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Dosage du polyisoprène et des résines de la biomasse de guayule (*Parthenium argentatum*) par spectroscopie proche infrarouge (SPIR) Méthodes d'extraction par solvant de référence

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Dosage du polyisoprène et des résines de la biomasse de guayule (*Parthenium argentatum*) **par spectroscopie proche infrarouge (SPIR) Méthodes d'extraction par solvant de référence**

Un protocole basé sur l'extraction accélérée (ASE) avec l'acétone (EA) (résine) puis l'hexane (EH) (polyisoprène, PI) a été sélectionné et optimisé (rendement maximal; adapté à l'analyse de séries) ; quantification d'abord basée sur le poids de l'extrait (gravimétrie). L'EH est maximal à 120°C après étapes avec l'acétone à 40°C (plan d'expérience). La contamination croisée a été confirmée par SEC et FTIR (5 à 29%), conduisant à une deuxième méthode basée sur résine et PI et non plus sur EA et EH, incluant le PI de faible masse molaire (*Mw*) de l'EA. Ces 2 méthodes de référence ont servi à calibrer la SPIR (chimiométrie/PLS, coeff. beta) afin de relier signature spectrale, PI, résine. ASE-SPIR, couplés ici pour la première fois, ont été plus performants (R^2 0.96; 0.98; RPD 4.8; 4.6; EA et EH resp.) que les méthodes de la littérature, grâce aux 215 échantillons représentatifs (génotypes, saison, âge du guayule, climat). La méthode tenant compte de la contamination est moins performante (erreur exp. due aux analyses SEC et FTIR; variation de composition des résines). Ayant montré la dégradation du PI au cours de l'extraction, un autre protocole a été étudié (biomasse humide, un solvant, une seule étape, 20°C) afin d'accéder au *Mw «*natif », donnant 2.10^6 g au lieu de 6.10^5 avec la biomasse séchée; il faut donc être prudent face aux *Mw* de guayule de la littérature. Ce travail montre la nécessité de tenir compte de la complexité de la biomasse de guayule (échelle cellulaire/PI vacuolaire, résine des canaux; moléculaire/instabilité chimique) lors de l'extraction du PI pour l'analyse structurale. Ces méthodes analytiques ont contribué à produire des prototypes (gant non allergisant, pneu) à haut *Mw* et à l'acclimatation en Europe dans le cadre du projet EU-Pearls.

Mots-clés: guayule, polyisoprène, résines, extraction, spectroscopie proche infrarouge

Measurement of resin and polyisoprene in *Parthenium argentatum* (guayule) biomass using near infrared spectroscopy (NIRS) -Associated solvent-based reference methods

A protocol based on sequential extraction with acetone (resin) and hexane (polyisoprene, PI) with accelerated solvent extraction (ASE) was optimized and selected (maximized yield; adapted to large series) instead of Soxhlet and homogenizer. Quantification was first based on extract weight (gravimetry). Hexane extract was maximized at 120°C, after acetone steps at 40°C, through an experimental design. Cross contamination was confirmed and quantified (5 to 29%; SEC and FTIR). This gave a second method based on resin and PI, instead of crude extracts, accounting for low average molar mass PI (*Mw*) extracted by acetone instead of hexane. Both reference methods were used for calibrating NIRS applied to powdered biomass, with chemometric tools (PLS loadings, beta coefficients) to interpret spectral bands vs PI-resin relationship. ASE, not used before as reference, is highly reliable, and calibration with gravimetry (R^2 0.96; 0.98; RPD 4.8; 4.6; for acetone and hexane extract) better than published data, thanks to the 215 samples covering genotypes, harvest date, plant age, climate. The method using cross contamination was less efficient because of higher experimental error induced by additional SEC and FTIR, and change in resin composition. Having set NIRS methods, a new protocol (single solvent THF, minimized processing, 20°C, fresh biomass) was designed to avoid degradation, yielding PI extracts with *Mw* above 2.10^6g/mole, closer to in vivo structure (6.10^5 when using dried guayule); caution to sample preparation in literature dealing with guayule PI structure. This calls for considering the complex structure of guayule biomass (PI in cells; resin in ducts; chemical instability) when extracting PI. These methods allowed producing high PI *Mw* glove and tire prototypes and domesticating this new crop in Europe within the EU-Pearls project.

Keywords: guayule, polyisoprene, resin, extraction, near infrared spectroscopy

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ABBREVIATION

ASTM	- American Society for Testing and Materials
^{13}C-NMR	- Carbon thirteen Nuclear magnetic resonance spectroscopy
FTIR	- Fourier transform infrared spectrometer
^{1}H-NMR	- Proton Nuclear magnetic resonance spectroscopy
IR	- Infrared Spectroscopy
GR	- Guayule rubber
GL	- Guayule latex
LOD	- limit of detection
LOQ	- Limit of quantification
MDS	- Molecular weight distribution
MMD	- Weight average molar mass distribution
Mn	- The weight-average molecular weight
M_w	- The weight-average molecular weight
NIRS	- Near Infrared Reflectance Spectroscopy
NR	- Natural rubber
MPLS	- Modified partial least squares
NIRS	- Near infrared reflectance spectroscopy
R^2	- Simple coefficient of determination
RMS	- Root mean square
SEC	- Standard error of calibration
SECV	- Standard error of cross validation
SEC-MALLS	- size exclusion chromatography coupled multi-angle light scattering detector
SEL	- Standard error of laboratory determination
SM	- Soft extraction method
SR	- Synthetic rubber
THF	- Tetrahydrofuran

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RESUME EN FRANÇAIS

Objectif de la thèse

L'objectif est la mise au point d'une méthode de quantification rapide de la teneur en poly-isoprène (PI) à partir de biomasse séchée et broyée, par spectroscopie proche infra-rouge (SPIR), élargie au dosage des résines associées (lipides, sesquiterpènes), afin de faciliter les études d'acclimatation, de sélection variétale et d'un procédé de bioraffinerie, dans le cadre du projet EU-Pearls. Si la méthode finale recherchée par SPIR est par essence non destructive, l'ambition du projet était aussi de pouvoir utiliser le protocole de référence de la calibration pour déterminer les caractéristiques structurales du PI extrait (masse molaire moyenne (*Mw*); taux de ramification); ce PI doit donc être aussi semblable que possible au polymère in vivo. Les contraintes contractuelles du projet EU-Pearls encadrant la thèse-le protocole par SPIR devant être mis en œuvre par les partenaires agronomes sur les parcelles expérimentales afin d'étudier l'évolution des plants- ont conduit à séparer les deux objectifs ci-dessus, donnant la priorité à la méthode de dosage sans chercher à éviter la dégradation du PI, cette dernière n'ayant pas a priori une forte influence sur le dosage. Le deuxième objectif (extraction de PI « natif ») n'a été poursuivi qu'au cours de la dernière année de la thèse.

Introduction

Le mémoire est organisé en trois parties. La revue bibliographique (première partie) comprend quatre chapitres, faisant le point sur le guayule en tant que végétal destiné à la bioraffinerie, et culture nouvelle en Europe (Chapitre I); les méthodes analytiques d'extraction et de dosage du PI et des résines (Chapitre II); la SPIR appliquée au cas de l'analyse de la biomasse de guayule et de ses dérivés (extraits) (Chapitre III); enfin le Chapitre IV porte sur les méthodes de caractérisation du PI, et d'analyse chimique des composants des résines et d'autres produits importants susceptibles d'être valorisés en sus des deux premières fractions.

La deuxième partie décrit les protocoles analytiques mis en œuvre pour le dosage du PI et des résines (Chapitre V), pour la mise en œuvre de la SPIR et l'analyse moléculaire du PI dans des conditions évitant sa dégradation (Chapitre VI).

Enfin les résultats sont exposés en troisième partie; méthode gravimétrique basée sur les extraits bruts (Chapitre VII); détection et quantification par SEC et IR moyen de la contamination croisée des extraits (Chapitre VIII); étalonnage de la SPIR à l'aide des deux

méthodes précédentes (Chapitre IX); méthode « douce » d'extraction et d'analyse du PI natif (Chapitre X).

Revue bibliographique

Le caoutchouc naturel (CN) est un polymère aux propriétés uniques et recherchées, sans équivalent de polymère de synthèse et irremplaçable dans de nombreuses applications, telles que les pneus d'avion. L'accroissement de la demande de la part des pays émergeants et les risques qui pèsent sur la production de caoutchouc d'hévéa conduisent à chercher de nouvelles sources de CN. Le *Parthenium argentatum* ou guayule, plante de climat semi-aride est une alternative crédible, étudiée, testée depuis plusieurs décennies aux Etats-Unis et au Mexique. La culture commence à se développer. De plus le guayule produit du CN non allergisant contrairement à celui de l'hévéa, apportant une solution au problème posé par la progression de l'allergie aux protéines qu'il contient (parmi le personnel hospitalier notamment). La viabilité économique de cette culture ne parait possible qu'en valorisant aussi d'autres composants du guayule, telles les résines.

La biomasse de guayule est composée d'environ 10% de cis 1-4 PI et d'autant de résines, ainsi que de protéines, de sucres, de polysaccharides, en sus de la matrice lignocellulosique. Le PI est situé dans le cytoplasme et les vacuoles, donc à l'intérieur de cellules spécialisées alors que les résines circulent dans des canaux résinifères. La susceptibilité du PI de guayule à l'oxydation ou à la dégradation thermique est attribuée à certains composants des résines, notamment les acides gras poly-insaturés des triacylglycérols.

Pour toutes ces raisons il est indispensable de savoir déterminer la teneur en résines et en PI.

Parmi les nombreuses méthodes proposées dans la littérature, bon nombre présentent des désavantages pour raison de toxicité et de coût, d'autres nécessitent des équipements qui ne sont pas disponibles dans tous les laboratoires. De plus leurs résultats n'ont été que rarement comparés les uns aux autres et, le cas échéant, des variations ont été constatées. Ce sont donc plutôt des méthodes d'estimation que de dosage, pouvant satisfaire agronomes et sélectionneurs, mais pas les technologues qui doivent déterminer avec exactitude la teneur en produits extractibles de la biomasse entrant dans l'usine.

Etude d'une méthode simple de dosage du PI et des résines (extraction, gravimétrie)

La première partie de l'étude porte sur le choix d'une méthode de référence de dosage du PI et des résines. Cette méthode servira ensuite à calibrer la SPIR. Le choix s'est porté sur la méthode gravimétrique qui est largement documentée dans la littérature (Chapitre I).

Les résines qui sont facilement extractibles via les canaux résinifères, sont extraites avec de l'acétone, puis le PI est extrait par l'hexane. Conformément à ce qui est communément admis dans la littérature, les résines et le PI sont quantifiés par simple pesée des extraits bruts secs. Trois techniques d'extraction ont été sélectionnées, Soxhlet, homogénisateur à grande vitesse (dénommée Polytron) et l'extraction automatisée (ASE).

Après optimisation séparée, les trois protocoles ont été appliqués à un même échantillon de biomasse de guayule. Pour ce qui est de l'extrait hexanique, les trois méthodes ne donnent pas des résultats statistiquement différents (9,01; 9,23; 9,05% ; résultats exprimés par rapport à, la biomasse sèche). Par contre le Polytron a conduit à un extrait acétonique plus faible que les deux autres (8.71% contre 10.63 et 10.56%). A taux d'extraction identique, le choix s'est porté sur l'ASE du fait de son adaptation au traitement de grandes séries d'échantillons (9 par jour), contrairement au Soxhlet. Cet aspect est d'autant plus important que les mesures ont été effectuées en triple.

L'ASE couplée à la gravimétrie sera donc une méthode de référence pour la calibration de la SPIR. Elle est pratiquée sur la biomasse séchée, débarrassée des feuilles qui contiennent peu de PI, broyée dans un moulin à café en présence d'azote liquide, en recyclant le refus du tamis 0,5 mm jusqu'à passage total. Après étude de la cinétique d'extraction, trois étapes sont pratiquées avec l'acétone, les extraits sont joints, puis 3 étapes sont effectuées avec l'hexane. Le solvant est éliminé des extraits à l'évaporateur rotatif sous pression réduite à 40°C et les ballons sont séchés jusqu'à poids constant en étuve sous pression réduite avant pesée.

Etude de la contamination croisée des deux extraits - deuxième méthode de référence

Il est généralement considéré dans la littérature que les extraits acétoniques et hexaniques sont représentatifs des teneurs en résines et en PI de la biomasse de guayule. La question de la sélectivité de ces deux solvants n'est que rarement évoquée dans la littérature. Certains résultats ont montré la présence de non-polyisoprèniques polluant l'extrait censé contenir le PI. Mais cela dépend de la méthode d'analyse (RMN ; IR) (Black et al., 1982); Meeks et al., 1947; 1951; Cornish et al., 2013). La présence de PI de faible Mw dans l'extrait acétonique dans un cas a été attribuée à la méthode par Soxhlet suite au chauffage prolongé susceptible de favoriser la thermodégradation (Ray et al., 2009). Cependant les résultats ne sont pas convergents et cette question relative à la sélectivité de l'extraction méritait donc d'être étudiée dans les conditions appliquées dans le cadre de la présente étude. En effet la distribution des masses molaires du PI inclue des macromolécules de taille modeste de

quelques milliers de grammes seulement et susceptibles d'être aussi solubilisées au cours de l'extraction par l'acétone, en sus des macromolécules de plusieurs centaines de milliers ou millions de grammes insolubles dans l'acétone mais solubles dans l'hexane.

Cette distribution laisse donc attendre un partage du PI, contaminant alors les résines très solubles dans l'acétone, d'une part. D'autre part la localisation du PI dans les cellules du parenchyme et celles voisines des canaux résinifères, mais aussi dans différents compartiments cellulaires (cytoplasme, vacuoles), et celle des résines dans des canaux mais aussi dans les cellules voisines excrétrices, est de nature à induire des modalités de dissolution et de transport très différentes. On peut en effet concevoir que les résines « libres », dans les canaux, seront extraites rapidement en milieu acétonique, alors que celles à l'intérieur de cellules peuvent être retardées en fonction de l'état des parois cellulaires résultant du broyage préalable de la biomasse et éventuellement au cours de l'extraction elle-même (Polytron). Or la sélectivité de l'extraction n'a été que peu étudiée par nos prédécesseurs. Compte-tenu de l'objectif de notre travail –la sélection d'une méthode de référence pour la calibration de la SPIR- l'influence de plusieurs paramètres expérimentaux sur la sélectivité de l'extraction a été étudiée via la détection de la contamination suspectée, puis en s'efforçant de quantifier ces contaminants.

La chromatographie d'exclusion stérique couplée à la diffusion de lumière laser multi-angles (SEC-MALS), mise en œuvre pour l'étude structurale du PI, a montré la réalité de cette contamination dans le cas de l'extrait hexanique, via la présence d'un pic de composés de très faible Mw, dont le spectre FT-IR après collecte de la fraction correspondante montre des bandes d'absorption de liaisons C=O vers 1.700 cm^{-1}, mais pas de bande caractéristique du PI (~835 cm^{-1}). Une situation symétrique a été observée pour l'extrait acétonique, lequel montre par SEC un pic de faible Mw, présentant quant à lui une bande caractéristique du PI en FTIR.

Ayant confirmé la contamination croisée des deux extraits, laquelle était très probable, deux méthodes de quantification de ces contaminants ont été recherchées et étudiées par FT-IR et par SEC, afin d'accéder à une teneur en résines et en PI la plus proche possible de la réalité.

Après étude de l'influence de plusieurs paramètres pour optimiser les protocoles expérimentaux (re-solubilisation complète des extraits dans le solvant d'analyse après évaporation du solvant d'extraction ; teneur en eau résiduelle de la biomasse), le dosage des

contaminants a été possible via l'obtention de droites d'étalonnage montrant des coefficients de corrélation très satisfaisants, sur la base des échantillons de biomasse déjà utilisée, et couvrant une large diversité de plants de guayule (âge, situation du champ expérimental, date de collecte …). Pour quantifier le PI contaminant les résines dans l'extrait acétonique, la méthode est basée sur l'injection d'une solution de l'extrait dans le THF en SEC (détecteur réfractométrique): $Y= 3.15x+0.004$, $R^2=0.999$. Pour les résines contaminant l'extrait hexanique, le spectre en FT-IR donne $Y= 2.86x+0.070$, $R^2 = 0.988$.

Ces deux méthodes maintenant disponibles et permettant de calculer le PI effectivement extrait via les deux solvants, comme alternative à la méthode gravimétrique basée sur la simple pesée de l'extrait hexanique, ont été appliquées aux extraits obtenus via le Soxhlet, le Polytron et l'ASE, à partir de la même biomasse et dans les conditions expérimentales optimisées (Chapitre VII). Sur la base de la combinaison des pourcentages de PI, contaminant l'extrait acétonique et constituant principal de l'extrait hexanique, l'ASE a été à nouveau sélectionnée, puis optimisée via un plan d'expérience afin de maximiser le PI extrait (ANOVA Response Surface Quadratic Model), prioritairement aux résines, les conditions optimales pour les deux constituants ne se recouvrant pas. Cette nouvelle méthode de référence pour calibrer la SPIR met en oeuvre l'extraction par l'acétone à 44°C, puis par l'hexane à 121°C, pour maximizer le PI extrait. L'allure relativement plate de la zone optimale se trouve être celle déjà sélectionnée pour la méthode gravimétrique et les conditions n'ont pas été changées. L'influence majeure de la température d'extraction a été notée, confirmant les observations antérieures.

Etalonnage de la SPIR par les deux méthodes de dosage optimisées

Les deux premiers chapitres de résultats (Chapitres VI et VIII) ont donc porté sur la recherche d'une méthode fiable de dosage du PI pouvant servir de référence pour étalonner et valider la méthode rapide par SPIR qui est recherchée.

Au fur et à mesure de leur collecte sur les champs d'expérimentation, les échantillons de biomasse de guayule ont aussi été étudiés en SPIR afin de renseigner la base de données. Après séchage et pulvérisation dans l'azote liquide, les échantillons étaient contrôlés pour leur humidité résiduelle (103°C jusqu'à poids constant). Celle-ci a été maintenue inférieure à 10%, conformément aux usages dans la littérature relative au guayule.

La première méthode d'étalonnage utilisée est la plus simple, par gravimétrie. Les données statistiques relatives aux 215 échantillons collectés à cet effet sont les suivantes (Tableau IX.2) : humidité (%) comprise entre 2,65 et 9,47; écart-type expérimental 0,1%; moyenne 5,94, écart type sur la série 1,72; extrait acétonique (%) compris entre 3,27 et 13,42; écart-type expérimental 0,3 ; moyenne 8,57; écart-type sur la série 1,92; extrait hexanique (%) : comprise entre 0 ,74 et 13,81; écart-type expérimental 0,4; moyenne 6,33; écart-type sur la série 2,33. Un sous ensemble de 33% des échantillons a été sélectionné au hasard pour la validation ultérieure soit 71 échantillons ; les 144 autres ont servi à l'étalonnage.

Avant l'étalonnage, l'analyse en composantes principales, avec détermination de la distance de Mahalanobis pour chaque échantillon et détection des atypiques pour H supérieur à 3. Quatre échantillons ont été trouvés extérieurs à la population. L'un correspond à un extrait acétonique « moyen », mais à un extrait hexanique très faible (0,74%), provenant de très jeunes plans, en début d'étude. Son spectre ne montrant pas de différence visuelle avec les autres, il a été conservé. Les trois autres ont aussi été conservés. Les trois composantes principales ont expliqué 49,8; 25,3 et 11,8% respectivement. Deux groupes peuvent être distingués (Figure IX.2) correspondant à l'origine géographique des échantillons (France, Espagne), sans discrimination claire en fonction de l'origine et/ou de l'année de collecte.

La calibration pour la teneur en eau a donné un R^2 de 0,99 et de 0,98 pour la validation ($RED_{prediction}$ 6,97 et SEP 0,25%), avec une pente de 0,97 entre les valeurs mesurées et la SPIR. Pour l'extrait acétonique, la calibration a été aussi efficace : R^2 0.96 pour calibration et validation ; $RPD_{prédiction}$ 4,80 ; SEP 0,40. La prédiction est faite avec une précision de 0,80%. La régression entre dosage et SPIR a donné une pente de 0,96 sans biais. Pour l'extrait hexanique, la calibration est aussi bonne : R^2 0.96 et 0,96 pour calibration et validation respectivement ; $RPD_{prédiction}$ 4,58; SEP 0,40. La prédiction est faite avec une précision de 0,88%. La régression entre dosage et SPIR a donné une pente de 1,00. Ces performances sont meilleures que celle publiées par Black et al. 1985; Cornish et al. 2004; Takeno et al. 2008).

En conclusion, le nombre d'échantillons et leur représentativité ont abouti à un modèle très fiable pour doser par SPIR les extraits acétoniques et hexaniques de la biomasse de guayule.

La dérivée seconde de la moyenne des spectres du guayule sec a montré les principales bandes d'absorption à 1.450, 1.490, 1.716, 1.940, 2.210, 2.308, et 2.380 nm. Ces bandes sont

en accord avec les observations de Black et al. (1985) et Cornish et al. (2004). Elles correspondent à la première harmonique de l'élongation C-H du CH_3 (1.716 nm), à la déformation et à l'élongation combinées du CH_2 des lipides (2.308 nm) et à celles de C=O carboxyliques vraisemblablement (2.210 nm). Les bandes à 1.450 nm correspondent quant à elles à la première harmonique de l'élongation O-H de l'eau.

Les loadings PLS pour les deux extraits ont montré que les trois premiers sont les mêmes (bandes et poids). Les premières sont proches du spectre du guayule sec. Les troisièmes sont liées aux bandes d'absorption utilisées pour la quantification de l'humidité résiduelle, 1.450, et 1.908 nm correspondant aux O-H (eau, cellulose, sucres, éventuellement phosphorique/ phospholipides). Les quatrièmes ont plus de poids pour l'extrait hexanique que pour l'extrait acétonique (bandes d'absorption à 2.308 et 2.268 nm; O-H combinés de la cellulose). Ce résultat a été confirmé par l'observation des coefficients beta en fonction de la longueur d'onde; certains sont spécifiques de l'extrait acétonique (1.492, 1.456 nm, première harmonique du N-H des amides; 1.528 : C-H des $=CH_2$; 2.084, 2.100 nm élongation C=O et déformation O-H combinés des sucres et des hydroxyles; 2.300 nm : élongation N-H et C=O combinés des amides plutôt qu'aminoacides ?; 2.300 nm : déformation O-H des alcools. La plupart des bandes correspondent à des fonctions chimiques attendues dans les résines, dans d'autres extractibles et parmi les polymères de la matrice lignocellulosique (phénoliques, sucres, amides/protéines).

Pour l'étalonnage avec les mesures de teneurs en PI et en résines déterminés par la deuxième méthode de référence, les données statistiques relatives aux 127 échantillons utilisés sont les suivantes : résines (%) comprises entre 3,61 et 12,71; moyenne 7,75; écart-type sur la série 1,75; PI (%) compris entre 1,18 et 14,17; moyenne 6,77; écart-type sur la série 2,72. La corrélation de Pearson (risque alpha 5%) entre extrait acétonique et résines est très significatif; $p < 0,0001$; r 0,955; et la dispersion de l'extrait acétonique vis-à-vis des résines, montre la forte corrélation, avec R^2 0,92 et une pente de 1,05.

La corrélation de Pearson (risque alpha 5%) entre extrait hexanique et PI est très significative ($p < 0,0001$; r 0,969) et cet extrait vis-à-vis du PI, montre une forte corrélation, avec R^2 0,94 et une pente de 0,89.

La calibration a été faite avec 2/3 des échantillons disponibles soit 96, le reste (31) a servi pour la validation du modèle. Pour la résine, la calibration a été efficace : R^2 0.96 et 0,92 pour

calibration et validation respectivement; $RPD_{prédiction}$ 3,05; SEP 0,53. La régression entre dosage et SPIR a donné une pente de 0,90 avec un biais faible. Comme ci-dessus, par comparaison aux performances du modèle gravimétrique simple, elles sont ici inférieures. Pour le PI, la calibration a donné: R^2 0.98 et 0,95 pour calibration et validation respectivement; $RPD_{prédiction}$ 4,13; SEP 0,62. La régression entre dosage et SPIR a donné une pente de 0,90 avec un biais faible.

En conclusion, l'objectif principal de la thèse a été atteint, via la mise au point d'une méthode rapide et fiable de dosage du PI par SPIR, à partir de la biomasse séchée et broyée.

Les performances du modèle basé sur la référence par gravimétrie sont supérieures à celles du modèle plus complexe, tenant compte de la contamination croisée. Dans tous les cas, le recours à la SPIR pour quantifier les deux composants dans la biomasse de guayule reste intéressant. L'erreur expérimentale supérieure, introduite par les dosages par SEC et par FT-IR peut expliquer la moindre performance du deuxième modèle. Il se peut également que cette technique soit plus sensible aux variations de composition des résines, ratio entre sesquiterpènes, triglycérides et autres molécules contenant un groupement carboxylique (cires, esters de stérols, acides gras libres) d'une part, et composants dépourvus de cette fonction d'autre part (partheniol, argentatines, stérols libres, alcools gras). En effet la méthode de quantification des résines dans l'extrait hexanique, par FT-IR ne prend pas en compte les composants dépourvus de fonction carbonyle. On s'attend donc à ce que la variation de composition des résines par rapport à celle utilisée pour l'étalonnage introduise une erreur, seule la bande C=O ~1730 cm^{-1} étant prise en compte pour les résines, alors que certains composants en sont dépourvus ; toutefois ces derniers représentent une faible proportion des résines d'après la littérature (Nakayama, 2004; Schloman et al., 1998).

Influence des conditions d'extraction sur les caractéristiques macromoléculaires du PI

Si l'objectif principal du travail était bien de mettre au point une méthode rapide par SPIR, comme déjà dit dans l'introduction, l'atteinte des objectifs du projet EU-Pearls comportait aussi l'évaluation de l'influence du génotype, des pratiques culturales et agronomiques, notamment celle du prétraitement et du stockage de la biomasse après la récolte, sur la valeur de la production d'une parcelle. Celle-ci est liée non seulement à la production de PI mais aussi à sa qualité, fonction des saisons et d'une façon générale des conditions climatiques. Les applications les plus courantes nécessitent du PI de Mw supérieur à 2 millions de g/mole.

La littérature fait état de la faible stabilité du PI dans la biomasse récoltée et de la nécessité d'utiliser des antioxydants (Keller et al., 1981; Bhowmick, 1985; Schloman et al., 1996). La probable dégradation, thermique notamment, de la Mw dans les conditions qui paraissaient nécessaires pour maximiser le PI extrait, dans la recherche d'une méthode de référence (extraction par l'hexane à une température supérieure à 100°C), le parti a été pris de maximiser l'extrait sans se préoccuper de l'influence sur les caractéristiques macromoléculaires du PI.

Il restait donc à vérifier dans quelle mesure les conditions thermiques de l'ASE dégradent le PI, en étudiant l'évolution de la structure du polymère en fonction de la température et de la protection contre l'oxydation, et le cas échéant, à rechercher un autre protocole, dédié à la détermination des caractéristiques moléculaires du PI.

Après avoir constaté la baisse du Mw dans les conditions d'extraction de la méthode de référence (ASE), un protocole très différent a été mis au point, opérant à 30°C, dans l'obscurité, avec ajout de BHT comme antioxydant, et avec le THF comme unique solvant. Ce dernier est en effet le solvant utilisé pour l'analyse en SEC-MALS de la mesure du Mw. Ceci évite les étapes d'évaporation du solvant d'extraction utilisé avec l'ASE (hexane). Etant donnée la température très basse, on s'attendait à une cinétique d'extraction assez lente, l'expérience acquise ayant montré que l'extraction par Soxhlet prenait déjà près de 48 heures. Le Mw de l'extrait à partir de biomasse fraiche (non séchée, analysée le jour même) est resté constant entre 6 et 24 heures, puis a graduellement baissé de 2,1 à 1,8 millions de g/mole au cours des 6 jours suivants. Avec la biomasse séchée, le Mw était déjà faible (1,6 million de g/mole) après la première journée. L'étude a été poursuivie afin de mieux connaitre l'influence des étapes les plus critiques sur la qualité du PI extrait. Avec ce nouveau protocole (« méthode douce » SM), le séchage de la biomasse a induit une baisse de Mw allant de 20 % à 61% suivant la date de récolte, passant de 2,1 à 1,7 millions de g/mole, et de 1,7 à 0,6 millions de g/mole, en mai et novembre respectivement (Figure X.5). Par contre avec la biomasse séchée, les deux protocoles SM et ASE ont conduit à des Mw voisins bien que faibles (1,1 et 1,2; 0,67 et 0,56 millions de g/mole pour juin et novembre par exemple). L'étape de séchage apporte donc une contribution majeure à la dégradation constatée.

Forts de ces résultats, un autre paramètre macromoléculaire a été mesuré, le rayon de giration qui traduit la compacité d'un polymère. A Mw égal, un rayon plus faible peut être relié à un taux de ramification plus élevée de la macromolécule. C'est bien ce qui a été constaté : le

rayon de giration est plus faible, pour l'ASE et pour la SM, en incluant l'étape de séchage de la biomasse.

En conclusion, le séchage de la biomasse doit être évité en vue d'approcher la structure du PI in vivo, et la SM mise en œuvre avec un seul solvant (THF) à 30°C via une seule étape de 24 heures sous simple agitation, via une simple microfiltration avec une système filtre-seringue avant injection en SEC-MALS, extrait la quasi-totalité du PI (par référence à l'ASE) tout en conduisant à des Mw supérieurs à 2 millions de g/mole, probablement représentatifs du PI existant dans la biomasse au moment de sa collecte.

Conclusion générale

Trois techniques ont été choisies parmi les nombreuses proposées dans la littérature. Elles sont basées sur l'extraction séquentielle par l'acétone puis par l'hexane des résines et du PI, jusqu'à épuisement de l'extrait en plusieurs étapes (Soxhlet, homogénisateur à grande vitesse, ASE). Après optimisation les résultats, sur la base du poids d'extrait sec, ont conduit à sélectionner l'ASE, jamais utilisée pour l'étalonnage de la SPIR avec le guayule. Les conditions optimisées font intervenir l'acétone à 40°C et l'hexane à 120°C.

Après avoir noté la contamination croisée des deux extraits –résines acétoniques par du PI de faible Mw ; PI hexanique par des composés de faible Mw assimilés à des résines- une méthode de dosage des contaminants a été mise au point, par SEC-MALS dans le premier cas et par FT-IR dans le second (R^2 voisins de l'unité). Ceci a débouché sur une deuxième méthode non plus basée sur les rendements gravimétriques en extraits bruts, mais sur les teneurs en PI et en résines, calculés en additionnant la contribution de chaque extrait (acétone et hexane) à la fraction considérée (résine ou PI).

Les deux méthodes ci-avant ont permis de réaliser deux étalonnages de la SPIR, chacun donnant des performances intéressantes. Cependant celles de la méthode gravimétrique sont supérieures. Deux explications sont avancées ; d'une part le recours à deux analyses chimiques en sus de la gravimétrie introduit donc une erreur expérimentale plus importante ; d'autre part l'étalonnage des méthodes de mesure de la contamination croisée (FT-IR et SEC) a été volontairement fait avec un seul type de biomasse, et la variation de composition des résines notamment d'un échantillon à l'autre doit introduire une déviation. L'amplitude de cette variation de composition, laquelle n'a pu être mesurée faute de temps dans le cadre de ce travail, dépend de la proportion de composés comportant une fonction C=O (~1.730 cm^{-1}

utilisée comme marqueur des résines), par rapport à celles des molécules ne comportant pas cette fonction, non prises en compte dans ce dosage. Il s'agit donc là de la limite de validité de la méthode. Toutefois cette incertitude n'empêcherait pas d'utiliser la deuxième méthode. Il faut donc noter que la démarche de prise en compte de la contamination croisée, justifiée a priori, s'avère ne pas apporter d'amélioration en l'état.

La faible stabilité du PI de guayule et sa sensibilité marquée aux conditions d'extraction ont montré la réduction du Mw au cours de l'extraction par le protocole ASE. Si cela ne jouait qu'à la marge pour la quantification des PI et résines dans la biomasse, dans le cas de la méthode gravimétrique, et pas du tout dans celui de la deuxième méthode (le PI de faible Mw issu de la dégradation étant aussi comptabilisé via l'extrait acétonique), cette dégradation a conduit à mettre au point une nouvelle méthode d'extraction dédiée à l'analyse structurale du PI. Elle est basée sur la réduction des étapes au strict minimum : un seul solvant (THF), une seule extraction (30°C, 24 h), suivie de la filtration indispensable avant injection en SEC-MALS, sans changement de solvant. C'est l'étape de séchage, même à 40°C et sous pression réduite (retour à la pression ambiante sous azote), qui contribuait le plus à la dégradation du PI. Elle est pourtant pratiquée couramment dans la littérature.

Les méthodes étudiées au cours de ce travail ont été transférées aux partenaires et appliquées dans le cadre du projet EU-Pearls, afin de connaitre les propriétés de la biomasse récoltée et celles du caoutchouc extrait et en préserver la qualité. Ce travail a précédé et a contribué à la production des premiers prototypes ex-guayule « made in EU » (gants, pneu). La prochaine étape vise le transfert de la technique au champ avec un appareil SPIR portable afin de mesurer directement sur les plants, avec le défi lié à la complexité de la biomasse de guayule.

INTRODUCTION

Natural rubber or polysioprene (PI) displays unique properties, and cannot be replaced in many applications. The increasing demands together with risk on the production of Hevea tree, the current source, ask for finding alternative sources. *Parthenium argentatum (*guayule), a plant from Mexico and the US, is a credible option. In addition, its rubber is said not to induce allergy, contrary to Hevea rubber. The industrial viability of guayule depends on the valorization of other compounds, the rubber making only about 10% of biomass dry weight; resin, also about 10% is the favored fraction for this purpose. In addition the presence of resin in the rubber is seen as oxidation promotor, therefore reducing its quality. Therefore it is necessary to determine PI and resin contents in guayule biomass. This has induced a continuous research effort for improving analytical methods and justifies the objective of the present work

The aim of the present work was to select and optimize a simple method based on Near infrared spectroscopy (NIRS) for fast determination of PI and resin contents in guayule biomass. In addition, there was also a need for having a method allowing to determine macromolecular structural features of PI under a form as close as possible to the in vivo form in freshly harvested biomass. On one side, it has been rapidly noted that due to degradation under common processing conditions, and the two objectives have been separated, priority being given to PI and resin quantification to provide as soon as possible an analytical tool to partners within the EU-Pearls project (http://www.eu-pearls.eu/). On the other side the very young plantations did not allow to collect samples covering a wide panel of composition at start of PhD work.

The present report is split in three parts. Part 1 deals with the literature review; general presentation of guayule (Chapter I); analytical methods for PI and resin quantification in guayule biomass (Chapter II); NIRS and applications to the present case (Chapter III); methods used for analysing the extractable fractions, especially resin, and for determining PI structure (Chapter IV). Part 2 details the analytical methods used within this work: protocols for extraction and associated measurement of PI and resin contents (Chapter V); NIRS spectra and calibration and PI analysis (Chapter VI).

Part 3 is devoted to results and discussion: Gravimetric method set for determining acetone and hexane extracts as a simple way to access resin and PI content in biomass, and further calibration of NIRS (Chapter VII); detection, quantification of cross contaminants in above extracts, and setting of a second reference method (Chapter VIII); NIRS calibration and efficiency of corresponding models (Chapter IX); setting of new protocol for avoiding PI degradation (Chapter X).

Chapter I

Natural rubber and guayule

I.1 Hevea and natural rubber market

The sole commercial source at present of natural rubber (NR) is *Hevea brasiliensis*. In addition to the allergy problem and to the need for latex-based medical products used in medical and personal hygiene applications, demand for NR is rapidly increasing. Thus there could be a shortage in world market NR supply (Ansell New, 2011; Carey et al., 1995; Cornish, 1998; Cornish and Lytle, 1999; Dawson, 2009; Siler and Cornish, 1994).

This is why guayule or *Parthenium argentatum,* a plant species producing hypoallergenic (non-allergenic) latex is being investigated (Cornish and Brichta, 2002; Cornish et al., 2001; Ray, 1993; Van Beilen and Poirier, 2007).

I.1.1 Hevea production and trends

NR is a strategic raw material used in more than four thousand applications, including four hundred medical devices (Mooibroek and Cornish, 2000). While over 2,500 plant species produce rubber-latex, almost all commercial NR comes from a single plant species, Hevea, often simply called rubber tree. It belongs to the botanical family o*f Euphorbiacea* and is of major *economic,* social, and strategic importance because it's sap-like extract, known as latex, is the primary source of NR (Samosorm, 2007). Hevea rubber (cis-1,4-polyisoprene) has a high molar mass (Mw) (>1 million g/mole), high performance properties and it cannot be replaced by synthetic rubber produced from petroleum in many uses (aircraft tires, medical devices). NR has a high resilience, a low heat build-up and high dynamic properties, due to its homogeneous macromolecular structure (99.8% cis-polyisoprene). It is the best polymer known for dynamic applications and has a strategic importance for the tire and automotive industry. Without NR, airplanes could not land safely. Thus there is still a need for NR (van Beilen and Poirier, 2007), although some products have been replaced by synthetic rubber (SR), for example cable insulators and golf, balls, that are produced from the trans-PI. Ten percent of NR is used as latex to produce i.e. gloves, condoms, catheters, tubing and other medical products. Hospitals depend on NR latex for protection gloves against HIV virus, although its use is presently limited due to allergenicity of protein in Hevea latex.

Currently, NR is mainly produced in Southeast Asia. In the short term, following the rapid recovery and growth seen in 2011, NR and SR consumption is forecast to reach 26 million tons, NR representing 11 million tons (46% of total world market) (IRSG, 2012) as shown in Figure I.1. Resource security supply of raw material is an increasing priority for the rubber

industry. Access to rubber raw materials at affordable price is a vital issue for the competitiveness and future of the automotive European industry. Prices have increased over 80% since 2005 and supply continues to tighten. The demand for NR latex is predicted to exceed 2 million tons by 2020, about the double of current consumption. In 2005, the International Rubber Study Group predicted a 2 million tons/year shortfall (Table I.1).

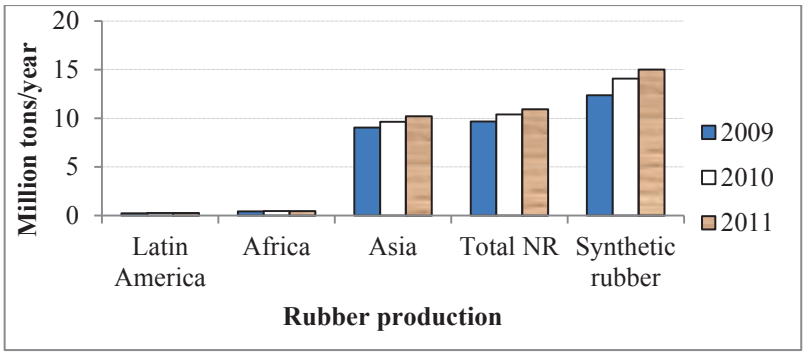


Figure I.1: Global production of SR and NR (2009-2011).

Table I.1: Annual NR production needed for different markets

Rubber market (tons)	2005	2020
Medical and high-end consumer products	350,000	2,000,000
All NR latex	1,000,000	6,000,000
All NR products	8,000,000	12,000,000
All rubber (NR and and SR)	20,000,000	30,000,000

Source: International Rubber Study Group, 2006

I.1.2 Allergy to Hevea rubber products

Allergy to Hevea rubber is a major problem for human suffering of life-threatening Type I *Hev-b* latex allergy (ALAA, 201; Ownby et al., 1994). Especially in the USA but also in Europe and Japan, an increasing number of people are allergic to proteins of Hevea derived products. The incidence of those reactions has increased dramatically in the last 15 years and 1-6% of the general population suffers from latex allergies (Cornish, 2001), up to 17% of healthcare workers are at risk of reactions. The American Society for Testing and Materials has developed a new standard (ASTM 1076-06) Category 4 NR latex in response to the allergy issue. While previous standards measured physical performance rather than protein content and applied only to Hevea-based raw materials, the new standard offers an opportunity for manufacturers looking to develop protein-free high performance latex from other sources. Guayule (Parthenium argentatum), a species producing hypoallergenic (non-allergenic) latex is being investigated (Cornish and Brichta, 2002; Cornish et al., 2001; Ray,

1993; Van Beilen and Poirier, 2007), thus justifying the topic of our work. It provides a new level of safety for medical product manufacturers, and they are now able to address these health issues by using alternative products (Nakayama, 2004).

I.1.3 Chemical structure of natural rubber

NR from Hevea and guayule are polymers of isoprene units (C_5H_8) (Tanaka, 2001; National Academic of Sciences, 2002). Figure I.2 shows the polymerization and the two polyisoprene (PI) stereo isomeric forms: poly(trans-1,4-isoprene) and poly(cis-1,4-isoprene). The cis form is a unique biopolymer with high-performance elastic properties. The isoprene units are linked in a head-to-tail configuration to form a macromolecule containing 400-50,000 isoprene units in a linear or branched chain (Figure I.3). The amount of branching and cross-linking between guayule PI macromolecules has been defined quantitatively, through the solubility in solvent. The presence of long chain branches increases the sensitivity to thermal relaxation (Santangelo and Roland, 1998). The lower Mw molecules and various isomers are part of the guayule extract called "resin".

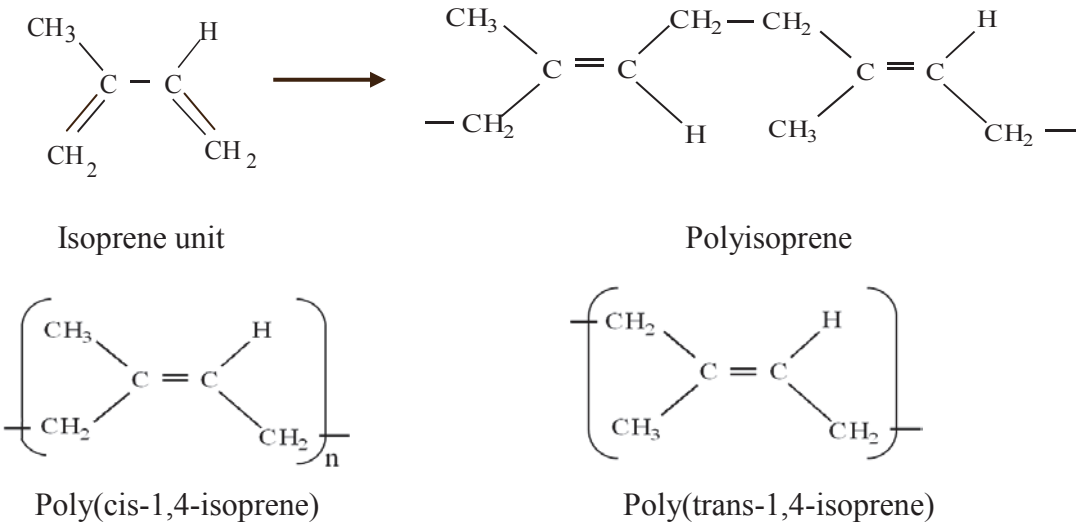


Figure I.2: Chemical structure of the NR from Hevea and guayule rubber

The gel is defined as a network of polymers formed as a result of extensive branching or crosslinking, which is swollen by solvents but does not dissolve (Eng et al., 1997). The macrogel is the part of NR that is visible and remains insoluble in a PI solvent. The microgel (or micro-aggregates) is contained in the soluble part. Regarding gel quantification the ASTM 2000 method of the swelling index is used to define the type of gel.

PI is produced by biosynthesis in plants in the form of latex. The insoluble rubber is encapsulated in ~1µm diameter latex particles with lipid monolayer membranes including various species, proteins, and other lipids.

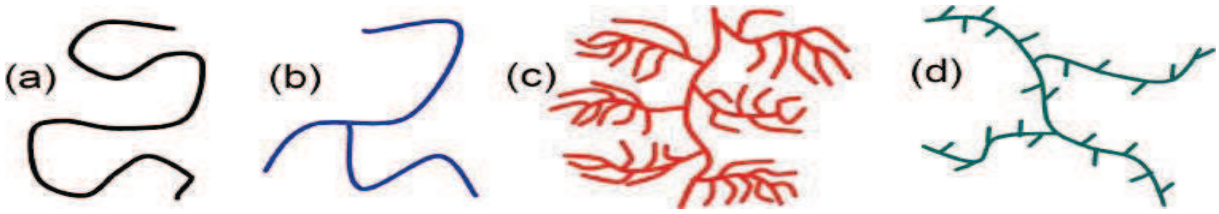


Figure I.3: Representation of a linear polymer chain (a) and branched structures with (b) long-chain, (c) short-chain, and (d) both short- and long-chain branches

I.1.4 Conclusion - Hevea

The sole commercial source at present of NR is Hevea and the demand for NR is rapidly increasing, thus there could be a shortage in world market. In addition, allergy problem and the need for latex-based medical products used in medical applications with Hevea, guayule is a species producing hypoallergenic latex is being investigated, justifying the topic of our work.

I.2 Guayule as an alternative rubber industrial crop

I.2.1 Rubber producing species

The Asteraceae, a large family with over 20,000 species, including sunflower (*Helianthus annuus)* produces PI. However the development of alternative annual rubber-producing plants, such as sunflower is not undertaken today because of the very low PI concentration contained within existing lines (0.1–1%, compared to ~10% in *P. argentatum*, on a dry weight (Cornish et al., 1999). Several species such as the Russian (Kazakhstan) dandelion (*Taraxacum kok-saghyz)* synthesize considerable amounts of PI and have been investigated as potential commercial sources (Buranov and Elmuradov, 2010). The genus *Parthenium*, a member of the Asteraceae family, is native to North America (Foster and Coffelt, 2005; Ray et al., 2005; Whitworth and Whitehead, 1991), and the most promising species NR crop is *P. argentatum* (Gray). The other species do not produce significant quantities of quality rubber and most are non-PI producers. *P. argentatum* name because of a silvery sheen on its gray-green leaves).

I.2.2 Guayule research and development history

Guayule is a desert shrub used by Amerindians in pre-Columbian times. It is a perennial shrub native to the Chihuahuan desert of Northern Mexico and to Texas. Guayule rubber (GR) has

been evaluated in the U.S and Europe as a potential commercial crop for at least four periods (Foster and Coffelt, 2005; Ray et al., 2005; Whitworth and Whitehead, 1991).

First use of guayule was witnessed in South America by the Spaniards in the 18th century. Llyod reported a first use by native populations. Pearson (1907), reported interested for guayule rubber (GR) at the centennial exposition in 1876. In the early 1900, wild guayule plants were harvested and latex extracted to produce "solid" rubber (Finlay, 2012; Hammond and Polhamus, 1965; Delafond, 1908). In 1902, guayule factories were built in Mexico and GR marketed in the USA as soon as 1905. In Texas, a commercial mechanical extraction started in 1909. The first extraction process by chewing was replaced later by trituration under water. From 1906 to 1912, 20 extraction plants operated in Mexico and 10,000 tons of GR were exported to the USA, the equivalent of 24% of total NR import in the US. Between 1900-1930, GR was used for cars and bicycles tires. This period ended with the great depression. In the 1920s, 3,200 ha of guayule shrubs were planted in the USA and 1,400 tons of GR produced. Guayule had passed from wild harvesting to an industrial crop.

The second period (1940-1945) was during World War II when rubber supplies were cut off from Southeast Asia plantations. In 1942, the Emergency Rubber Project (ERP) started with 13,000 ha of guayule plantations. In 1945, the ERP project stopped with the imports of Hevea rubber again from South-east Asia.

The oil embargo of the 1970s, with the quadrupling of crude prices, started a third period of research in the USA and Mexico on cultivation, processing, breeding and valorization of by-products. The two large US tires producers, Firestone and Goodyear showed an interest to develop GR rubber in Texas and Arizona. GR was recognized as an adequate substitute for tire applications and as a good domestic source in a case of emergency. With a world market of low NR price growing and a higher production costs to extract GR, guayule could not compete with Hevea rubber. New solvent extraction processes were developed but commercial stage was not reached (Jasso de Rodriguez et al., 2006).

During a fourth period (1990-2012), the GR interest was for latex (GL) rather than crude rubber. In 1997, production of a guayule hypoallergenic latex started with the Yulex Corp. in Arizona. Since 2006, a commercialized GL occupies the niche market of non-allergenic gloves. Yulex has demonstrated the technical feasibility of production, although commercial profitability remains questionable. In 2011, Yulex is factory with a capacity of 500 tons/year.

Although guayule studies were mainly done in North America, other guayule research programs were reported in Australia, New Zealand, South Africa, Argentina, Spain (1959), Italy and Morocco (1980), and Greece (1990). In 2004-2006, the EU Strategic Research

Agenda of the EPOBIO (EU/USA) concluded that development of an entire production chain from germplasm to end products, was needed (van Beilen, 2007). Since 2008, the European Union (EU) has financed a research project on alternatives for NR production, the EU-based Production of Alternative Rubber and Latex Sources project (EU-PEARLS), to experiment guayule in Spain, France and Mediterranean countries.

I.2.3 Ontogeny of PI synthesis in guayule

The plant material (Figure I.4) comprises distinct parts corresponding to different functions (Nakayama, 2005; Backhaus and Walsh, 1983). PI producing cells are mainly in the outer layers and mostly in new-grown tissues, but the old cells of the inner xylem produce PI for several years, PI being mainly found in stems and branches, with remainder in the roots (Whitworth and Whitehead, 1991) (Table I.2).

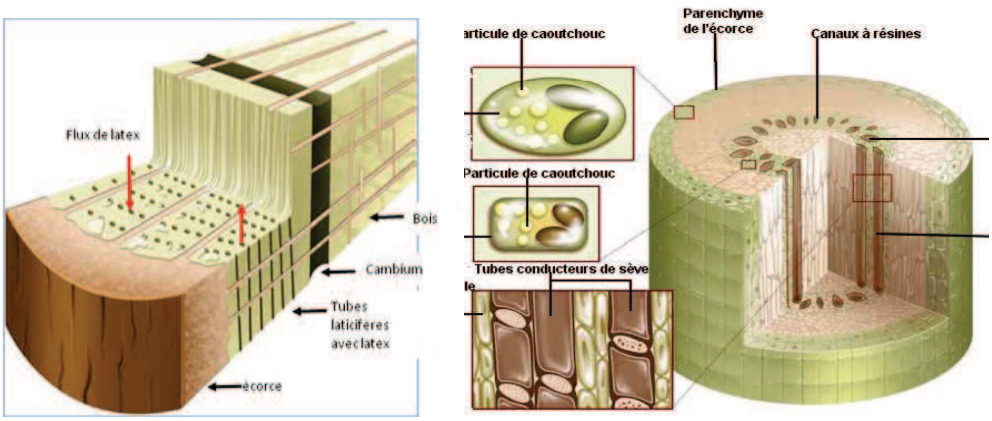


Figure I.4: Specific structure of the Guayule biomass (right) compared to Hevea (left)

(Source: Palu and Pioch, 2010)

Table I.2: Repartition of PI in the guayule plant (Teetor et al., 2009)

Season, extract type	Flowers	Leaves	Stems + branches
Biomass (% dry biomass)	4.80	30.6	64.5
PI (% dry biomass)			
-Fall	1.20	25.7	73.0
-Spring	0.80	7.9	91.2

While PI particles may appear in the vacuoles as well as in the cytoplasm, they are not visible in the vacuole at the initial stage of development. Backhaus and Walsh (1983) reported that the rubber formation in the stem first occurs in the cytoplasm of epithelial cells surrounding the resin ducts. PI formation starts when plants are about 2.5 months old. The epithelial cells at this age are highly vacuolated and contain the usual complement of organelles (nuclei,

plastids, mitochondria, microbodies, and endoplasmic reticulum). The PI particles are relatively small at this stage, the largest being of 1 µm in diameter. When the plants are 8 months old, parenchyma cells show accumulation of PI exclusively in the cytoplasm, and a few is observed in the vacuole. While the vacuole PI particles are almost perfectly spherical, cytoplasmic particles have an irregular, elliptical or globoid shape (Figure I.5). These differing shapes are maintained throughout the life of the plant. PI particles are roughly divided equally between vacuole and cytoplasm in the parenchyma cells, but in the epithelial cells, the majority is located in the cytoplasm. During this period of development, they grow up to 3-4 µm in diameter. The particle number also increases substantially compared to younger cells (Backhaus 1985; George et al, 2005).

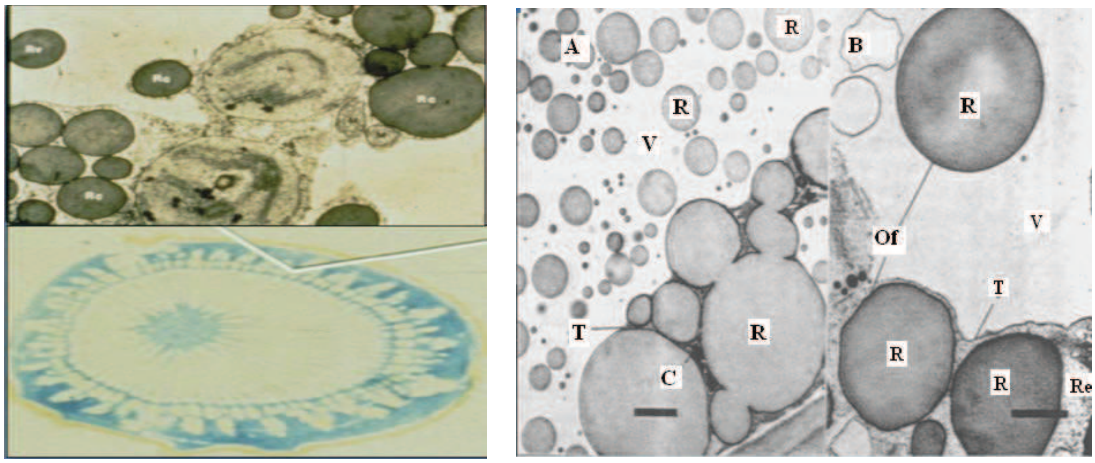


Figure I.5: Electron micrographs of PI bearing cells in guayule

(right) (A) Final stage of PI particles accumulation in cell (2.5 year old plant), mainly in the vacuole. (B) Parenchyma cell showing difference in shape of PI particles in vacuole and cytoplasm which contains endoplasmic reticulum. Horizontal bars = 1µm; R= rubber, V = vacuole, T = tonoplast, C = cytoplasm, Of = osmiophilic film, Re = rough endoplasmic reticulum. (Backhaus 1985 and George et al, 2005)

In 3 years old plants, PI accumulation proceeds further as cells mature, with more particles filling a greater volume of the available space in the cell. The largest individual PI particles are not larger than they were in 8 months old stems, but the total number and the proportion of large particles per cell increase tremendously. When the guayule shrubs are harvested and extracted for rubber, both the epithelial cells and the parenchyma cells are highly vacuolated and contain little residual cytoplasm. Consequently, nearly all of the PI particles occur in the vacuole and have the characteristic spherical shape peculiar to vacuole rubber (Backhaus and Walsh, 1983; George et al., 2005).

The rubber yield depends not only on genetic source but also on environmental conditions. When the plant is stressed, growth and products from photosynthesis are diverted into PI

production. Thus when plants grow slowly during cool weather because of reduced moisture, the PI content begins to increase (Whitworth and Whitehead, 1991).

I.2.4 Resin

Numerous studies have been made to characterize the resins, a mix of small molecules, located and free flowing in ducts (Figure I.4), and extractible with acetone. The composition has been determined to be very complex: sesquiterpene ethers, triterpenoids, and fatty acid triglycerides (Schloman, 1988; Nakayama, 2005). An abbreviated listing of the main resin components extracted from guayule is summarized in Table I.2 and shown at Figure I.6. At least 50 different types of chemicals have been identified in the guayule plant. Studies have focused on the whole plant (Banigan et al., 1982) including the leaves where about 20 compounds were identified with pinene, and terpenolene as the major components (Kumamoto et al., 1985). Bornyl acetate, a known cockroach attractant, was identified only in *P. argentatum* relative to six other *Parthenium* species (Kumamoto and Scora, 1984). When resin is present in the rubber, it decreases the quality of the materials. Resin can be valorized as a co-product.

Table I.3: Composition of guayule resin

Volatile fraction (3–5% in dry biomass)[a]	Hydrocarbon	α-and β-Pinene Camphene α- and β-Phellandrene Sabinene and β-Myrcene
Non-volatile fraction(85-97%)[a]		
Water soluble		Bornyl acetate Cinnamic acid Polyphenols Polysaccharides
Water insoluble (% in dry biomass?) Fatty acid triglyceride (20–25%)[a]	Hydrocarbon	β-Ocimene Limonene Linoleic (60–65%)[a] Linolenic (10–15%)[a] Palmitic (10%)[a] Oleic (10%)[a] Stearic (1%)[a]
Wax (in leaves)		
Sesquiterpene		Guayulin A, B, C, D, Partheniol
Triterpene		Argentatin A, B, C, D, E, F, G, H
Alkaloid		Guayulamine A, B

[a] *Percentage of total resins : Costa et al. (1992).*

Details on the resin composition were summarized by Schloman et al. (1983). The compounds identified include organic acids (cinnamic, *p*-anisic, palmitic, stearic, oleic, linoleic, and

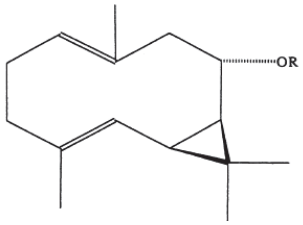
linolenic), sesquiterpene esters (guayulin A (cinnamoyl), B (*p*-aninoyl), C (cinnamate), and D (anisate)], triterpenoid esters (argentatin A, B, and C), and polyphenolics (tannin and flavonoid]. The presence of guayulin A and B have been confirmed in guayule latex and in cured guayule latex films (Stumpf et al., 2001).

