Chemistry of Hair Coloring

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Hair Colorant Product Safety

Chemistry of Hair Coloring
Types of Products

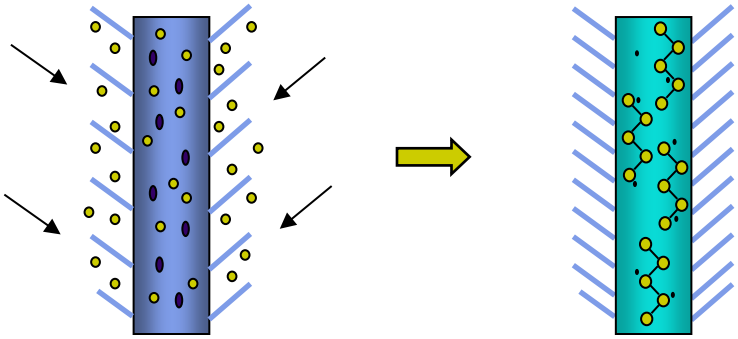
Oxidative Hair Dyes

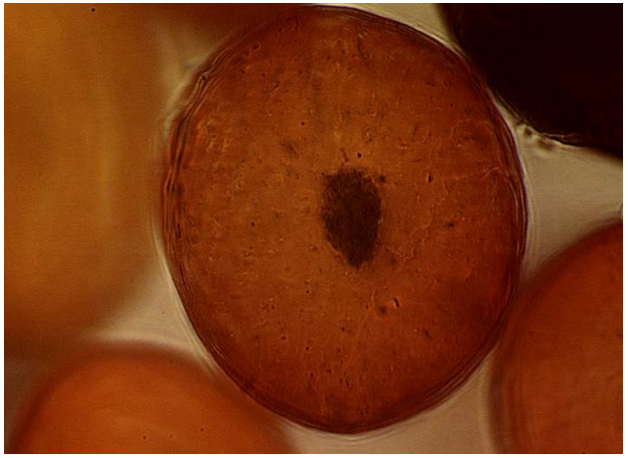
- Permanent
- Demi-permanent

Semi-permanent Hair Dyes

Types of Oxidative Hair Dyes: Permanent

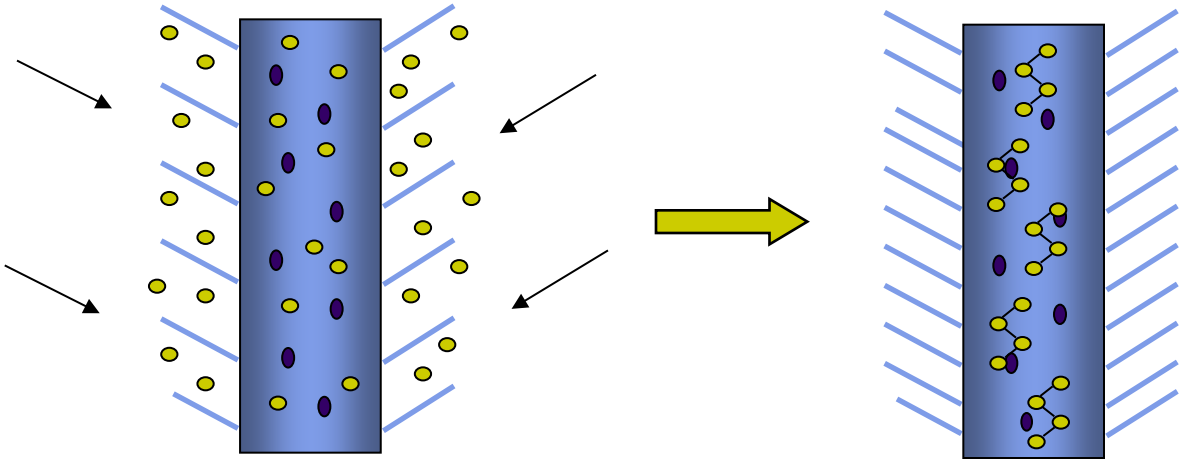
- >70% of the market
- Level of hydrogen peroxide used (3 - 4.5%) gives significant levels of bleaching
- Ammonia swells the hair but also aids the bleaching process
- Dyes penetrate the whole hair fiber
  - Color is therefore more wash-fast
- Can access a wide range of shades, e.g. blondes, vibrant red, etc.





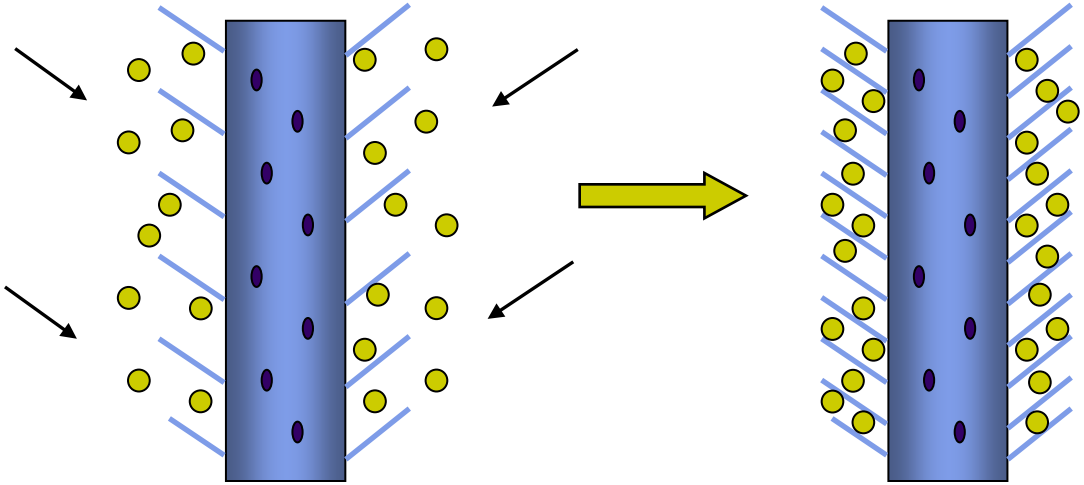
Types of Oxidative Hair Dyes: Demi permanent

- Low level of hydrogen peroxide (1 - 2%) delivers low bleaching of melanin
- Ethanolamine helps swell the hair & aid penetration of precursors (pH ~ 10)
  - Hair swelling is minimal
- Dye precursors oxidized by H_2O_2 forming large colored chromophores
  - Chromophores do not penetrate deep into the fiber
  - Dye penetration is less effective due to minimal hair swelling
- Limited shade range (no lightening of natural hair colour)



Semi-Permanent Hair Dyes

- Pre-formed Color – direct dyes
- Color trapped in cuticle, no significant penetration
- Color washes out after 6-8 shampoos
- No lift (hair lightening)

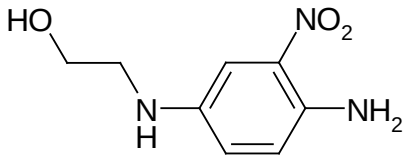


Note: some semi-permanent dyes are used in combination with oxidative hair dyes in permanent hair dye products to improve the tone of the final hair color

Structural Classes of Semi-Permanent Hair Dyes

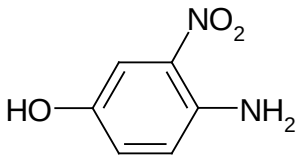
Examples of Commonly Used Classes

Nitro-phenylenediamines



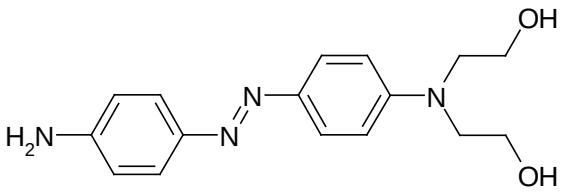
HC Red No. 7

Amino-nitrophenols



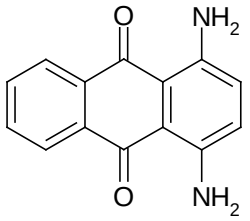
4-Amino-3-nitrophenol

Azo Dyes



Disperse Black 9

Anthraquinone Dyes

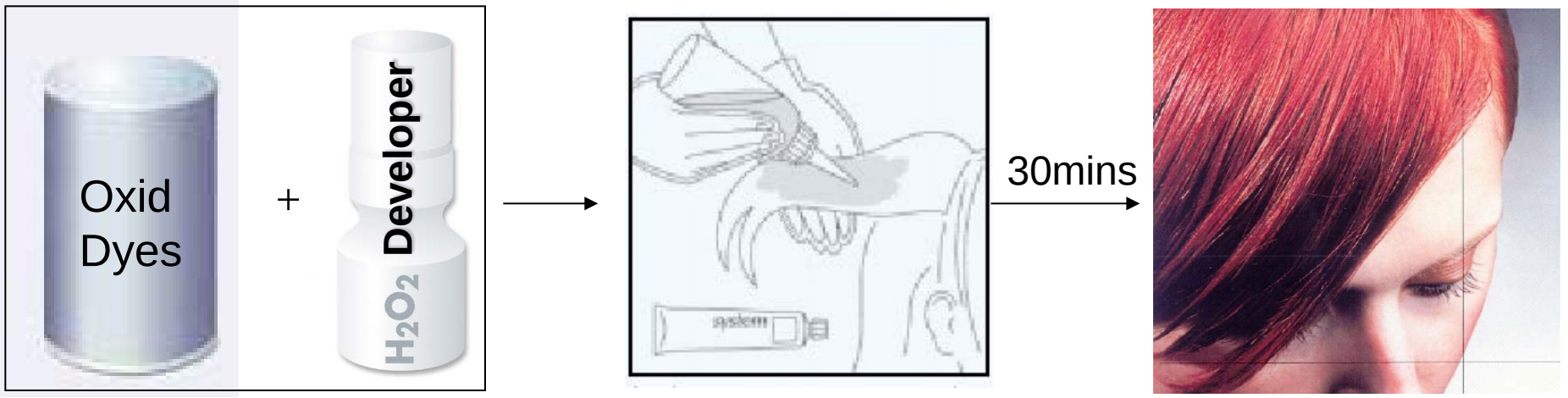


Disperse Violet 1

Oxidative Hair Dyes

- Hair dye market dominated by oxidative products (80% of market)
- Oxidative dyes are the predominant coloring technology

Typical Consumer Usage



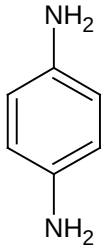
- General mode of action involves 2 simultaneous processes
  - Diffusion of small colorless molecules into the hair
  - Coupling reaction to form colored dyes in the hair

