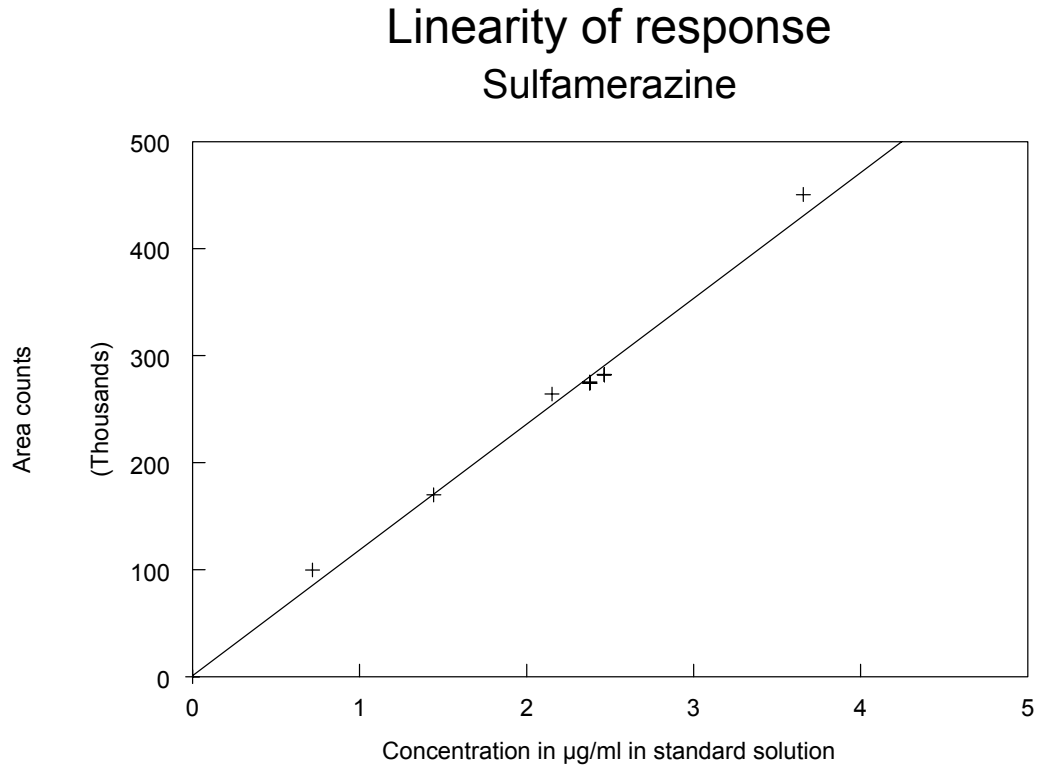
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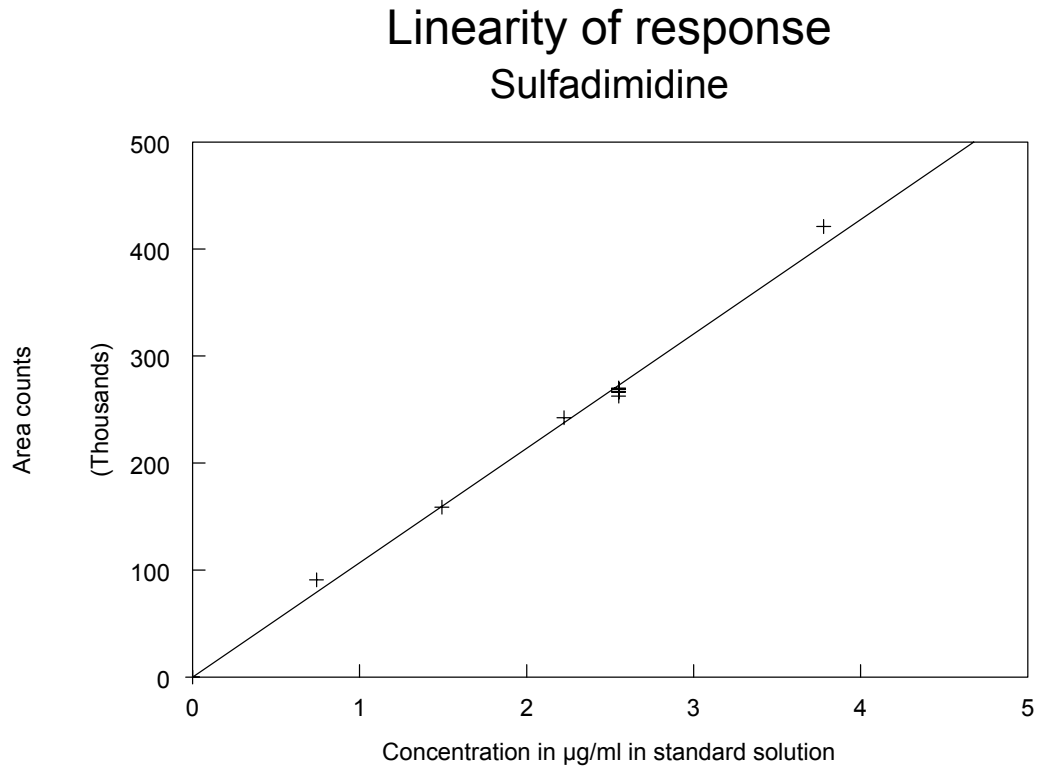
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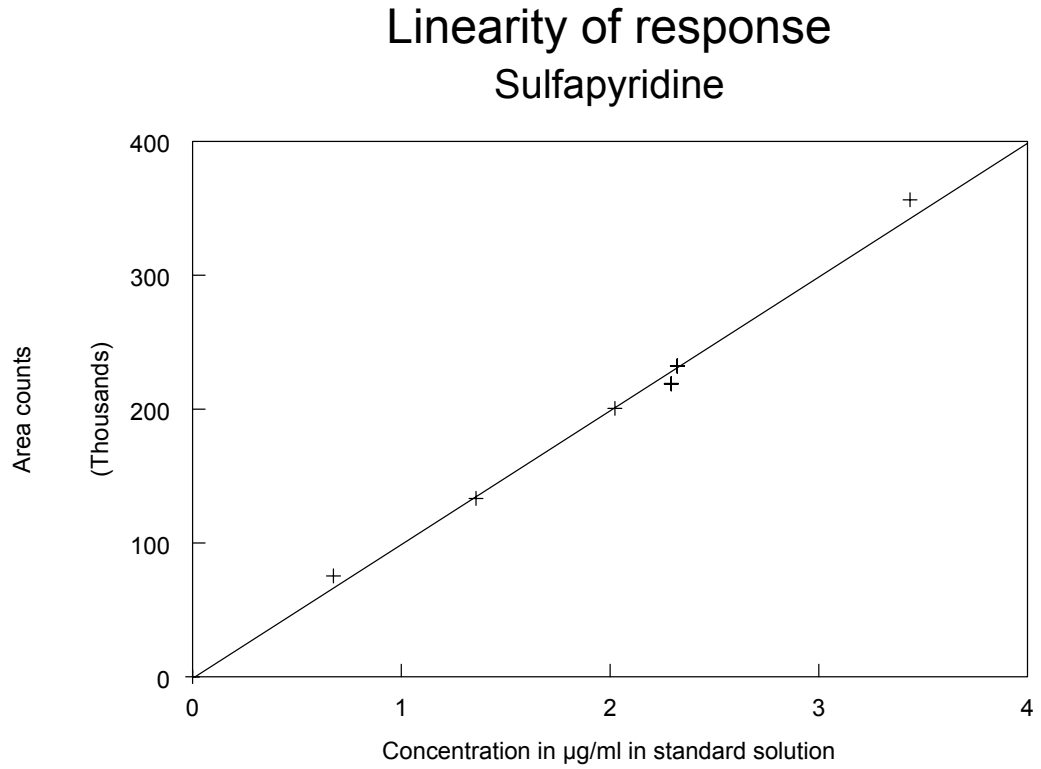