Resin eudesmanoid sesquiterpenes have been tested for antifungal activity (Maatooq et al., 1996). Physiological activity in humans and animals has been demonstrated for argentatins and derivatives (Calzada et al., 1995; Céspedes at el., 2001; Gutiérrez et al., 1999; Martinez-Vasquez et al., 1994; Parra-Delgado et al., 2005 and 2006).

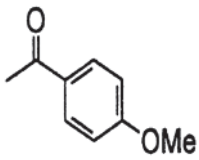
Localized lymph node assay and dermal test data reported by Cornish et al. (2009) indicate that it is "extremely unlikely" that guayule latex products will cause sensitization or irritation due to the presence of trace guayulins or other resin compounds co-extracted with GR latex.

Tests of the guayulins on humans did not indicate that dermatitis will be a major problem (Rodriguez, et al., personal communication, 2000).

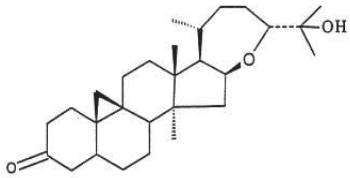
Among the sesquiterpenes, guayulines A and B are known as biological initiators in chemical hemi-synthesis of lychnostatine, an expensive antineoplastic drug, used in breast cancer treatment (Taxol®).



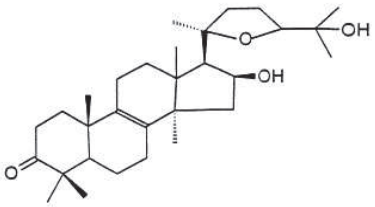
partheniol (**A**)



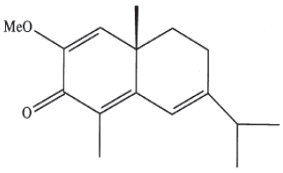
guayulin (**B**)



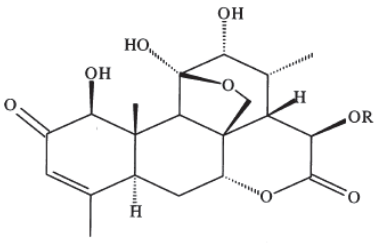
argentatin B (**C**)



incanilin (**D**)



argentone (**E**)



glaucarubolone glucoside (**F**),

Figure I.6: The chemical structure and compositions of the guayule resins

Resins composition varies with shrub line, cultivation site, harvest date, and processing (Schloman and Wagner, 1991). The recent ratios of resin/ PI are about 1 to 2.5. New varieties developed increases PI content, but even more the co-products in resin (Foster et al., 1981).

I.2.5 The guayule plant

The guayule plant is well adapted to arid environment: woody shrub, not exceeding 65 cm in height, and with a native dry weight of 1.0-1.5 kg wet sample. It is a perennial and, under undisturbed natural conditions, may live fifty years. In its natural habitat the growth is very slow (McCallum, 1926). It comprises the parts shown in Figure I.7 and Tables I.1 and I.4. Guayule plants are separated into several portions to display the distribution of rubber and resin in term of concentration or weight. When the plant is normally harvested, about two-thirds of the defoliated dry weight is in the branch stems above ground, with only one-third in the enlarged crown and root portion. A somewhat larger proportion of the total rubber is contained in the branches, for they usually carry a higher concentration of rubber than does not the combined root and crown (Teetor et al., 2009).

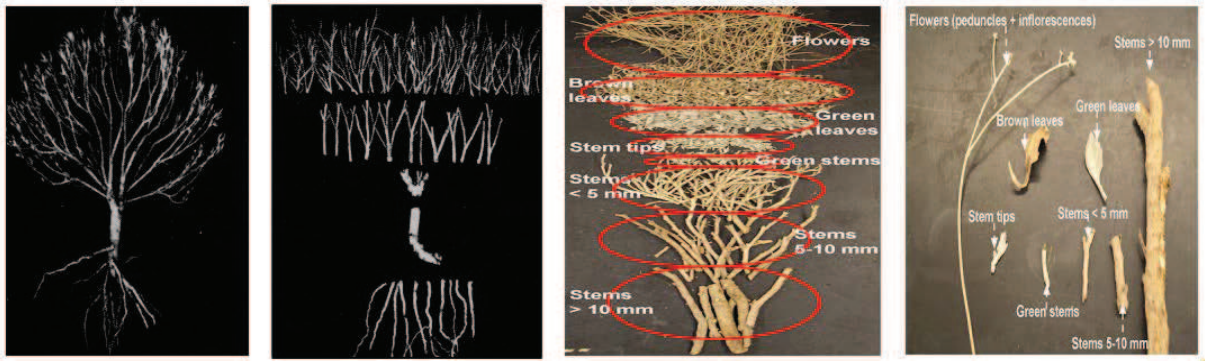


Figure I.7: Guayule tree, flowers, leaves, branches and shrubs

Table I.4: Components of guayule shrubs

Part	% dry weight
Wood (stem and branches)	30-50
Leaves	15-20
Bark	10-22
Cork Wood	3-10
Root	3-10

(Source: National academic of sciences 1977)

Flower and seeds. The guayule flowering occurs in periods of active growth when water is available in spring, but also through summer and fall. Although guayule is both wind and insect pollinated, it is mainly an apomictic plant. Seeds, very small, are produced at a prolific rate (Hammond and Polhamus, 1965). Flower peduncles yield only insignificant amounts of PI. Inflorescences can range from 5 to 22 cm with 1 to 4 branches.

Leaves. Guayule has alternate narrow to medium leaves, covered by a drought-protecting white wax. Leaves and flower peduncles yield only insignificant amounts of rubber. They do not contain sufficient PI to justify their inclusion in the extraction process. Also, exclusion of leaves tends to improve the final product because the leaves contain trace amounts of metals and resinous compounds that affect rubber quality (Hamerstrand and Montgomery, 1983)

Branches. The branch system is separated into the successive stem lengths corresponding to annual increments of elongation. Curtis (1947) recorded the distribution of PI and resin among the various segments of the root and stem. PI is concentrated in the bark and the rubber concentration expressed as percentage of dry weight. It is lowest in the root, higher in the branch, and much higher in the crown and branches. The oldest segments of the branches, stems formed during the first year (12.7±1%), are higher in PI content than the second year stems (11.5±1%). It is interesting to consider that although the first year stem contained a considerable percentage of PI before the growth of the second year stem even started, the two are now not so different. Jones (1948) studied differences in PI content in roots and mature shrub. When main stem of hand-defoliated shrub were cut just below the first branches and the two plants fractions were processed separately, the resin content of latex rubber recovered from branches was superior to that from roots (Hammond and Polhamus, 1965).

Stems. System of branching continues resulting in a symmetrical and closely branched shrub Dead shoots and branches are relatively low in PI as compared to live parts (Curtis, 1947).

Root System. The root system consists of the tap root, which loose prominence and give way to an intricate system of dense fibrous laterals and their branches (Muller, 1946). The depth of penetration of the taproot is determined by the depth of penetrable soil. Roots of plants in wild stands in Texas rarely penetrated the soil beyond 60 cm and the greatest concentration of fibrous roots is in the upper 15 cm of soil (Hammond and Polhamus, 1965). The proportion of PI is at minimum in the primary root. From the root upward the proportion of bark increases in the progressively younger parts with smaller diameters (Estilai, 1987; Curtis, 1947; Whitworth and Whitehead, 1991). Some strains have yielded up to maximum produce about 26% dry weight of PI after a 4 year growth (Rodríguez et al., 2006; Guayule, 1977; Whitworth and Whitehead, 1991). Mostly the PI in guayule shrubs is found in the stems, branches, roots, and a little bit in leaves (Table I.1). However, the contribution of leaves to high resin yield is low (Jasso de Rodriguez and Kuruvadi, 1991; Teetor et al., 2009). Jones (1948) reported that the dispersed root PI was highly unstable and contained a large quantity of resin, in line with the peculiar repartition of PI and resin in the roots compared to branches.

Jasso de Rodriguez and Kuruvadi (1991) analysed guayule biomass sectioned into root, stem and branch parts, by Soxhlet extraction. The values of the PI and resin percentage for the different plant parts increased from root to stem and from stem to branches. They found that PI varied from 2.2 to 8.5% and 2.7 to 8.4% in the stem and branch, more than in roots 1.4-4.6%; Stem contained 15.9% more PI compared to root. The resin content ranged from 4.9 to 8.8% in root, 6.9 to 10% in stem, and 9.2 to 10.8% in branch. The branches contained 19.9% and 52.3% more resin than the stem and the root tissues, respectively.

As rubber is more concentrated in the bark, the proportion of bark in the various segments of stem and root is a factor in their resultant rubber contents (Teetor et al., 2009). Contrast between the concentration in the bark and that in the remaining wood cylinder is most striking in the root where the concentration in the bark may be eleven times that in the wood. In the branches, however, the concentration in the bark is commonly only two or three times that of the wood; for the plant as a whole the bark has approximately three times the rubber concentration of the wood. In terms of amounts of rubber the bark is even more important, because the weight of bark tissue is somewhat greater than the wood. Resin (acetone soluble) concentration is also at a minimum in the root, but with only an insignificantly higher concentration in the crown and bark. The second year stem, formed during the past, shows distinctly more resin than the older stem (Curtis, 1947).

Evidence of the wood cylinder into annular parts and analyses of the variously aged components indicates that the cell of the xylem and pith continue to accumulate rubber for several years after they are formed. The increase rubber concentration occurring as the wood cylinder becomes older was found to be due to continued accumulation in the old tissues rather than to high rubber contents in the xylem added in later years. The annual rings of the xylem were much higher in rubber concentration than the outer ones, and higher than the corresponding annual rings of the young plants (Curtis, 1947).

I.2.6 Guayule cultivation

Guayule can grow naturally within a wide range of climatic conditions: precipitation 250-350 mm, temperature -23 to +49°C, and altitude 700-2,000 m. For commercial production, fields should be well-drained very fine sandy loams to silty loams. Minimum temperatures should be -9°C with 380-640 mm of rainfall for dryland production. Maximum yields occur when irrigation is similar to other crops grown in arid regions

Planting. Guayule stands have been established by: i) transplanting nursery-grown seedlings, ii) transplanting seedlings propagated in the greenhouse, and iii) direct seedlings in the field.

Switching acreage to guayule is easiest for growers with cotton experience as their existing equipment can be used for the production of guayule (plows, discs, bed shapers, cultivators, and module builders) (Whitworth and Whitehead, 1991; Yulex, 2012).

Growth cycles. Guayule matures for harvest within 12 to 20 months. Its subsequent re-growth can be harvested annually, producing high quality latex. High agronomic yields have been achieved through selective breeding. Since guayule is a perennial crop, less planting preparation, cultivating and harvesting is required compared to other crops. Also, owing terpene resins, which are natural pesticides, it is resistant to many pests (Yulex, Novenber, 2012). Potential benefit may be faster regrowth following harvest, allowing more frequent harvests than every 2 years (Foster and Coffelt, 2005; Coffelt and Nakayama, 2007).

Water supply**.** Once established, guayule requires less water than many other industrial crops. In addition, it is unrivaled compared to existing and alternative crops. Biomass yield reached up to 20 tons dry, and PI yield estimates approach 1,000 kg/ha.year. Past progress made with *Hevea brasiliensis* shows the potential of breeding in the PI yield increased from 300 to 3000 kg/ha.year. Although significantly greater improvements are yet anticipate, Yulex (2012) production in Arizona currently yields 40 tons fresh weight per ha.year (2 tons latex/ha.year).

I.3. Parameters acting on guayule rubber production and quality

I.3.1 Breeding

Llyod and McCallum were responsible for the initial cultivation of guayule for rubber production but were not able to increase the rubber content of their selections of plants beyong the wild state. In the late 70s, renewed interest for guayule brought breeding to the forefront. Guayule exists as both diploids (2n=36) and polyploids within the species. Tetraploids (2n=72) are the most common form. Polyploids are usually larger and more productive. Guayule reproduces by apomixis and corresponding seeds are an exact replica of the plant bearing them in regard of chromosome number and genetic makeup. But it can have both sexually and asexually formed seeds on the same plant, the later being predominant in spring and fall, while sexually produced seed are more frequent in the summer. In native stands PI content has been reported from 3.6-22.8%, while under cultivation, it has varied from 3-8% (Hammond and Polhamus, 1965). Guayule plants exhibits a great variation in biomass production. Since biomass is mainly bark and wood and PI in both tissues, breeding has focused of rubber and biomass yields (most known USDA guayule lines in Table I.5). Rubber quality, as the molar mass distribution (*Mw* and *Mn*), is not well defined for guayule selection and any early plant breeding assumed that any line of guayule will produce

acceptable quantity of rubber and therefore were not concerned with quality. High yielding, fast growing lines are needed commercially. The genetic improvement can be obtained when the selection for desired traits in a breeding is done when plants are 1-2 instead of 3-5 years old. The recent germplasm releases have not been fully evaluated for effects of environment on latex yield and plant growth (Ray et al., 1999). At present seeds are not marketed. Germplasm collections are maintained by the Land Grant Universities (Texas, New Mexico, Arizona, and California) as well as the USDA-ARS-USWCL (Phoenix, Arizona) (working germplasm collection) and recently with the EU-PEARLS project in Europe (Montpellier) and Spain (Cartagena).

Table I.5: Main USDA guayule lines tested in Arizona, Texas and California

Guayule line	PI (%)	Resin (%)	Dry biomass (kg/ha.year)	PI yield (kg/ha.year)
593	4.22	4.55	8.720	362
N565	4.42	7.82	7.950	348
11591	4.46	5.54	8.550	362
CAL-6	5.64	8.01	14.015	796
AZ101	2.60	8.50	9.760	217

*45 months old guayule plants; (*Schloman et al., 1983)

I.3.2 Agronomic parameters acting on PI production

Guayule is the sole non-tropical plant that has been used as a commercial NR alternative source. NR production from Hevea and guayule is compared in Table I.6. When a guayule plant is stressed, growth slows and the product from the photosynthesis is diverted to PI. The effect of moisture stress on the highest rubber yields were produced by plots with lowest moisture levels (Hunter and Kelley, 1946). The plants have been tested to high levels of soil water stress. Irrigation treatments significantly influenced rubber and resin yield by increasing the plant biomass (Rodríguez et al., 2006).

Table I.6: Compared Hevea and guayule PI yields

Rubber source	Mw (kDa)	Production (tons/year)	Yield (kg/ ha.year)
Hevea[a]	1,310	9.0-10	500 - 3000
Guayule[b]	1,280	0.01	300 - 1000

[a] *Black et al., 2006;* [b] *Mooibroek and Cornish, 2000; Polhamus,1962; Swanson et al., 1979*

Season and temperature. Guayule under cultivation exhibits a distinct seasonal cyclic pattern of alternate growth and PI accumulation. During cold weather or with reduced moisture supply, PI content increases (Benedict et al., 2011). As native plants withstand temperature fluctuation of -18^{o}C to 46^{o}C, guayule crop can survive -10^{o}C. Although guayule

may be considered drought tolerant, the productivity of the plant increases with irrigation. Backhaus and Walsh (1983) reported that guayule harvested in spring had higher content than in the fall months. Rubber percentage increases during the autumn-winter season and was at its highest peak in January and then decrease until October. The season of harvesting is an important parameter and good water management promotes biomass production during the spring-summer season allowing high PI production during winter synthesis cycles.

Plant age. Estilai (1987) showed that for a 20 months old N565 line guayule plant, the bark contains 75-80% of the plant's PI. Estilai (1988) reported that when plants aged, the bark/wood ratio decreased. Annual PI yield is genotype dependent with some giving their highest annual yield at the age of 21 months compared to 45 months (Estilai, 2003). The earliest harvest period with existing varieties is after two years of plant establishment.

Effect of harvesting. Guayule exhibiting a cyclical pattern of alternate growth and rubber accumulation, in contrast to latex concentration, maximum biomass occurs in the fall, following plant growth during the summer. Several methods of guayule harvest exist (Black et al., 1983; Estilai and Waines, 1987; Schloman, 2005; Cornish et al., 1999, 2000, 2004; Coffelt et al., 2009; Coffelt and Ray, 2010). Studies conducted with plants harvested by the recommended practice of harvesting the whole above ground portion of the plant (Foster and Coffelt, 2005; Coffelt and Nakayama, 2007) have indicated that the small to medium size branches contain most of the PI. The harvest method recommended is by cutting guayule plants of 50-60 cm height at 3-5 cm above the ground. Leaves are removed. The harvesting at pilot scale is based on the cotton harvesting method, making the transition from cotton to guayule easy and affordable for farmers.

Table I.7 Height and width of 1- and 2-year-old plants of four guayule germplasm lines grown at Maricopa and Marana, Arizona

Age (year)	Height (mm)	Width (mm)	PI, 1997 (%)	PI, 1998 (%)	Biomass, 1997 (kg)	Biomass, 1998 (kg)
1	428[b]	422[b]	0.93[b]		1.15[b]	
2	574[a]	572[a]	1.46[a]	1.06[b]	1.82[a]	3.16[a]
3				1.57[a]		3.79[a]

[a,d]*Data same letter within a given column are not statistically different to ANOVA test (p>0.05).*

Influence of storage. The effects of post-harvest storage on latex recovery, PI and resin contents, have been reported by Coffelt et al., (2005, 2009a, b); Dierig et al. (2001); Dissanayake et al. (2007). Taylor and Chubb (1952) showed that fresh shrubs gave the best PI

yield and high Mw with defoliated shrub. Storage of non-defoliated shrub gives a lowest PI yield and quality, and a slight increase in resin yield. (Coffelt et al., 2009) showed a decrease in the PI content and Mw after field storage. PI recovered increases with storage for three to six weeks but decreases with a longer storage. During the ERP project, the storage used was a prolonged field curing with storage of baled foliate shrubs indoor or outdoor, with curing of the shrubs before milling. A field curing of shrubs from 10 to 45 days, was the standard agronomic practice to reduce weight of material to be transported and to increase rubber content in the milling process (Taylor and Chubb, 1952). Several studies showed that the storage time has an effect on rubber quality (Taylor and Chubb, 1952; Black et al., 1986; Estilai and Hamerstrand, 1985; Coffelt and Ray, 2010). It is possible that in this group the enzyme machinery of rubber biosynthesis remained active, allowing the harvested shrub to continue to make rubber for a short period of time. Cornish et al. (2013) suggested a drying process at 50^{o}C for low rubber degradation prior to parameter control.

Antioxidants should be added to prevent rubber degradation during the extraction process, but just after harvest and prior to storage and transport to the factory. An increase of moisture extends the storage time of shrubs without negatively impacting Mw. However, under extreme conditions, high temperatures and extended dry storage times, a polymer molar mass reduction of up to 30% occurs. Storage conditions on the extractable latex, total rubber, resin contents, moist storage prior to dry chipping allows a higher yield and better of latex quality. A moist pretreatment of harvested shrub protected the latex fraction during dry chipping prior grinding processes. Storing harvested shrub under moist conditions allows a more flexible harvesting and processing schedules without significant latex loss (Coffelt, 2008).

The rubber is degraded by the resin constituent, most probably the unsaturated fatty acid and linoleic acid. The oxidation of unsaturated fatty acid forms hydroperoxide which in turn initiates the degradation of the rubber molecule. Linoleic acid can also act as a chain transfer agent and radical scavenger in the thermal degradation process (Keller, 1982). Bhowmick et al. (1985) observed that the degree of degradation based on molecular weight was higher when the greater is the number of double bonds of the unsaturated fatty acid, that is linolenic> linoleic > oleic acid. Stearic acid, a saturated fatty acid had the lowest rate of degradation. Rubber degradation can be retarded by cold storage and unsaponifiable fraction in resin (Keller, 1983). Black et al. (1986) found little change in rubber molecular weight, when the shrubs were stored in the freezer for one year. These characteristics influence the ability of the rubber product to resist oxidative and thermal degradation, chemical cross-links on vulcanized widen stress-strain, and performances characteristics of finished goods (Cantu et al., 1997).

I.3.3 Conclusion - Guayule production

The above bibliographical results show that assessing the PI and the resin contents in guayule biomass, before and after harvest, is of high interest for breeders, farmers and plant managers, due to the large number of parameters involved in biosynthesis and extraction.

I.4 Processing of the guayule biomass

In 1970, owing to high oil prices, the large US tire producers, Firestone and Goodyear, were interested for guayule development with pilot facilities (Fort-Stocktom, Texas; Sacaton, Arizona). In Mexico, in the 70s, a pilot unit was established and new extraction processes developed (Campos Lopez, 1978). With the development of a non-allergenic guayule latex, the Yulex Corporation started in 1997 and 2011 commercial plants in Arizona, to produce commercial latex. The technical feasibility of guayule latex production was demonstrated.

I.4.1 Guayule biomass processing methods

Several processes to extract rubber from guayule plants at pilot or industrial scale have been developed during the IRC period and the ERP project (1900-1945), then in the 80s and more recently since 2004. The main processes operated in Mexico and in the US are (Figure I.8):

- a latex and a solvent process plant of the ERP project in California at Bakersfield,
- a solvent process, Centro de Investigation en Quimica Applicada, Saltillo, Mexico.
- a solvent extraction plant of Bridgestone/Firestone (Sacaton, Arizona, 1980-1985),
- a solvent process pilot plant at Texas A&M University in the 80s,
- a latex plant by Yulex Corp., USDA station, Casa Grande, Arizona, since 2004.

Nowadays, the solvent extraction process is the more advanced one, but it does not meet environmental regulations due to losses of hexane and acetone from the extracted biomass (bagasse). A process under supercritical fluid (CO_2SC) has been investigated at laboratory scale for sequential recovery of resins and low Mw PI, but it is not adapted to extract high Mw PI. The hypoallergenic latex extraction developed by USDA and Yulex.

Flotation process. It is the oldest method. Plants were crushed and ground with crepers to produce pellets of branches, Pellets were crushed for two hours in pebble mills with a ratio water/biomass of 5:1. Rubber particles agglomerate into “worms”, a mixture of resin, fibers and PI separated by filtration and in a floating tank. A treatment in a pressure chamber at 90°C was applied to separate rubber and fibers; the GR was crushed and washed with detergent to eliminate remaining bark, resin, dried, pressed, baled and stored. GR produced by the ICRC process oxidized and aged badly. The quality was not constant due to harvesting of wild shrubs, presence of resins. The process was long and non-continuous (Lloyd, 1942).

Sequential extraction. Sequential extraction directly addresses the weakness of the flotation process: separating resin from rubber. Ground shrub is deresinated by extraction with polar solvent (acetone) (Kay and Gutierrez, 1985) and then processed with a second solvent

(hexane or pentane) to extract the rubber (Garrot et al., 1981; Nurthen et al., 1986; Schloman et al., 1987; Verbiscar et al., 1989; Wagner and Schloman, 1991). USDA evaluated this method at pilot scale (Wagner and Schloman, 1991). Defoliated shrub was then chopped and flaked. Batchwise deresination with acetone was carried out by a two-stage immersion and rinse, before continuous, countercurrent extraction of PI with hexane. Rubber recovery was 88 %. This method can be conducted in a single- or a multiple-step operation (Schloman et al., 1988). However, it appeared not to be economically viable (Wagner and Schloman, 1991).

Simultaneous solvent extraction. A mixture of acetone, and hexane or pentane is used (Wagner, 1988; Wagner and Schloman, 1991) to extract both PI and resin, then rubber is recovered upon addition of a polar non-solvent. Several solvent systems have been evaluated: toluene or hexane have been used as the rubber/resin solvent and methanol as the non-solvent component (Wagner and Parma, 1988; Wagner and Schloman, 1991). Other examples are the pentane-acetone azeotrope (78/22 weight %) and acetone as the non-solvent to coagulate the rubber (Kay, and Gutierrez, 1987).Different combinations of solvents and alcohols for rubber precipitation, antioxidants, extraction temperatures, shrub sources, storage conditions, and preparation methods were investigated; and their effects on rubber material properties and quality were evaluated (Wagner et al. ,1988). Other researchers used simultaneous extractions with pentane: acetone (82: 18 v/v) (Kroeger et al., 1999).

The advantage of simultaneous extraction is the capability of tailoring the physical properties of the rubber product. The rubber recovery step is based on coagulating the rubber by addition of a polar solvent. After the initial extraction, more acetone is added to coagulate the high molecular weight rubber. After separation the effluent consists of a solution of resin components (Beinor and Cole, 1986). While resin composition, including the levels of sesquiterpene esters, triterpene keto alcohols, and triglycerides, varies with the conditions of extraction, the total content of species bearing chemically reactive groups such as carbonyl shows little change (1.2–1.3 mmol/g). Rubber yield and molecular weight are unaffected by the choice of extraction medium (Schloman, et al., 2003).

Schloman et al. (1988) reported that the yield and composition of the resin extracted from guayule will depend upon the choice of extraction medium. Water-soluble components, primarily polysaccharides, are entrained by solvent when the shrub is deresinated with acetone by sequential extraction. As a consequence, resin yield is substantially higher with sequential extraction than resin from simultaneous extraction. The water-soluble material in resin from sequential extraction contains components suitable for commercial valorization.

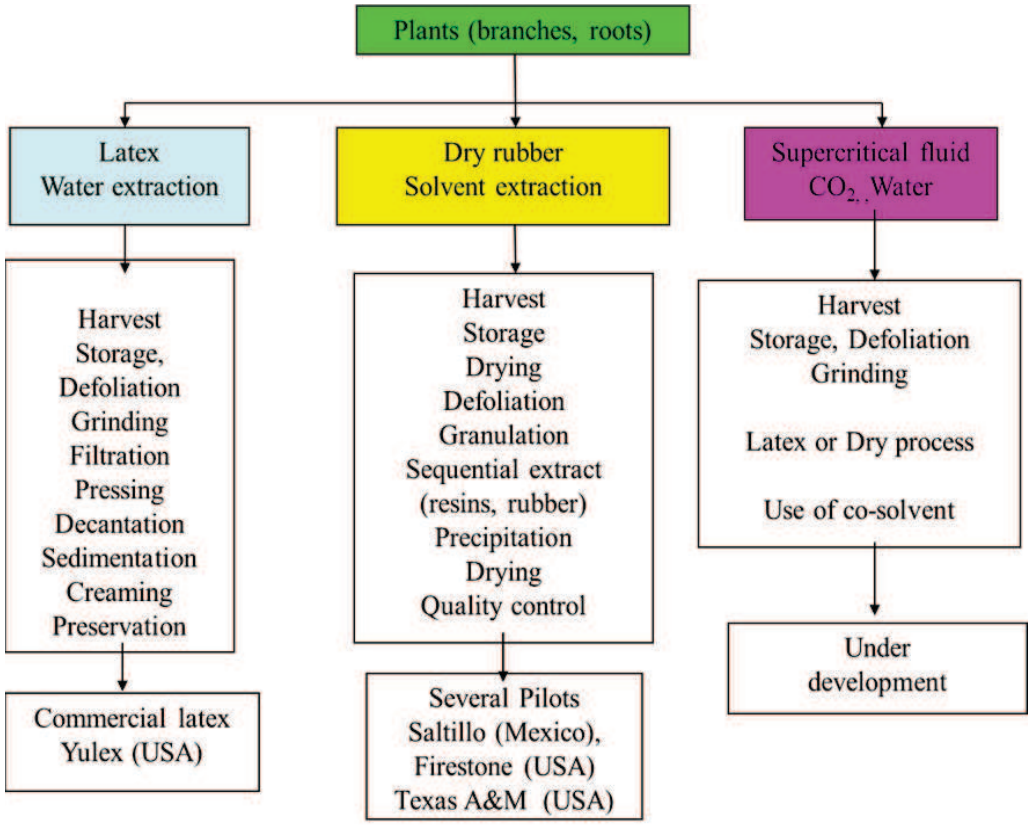


Figure I.8: The three types of extraction process for guayule rubber production

Rubber yield and molecular weight are unaffected by the choice of extraction medium. This method has been used at pilot level facility of Sacaton, Arizona by the Bridgestone/Firestone Corporation. Although, this method has been successful in extracting rubber, engineering difficulties in handling the shrub have plagued both facilities. It selectively eliminates low *Mw* of rubber. Simultaneous extraction appears to be the most acceptable basic for a commercial guayule processing industry.

In the frame of this thesis study, the sequential extraction method will be chosen with two solvents i.e. acetone and hexane because they are high efficiency and commercially available.

Supercritical fluid extraction (SFE). Supercritical fluid (CO_2, N_2O, SF_6, methanol, propane, water) is a state obtained at, or above, its critical temperature and critical pressure. This state is suitable to extract target substances in solid samples, owing the still high diffusivity of the fluid, comparable to the gas, and to the solving power high enough, although lower than the corresponding value for the liquid state. This is a relatively new method which has recently begun to be applied extensively. The sample is placed in an extraction chamber, through which the supercritical fluid is pumped, the target substances are extracted from the samples and trapped by condensing (reduced pressure). The most commonly used fluid is CO_2.

However, supercritical CO_2 (SC-CO_2) is non-polar, so modifiers (methanol, dichloromethane, acetonitrile, water, etc) are added to improve extraction efficiency for more polar compounds. Supercritical carbon dioxide (U.S. EPA Method 3561) is used as the extraction solvent for essential oils and caffeine. It is non-toxic and non-flammable, and separation of the extract from the starting material is much simpler than with traditional organic solvents, merely by allowing it to evaporate into the air recycling it by condensation into a cold recovery vessel. Its advantage over steam distillation is that it is used at a lower temperature, which can separate plant waxes from the oils. The low temperature of the critical point (31°C; 73 bar) and the stability of CO_2, allows extraction with little damage. In addition, the solubility in SC-CO_2 varies with pressure and temperature, permitting selective extraction. A patent relates the extraction, separation, fractionation and purification of resin and biopolymer from guayule plant materials using supercritical solvent extraction (Cornish et al., 2006). They have developed a method using an expanded hexane solvent and claim that the expanded hexane solvent results in a more efficient and rapid process over other SC-CO_2 extraction systems, including those systems using hexane as co-solvent. Extraction is achieved by pumping liquid hexane into the extraction vessel, introducing CO_2 into the extraction vessel, forming an expanded hexane solvent and transitioning the expanded hexane solvent to a two phase supercritical/liquid system wherein SC-CO_2 is saturated with the expanded hexane solvent, to finally extract the biopolymer from the plant material.

Extraction under the latex form. This procedure is drastically different from the pre-1980 water extraction method where the latex was coagulated to the solid form. As far as we know, at present, latex extraction from the guayule plant is based on a water extraction process (Cornish, 1998). Isolation of un-coagulated rubber particles in latex from the guayule shrub was originally described in 1948. Cornish et al. (1999) developed a new process. The plant material is chopped up, homogenized thoroughly in a basic buffer containing antioxidant using a hammer mill, and the mixture is pressed and filtered to remove plant fibers and other solid material. Subsequent clarification and purification steps are done using centrifugation for separating the latex particles from cell debris and resin. Thus, the shrub must remain in hydrated form throughout harvest, shipping, and storage until homogenized in the aqueous extraction medium. Alternatively, final concentration can be effected with a creaming agent. The concentrated latex can have a total solid content (TSC or DRC) as high as 50% by weight (Schloman et al., 1996). These methods have not been refined sufficiently and sometimes the GR does not coagulate, but remains as an emulsion in the latex (Cornish et al., 2005).

I.4.3 Valorization of non-rubber products

Current research on guayule co-products points a potentially profitable industry development from the resin, low-Mw PI, and bagasse. Their value may exceed that of PI and researchers now agree that the successful industrialization will be facilitated by the income derived from the co-products (Cornish, 2006). This valorization will be based on the biorefinery concept which is analogous to today's petroleum refinery, identified as the most promising route to the creation of a new domestic bio-based industry. A biorefinery might, for example, produces one or several low-volume but high-value chemicals and a low-value, but high-volume liquid transportation fuel, while generating electricity and process heat for its own use and perhaps enough for sale (Figure I.9). The high-value products enhance profitability, the high-volume fuel helps to meet national energy needs and the integrated power production reduces operating costs and avoids greenhouse-gas emissions.

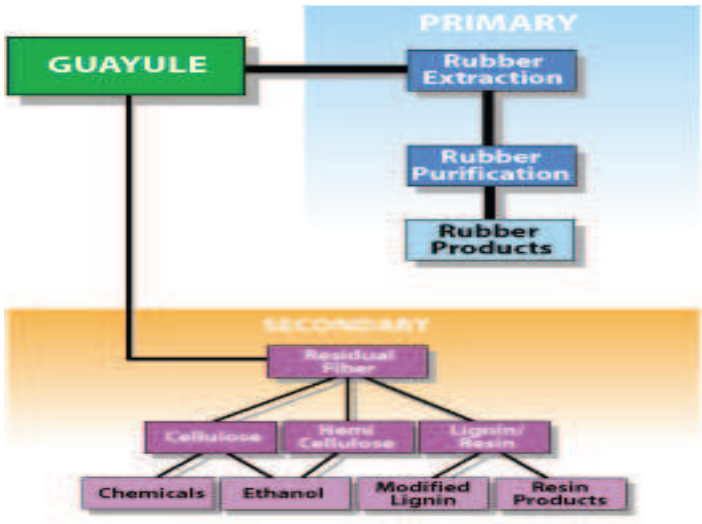


Figure I.9: Biorefinery processing scheme of guayule biomass
(Source: www.Yulex.com/technology, 2012)

Resin. Figure I.9 shows a large range of products some of them resin-derived to replace petrochemicals. Valorization of the resin will be with new applications as adhesives, pesticides against termites or crickets, shellac, paints, plastic, rubber chemicals (tackifier), wax, new polymers, and pharmaceuticals products, etc... This has been reviewed by Schloman and Wagner (1991) and Nakayama (2004). The bagasse originating from the latex extraction still contains resinous material in plant cells. It is worth to mention that compounds in latex makes only about 10% of the initial biomass. Especially in the case of latex extracted by a water-based process, the residual bagasse contains most useful resin compounds. The resin-containing bagasse can be used without additional chemical processing, for example, combined with a plastic binder to make high-density composite boards that are resistant to

termite degradation. This bagasse could also be blended with other types of wood sources to make boards that will have insect control properties (Cornish, 2006). The resinous material can be solvent extracted from either the whole plant or the bagasse. By impregnating wood with the crude resin extract, it can be protected against wood destroying organisms. Guayule-based resin and epoxy polymers have been combined to make strippable coatings that can be used for storage protection of equipment (Nakayama, 2004). Supercritical extraction was considered as a preferred method for collecting guayule resin (Cornish et al., 2007), and can be integrated into the PI extraction plant without incurring high capital costs.

Lignin and bagasse. Among the polymers making the wood structure, including cellulose and hemicellulose, the lignin fraction of guayule can be valorized. During delignification, the linkages within the lignin macromolecular structure "basically a complex mix of aromatic groups linked by C-O-C bonds" are hydrolysed with the release of aromatic fragments of varying molecular weight. Thermoplastic biopolymers can be developed through alkylation of lignin macromolecules and blending with Tg and Tm of polymers or plasticisers. Such lignin biopolymers have been demonstrated to achieve tensile properties like polystyrene. Schloman et al. (1983) found that the dilute-acid hydrolysis of guayule bagasse left after solvent extraction did not give a good yield of fermentable sugars (Schloman et al., 1983). Srinivasan and Lu-Kwang Ju (2010) converted the remaining bagasse to biorefinery feedstock for value-added products and evaluated the feasibility of including a SC-CO_2-based bagasse pretreatment method. The pretreatment involved: adding water to the bagasse, raising system temperature, pressurizing using supercritical CO_2, and quick release of pressure. The pretreated biomass was then subjected to enzyme hydrolysis. Supercritical method outperformed other methods and gave much higher sugar yields from guayule: 77% for glucose and 86% for total reducing sugars through both pretreatment and hydrolysis, as compared to 50% and 52% respectively with dilute-acid pretreatment, and 36% and 52% with the delignification pretreatment. The enzymatic hydrolyzates were tested on the cellulase-producing fungus *Trichoderma reesei* Rut C-30, with no inhibitory/toxic effect.
Industrialists are seeking replacement of urea-formaldehyde (UF) resins-not suitable for health-in the manufacture of wood panels. Guayule and resin can be used for that purpose.

Bioenergy production. The bagasse can be compressed into logs, briquettes, and pellets for energy production. Such combustible material has higher energy content than other wood sources because of the resin, which can make about 10% of the dry mass. Schloman and Wagner (1991) reported an energy content of resin of 38,200 kJ/kg, and a heating value of the

bagasse of 18,300 kJ/kg. Bagasse has also been converted into liquid fuel. With improved pyrolysis technology, it could become an economic source of diesel-type fuel (Nakayama, 2004). During the fermentation of effluents in the latex process, a large part of the organic substances are decomposed by bacteria, and biogas is generated and could be used for electrical production. The sugars present in the bagasse fraction of the guayule plant can be solubilised by enzymatic hydrolysis using commercially available preparations. The hydrolysates obtained, with a high content of glucose, xylose and other sugars, are a fermentation medium for production of ethanol, butanol, isopropanol and deresinated bagasse could be a source of ethanol and other liquid fuel or solvent (Nakayama, 2004; Srinivasan and Lu-Kwang Ju, 2010). Butanol is regarded as a fuel additive with better properties than ethanol, especially in diesel and kerosene. Isopropanol, which is a widely used alcohol in many applications (solvent in industry, anti-freeze agent) can be a precursor of propylene and has been regarded as a potential additive in gasoline (López-Contreras et al., 2010).

I.4.4. Conclusion - Guayule processing

The quality of guayule latex and rubber depends not only on biomass harvest conditions as seen in the previous section, but also on processing conditions. In addition to the two products containing PI -rubber and latex- a range of valuable compounds or extracts can be derived from resin. Keeping in mind that resin components may also affect the rubber properties, it is necessary to have achieved a sufficient chemical knowledge of biomass and product composition for monitoring this complex set of parameters. Successful commercialization of guayule depends on rubber product quality, but also on utilizing as much of the plant as possible to extract high value co-products (resin). From this it makes sense that accurate quantification of the extractable components (PI and resin) is needed for industrial processing.

I.5 Guayule rubber properties and manufactured products

GR is similar to Hevea rubber, a polymer of isoprene units (National Academy of Sciences, 1977) with the similar structure, stretch, bounce and general properties. GR is the only low protein, non or hypoallergenic NR Type I latex allergy safe on the market today, offering high performance physical properties and. GR proteins are not the same as those found in Hevea latex. The proteins content is lower than in Hevea latex and they do not cause any allergies (www.Yulex.com/technology, 2012), opposite to Hevea. In the mid-90s extensive allergy reactions were being observed in Hevea latex users (Finlay, 2012) and an increased demand has developed for latex medical products. However, with the increased use of latex medical

and hygienic products, the problem with allergies also increased and many lawsuits have been filed by persons who became sensitized to Hevea proteins (Steinauer, 1999; Cornish, 2001).

I.5.1. Guayule latex and derived rubber properties

Unlike Hevea, PI is formed and stored within individual cells in guayule, and tapping is not a viable option. Guayule extracted latex is a colloidal suspension of both PI and other components (Table I.7; National Academy of Sciences, 1977). GL meets the requirements of ASTM International for D1076 Category 4, for latex from alternative sources. Ammonium hydroxide is added as a pH modifier, emulsion stabilizer and microbial growth inhibitor. The origin and composition of the non-rubber components of guayule latex have been only partially described (Wagner and Schloman, 1991; National Academy of Sciences, 1977). The low molecular weight resin components act as plasticizers, and dry films of high-resin GR are less elastic and more easily distorted than HR films (Schloman, 2001). GL contains a substantially higher fraction of saponifiable lipids).

Table I.7: Guayule and Hevea latex specifications from ASTM D1076 category 4

Properties	Minimum	Maximum	Control HA hevea latex
Total Solids Content (%)	50.0	57.0	61.5
Dry Rubber Content (%)	49.0	56.0	60
Total Protein, µg/g dry wt. rubber	-	200	>600
Hevea Antigenic Protein, µg/g dry wt. rubber	-	ND	
Total Alkalinity, NH_4OH as % Latex	0.60	-	1,6 (min.)
Viscosity 43% TSC, cps	20	80	
Sludge, weight %	-	0.070	0.1
Coagulum, weight % max	-	0.020	0.05
pH	11.0	12.0	10.6
Mechanical Stability 43% TSC, seconds	100	600	540 (min.)
Copper (ppm) max.	-	6.0	8
Manganese (ppm) max.	-	6.0	8
KOH Number max.	-	0.75	1.0
Volatile Fatty Acids max.	-	0.05	0.2
Density (mg/m^3)	0.940	0.960	0.94-0.96
Color	Off-white, beige		Blue or grey tint
Odor	Ammonia		No putrefactive odor

For latex rubber coagulated directly from fresh shrub, PI can contain up to 20% of resin. When extracted from shrub by solvent extraction and deresination, the rubber bulk viscosities is similar to the viscosity of rubber obtained from coagulated latex. Factors that contribute to PI difference between latex and solvent extraction are a lower Mw, a broader distribution (Mn), and the resin content. Bulk GR viscosity similar to HR is obtained after selective coagulation of the high Mw fraction (Schloman et al., 1996) (Table I.8).

Table I.8: Characterization of various NR types (Schloman et al., 1996)

Origin	Mooney viscosity ML1+4 (100 °C)	Resin (%)	Reference
Hevea latex	100.4–109.0	1.9–2.3	Schloman et al., 1996
Guayule latex	49.9–69.2	8.2–9.9	Schloman et al., 1996
Guayule rubber unfractionated bulk	34.5–65.5	4	Wagner and Schloman, 1991
GR fractionated bulk	99.6	2,2	Bridgestone/Firestone Inc. Arizona, pilot plant
Raw GR	105	-	National Academy of Sciences, 2002
Hevea SMR 5	85	-	National Academy of Sciences, 2002

I.5.2 PI of raw guayule rubber

The Mooney viscosity and plasticity of GR are in the range of HR. It would probably be more difficult to process than synthetic PI rubbers. Both GR and HR have excellent tack characteristics, good green strength (Table I.9) (National Academy of Sciences, 2002).

Table I.9: Properties of GR (National Academy of Sciences, 2002)

Property	NR[a] Grade 20	FEMA[a]	Bridgestone/Firestone[b]	Guayule[c]	Hevea SMR5[c]
Dirt (%)	≤0.10	≤0.20	0.03 ± 0.04	-	-
Ash (%)	≤1.00	≤1.25	0.8 ± 0.5	-	-
Volatile matter (%)	≤0.80	≤1.50	0.5 ± 0.3	-	-
Manganese (%)	≤0.0015	≤0.0020	0.0010 ±0.0012	-	-
Acetone extract (%)	≤4.0	≤4.0	0.9 ± 0.6	2	2.8
Initial Wallace plasticity,P_0	≥35	≥30	37 ± 2	47.5	37
Plasticity retention, index PRI	≥40	≥40	82 ± 10	41	60
Mooney viscosity (ML 1+4 at 100 °C)	-	-		105	85
Antioxidant (%)	-	0.5 ± 0.2	-	0.6	-

[a] *Modified rubber,*
[b] *Means of test results for 61 bales produced 01-05/90 from Bridgestone/Firestone*
[c] *Data from* National Academy of Sciences, 2002 *(modified)*

Processing characteristics of extruded and flow property in molds, guayule differs slightly from Hevea in the ratio of chemicals needed to compound for curing rates. Both PI contain small amounts of moisture content, dirt, terpenes, triglycerides but guayule lacks proteins that are beneficial to the curing properties of Hevea (Table I.10). A rubber formulation curing analyzis on a Monsanto Rheometer at 140°C using ASTM D2084-71 shows that Guayule has a lack of vulcanization accelerators that gives a slower vulcanization than HR (Bridgestone/Firestone). Processing characteristics of aging properties of guayule has average physical properties (ASTM D 412) (Table I.11).

Table I.10: Comparison of vulcanized properties of GR and HR

Properties	GR	HR (SMR 5)
Initial viscosity (lbs-in)	5.0	5.5
Maximum viscosity (lbs-in)	25	35
T_s, min	10.5	7.0
T_c(90),Cure time at 140°C, min	25	19
Modulus at 300 % (psi)	1,050	1,770
Tensile Strength (psi)	3,645	4,050
Elongation (%)	635	490
Set at break (%)	14	13
Rebound (%)	40	48
Shore A Hardness	54	60
Tear Strength (psi)	178	436
Swelling Index (benzene)	3.44	2.94

(National Academy of Sciences, 2002).

Table I.11: Average physical properties of guayule aged properties (Yulex, 2009)

Guayule	Tensile (MPa)	Elongation (%)	500% Modulus (MPa)
Powdered - Unaged	27-31	900-975	1.8-2.3
Powder-Free -Unaged	26-30	930-1,000	1.6-2.1
Powdered - Aged	23-27	675-750	3.6-4.2
Powder-Free - Aged	23-26	700-775	2.3-2.8

Guayule rubber lacks the proteins and reactive functional groups present in both NR latex and bulk NR. As a result, guayule rubber is less stable to oxygen and heat. Bulk NR undergoes storage hardening with an increase in bulk viscosity when stored over long periods of time (Yulex, 2012). Cross-linking reactions produce branched polymer chains. In contrast, guayule rubber undergoes irreversible, heat-induced chain cleavage. Guayule produces no natural antioxidant and the rubber in its latex form rapidly degrades upon contact with air and heat or other parameter to degradation. Oxidation of the polymer chain is facilitated by the unsaturated fatty acid triglycerides present in the resin (Keller et al., 1981). Guayule rubber stability can be improved by combinations of an amine antioxidant and a zinc dialkyldithiocarbamate (Schloman and Hilton, 1992).

Molecular weight (*Mw*) and molecular weight distribution (*MwD*) of guayule rubber is an important characteristic to be considered. Agulo-Sanchez et al. (1995) showed that the molecular weight distribution of guayule rubber of young and old native plant prepared by socking grounded plants in the tetrahydrofuranne (THF) was multi-modal (up to three peaks). The *Mw* value of guayule rubber was comparable to *Mw* of Hevea rubber. Estelai (1987) indicated that *Mw* of the bark rubber was higher than *Mw* of the rubber in the wood. The *Mw*

of guayule rubber was in a range from $1x10^5$ to $2.5x10^6$. The average molecular weight rubbers showed distinct bimodal distributions with the positions of the maximum values at $0.1x10^6$ to $2.5x10^6$ g/mol (Liengprayoon, 2008).

McMahan et al. (2005) reported that rubber from guayule has a very high weight-average molar mass ranging from 500,000 g/mol to more than 1000,000 g /mol. Norton et al. (1991) studied tissue cultures found 1 to 10% of the rubber found in mature guayule stem tissue. The *Mn* distributions of rubber were bimodal in contrast with that of rubber from whole plant stems which is unimodal. The rubber from leaf tissue is bimodal with most of it with a peak of 22,300 g/mole. In contrast, the rubber peak in stems of young guayule plants was 500,000 g/mole. Rubber from shoot and root cultures had average $M_n s$ of 150,000 and 201,000 g/mole respectively, and normal and tumour callus cultures were 415,000 and 291,000 g/mole. McMahan et al. (2006) compared the M_w determined by size exclusion chromatography coupled to a multi-angle light scattering detector (SEC-MALS) of rubber of two lines of guayule (AZ-2 and 11591) harvested in latex form at different ages. The M_w of polyisoprene of older plants (3.3 years) were significantly higher than the one extracted from the youngest (1.7 years). Backhaus and Nakayama (1988) studied the effect of agronomic practices on macromolecular structure, especially irrigation frequency. Increasing irrigation frequency, for two cultivars tested, decreases M_w (from 580 to 480 kg/mol, for 11591 cultivars).

Backhaus and Nakayama (1986) studied the *Mw* of the rubber in different parts of the plants for 3 years with cultivars 593 and AZ101, harvested in March and May. The rubber is mainly in the stems and has a high *Mw* around 10^6 and a broad tailing shoulder of the low *Mw* rubber with an average *Mw* of $6x10^5$. The molecular weight distribution was bimodal. They found an increase of low Mw rubber when the plants were harvested in May. They linked it with an active growth and rubber synthesis at this of time of the year in the plants. They did not find a bimodal distribution with rubber extracted from wild plants which grow in high water stress conditions. Those plants grow very slowly compared to cultivated plant and consequently relatively little low *Mw* rubber would exist. The results indicate that the rubber *Mw* varies considerably with harvesting time and genetic origin of plants. The bimodal distribution is characteristics of plants in active growth stage and correspond to the synthesis of low *Mw* in new tissues. Guayule and Hevea rubber have a similar molecular weight as observed by SEC-MALS and an identical structure when checked by NMR and differential thermal analysis (DTA) (Black, 1986; Kim, 2008; Subramaniam, 1972). As a result, guayule rubber has the same structure, stretch, bounce and general properties as Hevea rubber (Figure I.2). Guayule

does not appear to be a highly cross-linked species. Little gel is produced during formation of rubber in the plant and little from after extraction.

I.5.3. Manufactured guayule rubber goods

Manufacturing of latex rubber products is by dipping, casting, and foaming. The process by dipping is the most used one. The process by casting requires a consistent, high quality material. GR emulsions is used for foam: sporting goods, bedding, pillow and apparel manufacturers require allergy-safe I Hevea latex-free materials while demanding high physical performance and highly improved tactile characteristics.

GR goods are used in many medical applications: exam gloves, surgical gloves, and protection systems, surgical drapes, urinary catheters, catheter balloons, condoms and oral barriers, and tools, elastics and straps, finger cots, hemodynamic devices, breather bags, hospital bedding, tubing, impregnated fabrics, intravenous systems, neurovascular and therapeutic delivery systems, bands and garrotes, respiration devices, septa, seals and gaskets, and specialty adhesives (Coffelt and Ray, 2010; Yulex, October, 2012). Products such as toys, bedding, pillows, yoga mats, "green" cleaners are also made with GR.

I.6 Conclusion - NR producing alternatives (Hevea and guayule)

NR is a unique biopolymer of strategic importance in many applications. New NR alternative feedstocks should be developed to bring answers to the present situation characterized by:

- increasing evidence of allergic reaction to the proteins of Hevea latex and immediate need to develop NR resources that do not cause such allergic responses,
- a cyclic increasing price of petroleum leading to an increase of synthetic rubber price,
- predicted NR shortage due to increasing demand of China and other emerging countries
- risk of development of the *Microcyclus ulei* responsible for the South American Leaf blight desease (SALB) that could spray on Asian Hevea plantations,
- global warming and difficult access to water supply in semi-arid lands,

GR technologies from breeding, cultivation, processing and manufacturing are available today. Nevertheless, it has still to be proven that guayule is commercially feasible and this depends on prices of Hevea and synthetic rubber mainly. Co-products of guayule processing can be valorized to increase the competitively of the starting GR industry. Therefore it is important to allow farmers and industrialists to determine the PI and resin contents in the biomass.

Chapter II

Methods for the quantification of PI and resin in guayule biomass

II.1 Introduction

Chapter I was devoted to the guayule plant and to rubber production. Chapter II deals with analytical techniques which are necessary for assessing plant composition for breeders, agronomists, and production managers (harvest date), plant operators (extraction yield), marketing and manufacturers (PI quality). While Hevea bears PI under latex form that can be easily recovered from ducts by tapping, guayule stores PI as μm-size particles in bark parenchymal cells, and this makes the analysis more difficult, namely introducing an extraction step (Estilai, 1987). This has been already discussed in the previous chapter when talking about rubber production. Here again, but for analytical purpose, the plant material is often mechanically disrupted to release the rubber particles from individual cells, although there are also non-destructive analytical ways.

This chapter deals with analytical methods: PI quantification in the biomass, as well as resin quantification, often performed within the same protocol; in most cases the procedure comprises two steps, extraction of PI and resin, then quantification of these components by gravimetry, by chromatography, or a spectral technique. Because of the diversity of these quantification methods, some combined to extraction and some not, for practical reasons, extraction protocols are first discussed, then results from the main extraction methods associated to gravimetry are compared, finally other non gravimetric quantification methods are discussed.

II.2 PI and resin extraction

Several analytical procedures have been described to measure resin and PI contents in plants (Buranov and Elmuradov, 2010; Van Beilen and Poirier, 2007; Kelley et al., 2004; Takeno et al., 2008; Cornish et al., 2004) especially guayule (Black et al., 1983; Nurthen et al., 1986; Jasso de Rodríguez and Kuruvadi, 1991; Wagner et al., 1988; Schloman et al., 2003). The measurement of resin and PI content in guayule tissues requires in most cases destructive methods for extracting, either with water or with an organic solvent. Therefore the extraction step is essential, and this first part of the Chapter II is devoted to it; in addition this part also includes gravimetry, because it is by essence coupled to an extraction step. Then other methods will be discussed, some of them also coupled to extraction, some not.

II.2.1 Water-based extraction

The isolation of un-coagulated rubber particles in latex from the guayule shrub was originally described in 1948 (Bonner, 1991), and modified and refined by Cornish et al., (2003) and other researchers (Diana and Rodriguez, 1991; Coffelt et al., 2009 a, b; Schloman, 2005; Wagner et al., 1988). Accurate determination depends upon stringent post-harvest and storage conditions to avoid rubber coagulation inside the cell or during extraction process, and to allow complete release of PI (Schloman, 2005; Cornish et al., 1999, 2000, 2005). Also water-soluble components, polysaccharides and proteins, are entrained by water, thus requiring a specific method to allow quantifying only PI. Coffelt and Nakayama (2007) have proposed a "standard" protocol from harvest of plants including obtention of the latex from fresh guayule biomass (stored maximum 2 weeks in refrigerator). Since the stem contains most of the PI, defoliation is desirable. Leaves break off after the shrub is partially dried, but if drying cannot be accomplished, chemical defoliation may be desirable before plant harvest. Chipping and processing shrubs without leaves gave a higher yield per weight of ground shrub (Van Staden and Gilliland, 1984). The shrub must remain hydrated until extraction. During the extraction, the biomass is homogenized thoroughly in a basic buffer containing antioxidant, using Waring blenders (Coffelt and Nakayama, 1991). An antioxidant is used to overcome easy oxidation of PI (Coffelt et al., 2007, Cornish et al., 2013). Then the mixture is pressed and the liquid fraction filtered to remove dispersed fibers. Clarification and purification are done using centrifugation for separating the latex particles from cell debris and resin (Cornish et al., 2005). The dispersion is concentrated in a series of centrifugation steps, until a total solid content (TSC) as high as 35-50% by weight (Jones, 1948; Schloman et al., 1996). Although used routinely in certain laboratories (Cornish and Schloman, 2004; Coffelt et al., 2005) according to Cornish et al. (2004), these methods are reliable.

Water-based extraction for analytical purpose does not serve for resin quantification, and even there is no assurance about total extraction of PI. But checking the PI extractable under latex form (Cornish et al., 2013), serves mainly to research programs devoted to extraction processes, and to plant operators, instead of breeders and agronomists.

II.2.2 Solvent extraction

Most methods published and used by guayule breeders and agronomists are based on extraction of rubber by a solvent (Black et al., 1983; Jasso de Rodríguez and Kuruvadi, 1991; Wagner et al., 1988; Schloman et al., 2003) -or even by a non-solvent (water), rubber being

extracted under latex form in the latter case (Cornish et al., 2000; Cornish and Brichta, 2002; Cornish et al., 2004; Kroeger et al., 1999; Beattie and Cole, 1986) as above detailed. As a general rule, resin and PI are sequentially extracted by a polar (acetone, ethanol) and a non-polar (hexane, cyclohexane, benzene, toluene, chloroform) solvent respectively (Black et al, 1983; Wagner et al, 1988; Salvucci et al., 2009; Teetor and Ray, 2004), although in some cases resin and rubber are extracted together prior to precipitating rubber by adding a non-solvent (methanol) (Wagner et al., 1991). All previous protocols are applied to powdered dry guayule tissues, consequently being of destructive type and the measured value is often the weight of the dry extract, PI or resin, thus being of gravimetric type (Black et al., 1983; Jasso de Rodríguez and Kuruvadi, 1991; Wagner et al., 1988; Schloman et al., 2003).

II.2.2.1 Moisture content

Biomass samples contain water that can prevent nonpolar organic solvents from reaching the target analytes. The use of more polar solvents (e.g., acetone, methanol) or solvent mixtures (e.g., hexane/acetone, methylene chloride/acetone) can assist in the extraction of wet samples. Drying is normally accomplished by oven (or freeze drying) prior to extraction; however, the recovery of volatile compounds may be compromised by these procedures.

Regarding freshly harvested guayule biomass, after the winter season the moisture content(MC) of fresh shrub ranges from 27% to 60% (Wagner et al., 1991). Also Black et al. (1983) reported high MC of 45-60% in fresh guayule samples. Therefore, these authors dried the ground guayule biomass in oven (105^{o}C) prior to analysis, to MC under 10%. Using an extraction method based on Soxhlet (Black et al., 1983; Schloman et al., 1988; Wagner et al., 1991), polytron (Jasso de Rodríguez and Kuruvadi, 1991), and ASE (Salvucci et al., 2009; Cornish et al., 2013) found the PI yield higher with shrub MC in the range of 5-20%. In pilot plant efforts are required to prevent shrub MC loss. Excessive field drying should also be avoided. Also the ASTM-297 determination recommends a MC of 5-6% for PI determination with hexane. Schloman et al. (1988) used samples with MC 14-17%, and Verbiscar et al. (1989) with MC as low as 4%.