Chemistry of Oxidative Hair Dyes and their Reaction Products

Two functional classes of oxidative dyes

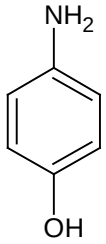
1. **Precursors** (Primary Intermediates, Developers) – 3 main chemical classes

p-Phenylenediamines



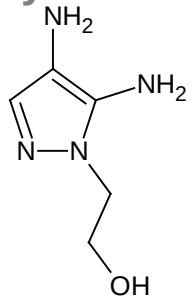
p-Phenylenediamine
A007

p-Aminophenols



p-Aminophenol
A016

Heterocyclic Diamines

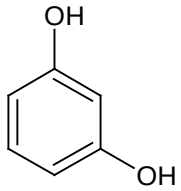


1-Hydroxyethyl-3,4-diaminopyrazole **A154**

Molecules capable of undergoing oxidation in presence of peroxide

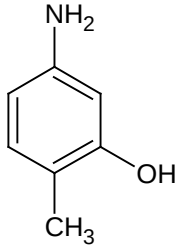
2. **Couplers** (Color modifiers) – 5 main chemical classes

Resorcinols



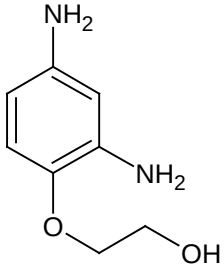
Resorcinol
A011

m-Aminophenols



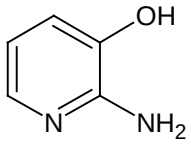
4-Amino-2-hydroxy toluene **A027**

m-Phenylenediamines



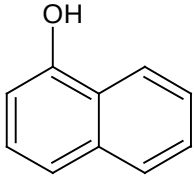
2,4-Diaminophenoxy ethanol **A042**

Pyridines



2-Amino-3-hydroxy pyridine **A132**

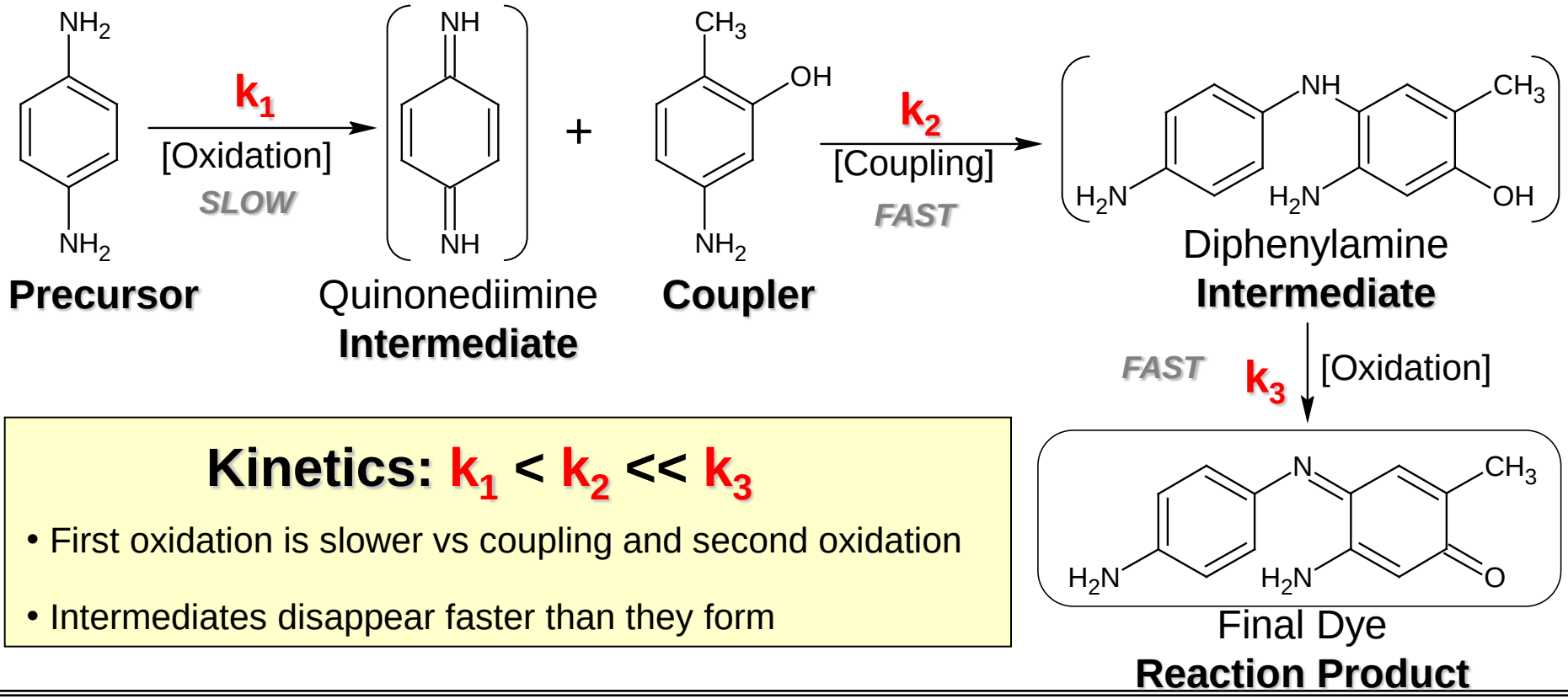
Naphthols



1-Naphthol
A017

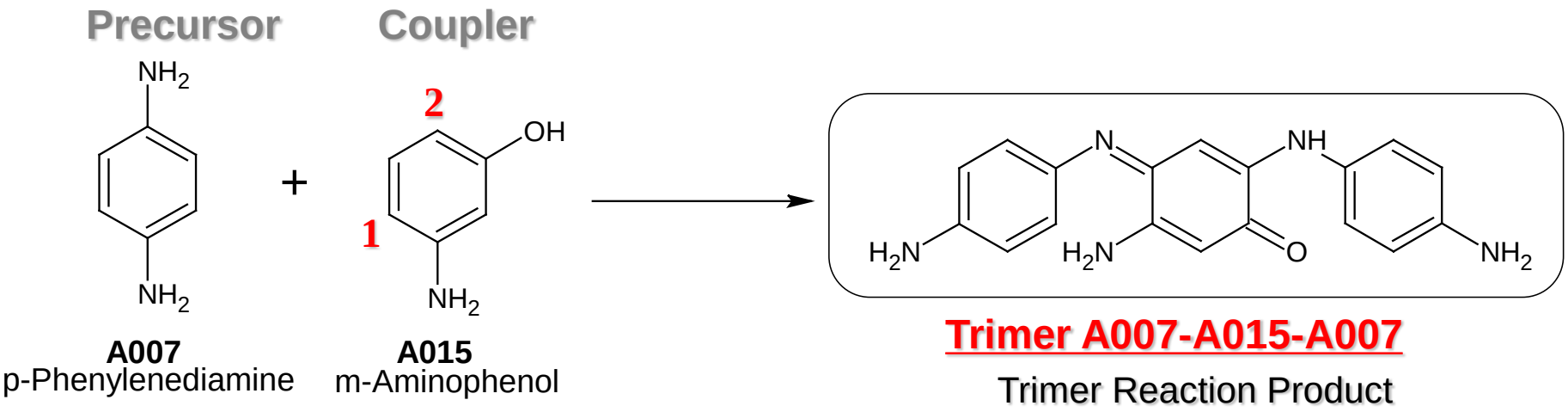
Molecules capable of reacting (coupling) with oxidized precursor

Chemistry of Oxidative Coupling Reaction

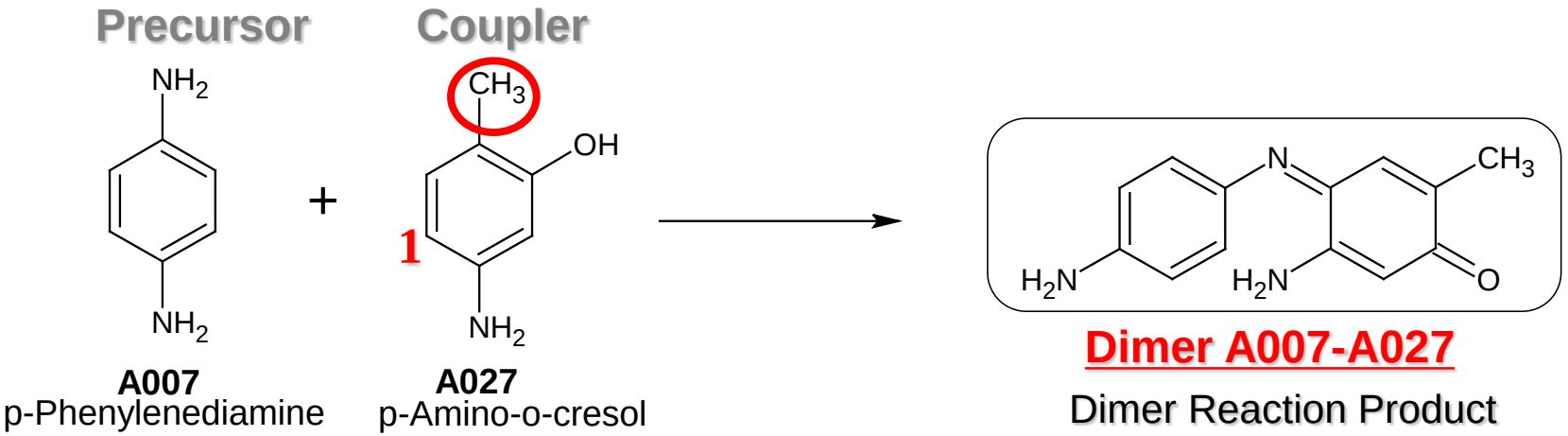


Chemical Principles of Oxidative Coupling

- Couplers with two coupling positions give trimer reaction products



- Couplers with blocked coupling position give dimer reaction products



Chemistry of Oxidative Hair Dyes and their Reaction Products

Background: Literature Review

- Wealth of information on oxidative chemistry in literature

- Series of papers published in 1970s (J Corbett, K Brown et al.)
    - Details of the chemical mechanism
    - Details of Reaction Products for selected reactions
    - Studies on Intermediate species
    - Kinetic studies detailing rates of reaction

- However studies mostly carried out under `non-relevant´ conditions
    - Studies run in solvents or solutions rather than in gel or cream formulation
    - Concentrations orders of magnitude lower than hair dye products
    - Studies run with artificial oxidants (e.g. Ferricyanide)

Chemistry of Oxidative Hair Dyes and their Reaction Products

New Industry Studies into Oxidative Coupling Chemistry

Objective: ***First Quantitative studies of oxidative hair dye chemistry and kinetics under consumer relevant conditions***

- **New quantitative HPLC analytical method developed to follow formation of reaction products**

- **Oxidative combinations studied under consumer usage conditions**
  - **Commercial cream formulation with high but realistic levels of precursors and couplers**
  - **3% Hydrogen peroxide as oxidant**
  - **Presence of human hair**
  - **30min reaction time at room temperature**

Chemistry of Oxidative Hair Dyes and their Reaction Products

- 26 precursor/coupler combination studied – representative of full range of chemistry in market
  - Highest tonnage oxidative dyes and most frequently used combinations
  - Main chemical classes of precursors and couplers
  - Formation of dimer and trimer reaction products with full range of sizes and solubilities