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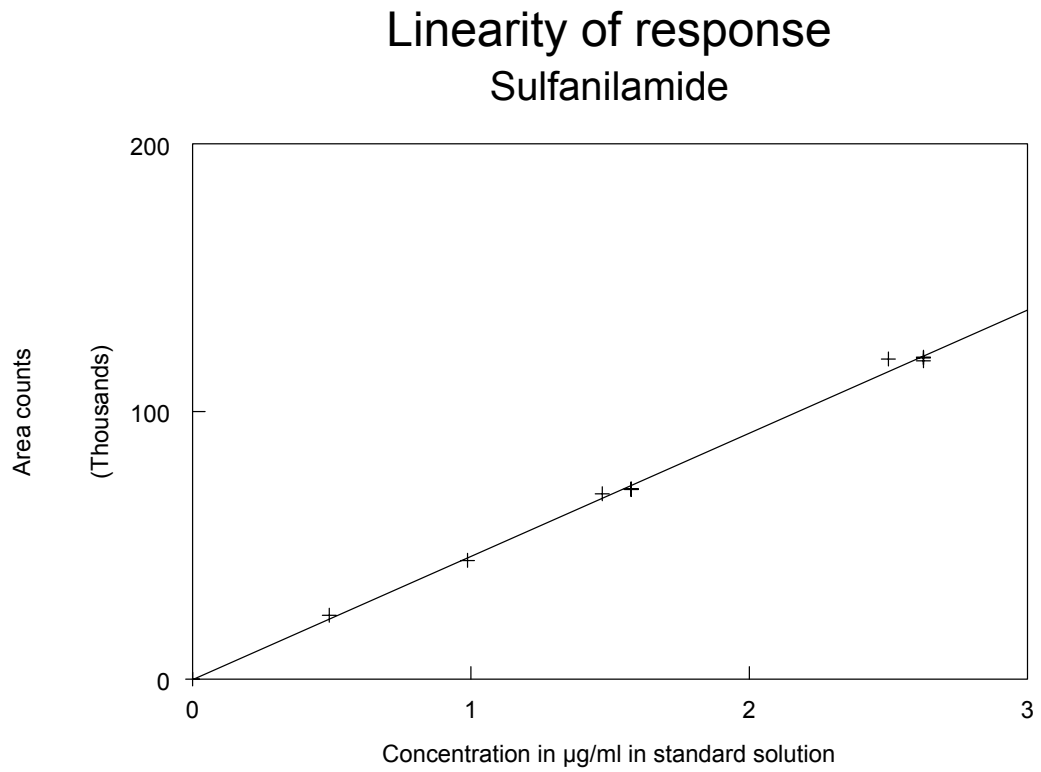
Concentration in µg/ml in standard solution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(Thousands)