II.2.2.2 Solvents

There are many classes of extractives in guayule biomass, covering a large range of molar weight (Mw) and molecular structures, from non polar, like terpenes and PI, to neutral lipids (triacylglycerols, free fatty acids) and sesquiterpenes, and even much polar compounds like

phospholipids. Therefore the yield and composition of the extracted fractions, namely resin and PI, will depend upon the choice of extraction medium. This brings the question about extraction selectivity. Polar and/or non-polar co-solvents can be used to enhance the selective extraction of resin and PI from the guayule shrub.

As a general rule, the resin is first extracted with acetone (Black et al., 1983; Hauser and Beau, 1944; Verbiscar et al., 1989), another polar organic solvent (ethanol, isopropanol, methyl ethyl ketone). Then the PI is extracted with hexane or a less polar organic solvent, like cyclohexane, chloroform, carbon tetrachloride, benzene, toluene, methylene dichloride, but also di-ethyl ether (Kay and Gutierrez, 1985; Verbiscar et al., 1989; Wagner et al., 1991). Cyclohexane, hexane, and ethyl ether is a good rubber solvent that extract less residual resin (Wagner et al., 1991). Hexane and cyclohexane gave higher PI quantity and quality. Benzene, also a good solvent, gave less resinous compounds than toluene. Chlorinated solvents are good solvents, but the extracts have more resin components as observed by infrared spectra analyses (Verbiscar et al., 1989).

II.2.2.3 Extraction protocols

In this case, and contrary to the water-based extraction, the biomass is here dried (developed in next section). Several types of apparatuses have been used, under atmospheric pressure (Soxhlet, Soxtec, agitated flask, high speed homogenizer-grinder (Polytron)), and under pressure, accelerated solvent extraction (ASE). The strategy for validating these methods is based on comparing to data obtained by applying other known methods, gravimetric and others.

Extraction of guayule dried and ground tissue had been reported, half a century ago, with Soxhlet (Holmes and Robbins, 1947), grinder (Hammond and Polhamus, 1965), other method (Spence and Caldwell, 1933; Bonner and Arreguin, 1947), and more recently again with Soxhlet (Black et al., 1983; Diana and Rodriguez, 1991), Polytron (Jasso de Rodriquez and Kuruvadi, 1991), Soxtec (Nurthen et al., 1986), ASE (Cornish et al., 2013; Salvucci et al., 2009; Teetor and Ray, 2004) for extracting and quantifying PI and resin from guayule.

Among the twenty-six reports in the literature on extraction-based protocols for PI quantification in guayule biomass, about half deals with Soxhlet; thus Soxhlet is considered as a “standard” option, (Black et al., 1983; Jasso de Rodríguez and Kuruvadi, 1991; ISO 1407-1976 F). This method together with the others above mentioned are presented and then compared in the next paragraphs. Need to recall that, once extracted, resin and PI are usually

determined gravimetrically, although other methods have also been proposed (described in the next sections).

Soxhlet extraction method. Before Soxhlet, the French Chemist Anselme Payen also pioneered with continuous extraction in the 1830s (Jensen, 1913). Also Wagner and Parma (2006) was pressing extraction step with a continuous extraction operation. Shrub preparation and extraction conditions were determined and found to be critically important for high rubber recovery efficiencies.

The Soxhlet extractor (Die, 1879; Harwood and Moody, 2007) was originally designed for the extraction of lipids from a solid material when the desired compound has a limited solubility in a solvent. When the Soxhlet chamber is almost full, it is automatically emptied by a siphon side arm, with the solvent running back down to the distillation flask. This cycle may be allowed to repeat many times. The advantage of this system is that instead of many portions of warm solvent being passed through the sample, just one batch of solvent is recycled. The solvent is heated to reflux, thus setting the extraction temperature.. The non-soluble portion of the extracted solid, remains in the thimble, eventually used for extracting a new fraction with a different solvent. Extraction of dried and ground tissue with a Soxhlet apparatus has been used in past studies with guayule (Spence and Caldwell, *1933*; Black et al., *1983*; Hammond and Polhamus, *1965*; Jasso de Rodriquez and Kuruvadi, *1991*; Coffelt et al., 2009 a, b). Once extracted, resin and PI are usually determined gravimetrically. Soxhlet was found by Black et al. (1983); Cornish et al. (2013) to allow degradation of PI, by the presence of low molecular weight PI continuously recovered, even after 48 h of extraction with acetone.

High speed homogenizer-grinder (Polytron) extraction method. A homogenizer, used for the preparing tissue, plant, food, soil, and many others, is able to perform blending, mixing, disrupting, emulsifying, dispersing, stirring processes, etc. The methods of homogenizing can be broken down into three categories, ultrasonic, pressure, and mechanical (blender or bead mills type). The mechanical homogenizers are rotor-stator or blade type. In last case, the blade (rotor) is being moved through the liquid at a high rate of speed generating cavitation. Rotor homogenizers (called colloid mills) generally outperform cutting blade-type blenders and are well suited for plant and animal tissue. However, the homogenized sample is rapidly contaminated with stainless steel particles due to abrasive wear. Appropriately sized cellular material is drawn up into the apparatus by a rapidly rotating rotor positioned within a static

head or tube containing holes. There the material is centrifugally thrown outward in a pump like fashion to exit through the slots or holes. Because the rotor turns at a very high rpm, the tissue is rapidly reduced in size by a combination of extreme turbulence, cavitation and scissor like mechanical shearing occurring within the narrow gap between the rotor and the stator. The process is fast and depending on the toughness of the tissue sample, desired results are usually obtained in 15-120 seconds. The variables to be optimized for maximum efficiency are as follows: design and size of generator, rotor speed, size of sample, time of processing, volume of medium and concentration of sample. Homogenization, often named "Polytron" in the literature after the name of a widely used model, is a very common sample preparation step prior to guayule analysis (Jasso de Rodriquez and Kuruvadi, 1991; Black et al. 1985). According to Diana and Rodriguez (1991), the Polytron extraction is a simple, rapid, cheap, and less labor-intensive method for assessing PI and resin content in guayule.

Accelerated Solvent Extraction method (ASE). In recent years, the ASE option was developed to increase the extraction speed and efficiency by using high temperature and pressure, under automatic extraction operation, while requiring lower solvent volumes than Soxhlet or Polytron (Grinberg and Shaubi, 1985; Jasso de Rodríguez and Kuruvadi, 1991; Salvucci et al., 2009; Teetor and Ray, 2004). ASE is a very efficient form of liquid-solid extraction, so all of the principles inherent to that technique apply. An important advantage of ASE systems over the previous ones is reduced handling of organic solvents. It is a quite a new technique, and its use has so far been limited due to its high cost, but being fully automated, it can be useful for routine analysis (apparatus and procedure described in the Material and methods Part).

To achieve efficient extraction, proper preparation techniques and operational parameters must be selected. As with Soxhlet, the ideal sample for extraction is a dry, finely divided solid. The influence of operating parameters is reviewed hereafter, in a more detailed way compared to above methods, because ASE was selected as the reference one during our experimental work.

Grinding. For an efficient extraction to occur, the solvent must make contact with the target analysts. The larger the surface area that can be exposed the faster the extraction. Samples with large particle sizes should be ground prior to extraction, generally smaller than 0.5 mm. Grinding can be accomplished with a conventional mortar and pestle or with electric grinders and mills. Because quantitative transfer of ground material can be difficult, it is

recommended that a large, representative sample be ground, and weighed portions of the ground sample be used for extraction. Compliant materials are best ground at reduced temperatures (e.g., liquid nitrogen).

Temperature. Temperature is the most important parameter in ASE. As the temperature is increased, the viscosity of the solvent is reduced, thereby increasing its ability to penetrate the matrix and solubilize the target analysts. The added thermal energy also assists in breaking analyst matrix bonds and encourages analyst diffusion to the matrix surface. Most ASE applications operate in the 75-125°C ranges. Note that not only does the analyst recovery increase, but the reproducibility improves as a function of temperature.

Pressure. The effect of pressure is to maintain the solvent as a liquid while above its atmospheric boiling point, and to rapidly move the fluids through the system. The pressure used in ASE is well above the threshold required to maintain the solvent under liquid state, so pressure adjustment for changing solvent is not required. In fact changing the pressure will have very little impact on analyte recovery, and it is not considered a critical parameter. Most ASE extractions are performed between 1000 psi (7 MPa) and 1,500 psi (10 MPa) the standard operating pressure.

Number of extraction steps. The use of static cycles was developed to introduce fresh solvent during the extraction process, which helps to maintain favorable extraction equilibrium. This effectively approximates dynamic extraction conditions without the need for troublesome flow restrictors to maintain pressure. When more than one cycle is used in a method, the flush volume is divided by that number. When the first static time is complete, the divided portion of the flush volume is delivered to the cell, with the "used" solvent directed to the collection vial. The system then holds the sample and solvent for a second static period. The nitrogen purge step is initiated only after the final static cycle.

Time. Increasing the static time at elevated temperature can allow compounds to diffuse into the extraction phase. The effect of static time should always be explored in conjunction with static cycles, in order to produce a complete extraction in the most efficient way possible. With proper sample preparation and optimization of extraction parameters, nearly any sample currently extracted with a liquid solvent can be performed in less time and with small volume of solvent using ASE.

According to several authors who optimized ASE protocols, this method gave accurate results when applied to guayule extraction (Salvucci et al., 2009; Teetor and Ray, 2004; Cornish et al., 2013). In addition, Cornish et al. (2013) reported that the amount of contaminating substances can be higher when the plant is actively growing but that is a reflection of processing method not of the rubber itself. They speculate that temperature above the bulk resin melt temperature (~60^{o}C) melt the resin in the ground material, allowing it to disperse and flow over the lignocellulosic fines in very thin films. It should also be noted that the ASE with acetone temperature used in these experiments was 100^{o}C. However, the protocol as used caused a loss of 26% of the high Mw PI fraction by thermodegradation into acetone soluble. Since the ASE is performed under nitrogen, this degradation can be attributed to thermodegradation, whereas the rubber "loss" in the pre-extraction drying oven is caused by a combination of oxidation degradation and thermodegradation. An acetone extraction temperature of 40^{o}C is recommended in alignment with the 50^{o}C drying temperature. It should also be noted that although sample preparation and resin extraction at relatively cool temperature protects the high Mw PI from degradation, during these process steps, the high 140^{o}C temperature used for hexane or cyclohexane to sequentially extract PI will degrade the polymer into lower Mw chains (Cornish et al., 2013).

II.3 Methods for the quantification of PI and resin

Methods proposed to quantify the content of PI and resin is based on:

- Real obtention of polymer by extracting with solvents, being named as gravimetric method,
- Extracting the PI but not weighing of the solvent-free extract, then measuring the turbidity of the liquid medium (photometry) or determining the rubber content by NMR or IR spectroscopy on film and solution,
- Methods applied to the crude solid biomass, based on spectral properties on a broad sense, like high resolution or low resolution NMR and NIRS (Traub, 1946; Hammond and Polhamus, 1965; Sundar and Reddy, 2001; Black et al., 1985).

II.3.1 Methods based on extraction and gravimetry

II.3.1.1 Comparison of extraction protocols

In most cases the protocols comprise, first the extraction of PI and resin, then the quantification of these components by gravimetry, chromatography, or a spectral technique.

As a general rule, guayule resin and rubber are sequentially extracted by a polar and a non-

polar solvent respectively, as summarized in Table II.1. However, in some cases resin and PI are extracted together prior to be separated in a subsequent step (Table II.2). Table II.2 shows a wide range of solvents for the sequential extraction; acetone for the first step (resin), hexane and cyclohexane for the second step (PI), although toluene was also used (Black et al., 1983; Salvucci et al., 2009; Teetor and Ray, 2004; Wagner et al., 1988). In the case of extracting both PI and resin simultaneously, the solvent is a mixture of acetone and of a hydrocarbon, like the azeotropic mixture of acetone and pentane (78/22; 82/18; 50/50 by Kroeger et al., 1999; Schloman et al., 1988; Teetor and Ray, 2004 respectively), hexane, or CH_2Cl_2. Then the rubber was precipitated by adding a non-solvent (methanol) (Wagner et al., 1991; Kay and Gutierrez, 1987; Wagner and Parma, 1988). This procedure also allows the separation of low and high *Mw* PI (Wagner and Schloman, 1991; Schloman, 1992). It yields a better quality rubber, but also gives rise to an additional co-product (Cole et al., 1991). Also the mixture of acetone and water 95:5 (Grinberg and Shaubi, 1985) was used, but for quantifying resin only. The single step extraction+ precipitation was used at pilot scale for simultaneous extraction of rubber and resin using an azeotrope of pentane and acetone. After separation of the solvent containing rubber and resin (miscella) from the bagasse, the high *Mw* PI was precipitated by the addition of acetone. The resulting swollen rubber cement was separated from the miscella, and desolventised. The (low *Mw*) rubber and resin remaining in the miscella were also desolventised in pot-type evaporators. Although the plant was successful in extracting and producing rubber meeting the specifications, engineering difficulties in handling the shrub plagued both facilities (Cole et al., 1991; Wagner and Schloman, 1991).

Table II.1: Methods for sequential extraction for quantifying rubber and resin in guayule

Extraction technique	Solvent	Quantification method	Quantified fraction	Reference
Soxhlet	Acetone	Gravimetry	Rubber	ISO 1407-1976 (F)
Soxhlet	Acetone, hexane	Gravimetry	Resin, rubber	Wanger and Schloman, 1991
Soxhlet	Acetone, toluene	Gravimetry	Resin, rubber	Cornish et al., 2004
Soxhlet	Acetone, hexane	Gravimetry	Resin, rubber	Schloman et al., 1988
Soxtec	Acetone, hexane	Gravimetry	Resin, rubber	Nurthen et al., 1986
Polytron	Acetone, hexane	Gravimetry	Resin, rubber	Garrot et al., 1981
Warring blender	Acetone, cyclohexane	Gravimetry	Resin, rubber	Black et al., 1983
ASE	Acetone, hexane	Gravimetry	Resin, rubber	Rath, 2005
Improved ASE	Acetone, cyclohexane	Gravimetry, UV, ELS	Resin, rubber	Salvucci et al., 2009
Supercritical CO_2	no solvent	Gravimetry	Resin, rubber	US Patent 7259231-2007

In the literature, the most widely employed extraction techniques are Soxhlet, Polytron and ASE, detailed in previous section. Although there are many options, a synthetic description is given in Figure II.5, showing the main steps and operating conditions (Garrot et al., 1981; Black et al., 1983; Salvucci et al., 2009). Advantages and drawbacks are summarized in Table II 3. It can be seen that ASE is the fastest method, requiring only 2 steps and being performed in 0.5 h, in addition to be automated (little operator time required, 0.1 h/sample).

Table II.2: Methods for simultaneous extraction for quantifying rubber and resin in guayule

Extraction technique	Solvent	Quantification method	Quantified fraction	Reference
Soxhlet	Acetone : pentane (78:22 v/v) precipitation with methanol	Gravimetry	Resin, rubber	Schloman et al., 1988
Soxhlet	Acetone : pentane (82:18v/v) precipitation with methanol	Gel permeation chromatography	Rubber, Mw	Kroeger et al., 1999
Soxhlet	Acetone : pentane azeotrope, precipitation with methanol	Gravimetry	Resin, rubber	Wagner and Parma, 1988
Soxhlet, centrifuge	Acetone : CH_2Cl_2, precipitation with methanol	Gravimetry	Resin, rubber	Cornish et al., 2001
Tissumize blender	Acetone : water (95:5 v/v), cyclohexane	Gravimetry	Resin, rubber	Banigan et al., 1981
Waring Blender	Acetone : water (95:5 v/v), hexane	Gravimetry, turbidimetry	Resin, rubber	Grinberg and Shaubi, 1985
ASE	Acetone : hexane (50:50 v/v) precipitation with methanol	Gravimetry	Resin, rubber	Teetor and Ray, 2004

Table II.3: Comparative advantages and disadvantages of extraction methods

Techniques	Advantage	Disadvantage
1. Soxhlet	Very inexpensive equipment Unattended operation Rugged, benchmark method	Slow extraction (up to 24-48 hrs) Large amount of solvent (up to 500 mL), mandatory evaporation of extract and larger samples
2. Polytron	Inexpensive equipment Low solvent (50 mL) Evaporation integrated Small sample (≥1 g)	Relatively slow extraction (0.1-0.5 hours) Technician required permanently
3. ASE	Fast extraction Very low solvent (11-40 mL) Small sample (≥1 g) Automated and easy to use	Expensive equipment Cleanup necessary Expensive

Polytron is also quite fast (0.3 h) although requiring substantial manpower with 3 steps (0.9 h/sample) and small sample. Soxhlet is the longest method (8-16 h), but does not require a permanent worker while refluxing (16 h/sample) but large amount of solvent for extraction.

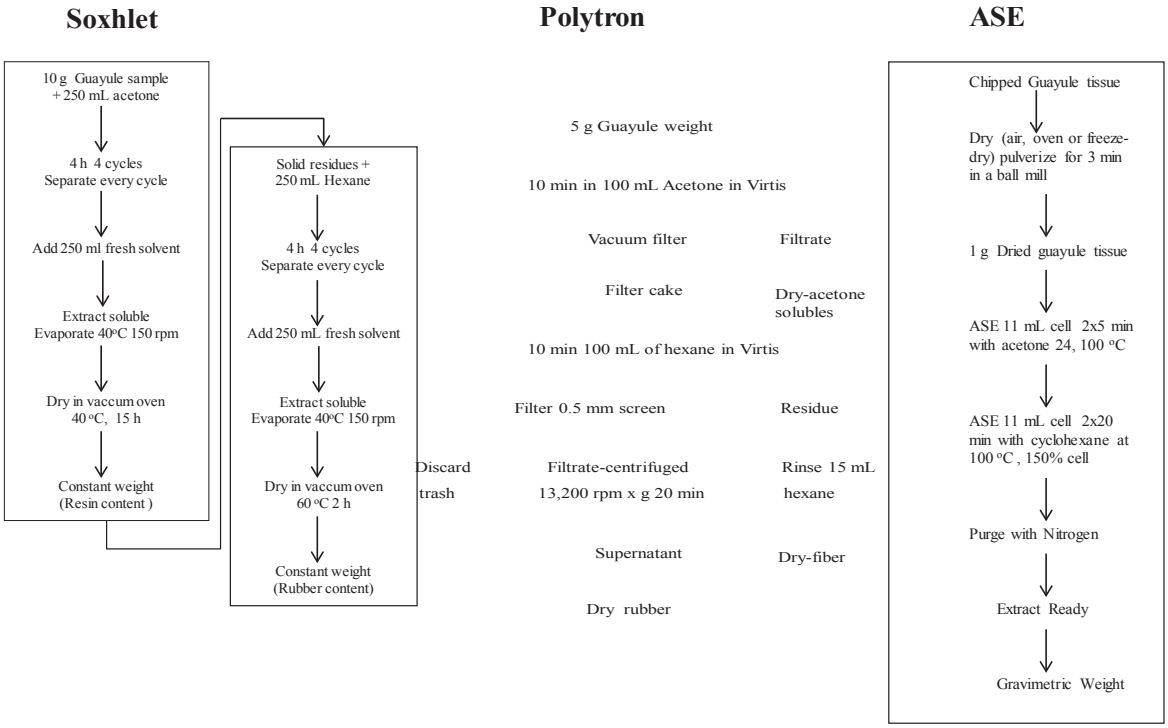


Figure II.1: Soxhlet, ASE, and Polytron extraction methods associated to gravimetry

II.3.1.2 Comparison of results from methods based on extraction and gravimetry

Earlier methods were principally based on gravimetry, because of their simplicity, although of uncertain selectivity: Tysdal, 1951; USDA, 1981; Banigan et al., 1981); Black et al. (1983); Kuruvadi (1991); Cantú et al. (1997); Teetor and Ray (2004); Michael et al. (2009). Several authors compared extraction+gravimetry when processing various biological samples (Zhuang et al 2004; Kim, 2009; Saito, 2004), including guayule (Teetor and Ray, 2004; Salvucci and Coffelt, 2009), and concluded to the high efficiency of the ASE method, as illustrated hereafter.

Zhuang et al. (2003) reported optimal conditions for ASE and Polytron two steps extraction for quantification of lipids and organochlorine in fish ground freeze-dried filets, at the temperature at 55°C and 100°C with ASE, and at room temperature with Polytron, and evaluated reproducibility. The mean values of lipid content were higher in the Polytron extraction than in the ASE. The statistical evidence against the null hypothesis for the

reference samples is not so strong (p=0.07). There is a good correlation between the two methods. The Polytron protocol may be more effective for fish of high lipid load while the ASE may be more sensitive for relatively clean fish samples.

Jasso de Rodríguez and Kuruvadi (1991) compared Soxhlet and homogenizer to determine PI and resin of guayule from Mexico in root, stem, and branch tissues, of ten genotypes. The analysis of variance indicated significant differences in percentage of PI and resin between genotypes, and individually in the root, stem, and branch tissues, and also between the two extraction procedures. They obtained contradictory results: the lower rubber yield was found with Soxhlet (Black et al., 1983).

Salvucci et al. (2009) used ASE with sequential extraction of resin with acetone, and of PI with cyclohexane, then followed by quantification of PI by gravimetry and by evaporative light scattering (ELS). They found very close data when working at low temperature during the acetone step (24°C), but this was not the case at 100°C because of PI degradation to acetone soluble lower *Mw* PI.

Teetor and Ray (2004) compared ASE with Sohxlet and homogenizer. Higher percent recovery of resin and PI in guayule can be obtained with the ASE than the homogenizer method, although the ratio of resin to PI remains the same. They investigated ASE for extracting resin and PI using simultaneous extraction by acetone:cyclohexane (volume ratio 1:1), followed by the rubber precipitation with methanol. Recently, while investigated the influence of sample drying prior to extraction. Cornish et al. (2013) used ASE, here with acetone and hexane for sequential extraction of resin and rubber in guayule biomass.

II.3.2 Non gravimetric methods

While gravimetry coupled to extraction is the most common way for quantifying PI and resin, as previously detailed, there are other ways, like (i) extracting but not weighing the solvent-free extracts, then measuring the turbidity of the solution (photometry) or determining PI by NMR or IR spectroscopy with films or solutions, and (ii) extraction-free methods applied to the crude solid biomass, like high resolution or low resolution NMR and NIR spectroscopy (the later will be detailed in the next chapter).

II.3.2.1 Mid-infrared spectroscopy

Infrared spectroscopy (IR) is the spectroscopy that deals with the infrared region of the

electromagnetic spectrum, with a longer wavelength, or lower frequency, than visible light. It covers a range of techniques, mostly based on absorption spectroscopy. As with all spectroscopic techniques, it can be used to identify molecules but also for quantification. IR exploits the fact that molecules absorb specific frequencies that are characteristic of their structure. A common laboratory instrument that uses this technique is a Fourier transform infrared (FTIR) spectrometer; Fourier transform turns the raw data into the sample's spectrum: light output as a function of infrared wavelength.

Each chemical bond in a molecule vibrates at a frequency which is characteristic of that bond. A group of atoms in a molecule (e.g. CH_2) may have multiple modes of oscillation caused by the stretching and bending motions of the group as a whole. If an oscillation leads to a change in dipole in the molecule, then it will absorb a photon which has the same frequency. In the mid-infrared region of spectrum, the wavelengths are between 4,000 cm^{-1} (2.5 μm) and 450 cm^{-1} (30 μm), used to study the fundamental vibrations and associated rotational-vibration structure (Kraft, 2006). The resonant frequencies can be related to the strength of the bond and the mass of the atoms. Thus, the frequency of the vibrations can be associated to a particular bond.

The "cast film" technique is used for analysis of polymeric materials. The sample is first dissolved in a suitable, non hygroscopic solvent. A drop of this solution is deposited on surface of a KBr cell. The solution is then evaporated to dryness and the film formed on the plate is analyzed directly. The film must not be too thick otherwise light cannot pass through. Liquid samples can be maintained between two plates of this salt, which is transparent to the IR light (Banigan et al., 1981). Grinberg and Shaubi (1985) indicated the presence of OH and C=O groups due to resin components, also Verbiscar et al. (1989) reported the absorption of C=O group attributed to resin at 1700 cm^{-1}, 1640, and 3200 cm^{-1} as C=O, OH respectively. Banigan et al., (1981); Black et al. (1983) considered the IR band characteristic of resin in guayule at 1700-1730 cm^{-1} corresponding to the C=O of resin and lipids and the band at 837cm^{-1} characteristic of cis-1, 4-polyisoprene). They used IR absorption for determining the area and intensity ratios of resin to PI (range of 10%) in a film derived from an extract obtained by fast homogenizer milling. They found the method simple, rapid and accurate, with a readily accessible instrument.

Buchanan (1981) also used IR for estimating rubber in plants. A film of the rubber extract is cast on NaCl plate and transmittance spectra peak heights are measured at 1730 cm^{-1} for resin and 830 cm^{-1} for PI. Quantitative results could be obtained by using PI solutions in fixed path

length cells for calibration. They observed problems for cleaning the cells. Gutierrez and Ransaw (1980) have reported attenuated total reflectance IR (ATR-IR) for analysis of guayule biomass containing 10-25% of PI. A test sample is prepared by pressing finely ground branch and measured absorption at 1030 cm^{-1}, corresponding to cellulose, the internal standard, at 830 cm^{-1} corresponding to PI, and at 1730 cm^{-1} for resin, with a calibration curve.

II.3.2.2 Nuclear magnetic resonance spectrometry

Nuclear magnetic resonance spectroscopy (NMR), exploits the magnetic properties of certain atomic nuclei to determine physical and chemical properties of the molecules in which they are contained, working solutions and solid samples. NMR is used to investigate the properties of organic molecules. Suitable samples range from small compounds analyzed with 1-dimensional proton or carbon-13 NMR spectroscopy (^{13}C-NMR) to large proteins or nucleic acids using 2, or 3, or 4-dimensional techniques (Hill and Koenig, 1998; Komoroski et al., 1986; Schirra et al., 2001). The ^{1}H NMR and ^{13}C-NMR were performed at 400 and 100 MHz, respectively. The ^{13}C-NMR spectra of ground rubber consist of lines with chemical shifts of 134.8, 125.3, 32.5, 26.7, and 23.6 ppm assigned to C1, C2, C3, C4 and C5 carbon atoms respectively in cis-1,4-isoprene units of PI and lines with chemical shifts of 129.6, 30.4 and 27.6 ppm assigned to carbons. The ^{1}H-NMR spectra of ground rubber samples display three dominant lines with chemical shifts of 1.6, 2.0 and 5.1 ppm assigned to the methyl, methylene and unsaturated methine protons of cis-1,4-isoprene units (Kovaľaková et al., 2011). NMR can be used for identifying molecules or functional groups, but also for a quantitative purpose, like the ratio of the cis/trans stereoisomers in 1,4-polyisoprene.

Liquid NMR analysis of guayule extracts. Tonnet and Downes (2006) used low-resolution proton NMR as a rapid screening method for estimating PI in guayule with acetone extract. Sample pretreatment was found to be important, because grinding and heating resulted in a loss of rubber, as determined by both proton magnetic resonance and extraction.

Need to recall that rubber quality depend on PI molecular weight distribution; a small portion of low-molecular weight may adversely affect the rubber quality. Therefore a few authors checked this. Meeks et al. (1947, 1951) identified typical isoprenoid carbon structure of low molecular weight acetone-soluble rubber, by ^{13}C-NMR. The yield percent agreeing with NMR did not indicate the presence of any resinous contaminates in the PI extract. Black et al. (1982) checked by ^{13}C-NMR both resin and rubber extracts for cross contamination. The resin fraction was found to contain a small percentage of PI, in the range of 5-26%, having a

molecular weight by GPC of 14,000 g/mole, which was subsequently identified by IR. This work showed the low molecular weight PI continuously when extracting with acetone in Soxhlet after 48 hours. This results of a continuous oxidative or thermal degradation of high molecular weight PI in hot acetone into low Mw PI. They also found that the resin is very difficult to remove quantitatively by Soxhlet extraction.

Solid NMR analysis of guayule biomass. Shoolery (1980) used ^{13}C-NMR for direct quantification of PI in ground guayule biomass, with a good agreement compared to values obtained by gravimetry (benzene extraction), range of 2-5% of PI in dry biomass. Visintainer et al. (1981) reported a sensitively of 1-2% of the absolute values. Komoroski et al., (1986) and The Los Angeles State (1983) have used ^{13}C-NMR for direct analysis of rubber.
Trautmann et al. (1991) reported ^{13}C-NMR to determine the rubber content of guayule stems in plant which was calculated to be approximately 11% (w/w). Banigan et al. (1981) investigated a gravimetric method based on sequential extraction of resin by acetone:water 95:5 and PI by cyclohexane with a Tissumizer blender, and also analysed these extracts by ^{13}C-NMR, turbidimetry and IR spectroscopy. Although based on a single extraction protocol, they found a PI content ranging from 8.7% up to 10.1% (NMR), thus a variation of about 15% between these four determinations based on 24 biomass samples.

II.3.3 Other methods for PI and resin quantification

All methods hereafter are coupled to prior extraction of resin and/or PI

Bromide method. This method consists in brominating the C=C double bond of PI, precipitating from the solution, collection, drying, and weighing the crystalline rubber bromide (Gowans and Clark, 1952). The bromide method is more selective of PI in the presence of resins, than gravimetric methods where rubber is weighed directly and contaminants are weighed together.

Turbidimetry. This method is based on controlled precipitation of rubber particles from a solution, and quantification by the photometric measurement of the resulting turbidity (Hammond and Polhamus, 1965; Grinberg and Shaubi, 1985; Sundar and Reddy, 2001; Traub, 1946). Accuracy by the photometric method was done.

Viscosimetry. The viscosity of cyclohexane solutions increases with rubber concentration (Smith, 1985), and this allowed rubber determination in guayule material. Oxidative degradation of the rubber solutions can affect viscosity but these effects can be minimized by avoiding light, by use of appropriate antioxidants, and by storage of samples under refrigeration. This is a simple and inexpensive method.

Evaporative light scattering. Salvucci et al. (2009) reported the evaporative light scattering (ELS) usually used as detector coupled to HPLC, was here used for quantifying rubber in extracts of rubber, to direct extraction of guayule with ASE (cyclohexane). The data show that the light scattering signal was linear over rubber concentrations from 0 to 2 mg/ml and departed slightly from linearity between 2 and 5mg/ml. Compared ELS and gravimetric determinations of rubber content was a linear relationship established with a slope of 1.15.

UV spectroscopy. Salvucci et al. (2009) used for resin content analysis after ASE (acetone and acetonitrile). Determination of resin was by absorbance at 272 nm for guayule complex mixture of terpenoid compounds. The relationship between absorbance and amount of acetone-soluble determined gravimetrically was linear with both spectrophotometers (absorbance 272 nm). UV method is more specific for resin than gravimetry. Two analysis methods for resin by UV and PI by ELS gave comparable results to gravimetric methods.

II.4 Comparison of results from gravimetric and non gravimetric methods

The viscometry gave results comparable to other methods to determine rubber content, such as the gravimetry and IR (Smith, 1985) but has a lack of accuracy. The turbimetric method may suffer the same drawback as well as high dependency on rubber coagulation conditions, which may not allow all PI to be separated depending on particle size distribution. There is a lack of accuracy especially in the case of quantitative IR analysis, and some other difficulties for setting up the analytical material, or when looking for the reference wavelength characteristic of PI only. Banigan et al. (1981) compared four methods, quite different. It is almost the sole work providing very useful data on this crucial point. They obtained the following PI content for the same sample (extraction of resin with acetone-water, then cyclohexane for PI): 8.7% for gravimetry; 9.2% for turbimetry; 9.3% for IR; 10.1% for ^{13}C-NMR. Worth noting from that data, there is a variation difference of about 15% between these four determinations based on an average which is of course not surprising. For example,

about gravimetry, one can expect incomplete rubber extraction, and or non selective extraction of PI. Considering the reference wavelength to be characteristic of PI only; same remarks apply to IR spectroscopy, turbidimetry and ^{13}C NMR. Although based on a single extraction protocol, they found a rubber content ranging from 8.7% up to 10.1% (NMR), thus a variation of about 15% between these four determinations based on an average of 24 biomass samples.

Taking advantage of spectral properties on a broad sense, like high resolution or low resolution and specialized and costly equipment (NMR, NIRS), of the biomass to assess the PI content directly on the biomass without applying an extraction step. But on one side, turbidimetry is highly dependent on rubber coagulation conditions, which may not allow all PI to be detected depending on particle size distribution. NMR needs expensive equipment (NIRS needs prior calibration). Owing to the current extended use and expertise of gravimetry on the other side, the last was recommended as preferable method for routine use.

II.5 Conclusion of Chapter II

Improved cultivation practices, and breeding, have been limited by the time required for sample processing and quantification of PI and resin. This has induced a continuous effort for improving the analytical methods. This chapter refers a very large number of publications on the topic, detailing many methods and options.

Water extraction suffers of incertitude: (i) uncomplete recovery of PI; (ii) pollution of the extract by water soluble compound (polysaccharides, sugars, proteins etc,) and non soluble resin (in the absence of preliminary selective extraction step). Water-based extraction can serve for checking PI extractable as latex (research on extraction processes, plant operators).

Organic solvent extraction was developed with three techniques -Soxhlet, Polytron, ASE. Soxhlet, was initially accepted as a standard extraction method, although suffering of several drawbacks. Polytron reduces the extraction time, but still presents some drawbacks. ASE brings(i) automation, saving operator’s time, and (ii) faster extraction rate, thanks to high temperature and pressure, although using an expansive equipment.

These three extraction methods require generally two solvents: acetone for extracting polar components (resin), and hexane for extracting non-polar components, especially PI. Both solvents are relatively safe, cheap and recyclable.

Alternative options were developed, based on simultaneous extraction of PI and resin, followed by precipitation and separation of PI. On a practical side, precipitation is as complicated as a second extraction step, requiring skills for achieving reproducibility, and needs at least two solvents, but generally three (acetone-pentane mixture for extraction, methanol for precipitation. Thus these alternative protocols did not prove to be very useful and were not widely used.

The extraction, by itself, does not provide the content of PI and resin. In most cases it has been combined to solvent evaporation and weighting (gravimetry). This method looks rather reliable although evaporation being time consuming. Many other methods were proposed, either coupled or not to extraction. NMR requires sophisticated equipment, bromination is not environmentally safe because of the reactant and of disposal of the brominated PI, ELS needs relatively expensive equipment, viscosimetry is not convenient for differencing samples having close composition (lack of accuracy).

In addition to above detailed gravimetry, a few methods look relatively attractive; low resolution NMR although not widely available in laboratories; same applies to ELS, and in addition it is not specific of PI, unless coupled to HPLC. Being widely used techniques, IR and UV looks attractive.

Regarding PI and resin quantification, above methods do not allow the same accuracy or the full quantification of the total PI content because calibration was not fully or properly achieved, the method not fully optimized, nor the results were compared to those provided by other methods. We found only three publications comparing the results obtained through different methods. Thus it is clear that data in the literature about breeding or agronomy, should not be compared when provided by differing methods. In addition, given the uncertainty about the selectivity, the question about their ability to provide reliable data relative to “real” PI or resin is pertinent.

NIRS was also used for determining PI and resin contents, but it needs to be calibrated with a reference method, thus depending on above listed options. This is detailed in the next chapter.

Chapter III

Near infrared reflectance spectroscopy method applied to polyisoprene and resin quantification

III.1 Introduction

Near infrared spectroscopy (NIRS) has become a very popular technique for a wide range of analyses in various industries and has been used for the characterization of different forms of biomass (Marten et al., 1985). Norris and his colleagues developed the first application of NIR spectroscopy to measure water in grains and seeds (Norris and Hart 1965). NIR spectroscopy has been used in a remarkably wide range of analytical situations. The technology uses simple sample preparation methods (drying and grinding), is very rapid (once the sample has been prepared, measurements are made in seconds), inexpensive, and nondestructive. This technique can be used to evaluate the quality of many agricultural, food and fruits products such as rice, tea, mango, peach, apple, kiwifruit, pulp, Safflower, Castor oil and ink, (Rudolphi et al., 2012; Fernandez-Cuesta et al., 2012; Raphael et al., 2001; Shou-He Yan, 2007). NIRS has been successfully applied to the rapid determination of various chemical components (Coppa et al., 2010; Laasonen et al., 2002; Kelley et al., 2004).

Indeed, several studies have demonstrated NIRS efficiency in characterizing the rubber and resin content in guayule (Black et al., 1985; Cornish et al., 2004; Kleine and Foster, 1992) and in other rubber containing biomass. In this last case Takeno et al. (2008) was reported the quantification of rubber, based on Fourier transform near infrared spectroscopy (FT-NIRS), in leaves of *Eucommia ulmoides*. In an early work, Black et al. (1985) showed the potential of NIRS to quantify rubber, water and resin in freshly ground guayule biomass. However, this pioneering works was based on few wavelengths, while the complex chemical composition of guayule biomass or extracts, makes the problem being a complicated one. For example, the resin fraction in guayule biomass is composed chiefly of hydrocarbons, terpenes, and lipids, all containing methyl groups, just like polyisoprene to be quantified as a separate class (Banigan, 1931; Thames and Wagner, 1991). Cornish et al. (2004) considered this important point, using multivariate analysis with various chemometric techniques, and comparing dry and wet guayule biomass homogenates. This allowed successful quantification of rubber not only in guayule, but also in *Heliantus annuus* biomass. However, although not relying on gravimetric determination, thus avoiding complete drying of extract, this procedure was based on analyzing a latex-impregnated cellulose paper. Thus this required water-based latex extraction from ground biomass obtained through several processing steps, prior to fast determination by NIRS. Dierig et al. (1989) studied the quality and quantity of guayule produced natural rubber under wet and dry growth conditions in Arizona. Rubber content was determined by NIR using Black et al, model.

III.2 Principle of NIRS

- ***Elements of near infrared reflectance spectroscopy***

Instrumentation for NIR spectroscopy is similar to instruments for the UV-visible and mid-IR ranges. Spectrometers are composed of a light source, a detector, and a dispersive element (such as a prism, or, more commonly, a diffraction grating) to allow the intensity at different wavelengths to be recorded. Fourier transforms NIR instruments using an interferometer are also common. Depending on the sample, the spectrum can be measured in either reflection or transmission.

- ***Measurement of the absorbance of radiation by a sample***

The Beer-Lambert law describes the relationship between the concentrations of a solute and the amount of light absorbed by the solution:

$$Cx = Ax / eL \qquad \text{(III.1)}$$

Where: Cx = concentration of the test solute

Ax = absorbance of the test solution

e = molar absorptivity of the test solute

L= path length travelled by the light through the solution

Important feature of this relationship is that it allows the measurement of Cx directly from Ax. When infrared radiation is incident on a solid sample, some of it is reflected from the surface of the sample (specular reflexion). Another proportion of the radiation enters the sample and may be absorbed. Radiation which is not absorbed may be transmitted through the sample or reflected from it (diffuse reflectance, Figure III.1).

The relationship between reflectance and analyte content cannot be directly described by any mathematical relationship. Thus while the characteristics of near infrared radiation reflected from a sample can be used to predict certain sample characteristics, each application of this type must be obtained by calibration. This introduces a number of complications such as the choice of wavelength, mathematical treatment of the reflectance data, methods of sample preparation and the effects of instrumentation differences. The amount of radiation reflected from the sample is quantified as the reflectance (R) of the sample. The value is usually expressed as log(1/R), which gives higher values at higher levels of absorbance (i.e. lower reflectance). There is an almost linear relationship between log(1/R) and the concentration of an absorbing component (Hruschka, 1987). The log(1/R) curve is comparable to an absorption

curve with peak values occurring at wavelengths which correspond to absorption bands in the sample (Norris *et al.,*1976).

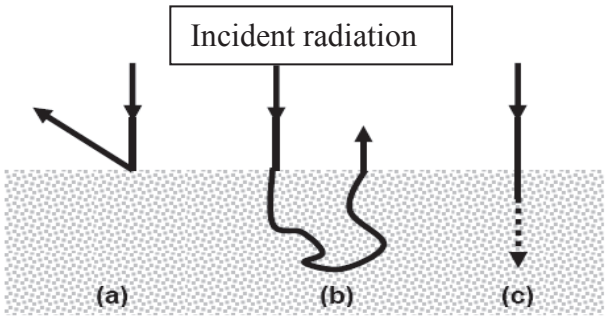


Figure III.1 Diagrammatic representation of specular (a) and diffuse (b) reflectance, and absorption (c) of near infrared radiation from a sample (Givens et al., 1997).

- ***Chemical bounds and wavelengths***

The near infrared region of the electromagnetic spectrum range is between 750 to 2500 nm. NIRS is based on vibration properties of organic molecule chemical bonds and their interactions with infrared radiation (Burns and Ciurczak, 1992; Davrieux et al., 2010). The chemical structures related to the NIR wavelengths are reported in Table III.1 (Barnes, 1988; Osborne and Fearn, 1986; Smith and Kelman, 1997). The NIR absorption spectrum is therefore correlated to the sample chemical composition (Pasquini, 2003; Sinnaeve et al., 1994). While the molecules of organic compounds absorb the near infrared light, the vibration and rotational energy increase. Fundamental vibration mode mainly consists of stretching vibration and deformation vibration. As was stated previously, absorption in the NIR region always occurs to the vibration caused by overtones or by combination of fundamental vibrations in the IR region. In particular, the absorption mainly occurs through functional groups that have a hydrogen atom such as C-H, O-H, and N-H bonds.

- **Mathematical treatment of the reflectance data**

NIR spectral are the resultant of all compounds elementary absorptions (which results in overlapping peaks) plus instrumental noise and samples preparation (especially water content and particle size) effects. There is usually a baseline variation such that log(1/R) values are greater at wavelengths 2500 nm. Further, reflectance at different wavelengths may be highly correlated. This collinearity is acceptable when it occurs at chemically-related wavelengths, should be corrected for if it occurs at unrelated wavelengths. Noise and baseline changes can be reduced by mathematic pre-treatments of the spectra such as scatter correction (standard

normal variate (SNV, Barnes et al. 1989), derivative and smoothing (Savitzky, A. and Golay, M.J.E., 1964). Variations in sample water content are important because water absorbs near infrared radiation strongly. Additionally, variations in sample particle size and temperature influence the scattering of radiation as it passes through the sample. Large particles do not scatter infrared radiation as much as small particles. More radiation is absorbed, giving higher log(1/R) values, and this effect is greater at those wavelengths which are absorbed more strongly.

Table III.1 Near infrared wavelengths and their association with chemical structures

Wavelength (nm)	Bond type	Vibration mode	Chemical Structure
1160	C-H	stretching 2nd overtone	$-CH_3$
1172	C-H	stretching 2nd overtone	HC=CH
1214	C-H	stretching 2nd overtone	$-CH_2$
1396	$-CH_2$	stretching	$-CH_2$
1450	O-H	stretching 1st overtone	H_2O
1724	C-H	stretching 1st overtone	$-CH_2$
1740	cis=C-H, C=C	stretching and deformation	HC=CH
1916	O-H	stretching and deformation combination	H_2O
2100-2200	C=C	stretching and deformation combination	HC=CH
	cis=C-H, C=C	stretching combination	HC=CH
2308	C-H	stretching and deformation combination	$-CH_2$
2294	C=O	stretching	-C=O

NIRS is based on molecular overtone and combination vibrations. As a result, the molar absorptivity in the near infrared region is typically quite small. One advantage is that NIR can typically penetrate much farther into a sample than mid infrared radiation. Near-infrared spectroscopy is, therefore, not a particularly sensitive technique, but it can be very useful in probing bulk material with little or no sample preparation. The molecular overtone and combination bands seen in the near IR are typically very broad, leading to complex spectra, it can be difficult to assign specific features to specific chemical components. Multivariate (multiple variables) calibration techniques (e.g. principal components analysis, partial least squares, or artificial neural networks) are often employed to extract the desired chemical information.

Development of a set of calibration samples and application of multivariate calibration techniques is essential for near-infrared analytical methods. Hruschka (1987) found the effect of using a second derivative to resolve overlapping peaks (Figure III.2). Norris, et al. (1976)

has been developed good prediction equations (coefficient of determination, R^2 = 0.85 to 0.99) for protein, Shenk, et al. (1981) recommended log(1/R) used for protein. Garcia-Cuidad et al. (1993) compared log(1/R), and the first and second derivatives, in the development of equations for proteins ingresses. Brown, et al. (1990) developed equations to predict protein of grass based on first and second derivatives of log(1/R).

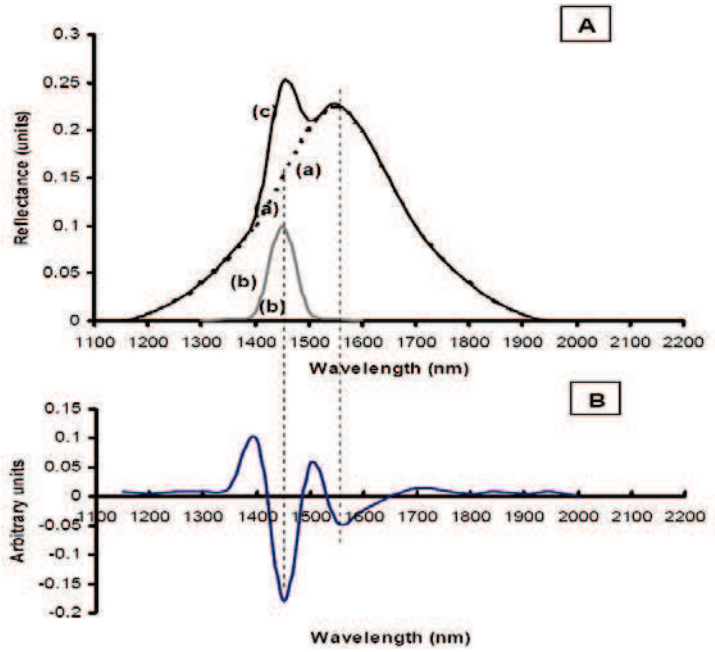


Figure III.2: (A) Reflectance spectra showing (a) and (b) overlapping spectra of two different compounds, and (c) the combined spectrum. (B) Second derivative of log(1/R) peaks separation

III.3 Calibration

Partial least squares regression (PLS regression) is a mathematic method which establish a linear regression model by projecting the predicted variables and the observable variables to a new space. Because both the X and Y data are projected to new spaces, the PLS methods are known as bilinear factor models. PLS is used to find the fundamental relations between two matrices (X and Y), i.e. a latent variable approach to modeling the covariance structures in these two spaces. A PLS model will try to find the multidimensional direction in the X space that explains the maximum multidimensional variance direction in the Y space.

PLS regression is particularly suited when the matrix of predictors has more variables than observations, and when there is multi-collinearity among X values. By contrast, standard regression will fail in these cases.

A PLS model expresses a relationship between a matrix of predictors X(m, n) like a set of NIR spectra and a property of interest Y(m, 1) (i.e. concentration of a chemical compounds measured in the sample). The PLS algorithm finds simultaneously important and related components of X and of Y. Final PLS model can be presented as (III.2):

$$Y = bX+e \qquad \text{(III.2)}$$

Where b(n, 1) is a vector of regression coefficients and e(m, 1) is vector of errors unexplained by the model. The model complexity (i.e. optimal number of PLS factors) is estimated based on cross-validation procedure.

III.3.1 Characteristics of the calibration data set

Equations are developed from a calibration data set, i.e. values for the analyst under consideration which have been generated by some reference method. Calibration data sets should be obtained from material which encompasses all of the chemical and spectral variation and the physico-chemical characteristics that are likely to be found in the population to be analyzed using the calibrations. This avoids any need to extrapolate beyond the boundaries of the calibration data. Calibration sets should have a wide range of variation in composition. Construction of a calibration set involves the balancing the cost of obtaining a widely representative data base, against the desirability of having the calibration set containing representatives of all the samples that are likely to be analyzed by the prediction equation. Several authors have commented that the removal of outliers from the calibration set improves (as would be expected) the goodness of fit of the resulting calibration equation. Shenk and Westerhaus (1991) suggested the calibration set should exclude samples with extreme values or similar. They suggested that, would help to reduce the cost of obtaining reference values.

III.3.2 Calibration procedure

NIRS spectra actually contain relevant information about the nature, the physical characteristics and the chemical composition of the studied samples. This wealth of information is both an advantage and a difficulty in NIRS analysis: the spectrum contains a lot of information, but it is all mixed up.

To overcome this difficulty, chemometric methods have to be used, which make it possible to link spectra to chemical analyses. Quantification of a constituent by NIRS requires a prior calibration stage, which establishes a linear model linking spectral data to quantifications obtained by a reference method. The prediction equation linking absorption values and reference values is of the multilinear type. It expresses constituent contents as a function of absorbance at certain wavelengths.

PLS regression is based on full spectrum, and allows finding patterns within spectra which explain the variation of a specific constituent.

The first step concerns the creation of a factor (PLS factor) result of linear combination of original variables (wavelength). The second step is the regression of the Y (constituent) variable using this PLS factor. The optimum number of PLS factor to introduce in the model is fixed by cross validation (Wold et al., 2001).

Overfitting can be avoided by choosing equations which give the lowest SEP close to the lowest SEC.

The sample outlier

Outlier detection is important during the calibration modeling and monitoring phases. True spectra outliers are considered to be samples whose spectral characteristics are not represented within a specified sample set. Outliers are not considered to be part of the group which is designated to be used as a calibration set. The criterion often given representing outlier selection is a sample spectrum with a distance of greater than three Mahalanobis distance from the centroid of the data. Another quick definition is a sample where the absolute residual value is greater than three to four standard deviations from the mean residual value (Draper and Smith, 1981).

III.4 Statistical assessments of the quality of NIR calibration

Several statistics are used to describe the quality of calibration equation, and the principal ones described by Williams (1987) are summarized in Table III.2.

The multiple linear regressions

The statistics were used to describe in calibration equations;

$$Y = a + b_1X_1 + b_2X_2 + b_3X_3 ..... + b_pX_p \qquad \text{(III.3)}$$

Where; X = independent value

Y = dependent value

Table III.2: Statistics criteria used for NIR calibration performances evaluation

Statistic	Definition
SEC	Standard error of calibration
SEP	Standard error of performance, or prediction, expressed as a difference between NIR analyses values and reference method values.
SEL	Standard error of laboratory or Standard error of difference between blind duplicates using the reference method.
SECV	Standard error of cross validation or variability in the difference between predicted values and reference values when the equation is applied to a subset of data during calibration procedure.
R^2	Coefficient of multiple determination or proportion of explained variance of Y data by the model.
RPD	The ratio of performance to deviation expressed as ratio of SD to SECV
Bias	The mean difference between the predicted and the reference values.

The variability in the difference between predicted values and reference calibration values when the equation is developed from the calibration data set is expressed trough SEC which is calculated as follows (Smith and Flinn 1991):

$$SEC = \{\Sigma(X_i - Y_i)^2 /(n - p - 1)\}^{0.5} \quad \text{(III.4)}$$

Where: X_i = predicted value of the i^{th} item in the calibration set

Y_i = reference value of i^{th} item in the calibration set

n = number of items in the calibration set

p = number of independent variables in this equation

Calibration equations are routinely validated against another data set in which reference analyst values have been determined. The samples in the validation set are normally different to those which were used to develop the prediction equation, usually a smaller set than the calibration set. The predicted values will normally differ from the reference values.

Williams (1987) noted that, this will introduce a systematic bias at either end of the range of predicted values. Predictions may be biased, i.e. displaced by a constant amount from the reference values. A standard error of prediction corrected (SEP) for bias can be calculated as follows (Smith and Flinn, 1991):

$$SEP = \{\Sigma (X_i - Y_i)^2 - (bias)^2/(n-1)\}^{0.5} \quad \text{(III.5)}$$

Where: X_i= predicted value of the i^{th} item in the validation set

Y_i= reference value of i^{th} item in the validation set

n= number of items in the validation set

bias= difference between overall means; it can determined in (III.6)

$$bias = \Sigma (X - Y)/n\} \quad \text{(III.6)}$$

Some workers use all the available reference data to construct the calibration set. Validation is then done by taking a series of randomly selected subsets of the calibration data and examining the distribution of differences between the predicted and reference values for each set. The statistic which describes the precision of the prediction is then the standard error of cross validation (SECV). The cross validation is performed with the exception that rather than sum of squares for residual as the reported results, cross validation uses the squares root of the mean squares for residual, using n-1 degrees of freedom for each model as:

$$SECV = \{\Sigma (X_i - Y_i)^2/(n-1)\}^{0.5} \quad \text{(III.7)}$$

Thus a cross validation report would traditionally include two columns; column one as number of factors, and column two as the standard error of cross validation.

Williams (1987) has provided rules for interpreting values for bias, SEP and correlation between predicted-reference values. Recommended the SEP should not be more than 3% of the mean reference value for that analyst. Stimson et al. (1991) reported that the SEP should be no greater than twice the SEL (Table III.3). Bias can be assessed by the size of the ratio of $bias^2$: SEP^2 in relation to the mean of the reference values, this ratio should be small. A uniform bias can be corrected by adjusting the regression intercept. Displacements of predicted values at either end of the reference range can be corrected.

The reference data with standard error laboratory (SEL) was estimated from these duplicates using (Urbano-Cuadrado et al., 2004) the following equation:

$$SEL = \sqrt{\frac{\sum_{i-1}^{n}\left(Y_{i1}-Y_{i2}\right)^2}{n}} \quad \text{(III.8)}$$

Where; n is the number of samples and Y_{i1} and Y_{i2} are values obtained for the replicates 1, and 2, respectively, of sample i.

Table III: 3 Displacements of predicted values of SEP with SEL

SEP values	The precision of the predicted values
SEP = 1.0-1.5 SEL	Excellent
SEP = 2.0-3.0 SEL	Good
SEP = 4.0 SEL	screening
SEP = 5.0 SEL	Poor

The ratio of performance to deviation was calculated as;

$$RPD_c = SD/SECV \text{ or } RPD_p = SD_{val}/SEP \quad \text{(III.9)}$$

Where; SD_{cal} = the standard deviation of calibration samples

SD_{val} = the standard deviation of validation samples

SECV = standard error in cross-validation

SEP = standard error of prediction

RPD_c and RPD_p were used to evaluate the general quality of the fit obtained for each equation.

Table III.4: The RPD as criterion of calibration performance (Williams, 1993)

RPD values	Classification	Application for quantity
<1.0	Very poor	Not recommended
1.0-2.4	Poor	Not recommended
2.5-2.9	Fair	Rough screening
3.0-3.9	Reasonable	Screening
4.0-5.9	Good	Quality control
6.0-7.9	Very good	Quality assurance
8.0-10.0	Excellent	Any application
>10.0	Superior	As good as reference

The Student (*t*) test was used to identify *t*-outlier samples during calibration development. Outlier detection was based on the standardized residuals (equal: error/SECV) with a cutoff of 2.5. Two outlier elimination passes were used**.** The ratio of performance to deviation of calibration was described in Table III.4.

II.5 Applications of Near Infrared Reflectance Spectroscopy

NIR spectroscopic analysis of chemical composition

Black et al. (1985) reported a study done on rubber and resin content determination in guayule using NIRS. Guayule plants were harvested by cutting plants at ground level. After removal of leaves and flower heads, plants were weighed, chipped and sampled for rubber and resin content determination. Rubber and resin contents were determined by the extraction procedure using acetone and cyclohexane to extract resin and rubber form finely ground shrub samples, respectively (Black et al., 1983). At riverside, the near-infrared reflectance spectroscopy (NIR) method was used to determine rubber and resin content. The two methods produced similar results. Black et al. (1983) determined rubber and resin contents in guayule by double extraction procedure using acetone and cyclohexane to extract resin and rubber from finely ground shrub samples, respectively. The near-infrared reflectance spectroscopy method was used to determine rubber and resin content (Black et al., 1985). The two methods produced similar results.

Kleine and Foster, 1992 improved the use of NIRS for guayule characterization using the latest chemometric tools (PCR, PLS and MPLS). They developed a calibration based on 67 samples selected (selection based on their Mahalonobis distance) out of 179 samples, the remaining 112 samples were used as validation. This calibration was high efficient and accurate use in routine.

Cornish et al. (2004) developed a NIRS calibration for rapid quantification of latex in both wet and dried guayule homogenate and purified latex samples. Guayule latex could be accurately quantified for a wide concentration range (0 to 25 mg/ml). The correlations between the measured and predicted rubber contents were respectively 0.96 and 0.91 for dry and wet samples (Table III.5). The procedure was tested on other latex samples such as *Ficuselastica* (Indian rubber tree), *Helianthus annuus* (sunflower), *Hevea brasiliensis* (Brazilian or para rubber tree) and *Taraxacumkok-saghyz* (Russian dandelion) with calibration models similar in terms of performances. Near infrared diffuse reflectance spectrophotometry in guayule was also investigated for prediction of moisture, organic C, total N contents. Wavelengths selected for moisture, organic C, and total N were respectively 1926, 1954, and 2150 nm, 1744, 1870 and 2052 nm, and 1702, 1870 and 2052 nm. The standard errors of prediction for finely ground samples were 0.58, 0.16, and 0.014% for moisture, organic C, and total N, respectively

Table III.5: Properties of PLS models developed for different rubber sources

Rubber source	Correlation coefficient	RMS error of prediction (mg/ml)	Concentration range (mg/ml)
All *P.argentatum*	0.96	2.7	0-35
P.argentatum (homogenate)	0.86	1.4	0-10
P.argentatum (latex)	0.96	1.2	0-10
Generalized model	0.82	4.5	0-35

Wagner et al., 1991 reported a comparison of shrub rubber content by NIRS analysis versus Soxhlet analysis. The study was conducted in order to compare a non obtrusive method to one that approximates the maximum amount of rubber attained using solvent extraction methods. The present rubber yield obtained by NIR analysis of samples compared to Soxhlet analysis. The Soxhlet data were generally lower (14 tests) than the NIR data.