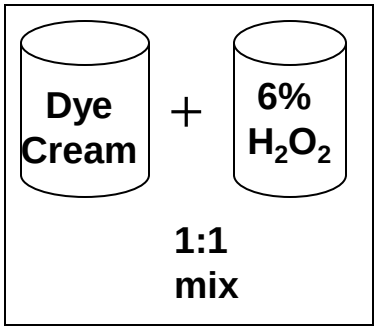
Precursor Class / Coupler Class	p-Phenylenediamines	p-Aminophenols	Heterocyclic Diamines
Resorcinols	✓ 4 examples	very slow	✓ 2 examples
m-Aminophenols	✓ 5 examples	✓ 3 examples	✓ 2 examples
m-Phenylenediamines	✓ 1 example	✓ 1 example	✓ 1 example
Pyridines	✓ 1 example	✓ 1 example	✓ 1 example
Naphthols	✓ 1 example	✓ 1 example	✓ 2 examples

Chemistry of Oxidative Hair Dyes and their Reaction Products

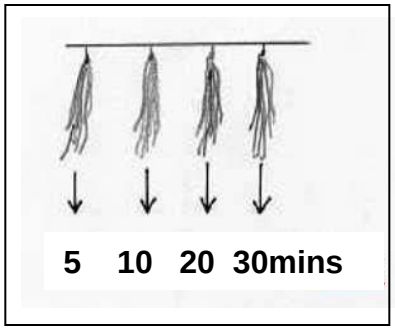
Quantitative Industry Studies into Oxidative Coupling Chemistry

Experimental Analytical Methodology:

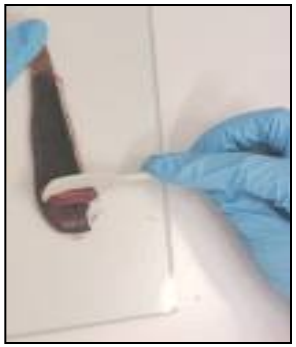
❶ **Dye cream mixed with peroxide**



❷ **Applied to human hair for upto 30mins**

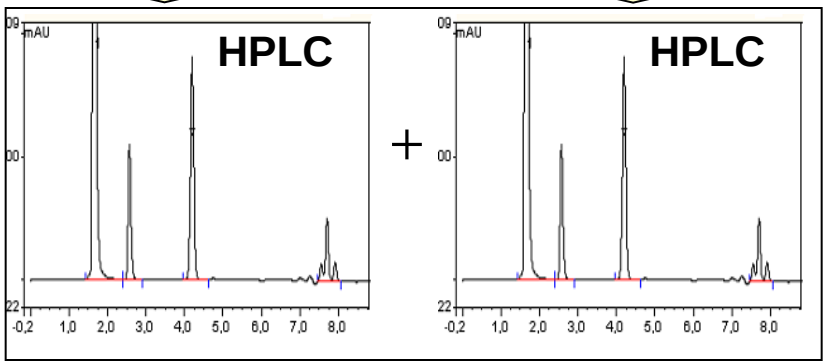


❸ **Cream scraped from hair**



❹ **Hair extracted for several hours**

- Realistic Formulation
- Replicates Consumer Use
- Formulation scraped from hair analyzed
  - ➢ Estimates consumer exposure
- Hair extraction analyzed
  - ➢ Experimental recovery

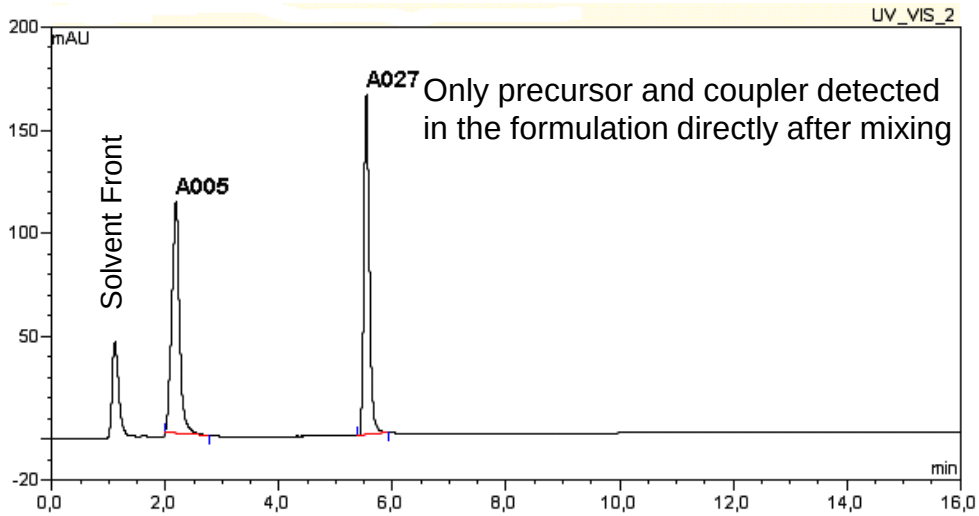


CALCULATIONS:
Concentration and Experimental Recovery

Chemistry of Oxidative Hair Dyes and their Reaction Products

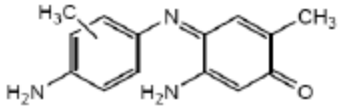
Quantitative Industry Studies into Oxidative Coupling Chemistry

Experimental Results: Example A005 + A027



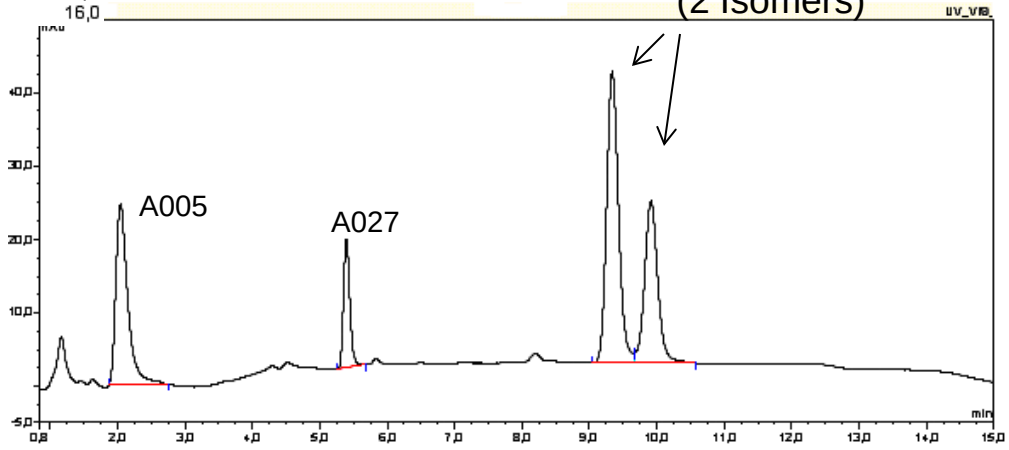
Only precursor and coupler detected in the formulation directly after mixing

HPLC analysis of cream formulation at time of application to hair (time = 0)



Reaction Product (2 Isomers)

HPLC analysis of cream formulation 30mins after application to hair

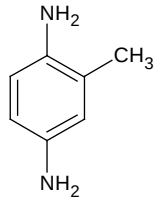


- Unreacted precursor and coupler plus expected reaction product detected
- No intermediates or self-coupling products detected under experimental conditions

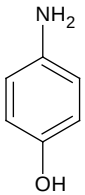
Effect of Multiple Precursors/Couplers

Commercial products can contain multiple precursors and couplers

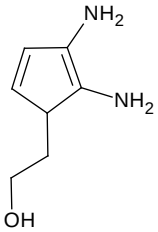
Oxidative Precursors



p-toluenediamine (A005)

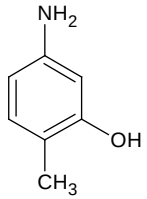


p-aminophenol (A016)

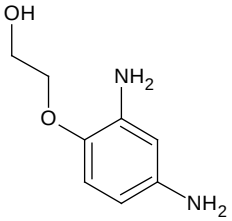


1-hydroxyethyl-4,5-diamino pyrazole (A154)

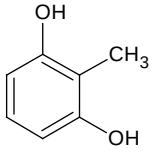
Oxidative Couplers



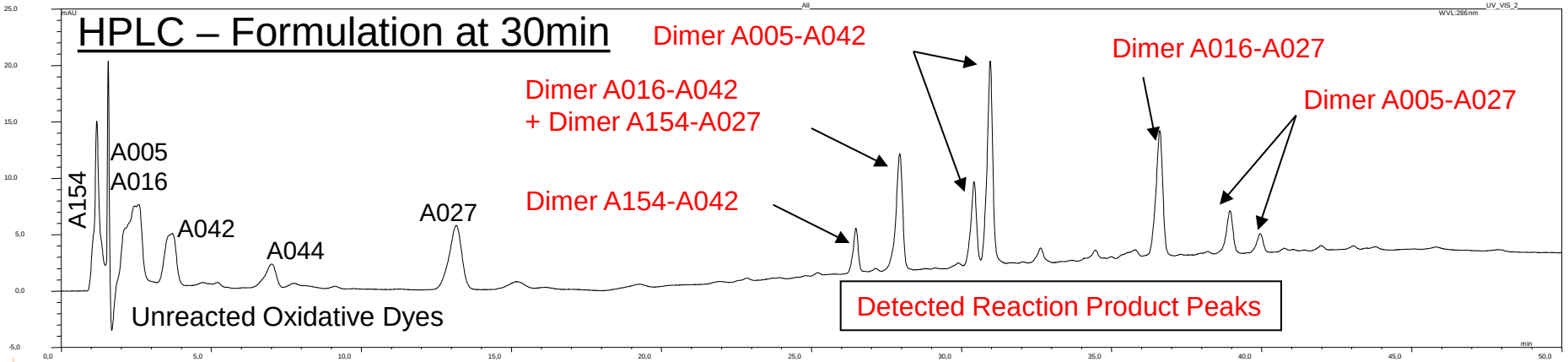
4-amino-2-hydroxytoluene (A027)



2,4-diaminophenoxy ethanol (A042)



2-methylresorcinol (A044)



Conclusion:

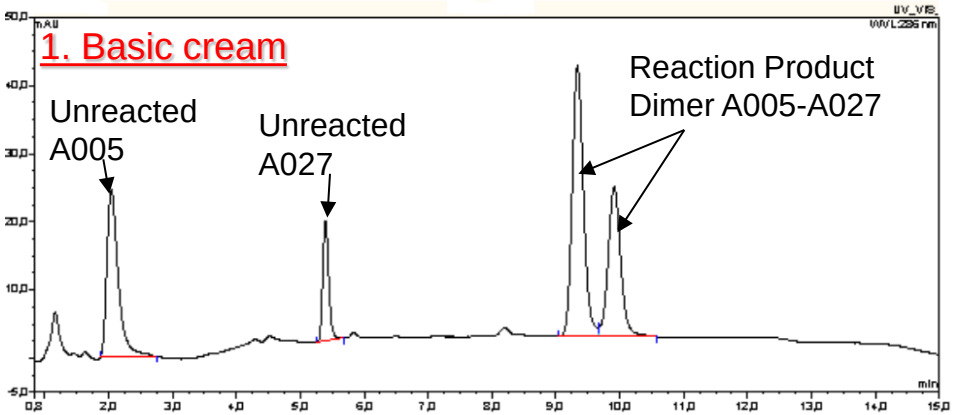
1. Major reaction products detected are those found in the relevant binary combination studies
2. Faster combinations dominate so that slower forming reaction products are not detected
3. No additional reaction products are detected
4. Binary combinations are highly predictive of more complex mixtures

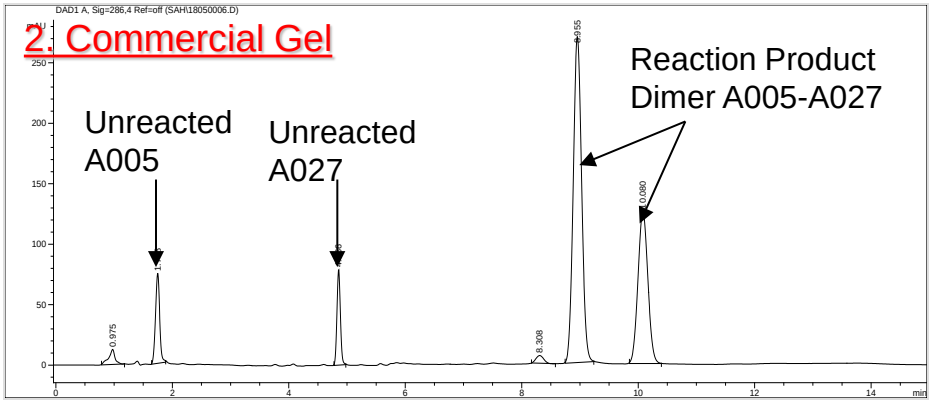
Influence of Formulation

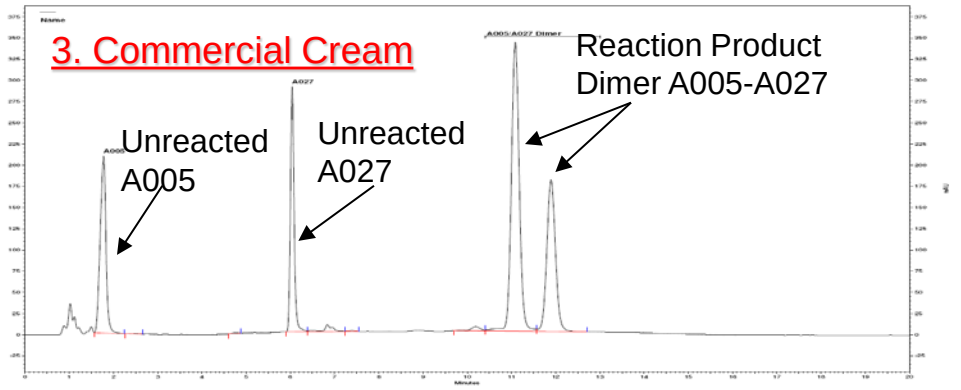
Oxidative combination between p-toluenediamine (A5) and 4-amino-2-hydroxytoluene (A27) studied in three different base formulations:

1. Basic cream formulation
2. Commercial gel formulation
3. Commercial cream formulation

All formulations analysed 30 min after mixing with peroxide and applying to human hair





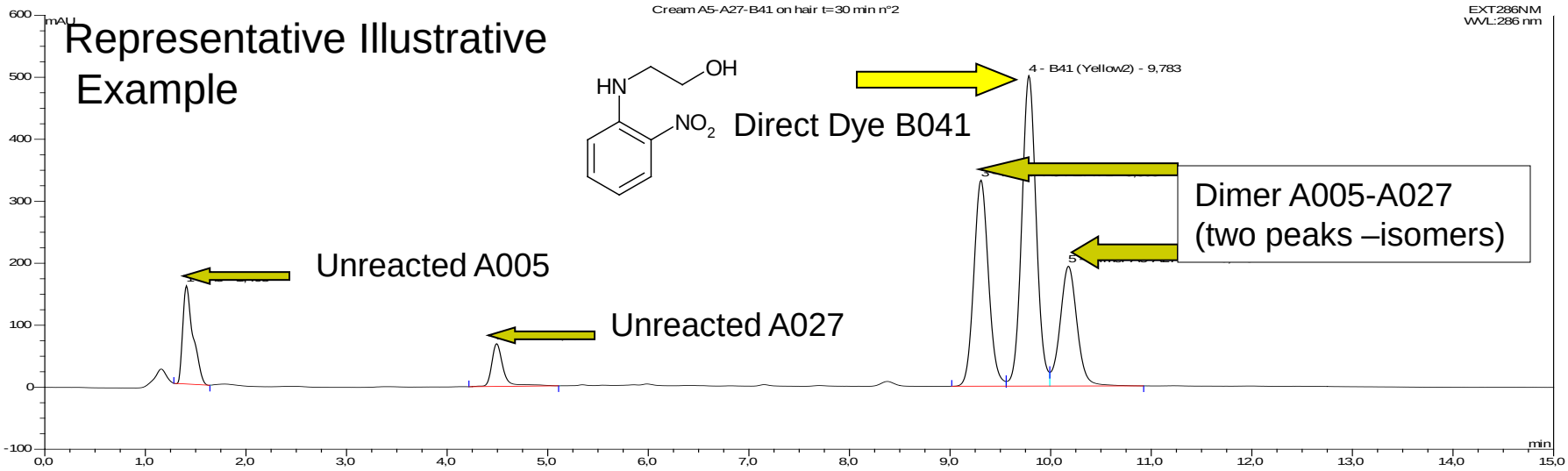


Conclusion:

- HPLC identical in all formulations
- Formulation has no effect on chemistry
- No additional reaction products

Effect of Additional Colorants – Direct Dyes

1. Limited number of direct dyes used with oxidative dyes
2. Stability of direct dyes to oxidative conditions already part of submitted dossiers
3. A005/A027 combination studied in presence of relevant direct dyes



Conclusion:

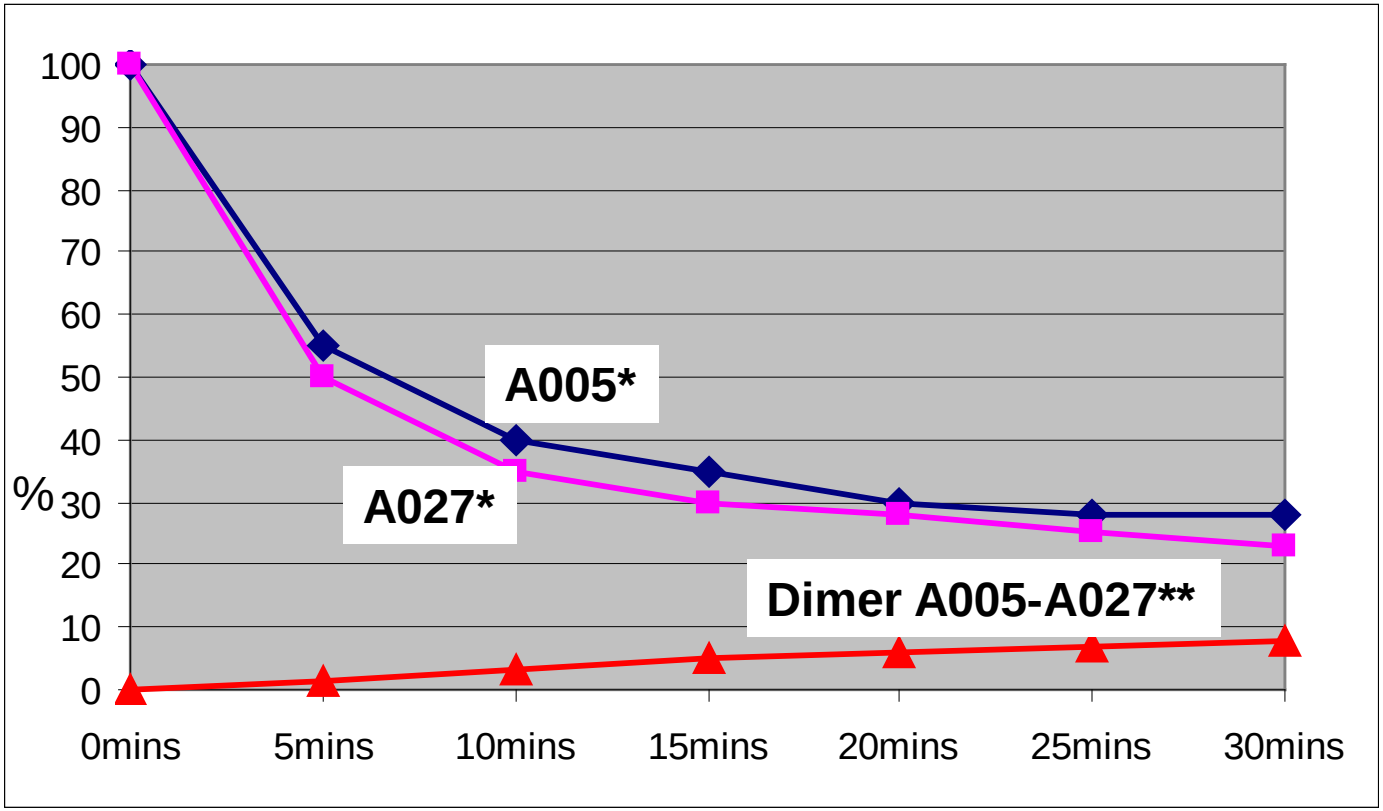
Direct dyes are inert to oxidative dyeing conditions and do not produce additional reaction products

Chemistry of Oxidative Hair Dyes and their Reaction Products

Quantitative Industry Studies into Oxidative Coupling Chemistry

Experimental Results:

- The kinetics also studied by analyzing **the cream formulation** at time intervals