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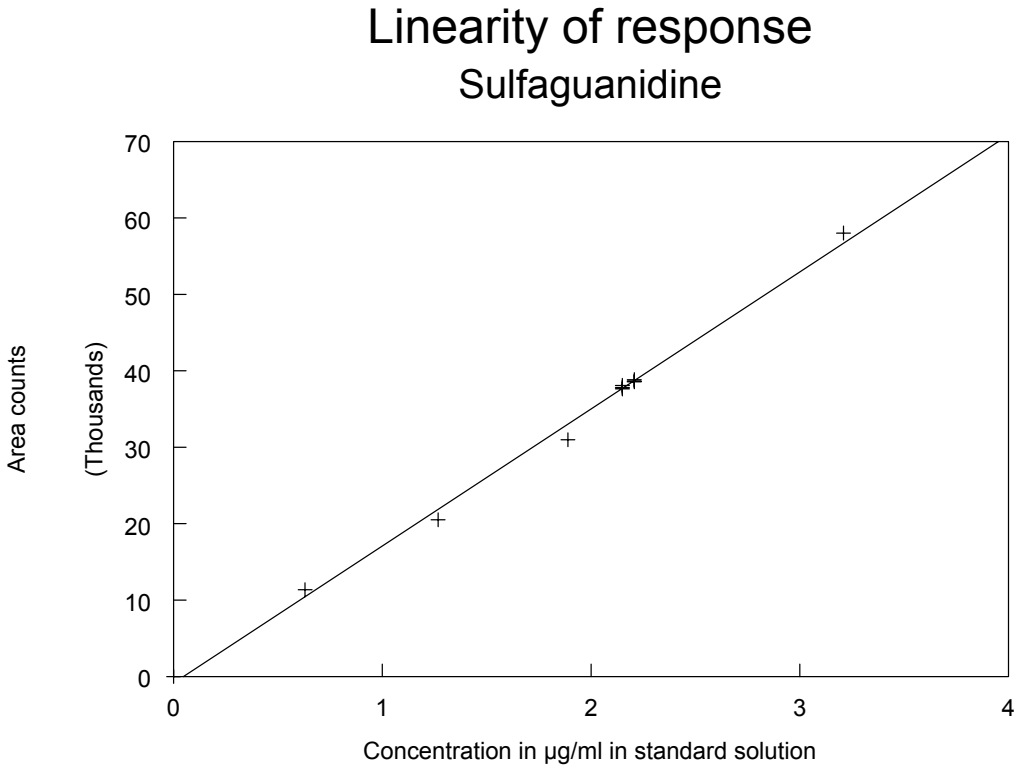
Concentration in µg/ml in standard solution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2
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Concentration