Takeno et al. (2008) developed NIRS-PLS model for selection of high-polyisoprene (PI) samples. Samples with PI contents ranging from 0.6% to 5.3% dry weight were analyzed by FT-NIR. The samples were divided into a calibration set and a validation set, consisting of 36 and 6 sets of data, respectively. The division was done to obtain similar mean values and standard deviations so that both sets spanned the full range of the PI contents. In the PLS regression, the data was preprocessed using auto scale (unit-variance scaling). The optimum number of components in the PLS model was determined by cross-validation. The FT-NIR spectrum in the region between 4000–6000 cm^{-1} gives the best PLS model for quantification of PI with high R^2 value (0.95), low RMSEC (0.25) and lowest RMSEP (0.37). Accordingly, the CH, CH_2, and CH_3 first overtones and CH stretching vibrations in the region between 4000 to 6000 cm^{-1} are the most significant for the PLS model.

Guilment and Bokobza (2001) reported the use of NIRS to study synthetic rubber (butadiene and polyisoprene), and compared the relationship between type cis or trans structures in mixtures of *Hevea brasiliensis* (cis-1,4-polyisoprene) and gutta-percha (trans-1,4-polyisoprene). Cornish et al. (2004) described the use of NIR for quantification of rubber content in biomass and of the ratio of *cis* to *trans* in rubber mixtures and copolymers. A recent study by Guilment and Bokobza (2001) compared Raman, mid-range IR, and NIR, this study showed the power of chemometric tools for measuring the *cis*, *trans* and vinyl contents of butadiene copolymers. Also, the *cis* to *trans* ratio in mixtures of rubber originated from *Hevea*

brasiliensis and gutta-percha could be accurately measured with NIR, (Marinho and Monteiro, 2000).

Raphael et al. (2001) studied the NIRS detection of monomer droplets in polymer latex. NIRS may be used to detect the presence of monomer droplets in polymer lattices directly from spectra, without any kind of previous calibration. Experiments involving the addition of methyl methacrylate (MMA) and butyl acrylate (BuA) to monomer-free lattices indicated that there exists a specific wavelength (1620 nm) where, at some instant of the addition process, spectra are subject to a marked change. It is shown that this abrupt spectral change is caused by the formation of monomer droplets in the reaction medium. This finding may significantly improve the controllability of emulsion polymerization processes and the quality of its products, as shown by actual MMA/BuA emulsion polymerization experiments, which reinforces the potential of NIRS as a multipurpose analytic tool for in-line and in-situ simultaneous monitoring of different properties in emulsion polymerizations.

James (2003) studied the reversion and curing characteristics of sulfur-cured guayule rubber with those of natural rubber. A series of formulated natural and guayule rubber compounds was studied by employing FTNIR and mechanical spectroscopy to characterize the cure and reversion behavior. The reversion process was found to be dependent on the amount of trans-methane structure formed during and after vulcanization. The appearance of this molecular species coincided with the onset of mechanical reversion. Two variables affecting the cure and reversion process were studied: the amount of carbon black loading, and the temperature of cure. As the cure temperature was increased, an increase in reversion was detected. In general, guayule rubber exhibited a slower rate of reversion than natural rubber; however, natural rubber exhibited a larger modulus than guayule rubber.

Pansuwan et al., (2005) developed a NIRS rapid analysis of pulp chemical compositions of for *Eucalyptus camaldulens* is plantation and paper industry in Thailand. The development of predictive models of pulp yield and lignin, glucose, glucan, xylose, xylan, pentosan, holocellulose, alphacellulose, ash and extractive contents was carried out.. The calibration equations were established using multiple linear regressions (MLR). The results showed that the multiple correlation coefficients, R^2 ranged from 0.80 to 0.98. The pentosan content were predicted with a R^2=0.99.

The United States Patent 7329547 referees to a FT-NIR fatty acids determination present in a fat and/or oil-containing material. This technique was developed by preparing a calibration matrix based on FT-NIR and Gas Chromatography (GC) analysis of known standards, and

subsequently using the calibration matrix to analyze the FT-NIR spectral data obtained from a sample to be tested.

Laasonen et al. (2002) reported a NIRS identification method for *Echinacea purpurea* dried milled roots. Method development was carried out using a PLS algorithm and pretreatment options. These demonstrated that NIR spectroscopy is a good tool for the fast identification of *Echinacea purpurea* roots if the samples are milled using the same procedure as for the calibration samples. The method is robust with respect to the origin of the samples and can be used routinely by the pharmaceutical industry or herbal suppliers to avoid mislabeling errors.

III.6 Comparison of NIRS studies applied to guayule

Table III.6: Comparison of the NIRS studies applied to rubber

Detail	Black et al., 1985	Kleine and Foster, 1992	Cornish et al., 2004	Takeno et al., 2008
Title of article	Analysis of rubber, resin, and moisture content of guayule by near infrared reflectance spectroscopy	Chemometric NIR calibration for guayule analysis	Latex quantification in homogenate, purified latex samples to various plant species using NIRS	A high-throughput and solvent-free method for measurement polyisoprene content in leaves by FTNIR
Biomass Sample	1-2 cm ground dried guayule biomass, 110 samples	1-2mm ground dried guayule 179 samples, two-six years	homogenate and purified latex, wet and dry guayule	42 samples leaf of *E. ulmoides,* 0.5-1-mm ground dried
Samples preparation	Homogenizer-grinder	cf. Black et al., 1983, Homogenizer-grinder	homogenate and purified latex	Waring blender
Chemistry method	Gravimetric method	Gravimetric method	Gravimetric method	PyGC/MS
Range of contents	Resin: 1.5 to 11% Rubber: 0.8 to 19%	Resin: 4.33 - 11.13% Rubber:3.10-12.05%	Latex concentration 0 to 25 mg/ml	PI: 0.6-5.3%
NIR instrument	A Neotec Model 6350 Mark II (Pacific,Silver Spring, Maryland)	NIR, Model 6500 scanning, IBM, ISI software, spinning-module,	the ASD absorbance spectra Unscrambler® (CAMO, Inc., Corvallis, Oregon).	Nicolet 6700 FT-NIR (Thermo Electron)
Scanning range	1100 - 2500 nm	1100 to 2500 nm	1100 to 2500 nm	4000-12,500 cm^{-1}
Mathematics	PCA and CRT	PCR, PLS, mPLS,	Multivariate analysis PCA and PLS	PLS
Wavelengths related to the rubber or resin	1716 nm of PI or rubber; 1708, 2170, 2208, 2322 nm resin	1714 nm of cis- PI; 1850 to 1970 nm water	Similar with Black et al., 1983; 1716 nm of PI; 2322 nm: resin	6500-7800 cm^{-1} For PI
NIRS equations	Resin; R =0.89; and rubber: R=0.95	Resin: R=0.96, Rubber: *R=0.98*	Rubber: R=0.96, 0.91 for dry and wet	The correlation; R =0.95 for PI
Efficiency	NIRS equations; accurate	High efficient and accurate, use in routine	NIRS equations; accurate	NIRS equations; accurate for trans PI

III.7 Conclusion of chapter III

The bibliography highlights that NIRS is currently apply for many various agricultural and natural products. This technology is a rapid and non-destructive alternative to classical methods which are time consuming and expensive. Different studies demonstrated the potential of NIRS for fast determination of resin and rubber contents in guayule biomass. These studies reached efficient calibration models based on wet chemistry values obtained by Soxhlet or Homogenizer-grinder extraction.

In our study we intend to demonstrate that similar or better NIRS calibration performances can be obtained using wet chemistry values obtained with a new extraction method. One knows that the final NIRS calibration is dependent of the reference method used to quantify the compound of interest. This is due to the selectivity, reproducibility and accuracy of the reference method. In this study the automatic solvent extraction (ASE) has been investigated and optimized then used to quantify rubber and resin content of various guayule biomasses. These wet chemistry results were introduced jointly to NIRS spectra in PLS regression models. Performances of regression models were compared to laboratory error and to previous NIRS models described in literature. A closer investigation was done using chemometric tools and their outputs, such PLS loadings and b coefficients, in order to interpret the relationship between spectral fingerprint (absorption bands) and rubber or resin contents.

Chapter IV

Methods for molecular analysis of PI, resin and other guayule components

IV.1 Introduction

The preceding chapters are devoted to the quantification of PI and resin by various methods, often including an extraction step (Chapter II), and to NIRS (Chapter III), presenting this technique and applications to quantifying these components. The above analysis are very useful for assessing plant overall production for breeders, agronomists, production managers (harvest date) and plant operators (extraction yield). But these determinations do not provide information about product composition to operators, nor to marketing and manufacturers. All of them need more detailed knowledge about the quality of the products, which in turn determine their value.

HR has specific properties that make it an "irreplaceable" product. Assessing and improving the quality of GR not only requires genetic variability, but it is also extremely dependent on the accuracy of the techniques used to evaluate that variability (Estilai and Ray, 1991).

Therefore, this chapter IV -the last of the bibliographical part (Part I)- deals with methods to be applied to the extracted fractions (PI and resin), as a second stage of analysis, in order to access to their chemical composition. For polymer analysis, these are general methods, here applied to the case of guayule PI. For resin, whose analysis is also recommended owing to its negative influence on rubber properties (Nakayama, 2004; Sholmane, 2012), the large number of classes present and their diversity requires specific methods. Some analytical techniques dealing with other components of guayule biomass, namely proteins, are included here, owing the central problem of protein-promoted allergy in HR and potentially in GR. The analyses of structural polymers (cellulose, hemicellulose, and lignin) are not included because it is out of scope of our work, although some are included in Chapter III devoted to NIRS. Last, this chapter is devoted to analysis techniques only; literature results about guayule are detailed in Chapter I.

IV.2 Determination of PI structure - Rubber quality

It has been reported that the properties of GR are very similar to that of HR, and the former may be used for similar applications (Cantu et al., 1997; National Academy of Sciences, 1997); thus techniques used for assessing the properties of guayule latex (GL) and GR are the same as those used for GR and HR. Properties of rubber isolated from guayule are dependent of the polymer structure, as formed in PI-bearing cells, and then possibly transformed during storage and processing (extraction from shrub tissue). Estilai (1987) found that the physical

and processing properties of GR are greatly influenced by PI *Mw* and by molar mass distribution (MMD), often being multimodal (Estilai, 2003).

According to Vaysse and Bonfils (2003), the complex structure of HR is described at 3 levels:

- Microstructure, concerns molecular features (chemical structure), and composition (lipids, proteins, etc.),
- Mesostructure, concerns macromolecular features, weight-average molar mass distribution (MMD), macrogel and microgel contents (Bonfils et al., 2005),
- Macrostructure, takes into account the bulk properties, rheology, breakdown behavior, vulcanization,

Hereafter analytical techniques refer to the micro- and meso-structure levels.

IV.2.1 Rubber degradation

The time and conditions of storage, either of harvested biomass, of raw rubber or latex, have a paramount importance on GR macromolecular structure (Black, 1986; Dierig, 1991; Schloman, 1996; Cornish, 2005; McMahan, 2006). Degradation can occur during processing and extraction; Black et al. (1985) noted that biomass handling, storage time and temperature contributed to degradation. Cornish et al. (2004), Coffelt et al. (2009) found that Mw and molecular radius both declined upon storage conditions. As detailed in a previous chapter, during hot solvent extraction, part of PI is converted to acetone-soluble materials (Cornish et al, 2013). This could be the result of oxidation of unsaturated fatty acid forming hydroperoxides which in turn initiate the degradation of PI (Curtis, 1947; Keller, 1982). This calls for obtaining a detailed knowledge of the chemical composition of resin.

On the applied side, with the aim of accessing a deep and representative knowledge of the chemical composition, PI degradation can be retarded by (i) cold storage (Keller, 1983); Black et al. (1986) noted only little change in Mw after storage of shrub in a freezer for one year; (ii) shortening storage time (Taylor and Chubb, 1952; Black et al., 1986; Estilai and Hamerstrand, 1985; Coffelt and Ray, 2010; (iii) high unsaponifiable fraction in resin found in the biomass (Keller, 1983); (iv) addition of antioxidants (Whitworth and Whitehead, 1991; Smith, 1985). To explain some variations, Estilai (1987) hypothetized that the enzyme machinery of PI biosynthesis remains active, allowing the harvested biomass to continue polymerizing for a short period of time, depending on genotype. Cornish et al. (2013) suggested performing the drying process at 50^{o}C for low PI degradation prior to quality control. These changes in PI characteristics influence in turn the ability of the rubber material to resist oxidative and thermal degradation, chemical cross-links during vulcanization changes

stress-strain performance characteristics in finished goods (Cantu et al., 1997). All this emphasizes on the importance of (i) performing a detailed chemical analysis of biomass and of the extracted fractions, for assessing the composition of PI and associated compounds in shrub, and the quality of derived rubber, and (ii) selecting experimental parameters for the whole processing chain from harvest down to the last sample preparation.

IV.2.2 Size exclusion chromatography

Size exclusion chromatography (SEC), also called gel permeation chromatography, informs on the size and shape of polymer molecules and has proven quite useful in characterizing PI (Cornish and Schloman, 2004). Based on molecular size, SEC separates molecules according to their hydrodynamic volume (proportional to *Mw*) in a liquid medium. It is based on the fact that the smaller a molecule, the easier it will enter the pores of a gel. Small molecules will therefore have to cover a longer distance during their migration in a column packed with such a gel. Macromolecular species in solution are thus eluted in decreasing order of their hydrodynamic volume (or *Mw*).

Associated detector (s) is a crucial aspect of SEC. Molecular characteristics of GR are often determined by SEC coupled to Multi-Angle Laser Light Scattering (MALS), called SEC-MALS (McMahan et al., 2005; Kim et al., 2008) In addition it can be coupled to a second detector, namely refractive index (DRI). The advantages of MALS coupled with DRI are to determine absolute Mw and to reach the radius of gyration (*Rg*). Determining both Mw and *Rg* allow to reach the branching features of the PI eluting of the SEC (Kim et al., 2009).

IV.2.3 Molecular mass distribution

GR quality is of course not defined by the PI content in biomass determined in Chapter II. The weight-average molar mass (M_w), number-average molar mass (M_n), molar masses distribution (MMD), polydispersity index (M_w/M_n), are commonly used to partially determine PI characteristics linked to rubber quality (Black, 1986). Usually, M_w is the main parameter for a quick view of PI macromolecular structure. The higher the M_w, the higher the mean length of chains, and therefore the better the quality of the rubber produced (Fuller, 1990; Ehabe, 2005; Kim et al., 2009). These characteristics are defined hereafter:

- $M_n = \Sigma N_i \times M_i / \Sigma N_i$; the denominator is the total number of rubber chains, it is called "number-average molar mass" and is the sole to have a clear signification among the three molecular mass descriptors.

- $M_w = \Sigma N_i \, x \, M_i^2 \, / \, \Sigma N_i \, x \, M_i$; the weight-average molecular weight. As the denominator of *Mw* is the total weight of the sample, it is called "weight-average".
- $M_z = \Sigma N_i \, x \, M_i^3 \, / \, \Sigma N_i \, x \, M_i^2$; called z-average molar mass.

The interest of M_w and M_z is that they overestimate the importance of long chains, and underestimate short chains in the determination of "average molar mass".

Logically, $M_n < M_w < M_z$. In practice, M_w is mainly considered, notably for the calculation of polydispersity. Values of 1.0×10^5 to 2.5×10^6 g/mol and 6.8×10^4 to 8.0×10^5 g/mol have been reported for guayule PI, for M_w and M_n respectively (Estilai, 1987; Cantu et al., 1997). An approximate limit between short chains and long chains is proposed at M_w 400x10^3 g/mol.

- MMD = M_w / M_n; Molecular weight distribution (MMD) or polydispersity.

In the case of HR, the data provided by SEC-MALS chromatograms are: MMD curve; average molar mass (M_n, M_w, M_z), root mean square radius of gyration (rms) or simply radius of gyration (R_g). The R_g is defined as the mean square of the distance between the monomers and the center of mass of the chain. The chain occupies a space of a sphere of radius *Rg*. SEC-MALS also provides the percentage of gel fraction (%), being the difference between (i) the total concentration of solutes of the prepared sample solution in tetrahydrofuranne, and (ii) the real PI concentration in the injected solution after the filtration step to remove insoluble material (gel containing PI) prior to injection. The MMD in GR determined by SEC-MALS may show different types (uni- or bi- or multimodal, Figure IV.1, depending on harvest date and plant age (Angulo-Sanchez et al., 1995).

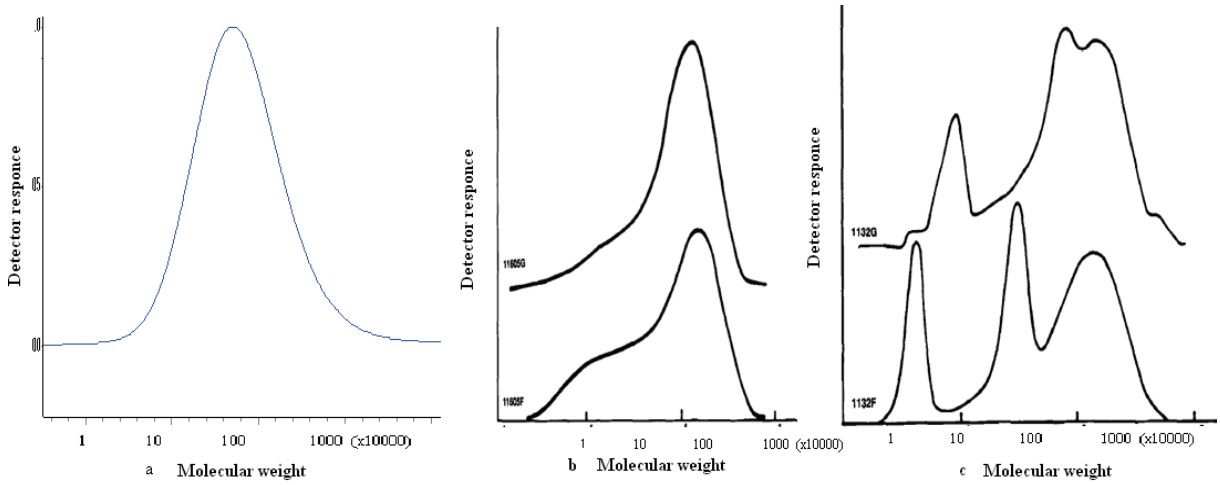


Figure IV.1: Unimodal (a), bimodal (b), multimodal (c) molecular weight distributions *(Angulo-Sanchez et al., 1995)*

The dependence of refractive index on concentration was assumed to be equivalent over the entire range of analysis (McMahan et al., 2005).

IV.2.4 Determination of branching of PI by SEC-MALS

To avoid the tedious fractionation steps necessary to obtain polymers with narrow molar mass distribution and structural modifications throughout the different fractionation stages, SEC-MALS is used for direct characterization of polymer branching. It is a very versatile technique since the light scattering detector provides absolute M_w and also a size parameter R_g the radius of gyration $(R_g^2)^{1/2}$ (certain authors use $<rms^2>^{1/2}$), throughout the chromatogram. This combination of a molar mass or chain lenght the molecular, and of a size parameter R_g, in the case of polydisperse polymers, is used to obtain information about the shape of polymer chains (Flory exponent, ν), and the distribution of branching, depending on size or molar mass. The conformation plot (R_{gi}=f(M_{wi}) (Kim et al., 2009) is significantly different between the PI of fresh biomass extracted with the soft method and the PI of dry biomass extracted by ASE. A reference polydisperse PI with linear chains, or a master curve obtained with standard linear PI, is needed to calculate the number of branching points per chain (m) using SEC-MALS and ASTRA Software (Kim et al., 2009). This method has the advantage of determining an absolute number of branching per chain and enables a better comparison with data in the literature.

However, Kim et al. (2008) showed that HR samples analyzed by SEC-MALS gave abnormal elution at high elution volume, probably highly compact microaggregates or micromicelles delayed by interactions with the column packing. This provides an additional argument about the extreme sensitivity of PI macromolecular data to operating conditions during processing steps when preparing samples from guayule biomass, as already pointed out in section IV.2.1.

IV.3 Resin analysis

Extracted latex makes at best only about 10% of the dry biomass and ways must be found to either dispose or develop useful co-products from the remaining 90% (Schloman and Wagner, 1991). The resin composition has been determined to be a very complex mixture (see Chapter I): volatile (α-and β-pinene, camphene, α- and β-phellandrene, sabinene, β-myrcene), and non volatile fractions, some water soluble (bornyl acetate, cinnamic acid, polyphenols (tannins, flavonoids), polysaccharides), some not : hydrocarbons (β-Ocimene, limonene), sesquiterpene esters of guayulins and partheniol, triterpenoids (argentatins), waxes and fatty acid triacylglycerols, alkaloids (guayulamine) (Schloman, 1988; Nakayama, 2005).

Also, resin present in compounded rubber decreases the quality of the final product (because of resin, GR is very prone to thermo-oxidation (Keller, 1981; Bhowmick, 1985; Schloman, 1996)), and resin components should find markets. Finally, the chemical composition of the sequiterpenoid group has been used as a tool for understanding taxonomy and evolution (Rodriguez, 1975; Dominguez, 1980; West et al., 1991).

The composition of the non-PI extractable in GR should be determined as comprehensively as possible, over time, by cultivar, etc, to establish the extent to which latex "resin" differs from material in the rest of the plant. These results should be compared with those obtained for whole-shrub resin (Schloman, 2012).

Owing to above reasons, numerous studies have been made to characterize the resinous material, although not being a well definite fraction. In fact this is the water insoluble fraction excluding PI whatever the Mw, composed of secondary metabolites.

Worth mentioning here, the method used by Liengprayoon et al. (2008) to extracted and quantify the non PI components in HL films. The aim was to provide a method suitable for comparing various sources, through an optimized total lipid extraction from NR either in the liquid state (latex) or in the dry state (unsmoked sheets). They checked combinations of organic solvents (chloroform/methanol and hexane/isopropanol mixes). Chloroform/methanol (2:1 vol/vol) was found to be the most suitable for lipid extraction from unsmoked sheet. The lipid extraction yield was improved by increasing the exchange surfaces by grinding rubber under liquid nitrogen and extracting the ground rubber for 6 h at room temperature, leading to 1.8% lipid extraction yield based on dry rubber. Concerning latex extraction, the problem of lipid entrapment in the coagulum from immediate coagulation of latex in the solvent was solved by preliminary two times dilution of latex, giving a 3.2% extract (dry rubber) containing a minimum quantity of contaminating PI. Concerning the nature of lipids, dilution increased mainly neutral lipid extraction, which may suggest that neutral lipids were those entrapped by coagulation. This method could be applicable to guayule biomass or derived extracts and films for accessing to the "total resin" content (neutral and polar lipids, terpenes and sesquiterpenes).

IV.3.1. Unsaponifiable fraction

The general strategy for analyzing resin from guayule involves the saponification of ester containing compounds, into soaps, and their separation from the unsaponifiable fraction. The last both contain compounds not bearing ester bonds in native biomass and the neoformed unsaponifiable products resulting from the reaction. A commercial lipase has been used for

triacylglycerol hydrolysis (Schloman and McIntyre, 2001), but the more conventional saponification using NaOH or KOH was also reported.

According to Scholman, 2012; the general approach described in Cornish et al. (2009) may be worth consideration, even though the reported ester hydrolysis was incomplete. The use of a carefully-selected phase transfer catalyst may provide a route to higher conversions (Bhatkhande and Samant, 1998; Entezari and Keshavarzi, 2001; Lele et al., 1983). Note that, first two references describe systems which required "sonication" to disperse the aqueous and non-aqueous phases, something that is undesirable and unnecessary in the case of stable GL.

Sesquiterpenes. Methods for the quantification of fatty acid triacyglycerols, guayulins, and triterpenoids (argentatins A and B) were reported by Schloman et al. (1986), Stumpf et al., (2001). Figure IV.2 shows an HPLC chromatogram for the analysis of guayulins, a family of sesquiterpene esters of cinnamic and *p*-anisic acids, under conditions reported by Schloman et al. (1983). Figure IV.3 also illustrates the susceptibility of guayulins A and B to oxidative and thermal degradation.

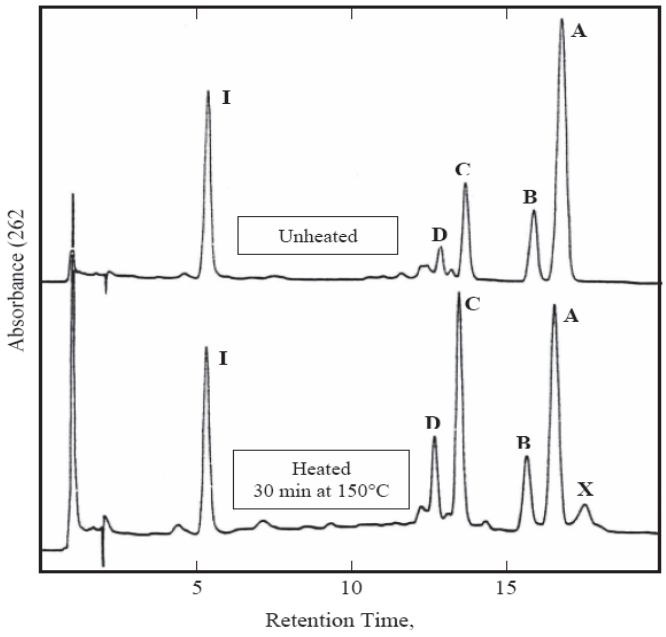


Figure IV.2: Representative HPLC chromatogram of sesquiterpene esters of guayule resin
I: ethyl p-anisate (internal standard), D: guayulin D, C: guayulin C, B: guayulin B, A: guayulin A, X: unknown.

Figure IV.3 shows the composition of resin is dependent on sample preparation conditions. As an example, oxidation of guayulin A in solution rapidly leads to the formation of a hydroxylated derivative (guayulin C). Guayulin D is the corresponding derivative of guayulin

B. Also the parent sesquiterpene, bicyclogermacrene, undergoes thermal rearrangements: a Cope reactions and *E* to *Z* isomerization of one double bond (or perhaps Cope reaction of the element derivative) to yield isobicyclogermacrene (Nishimura et al., 1973). The latter product is the most thermally stable isomer of the parent hydrocarbon. Bicyclogermacrene readily undergoes rearrangement at 200°C. Although guayulin A adopts a conformation suitable for Cope rearrangement, neither the corresponding elemene nor the "isoguayulin" has been reported to date. Molecular orbital calculations indicate that the energy difference between guayulin A and its Cope product may not favor rearrangement (Setzer, 2009). The minimum temperature necessary for thermal rearrangement has not been reported.

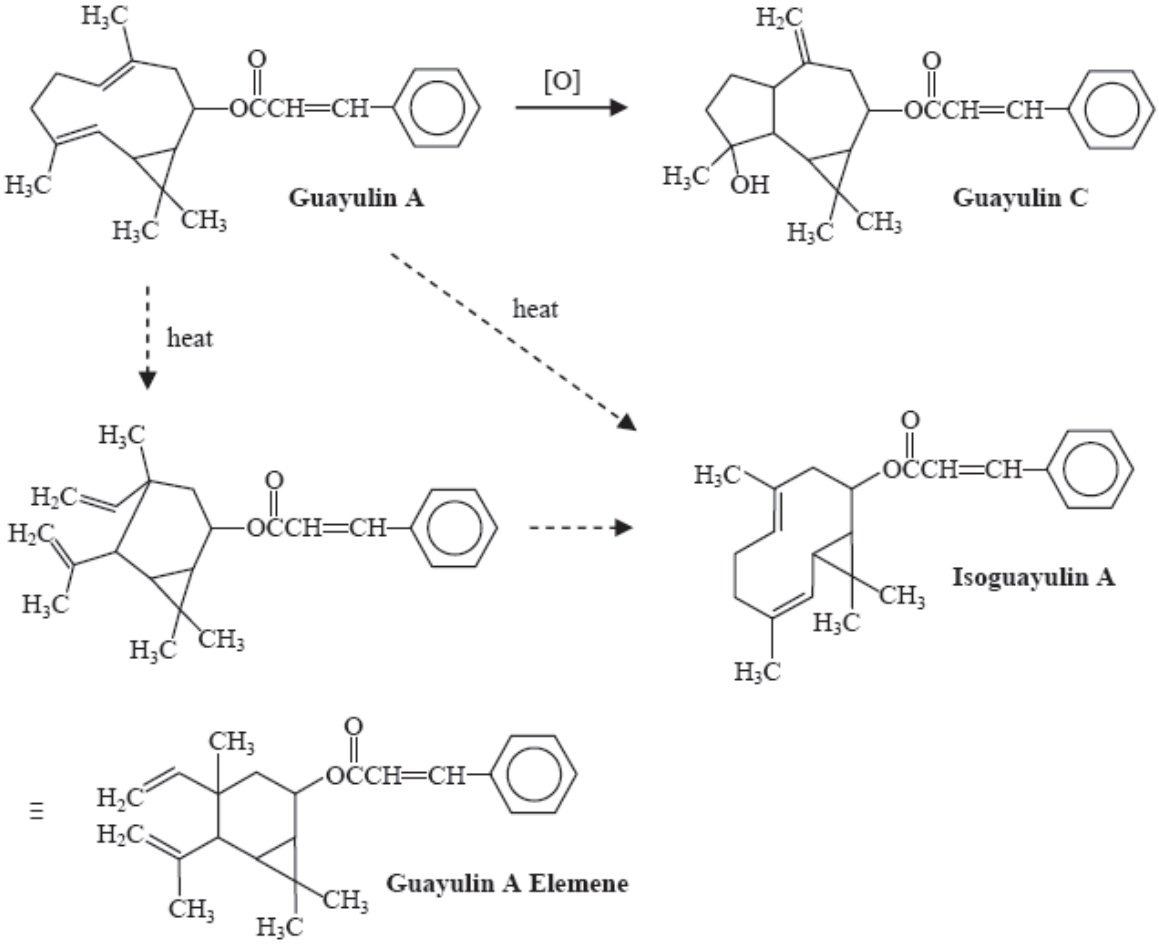


Figure IV.3: Guayulin A chemistry; possible rearrangement pathways (Schloman, 2012)

Figure IV.4 outlines these processes as they might occur with guayulin A. Thermal rearrangement of guayulin B has not been reported. Maatooq et al. (1996) identified a family of 5 eudesmanoids in resin extracted from cultivar AZ-101, a hybrid of guayule and *P.*

tomentosum, var. *stramonium* (Ray et al., 2005); it is possible that some of these substances may be unique to AZ-101.

Triterpenoids. Guayule produces a range of triterpenoids, about 10 of which have been characterized (Komoroski et al., 1986; Maatooq et al., 2002; Romo de Vivar et al., 1990). Argentatin A and B (Figure IV.3) have been widely quantified in resin samples (Schloman et al., 1983; Schloman et al., 1986). The analytical method was developed using authentic samples of argentatin A, argentatin B, and isoargentatin B. The relative contents were determined by direct HPLC analysis of the underivatized compounds (Schloman et al., 1983). Argentatins E-H, (Maatooq et al., 2002) have been isolated from AZ-101 genotype. The peaks for argentatin B DNPH and isoargentatin B are not resolved under these conditions.

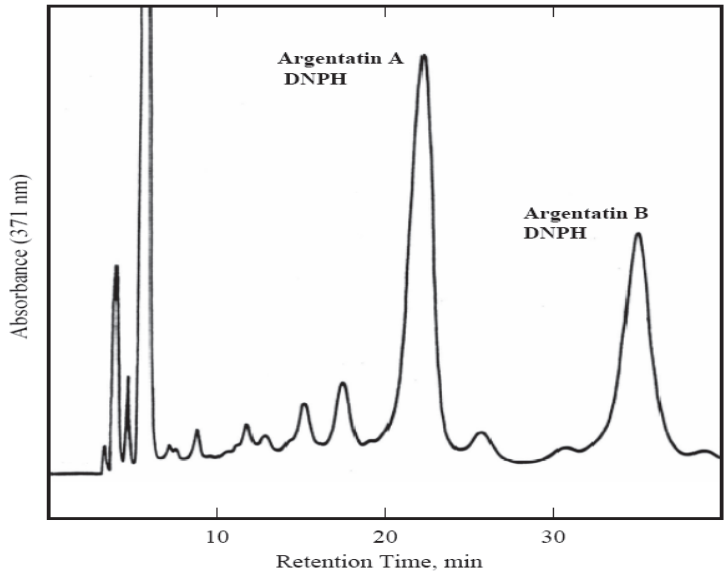


Figure IV.4: Representative HPLC chromatogram of 2,4-dinitrophenylhydrazone (DNPH) derivatives of guayule resin components.

IV.3.2. Carboxylic acid containing fraction (saponifiable)

The distribution of fatty acids has been determined by resin saponification followed by conversion of the acids to methyl esters (Schloman et al., 1983, and references therein). In addition to fatty acid esters, there are cinnamic and p-anisic acid esters, initialy associated to sesquiterpenes and produced by saponification of guayulin esters. Other aromatic acids of the same above family have been identified in the water extracts of guayule woody tissue and leaves (Schloman et al., 1991).

IV.3.4. Volatiles

The volatile fraction is made of hydrocarbon compounds, α- and β-pinenes being the major component, isoprene and limonene minor ones (Nakayama, 1984). Hydrocarbons, which include more than 11 compounds, are similar to the volatiles observed in pine and oak. This class can be extracted with a solvent, like other resin components (Nakayama, 2004).

IV.3.5 Miscellaneous

Water-soluble components, primarily polysaccharides are entrained by solvent when the shrub is first deresinated with acetone. But entrainment of water-soluble components does not occur when PI and resin are both removed with a solvent such as the acetone-pentane azeotrope (simultaneous extraction). Schloman et al. (1988) reported on the influence of extraction conditions on yield and composition of the resin extracted from guayule and found that it depends upon the choice of extraction medium.

Mears (1980) reviewed the flavonoid profiles of the genus *Parthenium*. Mabry (1982) reported of Goodyear Tire & Rubber Co.) identified four previously unreported flavonoids in the acetone extract (guayule cultivar 593). He also confirmed the absence of flavonoids in the hot water extract of fresh biomass.

Given the presence of various ultraviolet absorbing functional groups in resin components, especially aromatic rings, UV absorbance was used to determine the resin content in acetone extracts from guayule tissue (Michael et. al., 2009).

IV.4 Analysis of other components of guayule biomass

IV.4.1 Proteins

Proteins released from HL are now known to be highly allergenic, IgE-mediated hypersensitivity. The proteins in guayule latex are not cross-reactive with latex allergens. Devices that require the elasticity provided by rubber may be manufactured with guayule latex as an alternative source for patients with Hevea latex allergy (ALAA, 2011). It has been shown that individuals sensitive to HL allergies do not react to GL (Coates, et al., 2001).The amount of protein in the GL was quantified by using a Pierce micro-BCA protein assay kit. This procedure involves the dissolution of protein from latex (Siler and Cornish, 1995). The aqueous protein was removed from the latex, precipitated and re-suspended in solution. The solubilized protein was then mixed with the reagent, and along with blanks and standards, quantified using an Ultraspec-3000 spectrophotometer (McMahan et al., 2005). Guayule proteins have the potential to induce IgE immune responses, and they must be considered

potentially allergenic. The possibility that proteins from guayule latex would cross-react with other allergens, such as those on ragweed pollen, has not been investigated.

IV.5 Conclusion

These analytical methods complement the extraction and bulk quantification of PI and resin that was commented in Chapters II and III, designed for providing a detailed view of the molecular composition of these fractions. This chapter complements Chapter I for showing the complex nature of the guayule biomass, and especially the extractable classes. In turn it emphasizes on the difficult task when trying to quantify the resin components, either as a undesirable fraction to be separated before PI extraction, or as a pollutant of the so-named rubber fraction, extracted with a less polar solvent. Out of these problems of extraction selectivity, this chemio-diversity complicates the use of photometric methods like mid infra red (MIRS) or UV spectroscopy, given the presence of same chemical groups in both PI and resin classes (C=C). This aspect should be considered during our experimental work.

Chapter V

Materials and Methodology

V.1 Introduction

If the selectivity of solvents towards extraction of resins with acetone solvent, and of PI with hexane solvent is still pending, an adaptation of the gravimetric yields to determine contamination of resin and PI respectively in acetone and hexane extract was used. The methods deal with the following description:

- a first section , selection of analysis methods for chemical detection of resin and PI in both the extracts (paragraph V.4),
- a second section for setting methods for quantifying the contaminant accurately by FTIR (paragraph V.5.1) and SEC-MALS (paragraph V.5.2),
- a third section to check the presence of contaminating fractions and to estimate the extent of the contamination (paragraph V.6),
- a fourth section to test several methods to determinate the amount of contaminate using a calibration curve and to establish an equation for applying this methods to more samples and to compare to the real extractable data, thus based on "real" PI and resin contents by Soxhlet, Polytron and ASE,

- Finally, build a new calibration model again with the same samples of NIRS results.

V.2 Biomass samples

V.2.1 Guayule plants selection

We used a biomass of *Parthenium argentatum Gray* (guayule), harvested at three locations (France and Spain) for the rubber and resins content evaluation and for the NIRS calibration. The ccollected samples were used to set a reference method by acetone and hexane extraction. A total of 225 samples of guayule biomass were analysed, over the 2009 - 2011 period. The origins of the samples are:

- From the University of Tucson in Arizona (USA), AZ2 plant biomass,

- From France (Lavalette, center of transfer of Montpellier Sup Agro Montpellier), AZ2 line, 11 samples, from June 2009 until November 2011, also used to monitor PI increase with the plant age,

- From France (Lavalette center of transfer of Montpellier SupAgro, Montpellier), AZ2, AZ1, 11595, N565, 593, CAL-6 line, 52 samples from March 2010 until July 2011;

- From Spain (El Molinar, near Cartagena, Murcia province), from March 2010 to July 2011 with AZ2, AZ1, N565, 593, 639, 641, 642, 643, 644, 645, 646, 647, 648, 650,

651, 653, 654, 657, 658, 661, 662, 2241, 2247, 2249, 2252, 2253, 11591 lines samples (27 guayule genotypes) or 163 samples used for NIRS analysis.

All collected guayule samples were used for the NIRS calibration (Table V.1). The 225 samples cover three locations, many genotypes, and several seasons over more than two years old plants.

Table V.1: Guayule biomass harvested dates for the research study

Harvest date	Origin	Plant age (months)	Sample Number
June, 2009	France	8	1
October, 2009	France	13	1
February, 2010	France	17	1
March, 2010	France	18	1
June, 2010	France	21	20
October, 2010	France	25	1
February, 2011	France	30	27
May, 2011	France	32	5
June, 2011	France	33	1
October, 2011	France	37	1
November, 2011	France	38	1
March, 2010	Spain	18	2
June, 2010	Spain	21	104
October, 2010	Spain	25	1
June, 2011	Spain	33	56

V.2.2 Sample preparation

Sets of three to five guayule shrubs were selected randomly and harvested by cutting the plants at 3-5 cm above the ground. Leaves and flowers were removed manually in the field and the branches were cut into pellets of about 1 cm length with a manual cutter, before partial drying for two days at ambient pressure in open trays. Samples were frozen in liquid nitrogen and grounded (<0.50 mm mesh) using a coffee grinder and then, vacuum dried in an oven (Fisher Bioblock Scientific, Illkirch, France) for 48 hours (< 10 mmHg, 40°C). The

remaining moisture was kept below 10% (Black et al. (1983); Hamerstrand and Montgomery, (1984); Schloman et al. (1988); Verbiscar et al. (1989)). Dried ground samples were stored under nitrogen atmosphere in screw cap closed glass vessels, at 4°C in order to limit risk of oxidation.

V.3 Moisture content determination

Moisture content (MC) of 3.0 g ± 0.1 mg of each guayule sample was assessed by gravimetric analysis. Sample was grinded and put into recipients in an oven (Gefran 800, Chopin, Boulogne, France) at 103^{o}C for 15 hours, and then weighed with 0.1 mg of precision. The sample assay was made in triplicates.

From the Annual Book of ASTM (American Society for Testing and Materials) Standards, the total evaporable moisture content in Aggregate (C 566) can be calculated with the formula in equation V.1;

$$\%\,\mathrm{MC} = \frac{\mathrm{A} - \mathrm{B}}{\mathrm{B}} \mathrm{x}100 \qquad \text{(V.1)}$$

Where;

%MC: Percent of Moisture content is the fraction of total evaporable moisture content of sample (%),

A: the mass of the original sample weight (g), and

B: the mass of dried sample weight (g).

V.4 Resin and rubber extraction methods

V.4.1 Extraction methods

Three extraction methods, Soxhlet, high speed homogenizer (Polytron), and accelerated solvent extraction (ASE) were used. Experimental conditions were varied to determine optimum best a conditions and to allow comparison between methods. The efficiency was assessed based on the gravimetric yield of acetone and hexane extracts and expressed as per cent relative to the dry weight of the biomass sample. All solvents used were of analytical grade i.e. acetone (A.R., Carlo Erba), and hexane (A.R., Carlo Erba). Assays were carried on three replicates.

Soxhlet extraction. A sample of 10 g ± 1 mg of guayule was weighed into a standard extraction thimble, which was placed into the Soxhlet equipment (200 mL) with a condenser on top and a round bottom 500 mL flask; a volume of 250 mL of solvent (acetone or hexane) was placed respectively in the Soxhlet and in the flask with 3-4 boiling chips as pumice (Panreac Quimica, SA E-08211, Castellar del Vales, Barcelona, Spain) was used as boiling regulator.

The sample was allowed with a frequency of 6 refluxes per hour (scale number 4 of the heater) to reflux for 3, or 4, or 8 hours. Then, after cooling, the apparatus was fitted with another flask containing fresh solvent for a subsequent step. Evaporation of the solvent from the extract was done in a rotary evaporator (R-114, Buchi, Flawil, Switzerland) at 40°C, in a pre-weighed flask and taken to dryness under vacuum in an oven (Fisher Bioblock Scientific, Illkirch, France) at 40°C for 15 hours, and kept in a desiccator before weighing, to calculate the guayule extractable with Soxhlet by gravimetric method. Flow stages of the Soxhlet extraction procedure is shown at Figure V.1.

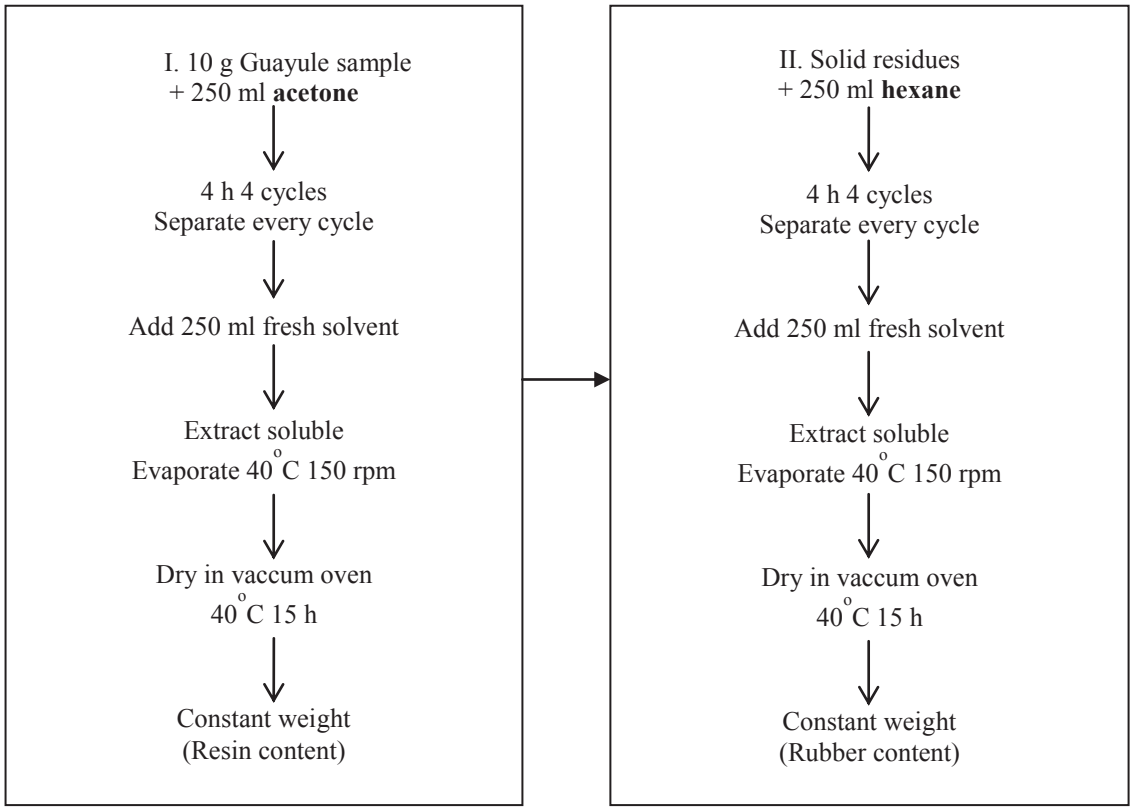


Figure V.1: Procedure diagram of the Soxhlet extraction method

Polytron extraction. A sample of 5 g ± 1 mg of guayule was weighed and put into a 200 mL of recipient of a Kinematica Polytron PT 3100, homogenizer (A.G., Littan/Luzern, Switzerland). With the Polytron homogenizer, we used 50 mL of solvent (acetone or hexane) and blended at 15,000 rpm speed for 12 min, in water bath under controlled temperature kept at 30°C. The extraction steps were repeated twice with fresh solvent, the kettle was rinsed with 10 mL of fresh solvent then added to the extract. After, the sample was poured with solvent into a pre-weighed 50 ml centrifuge tube. It was rinse with 10 ml fresh solvent and the rinse added to the sample tube. The tube was centrifuged at 7000 rpm for 15 minutes (changing the tube and re-centrifuge a second time if necessary). The supernatant was filtered through a filter paper (Whatman Grade No.1) in funnel apparatus into a pre-weighed balloon. After removing the filter paper, the glass funnel was rinsed with 2.5 mL of fresh acetone, and the rinse added to the evaporating balloon. The cake was air-dried. The extraction steps were repeated three times.

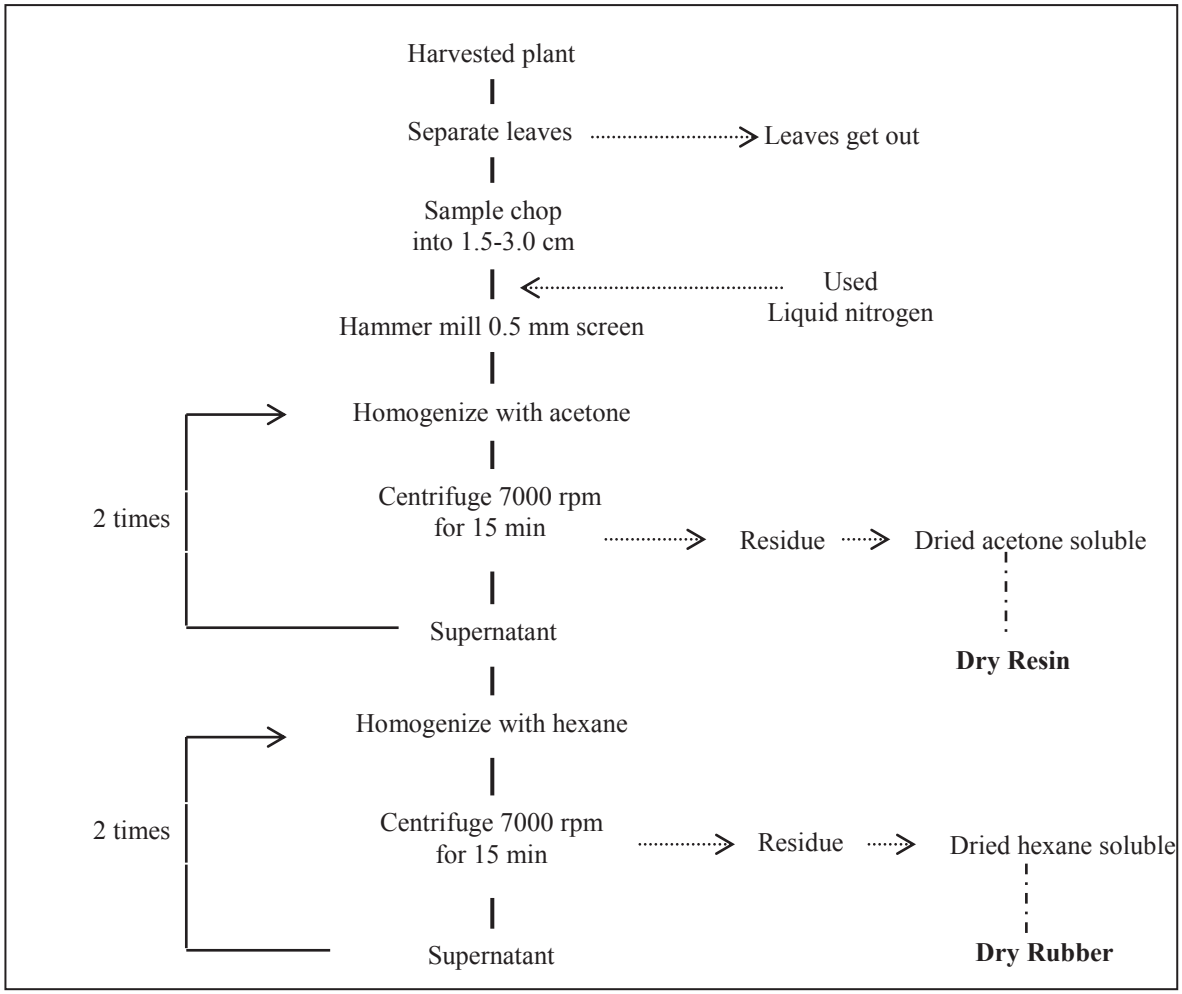


Figure V.2: Procedure diagram of the Polytron extraction method

The Polytron generator was cleaned between the three extractions, each of 30 seconds in acetone, followed by 30 seconds in distilled water and hexane. Hexane extraction was performed on the solid, after acetone extraction with the same procedure as for acetone extraction. The residue was re-extracted 3 times with hexane to determine percent recoverable rubber. Evaporation of the solvent of the three combined extracts to dryness was done in a rotary evaporator at 40°C in a pre-weighed flask and then kept in a desiccator before weighing.

Accelerated solvent extraction (ASE). A sample of 5 g ± 1mg of guayule was weighed and loaded into a stainless steel 22 mL cell of an ASE Dionex Model 350 (Sunnyvale, CA, USA) equipped with an auto-sample carousel, a solvent controller and a collection tray that allowed up to 24 separate samples to be extracted sequentially, connected to a nitrogen tank. A cellulose micro-filter (diameter 27 mm, Dionex Corp., Sunnyvale, CA, USA) was placed at the bottom of each cell. Glass collecting vials (250 mL) were used. Extraction was performed under the following conditions: the empty volume in cells was filled with solvent until pressure reached 10.3 MPa, a heating time of 6 minutes, static extraction period of 20 minutes for acetone extract, and 25 minutes for hexane extract, with a purge time of 60 s, a flush volume 85%, an extraction temperature and a number of static cycles which varied according to solvent (acetone or hexane). Following this, the extract was transferred into a pre-weighed flask. Evaporation of the solvent from the extract was done in a rotary evaporator at 40°C, in a pre-weighed flask and taken to dryness under vacuum in an oven at 40°C for 15 h, then kept 30 min in a desiccator before weighing.

The resin and rubber content was calculated as follows;

$$\frac{(M_C - M_B)}{M_A} x100 \qquad (V.2)$$

Where: M_A : the weight, in g, of the sample to be analysed by solvent extraction;

M_B : the weight, in g, of the recipient after evaporation;

M_C : the weight, in g, of the recipient remaining after extraction.

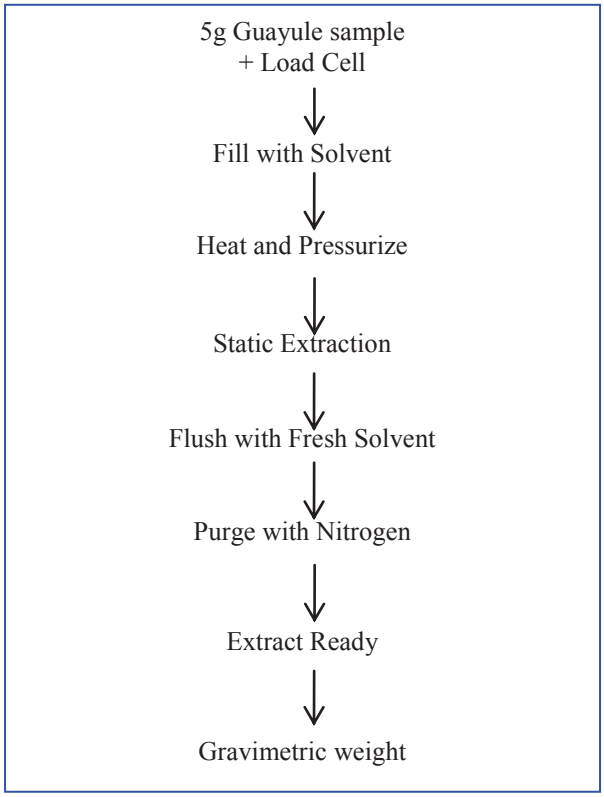
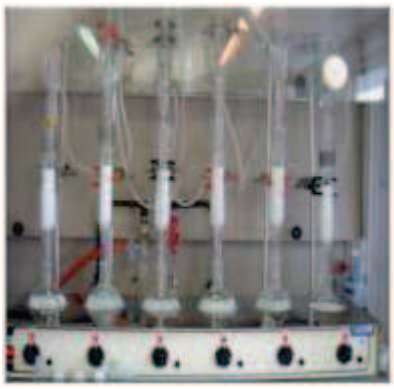


Figure V.3: Procedure diagram of the ASE extraction method

V.4.2 Comparison of the three extraction methods

The selected operating conditions for the three above protocols were then applied to the same batch of guayule biomass. The gravimetric methods have generally been used for determining resin and rubber content after extraction in suitable solvents (Black, et al., 1983, US. patent, 1986). Best conditions of each extraction method was studied, with Soxhlet, Polytron and ASE methods and the extractable yield controlled after three cycles, over 98% of acetone and hexane soluble. For Soxhlet, each extraction step was for 240 minutes, a weight of sample of 10.0±0.5 gand temperature of ebullition 56°C for acetone and 68°C for hexane. For Polytron each step was 12 minutes at a speed of 15,000 rpm, for 5.0 ± 0.5 g at a temperature of 30°C. For ASE, the weight per sample was 5.0 ± 1.0 g, extraction for 20 and 25 minutes at a temperature of 40°C for acetone and 120°C for hexane, with a pressure of 100 bar, and 85% flush with nitrogen. The three extractors apparatus used for guayule biomass, i.e. Soxhlet, Polytron and ASE are shown on Figure V.4. The influences of several parameters that could affect extracts quantities were studied.



Soxhlet

Polytron® PT 3100D

ASE/350 Dionex

Figure V.4: Three extraction apparatus on guayule biomass; Soxhlet, Polytron, and ASE

V.5 Quality analysis

Quality analyses included SEC-MALS and FTIR analytical measurement. The first step was to select an analytical method for the chemical detection of resins and PI in both the extracts (V.4), the second step was to set a method to quantify the contaminant, sufficiently accurate for FTIR (V.5.1) and SEC-MALS (V.5.2). The last stage, in the third section, was to check the presence of contaminating fractions and to estimate the extent of this contamination (V.6).

V.5.1 Determination of PI rate in the biomass measured by SEC-MALS

The quantity of polyisoprene (PI) extracted from the biomass (fresh or dried) was determined after integration of the PI peak on the chromatogram obtained from the refractometer detector, using the *dn/dc* ratio of PI. The *dn/dc* represented the incremental refractive index change (*dn*) of the solution for an incremental change of the concentration of the sample (*dc*). Knowing the volume injected in SEC-MALS and the exact quantity of biomass added in the solution and its moisture content, it was possible to calculate the rate of PI extracted from the biomass, expressed in dry biomass (%PI (g) per dry biomass (g)).

Size exclusion chromatography, couple with a multi-angle light scattering detector was used for the determination of molar masses distribution, weight average molar mass (*Mw*), number average molar mass (*Mn*).

Molar masses distribution. GR quality is not defined by the PI content in biomass determined in Chapter II. The weight-average molar mass (M_w), number-average molar mass (M_n), molar masses distribution (MMD), polydispersity index (M_w/M_n), are commonly used to partially determine PI characteristics linked to rubber quality (Black, 1986).

Determination of branching of PI by SEC-MALS. To avoid the tedious fractionation steps necessary to obtain polymers with narrow molar mass distribution and structural modifications throughout the different fractionation stages, SEC-MALS is used for direct characterization of polymer branching. It is a very versatile technique since the light scattering detector provides absolute M_w and also a size parameter R_g the radius of gyration $(R_g^2)^{1/2}$ (other authors use $<rms^2>^{1/2}$), throughout the chromatogram. This combination of a molar mass or chain length, and a size parameter $R_{g,}$ in the case of polydisperse polymers, is used to obtain information about the shape of the polymer chains (Flory exponent, ν), and the distribution of branching, depending on size or molar mass. The conformation plot (R_{gi}=f(M_{wi}) is significantly different between the PI of fresh biomass extracted with the soft method and the PI of dry biomass extracted by ASE.