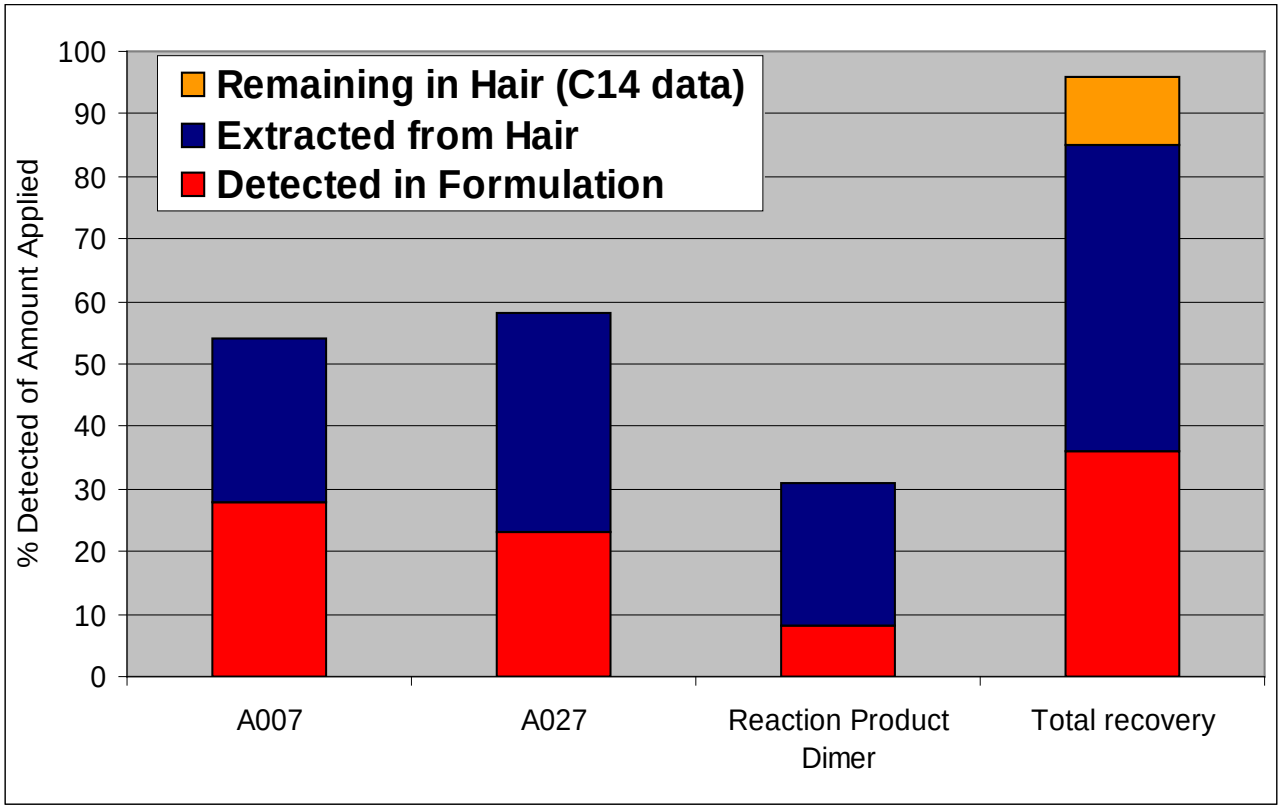
- Rapid decrease in precursor and coupler levels due to **diffusion into hair**
- Slow increase in reaction product level

*Percentage values based on the applied amount at time=0 min.

**Expressed as percentage of the theoretical maximum that would form if 100% of the A005 applied reacts with 100% of the A027 applied to the hair

Industry Studies – Experimental Results

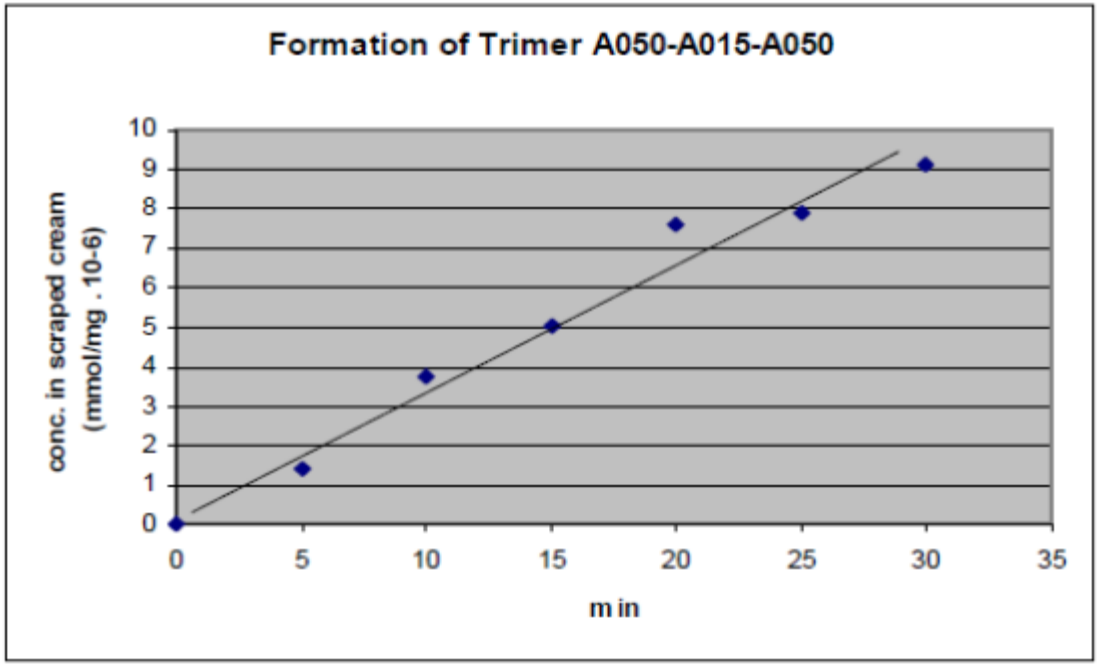
Combination A007 + A027 – Recovery and Reproducibility



Lab	Dimer (umol/g)	Recovery (%)
1	10	103
2	3	96
3	8	98
4	7	93
5	10	85
Ave	**7**	**95**

- High correlation between results of all five Industry laboratories
- Overall Recoveries excellent (85-103%) for all 5 labs
- Method reproducible

Rate of Formation of Reaction Products in Hair Dye Formulation



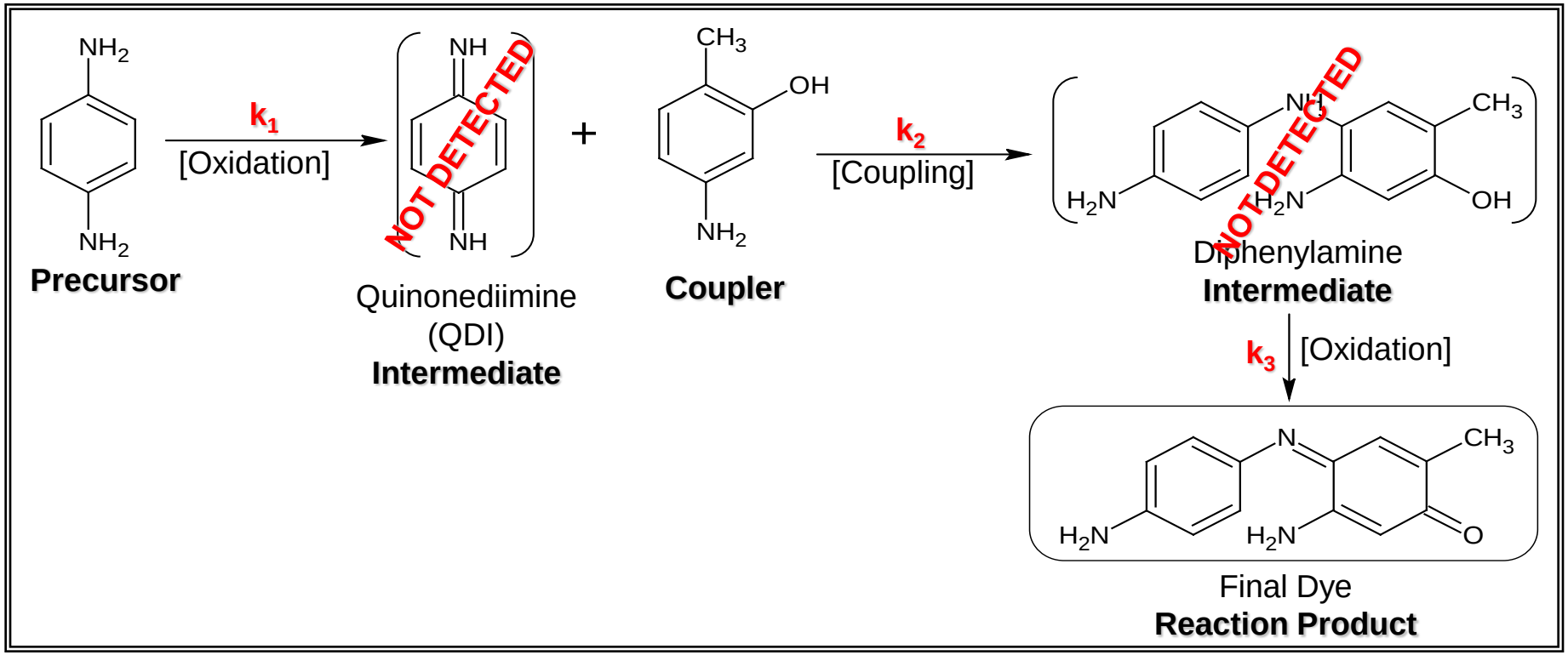
- Linear rate of formation for relatively fast coupling reactions
  - Average concentration over application time is ~50% of maximum concentration at 30 min

- Slower forming reaction products may not show linear rate of formation
  - Assume average concentration is the maximum concentration at 30 min

Chemistry of Oxidative Hair Dyes and their Reaction Products

Overall Summary:

- Industry studies are representative of the full range of chemistry found in the market
- Chemistry of oxidative coupling under consumer relevant conditions has following mechanism:



- Consumer exposure limited to unreacted precursors and couplers plus expected reaction products
- Exposure to self coupling products and intermediates (QDI) can be ruled out.
- Most of the chemistry takes place in the hair shaft
- Maximum external exposure of consumers to reaction products in range of **0.02-0.33%** (conc. of RP in formulation)

Measurement of Systemic Exposure to Reaction Products

In Vitro Dermal Penetration Studies

Dermal penetration studies on 14 RPs using OECD methodology but conducted to allow:

- Realistic on-head exposures to reaction products and differentiation between epidermal and dermal compartments
  - ✓ Three different concentrations tested including the maximum in-product level produced with a minimum of 12 samples from at least 4 donors/concentration
  - ✓ Sampling period extended to 72 hours for kinetics of penetration into the receptor fluid and for determining any potential for a reservoir effect in the skin
  - ✓ Heat separation of skin for improved quantification in different biological compartments
- Huge efforts invested in increasing sensitivity of analytical methods (trace analytics) utilizing state-of-art HPLC, LC-MS or radiolabelling techniques

Typical Formulation used for Dermal Penetration Studies

Ingredient	Concentration in Cream Formula (%)
Cetearyl Alcohol	7.0
Lanolin Alcohol	1.0
Sodium Laureth Sulfate (28%)	10.0
EDTA	0.1
Sodium Sulfite	0.4
Ascorbic Acid	0.3
Ammonia (25%)	4.55
Water	Up to 100

NOTE: This formulation is representative of commercial hair dye formulations. The reaction product to be studied is added to this formulation followed by mixing in a 1:1 ratio with a developer formulation containing 6% aqueous peroxide (or placebo developer without peroxide). The pH of the formulation is ~10.