in µg/ml in standard solution

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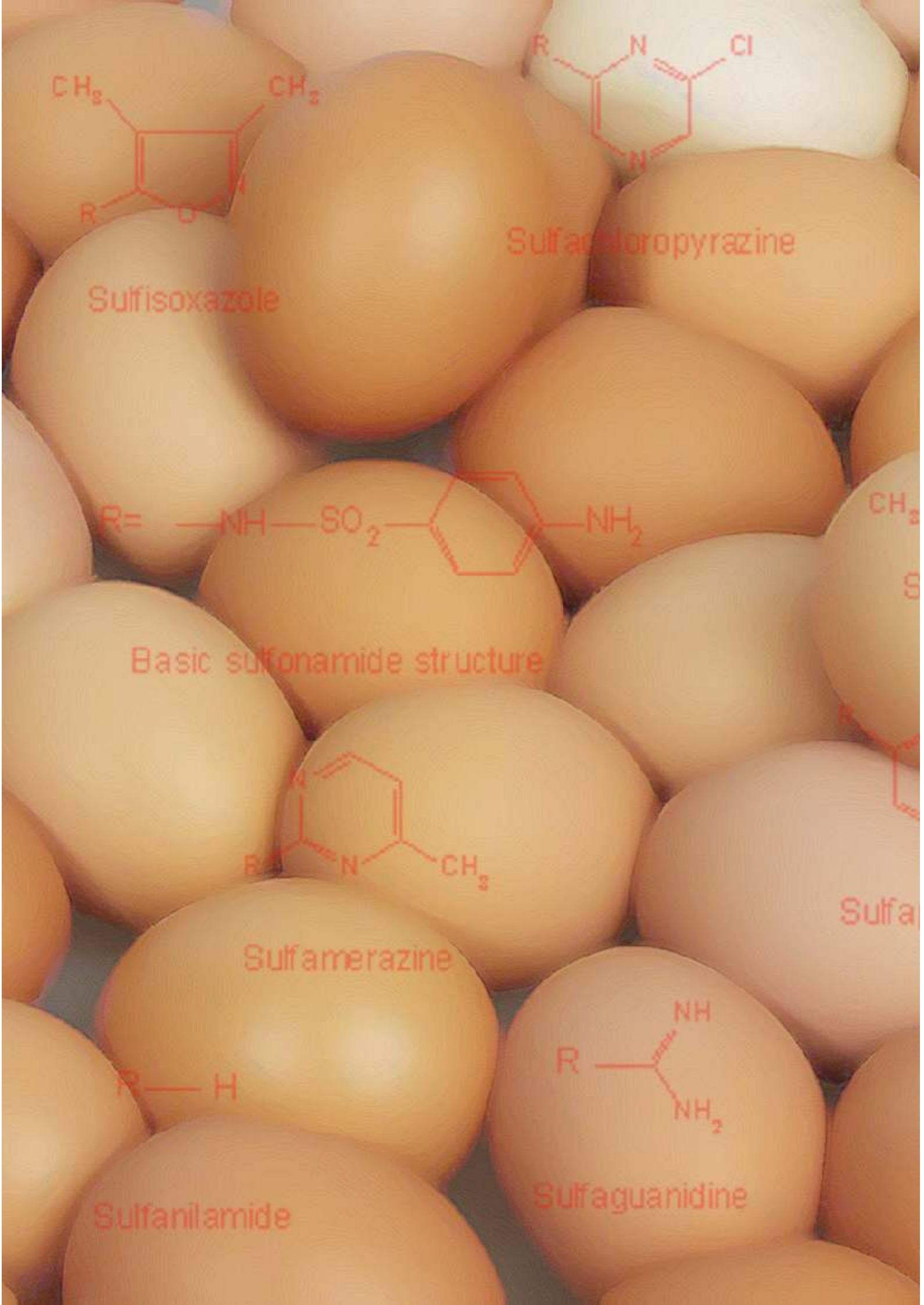
Sulfisoxazole
Basic sulfonamide structure
Sulfamerazine
Sulfanilamide
Sulfaguanidine