-$M_n = \Sigma N_i$ x $M_i / \Sigma N_i$; the denominator is the total number of rubber chains, and it is called the "number-average molar mass". It is the sole to have a clear signification among the three molecular mass descriptors.

- $M_w = \Sigma N_i \, x \, M_i^2 / \Sigma N_i \, x \, M_i$; the weight-average molecular weight. *Mw* is the total weight of the sample and it is called "weight-average".

- MMD = M_w / M_n; Molecular weight distribution (MMD)

A reference polydisperse PI with linear chains, or a master curve obtained with standard linear PI, was needed to calculate the number of branching points per chain (m) using SEC-MALS and ASTRA Software (Kim et al., 2009). This method has the advantage of determining an absolute number of branching per chain and enables a better comparison with data in the literature.

However, Kim et al. (2008) showed that Hevea rubber (HR) samples analyzed by SEC-MALS gave abnormal elution at high elution volume, probably due to highly compact micro-aggregates or micro-micelles delayed by interactions with the column packing. This provides an additional argument about the extreme sensitivity of PI macromolecular data to operating conditions during processing steps when preparing samples from guayule biomass, as already pointed out in section IV.2.1.

V.5.1.1 Sample preparation for SEC-MALS analysis

Sample preparation

A weigth of 50 mg of guayule biomasses was extracted with acetone or hexane solvents and kept after evaporation and weighing under nitrogen gas.

- Dissolving operation

Samples of 50 mg of the residual biomass after acetone or hexane extraction were dissolved in tetrahydrofuran (50 mL THF, HPLC grade density of THF of 0.887 at 25°C) and stabilized with 2,6-di-tert-butyl-4-methylphenol (BHT). The sample was put in solution in THF at a concentration of 1 mg/ml (THF solution stabilized with 100 mg BHT per Litter), then filtrated with 0.1 micrometer glass fiber filter. For a better repeatability, three solutions were prepared for each sample and each solution was injected once. The value of average molar masses is the mean of the three injections for each sample. The solutions were stored 3 days at 30°C.

- Filtration

After maturing with storage and the solution are filtrated through 1 µm disposable filters whose glass fiber membrane had a porosity of 1 µm (VWR filter: Acrodisc, Pall 516-7668).

V.5.1.2 SEC-MALS injection

A 150 µl volume of each solution was injected for a standard polyisoprene (PI) with *Mw* = 1860; 200; 4 kg/mol, Polystyrene (PS) with a *Mw* = 30.3 kg/mol, and natural rubber (TSR 5 grade) section V.5.1.1). The *dn/dc* was 0.185 for the PI and 0.130 for PS.

The filtered solution is collected directly into a 1 ml sample bottle for automatic injection into the SEC equipment, and injected in the SEC-MALS apparatus as the extract initial concentration of the sample solution was known and the injected quantity was determined.

V.5.1.3 SEC-MALS equipment

The SEC equipment consisted of an online degasser (Elite ^{TM}, Alltech), a Waters 515 pump, a refractive index detector (Optilab Rex, Wyatt technology 2410) and a multi-angle light scattering detector (Dawn DSP, Wyatt technology Corp.). The columns were two or three Styragel (Waters Corporation) and a PLGEL (Polymer Laboratories) Mixed-A mixed bed columns (3 columns: 1 column PL gel mixed-A (300 × 7.5 mm, porosities de 20 µm) 2

column STYRAGEL waters HMW6E 20µm (7.8 x 300 mm) I.D., Waters Corporation) with a guard column. These columns were maintained at 45°C. The mobile phase was THF at a flow rate of 0.65 mL/min; the injected volume was 150 µL.

All diode detectors at all 18 angles in the MALS instrument were normalized using a THF solution of a low polydisperse polystyrene standard (*Mw* = 30.3 kg/mol, Wyatt technology). The same solution was used to determine the interconnection volume between the two detectors (Differential refractive index detector (dRI) and MALS detector).

The M_w, the M_n and radius of gyration (R_z or R_g) at each slice of the chromatogram were calculated using Berry method for extrapolation in ASTRA software version 5.3.1 (Wyatt technology Corp.). The order of polynomial fit used with Berry method was two. Fourteen angles, from angle 4 (32°) to angle 16 (134°), were used for calculation.

The differential refractive index increment (*dn/dc*) value used was 0.13 mL/g at 633 nm as determined by Kim et al. (2009).

For the Berry fit method the square root of $Kc/\Delta R(\theta)$ was plotted against $\sin^2(\theta/2)$. The order polynomial fit used in the Berry method was two. The angles no. 2, 3, 17, and 18 were excluded from the calculation, for all samples and fitting methods, because of erroneous fitted. $\Delta R(\theta)$ is the excess Rayleigh ratio, the ratio of scattered and incident light intensity; C is the solute concentration in g/mL, K is an optical constant, θ is the scattering angle, M_{wi} and R_{gi} are, respectively, the weight-average molar mass and z-average radius of gyration for elution slice i.

V.5.2 Infrared spectroscopy

The infrared portion of the electromagnetic spectrum is usually divided into three regions; the mid-infrared, approximately 4000–450 cm^{-1} (30–2.5 µm). Thus, a Fourier transform infrared (FTIR) spectrometer (Perkin Elmer model Spectrum) was used. The fundamental vibrations and associated rotational-vibration structure were compared with reference handbook IR. The tarea under the peak corresponding to resin and PI were calculated. The ratio : resin peak to PI peak was used to determinate the contamination and to set up the IR calibration curve.

V.5.2.1 Preparation of solution

The guayule sample extracted by solvent was dissolved in chloroform, a non-hydroscopic solvent, at a concentration of 1% of guayule extracted, and then stored 3 days. For a better

repeatability, three solutions were prepared for each sample and each solution was scanned once. The value of average is the mean of the three spectrums for each sample.

V.5.2.2 Preparation of KBr cell

A weight of 150 mg of KBr in solid powder form was pressed at 10 psi to produce a translucent pellet KBr cell, through which the beam of the spectrometer can pass.

V.5.2.3 Preparation of casting film

Four drops of the 1% solution is deposited on the KBr cell surface. The solution was evaporated and a dry film formed on the cell and analyzed directly. It is important to ensure that the film is not too thick to permit light to pass through in the technique of cast film. This technique is suitable for qualitative analysis. It is important to note that spectra obtained from different sample preparation methods will look slightly different from each other due to differences in the samples' physical states.

V.5.2.4 Infrared spectrum test

The film formed on KBr cell, was analyzed in the infrared electromagnetic spectrum regions 4000-450 cm^{-1}. The ratio of the surface area under peak of the resin peak is measured and divided by the PI peak. The result is the mean of the three spectrums of each sample.

Calculate for IR: $A = \log (I_O/I) = \varepsilon LC$ (V.3)

Where

A : absorbance

Io : The intensity of the incident light.

(or the intensity of the light passing through the cell reference)

I : The intensity of the light passing through the sample to detector

ε : Molecular absorption coefficient (L/g.cm)

L : The path length of the sample

C : Concentration (g/L) of the solute

V.6 Determination of amount of contaminants in the extract

The level of contamination of PI in resins was measure with a cross-contaminated method using IR for acetone extract and SEC-MALS for hexane extract and setting up a standard

curve. A standard curve was repeated about 10 times, as there were many steps in the process and it was time consuming. However, the obtained standard curve was of good quality and could be used to determine the quantity of the contamination in all extracts.

By quantifying the resin and PI content in solution extracts, it is possible to evaluate the real resin and PI content in the biomass.

V.6.1 Detection of cross contamination in acetone and hexane extracts

Essentially, two methods were used to achieve the goal to quantify the cross contamination of PI in acetone extract by FTIR (V.6.1.2) and resins in hexane extracts by SEC-MALS (V.6.1.1)

The SEC-MALS quantification derives from the analysis of the Mw of PI. In both cases, we checked separately several parameters to access an acceptable calibration curve.

V.6.1.1 SEC-MALS

The method of preparation of the solution for the contaminants determination in extract was as follow:

Master solution. In the first step, the sample to be analyzed is by preparation of a master solution at a concentration 1 mg/ml of THF solution (100 mg BHT into 1 L of THF).

Solution of acetone or hexane extract and synthetic polyisoprene. Samples were prepared as a master solution at a concentration ranging from 0% to 16%. Solutions were left to mature for one day in the dark. The intensity of concentrate from 0 to 16% was then measured

V.6.1.2 Determine the amount of PI contaminants in acetone extracted by SEC-MALS

The SEC-MALS derives from the analysis of PI molecular weight. Ffigure V.5 shows the acetone and hexane extracts, and the standard PI. The peaks at 28 to ~43 minutes elution time and above 44 minutes correspond to the PI (compared to a standard) and resin/ It shows several shoulders. Figure V.5 shows even more clearly the complex composition of another acetone extract. The PI standard of Mw ~4700 g/mole let think that this extract may contain low Mw PI.

To be detected the contamination of acetone extract by high MW PI. Profiles showing cross contamination are noted in many cases

The level of contamination of the PI content in acetone extracted by SEC/MALS and the contamination of the resin in hexane extract were described in IR paragraph.

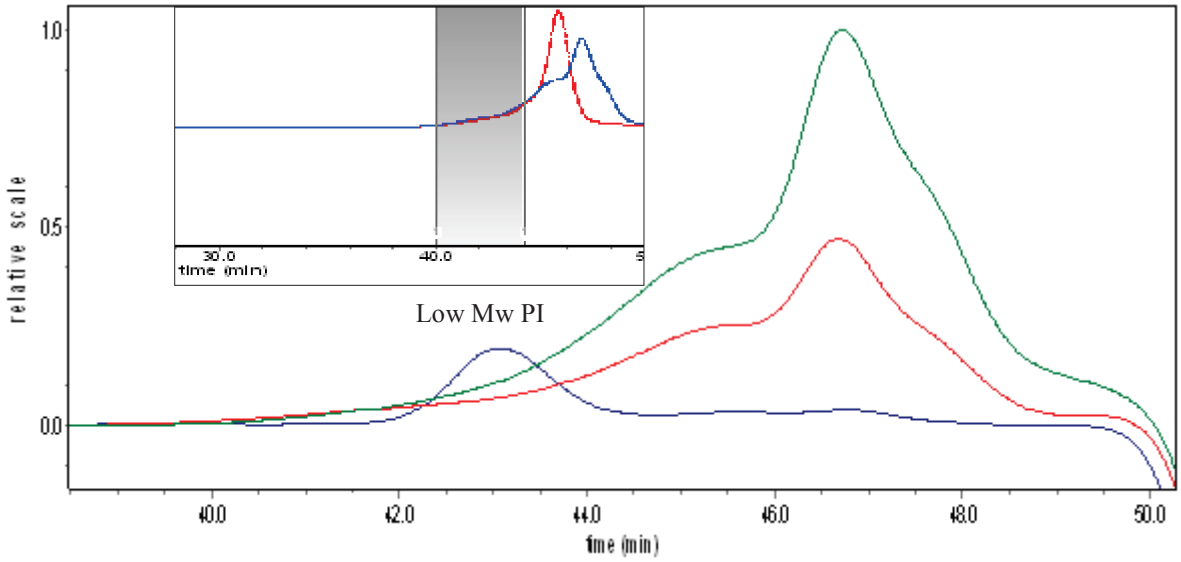


Figure V.5: SEC/MALS cross section of acetone extraction with low molecular weight PI *(Sample: SpMarch-2010)*

The SEC/MALS graph (Figure V.5.2) shows an overlap (or cross section) of PI peak and resin peak at retention time of 40-44 min. However, in acetone extract there is a low molecular weight PI contamination. These surface areas were the Y value in standard calibration equation. The method allowed to determine a percentage value of contaminants in acetone extract, and then to quantify the amount of pure resin in the acetone extract.

At the start, we detected the surface area of the shoulder in the graph of the acetone extract, by setting the beginning point and end point of shoulder in the graph. The begin point of the retention time at was set at 40 min and 44 min for the end point of the graphs.

V.6.1.3 Determination of the amount of resin contaminants in hexane extracted by IR

Master solution. In the first stage, a master solution at a concentration of 1% was prepared and then resin at concentrations ranging from 0% to 16%.

IR spectroscopy calibration curve. A reference curve for area ratio of the bands at various rubber concentrations in an extract containing a known amount of polyisoprene (PI) was set. The standard reference curve is made from films cast from solutions of polyisoprene at a

concentration ranging from 0% to 16%. The peak absorbance is measured at 1730 cm^{-1} for the resin and at 830 cm^{-1} for the PI. The ratio of resin peak area per PI peak area was measured and the concentration estimated.

For the reference curves ratios of absorption peak areas of resin/PI values were established and used to determine the contamination in the sample. The horizontal axis (x-axis) is the ratio of concentration of resin/PI and vertical axis (y-axis) is the ratio of absorbance of resin/PI.

V.7 Polyisoprene and resin purity

The experiments in the last chapter are based on measurements of extraction by gravimetric method. The experiment was used in the extraction of a large number of samples leading to a construction of an appropriate NIRS equation. However, it was occasionally mentioned that the extract was not as pure as expected. This study subsequently focused on an improvement method to look for the quantity of contaminates in each extraction in order to find a yield of non-contaminates. The purity of the extracts can be done by two ways: the first way is by precipitation of hexane extracted by methanol solvent. The second way is to determine the amount of contamination of the PI content in acetone extracted by SEC/MALS and the contamination of the resin content in hexane extracted by IR as explained in former paragraph. Finding the real PI and resin components was used to define the level of cross contamination. The method developed is the following:

V.7.1 SEC-MALLS

The first step is to find a surface area of the shoulder in the graph of acetone extracted of the unknown sample, and then set the beginning point and end point of shoulder in the graph of acetone extract. The begin point of the retention time at 40 min and 44 min as the end point of the graphs as described before.

The surface areas were the Y value in the standard calibration equation. It is used for determine percentage value of contaminants in acetone extracted, after that determines the amount of pure resin in acetone extracted. Calculate the purity values of the relationship with the contamination from the calibration equation with the gravimetric weight.

Finding the real PI components was used to cross contamination. The sum of the total resin was combine the percentage of contaminants in hexane extracted (resin from IR analyzed) with the amount of pure resin in acetone extracted.

Total resin$^{(1)}$ = %Resin in acetone extract$^{(2)}$ + %Resin in hexane extract$^{(3)}$ (V.4)

(1) percentage of resin in biomass (dry weight)
(2) = [percentage of resin in acetone extract] x [yield of acetone extract per dry weight of biomass]
(3) = [percentage of resin in hexane extract] x [yield of hexane extract per dry weight of biomass]

The standard calibration curve for resin contaminating in hexane extract was used resin in PI curve by IR analysis, it was; Y= 2.8567+0.0701, R^2 = 0.9795, for suitable to use the resin curve determine resin contaminating in hexane extraction.

V.7.2 IR spectroscopy

To find the ratio of surface area of resin peak with PI peak was done by IR. The ratio of surface area was in the Y value in the standard calibration equation. The result shows the percentage value of contaminants in hexane extract. Finding the real PI components was used to evaluate the cross contamination. The sums of total pure PI were combine the amount of pure PI in hexane extracted and the percentage of contaminants in acetone extracted.

Total PI$^{(1)}$ = %PI in hexane extract$^{(2)}$ + %PI in acetone extract$^{(3)}$ (V.5)

(1) percentage of PI in biomass (dry weight)
(2) = [percentage of PI in hexane extract] x [yield of hexane extract per dry weight of biomass]
(3) = [percentage of resin in acetone extract] x [yield of acetone extract per dry weight of biomass]

%PI contaminate in acetone extract (SEC) by the standard calibration curve for PI contaminating in acetone extract was used PI by SEC-MALS, RI detector, it was Y=3.1532x+0.0043, for determine PI contaminating in acetone extraction.

Chapter VI

Materials and methods of analytical methods and pollutions

VI.1 Introduction

For the characterization analysis of rubber polymeric properties we used a size exclusion chromatography coupled with a multi-angle light scattering detector (SEC-MALLS), infrared spectroscopy (FTIR) and structure conforming by nuclear magnetic resonance spectroscopy (NMR). To detect contamination in guayule extracts, two methods have been used one by IR spectroscopy and one by SEC-MALLS. In both cases, we checked several experimental conditions to access an acceptable calibration curve.

VI.2 Soft extraction method (SM)

VI.2.1 Guayule preparation in SM

The guayule biomass, cultivar plants of AZ-2, was planted in September 2008 in Montpellier at Lavalette (Campus Agropolis), France. The plants, when nearly 3 years-old, were cut at ground level and only the branches without the leaves, were used. The fresh biomass was harvested from May 2011 to November 2011 respectively. Moisture content was measured on fresh biomass and dry biomass. To prepare uniform ground guayule sample, the biomass was milled in a coffee grinder with liquid nitrogen until pellets passed through a 0.5 mm mesh screen. Fresh biomass powder was stored in a fridge and dry biomass powder was dried in vacuum oven at 40°C.

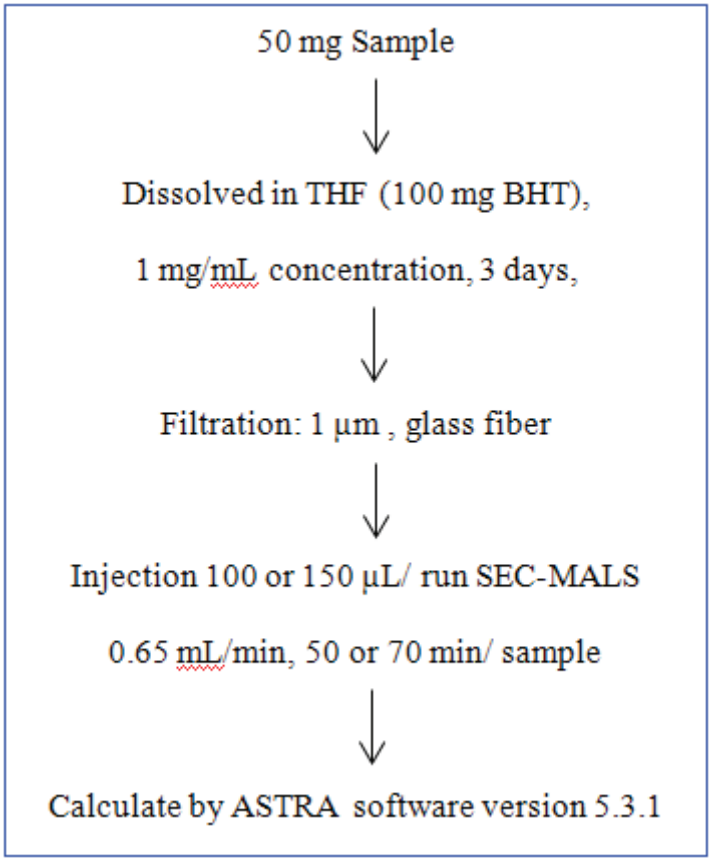
VI.2.2 Biomass solution preparation

The guayule samples, fresh or dry (1.5 g±0.1 mg), were dissolved in tetrahydrofuran (THF, 40 ±1 mL, HPLC grade) stabilized with 2,6-di-tert-butyl-4-methylphenol (BHT). To evaluate the variability of the measure, three solutions were prepared for each sample. The solutions were kept in a water bath at 30°C during various times (6, 24, 72, or 168 hours). The solutions were filtered through a filter type Acrodisc (1 µm, glass fiber, Pal) and injected in SEC-MALS.

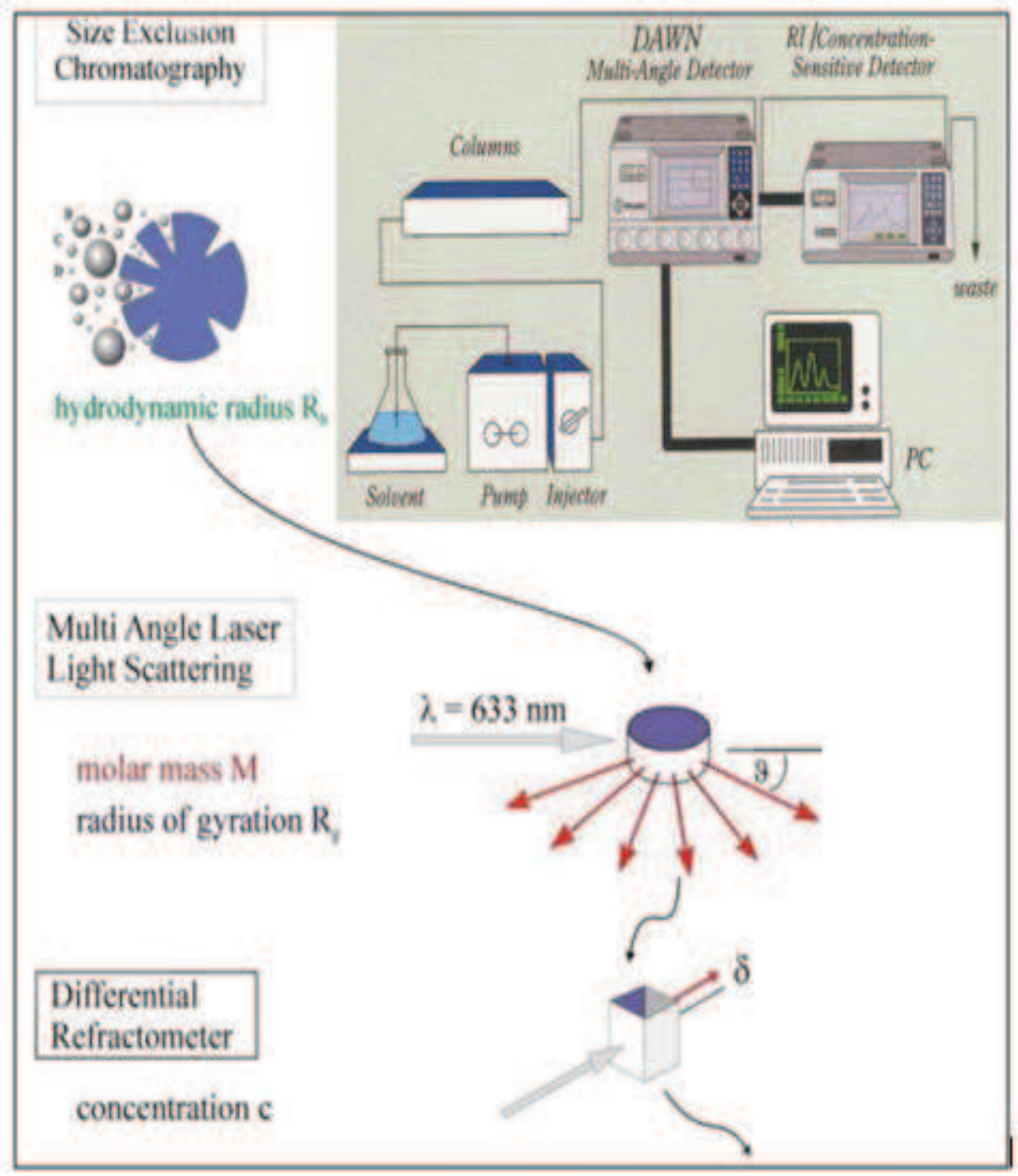
Three types of adsorbents such as alumina, silica or activated carbon, were tested to improve extraction conditions of the fresh biomass. Each adsorbent was added at a rate of 0.5±0.05 g and solutions were stored 24 hours at 30°C. Afterwards the solutions were filtered on an Acrodisc filter and injected also in SEC-MALS.

VI.3 Macromolecular structure analysis by SEC-MALS

The SEC equipment consisted of an online degazifier (Elite TM, Alltech), a Shimadzu pump (LC2A), a refractive index detector (Optilab Rex, Wyatt technology) and a multi-angle light scattering detector (Dawn DSP, Wyatt technology). The columns were two Varian columns (porosity 20 µm, 300 mm × 7.8 mm I.D.) (Varian, Walnut Creek, USA). The columns were maintained at 45°C. The mobile phase was stabilized THF at a flow rate of 0.65 mL/min; the injected volume was 100 µL. All diode detectors at all 18 angles in the MALS instrument were normalized using a THF solution of polystyrene standard (M_w = 30.3 kg/mol, Wyatt technology). The same solution was used to determine the interconnection volume between the two detectors. The weight-average molar masses (M_w) and radius of gyration (R_g) at each slice of the chromatogram were calculated using Berry method for extrapolation in ASTRA software version 5.3.1 (Wyatt technology). The order of polynomial fit used with Berry method was two. Twelve angles, from angle 5 (38.8°) to angle 16 (134°), were used for calculation. The differential refractive index increment (*dn/dc*) used was 0.130 mL/g (Kim et al., 2009).



a. Flow chart of SEC-MALS measurement



b. Flow chart of SEC-MALS system

Figure VI.1: SEC-MALS measurement of guayule plant;

a. Flow chart of SEC-MALS measurement

b. Flow chart of SEC-MALS system

VI.4 NIRS procedures and analysis

A XDS monochromator (Foss NIR Systems, Silver Spring, MD) was used to scan reflectance from 400 to 2500 nm at 2 nm intervals, using ring cups (50 mm in diameter) with about 3 g of ground and sieved (< 0.5 mm) guayule. Data were saved as the average of 32 scans and stored as log (1/R), where R was the reflectance at each wavelength and 1 the reflectance of a standard ceramic reference. Spectra were acquired randomly, each sample being measured twice, and the average spectrum stored. Statistical analyses were performed using Win-ISI II software (Infrasoft International, Port Matilda, PA, USA) for spectra treatments and calibration developments, and XLSTAT 7.1 software (Addinsoft, Paris, France). Spectra were mathematically corrected for light scattering by using the standard normal variate and detrend correction. The second derivative was calculated on five data points and smoothed using Savitzky and Golay polynomial smoothing on five data points.

First a NIR calibration was developed using 215 samples and reference values obtained by gravimetry, and then a new NIR calibration was developed using 127 samples randomly picked out of the 215 for which pure PI and resin content was determined by SEC-MALS and FTIR.

In order to assess the performances of the predictive equations in the first step the 215 samples were split into a calibration subset (Cal) and a validation subset (Val). The validation set was created by selecting about 33% of the 215 samples randomly. Doing this way, the calibration set comprised 144 samples and the validation set 71 samples. From these selections all the samples were analyzed for MC, 183 samples (127 in calibration set and 56 in validation) were analyzed for resin content and 174 samples (119 in calibration set and 55 in validation) were analyzed for rubber content.

In the second step; 127 samples were selected randomly out of the 215 samples in order to test the effect of purity correction. The 127 data were split into 2 data groups: calibration and validation sets. Validation set was created by selecting about 33% of the 127 samples randomly, doing this way, two third of samples were randomly selected for calibration set. Moreover, the calibration set were comprised 96 samples and the validation set 31 samples.

Calibration equations for the PI and Resin were developed using the modified partial least squares regression (mPLS) algorithm of WinISI software. Calibration statistics included the following parameters: standard deviation (SD), coefficient of determination (R^2), standard error of calibration (SEC), and standard error of cross-validation (SECV). Cross-validation was used during calibration development in order to select the optimum number of latent

variables and to minimize over-fitting of the equations. For SECV estimation, 25% of the samples (randomly selected) were predicted using a calibration model developed with the other 75%, SECV estimation was repeated four times and the average calculated.

In addition to R^2_c, the ratio of performance to deviation for calibration ($RPD_c=SD_{cal}/SECV$) was used to evaluate the general quality of the fit obtained for each equation. The Student (*t*) test was used to identify *t*-outlier samples during calibration development. Outlier detection was based on the standardized residuals (= error/SECV) with a cutoff of 2.5. Two outlier elimination passes were used.

The standard error of prediction (SEP), corresponding to the standard deviation of residuals, was estimated by predicting the validation subset using a model developed on the calibration subset. The ratio performance to deviation of prediction (RPD_p) was also calculated as SD_{val} / SEP (where SD_{val} was the standard deviation of validation samples). The quality of the fits between values from reference analytical data based on extraction and gravimetry (moisture, resin and rubber percentages) and NIRS-predicted values was evaluated from the linear regression slope, the R^2_p and the bias.

The flow charges of the NIRS procedure shows in Figure VI.2.

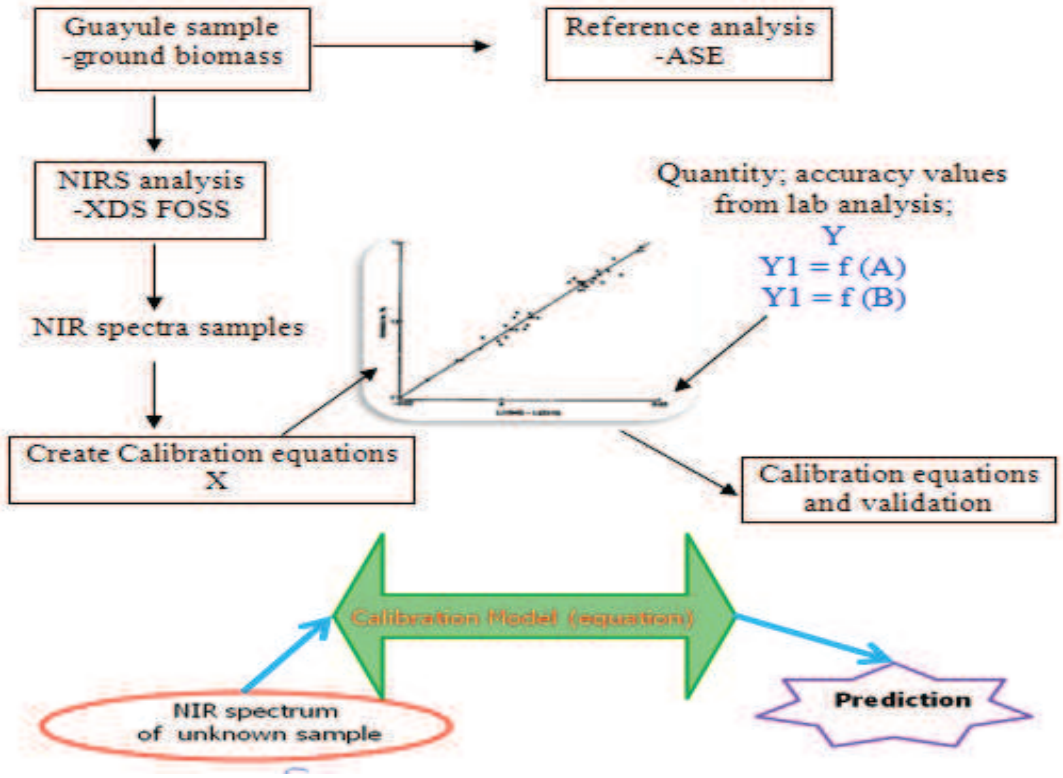


Figure VI.2: Flow chart of NIRS measurement of rubber and resin contents in guayule plant

V.5 Statistical analysis

V.5.1 Data analysis for chemical analysis and NIRS

The effect of the extraction method on rubber and resin extract yields was studied by an analysis of variance (ANOVA). The calculations were carried out with XLSTAT 7.1 software (Addinsoft, Paris France). The differences between means were tested by the Newman and Keuls test at 5%.

V.5.2 Data analysis for SEC-MALS and Soft method

Statistical analyses (one way analysis of variance, ANOVA) were performed using JMP software v.5.1.2. (SAS Institute, USA). Standard deviation (SD) bars and SD values are mentioned in figures and tables, respectively. Significance level α of statistical analysis was set to 0.05.

V.5.3 Experimental design

In addition to find optimized conditions of a selected method to maximize yield extraction. Resin and rubber content were solvent-extracted (acetone and after that hexane) from guayule biomass powders, using an ASE extraction automatic method, after gravimetric quantification. In order to find optima conditions for rubber and resin extractable with ASE, optimized conditions of extraction according to an experimental design by ANOVA, a response surface quadratic model has been experimented for different temperatures by acetone extract (40-64^{o}C) and hexane extract (118-130^{o}C) for resin and rubber contents measured.

VI.5.4 LOD and LOQ

The limit of detection (LOD) and the limit of quantification (LOQ) in the FTIR and SEC-MALS apparatus were done, also by NMR and NIRS.

The LOD and LOQ were calculated using equation (1) and (2), respectively **(**Gadape and Parikh, 2011). The theoretically determined values of detection and quantitation limits were crossed checked by actual analysis of these concentrations using proposed methods:

$$LOD = 3x\ SD/\alpha \quad (1)$$

$$LOQ = 10x\ SD/\alpha \quad (2)$$

Where: SD is the standard deviation of curve and α is the slope of curve.

The analytical responses of blank ten times is a statistical reality and based on 95% confidence limits, negative samples may be falsely detected or positives may be falsely negative or undetected. The LOQ not only sets the point at which a sample is consistently detected (≥95% of the time) but is also reproducibly reported as a given concentration, within a stated error, 95% of the time.

Analyses of ten times of blank in apparatus and the LOD and LOQ were calculated using equation (1) and (2).

Chapter VII

Selection and optimization of a reference method based on sequential extraction of resin and polyisoprene with solvents

VII.1 Introduction

The selection of a reference method for assessing PI and resin contents in guayule biomass has been the first part of the PhD work. The bibliography has shown that attempts to achieve this goal has been done over half a century or more through a wide panel of ways, depending on objectives, raw materials, applicable experimental conditions.

The literature review in Chapter II justifies the objective of the present study i.e. to set up a quick method based on NIRS for fast determination of rubber and resin in guayule biomass. To achieve this goal there was a need for selecting a reference method among the wide panel already proposed, for calibrating the NIR spectroscopic one. Facing a large number of methods and experimental protocols, we concluded that this important step, on the way to the targeted NIRS-based method, justified a dedicated part of the work and therefore a chapter; this for allowing to choose a reference method based on sound arguments. In spite of large amount of literature results, there was little attention paid to compare the many methods based on efficiency and the sole works devoted to this important point showed some variation between the four methods that were compared (cf Chapter II.2.3). Based on above considerations, priority was given to gravimetry, at least as first priority, but still there were many alternatives within this option.

This first part of the PhD work was devoted to optimizing dual solvent extraction protocols (acetone and hexane), by investigating the influence of relevant experimental parameters, trying to find suitable conditions close to optimum, yielding reproducible results for PI and resin fractions. Three extraction devices “Soxhlet, high speed grinder (named Polytron) and automated extraction (ASE: Accelerated Solvent Extraction)” were tested. Then the results obtained under these (partially) optimized conditions, together with advantages and drawbacks, were compared. One technique was selected (ASE) and further optimized to serve as reference method (Reference Method 1; RM1), because additional investigations have led to set another one. This makes the Chapter VII.

This RM1 will then be used in Chapter VIII to calibrate a NIRS-based method.

It should be mentioned that, although it was initially planned to find a protocol suitable for both (i) extracting quantitatively PI, and (ii) using the extract to investigate its molecular structure, it has become clear that these objectives would not be achieved simultaneously. Nonetheless, the conditions were kept as soft as possible whenever possible to avoid PI degradation and possible adverse effect on extraction.

VII.2 Selection of three preferred options to quantify PI and resin in guayule biomass

As a summary of the bibliography synthesized in Chapter II, methods proposed to quantify non polar extractable, namely PI and resin, are based on the following:

(i) Obtaining with solvents the non-polar extractable fractions, followed by elimination of the solvent, here named gravimetric methods; it should be stressed that the close solubility of these compounds do not allow one step selective extraction of the PI,

(ii) Extracting these compounds but not weighing the solvent-free extract, then measuring the turbidity of the liquid medium (photometry) or determining PI content by NMR or IR spectroscopy on films,

(iii) Taking advantage of spectral properties on a broad sense, like high resolution or low resolution NMR, of the biomass to assess the PI content directly on the biomass without applying an extraction step. Note that NIRS which falls in this group requires a calibration step with a reference method.

Banigan et al (1981) compared four methods and obtained the following rubber content for the same sample: 8.7% for gravimetric (two solvent extraction, acetone-water for resins, cyclohexane for PI), 9.2% for turbimetry, 9.3% for IR and 10.1% for NMR.

Worth noting from these data, there is a variation of about 15% between these four determinations based on an average of 24 biomass samples, which is of course not surprising. For example, about gravimetry, one can expect incomplete rubber extraction, and/or unselective extraction of PI. The turbimetric method may suffer the same drawback as well as a high dependency on rubber coagulation conditions, which may -or not- allow all PI to be separated depending on particle size distribution. Also there is a lack of accuracy especially in the case of quantitative IR analysis, because band broadening and some other difficulties for setting up the analytical material, or when choosing for the reference wavelength characteristic of PI only.

Thus which method gives the result closest to the real PI content?

Because above methods suffering of several drawbacks, like improper calibration, lack of optimization, no comparison of results between methods, we considered that, until more detailed investigations, the results obtained through most of above methods should be considered as estimations. This is why we are aiming to choose a reference method based on sound arguments; also taking into account the availability of highly specialized and costly equipment on routine

basis (NMR and IR) and existing extended use and expertise from the literature (gravimetry). Based on above considerations, the gravimetric method was chosen as the first priority. Still there are many alternatives within this single option; PI can be either:

(i) extracted together with resin with a solvent and then coagulated,

(ii) extracted with a non polar solvent after deresinated with a more selective solvent.

After preliminary trials, having noted some difficulties for achieving a reproducible coagulation and rubber separation, it was decided to focus on sequential extraction.

Several published methods, summarized in Chapter II.3, for determining resin and rubber content of guayule were available when this project began. Each method required different specialized equipment and varying inputs. There was a need to develop a technique suitable to conditions that would allow rapid and reliable determination of resin and rubber content in guayule, hopefully to develop a best technique.

In the course of this part of the work, devoted to selecting a reference protocol for extracting resin and rubber, to be used then for calibrating the NIRS method, three extraction methods were tested. Based on literature review, one of the most widely used method involves a two-step extraction, first of the resin fraction with a medium polarity solvent, and then of the rubber with a non-polar solvent (Wagner et al., 1988; Black et al., 1983; Jasso de Rodríguez and Kuruvadi, 1991; Schloman et al., 2003; Wagner et al., 1991); acetone and hexane were chosen in the present case, according to Garrot et al. (1981) and Peason et al. (2010). Then the solvent of each extract was evaporated and the dry extract was weighed; thus the three investigated protocols are based on gravimetry. As generally admitted in the literature, we consider here that the weight of the acetone and hexane extract corresponds to the yield of resin and rubber respectively (Teetor and Ray, 2004).

The three methods include:

(i) Soxhlet, a standard option, although it has never been officially standardized (Black et al., 1983; Jasso de Rodríguez and Kuruvadi, 1991);

(ii) high speed homogenizer, Polytron, potentially well adapted to the fact that rubber is located inside the guayule cells, enabling to speed up the process, and used by Salvucci et al. (2009); Garrot et al. (1981); Jasso de Rodríguez and Kuruvadi (1991).

(iii) ASE, shown to increase extraction speed by using high temperature and pressure, while presenting other advantages (low solvent consumption, automated operation) (Grinberg

and Shaubi, 1985; Jasso de Rodríguez and Kuruvadi, 1991; Teetor and Ray, 2004; Salvucci and Coffelt, 2009);

These three protocols were applied under varied experimental conditions to determine the optimum, and then to select the one enabling to maximize the yield of hexane extract as the reference protocol for NIRS calibration. Note that if needed, the priority should be given to setting the PI content, and not resin. These three extraction methods were carried out using 2 solvents; (1) acetone which was best for extracting component with a polar, followed by (2) hexane as the proper solvent for extracting the non-polar component. Both solvents were shiwn to be efficient and are easy to find and (relatively) cheap.

In summary, the general strategy to meet the goal includes the following experimental steps: partial optimization of the competing techniques to measure acetone and hexane extracts - Soxhlet, Polytron, and ASE; choosing and further optimizing one of above gravimetric techniques (for example kinetics i.e. duration and number of extraction steps), for measuring acetone and hexane extracts representing respectively resin and PI contents, according to literature (Cornish et al. 2013; Garrot et al.,1981; Grinberg and Shaubi, 1985; Jasso de Rodríguez and Kuruvadi, 1991; Salvucci et al., 2009; Teetor and Ray, 2004).

VII.3 Biomass samples

VII.3.1 Collected samples

For setting Reference Method 1, eleven samples of guayule biomass were collected or received from partners, over the 2009 - 2010 periods, from the several guayule lines AZ2, and from the following locations:

- USA (Arizona), four samples of plants aged of more than 20 months, lines AZ2, analyzed while waiting for aged enough plants from France or Spain,
- France (Agropolis campus, Montpellier), 1 sample AZ2 line, June 2009; one sample AZ2 Oct.2009; and two sample AZ2 in Feb. and March 2010.
- Spain (El Molinar, Murcia province), three samples March 2010 line AZ2.

In order to simplify the text, samples are generally named with the following rule: Country of origin (Az for Arizona, Fr for France, Sp for Spain)-Harvest month-Harvest year (decade); for example the results in Table VII.1 were obtained with Sp-March-2010.

In some cases, upon harvest, leaves, big branches and small branches, were separated and analysed individually for moisture content (MC). A typical composition is given in Table VII.1. MC of the small branches is higher than for big branches, 41.6 and 38.6 % respectively. For the leaves was lowest of MC and weight percent. The ratio of discarded leaves is low on dry weight basis. MC is higher in big branches, and the big/small ratio is about haft of fresh weight and accordingly to author (Black et al., 1983; Black et al., 1985; Hamerstrand and Montgomery, 1984; Schloman et al., 1988; Verbiscar et al., 1989; Cornish et al., 2013) were investigated.

Table VII.1: Composition of the biomass (Sp-March-10)

Sample % in received plant	Big branches	Small branches	Leaves	Whole Average
Average weight/plant (g)	420	810	320	1550g
MC in the fresh plant (%)	31	49	20	
MC in dry weight (%)	29	41	26	
dry weight basis (g)	130	367	64	

While, that results from Table VII.1 and Figure VII.1 shown the part of branches not effect of MC and weight percent, then we were chosen mixture of big branches and small branches.

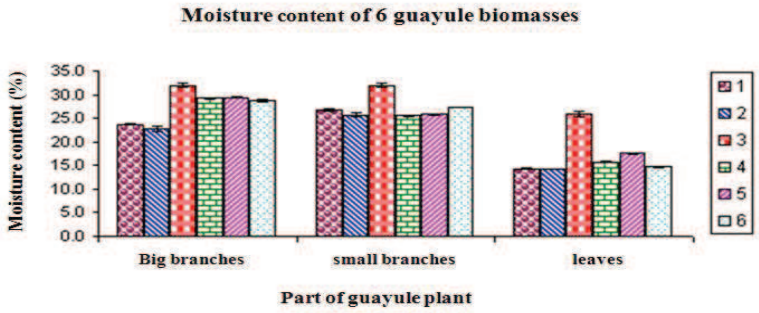


Figure VII.1: Moisture content percent of guayule part from Fr-March-10
(Sample Fr-March-10; Drying: 103°C, 14 h in moisture oven)

Evaluation the moisture content percent of guayule in three parts (small branches, big branches and leaves) from Montpellier sample on March 2010, were harvested at random triplicates 6 plants. The MC of small and big branches have not significant different (27.18±0.21 and 27.65±0.29%), except for leaves was lowest (17.65±0.29%) in Figure VII.1. In the literature

review (Curtis, 1947 and Hamfrstrand and Montgomery, 1983) was reported the leaves do not contain sufficient rubber to justify their inclusion in the extraction process. Moreover, the leaves tend to improve the final resin product because the leaves contain trace amounts of metals and resinous compounds that could affect rubber quality. Followed the result can suggest for this studied to focus of rubber will be used branches part of guayule (combine on small and big branches) without leaves guayule plant part.

VII.3.2 Sample preparation

Sets of three to five guayule randomly selected shrubs of the same genotype were cut above ground level. Curtis (1947) reported that the leaves have a low content of PI, but a rich in resins and together with some metals that could affect rubber quality (Hamfrstrand and Montgomery, 1983). Given the focus of this study, leaves and flowers were removed manually in the field. Stems and branches were cut into pellets before partial drying in open trays. Samples were frozen in liquid nitrogen, grounded (<0.50 mm) and vacuum dried in an oven for 2 days at 40°C. The whole processing was performed as quickly as possible right after harvest, the bibliography showing that this was preferable to avoid degradation (Schloman et al., 2009). The remaining moisture was kept below 10% following Black et al. (1983). Dried ground samples were stored under nitrogen atmosphere in screw cap closed glass vessels, at 4°C in order to limit risk of oxidation (Schloman et al., 2003; Wagner et al., 1988). Figure VII 1 shows the biomass along the processing line, from field to dry powder which has a brown colour.

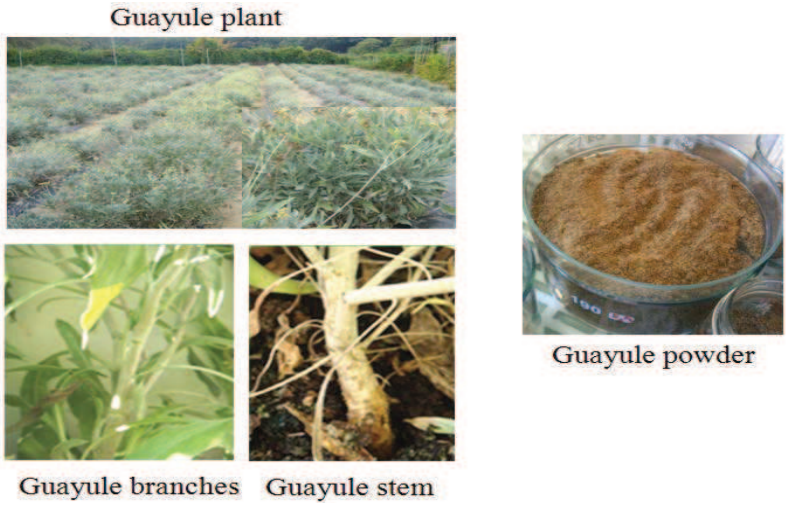


Figure VII.1: Samples of guayule, from field to powder

VII.3.3 Drying conditions

Whereas PI degradation is not important when aiming only a quantitative determination of PI contents, this must be avoided when targeting its molecular structure; we paid attention to drying conditions for some of them were used to further analyze the extracts (Chapter IX). In addition MC has been shown to be an important parameter, regarding extraction yield. In the literature review MC was kept below 10% (Black et al., 1983; Hamerstrand and Montgomery, 1984; Schloman et al., 1988; Verbiscar et al., 1989); under this level MC did not affect the gravimetric results nor the NIRS (Table VII.2). Black et al. (1985) reported that at 49°C drying had no effect on Mw of guayule PI. Cornish et al (2013) recommend a drying temperature than 60°C.

Thus, in order to limit the thermal degradation the temperature was kept as low as possible while still the duration was affordable, and the MC was decreased by drying in vacuum oven at 40°C, thus reducing contact with oxygen while avoiding high temperature. The kinetics was investigated by weighing the sample every 6 hours (Figure VII.2). Starting from fresh biomass having 33-42% of moisture, the first part of the drying process consist of a linear decrease of MC (constant slope~0.66 °C/h) until 6-8% process, in about 48 hours, corresponding to free water. Then MC decreases at a much slower rate as noted elsewhere (Black et al., 1983; Black et al., 1985; Hamerstrand and Montgomery, 1984; Schloman et al., 1988; Verbiscar et al., 1989). From this we conclude that these conditions (40°C, under reduced pressure, 2 days) are suitable for lowering the MC under 10% as advised by some authors, although mobilizing an oven almost continuously, given the number of samples to be prepared in triplicate.

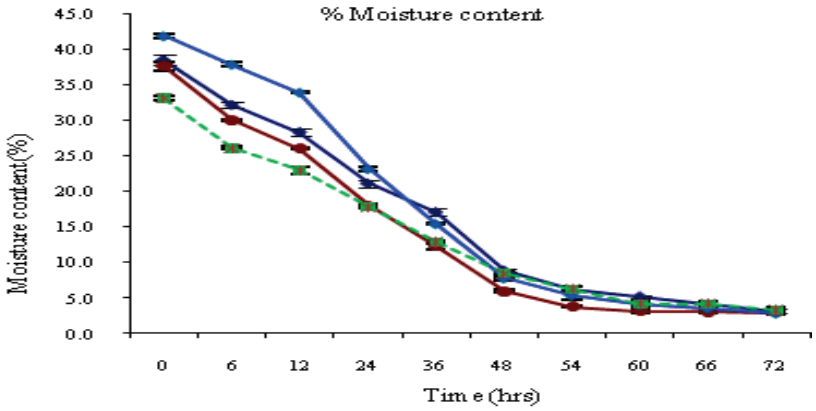


Figure VII.2: Evolution of MC of guayule biomass in a vacuum oven at 40°C

(*4 samples analyzed in triplicate and harvested aged on Fr-March-10*)

VII.3.4 Influence of residual moisture content on extract yield

The influence of residual moisture content on extract yield was checked with a sample rich in resin and PI (Sp-July-11), partially dried during transportation, and dryied then in the laboratory (Cirad) in the vacuum oven at 40°C, down to about 9%), under same soft conditions (vacuum oven, 40°C). Table VII.2 shows that, the MC does not affect the extract yield, in the MC range of 3.3-9.5%, in the case of acetone. In the case of hexane, there might be a tendency of getting a slightly higher result when MC is less than 9%, but this is within the limits imposed by SD. Thus let us take this percentage (9%) as the upper MC limit for applying our protocol.

Table VII.2: Influence of MC of biomass on extract yield with acetone and hexane

Drying time (h)	MC (%)	Acetone extract (%)	Hexane extract (%)
0	9.05±0.19	9.18±0.37[b]	9.41±0.13[b]
6	6.48±0.21	9.68±0.16[a]	10.19±0.25[a]
12	3.28±0.15	9.37±0.28[b]	9.81±0.09[a]

Sample Sp-July-11; Drying: vacuum oven at 40°C; Soxhlet 4 steps/solvent, 4 h each

[a, b] Data bearing same letter within a column are not statistically different according to ANOVA test (p>0.05).

Mean ± SD: Standard deviation values based on triplicates.

VII.4 Optimization of the Soxhlet extraction protocol

The efficiency was assessed in terms of gravimetric yield of acetone and hexane extracts, expressed as percent relative to the dry weight of starting biomass sample, as indicated in the Material and methods Chapter. The operating conditions for the Soxhlet method were investigated for selecting the convenient number of steps and the duration of each one.

The operation conditions for the Soxhlet method were investigated for selecting the convenient number of steps and the duration of each step. The settings of the heaters were checked individually for the six flasks forming the ramp, allowing a cycling time of about 12 minutes (5 cycles/h), thus providing close working conditions to all runs (results in Annex 2). Table VII.3 shows that a single step of 4 h and even 8 h is not sufficient for maximizing the yield of acetone or hexane extracts. A series of four steps of 4 h each and of 2 steps of 8 h each, thus making a refluxing time of 16 h in both cases, gives the same yield of acetone extract within the

experimental error. But four steps with hexane allow a slightly higher yield of this fraction, statistically significant, in comparison to 2x8 h. Thus for the same total extraction time, it looks that there is an effect of combined step number and step duration. This was also noted during preliminary trials; although reasons remain unclear, this may be due to the fact that the total contact time between the solid and hexane is slightly higher owing to the necessary cooling step before changing the solvent, in the case of 4 h steps.

But a fifth step of 4 h did not give higher yields (8.07±0.25 and 1.22±0.10%). This is a consequence of the kinetics of extraction, the first two steps allow to extract 1.10% over a total hexane extract of 1.29%, and 7.54% over the 7.99% of acetone extract, representing 4.15% and 13.78% relative to each extract respectively. Thus it is clear then that the third and fourth steps can make only a slight contribution, and this explains that a fifth step should not be necessary.

Table VII.3: Soxhlet yield vs duration and number of extraction steps

Duration of each extraction step (h)		Step number[a]				Total extractable
		1	2	3	4	
8	Acetone	6.84±0.75	1.22±0.18	-	-	8.06±0.93
	Hexane	0.65±0.08	0.33±0.03	-	-	0.98±0.11
4	Acetone	6.74±0.17	0.80±0.15	0.32±0.15	0.13±0.02	7.99±0.12
	Hexane	0.79±0.19	0.31±0.11	0.12±0.11	0.07±0.04	1.29±0.18

[a] *Percent weight of dry biomass, sample Fr-Oct-09. Mean ± SD based on triplicate of* [a] *Step number*

It should be pointed that these experiments were performed at the start of the study, with a biomass sample from very young guayule plants, thus having a low content of PI, while the percentage of resin was already much higher. This is why above trials were done again with samples provided by the University of Arizona, and later collected in Spain, yielding an hexane extract of 5.2 % and 9.0 % respectively. Tables VII.4 (Az) and VII.5 (Sp), obtained under same extraction conditions, confirm the conclusion from Table VII.3. Also, Table VII.4 and Figure VII.3 corresponding to Table VII.5, shows this tendency of PI to be extracted at a slower rate in comparison to resin, this during steps 1 and 2, while steps 3 and 4 make a very small contribution. For example, the hexane extractable stepwise is 92; 8; ~1; ~0.5 % of total extract respectively for steps 1; 2; 3; 4 respectively (Table VII.4).

In conclusion, in order to stay as close as possible to the maximum of extractable with Soxhlet, it was decided to perform four extraction steps of 4 h each, for both the solvents (10 g of sample). Four steps were kept instead of two of 8 h each, for 16 has total refluxing time in both cases, in an attempt to minimize PI degradation in the collection flask (extract withdrawn after each step).

Table VII.4: Soxhlet extract yield vs number of extraction steps

	Step number a				Total
Yield of extraction	1	2	3	4	extractable
Acetone extract (%)	11.59±0.25	0.93±0.13	0.3±0.10	0.1±0.09	12.92±0.15
% per step relative to total extract	91.52	7.5	0.7	0.4	100
Hexane extract (%)	4.16±0.16	0.8±0.08	0.2±0.11	0.1±0.07	5.26±0.08
% per step relative to total extract	83.93	14.1	1.5	0.6	100

Percent weight of dry biomass, Soxhlet extract 4 steps, 4 h per step, Sample Az-Oct-09

Table VII.5: Soxhlet extract yield vs number of extraction steps

Solvent	Step number[a]				
	1	2	3	4	Total
Acetone	9.75±0.10	0.48±0.02	0.19±0.04	0.21±0.02	10.63±0.11
% per step relative to total extract	91.72	4.52	1.79	1.98	100
Hexane	7.91±0.05	1.06±0.27	0.04±0.04	0.01±0.00	9.01±0.16
% per step relative to total extract	87.79	11.76	0.44	0.11	100

[a]*Percent weight of dry biomass, 4 steps, 4 h per step, Sample Sp-March-10, Mean ± SD on triplicate*

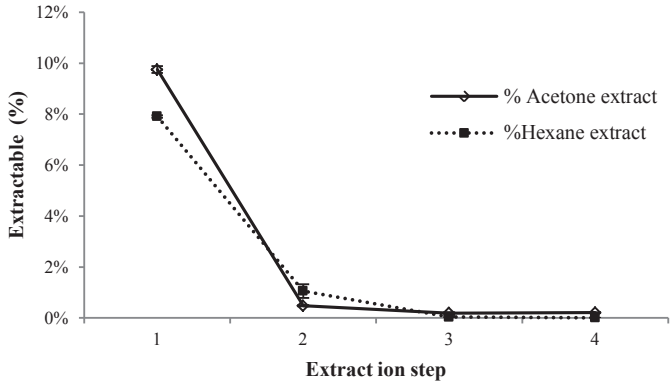


Figure VII.3: Soxhlet yield of hexane and acetone extracts vs step number

(Experimental conditions: see TableVII.5)

VII.5 Optimization of Polytron homogenizer extraction protocol

The number of parameters investigated in the case of the homogenizer protocol included the volume/weight ratio of solvent to powdered sample (mL/g), operation temperature, grinding speed, and duration of each step (number of steps was set at 3 after preliminary trials, results not shown). The temperature is that of the solvent at the start of the trial; it must be kept low enough for avoiding safety problems. Because of this the temperature was limited to 50°C. In order to overcome the problem noted when working with Soxhlet at the beginning laboratory work (low PI content because of very young guayule plants), we used the sample from the University of Arizona (Az-Oct-10). There was no previous expertise at Cirad about extracting rubber with a high speed grinder, therefore several parameters were investigated in preliminary trials, using 100 mL of solvent, 3 extraction steps of 10 minutes each (experiments in triplicate). Although the parameter investigated is the sample to solvent ratio, the volume of solvent was kept constant to avoid disturbing the fluid mechanics between trials (vortex, etc).

Table VII.6 shows that the total of acetone and hexane extractable, based on the sum of three replicates, and increases with sample load, at least 3 g until 12 g. Therefore it would be useful to further investigate this point. From Figure VII.4 derived from Table VII.6 it seems that the third step completes the extraction process. The relatively high SD is similar to that reported by Donald (1984) with Polytron and benzene or hexane (~91).

A new set of experiments was then launched for investigating the main important parameters governing the extraction with Polytron. The results shows in Table VII.7 that, within the range of investigated values, the solvent/sample ratio and the temperature look to be important parameters for extracting with acetone (conditions 1; 2; 8), while the yield with hexane is influenced mainly by the agitation speed (conditions 3; 6). Consequently, the selected conditions are the following: ratio of 10/1 mL/g (10 g sample load), speed 15,000 rpm, temperature 30^{o} and extraction time 12 min per step (3 extraction steps).

Of the three hexane extractions made on each sample, most the hexane extractable (averaging 81%) is removed in the first extraction step. Small sample size provided higher in the first extraction percentages than large size (Table VII.8). Additional improvements in technique have increased that to an average of 89% with the first extraction of 6 g sample, and provide improved extraction percentages. The three extraction steps still remaining ingredients. Indeed, it was extracted not more than three times.