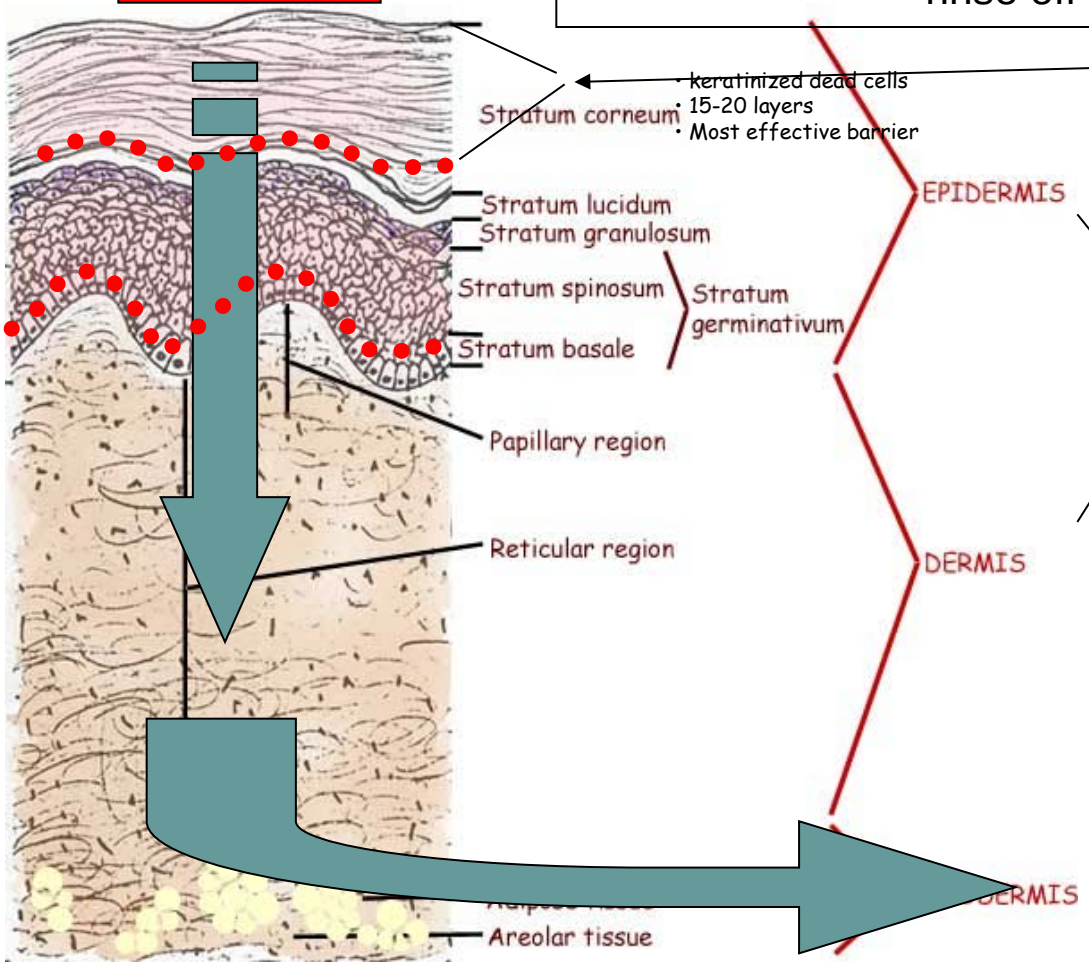
Dermal Penetration Study Design

Exposure - 20mg/cm² [hair dye formulation containing RP mixed1:1 with (placebo) developer]

(non-viable human skin)

-rinse off after 30 min

2. Tape stripping



3. Heat Separation

1. Receptor fluid Samples collected over 72 hours (flow through)

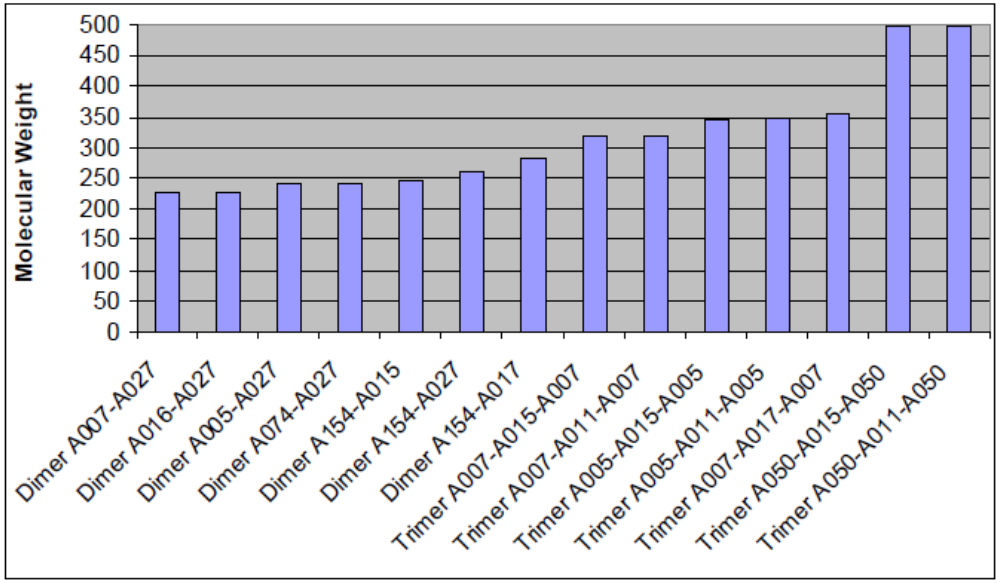


Figure 5: Range of reaction product molecular weights covered by studies

Reaction products studied for dermal penetration cover a MW range of 229 to 490 and Log D values at pH 10 of +1.8 to -3.5

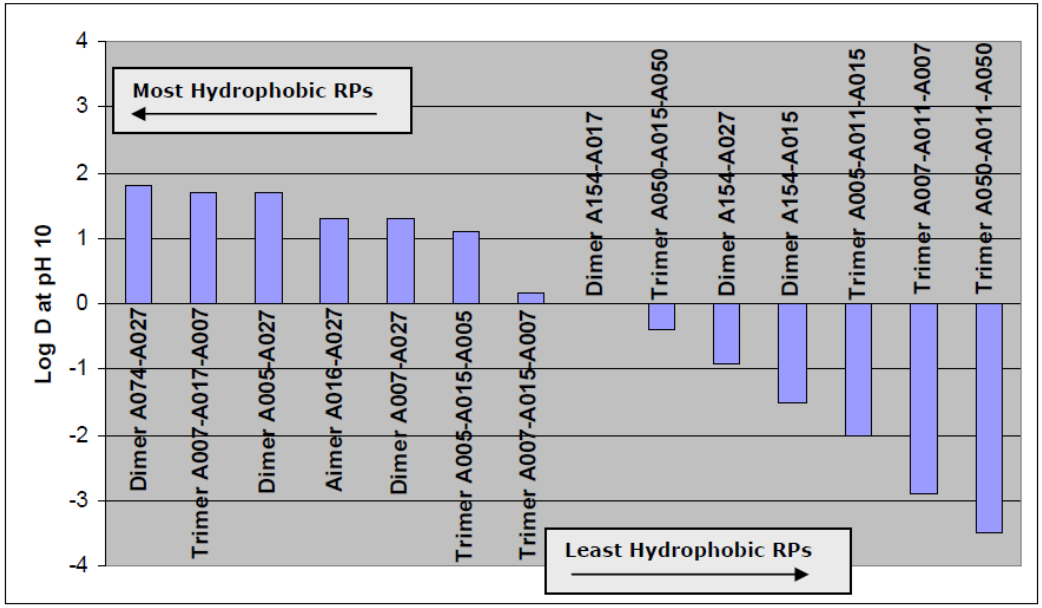


Figure 6: Range of hydrophobicities (based on Log D at pH 10) covered by studies

Calculated Human Exposure to Reaction Products from Dermal Penetration Studies

Exposure per day = bioavailable dose (ng/cm^2) X scalp surface ($580\ cm^2$) X frequency per day (1/28 days)
(receptor fluid + dermis (+epidermis)[a]

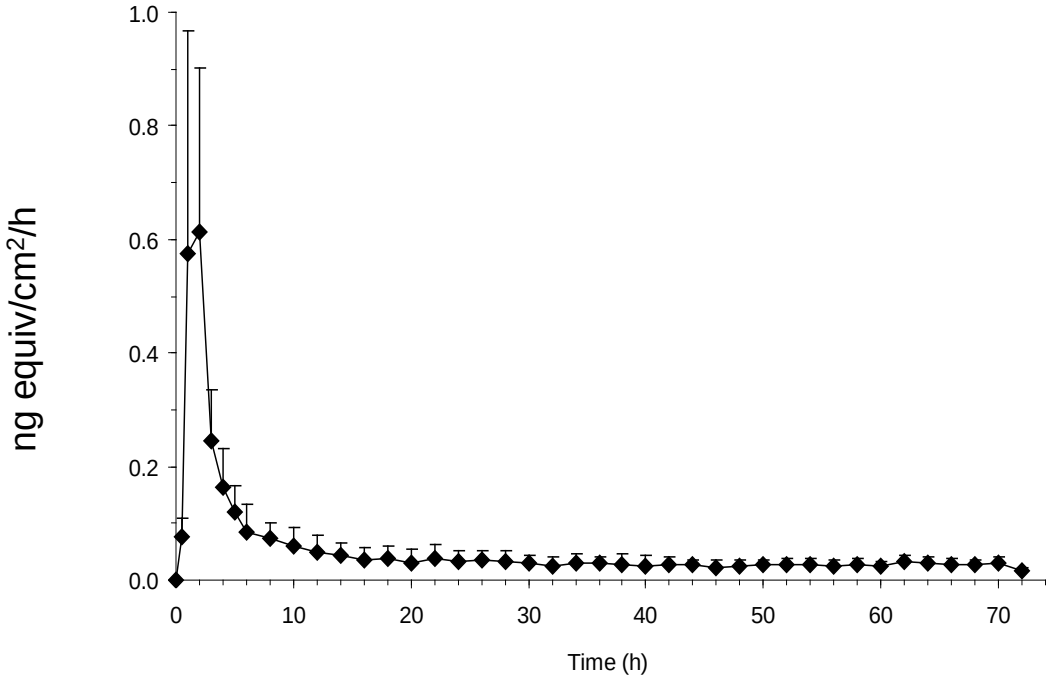
Reaction Product	Quantity of RP in the final formulation applied to consumer (based on kinetic studies) (%)	Most relevant tested RP concentration (%)	Estimated Bioavailability (receptor fluid + dermis) ($ng/cm2$)[a]		Exposure per day (µg/day)	
			Mean	Mean+1SD[b]	Mean	Mean+1SD
A050-A015-A050	0.25	0.25	2.27	3.27	0.05	0.07
A050-A011-A050	0.016	0.05	2.67	4.30	0.06	0.09
A154-A027	0.33	0.35	2.76	12.53	0.06	0.26
A007-A011-A007	0.03	0.03	4.54	7.54	0.09	0.16
A154-A015	0.16	0.16	4.72	10.54	0.10	0.22
A005-A011-A005	0.03	0.1	6.75	16.21	0.14	0.34
A005-A027	0.12	0.1	7.08	17.78	0.15	0.37
A007-A015-A007	0.03	0.1	7.79	11.61	0.16	0.24
A154-A017	0.16	0.15	27.87	7.79[c]	0.58	0.16[c]
A007-A027	0.08	0.1	28.0	44.30	0.58	0.92
A007-A017-A007	0.14	0.15	64.66	95.25	1.34	1.97
A074-A027	0.08	0.1	175	263.7	3.64	5.46
A005-A015-A005	0.07	0.1	305	461.9	6.32	9.57
A016-A027	0.07	0.1	436	717.8	9.04	14.87