Table VII.6: Influence of sample load on extraction yield with acetone and hexane

Sample load ratio solvent/solid (g/100mL)	Acetone extract (%)[a]	Hexane extract (%)[a]
3	12.39± 0.15	4.130 ± 0.61
6	14.33± 0.32	5.096 ± 0.35
12	11.76± 0.24	5.723 ± 0.42
16	11.77± 0.26	6.379 ± 0.33

[a]*Percent weight of dry biomass. Mean ± SD based on triplicate*

Az-Oct-09, solvent 100 mL, speed 15000 rpm, 30°C, 3 steps of 10 min with each solvent

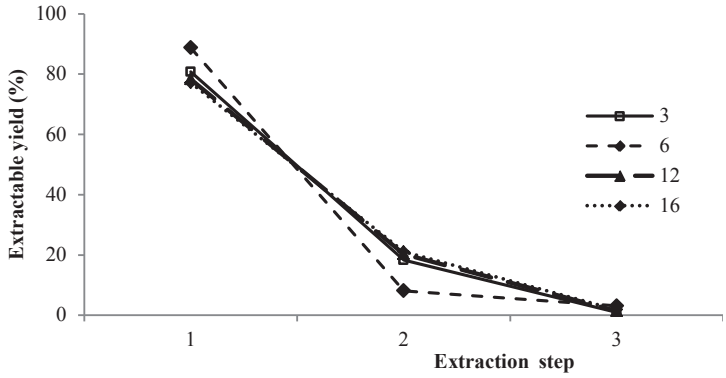


Figure VII.4: Polytron yield of hexane extract vs step number

(Conditions: see Table VII.6; average of data from Table VII.6)

A new set of experiments was then launched for investigating the main important parameter governing the extraction with Polytron. The results shown in Table VII.9 that, within the range of investigated values, the solvent/sample ratio and the temperature look to be important parameters for extracting with acetone (conditions 1, 2, and 8), while the yield with hexane is influenced mainly by the agitation speed (conditions 3 and 6). Consequently, the selected conditions are the following: ratio of 10/1 mL/g (10 g sample load), speed 15,000 rpm, temperature 30^{o}C and extraction time 12 min per step (3 extraction steps).

Table VII.7: Influence of operation parameters on Polytron extract yield

Condition	Ratio*	Speed (rpm)	Temp. (°C)	Step duration (min)	Acetone extract (%)	Hexane extract (%)
1	10/1	15000	40	12	8.65±0.13^{ab}	7.79±0.22^{ab}
2	6/1	15000	40	12	7.73±0.26^{d}	7.38±0.05^{ab}
3	10/1	6000	40	12	8.34±0.07^{bc}	6.93±0.04^{c}
4	10/1	10000	40	12	8.75±0.13^{ab}	7.66±0.04^{ab}
5	10/1	19000	40	12	9.01±0.08^{a}	7.94±0.25^{a}
6	10/1	15000	30	12	8.86±0.05^{ab}	7.70±0.19^{ab}
7	10/1	15000	50	12	8.83±0.29^{ab}	7.83±0.30^{ab}
8	10/1	15000	40	4	8.02±0.20^{cd}	7.26±0.31^{bc}
9	10/1	15000	40	8	8.32±0.42^{bc}	7.52±0.30^{ab}

Sp-March-10; Total extract mean ± SD based on triplicates
**Ratio of solvent per sample weight. Mean ± SD: Standard deviation values are based on triplicates.*
a,b,c,dData same letter within a given column are not statistically different to ANOVA test (p>0.05).

VII.6 Optimization of ASE protocol

By essence this protocol is the most complex from the experimental standpoint, owing to the marked difference with the two previous protocols, i.e. the ability to operate at a temperature higher than the solvent boiling point; also the automated procedure allows to set other parameters like pressure, heating time, purge time, volume of extraction solvent, flush volume, in addition to the obvious parameters like extraction temperature, number of extraction steps and duration (static extraction period). The study was based on data reported by Cornish et al. (1999); Salvucci et al. (2009); Salvucci and Coffelt (2009); Teetor and Ray (2004). Compared to previous options, the results are presented in more details, because this method was then chosen as the reference one at the end of this part of the work. Several experiments were run, like for Soxhlet, some of them with low rubber content biomass; we report here only the results obtained with the highest rubber content.

VII.5.1 Number of extraction steps and extraction time

The first parameter to be checked was the number of static extraction steps; from data in Figure VII.6, the kinetics of the extraction with acetone is slower compared to hexane; indeed the fourth

step does not increment further the yield of hexane extract (Table VII.8) while the extract with acetone is still noticeable at fourth step, although very small. Thus from Figure VII.6 and Table VII.8 we conclude that the extraction can be satisfactory performed with 3 steps lasting 20 min each with acetone, and 25 min each with hexane. This will be used for setting the next trials.

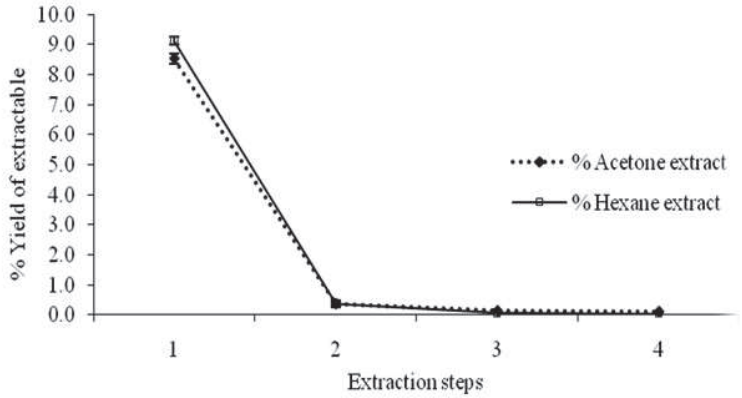


Figure VII.5: ASE yield of acetone and hexane extract vs step number
(Sp-May-10; static extraction time with acetone 20 minutes/ step at 40°C; *static extraction time with hexane 25 min/step performed after the 4th step with acetone, temp.120°C)*

Table VII.8: ASE yield vs step number

Solvent	Step number[a]				Total
	1	2	3	4	extractable
Acetone	8.52±0.17	0.37±0.10	0.13±0.04	0.10±0.01	9.12±0.11
%/ step relative to total extract	93.42	4.06	1.43	1.10	100
Hexane	9.12±0.13	0.36±0.06	0.05±0.01	0.04±0.01	9.57±0.05
%/ step relative to total extract	95.30	3.76	0.52	0.42	100

Conditions: see Figure VII.5; Mean ± SD of samples analyzed in triplicate.

[a]*Percent weight of dry biomass, sample Fr-Oct-09. Mean ± SD based on triplicate*

In addition another run allowed seeing a decrease in Mw at the fourth step, 770.000 instead of ~1060 g/mole (results discussed in last next chapter). This is likely to be due to degradation with time under these adverse thermal conditions (120°C), and does not play in favour of expanding neither duration nor number of steps.

VII.5.2 Solvent for deresination

Wagner et al. (1988) used solvent mixtures for extracting resin. In addition to acetone which is the most common solvent reported for guayule resin recovery (deresination), we checked acetone with 5% of water or methanol (acetone 40ºC, 4 steps, 20 min/step; hexane 60ºC, 4 steps 25 min/step). From Figure VII.6 the "acetone" extract obtained in the presence of water is larger than the extract obtained with the acetone or acetone+ methanol. However, there is no change on the hexane extract within experimental error, and it was decided to stay with pure acetone.

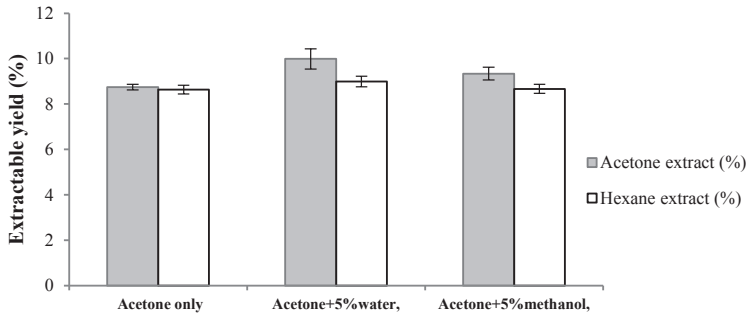


Figure VII.6: Influence of polarity of deresination solvent on extract yield with ASE

(SampleSp-March-10, acetone 40ºC, 4 steps, 20 min/step; hexane 60ºC, 4 steps 25 min/step).

VII.5.3 Effect of extraction temperature

Although obtained with a low rubber biomass (sample Fr-Oct-09), we report here on the first experiment performed at the same temperature for all acetone and hexane steps (sample load 5 g, a step is 20 and 25 minutes, temperature 40°C for acetone and 120°C for hexane, pressure 100 bar, 85% flush with: 40°C, 60°C, 80°C, and 100°C. Although, we noted a permanent increase of total extraction yield for both the solvents in Table VII.9, this lowers the extraction speed, thus corresponding to a decreased efficiency of the first extraction step (this run was performed over 6 extraction steps). While this is clearly apparent for acetone in Figure VII.7, derived from above table, the observed variation stays close to experimental error because of the low rubber content. Still this point out the need of achieving a refined optimization of operating conditions with ASE, given the importance of temperature. Worth noting that above investigated 40-100°C segment does not cover the range reported in literature. In another run we noted a slight decrease of hexane extract at 160°C, while the acetone extract was still increasing.

Table VII.9: ASE yield vs extraction temperature (same for both solvents) ASE yield vs extraction temperature (same for both solvents)

Temperature (°C)	Solvent	Extraction yield (%)	1st step relative to total (% yield)
40	Acetone	5.01 ±0.12	90.0±0.24
	Hexane	0.92±0.09	72.2±0.13
60	Acetone	5.38±0.27	92.7±0.21
	Hexane	1.04±0.10	66.8±0.09
80	Acetone	6.54±0.22	85.5±0.20
	Hexane	1.14±0.08	46.0±0.10
100	Acetone	8.02±0.29	81.2±0.31
	Hexane	1.23±0.11	43.5±0.09

Sample Fr-Oct-09 4 steps, 20 min, temperature 40°C for acetone and 25 min, 120°C for hexane

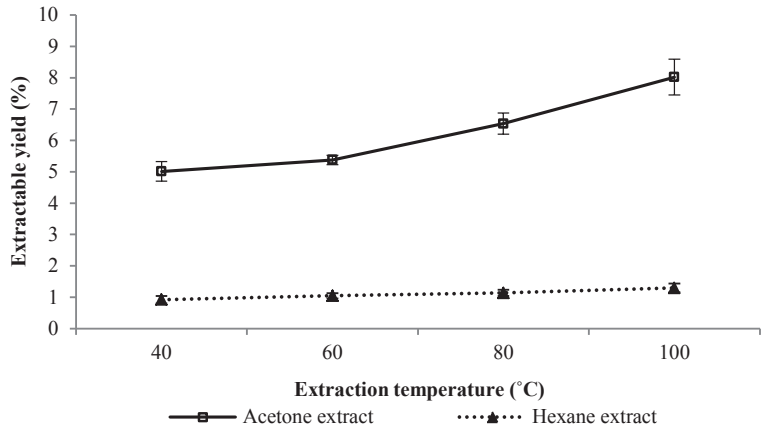


Figure VII.7: Evolution of acetone and hexane extract yield vs temperature

(Conditions: see Table VII.9)

In order to refine the study, runs were then performed while kipping constant one of the solvent temperature. Rath et al. (2009) and Teetor and Ray (2004) having performed ASE at 140°C with hexane, Figure VII.8 shows a strong increase of the efficiency of acetone, associated to similar but negative effect on the hexane extract, while kipping the total acetone+hexane almost constant (give average total with SD). In addition to a possible speed up of the extraction of resin, with temperature during the extraction with acetone, to confirm the extraction of low molecular weight PI, because of degradation, as already noted by Cornish et al. (2013).

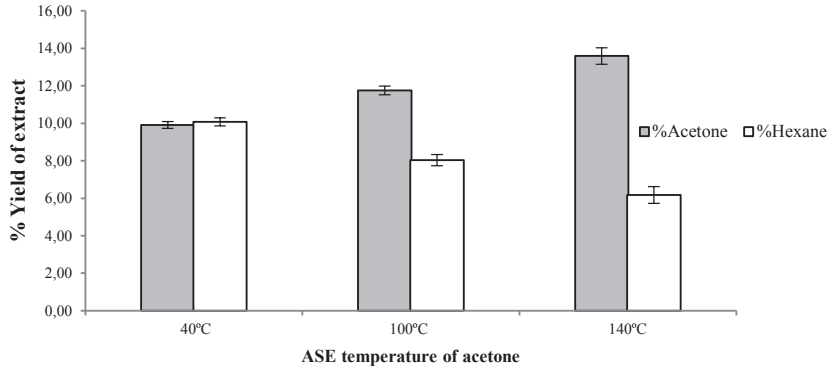


Figure VII.8: Effect of increasing acetone temperature combined to preset hexane temperature at 140°C, with ASE *(Sample Sp-March-10, 4 steps, 20 min/step for acetone, 25 min/step for hexane)*

Finally, above results led to perform another set of trials while varying the two temperatures independently (while kipping all other conditions at set points). Previous results led to kip the acetone temperature in the range 40-64 °C; this to allow setting a higher temperature only for extraction steps with hexane.

On one side, Table VII.10 shows that the hexane extract is maximized in the range of 120-130°C, but under low temperature conditions for the preceding acetone steps (40°C). This confirms above notes, and could be the sign of changes, if not degradation, occurring in the vegetal matrix with acetone, then impeding the yield obtained with hexane. On the other side, the acetone extract is increasing slowly with temperature as expected.

Thus this led to choose between maximizing acetone or hexane steps. Given the aim of the work i.e. to allow fast measurement of rubber in biomass, the conditions selected for the ASE protocol are the ones corresponding to the highest hexane extract yield: performing extraction with acetone at 40°C and with hexane at 120°C. Here again (see previous paragraph), in spite of diverging results regarding optimum temperature for the acetone and hexane extract, it is clear that the sum of both extracts does not vary, within experimental error, possibly benefiting of a kind of compensation between efficiency of the two solvents.

These conditions confirm results published by Pearson et al. (2010); Teetor and Ray (2004). In spite of diverging data regarding optimum temperature for the acetone and hexane extract, it is clear that the sum of both extracts does not vary, within experimental error, bringing a kind of

compensation between efficiency of the two solvents (Table VII.10), but care should be taken to these phenomena when trying to quantify the rubber through the hexane extract.

Table VII.10 ASE yield vs temperature

Temperature acetone steps (°C)	Total acetone extract (%)	Temperature hexane steps (°C)	Total hexane extract (%)	Total Acetone + hexane (%)
40	9.30±0.04^{c}	120	9.05±0.06^{a}	18.34±0.10^{abc}
	9.35±0.03^{c}	125	8.94±0.17^{ab}	18.29±0.14^{bc}
	9.39±0.01^{c}	130	8.85±0.19^{ab}	18.24±0.19^{b}
50	9.81±0.03^{b}	118	8.55±0.17^{abcd}	18.35±0.16^{abc}
	9.48±0.05^{c}	125	8.51±0.13^{bcd}	17.98±0.12^{bcd}
	9.85±0.13^{b}	130	8.65±0.20^{abc}	18.50±0.31^{a}
60	9.96±0.08^{a}	120	7.93±0.20^{e}	17.89±0.28^{e}
	9.91±0.06^{a}	130	8.27±0.17^{cde}	18.18±0.11^{bcd}
64	10.32±0.21^{a}	125	8.62±0.05^{abc}	18.94±0.25^{a}

[a, b] Data bearing same letter within a given column are not considered statistically different according to ANOVA test (p>0.05).Mean ± SD: Standard deviation values are mean of samples analysed in triplicate.
Sample from Sp-March-10 by ASE 3 steps 20 and 25 minutes 40°C for acetone and 120°C for hexane, pressure 100 bar, 85% flush

In conclusion about optimizing ASE, the extraction can be satisfactory performed with 3 steps lasting 20 min each with acetone, and 25 min each with hexane, the fourth steps accounting for a negligible amount of extract, falling within the standard deviation found on the total extract. Therefore, the optimal chosen protocol uses 40°C with acetone and 120°C with hexane. Also, Rath et al. (2007); Salvucci et al. (2009); Teetor and Ray (2004) have been to used 2-3 extraction steps and extraction time in the range 16-25 min per step, and the following temperature range, 24-100°C with acetone and 40-140°C with hexane (or another solvent), although the optimization was less detailed than in the present chapter.

VII.9 Comparison of the three selected methods under optimized conditions

The parameters maximizing the hexane extract were as follows, respectively for Soxhlet, Polytron and ASE:

- Soxhlet: 10 g sample load, 4 extraction steps of 4 h per step with each solvent, temperature (boiling point, 56°C for acetone and 68°C for hexane).
- Polytron: 5 g sample load, 3 extraction steps of 12 min per step with each solvent 100 mL and solvent/biomass weight ratio of 10/1 mL/g, speed 15,000 rpm, at temperature 30°C.
- ASE: sample load 5 g, a step is 20 and 25 minutes at temperature 40°C for acetone and 120°C for hexane, pressure 100 bar, 85% flush with nitrogen.

The selected operating conditions for the three above protocols were then applied to the same batch of guayule biomass (Table VII.11; Sp-March-10).

The highest SD is found when using the Polytron protocol, both with acetone and hexane extraction, whereas Soxhlet is the most reproducible one (lowest SD). Regarding the first part of extraction, with acetone, ASE and Soxhlet yield the same results, while Polytron is less efficient. But the three methods yield the same percent of hexane extract within the experimental error (Multiple comparisons using the Newman and Keuls (SNK) Test at 5%). With regard of the large number of publications on the topic (guayule), only a few authors did compare several extraction methods for processing various biological samples (Kim, 2009; Saito et al., 2004; Zhuang et al., 2004), including guayule (Salvucci et al., 2009; Teetor and Ray, 2004), and also concluded to the high efficiency of the ASE method.

In Table VII.11, we note that the highest SD is given by the Polytron protocol whatever the solvent, whereas Soxhlet is the most reproducible protocol. Regarding the first part of extraction, with acetone, ASE and Soxhlet yield the same results, while Polytron is less efficient. But the three methods yield the same percent of hexane extract within the experimental error (ANOVA test at risk level of 5%). In addition, we found a lower Mw for the PI extracted with Polytron, in comparison to ASE and Soxhlet (about 180.000 and 250.000 g/mole respectively).

Moreover, ASE provides known advantages, automatic operation, low solvent consumption (2 L; 600 mL; 600 mL respectively for Soxhlet, Polytron, ASE), higher safety (closed stainless steel vessels) and less time consuming (extraction steps only: 4 days; ~1.5 h; 2.5 h for Soxhlet, Polytron, ASE respectively), than the two other protocols. Still the efficiency for hexane extract of the high speed homogenizer should be pointed out, thanks to the mechanical action, in spite of the much lower operating temperature (40°C vs 120°C for hexane steps with ASE), and it could be useful as a soft method for investigating the rubber structure (under specific conditions to be determined).

Other authors did compare several extraction methods recently, for processing various biological samples (Kim, 2009; Saito, 2004; Zhuang et al 2004), including guayule (Salvucci and Coffelt, 2009; Teetor and Ray, 2004), and also concluded to the high efficiency of the ASE method.
Therefore, based all on above arguments, ASE was retained as the reference method for NIRS calibration.

Table VII.11 Comparison of acetone and hexane extract yield obtained by 3 optimized methods

Extraction method	Acetone extract (%)	Hexane extract (%)	Total extract (%)
Soxhlet[b]	10.63 ±0.10[d]	9.01 ±0.16[d]	19.64 ± 0.23[d]
Polytron[c]	8.71 ±0.70[e]	9.23 ±0.67[d]	17.94 ±0.58[e]
ASE[a]	10.56 ± 0.41[d]	9.05 ±0.30[d]	19.61 ± 0.53[d]

[a] *ASE: 3 extraction steps; acetone extract 40°C, 20 min, and hexane extract at 120°C, 25 min.*
[b] *Soxhlet: 4 extraction steps and 4 hours per steps and refresh solvent each step.*
[c] *Polytron: 3 extraction steps, 12 minute per steps, ratio of solvent per sample weight 10/1 mL/g, and speed 15,000 rpm, 30°C.*
[d, e] *Data bearing same letter within a given column are not considered statistically different according to ANOVA test (p>0.05).*
Mean ±Standard deviation values are expressed as mean of samples in triplicate.
Sample Sp-March-10

VII.10 Conclusion of Chapter VII

This first part of the thesis focused on the development of a reliable method for determining rubber content in guayule biomass for use as reference method to calibrate the NIRS method. The gravimetry, widely used in the literature, was selected as a simple way for quantifying the targeted components, resin and PI, and the above work was devoted to selecting the best extraction conditions with the dual solvent option (acetone, then hexane), prior to comparing the three selected techniques, Soxhlet, Polytron and ASE. Soxhlet is a standard option; high speed homogenizer Polytron, could be potentially well adapted to the fact that rubber is located inside the guayule cells; ASE is a modern technique.
In spite of early samples from the EU-Pearls project having low rubber content (~1 %), the work was developed also with samples from Arizona, and later the results could be confirmed with

older samples from the project, containing up to ~10% of rubber, thereby minimizing experimental error.

The preliminary investigation of the main experimental parameters showed the very high influence of temperature on extract yield, not only because of the complex structure of the vegetal matrix, but also more specifically, because of the low PI stability which causes the degradation and, incidently, while the total (acetone + hexane) extract remained constant, the acetone extract increased symmetrically with the decrease of the hexane extract. These bring the question about the selectivity that will be handled in next chapters.

These chemical and biological features complicates the optimization of the experimental conditions for maximizing both resin and PI extracts in the same run, and led to choose to maximize the hexane extract in priority, given the applied purpose of the PhD aim, ie to provide a quick method for rubber quantification, to be used by agronomist partners as soon as possible (by mid-term of the project).

The above work allowed comparing the results obtained by the three selected techniques, in terms of gravimetric yield of acetone and hexane extracts (expressed as % relative to the dry weight of starting biomass) considered to be representative of resin and rubber content in the literature. Regarding the yield of hexane extract, the three methods gave results not statistically different. The choice of ASE was based on practical arguments: safety, lo solvent consumption, quick extraction time and consumption of operator's time (automated), availability of suitable equipment in all partners' laboratories. Also Polytron gave significantly lower yield of acetone extract. ASE allows processing 9 guayule samples in triplicate per day (24 h).

Being a relatively new technique, ASE was never reported as reference method for NIRS calibration with guayule.

Chapter VIII

Assessment of cross contamination of acetone and hexane extracts; based on PI and resin content

VIII.1 Introduction

The preceding Chapter VII resulted in setting-up a reliable quick method based on NIRS, for quantifying acetone and hexane extracts, then transferred to agronomists and breeders within the EU-Pearls project, as a contractual deliverable.

However the question coming from the bibliographical synthesis (Chapter II) about the selectivity of these solvents towards the extraction of some PI with acetone, and of resin with hexane, is still pending, although the assimilation of the gravimetric yields to resin and PI respectively be widely used (Black et al., 1983; Cornish et al., 2013).

This chapter deals with the following:

- Selecting analysis methods for chemical detection of resin and PI in both the extracts
- Checking the presence of contaminating fractions and estimating the extent of contamination
- Setting methods for quantifying the contaminant, accurate enough for the purpose (IR, SEC-MALS)
- Building the calibration lines
- Applying the methods to further optimize the ASE reference methods, and compare to Soxhlet, Polytron
- Build a new calibration model again with the same NIRS results (NIRS2), this time based on "real" PI and resin content.

The aim of quantifying the resin and PI content in guayule extracts, is to come as close as possible to the real resin and PI content in the biomass.

VIII.2 Bibliographical synthesis about the cross contamination of extracts

Only a few publications deal with this topic. Black et al. (1982) checked the extracts for cross contamination by ^{13}C NMR. The resin fraction was found to contain a small percentage of low MW PI in the acetone-soluble extract analysed by SEC, in the range of 14,000 g/mole, subsequently identified by IR. Such low Mw acetone-soluble rubber has been reported by other workers (Meeks et al., 1947; 1951). However, for the rubber fraction, NMR analysis yielded δ values agreeing with typical isoprenoid carbon structure, and within the limit of the detection, the analysis did not indicate the presence of resinous contaminants. Their results indicate that degradation of high MW PI in hot solvent with Soxhlet extraction might be due to oxidation; PI was still present in fractions obtained at 48 hours, being continuously degraded. Also, Cornish et

al. (2013) reported that the amount of contaminating substances can be higher when the plant is actively growing. They speculate that above the bulk resin melting temperature (~60^{o}C) the resin melt in the ground material, allowing it to flow and disperse over the lignocellulosic fine particles as a thin film. Under this conformation, the resin is faster to extract during ASE steps than under the initial form (un-heated biomass). The ASE acetone extraction temperature used in these experiments was 100^{o}C. However, then the Mw of the extracted PI decreased by 26% by thermodegradation into acetone soluble. Since the ASE is performed under nitrogen, this degradation can be attributed to thermodegradation, whereas the rubber loss in the pre-extraction drying oven may be caused by a combination of oxidation and thermal degradation.

The biomass structure may not help the selectivity. Backhaus and Nakayama, 1986) indicated high-MW PI is present only in the extract from the stem, thus olther parts containing low MW acetone soluble PI, or close to be so, might contribute to finding PI in acetoine extract. Ray et al. (2009), noted that the leaves and flowers have essentially no rubber; this is why we routinely discard them before analysis. Also, they found rubber content by hexane extract then in hexane extract have something inside not only rubber, may be the polution in hexane extraction or contaminated in hexane or may be has the low Mw PI contaminated in acetone extract, be expecting at the high temperature of acetone extracted also in drying process avoided of PI degradation in guayule biomass.

From above and from our first results (Chapter VII), need to recall that the acetone extraction temperature with ASE is 40^{o}C for more accurate resin and rubber quantification-in alignment with the 50^{o}C drying temperature indicated in the present study. However it should be noted that although sample preparation and resin extraction is performed at relatively low temperature, the extraction of PI with hexane or cyclohexane, is performed at a much higher temperature, 140^{o}C and 120°C for Cornish et al (2013) and present work, respectively. This, to maximized the extract yield, in an attempt to quantify PI. Therefore we might expect some degradation of PI during these last steps, but so far this as not reported with ASE.

It should be noted that, while the gravimetric method allows obtaining real extract for further analysis, because of the pending question about the selectivity of the solvents for the sequential extraction option, the solvent-free extract might be free of contaminant; the extent of which could affect the accuracy of the method. But there is no certitude neither with the turbidity of the liquid medium (photometry).

This is not the case of methods like NMR, and IR spectroscopy on films for example, then measuring signals which can be attributed with certitude to the targeted compounds.
Therefore all above methods do not allow neither the same accuracy, nor the full quantification of the total rubber content, because calibration might not be fully or properly achieved, the method optimized, nor the results compared to those provided by other methods, This applies to the gravimetric method proposed in the preceding chapter, not only because some PI might not be extracted at end of process, but also if the resin extracting steps take some low MW PI.
Thus, contrary to what is generally found in the literature, we consider absolutely required to assess the purity of the extract, i. e. to check and quantify the presence of PI in the resin extracts and of resins in the PI extracts. All this is linked to the selectivity of extracting method.

VIII.3 Detection of cross contamination in acetone and hexane extracts

Chiefly two methods have been used to achieve this goal, by SEC-MALS and by FTIR spectroscopy. SEC-MALS is derived from the analysis of PI MW. IR was used by Banigan et al. (1981) and Bultman et al. (1998), to estimate PI in vegetal extracts. The last used polystyrene as internal standard. In both cases we checked separately several parameters to access an acceptable calibration curve. As detailed in Part 2, SEC-MALS was coupled to two detectors, differential refractive index (dRI) and light scattering (LS11). Note that, molecular structure of PI extracted by hexane was identified by ^{1}H-NMR and ^{13}C-NMR (results in Chapter X).

VIII.3.1 Detection of cross contamination by SEC-MALS

The SEC-MALS derives from the analysis of PI molecular weight. Figure VIII.1 shows the acetone and hexane extracts, and standard PI. The peaks at 28 to ~41 minutes and above 41 minutes correspond to polyisoprene (compared to a standard) and resin; the last one shows several shoulders. Figure VIII.2-left shows even more clearly the complex composition of another acetone extract. The PI standard of Mw ~4400 g/mole let think that this extract may contain low MW PI. The hexane extract in Figure VIII.2-right shows a peak centered at 44-45 minutes, just like the one in Figure VIII.1, thus corresponding to resin. This makes sense because the MW of resin components is less than 1000 g/mole (Chapter I). Figure VIII.3 allows better detection of the contaminant high MW PI, when increasing the concentration of PI standard in

the injected solution. Last, adding increasing amounts of acetone extract in to PI solution, induce a clear increase of peak height.

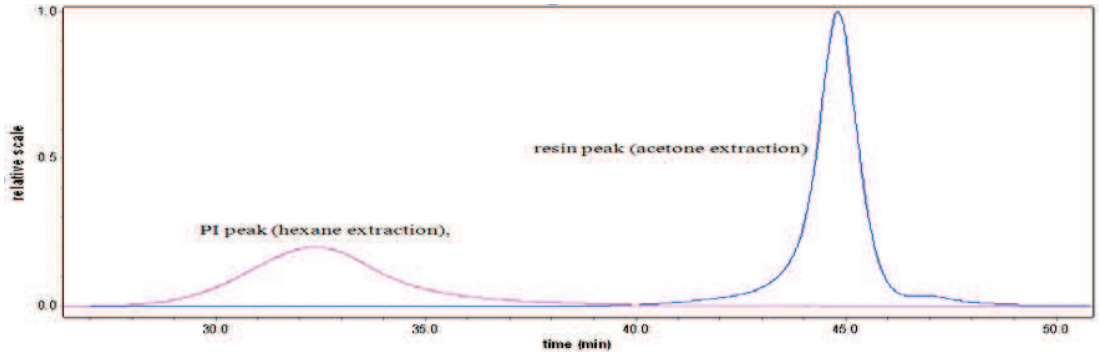
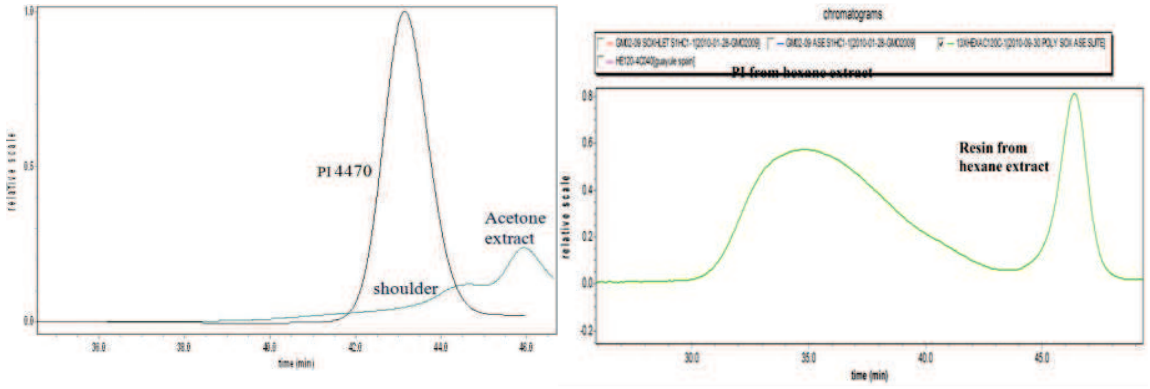


Figure VIII.1: Example of SEC-MALS chromatograms by Rayleigh ratio of acetone extract and PI standard, blue and pink lines respectively



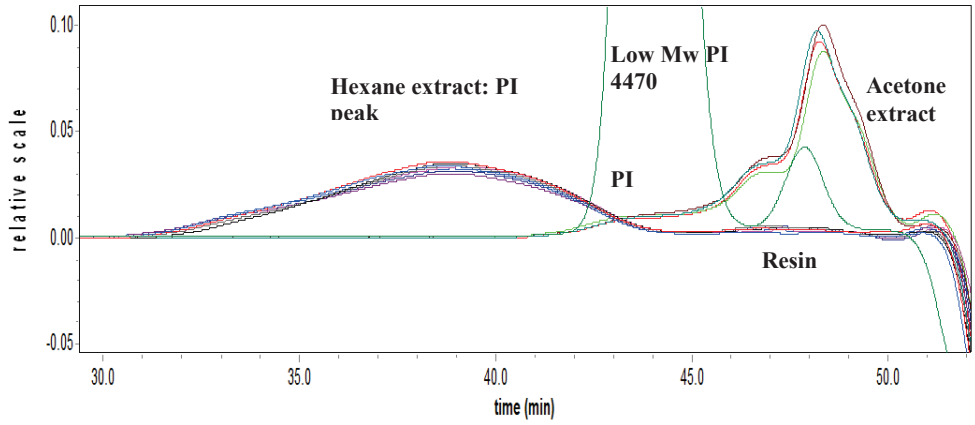


Figure VIII.2: SEC-MALS chromatograms; (a) acetone extract and of a low MW PI standard, (b) hexane extract by dRI detector, button, (c) compare of low *Mw* PI with both extracts

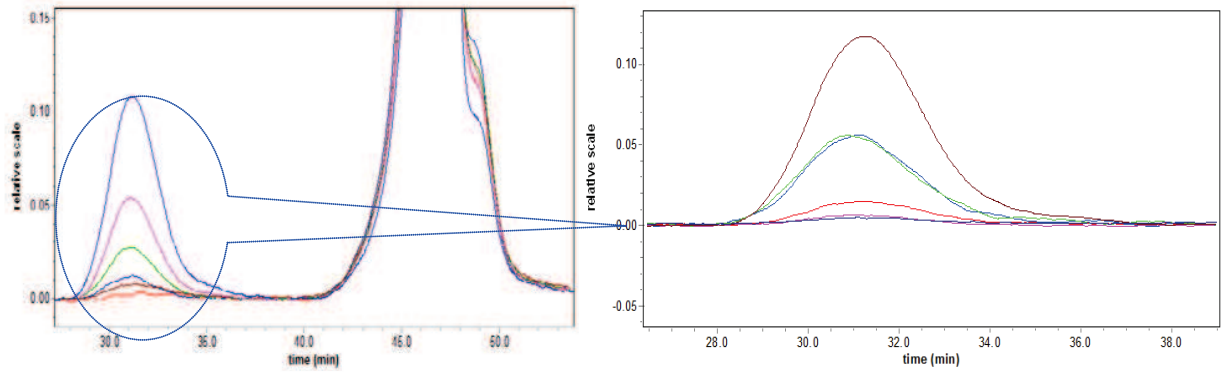


Figure VIII.3: SEC-MALS chromatograms of acetone extract with increasing concentration of amounts of PI standard in the injected solution

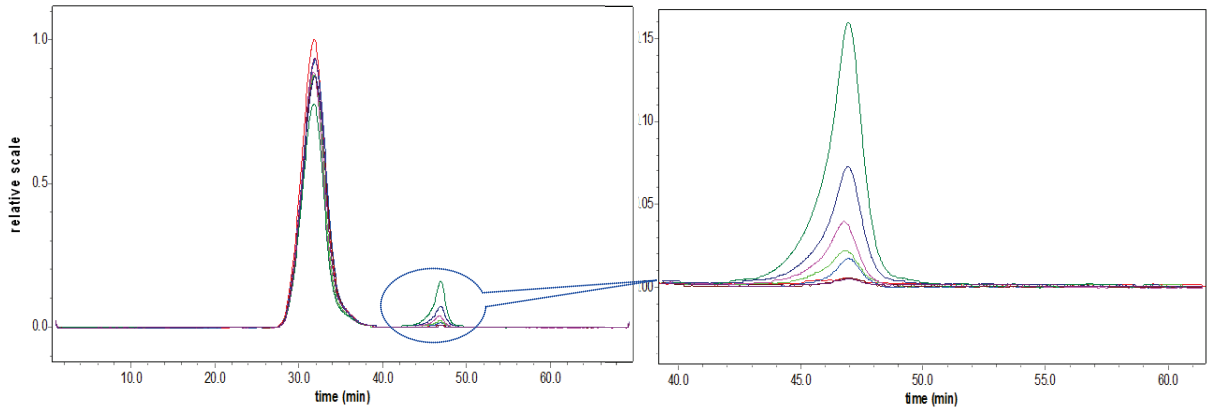


Figure VIII.4: SEC-MALS chromatogram of hexane extract doped with increasing amounts of resin added to the injected solution

In addition to injecting very low *Mw* standard PI (see above), to help attributing peaks to PI or to resin (main component of extract or contaminant), we collected 4 fractions in the critical area of the SEC-MALS chromatogram of an acetone extract (relative vs times of corresponding to the fractions at 4 times). Coupled to FTIR, and NMR this trial confirmed the presence of PI and resin as main component in fraction 30-45, and 45-50 minutes respectively, but only as traces in fraction having a larger retention time. On the other side, FTIR control showed the presence of C=C and C=O groups in fractions 830-840 and 1700-1730 cm^{-1} but not in fractions with a lower retention time.

Although still leaving some error because of this sharp cutting of the chromatogram, these results should allow a better quantification of the contamination in acetone extract.

In conclusion, SEC-MALS shows contamination of resin by low *Mw* PI in acetone extract, but not high PI in this extract although we could check that detection was possible. A symmetric situation is found for hexane extract, containing a distinct peak of low *Mw* component, possibly resin. The nature of the contaminant needs confirmation.

TableVIII.1: Four fractions and times

Acetone extraction at	Fraction at retention time (min)			
	1	2	3	4
Start point	40	43	45	47.5
Stop point	43	45	47.5	50.4

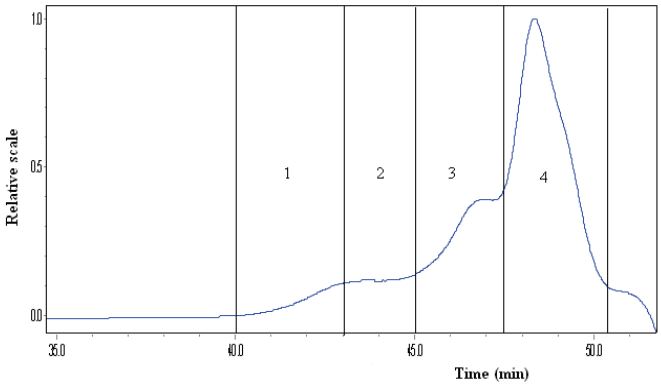


Figure VIII.5: SEC-MALS chromatogram of hexane extract separate the 4 relative times after injected

VIII.3.2 Detection of cross contamination by FTIR

The recording of FTIR spectra of guayule (Figure VIII.6) allowed to detect C=O of resin and lipids specify which vibration mode band (s) (1700-1730 cm^{-1}) as a principal group in acetone extract, according Buchanan (1981) used and infrared method for estimating rubber in plants, peak heights are measured at 1710-1730 cm^{-1} for resin and 830-840 cm^{-1} for rubber. And Grinberg and Shaubi (1985) to studied infrared spectroscopy of the solution indicated the absence of OH and C=O groups due to resin components, also Verbiscar et al. (1989) reported the absorption of C=O was resin at 1700 cm^{-1} and C=O, OH at 1640, 3200 cm^{-1}. And also as minor component in hexane extract; this is, based on the fact that pure PI does not have such a band. Similarly a strong band characteristic of cis-1, 4-polyisoprene at 837cm^{-1} (Banigan et al., 1981; Black et al., 1983) and 830-840 cm^{-1} (Buchanan (1981) was found as main group in hexane extract, and possibly in an acetone extract, as a minor group, due to the presence of other small bands in the region. However such a band becomes clearly visible when PI is added to a hexane extract (Figure VIII.7-left), at a ratio representing 16% of the extract in this example. From this it seems that FTIR is not suitable for detecting. Conversely, Figure VIII.7-right a real flat baseline the C=O region, bringing suitable conditions for detecting resin in hexane extracts.

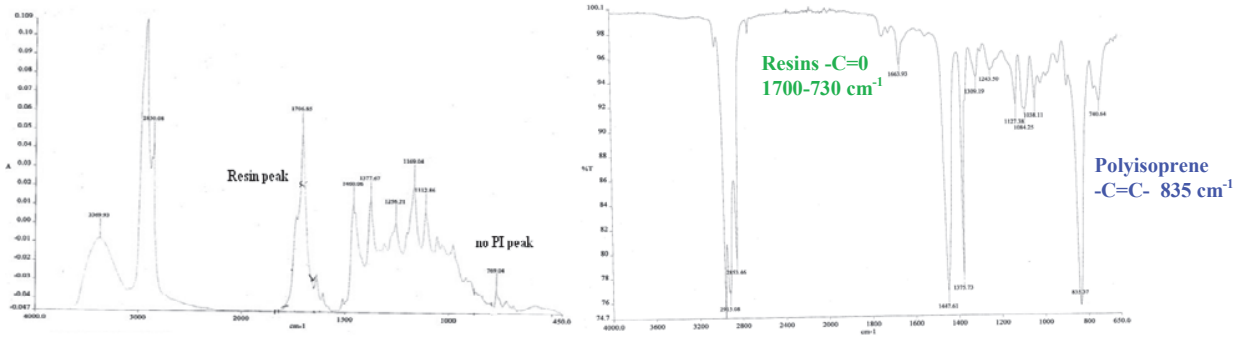


Figure VIII.6: FTIR spectra of acetone (left) and hexane (right) extracts from guayule

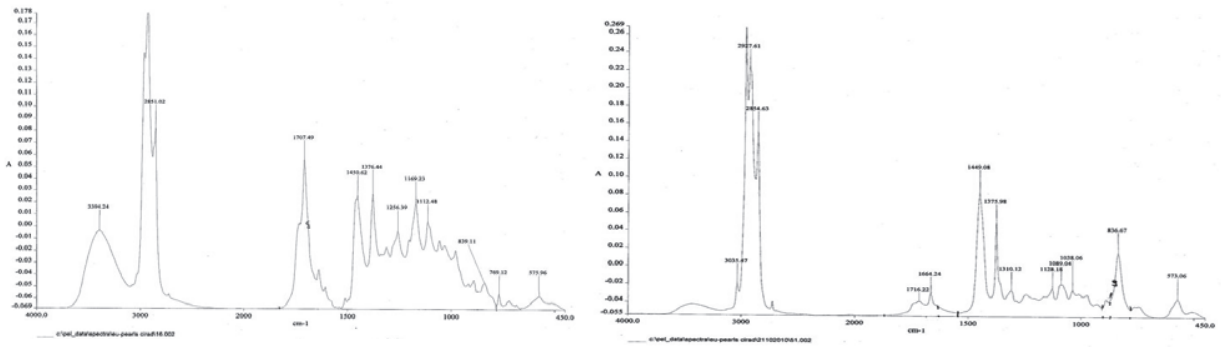


Figure VIII.7: FTIR spectra of acetone (left) and hexane (right) extracts from guayule
(Both 1% extracts in $CHCl_3$ solution Sp-21-10-10)

Characteristics studies by FTIR absorbent bands in acetone extraction, they have limited to detecting PI peak because we cannot looking the PI peak at the PI position of acetone extract on guayule biomass (Figure VIII.8). (Found resin peak but no visible PI peak in acetone extract).

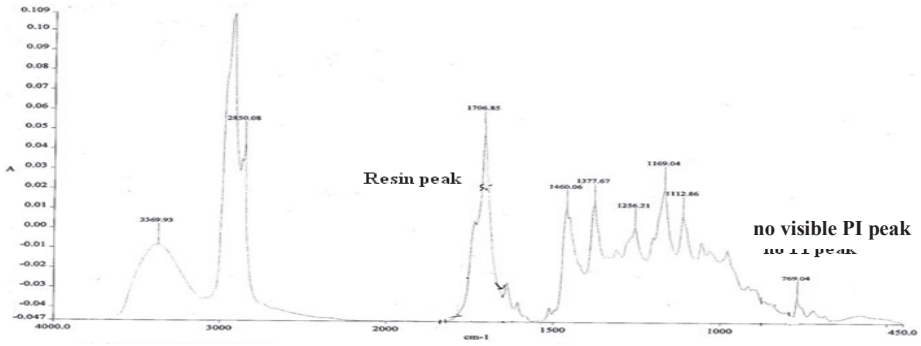


Figure VII.8: FTIR absorbent band characteristic of acetone extracts
(1% Sample Sp-10-10 in $CHCl_3$ solution)

While, to adding some of PI solution into acetone solution found that the band had PI peak at 839 cm^{-1}, however it is a small amount of PI peak (Figure VIII.9). This research was interesting to build the standard curve various the PI concentration solution; the PI concentration between 0-16% for determine the peak area of PI and used that to finding the contaminating of PI in acetone extraction in the pollution section.

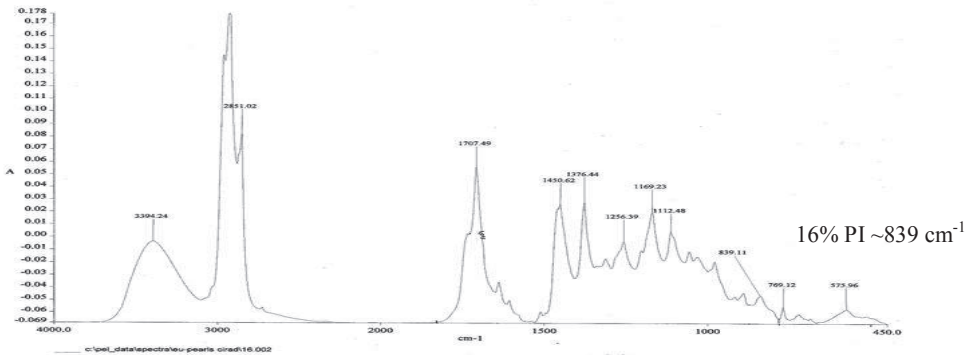


Figure VII.9: PI peak at 839 cm^{-1} while 16 %PI added in resin acetone extract solution

- Hexane extraction

The characteristics studies by FTIR absorbent bands of PI (Figure VIII.10) shown they have not resin peak (about 1710 cm^{-1}). It is made known PI peak at 836.63 cm^{-1} according the reference cis-1, 4-polyisoprene (PI) (Banigan et al., 1981).

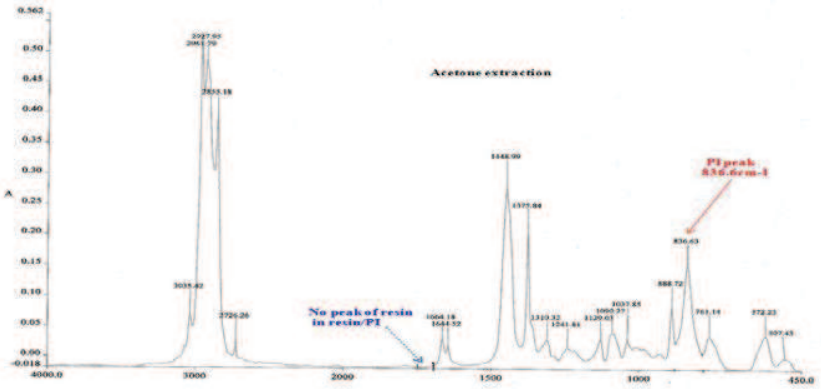


Figure VIII.10: FTIR absorbent band characteristic of PI solution

Whereas, to adding some part of resin solution into PI solution, found that the FTIR band had resin peak at 1716.22 cm^{-1}, conversely it is a small amount of resin peak (Figure VIII.11).

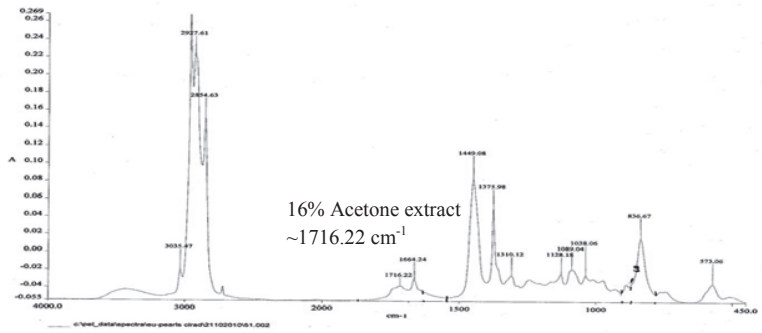


Figure VIII.11: FTIR band of 16% resin adding in PI solution found the resin peak at 1716 cm^{-1}

In conclusion, we confirm that there is some contamination in both the extracts. Therefore, it would be very useful to quantify these contaminants, in order to take them into account when calibrating the NIRS method.

VIII.4 Optimization of FTIR and SEC-MALS protocols to quantify contaminants

The above results indicate that FTIR and SEC-MALS could be used not only for detecting the contamination of extracts, but possibly to quantify these contaminants, or in other words to access the purity of the main class supposed to be extracted by each of the two solvents. This section deals with checking the influence of some important parameters in view of building calibration lines.

VIII.4.1 Preparation of the solutions of extracts

After the extraction steps with acetone or hexane, the solvent is evaporated, and the extract dried until constant weight prior to weighting. This provides the data for the gravimetric determination of acetone and hexane extracts. In the course of the present Chapter, aiming at obtaining the purity of the main component in the extract, the dry extract must be solubilize again prior to FTIR or SEC-MALS analysis. However, we noted that it was impossible to achieve this goal when trying to dissolve again each extract in the same solvent used for the extraction. In fact, there were particles sticking on the glass wall, even after one day of stirring. This might be due to physicochemical, if not chemical, changes given the low stability of some components, PI and PI particles, and resin components, highly sensitive to oxidation. Thus several trials were done in order to check the completeness of the transfer of the extract solution in THF, the solvent for

injection for SEC-MALS, and in chloroform for IR analysis. The result showing that the transfer of the extract is complete in the most relevant cases of THF of acetone extract (residual difference weight 0.0020±0.0003%), and chloroform of hexane extract (residual difference weight 0.0017±0.0002%), in terms of weight of the flask used for evaporating the extraction solvent (initial weight of empty flask and weight of flask after transfer of the new solution).

TableVIII.2: Efficiency of dissolution of acetone and hexane extract prior to analysis sample

Solvent; ASE	Gravimetry (%dry weight)	Solvent solution (mL)	Difference weight (% dry weight)
Acetone	8.34±0.13	THF	0.0020±0.0003
Hexane	6.34±0.08	$CHCl_3$	0.0017±0.0002

(Sample Sp-May-10)

VIII.4.2 FTIR protocol

The analysis was made on films of extract formed by casting the chloroform solution on a KBr plate. The first step was devoted to evaluating the number of drops for FTIR analysis; there was no difference on the result based on the resin/PI ratio in terms of peak area (Table VIII.3). The SD was highly improved when deposing 4 or 8 drops, and thus 4 drops were chosen for the rest of the study.

Table VIII.3: Influence of drop number on KBr plate for FTIR analysis

Drop No.	Resin Area	PI area	Ratio of resin/PI (Avg.)
2	0.18±0.10	5.02±0.16	0.04±0.13
3	0.20±0.13	4.83±0.20	0.04±0.16
4	0.25±0.09	5.04±0.08	0.05±0.08
8	0.27±0.11	5.05±0.14	0.05±0.12

(Sample Fr–May-10, acetone extract /PI ratios in $CHCl_3$ solution)

The effect of the concentration of resin or PI was also tested, as well as the resin expressed using the ratio of peak area. The concentration was limited to 1% of PI or acetone or hexane in chloroform ($CHCl_3$) because of limited solubility (3 days at room temperature). The ratio of resin/PI (g/L) was set at 5% to 50% (Figure VIII.3). The ratio of peak areas was chosen

because the principle to determine of concentration of sample by IR peak areas (IR hand book). Thus the protocol for FTIR analysis of resin in PI can be summarized as follows: the acetone and hexane extracts by ASE and PI synthetic. Each sample was dissolve in chloroform solvent with the concentration as 1%, storage 3 days for complete dissolve solution. For a better repeatability, three solutions were prepared for every samples and each solution was the infrared electromagnetic spectrum scanning once. The value of average is the mean of the three spectrums for every sample. Followed by, detecting PI or resin peaks at the PI and resin absorbent positions. Determine the ratio of resin/PI peak areas and the concentration of resin/PI: that in the first we need percent added in solution and then calculate to concentration in g/L for determine the amount of contaminate using the calibration equation.

The ratio of peak areas was chosen because the principle to determine of concentration of sample by IR peak areas and the concentration of Resin/PI: that in the first we need percent added in solution and then calculate to concentration in g/L for determine the amount of contaminate using the calibration equation. I can show the table for calculated.

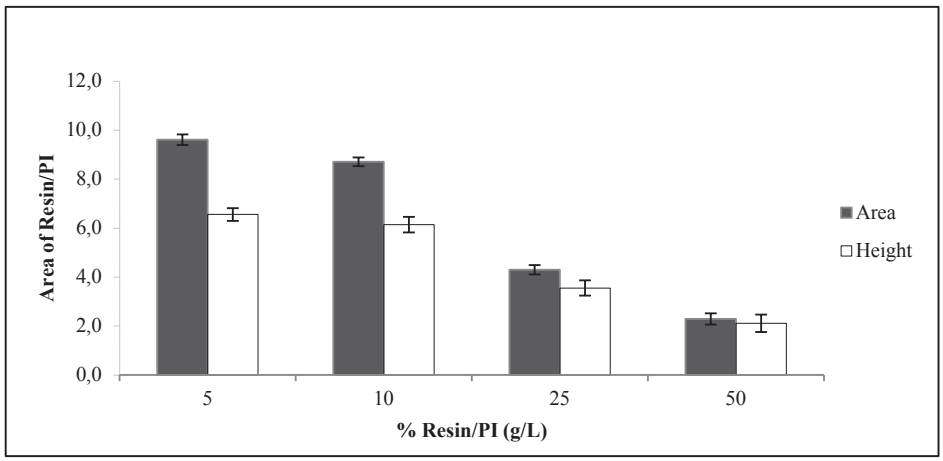


Figure VIII.12: IR response for various resin/PI ratios in the solution

(Sample Fr–May-10, acetone extract /PI ratios in $CHCl_3$ solution)

Then, following above protocol, the results were investigated over the range from 0% of PI to pure PI, resin making the balance to 100% in the prepared standard solution. "%" refers to the weight percent of PI in the total (resin plus PI) prepared standard mixture, to simulate a

contamination of PI in acetone extract for example. From Table VIII.4, we see that the area ratio of PI/resin increases with % PI. In fact the contamination of low MW PI in acetone extract does not overpass 50% (Black et al. (1983); Cornish et al. (2013). Same figure for resin in hexane extract (Cornish et al., 2013). In fact, we determine resin contaminate in hexane extract not so much not more than 26% of low Mw of PI contaminated but for resin contaminate was less than PI contaminate (Black et al. (1983); Cornish et al. (2013).

Table VIII.4: Influence of acetone percent to various PI/resin ratios

PI concentration (%)	IR ratio PI/resin
5	9.61±0.23
10	8.71±0.39
25	4.29±0.02
50	2.29±0.07

(Sample Fr–June-10, acetone extract /PI ratios in $CHCl_3$ solution)

VIII.4.3 SEC-MALS protocol

A simple calibration experiment was launched with SEC-MALS, with two standard samples of PI, 1,896,000 and 4,470 g/mole, and each entire calibration run was triplicated (Figure VIII.13).

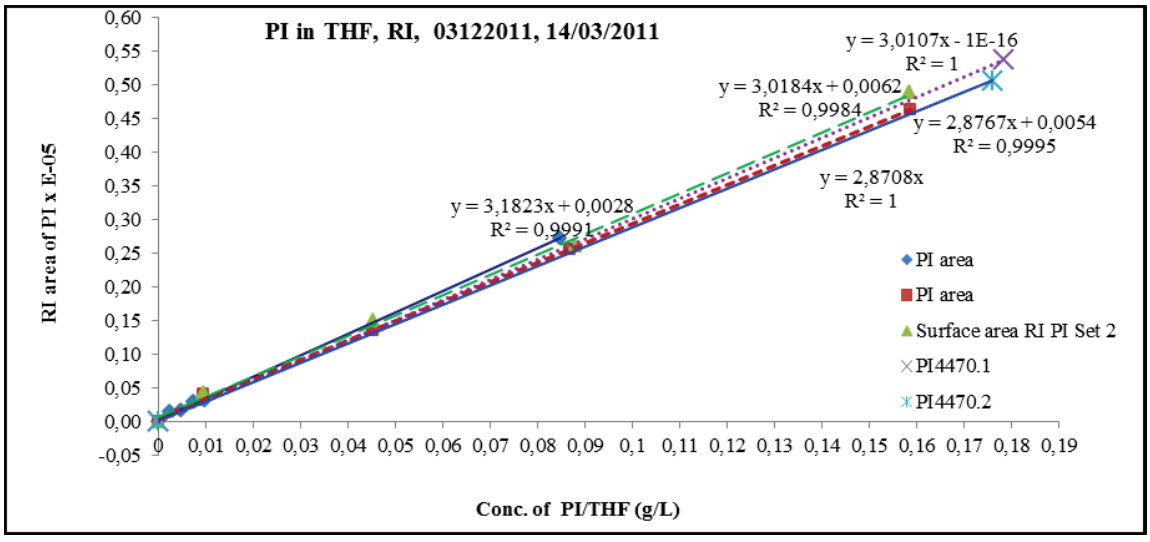


Figure VIII.13: Response of SEC-MALS (dRI detector) with standard solutions of PI having low or high MW

For high Mw PI $y_1 = 3.1823x + 0.0028$, $R^2 = 0.9991$; $y_2 = 3.0184x + 0.0062$, $R^2 = 0.9984$; $y_3 = 2.8708x$, $R^2 = 1.000$, with coefficient averages of a =3.02±0.16, b=0.003±0.003. For low Mw PI, found the lines are very also close ($R^2 = 0.9995$), with average coefficients of a=2.97±0.08, and b=0.005±0.00. From this we can quantify both low and high Mw PI, respectively in acetone and hexane extracts, whatever the MW of PI used for calibration. This experiment also shows a good repeatability of the measurement.