[a]If a reservoir effect must be assumed based on skin penetration results, epidermis is included in bioavailable dose

[b]SCCS opinion used mean+1SD values whereas industry submission used mean values for exposure calculations

[c]For this RP, SCCS apparently assumed no reservoir effect, so epidermis was excluded from mean +1SD, whereas industry assumed a reservoir effect

Representative Dermal Penetration Profile
No Reservoir Effect



Penetration profile (ng equiv/cm²/h) in receptor fluid following topical application of a typical hair dye formulation containing [^{14}C]-A007-A015-A007 (0.1%) to human split-thickness skin (mean + SD, n=11 from 6 donors)

In Vitro Dermal Penetration Studies with Reaction Products Summary

- 14 Reaction products representing the full range of physico-chemical properties (molecular size, molecular weight, and hydrophobicity) were studied

- Amounts of bioavailable reaction product ranged from:
  - 2.27 - 436 ng/cm^2 (mean values)
  - 3.27 - 718 ng/cm^2 (mean +1SD values; reported in SCCS/1311/10)

- Calculated exposure per day for reaction products ranged from:
  - 0.05 - 9.04 µg/day (mean values)
  - 0.07 – 14.87 µg/day (mean +1SD values; reported in SCCS/1311/10)

- Dermal penetration of reaction products is signficantly lower than precursors/couplers
  - Mean dermal penetration of PPD (Hueber-Becker et al. 2004): 10.6 ± 6.7 µg/cm^2
  - Mean dermal penetration of reaction products: 0.0023-0.436 µg/cm^2
  - Dermal penetration of reaction products ranges from ~24 –4600 fold lower than dermal penetration of PPD

Human Exposure Study with a ^{14}C-PPD-Containing Hair Dye

Study Design

- Sixteen male volunteers had their hair dyed with a formulation containing [^{14}C]-PPD (final on-head concentration of 1%) with a total contact time of 30 minutes
- Formula also contained unlabeled resorcinol (0.5%) (A11) and m-aminophenol (0.5%) (A15) as couplers
- Hair shampooed, rinsed, dried, and clipped; protective cap worn from the time after hair clipping until the morning of Day 2; scalp washed on Day 2(~24 hours post clipping)
- Blood samples drawn at 2, 4, 6, 10, 24, and 48 hr after hair dyeing
- Urine sampling: 0-12 , 12-24, and 24-28 hr
- [^{14}C] measured by liquid scintillation counting: clipped hair, wash water, caps, materials (comb, brush, towels, gloves), plasma, and urine – mass balance determined
- HLPC –MS/MS methods also used for analysis of plasma and urine
  - Quantification of reaction products (A7-A11-A7 and A7-A15-A7) and their mono- and diacetylated metabolites of both reaction products
  - Quantification of PPD (A7) and its mono- and diacetylated metabolites

Reference: Nohynek et al., Food and Chemical Toxicology 81 (2015) 71–80

Human Exposure Study
Mass Balance Results

COMPARTMENT	(1.0% [^{14}C]-PPD on-head)	
	Mean	Range
Washing water	64.91 ± 6.23	58.4 – 74.8
Hair	30.25 ± 4.58	19.9 – 36.1
Coloring materials *	14.30 ± 10.40	3.6 – 36.2
Drying materials **	0.17 ± 0.09	0.08 – 0.5
Scalp rinse	0.14 ± 0.06	0.06 – 0.26
Protective cap	0.017 ± 0.012	0.01 – 0.006
Urine (0-48hours)	0.88 ± 0.46	0.40 – 2.06
Mass balance	96.21 ± 1.57	93.59 – 98.59

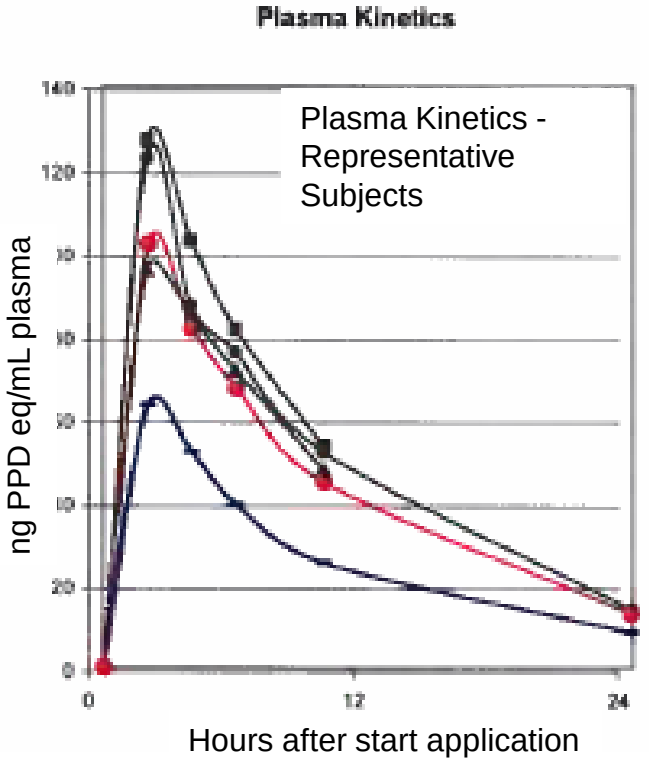
* Mixing bowl, brush, gloves
** Towels and gloves

Reference: Nohynek et al., Food and Chemical Toxicology 81 (2015) 71–80

Human Exposure Study
Plasma Kinetics [^{14}C]-PPD$_{eq}$

Parameter	Unit	Mean	S.d.	Min	Max
C_{max}	ng PPD_{eq}/mL	97.4	61.5	20.9	234.0
T_{max}	h	2.0	0.0	2.0	2.0
$K_{e\ (elimination)}$	/h	-0.109	0.044	-0.201	-0.027
$T^{1}/_{2}$	h	7.8	5.1	3.5	25.8
$AUC_{\ 0\text{-}24\ h}$	ng PPD_{eq}/mL.h	762	401	410	1727
$AUC_{\ 0\text{-}\infty}$	ng PPD_{eq}/mL.h	966	575	228	1946

Plasma kinetic data for [^{14}C]-PPD$_{eq}$ show a mean C_{max} of 97.4+61.5 ng/mL, a T_{max} of 2 hrs and a mean $AUC_{0\text{-}\infty}$ of 966 $\pm$ 575 ng/mL·hr.

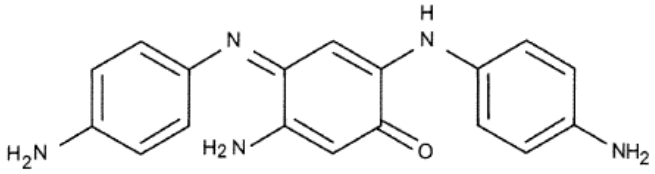


Reference: Nohynek et al., Food and Chemical Toxicology 81 (2015) 71–80

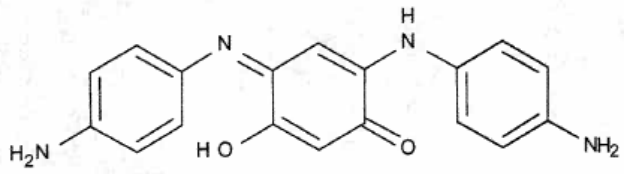
Human Exposure Study Analytes Measured by HPLC-MS/MS

Reaction Products and Metabolites

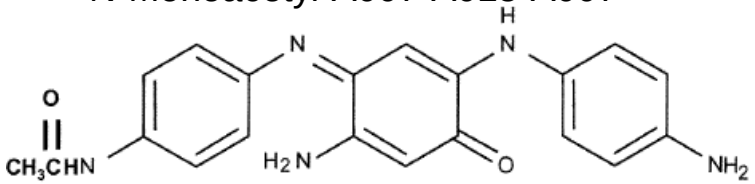
A007-A015-A007



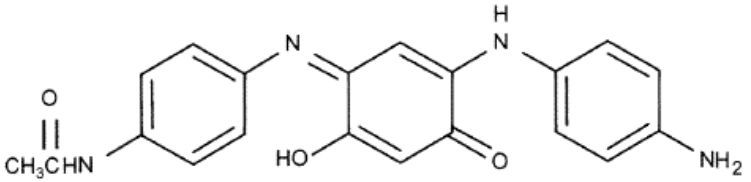
A007-A011-A007



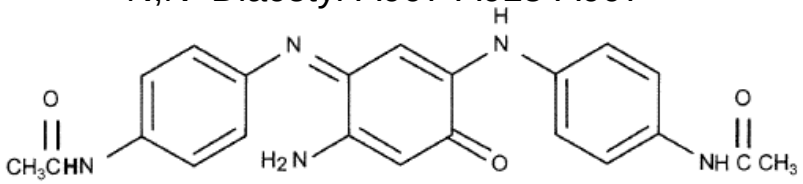
N-Monoacetyl-A007-A015-A007



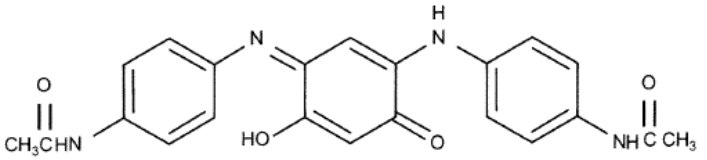
N-Monoacetyl-A007-A011-A007



N,N'-Diacetyl-A007-A015-A007

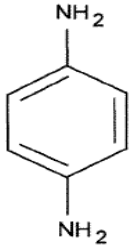


N,N'-Diacetyl-A007-A011-A007

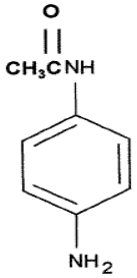


PPD and Metabolites

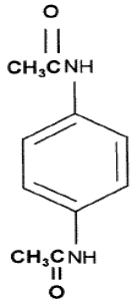
PPD



N-Acetyl-PPD



N,N'-Diacetyl-PPD



Lower Limits of Quantification (LLOQ) of the HPLC-MS/MS Analytical Methods for Reaction Products and their Potential Metabolites