Figure VIII.5 that, shows the low and high Mw PI gave no different of calibration, the red and blue line that used the high Mw PI and other were low Mw PI for calibration and you want to know how are the effect of low or high Mw PI standard to the calibration. Then, conclusion was it has not effect of low or high Mw PI.

VIII.5 Set-up of calibration equations for contaminants in acetone and hexane extracts

The very encouraging preceding results led to set calibration lines for quantifying the cross contamination of obtained extracts, by resin in PI and PI in resin as a first aim, then to be used for calibrating NIRS methods, together with gravimetric data already detailed in Chapter VII. Calibration lines were performed in triplicate, but in addition, each individual analytical point (concentration) was performed several times (x injections in HPLC and y determinations for separate deposits on KBr cell plate for FTIR). In addition several biomass samples were also checked.

VIII.5.1 Calibration for analysis of PI in acetone extract

VIII.5.1.1 PI in acetone extract by IR

As said in VIII.4, the FTIR analysis is based on the ratio of analyzed compounds, here PI/resin, and the result shows an acceptable straight line only at 4 % and above (Figure VIII.14), because of a lack of linearity at lower concentration. The whole calibration was performed four times over a period of 6 months, and “a” coefficient (the slope) varied from 0.67±0.10 to 1.37±0.11, thus showing a large variation; moreover R^2 was quite low in some cases (0.87). Therefore this protocol was not selected. However it could be used for high contamination, the linearity being noted until a concentration equivalent to 25 % of PI in acetone extract, which is the point on the far right of the line.

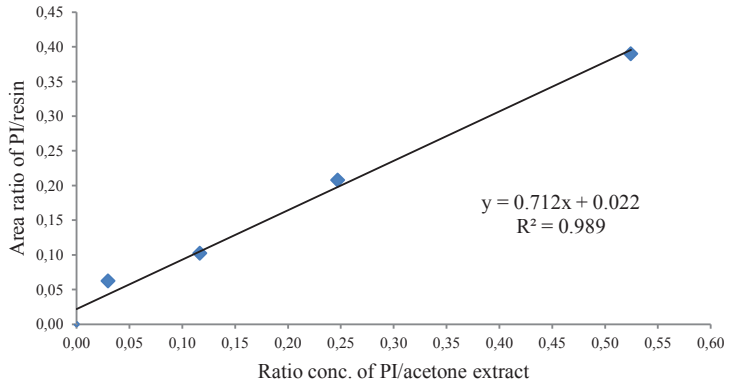


Figure VIII.14: FTIR calibration equation for PI in acetone extract

(Sample Sp-Oct-10)

Standard calibration curve of PI in resin extracted 3 replicates per time. PI in resin (various PI concentrations in resin extracted; 0, 16 % PI concentration)

Table VIII.5: Resin contaminate in PI solution

Date	a	b	R^2
5/5/2010	0.77±0.19	0.57±0.18	0.87±0.04
29/6/2010	1.37±0.11	0.15±0.10	0.96±0.03
1/11/2010	0.68±0.05	0.01±0.00	0.99±0.00
9/12/2010	0.67±0.10	0.00±0.00	0.99±0.00
Total	0.87±0.06	0.18±0.09	0.95±0.02

VIII.5.1.2 PI in acetone extract by SEC-dRI

Standard calibration curve by SEC coupled to dRI detector was first checked with a solution containing PI and PI-free resin to modelise a real acetone extract, with PI concentrations up to 16 % of contamination (0, 0.25, 0.5, 0.75, 1, 4, 8, 16%. Although the linearity was very good, down to zero (Figure VIII.15), we noted a variation of the slope "a" for the three whole experiments performed within one month, between 3.11±0.15 and 3.53±0.73, while "b" stayed at 0.00 and R^2 at 0.997-0.999.

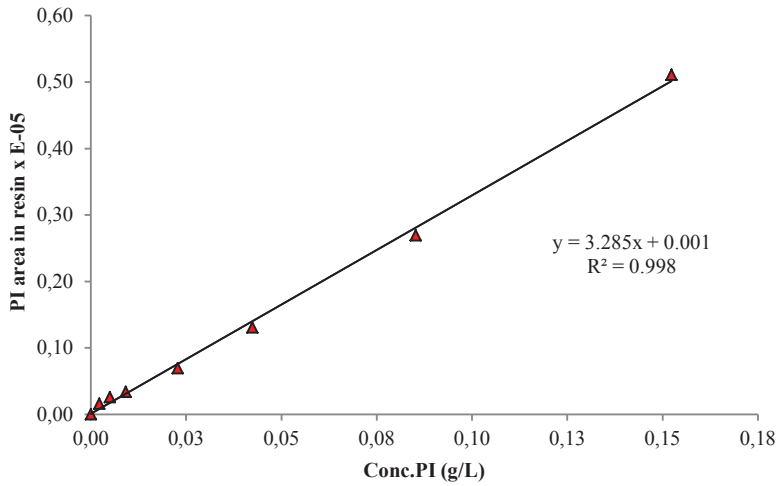


Figure VIII.15: SEC-MALS (dRI detector) calibration for PI (with acetone extract)
(range 0-16 %); Sp-March-10, total concentration 1 mg/mL in THF)

Then we performed again the calibration with PI only (no resin) and obtained a similar line but with a better reproducibility of the slope as shown in Figure VIII.16 and Table VIII.6: a: 3.06±0.08; b: 0.007±0.02; R² 0.999±0.0. The LOQ is about 0.25 % in acetone extract. This equation can serve as reference method.

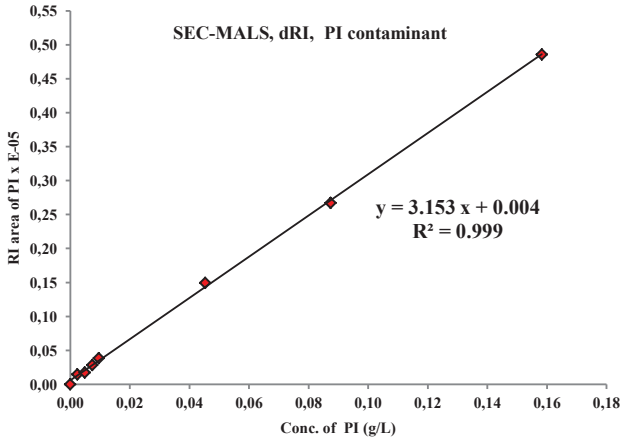


Figure VIII.16: SEC-dRI calibration for PI with acetone extract (no resin) range 0-16 %

Table VIII.6: SEC-dRI calibration for PI in THF

Date	a	b	R^2
30/9/10	3.00±0.02	0.006±0.01	0.999
03/12/10	3.15±0.10	0.004±0.04	0.999
21/12/10	3.03±0.09	0.006±0.004	0.999
Average	3.06±0.08	0.007±0.02	0.999 ±0.0

VIII.5.2.1 Resin by FTIR. Similarly to VIII.5.1 also dealing with FTIR, four independent calibrations were done over a period of 6 months (Figure VIII.17, Table VIII.7). Although with some noticeable variation for example on the slope from 2.29±0.12 up to 3.54±0.19, the LOD and LOQ is quite low (0.015 and 0.045) and, after averaging each individual concentration point -a: 2.86±0.25; b: 0.07±0.04 and R^2: 0.979 ±0.17-, we conclude that this protocol could be used for assessing resin in the reference method needed for NIRS.

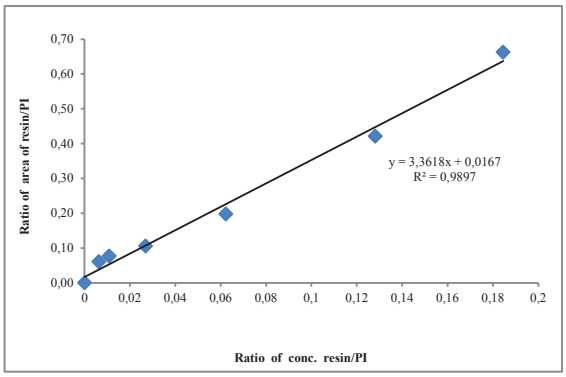


Table VIII.7: FTIR calibration resin in acetone extract *(Average 4 independent calibrations over 6 months; 0 16%)*

Date	a	b	R^2
17/6/10	2.86±0.63	0.06±0.10	0.975
29/6/10	3.54±0.19	0.05±0.03	0.963
1/11/10	2.73±0.06	0.05±0.02	0.999
9/12/10	2.29±0.12	0.03±0.01	0.998
Average	2.86±0.25	0.07±0.04	0.979 ±0.17

Figure VIII.17: FTIR calibration equation for resin in hexane extract

VIII.5.2.2 Resin by SEC

The last contaminant / protocol combination to be discussed deals with the quantification of resin by SEC. Independent calibrations were done either with PI in THF also containing resin (PI-free acetone extract) for complementing PI as a model of contaminated acetone extract, or with PI alone in THF (four in each case), over a concentration range equivalent to a contamination of 0, 0.25, 0.5, 0.75, 1, 4, 8, 16 % (up to 100%). In the first case the slope varied quite a lot from 2.66 to 3.61 (one month), and averaged data are a: 3.09±0.56; b: 0.30±0.28; R^2:0.988±0.03 (Table VIII.8 and Figure VIII.18).

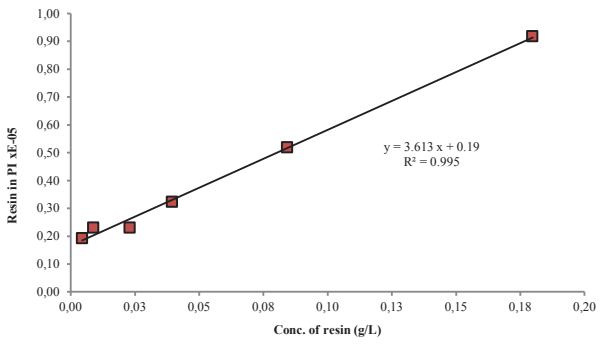


Table VIII.8: SEC calibration for resin with PI

Date	a	b	R^2
25/06/2010	3.46±0.40	0.43±0.46	0.985
13/07/2010	3.16±0.79	0.51±0.32	0.988
26/07/2010	2.66±0.61	0.15±0.05	0.991
Average	3.09±0.56	0.30±0.28	0.988±0.03

Figure VIII.18: SEC calibration for resin with PI *(3 independent calibrations over one months; 0-16%)*

In the second case (PI alone in THF) the slope did not vary a lot (3.03±0.02 and 3.36±0.07; two separate calibrations), and averaged data are a: 3.195±0.045; b: 0.405±0.08; R²:0.994±0.07. (Table VIII.9 and Figure VIII.19). This is better than in the first case but still the B value is far from zero and we were not confident in the reliability of this protocol, at least for low contamination.

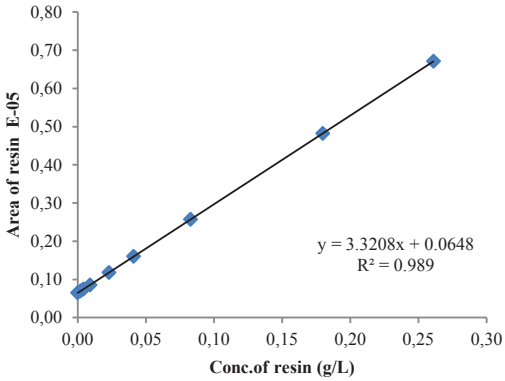


Table VIII.9: SEC calibration equation for resin in THF solution (no PI added)

Date	a	b	R^2
30/9/2010	3.03±0.02	0.38±0.06	0.998
21/12/2010	3.36±0.07	0.43±0.10	0.989
Average	3.195±0.045	0.405±0.08	0.994±0.07

Figure VIII.19: SEC calibration for resin in THF solution (no PI added)

VIII.5.3 Conclusion about calibration equations for quantifying the cross contamination

The four possible combinations were investigated. FTIR was not selected for PI in acetone extract because of high LOQ, while SEC-dRI gave a very good equation for PI in THF (a: 3.15±0.08; b 0.004±0.02; R^2 0.999) and was selected for determining PI as contaminant in acetone extract.

Regarding PI, although it would have been possible to determine it by SEC, it was not selected for practical reasons, the HPLC equipment being located in another campus, although still belonging to Cirad, and being overbooked and used for quantifying PI in acetone extract in addition to structure determination of many polymers. Thus we chose FTIR for determining resin as PI contaminant in hexane extract, a: 2.86±0.25; b: 0.07±0.04 and R²: 0.979.

Improving above data would have required addition experiments, whereas time was short, because the calibration equations were needed by other teams within Cirad and of partners for quantifying the composition of harvested biomass before processing and cropping practices.

VIII.6. Comparison of results obtained by gravimetry vs total extracted PI

VIII.6.1. Hexane extract vs PI with Soxhlet and Polytron methods

The above work has shown the cross contamination of the extracts obtained with acetone and

hexane. The analytical method now selected for assessing the PI content in place of the weight of hexane extract was applied to the extracts obtained under the extraction conditions optimized in Chapter VII, with Soxhlet, Polytron and ASE (each run in triplicate). After having (i) determined the percentage of resin contaminating PI in the hexane extract by using the FTIR calibration set erlier (Chap. VIII), (ii) obtained the purity of the PI being the complement to 100%, we applied it to the gravimetric data.

Under the optimal conditions selected for the Soxhlet method in Chapter VII, while the gravimetric yield of hexane extract was 9.0%, we found that the PI extracted by hexane was only 7.0 %, because of the quite large contamination by resin (22%). Thus the two yields are not in the same range.

Table VIII.10: Influence of operating conditions with Polytron on hexane extract and PI yields

Experimental conditions	Yield of hexane extract (%)	Resin contaminant in hexane extract (%)	PI extracted by hexane (%)
1	7.79±0.22^{ab}	20.0±1.3	6.13±0.24^{bc}
2	7.38±0.05^{ab}	21.0±1.3	6.48±0.01^{ab}
3	6.93±0.04^{c}	15.7± 0.4	5.69±0.22^{c}
4	7.66±0.04^{ab}	18.2± 0.1	6.27±0.09^{ab}
5	7.94±0.25^{a}	14.7± 0.2	6.74±0.03^{a}
6	7.70±0.19^{ab}	10.9± 0.1	6.81±0.09^{a}
7	7.83±0.30^{ab}	14.6± 0.0	6.60±0.14^{ab}
8	7.26±0.31^{bc}	13.0± 0.3	6.29±0.02^{bc}
9	7.52±0.30^{ab}	19.6± 0.0	6.04±0.14^{bc}

[a, b, c] Data bearing same letter within a given column are not statistically different according to ANOVA test (p>0.05).
Mean ± SD: Standard deviation values are based on triplicates.
Experimental conditions: see Table VII.7

Regarding Polytron, Table VIII.10, derived from Table VII.7 in the gravimetry chapter, shows also the strong influence of experimental conditions on the contamination of the hexane extract by resin, which varies from 10.9 to 21.0%, making the PI extracted by hexane significantly lower than the gravimetric result. In the righted column the maximum of hexane extract is noted in lines 5; 6; 7. Having in mind that we selected conditions of line 6 in the preceding chapter, we

can note as a broad conclusion, that considering the contamination by resin does not change the conclusion; still we should also consider the PI possibly extracted by acetone. This will be done later with the same guayule sample for comparing the three extraction methods.

VIII.6.2 Hexane extract vs PI with the ASE method

The ASE method leads to a similar conclusion. For example, in the case of acetone temperature set at 40°C, we note in Table VIII.11, that the contaminant (resin) in the hexane extract found by the selected FTIR analysis, increases almost linearly from 3.0 to 20.9 %, over the temperature range 40 – 180 °C. This increase is by far much bigger than the one observed for the gravimetric yield and thus overpasses it; the result is a moderate increase of the percent of PI extracted by hexane, although staying close to the experimental error. This cannot be attributed to degradation of PI into acetone soluble polymer, because we are here only talking about the second part of extraction, with hexane. While it looks that at 120°C and above, all recoverable PI is recovered, there must be a phenomenon enhancing the extraction of non PI compounds, not extracted at lower temperature by acetone and hexane. Nonetheless it is clear that, especially at the optimum temperature of 120°C and above, the extracted PI does not match the gravimetric yield.

On the other hand, the SEC-MALS analysis of hexane extracts obtained by ASE under the same above conditions (acetone 40°C, hexane 120°C) and collected stepwise, shows a decrease of Mw at the third and fourth steps (Table VIII.12). This is consistent with the degradation observed by Cornish et al. (2013), because of longer exposure to solvent and temperature of fraction most difficult to extract, although this could be due to the specific location of the lower Mw PI inside the complex vegetal matrix.

By the way the hexane extract being analyzed also by SEC-MALS for accessing the Mw, in addition to FTIR, Table VIII.11 allows to compare the contamination measured by FTIR to the value computed from the “real” PI measured by SEC-MALS. Data are in agreement within experimental error, except in the case of 120°C (9.6 and 5.7% for FTIR and SEC-MALS respectively). However this “local” discrepancy does not alter the main conclusion already drawn from the table. From the regular shape of the variation in the FTIR series it looks that the value measured by SEC-MALS might not be reliable, although being confirmed by several injections. Table VIII.12 also shows that there is no more PI if any left in the bagasse, recoverable by ASE.

Table VIII.11: Influence of hexane temperature on resin contaminant by ASE

Hexane temperature (°C)	Yield of hexane extract (% dry biomass)	Resin contaminant in hexane extract (% of hexane extract) FTIR *(SEC-MALS)*	PI extracted by hexane) (% dry biomass) FTIR *(SEC-MALS)*
40	7.80±0.13	3.02±*0.11 (4.5±0.10)*	7.57±0.16 *(7.4±0.13)*
80	8.34±0.28	5.43±*0.31 (5.0±0.18)*	7.89±0.31 *(7.9±0.31)*
120	9.08±0.27	9.60±*0.16 (5.7±0.10)*	8.19±0.24 *(8.5±0.14)*
140	9.34±0.39	12.14±*0.25 (12.6±0.21)*	8.20±0.45 *(8.2±0.26)*
180	10.42±0.43	20.21±*0.41 (22.3±0.34)*	8.31±0.49 *(8.1±0.41)*

Acetone extraction temperature 40°C; Sample Sp-Mar- 2010

Table VIII.12: ASE yield with hexane, for each successive step and associated Mw

Extraction step	Extract per step (% of total hexane extract)	Mw (Kg/mol)
1	84.34±0.01	1158± 39
2	10.45±0.00	1139± 25
3	4.30±0.01	1060± 30
4	0.27±0.01	769± 33

The influence of temperature on the contamination of acetone extract was also checked. Need to recall that we observed in Chapter VII an increase of the yield of acetone extract from 5 to 8 % between 40 and 100°C. The corresponding SEC-MALS analysis of these acetone extracts showed a clear increase of contamination from 3.6±0.20 to 13.6±0.31 and 18.1±0.11% while extracting at 40; 120 and 140°C respectively. In the mean time the amount of extracted resin remained almost constant. This is in line with the degradation of PI observed by Cornish et al. (2013) when exposing guayule biomass to temperature and solvent. Thus all above analysis confirm the cross contamination and there was a need for further comparing the three methods.

VIII.6.3 Comparison of the three selected methods with the same guayule sample

The following results were obtained respectively for gravimetry and real PI in extracts (% of dry

biomass): 9.01±0.20 and 7.01±0.02 for Soxhlet; 9.23±0.67 and 8.22±0.65 for Polytron; 9.08±0.27 and 8.25±0.25 for ASE. Thus we note an important drop in each case, and PI extracted by ASE with hexane is ahead significantly. This is the result of the contamination of respectively 22.2; 10.9 and 9.1% (relative to extract). Thus, Polytron and ASE provide the same PI % extracted by hexane, and the last was chosen again for practical reasons already listed in Chapter VII. We could explain this by taking into account the much longer extraction time with Soxhlet (16 h) which could allow diffusion or dissolution of resin compounds difficult to remove with acetone, thus leading to a higher contamination in the hexane extract. Extraction time is much shorter (~1 h) for ASE and Polytron, and even the last is operated close to ambient temperature even with hexane (30°C).

Table VIII.13: Comparison of percent yield PI content obtained by 3 optimized methods

Extraction method	Yield of hexane extract (%)	Resin contaminant in hexane extract (%)	PI extracted by hexane (%)
Soxhlet[a]	9.01±0.20[d]	22.2±0.30[d]	7.01±0.20[e]
Polytron[b]	9.23±0.67[e]	10.9±0.18[e]	8.22±0.65[d]
ASE[c]	9.08±0.27[d]	9.1±0.42[f]	8.25±0.25[d]

[a] *Soxhlet: 4 extraction steps of 4 hours per steps and refresh solvent after each step.*

[b] *Polytron: 3 extraction steps, 12 minute per steps, ratio of solvent per sample weight 10/1 mL/g, and virtis speed 15,000 rpm, 30°C.*

[c] *ASE: 3 extraction steps of 60 min by acetone at 40°C, and then X hexane steps of 75 min at 120°C*

[d, e,f] *Data bearing same letter within a given column are not considered statistically different according to ANOVA test (p>0.05).*

Sample Sp-March-10

VIII.7 Optimization of the reference ASE method, based on total extracted PI

At this point the ASE, given the complex interactions between extraction conditions and cross contamination, the ASE method was gain optimized, as in Chapter VII, but now taking into account the cross contamination.

VIII.7.1 Effect of deresination solvent on contamination and on extracted PI

In addition to acetone which is the most common solvent reported for deresination of guayule, acetone mixed to water or methanol (5% volume) following Wagner et al. (1991). This work has been partly commented in Chapter VII (dealing with gravimetry only), but here we add the results about the contamination. To simplify Table VIII.14, we focus on the hexane extract because the contamination in the acetone extract was low, due to the higher polarity of the mixtures. The presence of water or methanol does not act on the gravimeric yield but increases sharply the contamination by a factor of ten in the shown example, resulting in a small drop of extracted PI, although with little significance with regard of the experimental error. Therefore it was decided to stay with acetone. A part of this, but still about the effect of water, the MC did not show a clear influence on cross contamination, the difference on total extracted PI staying within experimental error, in the investigated range 3-8% (10.0±0.4; 10.6±0.4). On one side the effect of water left in the matrix is not linked to the observed effect of when provided in large amount through the solvent, and on the other side, this confirms the recommendation by Black et al., 1983, and our fixed experimental procedure to reduce MC to less than 10% when preparing the guayule samples.

Table VIII.14: The effect of the deresination solvent on resins and PI yield

Yield of extract	Acetone	Acetone/methanol 95/5	Acetone/water 95/5
Yield of acetone extract (%)	8.74±0.11	9.34±0.24	9.99±0.36
Yield of hexane extract (%)	8.64±0.25	8.66±0.21	8.99±0.18
Resin contaminant in hexane extract (%)	0.63±0.11	1.20±0.24	2.20±0.36
PI extracted by hexane (%)	8.58±0.25	8.12±0.21	8.34±0.18
Total PI (acetone+hexane) (%)	9.21±0.18	9.32±0.22	10.54±0.28

VIII.7.2 Experimental design

The above results indicate that resin should be extracted at low temperature while it is not necessary to extract PI at a temperature exceeding 120°C. Therefore we investigated the same temperature range used in Chapter VII: 40-64°C; 118-132°C with acetone and hexane respectively, with an experimental design by ANOVA response surface quadratic model use

correct name). Other conditions were left as set in Chapter VII, and also used in the preceding study: Four extraction steps with each solvent (20 min and 25 min each for acetone and hexane respectively), 85 % of volume flush. Table VIII.16 summarizes the main results: resin from acetone extract and resin from hexane extract making the total resin extract, to be compared to the acetone extract, and similar data related to PI and hexane extract. As a broad comment, the total extracted PI shows a maximum when acetone temperature is 40-50°C and hexane temperature 125°C or lower. These conditions match also the maximum for the hexane extract. The contribution of the PI contaminating the acetone extract is not negligeable although making about 10% of total extracted PI or less in most cases. The maximum of resin extract is obtained at higher acetone temperature (64°C), as expected, thus in a region situated out of the maximum for PI extract. Therefore, as already decided under similar conditions in Chapter VII, the priority must be given to maximizing the PI extract. The complete data, including standard deviation, are given in annex.

Table VIII.15: Extraction yield at variable temperature of acetone and hexane extraction in experimental design

ASE condition	Acetone extract (%)	Hexane extract (%)	Total % gravimetric
Ac60 hex130C	10.02 ± 0.25	8.27± 0.21	18.29± 0.23
Ac50 hex 125C	9.48 ± 0.44	8.51± 0.23	17.99± 0.31
Ac50 hex 118C	9.82 ± 0.32	8.55± 0.20	18.37± 0.26
Ac40 hex 120C	9.41 ± 0.12	9.05± 0.26	18.46± 0.13
Ac64 hex 125C	*10.30 ± 0.46*	*8.20*± 0.55	*18.50*± 0.50
Ac40 hex 130C	9.33 ± 0.62	8.85± 0.24	18.18± 0.35
Ac40 hex 125C	9.32 ± 0.16	8.94± 0.21	18.26± 0.19
Ac50 hex 132C	9.84 ± 0.26	8.65± 0.25	18.48± 0.25
Ac60 hex 120C	9.86 ± 0.31	7.94± 0.30	17.81± 0.32

This led to the diagram in Figure VIII.20 showing a maximum, although being relatively flat in the foreseen region. The consequence of this shape of the response surface, is that, given the experimental error, there is no need of being situated very accurately at a given point. This is in

line with the flat observed in Table VIII.12, above 80°C for the PI extracted by hexane. Therefore the reference method for building the second NIRS calibration will involve acetone at 40°C and hexane at 120°C. The fact that optimal experimental conditions for maximizing both the hexane extract and PI are located in the same region is –to some extent- a coincidence, because the last parameter, being the sum of two factors attached to each extraction solvent and to the complex vegetal structure, is the result of several phenomena, some having antagonistic effects. This is visualized in the hereafter formula, taken from Part 2, used to compute PIt, the value of the total extracted PI:

$$PIt = [PIc \,.\, ACE] + [(100 - RSc).Hex]$$

PIc: % of PI contaminant in acetone extract, relative to this extract, determined by SEC-MALS
ACE: acetone extract in % weight related to the dry weight of guayule biomass
RSc: % of resin as contaminant in the hexane extract, relative to this extract, determined by FTIR
Hex: hexane extract in % weight related to the dry weight of guayule biomass

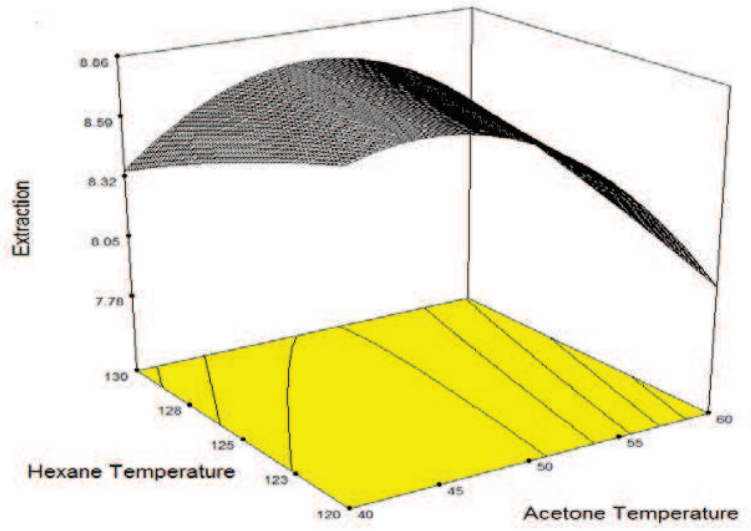


Figure VIII.20 Diagram by ANOVA experimental design response surface quadratic model for maximizing the total PI extract from guayule biomass.

VIII.8 Conclusion of chapter VIII

Based on scarce information available in the literature about the lack of selectivity of the extraction solvents towards the two targeted fractions of guayule biomass –resin and PI-, and given the aim of our work, this chapter investigated the influence of extraction selectivity on several parameters linked to the quantification of "rubber" and "associated resin".

SEC-MALS shows the contamination of resin by low MW PI in acetone extract. A symmetric situation is found for hexane extract, containing a distinct peak of resin, also detected by FTIR in the C=O region, free of PI bands, bringing suitable conditions for quantifying resin.

Having confirmed the contamination in both the extracts (cross contamination), as expected, two analytical methods were selected to quantify these contaminants, in order to take them into account when calibrating the NIRS method. Several parameters were checked and optimized to adjust the experimental protocols (like complete transfer and re-solubilization of extracts after evaporation of extraction solvent; deresination solvent; residual MC of biomass).

Calibration equations were set satisfactorily, based on wide statistical data including samples covering various plant ages, production place and harvest date. For quantifying PI contaminating resin in acetone extracts, the method is based on injecting a THF solution in SEC (dRI detector): $Y=3.1532x+0.0043$, $R^2=0.999$ For resin contaminating hexane extracts the FTIR analysis gave $Y= 2.8567+0.0701$, $R^2 = 0.988$, also choose the values corresponding to the best R^2 (0.99).

These analytical methods now selected for assessing the PI content as an alternative to the weight of hexane extract, was applied to the extracts obtained under the conditions optimized in Chapter VII, with Soxhlet, Polytron and ASE. After having (i) determined the percentage of resin contaminating PI in the hexane extract (by FTIR), (ii) obtained the purity of the PI being the complement to 100%, we applied it to the gravimetric data. The comparison lead to select (again) ASE as the most suitable method, which was optimized again, this time not for maximizing the yield of hexane extract but the percentage of total extracted PI (by acetone and by hexane). The reference method for building the second NIRS calibration involves acetone at 40°C and hexane at 120°C, for maximizing PI extract, the sum of the two contributions, being incidently the same as the ones selected in Chapter VII for the gravimetric method.

Chapter IX

NIRS calibrations for resin and rubber contents

IX.1 Introduction

The selection of a reference method for assessing PI and resin contents in guayule biomass has been the first part of the PhD work. The bibliography has shown that attempts to achieve this goal has been pursued over half a century or more, through a wide panel of ways, depending on objectives, raw materials, applicable experimental conditions.

The literature review in Chapters II and III justifies the objective of the present study i.e. to set up a quick method based on NIRS for fast determination of PI and resin in guayule biomass. NIRS has been successfully applied to the rapid determination of various chemical components in other rubber containing biomass. To achieve this goal there was a need for selecting a reference method among the available wide panel, for calibrating the NIR spectroscopic one.

The accelerated solvent extraction (ASE) method was selected and optimized in Chapter VII, among three alternatives which also included Soxhlet and Polytron. Facing a large number of methods for quantifying the targeted compounds, we concluded that this important step, on the way to the targeted NIRS-based method, justified a dedicated part of the work (Chapter VIII); this for allowing choosing a reference method based on sound arguments. In fact, in spite of a large amount of literature results, there was little attention paid to comparing the results obtained through the many methods and the sole works devoted to this important point showed some variation between the four protocols that were compared (Chapter II). Based on above considerations, priority was given to gravimetry, at least as first priority, but still there were many alternatives within this option. Further work in Chapter VIII led to consider also the cross contamination of the acetone and hexane extracts, quickly mentioned in a few early works (Black et al., 1982 and Cornish et al., 2013), and a second method has been set. The present chapter is devoted to investigating the above two protocols for calibrating the NIRS method, and associated chemometrics.

IX.2 Biomass samples used for calibration

For setting the NIRS calibration, 215 samples were collected over two years from 2010 to 2011 in Spain (El Molinar, near Cartagena, Murcia province) and in France (Lavalette in Agropolis campus, Montpellier) (Table IX.1). The samples were collected from the following guayule lines: AZ2, AZ1, 11595, N565, 593, and CAL-6. Each of the 215 collected samples was prepared according to the procedure detailed in Part 2 (Figure IX.1).

Table IX.1: Guayule biomass samples used for NIRS calibration

Harvest year of samples	Location		Total
	Spain	France	
2010	107	20	127
2011	56	32	88
Total	163	52	215

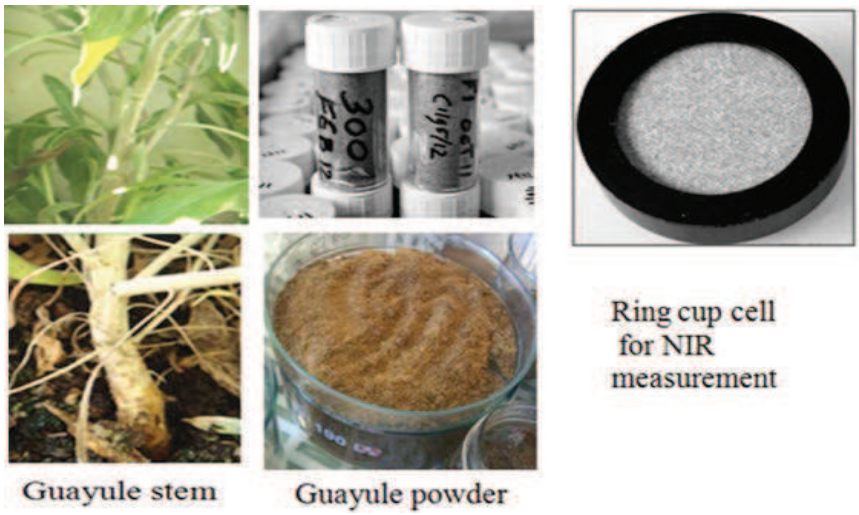


Figure IX.1: Guayule samples preparation for NIRS measurement

IX.3 Analysis of the whole set of guayule samples by the reference gravimetric method

The reference gravimetric method involving ASE was applied to the entire set of 215 samples. It was necessary to assess the content of residual moisture, in order to express the weight of the acetone and hexane extracts based on dry weight of each biomass sample. Statistical results regarding MC, and acetone and hexane extracts are summarized in Table IX.2.

MC ranged from 2.65% to 9.45%, with an average value of 5.94% and associated SD of 1.72%. Acetone extract, which is supposed to represent the resin yield, has an average value of 8.57%. The highest value is 13.42% and the lowest is 3.27%, with a dispersion of 1.92% corresponding to a relatively low figure. PI content expressed as hexane extract is ranging between 0.74% and 13.81% (average value of 6.34%, data dispersion of 2.33%). Thus the analytical work shows that this set of 215 samples covers the usual range of variation of these extractible biomass components (Nakayama, 2004, Scholman et al., 1998). The distributions of acetone and hexane extracts were suitable for NIRS calibration development.

Table IX.2: Descriptive statistics for moisture, acetone and hexane contents

Prediction	Moisture (%w/w)[a]	Acetone extract (%w/w)[a]	Hexane extract (%w/w)[a]
Minimum	2.65	3.27	0.74
Maximum	9.47	13.42	13.81
Mean	5.94	8.57	6.33
Standard Deviation	1.72	1.92	2.33
Standard error lab	0.1	0.3	0.4
Number of samples	215	193	193

IX.4 Optimisation of NIR measurement protocol

The main objective of this first step was to select the best conditions for guayule NIRS analysis and evaluate the repeatability through the root mean square error (RMS). This error was estimated by calculation of the mean and standard deviation at each wavelength for a set of replicates.

$$\overline{X}_j = \frac{1}{n}\sum_{i}^{n} X_{ij}$$

Mean of absorbance of wavelength j.

$$RMS(i) = \sqrt{\sum_{j}^{p} \frac{(\overline{X}_j - X_{ij})^2}{p}}$$

Where n: Number of spectra compared (i ranges from 1 to n),
p: Number of wavelength (j ranges from 1 to p),
Xij: Absorbance value of spectrum i at wavelength j.

This calculation aims to evaluate the repeatability of the NIR measurement including the instrumental noise, the homogeneity of the sample, and operator variability.
The test was carried out on:

- Fresh material. The fresh guayule samples were cut (about 1 cm in length) and about 5 g of the biomass were analyzed by NIRS using a rectangular cell.

- Dried material. The dried guayule samples were ground (particle passing 0.5 mm screen), and about 3 g of powder were analyzed in NIRS using a ring cup cell.

Nine replicates (different cells, new test sample, with no reset) were carried out for both samples, and root mean squares (RSM) based on spectra second derivatives were calculated for each replicate (Table IX.3).

Table IX.3: the root mean squares of fresh and dried guayule samples

Guayule samples	RSM[1]; range (mean)
Fresh	69-144 (97)
Dried	41-112 (67)

[1] *Root mean squares expressed in micro absorbance units*

According to RMS values the measurements of fresh and dried guayule biomass can be done with a high repeatability and a sufficient representatively of the homogeneity of the sample. These low RMS values allowed a repeatable measurement of biomass using only two replicates representative of a homogeneous sample.

We decided to work on dried ground guayule to simplify the transfer of samples from the field in Spain to the laboratory in France, and insure the stability of the samples.

Working on dry samples was also a guarantee to avoid problems with water bands absorption in NIR and, by the way, to make it easier to develop efficient models to be applied by the Europeans partners involved in the EU-PEARLS project.

IX.5. Principal Component analysis

Prior to calibration development, a principal component analysis (PCA) was used to extract relevant information from the spectral matrix (n = 215). The generalized Mahalanobis distance (H) was calculated on the extracted Principal Component (PC) for each sample. This statistical distance is useful for defining boundaries of the population and a similarity index between spectra (Shenk and Westerhaus, 1996). The Mahalanobis distance limit was fixed as $H > 3.0$ for outliers detection. According to this analysis, 4 outliers consisting of 3 samples from France

(2011) and one sample from Spain (2011) were identified.

The GH values were quite low for these outliers 3.0 and 3.4, except for one French sample (reference 345/11) with GH value equal to 6.0. This sample presented average moisture content (7.2%) and acetone extract (4.1%) but low hexane extract (0.74%). This sample was obtained from a young plant, thus its origin explains the low hexane extract value, but the spectrum of this sample was not visually different from others. We decided to keep all the samples in the data and no outlier was removed.

The first three PCs extracted from the PCA carried out on the 215 samples explained 49.8%, 25.3% and 11.8% of total inertia respectively. The three dimensional representations of the samples scores for the first three PCs is shown in Figure IX.2. Two groups can be distinguished, and correspond to geographical origin of the samples (France and Spain), without clear discrimination according to origin and/or harvest year.

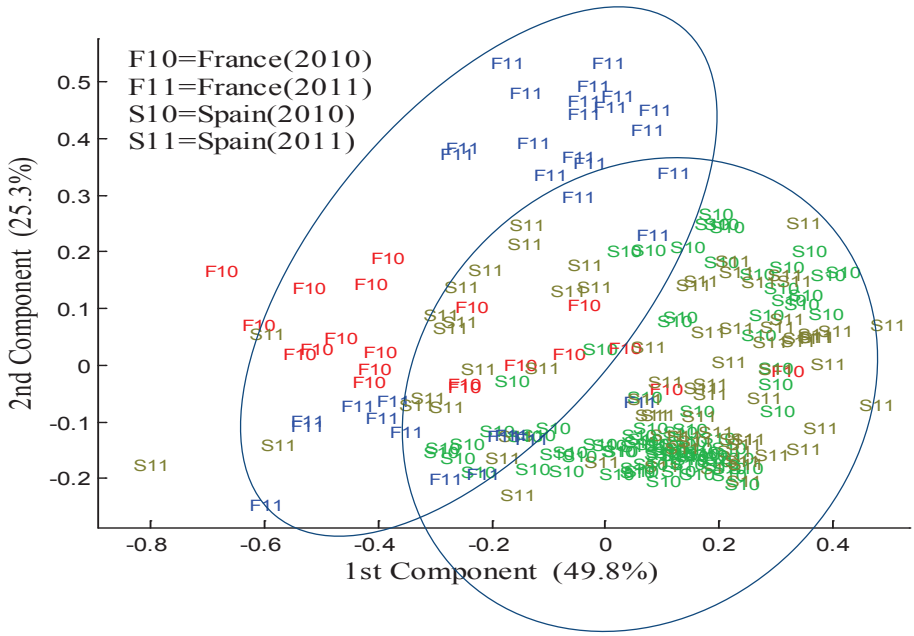


Figure IX.2: Samples scores for the first three principal components

IX.6 Near infrared spectroscopy calibration based on gravimetry (ASE)

IX.6.1 Samples selection for calibration and validation sets

In order to assess the performances of the predictive equations, the 215 samples were split into a

calibration subset (cal) and a validation subset (val). The validation set was created by selecting about 33% of the 215 samples randomly. Consequently, the calibration set comprised 144 samples and the validation set contained 71 samples. The spectral variability of the validation sample was representative of the whole data set (Figure IX.3), the GH values of validation samples compared (as supplementary) to the calibration data base were on average equal to 1.2, four samples were outliers (maximum GH value was 5.1). The projection of calibration samples (144 samples) on the PCs extracted from calibration data set (71 samples) confirmed this result. The average GH was 1.1, only four samples were outliers with a maximum GH value of 6.1. The random selection of validation samples was efficient and insured an accurate evaluation of equations performances; validation samples covered MC ranges, acetone and hexane extracts.

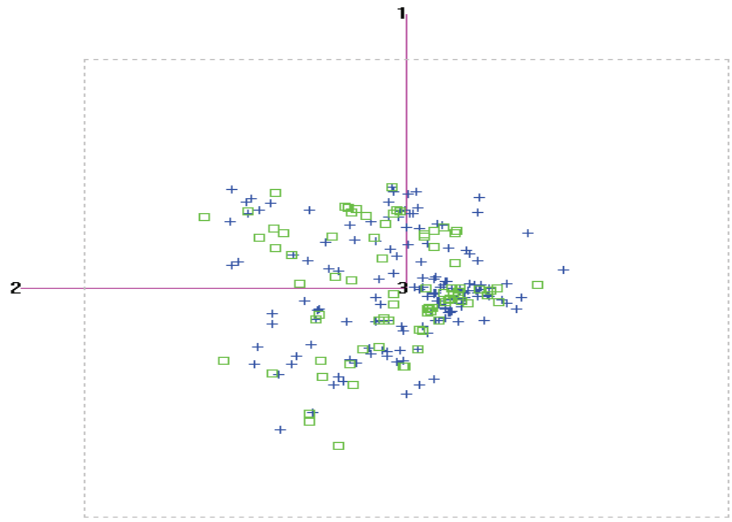
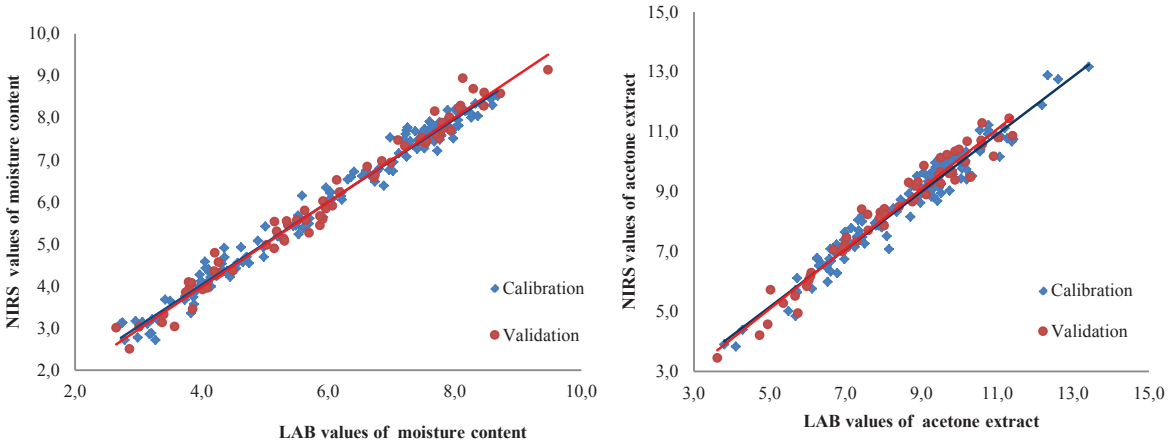


Figure IX.3: Validation samples (green) scores for the 2 first PCs against the calibration samples (blue) scores

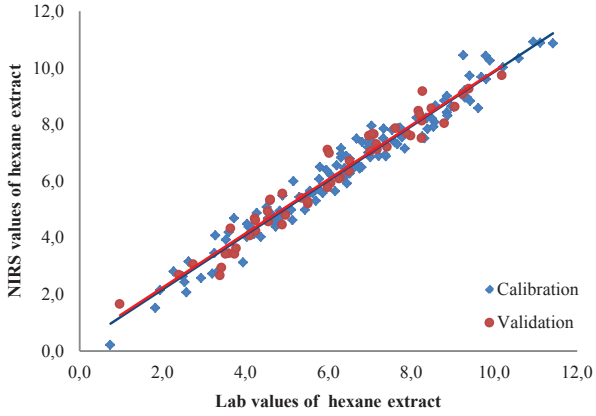
IX.6.2 Moisture content calibration

Calibration for MC gave both R^2_c (for calibration) and R^2_p (for prediction) of 0.99 and 0.98, respectively. According to this model, RPD_p was 6.97 and SEP was 0.25% (Table IX.4) with a regression slope of 0.97 between laboratory and NIR-predicted values (Figure IX.4a). Thus this calibration can be used accurately to determine the MC in routine analysis. Compared to other models obtained in the literature (Black et al., 1985), this MC calibration model appears to be highly accurate (low bias and low SEP).



a. Moisture content

b. Acetone extract



c. Hexane extract

Figure IX.4: Scatter plots of laboratory values versus NIR-predicted values: moisture content (a), acetone extract, (b) and hexane extract (c).

IX.6.3 Acetone extract calibration

Calibration for acetone extract (resin) was also efficient when both of the R^2_c and R^2_p were 0.96, RPD_p was 4.80 and the SEP was 0.40% (Table IX.4). The prediction was carried out with an accuracy of ±0.80%. The regression between laboratory and NIR-predicted values (Figure IX.4.b) led to a slope equal to 0.96 with no bias. This calibration was efficient and accurate for the whole range of the resin content observed in the guayule samples. The model obtained using

Table IX.4: NIRS equations statistic parameters for gravimetry as reference method

	Constituent	N	Mean	SD_{cal}	SEC	R^2_c	SECV	RPD_c	SEL
Calibration	Moisture content	141	5.91	1.73	0.23	0.99	0.26	6.79	0.13
	Acetone ext.	127	8.68	1.81	0.38	0.96	0.43	4.23	0.39
	Hexane ext.	119	6.47	2.27	0.40	0.98	0.43	5.28	0.47
	Constituent	N	Mean	SD_{val}	SEP	R^2_p	RPD_p	Slope	Bias
Validation	Moisture content	68	5.92	1.76	0.25	0.98	6.97	0.97	0.00
	Acetone ext.	56	8.35	1.93	0.40	0.96	4.80	0.96	-0.09
	Hexane ext.	55	5.99	2.01	0.44	0.96	4.58	1.00	-0.07

All values are expressed in % of dry matter; Acetone ext.: acetone extraction; Hexane ext.: hexane extraction; N: number of samples retained in calibration (t-outlier samples); SD_{cal}: standard deviation for calibration subset; SEC: standard error of calibration; R^2_c: coefficient of multiple determinations for calibration; SECV: standard error of cross validation; RPD_c (ratio performance to deviation) = SD_{cal}/SECV; SEL: standard error of laboratory; SD_{val}: standard deviation for validation subset; SEP: standard error of prediction; R^2_p: coefficient of multiple determinations for prediction; RPD_p (ratio performance to deviation for prediction) = SD_{val}/SEP.

IX.6.4 Hexane extract calibration

The calibration developed for hexane extract (rubber) quantification showed R^2_c, R^2_p and RPD_p at 0.98, 0.96, and 4.58, respectively. The regression slope between laboratory and NIR-predicted values was 1.00 (Figure 4.c). The estimated SEP was 0.44%, enabling determination with an accuracy of ±0.88%. These performances were better than those found in literature (Black et al., 1985; Cornish et al., 2004; Takeno et al., 2008) related to guayule and in accordance to results from Kleine and Foster (1992) also with guayule, and from Takeno et al. (2008) *relative* to *Eucommia ulmoides* leaves using FT-NIR on a small set of samples (42).

Conclusion. The large set of samples used in the present study from two harvest cycles, two geographical origins, and six guayule genotypes, and the performances of the calibration make it possible to apply NIRS as a routine analysis for acetone and hexane extracts in guayule biomass.

IX.6.5 Near infrared spectra interpretation

The second derivative of the average spectrum of dried guayule (Figure IX.5) highlighted principal absorption bands at 1,450, 1,490, 1,716, 1,940, 1,708, 2,210, 2,308, and 2,380 nm. These bands are in accordance with observations done by Black et al. (1985) and Cornish et al. (2004). The bands for PI and resin where mainly related to C-H stretch first overtone (OT) of -CH_3 (1,716 nm), C-H stretch and deformation combination of -CH_2 from lipids (2,308 nm) and C-H/C=O stretch combination of aldehyde structure (2,210 nm) (Osborne et al., 1993). Moreover, these bands at 1,450 nm and 1,940 nm correspond to O-H stretch first OT from H_2O.

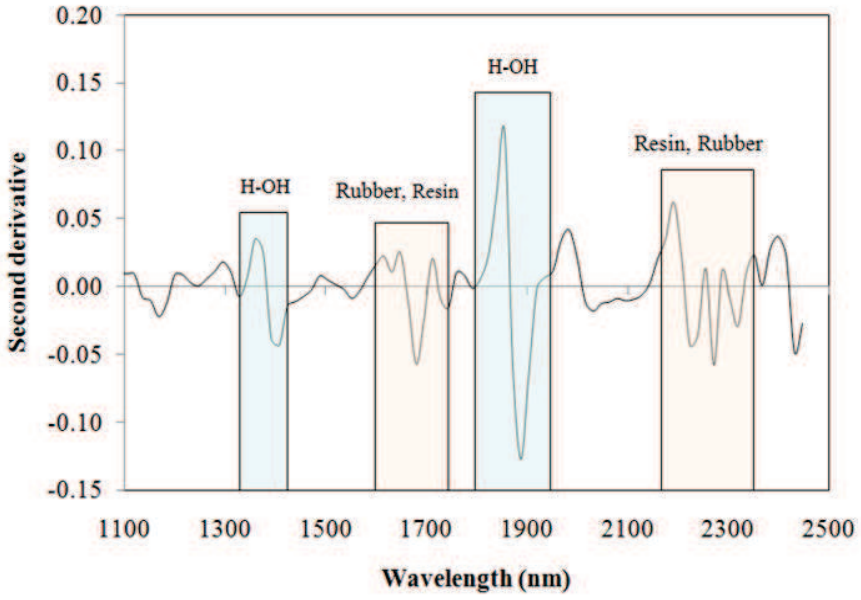


Figure IX.5: Second derivative of log (1/R) of dried guayule NIR average spectra

The examination of PLS loadings (Williams, 2001) found for acetone and hexane extracts showed that the first third loadings (Figure IX.6.abc) where similar (bands and weights) for quantification of both extracts. The first ones were close to the NIR spectrum of dried guayule. The third ones were related to absorption bands similar to those used for the moisture content quantification: 1,450 nm and 1,908 nm which correspond to O-H stretch first OT from H_2O, cellulose and carbohydrates and to O-H stretch first OT of P-OH, respectively. The fourth ones (Figure IX.6.d) showed the highest weights for hexane than for acetone extract quantification for absorption bands at 2,308 nm and 2,268 nm (O-H stretch combination of cellulose).

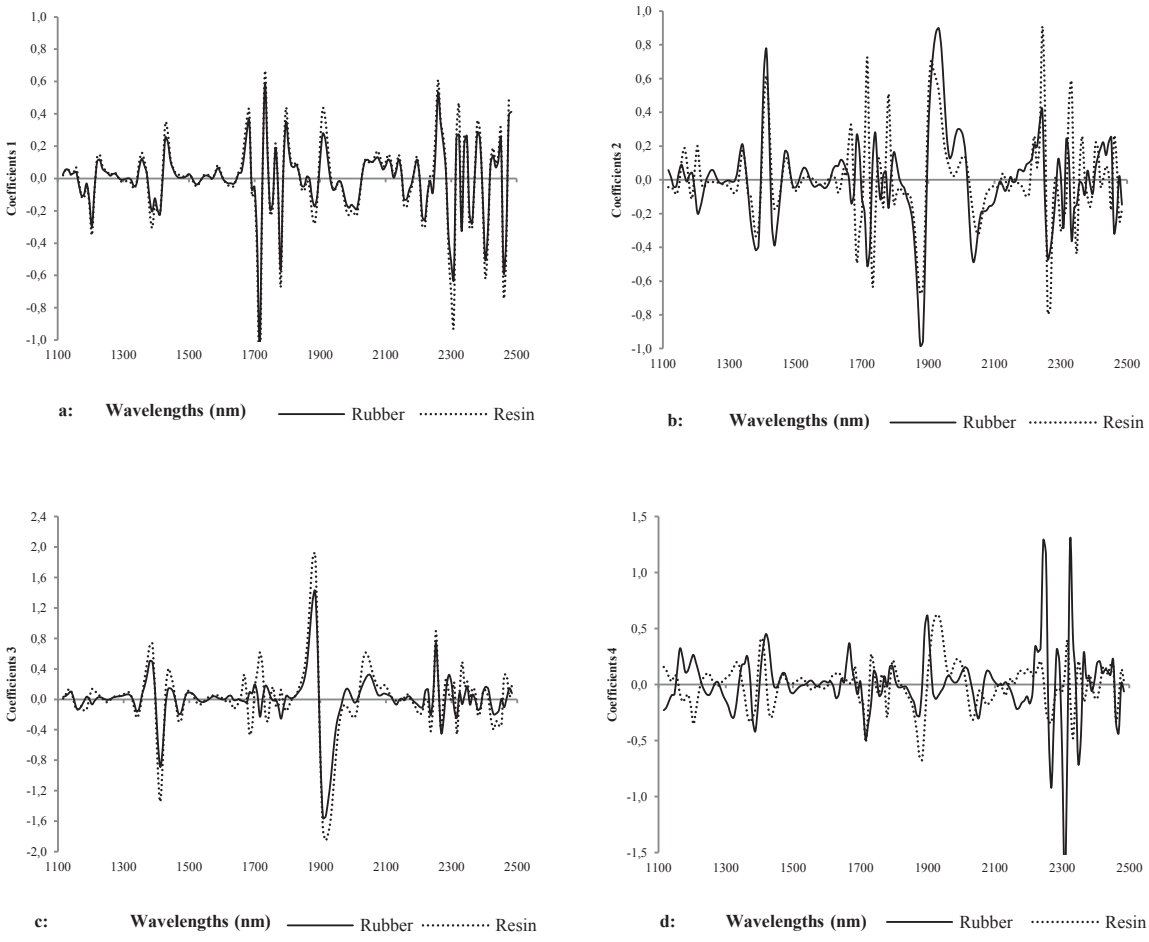


Figure IX.6: Comparisons of the hexane extract and acetone extract loadings as a function of wavelengths: first loadings (a), second loadings (b), third loadings (c) and fourth loadings (d).

The Pearson correlation coefficient between PI and resin wet chemistry values was 0.42 (different from zero at significant level 0.05), and the correlation coefficient between PI and resin NIRS predicted values was 0.52 (different from zero at significant level 0.05).

The absence of a high correlation between Y predicted values for PI and resin (close to the correlation observed between wet chemistry values) indicated that, despite of common loadings, there is no interaction between PI and resin b coefficients, and suggested that PI and resin cannot be used as indicators of one another. The similarity within loadings was probably due to similar functional groups, and long $-CH_2-$ chains in both resin and PI structures, and interferences with cellulose, which is probably correlated to the quantified compounds.

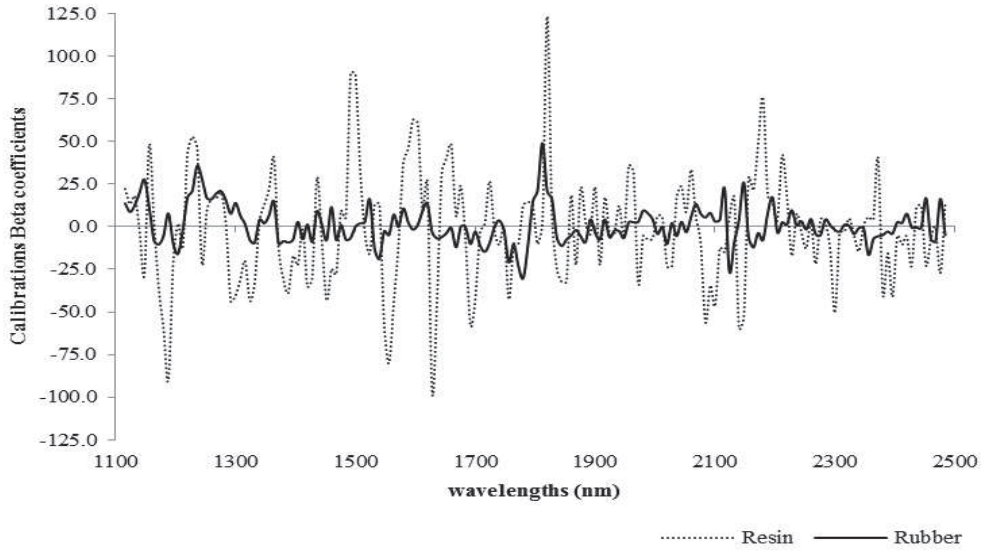


Figure IX.7: Acetone and hexane extract calibrations beta coefficients as a function of wavelength

This result was confirmed by observation of calibration beta coefficients as a function of wavelengths (Figure IX.7). Some coefficients were specific to the acetone extract (1,492 nm and 1,156 nm: N-H stretch first OT of $-CONH_2$ and -CONH-, 1,628 nm: C-H stretch first OT of $=CH_2$, 2,084-2,100 nm: C=O stretch and O-H deformation combination of carbohydrates and R-OH, 2,300 nm: N-H and C=O stretch combination from amino acid and 2,380 nm: O-H deformation from R-OH). These coefficients were in accordance with the functional groups expected in resin, being mainly constituted of triacylglycerols and sesquiterpenes, but also phenols and carbohydrates, as mentioned by Nakayama (2005) and possibly of amide-containing compounds although not mentioned in literature.