Substance	Plasma (pg/mL)	Urine (pg/mL)
A007-A011-A007	100	400
N-monoacetyl-A007-A011-A007	160	500
N,N'-diacetyl-A007-A011-A007	800	4000
A007-A015-A007	100	1280
N-monoacetyl-A007-A015-A007	160	1280
N,N'-diacetyl-A007-A015-A007	320	800

Human Exposure Study
Results of Plasma and Urine Analyses for Reaction Products

Plasma

- RPs were not detectable in most plasma samples
- Traces of RP or acetylated metabolites were detected in 4/96 plasma samples from 4/16 volunteers
  - Concentrations at or slightly above the relevant $LLOQ_{plasma}$
  - Concentrations were ~1000 fold lower than the C_{max} for N,N'-diacetyl-PPD (parent PPD was not detected in plasma)

Urine

- RPs were not detectable in most urine samples
- Traces of RPs or acetylated metabolites were detected in 4/48 urine samples – all samples were from the 0-12h collection
  - Concentrations were slightly above the relevant $LLOQ_{urine}$

Comparison of Human Exposure to Reaction Products
Human Study Results vs In Vitro Skin Penetration Results

Exposure Model	Reaction Product Exposure			
	A007-A015-A007 (ng/event)	A007-A011-A007 (ng/event)	A007-A015-A007 (ng/day) †††	A007-A011-A007 (ng/day) †††
In vitro Skin penetration study Mean absorbed dose	4518	2633	161	94
In vivo Maximum excretion found in the human study†	1881	1462	67	52
Mean exposure values estimated for the human study taking into account samples without detectable levels††	826	417	30	15

† Results from Subject #13 who had the highest levels of RP A007-A015-A007 plus mono-acetylated metabolite and the highest level of RP A007-A011-A007 in the 0-12 hr urine sample

†† For the volunteers with urine concentrations below the LOQ_{urine}, 0-12 hr samples were assumed to contain RP A007-A011-A007 or A007-A015-A007 at 50% LOQ_{urine} according to Beal (2001). For the few subjects with urine levels above the LOQ_{urine} in 0-12 hr urine samples, the measured values were used.

††† use frequency of oxidative hair dyes of once every 4 weeks (28 days)

Measured exposure from the subject with the highest excretion of reaction products was ~2 fold lower than mean exposure levels estimated from in vitro dermal penetration studies. Conservative estimates of mean exposure in study subjects was ~5-6 fold lower than the in vitro study estimates. The in vitro studies overestimate actual human exposure

Comparison of Human Exposure to PPD and Its Metabolites vs. Reaction Products and Their Metabolites

PPD and Metabolites

- Mean Total Urinary Excretion (N=16)

N,N'-Diacetyl-PPD	3067 µg
N-Acetyl-PPD	4.4 µg
PPD	8.8 µg
Total	3080 µg

Human exposure to reaction products of PPD formed during hair coloring was 3 orders of magnitude lower than exposure to PPD precursor based on urinary excretion measurements

Reaction Products and Metabolites

- Maximum Total Urinary Excretion (Subject 13)
  - A007-A015-A007 0.96 µg
  - N-Acetyl-A007-A015-A007 0.92 µg
  - N,N'-Diacetyl-A007-A015-A007 Not Detected
  - Total 1.88 µg

  - A007-A011-A007 1.46 µg
  - N-Acetyl-A007-A011-A007 Not Detected
  - N,N'-Diacetyl-A007-A011-A007 Not Detected
  - Total 1.46 µg

Conclusions of Industry Studies on Exposure to Reaction Products

- **Chemistry investigation of 26 representative oxidative coupling reactions**
  - Identification and quantification of major reaction products formed
  - Results used to select relevant reaction product concentrations for *in vitro* dermal penetration studies
  - Analytical studies showed exposure to intermediates can be ruled out
- ***In vitro* dermal penetration studies with representative set of 14 reaction products**
  - Very low levels of exposure
  - Dermal penetration of reaction products under conditions relevant to hair dyeing is substantially lower than dermal penetration of precursors/couplers
- **Human exposure study**
  - Extremely low exposure to reaction products confirmed
  - *In vitro* dermal penetration studies shown to over-estimate actual human exposure (~5-6 fold)
  - Confirmed that exposure during hair dye use is predominantly to precursors and couplers – human exposure to reaction products was 3 orders of magnitude lower than exposure to PPD precursor

Genotoxicity Testing of Reaction Products

Selection of Reaction Products for Genotoxicity Testing

- Selected reaction products at the upper end of the range of systemic exposures estimated from *in vitro* dermal penetration studies
- Both dimers and trimers are represented
- Contain the structural alerts for carcinogenicity and mutagenicity/genotoxicity consistently present across the spectrum of RPs formed from all major classes of oxidative hair dye precursors/couplers
- Major classes of precursors (p-phenylenediamines, p-aminophenols) and couplers (m-aminophenols, naphthols) are represented

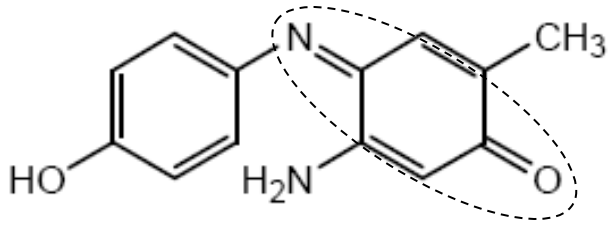
Structure Activity Relationship (SAR) Analysis of RPs for Carcinogenicity & Genotoxicity Potential

- DEREK analysis identified no additional alerts for carcinogenicity involving genotoxic mechanism beyond those already identified in precursors/couplers (aromatic amines)

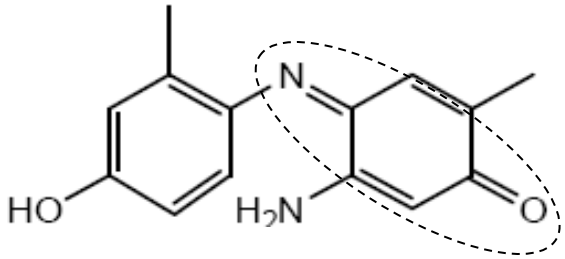
- Only one additional structural feature found in RP not found in P/C i.e. the benzoquinone imine

  - This gives rise to DEREK alerts for mutagenicity/genotoxicity
  - Highly stabilized by conjugated reaction product structure – not expected to behave like a free benzoquinone imine
  - If the benzoquinone imine alert in the RPs behaved as a genuine quinone imine, the RPs would continue to react
  - Potential for genotoxicity associated with this structural feature was addressed by testing four representative reaction products

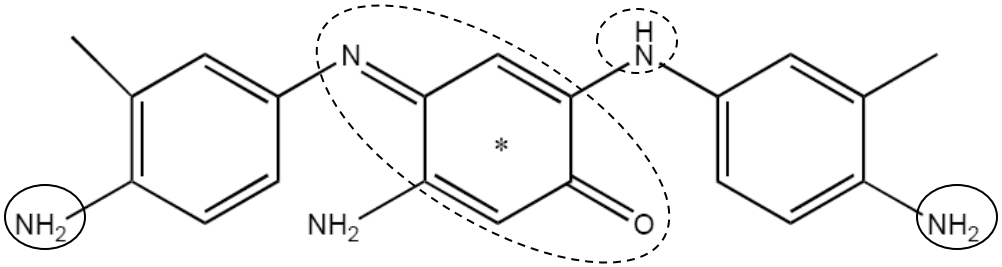
Reaction Products Tested in Genotoxicity Assays



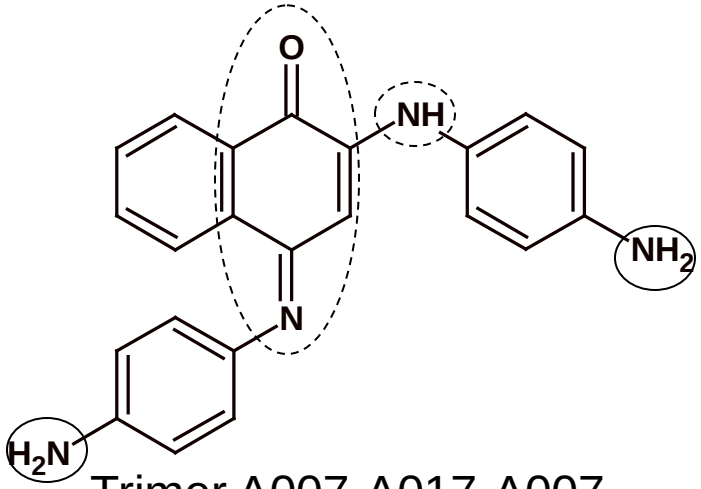
Dimer A016-A027



Dimer A074-A027



Trimer A005-A015-A005



Trimer A007-A017-A007

DEREK Structural Alerts: Solid circles – primary aromatic amine
Dashed circles – secondary aromatic amine
Dashed ovals - benzoquinone imine

Results of Genotoxicity Testing of Selected Reaction Products

Table 14: Overview of genotoxicity test results of the four reaction products tested

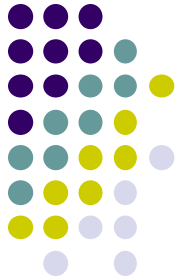
Reaction Product	*In vitro* Tests			*In vivo* Tests	
	Ames	hprt	MN	MN	UDS
A016-A027	+	-	-	-	-
A074-A027	-	-	+	-	Not performed
A005-A015-A005	+	-	-	Not performed	-
A007-A017-A007	+	-	-	Not performed	-

Ames: Bacterial Reverse Mutation Test
hprt: mammalian cell gene mutation assay (hprt locus)
MN: Micronucleus test
UDS: In vivo unscheduled DNA synthesis

Qualitatively similar profile of genotoxicity testing results for RP compared to precursors and couplers:

- Positive in some *in vitro* genotoxicity assays; no evidence of genotoxicity *in vivo*
- Suggests that benzoquinone imine DEREK alert for reaction products does not confer a concern for *in vivo* genotoxicity

Overall Summary/Conclusions



Reaction Products:

- **Identified and Quantified**
- **Extremely Low Exposure**
- **No Evidence for In Vivo Genotoxicity**

Safety assessment of oxidative hair dyes is driven by the toxicological evaluation of the ingredients (i.e., precursors and couplers) rather than by the reaction products formed during use