IX.6.6 Conclusion about calibration with the gravimetric method

The high performances of calibrations for MC (RPD_p= 6.97; R_p^2=0.98) and relative acetone extract (RPD_p = 4.59; R_p^2=0.96) and hexane extract percentages (RPD_p=4.87; R_p^2=0.96) indicated their suitability for fine determination. NIR calibration enables accurate characterization of acetone and hexane extracts, as a rapid (less than 1 min/sample) nondestructive and reliable one shot determination of moisture, resin (acetone), and PI (hexane) of guayule. Thus these models can now be applied for cheap and high-throughput characterization of guayule biomass for breeding programs and production. The efficiency of

NIRS models confirms that extraction steps and methodology provided repeatable and accurate results.

The use of factorial regression such as PLS regression allowed developing accurate and independent models for acetone and PI contents despite similar chemical backbones structures (mainly CH_2 chains).

However, it was already found in Chapter VIII, that, doing this way, the extraction is not as selective as expected. The next sections subsequently focus on an improved –although more complex- method accounting for cross contamination of each of above extracts (acetone, hexane) as detailed in Chapter VIII. The extraction selectivity for resins & PI with acetone and hexane, was determined by size exclusion chromatography coupled to a multi-angle light scattering detector (SEC-MALS), and Fourier transform infrared (FT-IR) spectrometetry, as a routine control of extracts compositions obtained using ASE.

IX.7 NIRS calibration taking into account cross contamination of extracts

The purity of hexane and acetone extracts was controlled using SEC-MALS and FTIR, as described in Part 2, on 127 samples randomly selected within the 215 guayule samples already used for NIRS calibrations with acetone and hexane extracts (gravimetry). The objective of this complementary study was to evaluate the impact of contamination on NIR equations. Samples were found to be adulterated by low *Mw* PI in resin (acetone extract) or by resin in PI (hexane extract).

IX.7.1 PI and resin determined from chemical analysis of acetone and hexane extracts

The descriptive statistics for the resin and PI contents determined for the 127 selected samples are reported in Table IX.5. Resin content (total) presented an average value of 7.75%. The highest value was 12.71% and the lowest one was 3.61%. Data dispersion of the resin content was relatively small (1.75%). PI ranged between 1.18% and 14.71% with average value of 6.77% and data dispersion value was 2.72%.

Total PI and resin contents of the 127 control samples cover the range observed for these two compounds in natural guayule biomass, and were representative of the 215 samples.

Table IX.5: Descriptive statistics for resin and PI contents

Constituents	Number sample	Min. value*	Max. value*	Mean value*	SD*
Resin content	127	3.61	12.71	7.75	1.75
PI content	127	1.18	14.17	6.77	2.72

**Percentages of dry matter*

The Pearson correlation test (risk α = 5%) between acetone extract and pure resin contents was highly significant (p value < 0.0001) and R^2 = 0.955. The scatter plot (Figure IX.8) of acetone extract versus resin contents highlights this strong relation with an R^2 = 0.922 and a slope of 0.894. The Pearson correlation test (risk α = 5%) between hexane extract and pure PI contents was highly significant (p value < 0.0001) and R^2 = 0.969. The scatter plot (Figure IX.8) of acetone extract versus pure resin content highlights this strong relation with R^2 = 0.946 and a slope 1.06.

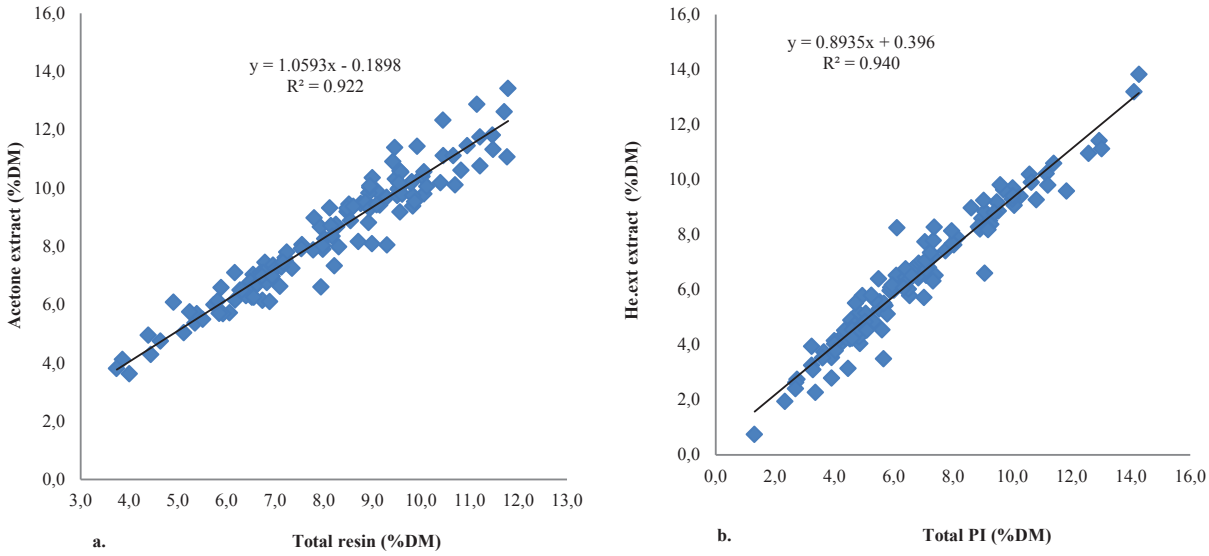


Figure IX.8: Scatter plots of acetone extract (a) and hexane extract (b) contents versus total rubber and resin contents

IX.7.2 NIRS calibration based on resin and PI

The objective of this complementary study was to compare the performances of the corresponding calibration models, to the highly performing models set with acetone and hexane

extracts. One can imagine that this should be certain according to high correlations existing between set of data compared in Figure IX.8. But, it is well known that NIRS calibrations are highly dependent on reference values and especially on the methodology chosen among others in terms of accuracy. That is why we decided to develop NIRS models based on new investigations and determinations of resin and PI contents, and to compare them with previous ones. The data set (n=127) was separated in 2 sets: calibration and validation sets. One third (31 samples) was randomly selected for validation and the remaining was used for calibration (96 samples).

Resin. Calibration for pure resin content was efficient with R^2_c and R^2_p of 0.96 and 0.92, RPD_p was 3.05 and the SEP at 0.53% (Table IX.6). The regression between laboratory and NIR-predicted values (Figure IX.9.a) led to a slope equal to 1.08 with no bias. Compared to previous calibration for acetone extract (contaminated resin), RPD_p and SEP were slightly degraded in this case (SEP was 0.40 % and RPD_p was 4.80).

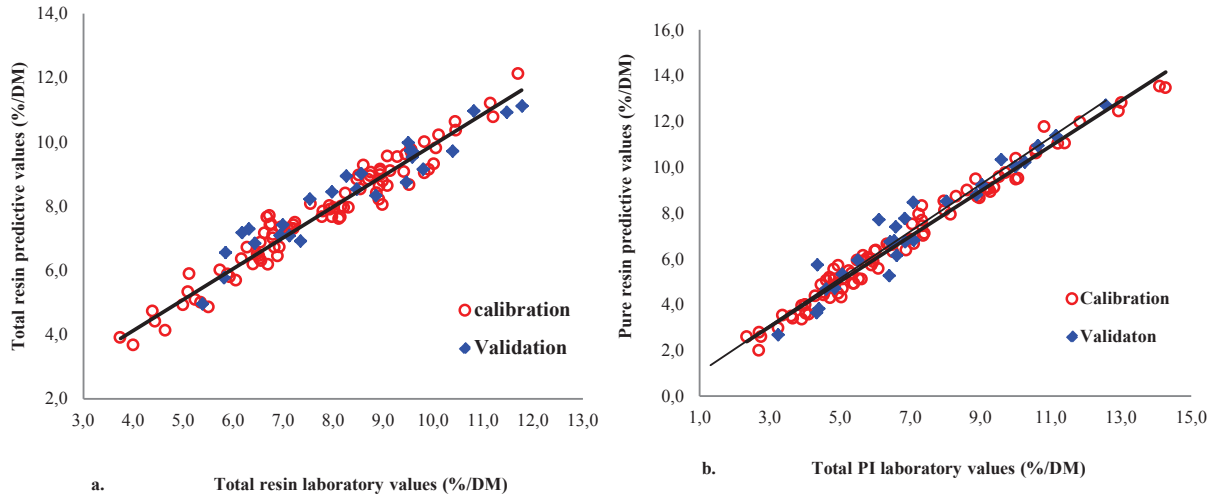


Figure IX.9: Scatter plots of total resin values (a) and total PI values (b) versus NIRS predictive values for both calibration and validation sets

PI. The calibration developed for PI content quantification showed R^2_c, R^2_p equal to 0.98, 0.95 and RPD_p was 4.13 with an SEP equal to 0.62% (Table IX.6). The regression slope between laboratory and NIR-predicted values led to a slope of 0.90 with low bias (Figure IX.9.b). Compared to previous calibration for hexane extract (contaminated PI), RPD_p and SEP were slightly degraded in this case (SEP was 0.44 % and RPD_p was 4.58).

Table IX.6: NIRS equations statistic parameters for resin and PI calibrations

Statistic	**Constituent**	**N**	**Mean**	SEC	R^2_c	SECV	RPD_c	SEL
Calibration	Resin	89	7.75	0.33	0.96	0.40	4.39	0.51
	PI	89	6.77	0.36	0.98	0.41	6.88	0.59
Statistic	Constituent	N	Average	SEP	R^2_p	RPD_p	Slope	Bias
Validation	Resin	26	8.39	0.53	0.92	3.05	1.08	-0.07
	PI	26	7.41	0.62	0.95	4.13	0.90	-0.15

Note; [a]MC: moisture content. N: number of samples. SEL: standard error of laboratory. SD: standard deviation for calibration subset. SEC: standard error of calibration. R_c^2: coefficient of multiple determinations. SECV: standard error of cross validation. RPD_c (ratio performance to deviation) = $SD_{cal}/SECV$. SD_{val}: standard deviation for validation subset. SEP: standard error of prediction; R_p^2: coefficient of multiple determinations for prediction. RPD_p (ratio performance to deviation for prediction) = SD_{val}/SEP.

IX.8 Conclusion

The above results of calibrations based on resin and PI contents indicate that NIRS can be used for routine analysis of guayule biomass samples. However lower performances in terms of SEP values compared to those obtained for the calibration based on contaminated resin and PI (acetone and hexane extracts respectively) reflect the impact of the analytical reference methodology on models. In this case, SEC-MAL and IR quantification increase laboratory errors (SEL > 0.5) and thus the standard error of prediction.

In both cases, simple gravimetric protocol and the one based on cross contamination results, the use of NIRS for resin and PI determination remains efficient and allows fast and accurate characterization of guayule biomass. This high throughput application will accelerate and facilitate guayule breeding programs for selection of high PI producing plants.

The next step should be the application of Near Infrared Spectroscopy directly on growing guayule in the field.

Chapter X

Influence of the extraction protocol on polyisoprene macromolecular structure

X.1. Introduction

As detailed in Chapter IV, PI from HL has very long chains, characterized by high average molar mass (M_w) (Subramaniam, 1972; Kim et al., 2008), whereas few plants are able to achieve a Mw above 1,000 kg/mole (Swanson et al. 1979). As a general rule, the higher the M_w, the higher the mean length of PI chains and therefore the better the quality of the produced rubber for the main market outlets, like tires and gloves (Fuller, 1990; Ehabe, 2005; Kim et al., 2010) Several parameters can influence the M_w of PI extracted from guayule. McMahan et al. (2006); Mcmahan et al, (2006) compared the M_w determined by size exclusion chromatography coupled to a multi-angle light scattering detector (SEC-MALS) of PI extracted under latex form (lines AZ-2 and 11591), and found that M_w in 3.3 years old plants was significantly higher than the one extracted from 1.7 years old plants. Also, Backhaus and Nakayama Backhaus, 1988) studied the effect of agronomic practices on PI macromolecular structure. In some cases, increasing irrigation frequency induced a decrease of M_w (from 580 to 480 kg/mole; 11591 genotype), but depending on the harvest date, one could observe the contrary. Therefore, during guayule breeding experiments, or before deciding the harvest date for industrial processing, it is of course very important to know about the quantity which can be achieved or expected from the biomass, in order to meet PI quality according to the demand.

The whole history of the sample, from harvest date, storage time and conditions, down to processing conditions, are of paramount importance when studying the macromolecular structure of guayule rubber, as pointed by several authors (Black et al., 1986; Dierig, 1991; Schloman, 1996; Cornish et al., 2005; McMahan et al., 2006). The calibration of the NIRS method with a laboratory reference method like the ASE, as did Salvucci et al., 2009 Cornish et al., 2013; Grinberg and Shaubi, 1986; Jasso de Rodríguez and Kuruvadi, 1991; Salvucci et al., 2009; Teetor and Ray, 2004, and also used in previous chapters, is not supposed to be a function of Mw (except for very low Mw). However, one possible consequence of any extraction procedure devoted only to assessing the PI content in biomass, is to obtain PI which could have been degraded by the extraction process. Thermal conditions or exposure to oxygen are among the most obvious parameters responsible for degradation.

Therefore to know whether the ASE routine protocol set for calibrating the NIRS method was suitable or not for assessing the macromolecular structure of PI in the plant, it was necessary to investigate this point. Indeed, to compare different line of guayule or other PI producing

plants, being aware of the sensitivity of PI to thermo-oxidation, it is important to preserve the native macromolecular structure during harvest, storage and extraction steps.

After having checked the influence of experimental conditions for the routine ASE method, we developed a one-step "soft" method (SM) to determine the PI macromolecular structure in guayule biomass. This makes the present and last chapter of our PhD work.

X.2. Experimental strategy

In the course of a follow-up of an experimental guayule field throughout the year, samples are periodically harvested, extracted by the ASE method described in previous chapters based on extracting resin with acetone, then PI with hexane (Suchat et al., 2012), and finally the hexane extract is analyzed by SEC-MALS. In addition to the gravimetric estimation of the extract, the analytical procedure provides structural parameters relative to the extracted PI. Due to large number of samples, these are dried and stored until analysis. In order to check the influence of the whole analytical procedure -from harvest to SEC-MALS- on PI structure, another method was partially optimized and applied in parallel to the routine one.

This second extraction method was set with the aim of providing conditions expected to preserve PI structure better than those applied during the routine method, which involves numerous steps. Although the sample is exposed to air most of the time, and in spite of adding an antioxidant (BHT) to the extract, several steps like drying of biomass, extraction at temperature of 120°C, evaporation of the solvent, preparation of the solution by stirring for one week, final filtration before injection, could be seen as the most probable causes of PI degradation. The new method was set in order to provide much softer operating conditions, and so named (soft method (SM)). In particular, the number of steps was reduced to the minimum: a single low temperature extraction step under nitrogen atmosphere in the dark, and microfiltration prior to injecting into the SEC-MALS apparatus. Initially the drying step was optionally kept in the soft protocol, because of the possible adverse effect of the water contained in the biomass against PI extraction yield. The extracting solvent, tetrahydrofuran (THF), was selected because routinely used as the injection solvent for SEC-MALS, thus avoiding unnecessary evaporation of the extraction solvent prior to injecting. The solvent/biomass ratio (40 mL/1.5 g) was adjusted for yielding extracts falling in the useful PI concentration range for SEC-MALS, thus not requiring a new dilution or concentration step.

The SM was set with one guayule sample (spring harvest; May 2011), then applied to other samples harvested periodically, in parallel to the routine procedure for allowing to compare the results.

Table X.1: Guayule samples used for investigating the soft method vs fresh and dry biomass

Harvest date	Sample code	Type of biomass	Moisture content[a] (%)	Resin content (%)	Drying time[b] (day)
18/05/2011	245/11	Fresh	49.9±0.4	5.57±0.02	-
	321/11	Dry	6.9±0.2	5.81±0.94	1.5
24/06/2011	370/11	Fresh	53.5±0.9	6.11±0.85	-
	371/11	Dry	9.7±0.3	5.85±0.08	2
18/11/2011	739/11	Fresh	51.1±0.2	8.83±0.19	-
	740/11	Dry	5.7±0.0	7.0±0.1	2.5

[a] *Samples dried for 15 h at 103°C;*
[b] *Drying in a vacuum oven at 40°C*

X.3. Influence of the extraction time on the PI yield with the soft method

The SM could be used for determining the molecular structure, only, if it allows extracting a significant amount (if not all), compared to the PI extracted by the routine ASE method taken as reference. The extraction was performed at 30°C, and extraction kinetics with THF was recorded from 6 hours up to 7 days, for both fresh and corresponding dried biomass. After the extraction with THF during the chosen time, the solution was filtered on a disposable filter (pore diameter 1 µm) and injected. The analysis by SEC-MALS allowed determining the PI content in starting biomass (% w/w dry biomass) and analyzing the macromolecular structure of the samples, the later will be commented in the next section.

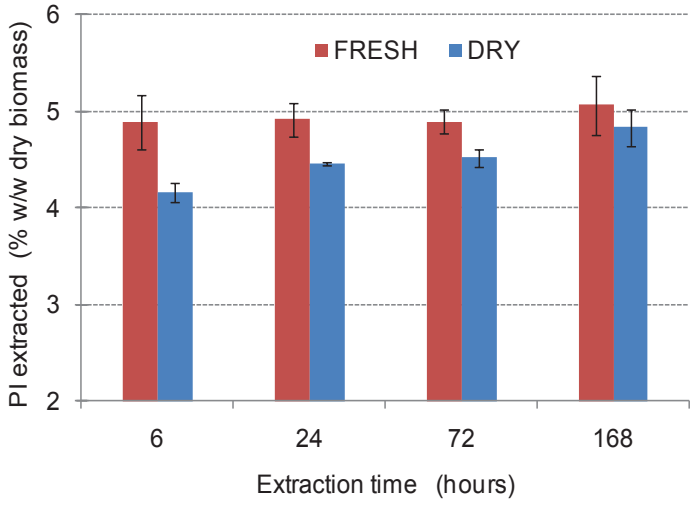


Figure X.1: Yield of extracted PI with SM determined by SEC-MALS vs extraction time

Fresh sample 245/11; dry biomass sample 321/11). Data are the mean of 3 independent extractions. Error bar indicates standard deviation.

For the fresh biomass, Figure X.1 shows that THF allowed extracting the maximum quantity of PI in 6 hours. The percentage of extracted PI was 4.9±0.3% (w/w dry biomass) after 6 hours and 5.1±0.3% after 168 hours (7 days). Thus increasing the extraction time, above 6 hours did not improve PI yield from the fresh biomass. On the contrary, for the dry biomass, the extracted PI increased significantly from 4.2±0.1% after 6 hours of extraction to 4.8±0.2% after 7 days. Thus with the SM, here applied to the dried biomass, it took one week to reach the yield obtained in one day with fresh biomass. This could mark a change in biomass structure impeding the mass transport through the vegetal matrix. It should be noted that, in spite of above observation regarding the shift of mass transfer properties of the biompass, the working conditions with ASE (high pressure, higher temperature) should enable to overcome this effect brought by the drying step and revealed by the SM. Thus, with the SM the extraction was faster for the fresh biomass than for the dried one.

X.4 Influence of extraction time on PI macromolecular structure with the soft method

Figure X.2 gives the molar mass distribution (MMD) obtained by SEC-MALS from fresh and dry biomasses after 6 hours of extraction. It can be noticed that the MMD was unimodal in both cases, and the molar masses were lower for PI extracted from dry biomass. Figure X.3 shows the evolution of the weight-average molar masses (M_w) in function of the extraction time for the fresh and dry biomass. For the fresh biomass, the M_w of the extracted PI remained constant between 6 and 24 hours, then decreased slowly with the extraction time from 2,130±30 kg/mole (6 hours) to 1,800±70 kg/mole (168 hours). Having already shown that the extraction yield did not progress during these six additional days, it can be concluded that the marked decrease of M_w cannot be attributed to a change of selectivity with time during the extraction process, but rather it is the result of structural changes even under these favorable conditions (30°C, BHT added to THF, dark). For the dried biomass, the M_w of extracted PI for the shorter extraction time is close to the one found after 168 hours for the fresh biomass. The M_w of extracted PI from dried biomass again shows a tendency to decrease with time (Figure X.3). Even, the M_w for the extracted PI from the fresh biomass after 168 hours of extraction, was still higher than the Mw of the PI extracted from dry biomass after 6 hours.

This shows the effect of drying, while by-passing this step (fresh biomass) allows to extract all extractible PI in biomass within one day, having a M_w of more than 2,100 kg/mol. The

drying step enabled the same yield after one week, but then the *Mw* was only 1,600 kg/mol, thus suffering a 25 % reduction, although extracted from the same biomass.

These results show the differing behavior of one important processing parameter, i.e. the M_w lowering effect of the drying step. Accordingly it was decided to set the SM with the following conditions: quick processing right after harvest, no drying step, extraction with THF (+ BHT) at 30°C for one day (for practical convenience, regarding this last point).

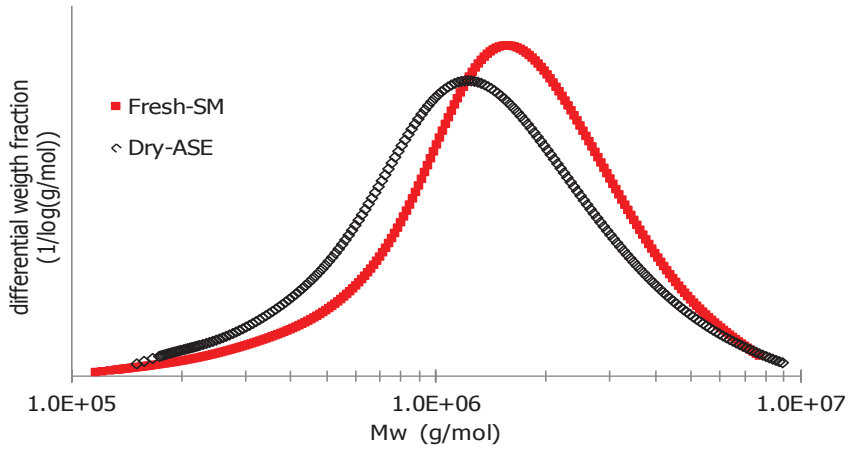


Figure X.2: Molar mass distribution for extracted PI determined by SEC-MALS for fresh (sample 245/11) and dry biomass (sample 321/11) *(Extraction with the soft method)*

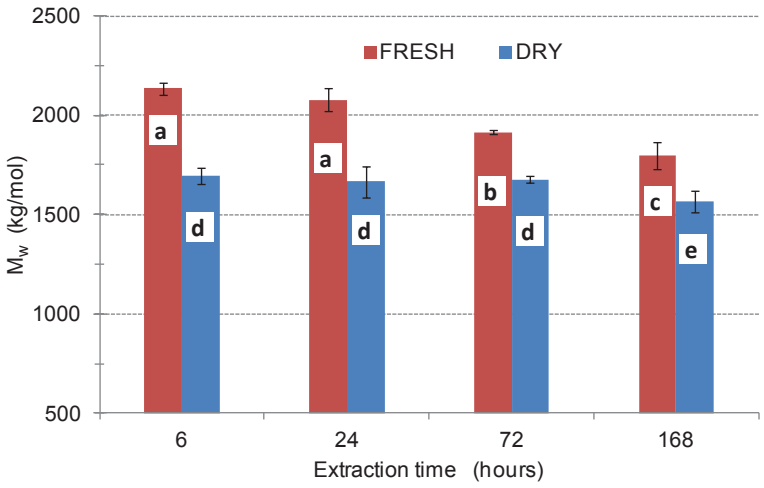


Figure X.3: *Mw* of extracted PI vs extraction time for fresh and dry biomass

Extraction with the SM, Mw determine by SEC-MALS. Data are the mean of 3 independent extractions. Error bar indicates standard deviation. Results bearing the same letter were not significantly different (P≤0.05).

X.5. Comparison of molecular structure of PI extracted by soft and ASE methods

The SM (one day extraction time) was applied to two additional biomass samples from the same field, harvested in June and in November (Table X.1) in addition of the sample

harvested in May. Figure X.4 gives the yield of PI extracted from the six samples (fresh and dry) depending to the extraction method, SM and ASE. Again, the yield of PI with the SM from fresh biomass was significantly higher than that from dry biomass. Thus the dry biomass option cannot be regarded as a suitable material for SM, because the corresponding extract may differ from the PI contained in the guayule biomass (lower yield, thus possible selective extraction). Also, whatever the harvest date, the yield of PI extracted from the fresh biomass with the SM was always the same as the one given by the reference ASE method. This confirms the conclusion from section X.3 and entitles the SM for further investigating the molecular structure of PI from guayule.

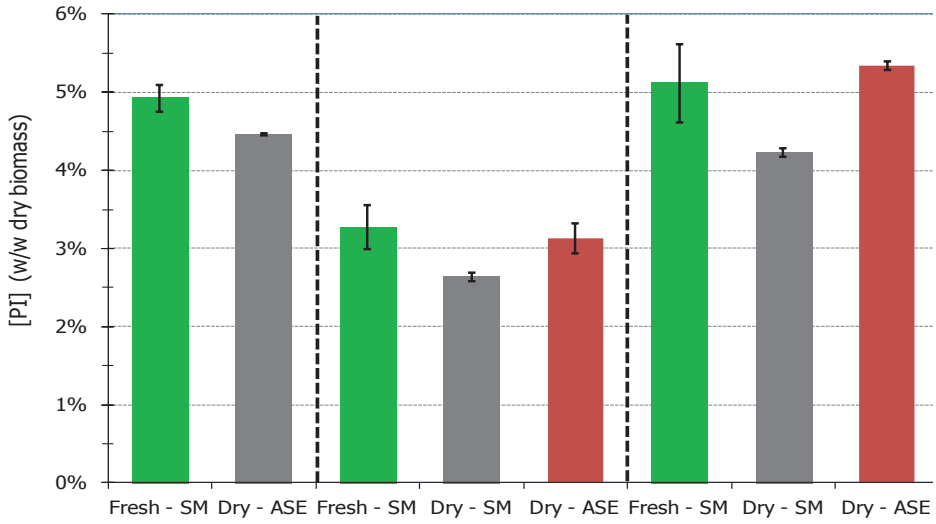


Figure X.4: Yield of extracted PI with SM and ASE methods vs harvest date

SM: THF, 24 hr, 30°C; ASE method: hexane, 3x25 min, 120°C); Data are the mean of 3 independent extractions. Error bar indicates standard deviation.

Figure X.5 gives the M_w of extracted PI from fresh and dry samples, according to the extraction method (same above samples and same experimental conditions). Whatever the harvest date, drying led to a significant decrease of the M_w, although to a variable extent. The decrease of the M_w was 20 % for the May sample (from 2,080 to 1,665 kg/mol); 36 % for the June sample (from 1,625 to 1,035 kg/mole) and even up to 61 % for the November sample (from 1,710 to 670 kg/mole). Worth noting from this new set of samples in Figure 5, that the PI extracted from dry biomass with ASE and with SM had very close M_w values (1,060±60 vs 1,170±20 kg/mole, and 670±25 vs 560±10 kg/mol for June and November samples respectively). In spite of the slightly slower extraction kinetics for the SM applied to dry biomass which allowed extracting about 90% of the PI content, this confirms the negative

effect of the drying step already noted. Thus the main degradation of PI takes place during this drying step, and not during the extraction step by the routine ASE.

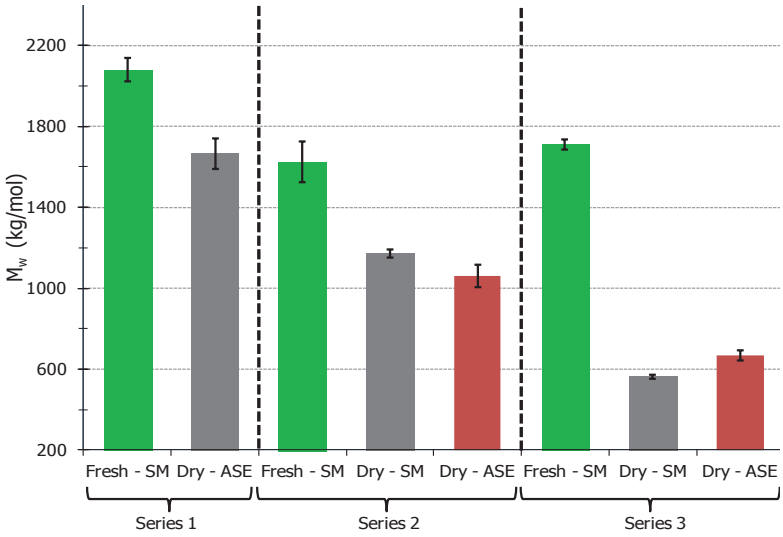


Figure X.5: Evolution of M_w of PI extracted from fresh and dry samples vs harvest date

(Same conditions as in Figure X.4)

The above results confirm that the conditions used for the SM with fresh biomass allow the extraction of large PI chains. Indeed, Figure 5 provides a clear picture of the influence of the harvest season: the SM shows a quick but moderate decrease of *Mw* from above 2,100 kg/mole in May to about 1,700 kg/mole in June and in November, whereas the ASE method would lead to conclude a strong trend of *Mw* to decrease over the six months period covered by this example.

Owing to the encouraging results obtained through the SM, another structural macromolecular parameter was investigated. Figure X.6 shows that the conformation plot (R_{gi}=f(M_{wi}) is significantly different between the PI from fresh biomass extracted with the SM and the PI from dry biomass extracted by ASE. For a same M_{wi} the radius of gyration (R_{gi}) of PI from dry biomass is significantly lower. A smaller R_{gi}, (a smaller size) for the same M_{wi} means that the macromolecules after drying and/or after ASE are more compact and probably more branched. Indeed, as shown in Figure X.5 the M_w of PI from dry biomass is quite low (668 kg/mole) indicating a dramatic degradation probably due to oxidation during biomass drying. It is well known that (i) PI is rather sensitive to oxidative degradation in guayule Keller, et al.,1981; Bhowmick, 1985; Schloman et al., 1996), and (ii) the oxidation leads to radical scission of PI chains which can recombine to create branching (Bevilacqua, 1965; Ehabe,

2005; Colin, 2007). This increase in branching of PI induces an increase of the compactness of macromolecules (lower R_{gi} for the same M_{wi}).

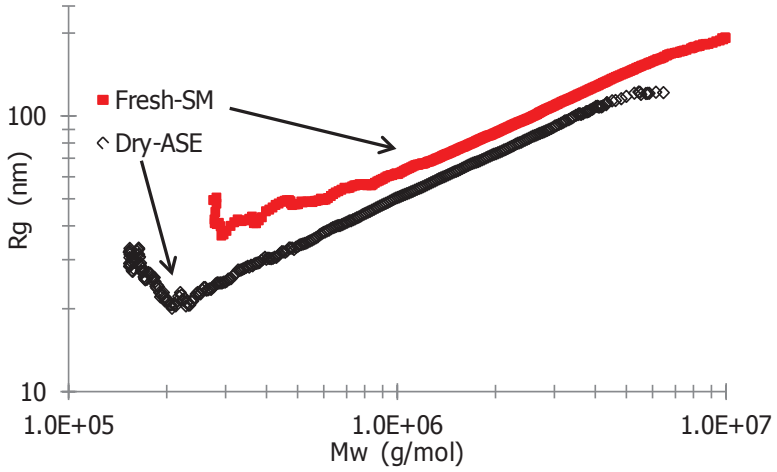


Figure X.6: Conformation plots (R_{gi}=f(M_{wi}) obtained from SEC-MALS
(Sample biomass harvested in Fr-Nov-2011)

These results justified our initial doubt, based on literature data, about the aptitude of the routine gravimetric method to extract native PI and keep it as close as possible to its native form. While the effect of storage conditions had been already pointed, especially for extracting GR under latex form, the effect of storage and of drying was less known. However Cornish et al. (2013) have already found that temperature above the bulk resin melt temperature (~60^{o}C) melt the resin in the ground material, allowing it to disperse and flow over the lignocellulosic fines in very thin films. This resin conformation is faster to extract during the subsequent ASE extraction than the initial bulkier resin conformation in the ground material as the melt temperature effect. Since the ASE is performed under nitrogen, this degradation can be attributed to thermodegradation, whereas the rubber loss in the pre-extraction drying oven is caused by a combination of oxidation degradation and thermodegradation. Fortunately, accurate resin and rubber quantification-in alignment with the 50^{o}C drying temperature indicated in the present study. It should also be noted that although sample preparation and resin extraction at relatively cool temperature protects the high molecular weight rubber from degradation, during these process steps, the high temperature hexane to extract the high molecular weight rubber for quantification also will degrade the polymer into lower molecular weight chains. The loss of resin and degrade rubber, at high temperature. Still this is the first report to our knowledge of a so marked effect

of drying guayule biomass even under reasonably favorable conditions (40°C; vacuum oven; 1-2 days). In fact this step, which then allows storing the biomass for long periods prior to analysis, is widely performed. Only Coffelt and Ray (2010) and Cornish et al. (2013) did avoid drying, as well as other questionable processing options, in view of preparing extracts

prior to molecular analysis of PI. During drying, not only the *Mw* was strongly decreased -up to 60%- but also molecular packing. It is well known that (i) PI is rather sensitive to oxidative degradation in guayule, and (ii) the oxidation leads to radical scission of PI chains which can recombine to create branching. This increase of branching of the macromolecules induces an increase of the compactness of macromolecules (lower R_{gi} for the same M_{wi}).

X.6. Conclusion of Chapter X

In order to obtain macromolecular data as close as possible to the PI structure in fresh guayule biomass, an alternative method was evaluated. This "soft method" (SM) was set for reducing the number of steps to the minimum while operating under low temperature conditions (extracting PI with THF at 30°C for 24 hours using fresh -not dried- biomass; filtration; SEC-MALS analysis). After having compared to the results given by the routine gravimetric two-solvent protocol, this SM can be used successfully to estimate both quantity and *Mw* of PI.

The most interesting features lye in the fact that (i) THF enables to extract biomass without drying, giving the same extraction yield than the routine method (ASE with acetone then hexane at 120°C), and (ii) the drying step even performed under vacuum and at 40°C induces a dramatic change in the PI macromolecular structure. The relatively long drying time (2 days) may play adversely.

Under our experimental conditions, the use of SM seems to bring a real advantage. However it would be necessary to explore the influence of other parameters in order to achieve a better understanding of the phenomena responsible for the observed effects. In particular the used fresh samples were not stored before analysis. However, although useful under the scope of the EU-Pearls project, this study was not the core of the PhD program, and thus was performed at the end of the period.

After being tested on a larger panel of samples covering a wider range of guayule lines, PI content, and field and experimental conditions, this protocol could be used for routine and large scale determination of PI structure for agricultural and breeding investigation, this analysis being performed only at small scale today, thus limiting research programs.

GENERAL CONCLUSION

Among the many techniques proposed in the literature for accessing to the resin and PI content in guayule biomass, we selected three methods based on sequential extraction with two solvents -acetone and hexane- each involving several steps until no more extract could be obtained, and compared the results in terms of gravimetric yield.
This led to choose ASE (Chapter VII), not only because of the maximized extract yield but also owing to its ability to process large series of samples. Worth noting that the maximum yield of hexane extract, representing PI, could be obtained only under low temperature conditions with acetone, confirming some earlier works, due to combined effects of (i) the complex nature of the guayule biomass at cellular and subcellular levels (PI entrapped inside cells), and (ii) the rapid degradation of PI at molecular level.

Regarding the accurate quantification of PI (and resin) in biomass, the possible cross contamination of acetone and hexane extracts mentioned in the literature only by a few authors, led to check this phenomenon in Chapter VIII. It was confirmed to be real and not negligeable, although of varying amplitude (11% to 26% of impurity) for SEC-MALS. Include here approximately the max 29% and 6% minimum of contamination we were found by IR. Of the interesting in Chapter VIII, we found the total resin content range from 3.74 to 11.79% and the total PI range from 1.31% up to 14.27%.
Proper analytical procedures were set, based on SEC-MALS and FT-IR, for estimating the cross contamination, and this gave access to a second set of data, this time called “resin” and “PI”, instead of “acetone and hexane extracts”. The optimum extraction conditions which turned to be almost the same as the ones set for the gravimetric method were determined with an experimental design. Need to note that in practice, above mentioned PI degradation which was then confirmed in Chapter X, should not affect the quantification of PI in guayule biomass, because this second protocol takes into account any low molar mass PI resulting from degradation, even when extracted by acetone instead of hexane.

Chapter IX, which is at core of this work, has shown that, (i) ASE, a modern technique not previously used as reference extraction method for NIRS calibration by our predecessors, is highly reliable, and (ii) obtained NIRS calibration performances are better than the ones previously published. It is known that the NIRS calibration depends on the reference method used to quantify the targeted compound (selectivity, reproducibility, accuracy), but also on the number and coverage of the set of samples used. In the present cases the work involved a quite large number of samples (215) covering several genotypes, harvest seasons, plant ages

(more than two years), and fields under differing climates. These wet chemistry results were introduced jointly to NIRS spectra in PLS regression models. Performances of regression models were compared to laboratory error and to previous NIRS models described in literature. A closer investigation was done using chemometric tools and their outputs, such PLS loadings and b coefficients, in order to interpret the relationship between spectral fingerprint (absorption bands) and rubber or resin contents.

The low stability of PI, noted at start of the PhD work, led to separate the initial ambitious objectives of achieving PI quantification and PI molecular structural analysis, through a single extraction protocol. After having reached the first aim, as above detailed, we set a second and new protocol based on single solvent extraction and minimized sample processing steps, performed at room temperature and with fresh (non dried) biomass right after collection, design to avoid rapid PI degradation (Chapter X).
This so called "soft method" enabled to obtain PI extracts showing an average molecular mass above 2 million g/mole, probably close to in vivo structure, while the most widely used protocols yielded about 600 thousand g/mole, only. The biomass drying step, although initially performed at 40°C and under reduced pressure (Chapters VII and VIII) was identified as the major contributor to this drastic drop of PI molar mass. Thus, it could be possible that, either (i) the drying step changed cell wall properties (biomass particle size of 0.5 mm might not have all cells opened by grinding), being then less permeable to large macromolecules, and/or (ii) the dissolution of PI molecules or their transport through cell walls is more difficult, because of higher branching resulting from oxidation or "in-cell gelation" or less favorable packing. Imaging would help understanding the phenomena responsible for this effect; this is being undertaken in another PhD work.
On the basis of these results, caution should be given to sample preparation conditions in literature dealing with PI macromolecular structural parameters in guayule biomass.

The above soft method could be used as reference for a NIRS calibration, after further investigation regarding selectivity and total PI extraction through multiple steps. About this last point, recent ^{13}C NMR (solid) work on guayule biomass, started just before closing the present report, tend to show that even after having noted the end of extraction kinetics after the third hexane extraction step, there would be still some PI left in the bagasse (about 5% of the solvent extracted PI, to be confirmed). This emphasizes on the many constraints brought by the unique and complex structure of guayule biomass at cellular and molecular levels (PI

in many locations, entrapped in cells; resin free flowing in ducts; chemical instability), and the need to take this into account when extracting macromolecules such as PI, which was seldom considered in literature dealing with analytical work on guayule.

The NIRS method is being transferred to partners at Wageningen University (WUR) under the frame of the EU-Pearls project, and it has been already used extensively in our team, in collaboration with agronomists from UR 34 of CIRAD together with researchers from WUR, in charge of domesticating guayule in Europe.

These gravimetric and soft methods are being applied within the EU-Pearls consortium for monitoring agronomic and pilot extraction trials, and thanks to this, high PI molecular mass gloves and tires were produced by CIRAD (UR 40-Biorefinery team) and industrial partners, and presented at the seminar in Wageningen, The Netherlands. (http://www.eu-pearls.eu/UK/November,09, 2012; Apollo Vredestein Press, 2012; Palu, S., 2012)

In the near future the next goal will be "transferring this technique to the field", i.e. calibrating with a portable NIRS device to provide an even more efficient analytical tool for researchers and farmers.

Ms. Ref. No.: INDCRO-D-12-00390R1
Title: Fast determination of the resin and rubber content in Parthenium argentatum biomass using near infrared spectroscopy
Industrial Crops and Products

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Annex

Annex 1: Influence of the extraction step in Sp-Oct.-2009

ASE	Sample	Extraction cycle time (cycles)						Total
	Weight (g)	3Ac1/1	3Ac1/2	3Ac1/3	3Ac1/4	3Ac1/5	3Ac1/6	Extracts
Extract Weight (g)	5.3421	0.2662	0.0145	0.0066	0.0035	0.0033	0.0005	0.2873
STD		0.0137	0.0025	0.0021	0.0016	0.0016	0.0014	0.0166
Resin Content (%)		4.9819	0.2707	0.1241	0.0651	0.0614	0.0102	5.3767
STD		0.1903	0.0461	0.0388	0.0307	0.0311	0.0271	0.2484
%Yield of		92.6836	5.0198	2.2965	1.2282	1.1603	0.2015	100
STD		1.2928	0.6607	0.6341	0.6129	0.6280	0.5236	0.0000

ASE	Sample	Extraction cycle time (cycles)						Total
	Weight (g)	*3*He1/1	*3*He1/2	*3*He1/3	*3*He1/4	*3*He1/5	*3*He1/6	Extracts
Extract Weight (g)	5.3421	0.0373	0.0088	0.0037	0.0024	0.0028	0.000715	0.0558
STD		0.0021	0.0018	0.0011	0.0014	0.0020	0.0006	0.0036
Rubber Content (%)		0.6980	0.1657	0.0694	0.0457	0.0527	0.0134	1.0449
STD		0.0323	0.0355	0.0217	0.0270	0.0380	0.0108	0.0815
%Yield		67.2214	15.7988	6.5642	4.3720	5.0421	1.2575	100
STD		8.2736	2.5698	1.6011	2.4384	46.6678	1.0169	0.0000

Annex 2: The extraction yield with age and location of guayule biomass by SM

Sample harvested date	Location	MC (%w/w)	M_w (kg/mol)	PI (%)
June, 2009	France	7.92 ±0.05	216±5.46	0.44±0.07
October, 2009	France	6.95 ±0.08	326±11.17	1.81±0.05
March, 2010	France	6.80 ±0.06	415±7.71	2.55±0.11
June, 2010	France	4.51 ±0.08	352±4.04	3.60±0.12
October, 2010	France	5.59 ±0.11	497 ±2.85	4.08±0.33
May, 2011	France	9.00 ±0.02	494 ±8.18	4.30±0.08
June, 2011	France	9.67 ±0.04	550 ±18.69	3.71±0.04
October, 2011	France	6.08 ±0.03	714 ±10.53	4.73±0.04
October, 2009	Spain	5.24 ±0.09	405 ± 24.45	1.45±0.02
June, 2010	Spain	9.19 ±0.05	531 ±2.77	6.26±0.04
October, 2010	Spain	5.23 ±0.07	611 ±13.46	8.22±0.20

Annex 3: Influence of purification methods to % yield PI on different guayule 3 plant ages

Guayule samples (Code #, date)	PI, he.ext (Gravimetric) (%)	PI (total pure) (%)	PI (SM) (%)
France samples			
345/11, June 09	0.85±0.12	1.22±0.02	0.44±0.07
319/11, Oct. 09	1.97±0.21	2.18±0.12	1.81±0.05
18.2/10, June 2010	3.24±0.33	4.07±0.11	2.55±0.11
741/11, Oct.10	4.44±0.30	4.17±0.21	3.60±0.12
371/11, June 11	3.30±0.15	3.47±0.06	4.08±0.33
738/11, Oct.11	5.59±0.31	5.07±0.16	4.30±0.08
320/11, March 10	3.99±0.16	3.66±0.17	3.71±0.04
232/11, May 11	5.23±0.22	3.99±0.33	4.73±0.04
Spain samples			
394/11, Oct.09	1.77±0.22	1.79±0.25	1.45±0.02
47/10, June 10	6.18±0.16	6.45±0.06	6.26±0.04
399/11, Oct. 10	8.53±0.12	8.63±0.34	8.22±0.20

Annex.4: Influence of guayule extraction methods to molecular weight

Sample detail	Harvested date	M_w (kg/mol)	M_n (kg/mol)
Soft method, fresh biomass;			
France, branches, fresh	17/04/2012	1812±37	1118±36
Spain, branches, fresh	12/04/2012	1995±73	1220±167
Spain, root, fresh	12/04/2012	1960±55	1299±94
Spain, branches, fresh CAL6	12/04/2012	1909±13	1284±19
Spain, branches, fresh 11591	12/04/2012	1832±60	1191±46
Spain, branches, fresh AZ	12/04/2012	1760±63	1109±24
Other extraction method			
Spain, branches, AZ	12/04/2012	905±46	445±20
Waring Blender (Lab scale)	14/02/2012	325±67	114±67
Pilot scale	20/02/2012	235±24	101±27

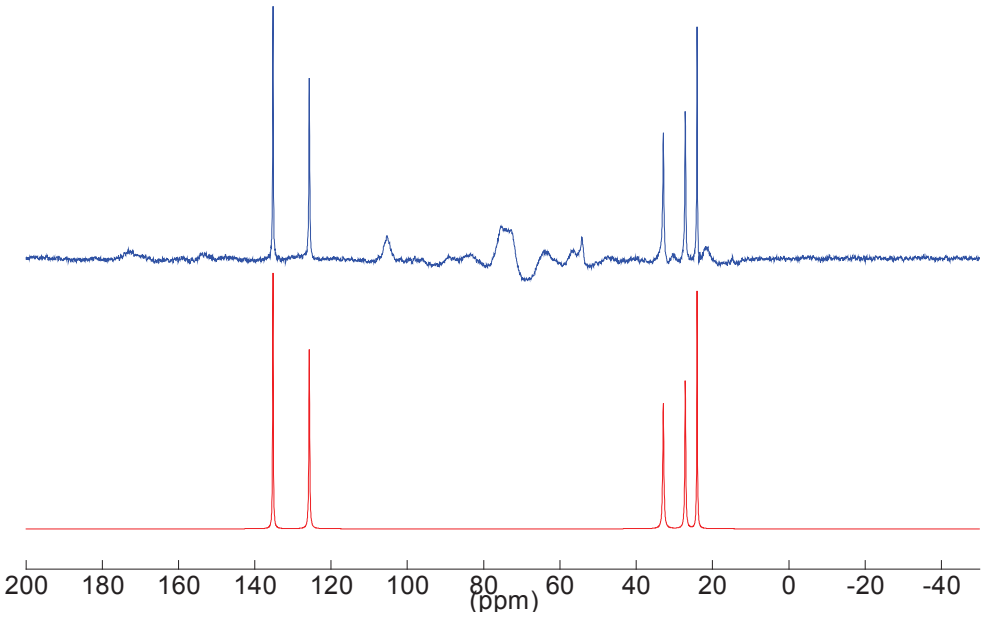
Waring Blender (Lab scale)	05/03/2012	629±145	253±100
Pilot scale	05/03/2012	683±116	197±28

Note: Data from Amor A., 2012.

Annex 5: Comparison the yield percent of precipitated of guayule with bulk guayule rubber

Sample types	Initial weight (g)	Extracted weight (g)	Weight of extracted (%)	Weight of precipitate (g)	Weight of precipitate (%)
Bulk guayule	0.63±0.12	0.63±0.02		0.54±0.05	86±0.52
Hexane extract (+Ac)	10.01±0.30	0.05±0.014	0.50±0.14	0.02±0.02	40±0.17
Residues liquid	10.01±0.30	0.05±0.014	0.50±0.14	0.01±0.00	11±0.03
Hexane extract	15.30±0.25	0.62±0.35	4.12±0.02	0.05±0.04	8.07±0.42
Residues liquid	15.30±0.25	0.62±0.35	4.12±0.02	0.01±0.00	1.79±0.01

Sample Ar-Oct.-2009 and Fr-Oct.-2009



Annex 6 : Carbon 13 magnetic resonance spectrum of raw guayule structure (before extraction) with correction base and simulation.

This study to identifying the guayule structure in solid ^{13}C-NMR in the solid form, and it did not preparation for dissolving the extracts into Barycentre 5358.24 Hz /71.03 ppm at 300

MHz, MAS 7.5 mm, rotation of 5kHz, and time of recycle of 30s. Analytical integration accounts for different G/L ratios Barycentre 5358.24Hz / 71.03ppm.

Annex 7: results of samples from Spain by AES and by NIRS

Sample Number	Resin pure	Rubber pure	Resin	Rubber	resin residua	rubber residua
319/11	6.7	3.7	6.1	3.7	-0.6	0.1
342/11	8.0	10.8	8.3	9.3	0.2	-1.6
345/11	3.9	1.3	4.1	0.7	0.3	-0.6
393/11	9.3	10.6	9.7	10.2	0.4	-0.4
394/11	6.8	2.3	7.4	1.9	0.7	-0.4
396/11	9.5	10.3	10.3	9.4	0.8	-0.9
397/11	8.8	8.9	9.5	8.2	0.7	-0.7
398/11	8.9	8.1	8.8	7.9	-0.1	-0.3
399/11	7.6	9.0	8.1	8.6	0.5	-0.4
400/11	10.1	10.6	9.8	9.9	-0.3	-0.7
401/11	8.9	9.5	9.5	8.9	0.7	-0.7
402/11	6.9	6.0	7.0	6.0	0.0	0.0
403/11	7.9	9.2	8.7	8.5	0.7	-0.7
404/11	8.3	8.9	8.7		0.4	
405/11	9.1	11.4	9.9	10.6	0.8	-0.8
406/11	9.6	10.1	10.6	9.1	1.0	-1.0
407/11	8.9	9.3	9.8	8.4	0.9	-0.9
408/11	8.5	10.0	9.2	9.3	0.7	-0.7
409/11	8.6	10.0	8.9	9.7	0.3	-0.3
410/11	9.6	10.0	9.2	9.4	-0.4	-0.6
411/11	7.2	8.9	7.6	8.3	0.4	-0.6
412/11	8.6	6.5	9.3	5.8	0.8	-0.8
413/11	8.3	5.3	8.7	4.8	0.5	-0.5
414/11	9.9	12.9	11.4	11.4	1.5	-1.5
415/11	10.4	13.0	12.3	11.1	1.9	-1.9
416/11	9.6	11.1	10.5	10.2	0.9	-0.9
417/11	10.0	11.2	10.4	9.8	0.4	-1.4
418/11	9.0	14.1	10.3	13.2	1.3	-0.9
419/11	8.5	14.3	9.4	13.8	0.9	-0.5
420/11	11.8	12.6	13.4	10.9	1.6	-1.6
421/11	8.0	6.0	7.9	6.0	-0.1	0.0
422/11	8.9	5.1	10.0		1.1	
424/11	9.3	4.7	8.0	4.6	-1.3	-0.2
425/11	9.8	8.0	10.2	7.6	0.4	-0.4
427/11	6.6	6.5	7.0	6.0	0.5	-0.5
428/11	8.5	4.9	9.3	4.0	0.8	-0.8
429/11	8.1	4.8	8.7	4.3	0.6	-0.6

430/11	9.6	5.1	10.0	4.6	0.5	-0.5
431/11	9.5	8.3	10.0		0.4	
432/11	7.2	6.5	7.5	6.4	0.3	-0.1
434/11	8.6	10.6	9.4		0.8	
435/11	7.3	6.8	7.2	7.0	-0.1	0.1
436/11	7.0	6.1	7.3	6.5	0.4	0.4
232/11	6.1	4.8	5.7	5.2	-0.3	0.3
301/11	5.3	4.1	5.4	4.1	0.0	0.0
302/11	11.5	7.8	11.8	7.4	0.3	-0.3
303/11	6.7	3.9	7.1	3.5	0.4	-0.4
304/11	5.1	4.1	7.2	3.4	2.1	-0.7
305/11	7.8	5.7	9.0	3.5	1.2	-2.2
306/11	5.4	3.2	5.7	3.9	0.3	0.7
307/11	4.0	2.8	3.6	2.7	-0.4	0.0
308/11	11.2	3.4	11.8	2.3	0.5	-1.1
309/11	3.7	2.7	3.8	2.6	0.1	-0.1
310/11	8.9	7.1	9.5	6.5	0.7	-0.6
311/11	5.9	4.9	5.7	5.8	-0.3	0.8
312/11	6.5	4.5	6.3	4.2	-0.3	-0.3
313/11	6.8	4.3	7.0	4.1	0.2	-0.2
314/11	6.4	6.4	6.4	6.4	-0.1	0.0
316/11	5.1	6.7	5.0	6.8	-0.1	0.1
317/11	4.4	4.0	4.3	4.1	-0.2	0.1
318/11	11.8	8.0	11.1	7.7	-0.7	-0.3
320/11	6.2	4.3	6.2	4.4	0.0	0.0
321/11	5.5	5.5	5.5	5.6	0.0	0.0
340/11	5.7	9.1	6.0	8.8	0.3	-0.3
341/11	8.9	3.9	10.1	2.8	1.1	-1.1
343/11	7.5	7.2	7.9	6.8	0.4	-0.4
344/11	6.5	9.5	6.8	9.2	0.3	-0.3
346/11	4.4	2.7	4.9	2.4	0.6	-0.3
38/10	11.1	7.0	12.9	5.7	1.7	-1.3
41/10	9.5	4.4	9.7	4.2	0.2	-0.2
59/11	6.5	3.2	6.5	3.3	0.0	0.0
60/11	8.1	3.3	9.3	3.1	1.2	-0.2
61/11	8.3	4.6	8.0	4.9	-0.3	0.3
62/11	5.8	4.3	5.7	4.5	-0.2	0.2
63/11	7.2	4.0	7.8	4.0	0.6	0.0
72/11	4.9	5.6	6.1	4.5	1.2	-1.1
73/11	6.4	4.7	6.3	4.7	-0.1	0.0
74/11	7.0	5.5	7.2	5.3	0.2	-0.2
75/11	6.6	4.6	6.7	4.5	0.1	-0.1
76/11	6.8	5.1	6.8	5.1	-0.1	0.1
77/11	6.3	4.9	6.5	4.7	0.2	-0.2
78/11	7.1	6.4	7.3	6.3	0.2	-0.2

79/11	7.2	5.7	7.4	5.4	0.3	-0.3
80/11	5.9	5.8	6.6	5.1	0.7	-0.7
81/11	5.2	4.0	5.8	3.8	0.5	-0.3
82/11	5.8	4.8	6.1	4.5	0.3	-0.3
83/11	6.2	7.4	7.1	6.5	0.9	-0.9
84/11	4.6	3.6	4.7	3.5	0.1	-0.1
85/11	6.8	4.5	7.1	3.1	0.3	-1.3
152/10	6.3	6.8	6.5	6.4	0.2	-0.4
153/10	8.2	5.7	8.3	5.5	0.2	-0.2
155/10	6.7	5.4	7.0	5.6	0.2	0.3
163/10	8.9	6.9	9.3	6.5	0.3	-0.3
164/10	9.6	9.1	9.8	6.6	0.2	-2.5
171/10	7.9	7.3	6.6	7.2	-1.3	-0.1
8_2/10	6.7	5.4	8.4	3.6	1.7	-1.8
180/10	9.1	7.1	9.4	6.7	0.3	-0.4
181/10	6.9	7.1	7.0	7.0	0.0	0.0
184/10	7.8	9.7	7.9	9.6	0.1	-0.1
186/10	10.5	8.6	11.1	9.0	0.7	0.3
187/10	11.2	5.0	10.8	4.8	-0.5	-0.2
192/10	10.7	6.6	10.1	6.7	-0.6	0.1
198/10	9.1	7.1	9.4	7.7	0.3	0.7
199/10	9.8	5.9	9.7	6.0	-0.1	0.1
200/10	11.7	7.2	12.6	7.3	0.9	0.1
202/10	9.8	4.6	10.2	4.2	0.4	-0.4
204/10	9.6	5.9	10.2	6.1	0.6	0.2
206/10	9.8	7.3	9.4	7.8	-0.5	0.4
207/10	9.9	6.4	9.5	6.8	-0.3	0.3
208/10	10.8	9.0	10.6	9.2	-0.2	0.2
210/10	10.7	9.1	11.1	8.3	0.4	-0.9
212/10	8.2	5.5	7.3	6.4	-0.9	0.9
213/10	10.9	9.2	11.4	8.2	0.5	-1.0
214/10	10.4	9.6	10.2	9.8	-0.2	0.2
215/10	10.1	8.0	10.6	8.1	0.5	0.2
216/10	6.9	4.7	6.1	5.5	-0.8	0.8
218/10	9.0	7.4	8.1	8.3	-0.9	0.9
219/10	11.5	6.1	11.3	8.3	-0.2	2.1
220/10	8.7	5.3	8.2	5.8	-0.5	0.5
30/10	9.5	7.3	11.4	6.3	1.9	-1.0
36/10	10.1	9.4	10.1	8.8	-0.1	-0.6
47/10	8.0	6.6	8.0	6.5	0.0	-0.1
52/10	7.1	5.1	6.6	4.5	-0.5	-0.6
42/10	6.6	5.9	6.3	6.2	-0.3	0.2
50/10	9.4	6.3	10.9	6.1	1.5	-0.3